New Frontiers of Environmental Biotechnological Application

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Preface

Environmental biotechnology is the application of all components of biotechnology to solve environmental problems. The prime target of this science is the abatement of pollution through bioremedation/ biotreatment or supporting as resources for human use in non polluting ways. It can also help in cleaner production of existing products. On the whole it encompasses aspects of natural resources management, the treatment of waste and control of pollution. Thus the major areas of understanding are environmental pollution abatement through biodegradation, biotranformation, bioaccumulation of toxicity like organics, metals, oil & hydrocarbons, dyes, detergents etc., Energy management through production nonconventional nonpolluting energy like biodeasel methanol, biogass, biohydrogen etc., Agricultural application of biofertilizer, biopesticides, or bioorganics of multiple users, Recovery of resources from toxic or nontoxic wastes through biotechnological approach, Biosensor approach of pollution monitoring and several other allied issues.

Environmental Biotechnologies are competing with great success aginst traditional technologies recent days and are providing solutions to acute problems through the so called 'end of pipe' treatment technologies and bioremediation.

Environmental biotechnology can also provide a natural way of addressing mainly environmental problems ranging from the identification of biohazards to bioremediation techniques for industrial, agricultural and domestic like municipal effluents and residues. Thus environmental use of biotechnology includes the development, biosecurity use and regulation of biological systems for remediation of contaminated environments like land, water, air as well as for use environmentally sound processes leading to clean technologies and sustainable development.

Virtually all types of human activity generate wastes and this places a heavy burden on the environment. So far we have relied more upon the physical and chemical methods of pollution control. However microorganisms are likely to prove as more suitable tools for pollution control due to their versatility and adaptability. In the field of environmental management biotechnology have helped in environmental monitoring, degradation of wastes, substitution of non renewable resource base with a renewable and production of ecofriendly products and processes.

The application of biotechnologies with its risks that is difficult to define, much less to assess. The introduction of genetically engineered microbes can create new ecological niches and bring about large-scale transformations in the structure and function of the ecosystem as a whole. There are many unknown risks and hazards associated with the accidental or deliberate release of self-propagating, genetically engineered novel forms of life into the biosphere. In the event of disastrous consequences, it is likely that people will be harmed who had little gain from the use of biotechnology.

In recent years, considerable development in terms of R&D and its application on "Environmental Biotechnology" is made round the world. The major areas of application are microbial biodegradation of pollutants, development appropriate technology for biofertilizers and biopesticides production and its use in the field of agriculture and

horticulture; production of single cell protein etc. Considerable discussion occurred in recognition and support of the tremendous potential that environmental biotechnology offered particularly in the development of the next generation of pollution prevention, pollution abatement, and sustainable development of green technologies. The emergence and acceptance of the concept of sustainable development warrants that the scope of environmental biotechnology be enlarged to address issues like environmental monitoring, restoration of environmental quality, resource/residue/waste-recovery/utilization/treatment, and substitution of the non-renewable resource base with renewable resources. Different articles delineates the current and prospective applications in these sub-areas of environmental biotechnology, and documents, case studies on environmental monitoring, restoration of environmental quality, resource recovery etc.

The book intends to present some new frontiers of environmental biotechnological applications and their importance as feasible for environmental problem solving. The chapters deal with different aspects of microbial degradation and interactions with environmental pollutabts like heavy metals, harmful chemical from wastewater. Development of molecular probe for identification of harmful microorganisms has been put to looked upon. Chapters also describe the novel biofiltering technique to remove air borne contaminants creating pollution free atmosphere. Integrated process biotechnology has also been emphasized in one chapter.

First of all I would like to express my appreciation and thanks to Ministry of Environment and Forest, Govt. of India for providing financial support through ENVIS Centre towards publication of this book. My extreme thanks will goes to all the contributors of this book without their support this publication couldn't be possible. I am also thankful to Department of Environmental Science, University of Kalyani and University authority for providing support and functioning of the ENVIS Centre. Lastly I would like to thank our editorial staff Dr. A. C. Samal, Dr. J. P. Maity, Mr. S. Kar and Mr. S. Banerjee for their enormous work during preparation of the manuscript.

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Chapter-1

Environmental Biotechnology – A Critical Appraisal

S. C. Santra, A. C. Samal, J. P. Maity and S. Kar

1.1 Introduction

"Biotechnology means any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use."

Biotechnology as an art has been practiced for a long time in traditional activities such as fermentation and brewing. It has now emerged as a separate discipline, pointing to future revolutionary developments for the good of mankind. Spectacular advances that have taken place in the field of biotechnology in the last two decades have a profound impact on the socio-cultural scene, life-style, health care system, and availability of food, employment opportunities, energy and clean environment. The promise of biotechnology offers hope to the most destitute people on the earth. Through its miracle we can conceive of winning the eternal struggle against hunger and disease.

Micro-organisms are the oldest inhabitants of earth. They are masters in versatility and adaptability to the changing environment. They will definitely prove to be most cost effective partners in our efforts for sustainable development. So limitations faced by physical and chemical methods of pollution control will be overcome if we take the help of these environmental masters (micro-organisms).

Biotechnology stands on the understanding of molecular basis of biological cell functions and the ability of mankind to alter cell functions to make it produce products required by society. New techniques available with biotechnology holds potentials for developing products and processes in various sectors of agriculture, horticulture, floriculture, forestry, animal husbandry, health care, energy generation and environmental protection.

Virtually all types of human activity generate wastes and this places a heavy burden on the environment. So far we have relied more upon the physical and chemical methods of pollution control. 'However, micro-organisms are likely to prove as more suitable tools for pollution control due to their versatility and adaptability. In the field of environmental management biotechnology have helped in environmental monitoring, degradation of wastes, substitution of non-renewable resource base with a renewable resource base, and production of eco-friendly products and processes.

The application of biotechnologies with its risks those are difficult to define, much less to assess. The introduction of genetically engineered microbes can create new ecological

niches and bring about large-scale transformations in the structure and function of the ecosystem as a whole. There are many unknown risks and hazards associated with the accidental or deliberate release of self-propagating, genetically engineered novel forms of life into the biosphere. In the event of disastrous 'consequences, it is likely that people will be harmed who had little to gain from the use of biotechnology.

Biotechnology is an agenda for development, biotechnological boom and ecological doom, Biohazards and Bio-safety. This is followed by conventional fuels and their environmental impacts, non-conventional fuels and their environmental impacts, Transgenic plants with an emphasis on improved fiber quality, Transgenic animals with an emphasis on improved wool production, Biotechnology for pollution abatement, Bioremediation, Biomineralization, Thuringiensis toxin as a natural pesticide, Biological control of insects swarming agriculture fields, Biofertilizers, and Biotechnology of food etc. Thus the major areas of understanding can be grouped as follows:

i) Environmental pollution abatement through biodegradation, biotranformation, bioaccumulation of toxicity like organics, metals, oil & hydrocarbons, dyes, detergents etc.
ii) Energy management through production nonconventional nonpolluting energy like biodeasel methanol, biogass, biohydrogen etc.
iii) Agricultural application of biofertilizer, biopesticides, or bioorganics of multiple users,
iv) Recovery of resources from toxic or nontoxic wastes through biotechnological approach,
v) Biosensor approach of pollution monitoring and several other allied issues.

There are also applications of biotechnology that do not use living organisms. Examples are: DNA microarrays used in genetics and radioactive tracers used in medicine.

i) Red biotechnology is applied to medical processes. Some examples are the designing of organisms to produce antibiotics, and the engineering of genetic cures through genomic manipulation.

ii) White biotechnology, also known as **grey biotechnology**, is biotechnology applied to industrial processes. An example is the designing of an organism to produce a useful chemical. White biotechnology tends to consume less in resources than traditional processes used to produce industrial goods.

iii) Green biotechnology is biotechnology applied to agricultural processes. An example is the designing of transgenic plants to grow under specific environmental conditions or in the presence (or absence) of certain agricultural chemicals. One hope is that green biotechnology might produce more environmentally friendly solutions than traditional industrial agriculture. An example of this is the engineering of a plant to express a pesticide, thereby eliminating the need for external application of pesticides. An example of this would be Bt corn. Whether or not green biotechnology products such as this are ultimately more environmentally friendly is a topic of considerable debate.

iv) Bioinformatics is an interdisciplinary field which addresses biological problems using computational techniques. The field is also often referred to as computational biology. It plays a key role in various areas, such as functional genomics, structural genomics, and proteomics, and forms a key component in the biotechnology and pharmaceutical sector.

v) The term **blue biotechnology** has also been used to describe the marine and aquatic applications of biotechnology, but its use is relatively rare.

1.2 Biotechnology for Environmental Protection

Industrial growth, economic development, consumerisation indicate a country's progress and life standard of individuals. Industrial growth in the 20^{th} century has brought along with it new problems, too. Water pollution, air pollution, land pollution, noise pollution, radioactive pollution, solid wastes, depletion of resources, scarcity of good quality water, spreading health hazards, are all the result or consequences of stupendous industrial activities with less attention to its negative impacts on man and his environment. Nature's built-in mechanisms and self-regulation ability has been thrown out of gear by the quantity and complexity of wastes generated by the modern society. In economic terms, we only consider material costs and energy costs involved and fail to pay attention to the costs involved in the form of loss of environmental quality. Urbanization, wrong agricultural practices etc., are responsible for pollution. *As* technological progress has followed the industrial revolution, solving of environmental problem must follow technological progress. Industrial processes and products thereof both must become environmentally friendly and least damaging. .

Pollution may be defined as an undesirable change in physical, chemical or biological characteristics of air, water, land that can harm human life, the lives of desirable species, our industrial processes, living conditions and cultural assets; or waste our raw material resources.

Environment protection means limiting the impairment of environment and it includes conservations of resources. 'Environment protection' has three main objectives:

- To prevent damage and discomfort;
- To improve productivity and pleasure; and
- To maintain balances of the ecosystem.

Environment protection efforts will pay us back in terms of money, economy, productivity, social justice, cleaner surrounding and sound health. Environmental problems only differ a little with respect to a country, so the problems faced else where and controls applied are equally applicable. Awareness, participation and action in a concerted manner by all who are connected will be needed to solve the pollution problems.

'Environmental Biotechnology' involves specific applications of biotechnology to the management of environment and related socio-economic and developmental issues, keeping in view the concept of sustainable development. 'Environmental biotechnology' encompasses issues like:

i) Environmental monitoring;
ii) Restoration of environmental quality;

iii) Resource/residue/waste-recovery /utilization/treatment application of r-DNA technology;
iv) Substitution of non-renewable resources by renewable ones;
v) Strain improvement for degradation of highly-toxic pollutants with the production of chemicals;
vi) Global changes;
vii) Biological diversity; and
viii) Risk management.

Industrial pollution management is thus one amongst the many issues that environmental biotechnology addresses to. For a long time, the point of discussion in environmental pollution as an issue has been symptoms rather than causes of pollution. This naturally influenced our thinking, and emphasis was given on measurement and removal (treatment technologies). Then slowly attention was directed towards the Environment Impact Assessment (EIA) and this could add to the better planning and possibility of better Control. And today - we have started to attack the root cause of pollution. We have started discussing of what is described as 'clean technologies' which believe in process development and modifications to minimize pollution.

The points useful in effective pollution management are:

(a) In-process treatment.
(b) End-of-pipe treatment.
(c) Remediation of polluted sites.
(d) Modification of existing processes.
(e) Introduction of new processes and products.

Though many technologies have been available for clean-up purpose, only a few of them have been proved to be of routine application value. In 1989, the US Environmental Protection Agency published a broad assessment of international technologies (particularly those from Europe, Canada and Japan) for remediation of superfund sites. The criteria used by them for assessment, apply in general also, while, evaluating different technologies for pollution abatement purpose. The criteria are:

i) Function - object and applicability of technology;
ii) Description - details of operating principles and design features;
iii) Performance - demonstration of effectiveness;
iv) Limitations;
v) Economics; and
vi) Status of research, development and availability.

In addition to the above-mentioned points, the choice of technology is also influenced by the social, political, geographical considerations. The criteria apply to the assessment of technologies in other environmental clean up work, too.

1.3 Biotechnology for pollution abatement

We are so much obsessed with and possessed of industrial growth that our ecosystem has undergone many ramifications and diversions; its pristine glory and vigor, vitality and utility has been completely lost. Therefore, it is the bounding duty of us all to be aware of what is happening around us and how this dither-down can be halted. In the initial stage of industrialization, more importance was attached to stepping-up of production-regardless to the need to install pollution free technologies. While some form of environmental pollution has always existed and will continue to exist, it is only in the last two decades or so that we have found the concern for environmental pollution becoming more and more pervasive. The time clearly is now to have a look at our technological capabilities to protect our environment (Agarwal, 1996).

So far we have relied more upon physical and chemical methods of pollution control. Today, biotechnology is being considered as an emerging technology in environmental protection. It involves the use of micro-organisms, the oldest inhabitants of the earth, which are likely to prove as more suitable for pollution control due to their versatility and adaptability to changing environments.

The application of biotechnology in the field of air pollution abatement and water pollution abatement shall be discussed here in more details.

1.3.1 Biotechnology for Air Pollution Abatement

Sulphur dioxide, Nitrogen oxides, Volatile Organic Compounds and Particulates are the four major components of air pollution and are responsible for environmental hazards. Sulphur dioxide and Nitrogen oxides gases are just two of the four main pollutants known to be important determinants of local air quality.

Increase in environmental awareness has resulted in more attention of people to air pollution. People sense pollution by offensive odour just before their receiving the damage. Waste gases with an offensive odour may be generated during the production process or they may generate from open waste water treatment plants and garbage composting plants.

Three types of biological waste gas purification systems are in operation. These are: (i) Bioscrubbers, (ii) Biofilters, and (iii) Biotrickling filters.

(i) Bioscrubber

A typical bioscrubber consists of an absorption column and one or more bioreactor (Figure 1). Biological oxidation tales place in these bioreactors. The reaction tank are aerated and supplied with a nutrient solution. The microbial mass mainly remains in the circulating liquor that passes through the absorption column. Circulation rate is fast and not much of biofilm will develop in the absorption column. The biofilm is removed from time to time.

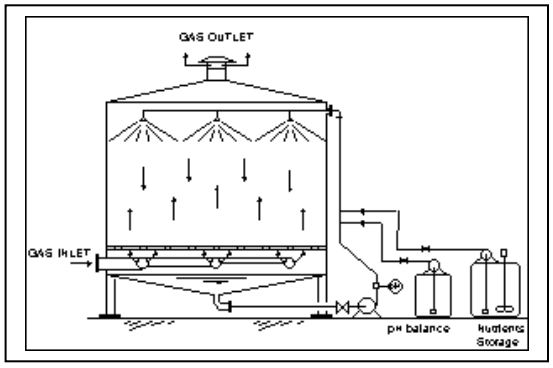

Figure 1 Bioscrubbers

(ii) Biofilters

Many micro-organisms have the ability to oxidise volatile organic compounds. Biofilters may be of open type or closed type (Figure 2). Soil, compost, peat, heather, bark etc are used in combination in biobeds. Uniformity, permeability of biobeds will decide the proper gas treatment and bypassing or chocking should not occur. Biobeds require a lot of space; they are turned 2-3 times in a year. Proper drainage is essential at the bottom. Height of packing of bed is 1 m and flow rate of gas is 130 m^3/hm^2. Microorganisms used in biofilters are mesophilic. Temperature 15-40.C, moisture 40-60% and gas contact time 10-30 seconds are maintained for biofilters.

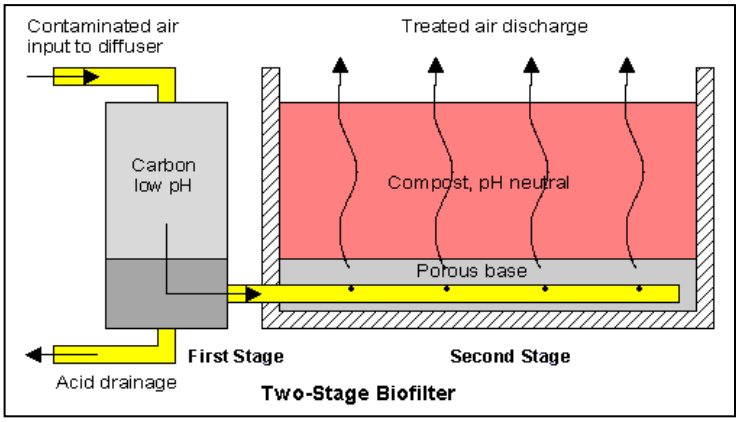

Figure 2 Biofilters

(iii) Biotrickling filters

They possess a biofilter packing through which gases are passed, water trickles down dissolving the gas, and the pure air comes out (Figure 3). In trickling filters the dissolved gas is unable to form acids.

Biosorption of polluted air have been increasing in recent past. A large number of micro-organisms have been observed to degrade specific air pollutants. Pure culture of *Pseudomonas* sp degrades Trichloroethylene (TCE). Genetically engineered *E. coli* has been found to be efficient TCE degrader. Volatile organic compounds are degraded and end products are carbon dioxide, water, biomass and inorganic salts. The process is cheaper than thermal and catalytic oxidation. *Chiobacillus ferroxidans* is used for treatment of Hydrogen sulphide and Sulphur dioxide, and gives solid sulphur.

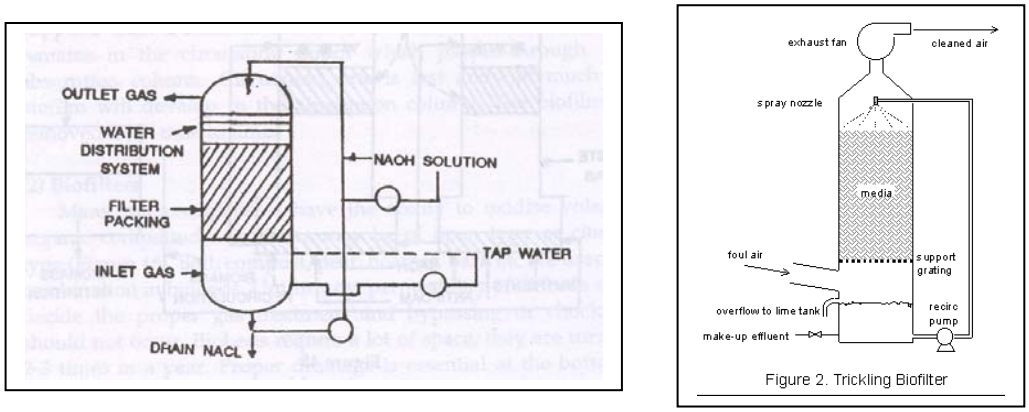

Figure 3 Biotrickling filters

1.3.2 Biotechnology for Water Pollution Abatement

The undesirable waste characteristics of polluted water include the following: (i) Suspended solid and soluble organic compounds, which undergo progressive decomposition and thus result in oxygen depletion and production of noxious gases; (ii) Heavy metals, cyanides and other toxic organics which are deleterious to aquatic life; (iii) Undesirable levels of nitrogen and phosphorus, which enhances eutrophication (excessive plant growth, which kills animals due to deprivation of oxygen) and stimulate undesirable algal growth; (iv) Non-biodegradable chemical and volatile materials like Hydrogen sulphide and Sulphur dioxide.

The objective of biological treatment of waste waters are to coagulate and remove the non-settleable colloidal solids and to stabilize the organic matter. They are considered better because they not only remove colour, but help in removal of carbonaceous organic matter in waste water which is measured as BOD (Biological Oxygen Demand), or COD (Chemical Oxygen Demand), or TOC (Total Organic Carbon), nitrification, denitrification and stabilization are the purposes of biological treatment.

Biological treatment can be carried out if the effluents are rich in unstable organic

matter. The microbes break-up these unstable organic pollutants into stable products like carbon dioxide, carbon monoxide, ammonia, methane, hydrogen sulphide etc.

Industrial effluents vary in load, concentration of pollutants, toxic materials and are often nutritionally unbalanced. They may contain biodegradable or non-biodegradable or both types of pollutants. They show seasonal variations related with production.

Biological treatment of wastewater is a relatively neglected area and is also less properly understood and controlled. Many people do not give importance to choosing of right biological treatment method. Many people handle biological treatment in the crudest manner. Ignorance about the composition of effluents just cannot be excused.

Effluent treatment systems broadly fall into two categories: (i) Aerobic, and (ii) Anaerobic

Aerobic Biological Treatment-

They are based on the system of sewage treatment and arc meant to handle easily biodegradable organic matter. When applying to industrial effluents, a careful treatability study should be done to determine the design and working parameters. The basic reaction in aerobic treatment plant is:

$$\text{Organic material} + O_2 \xrightarrow[\text{Other Nutrients}]{\text{Cells}} CO_2 + H_2O + \text{New Cells}$$

Microbial cells undergo progressive auto-oxidation of the cell mass:

$$\text{Cells} + O_2 \longrightarrow CO_2 + H_2O + NH_3$$

Lagoons and low rate biological filters have limited industrial applications but activated sludge and fixed film systems of different types are widely used for industrial effluents. Advanced activated sludge systems use pure oxygen instead of air and can operate at higher biomass concentration.

Activated sludge process-

It operates as a homogeneous continuous culture. Biosorption and flocculation removes the organic matter rapidly while oxidation and biosynthesis proceed at a lower rate. Subsequently flocs settle into the next stage of secondary sedimentation tank. A portion of this floc may be returned as inoculum. BOD and suspended solids are reduced by 85-95 per cent. Organisms in activated sludge are similar to that in percolating filters. In the activated sludge, conditions are not suitable for macro-invertibrates grazer population. Resultantly, activated sludge plants do not suffer from nuisance by flies. Also fungi are less dominant. So less sludge bulking. Protozoans are abundant in activated sludge. Nematodes and rotifers are small in number.

The contents of the reaction vessel are referred to as Mixed Liquor Suspended Solids (MLSS) or Mixed Liquor Volatile Suspended Solids (ML VSS) and consists mostly of microorganisms and inert and non-biodegradable suspended matter. The original activated sludge process was introduced in 1914. It has certain drawbacks: (1) High running costs; (2) Difficult to operate and maintain; (3) Produce large surplus biomass. There are various modifications of activated sludge process:

(A) ***Tapered aeration:*** Here aeration capacity is related to demand and it is less at the outlet than at the inlet.
(B) ***Step aeration****:* Here feeding as well as aeration is done at steps in the system throughout length of the tank.
(C) ***Contact stabilization:*** Here returned sludge is aerated to encourage organisms to utilize any stored nutrients. More wastes digested. Sludge volume is reduced through aerobic digester stage. It is similar- in principle to the extended aeration treatment. For extended aeration treatment, aeration and mixing of sludge and effluent is done in the same unit that is conducted separate in contact stabilization.
(D) ***Advanced activated sludge process:*** Most of them operate with pure oxygen. So they can operate at a higher biomass concentration. This reduces residence time and bulking (i.e. excessive growth of filamentous bacteria and fungi which may inhibit sludge setting) is inhibited.

Biological filters-Fixed film systems-

Microorganisms are attached to an inert supporting medium which is packed into a tower or tank. There are some variations like rotating biological contactors which basically are aerobic systems with fixed film of microorganisms growing on discs on a rotating shaft. Even distribution of effluent is done in the reactor and air is introduced from the bottom vents and' passes it across the media bed as effluent percolates or distributes.

Microbial slime develops on media support by using organic matter and oxygen. When the thickness of the slime increases, extra biomass sloughs off. This sludge is collected by gravity in a sedimentation tank. Sludge is later on treated and disposed off the same way as for activated sludge.

The first trickling filter was put in operation in England in 1893. Filter media used in trickling filter normally consists of rocks varying in size from 25-100 mm in diameter. The depth of " the rock varies with design and ranges from 3 to 8 ft. The use of plastic medium in trickling filters is relatively a recent' innovation and tanks are square-shaped with depths of 30-40 ft. Rock filter beds are circular with a rotating arm distributing liquid effluent at the top of the bed. Filters have an under drain system to collect sludge and treated effluents. Organic matter is decomposed by microorganisms grown as film on inert support. Development of slime, metabolic activities of microorganisms in slime, increase in thickness of slime, detachment of slime when micro-organisms near to medium reach endogenous phase (lose the ability to cling), formation of new slime is a continuous process occurring in a cyclic manner. Facultative bacteria like *Achromobacter, Flavobacterium, Pseudomonas, Adcaligenes,* filamentous forms like *Sphaerotilus natans, Baggiatoa* and at lower levels of bed nitrifying bacteria like *Nitrosomonas, Nitrobacter* are

common. Also few fungi, algae and protozoa are present.

Percolating liquid washes the slime off the medium. Sloughing off depends on organic and hydraulic loading of the filter. Hydraulic loading accounts for shear velocities and organic loading accounts for the rate of metabolism in the slime layer. On the basis of hydraulic and organic loading rates, filters are divided into two classes: low rate and high rate.

Rotating Biological Contactors (RBC)

This is one of the principle types of fixed film-moving medium systems used as a digester. Biological growth is established on disc surface made of polystyrene, polyethylene, polypropylene, stainless steel, cement, aluminium, glass, PVC, rubber, teflon, wood, wire screens etc.

Discs are 2-3 meter in diameter, 10-20 mm wide and mounted on a horizontal shaft. The distance, between adjacent discs is 20 mm. Discs are partly submerged (40% area in the medium). Discs are rotated at 1-7 revolutions per minute. Air is sparged to reduce the risk of anaerobiosis in multiple unit systems. Retention time of medium is comparable with percolating time. Biomass on discs is 200 gm dry weight per square metre of disc surface. Biological growth is 2.4 mm thick (Figure 4).

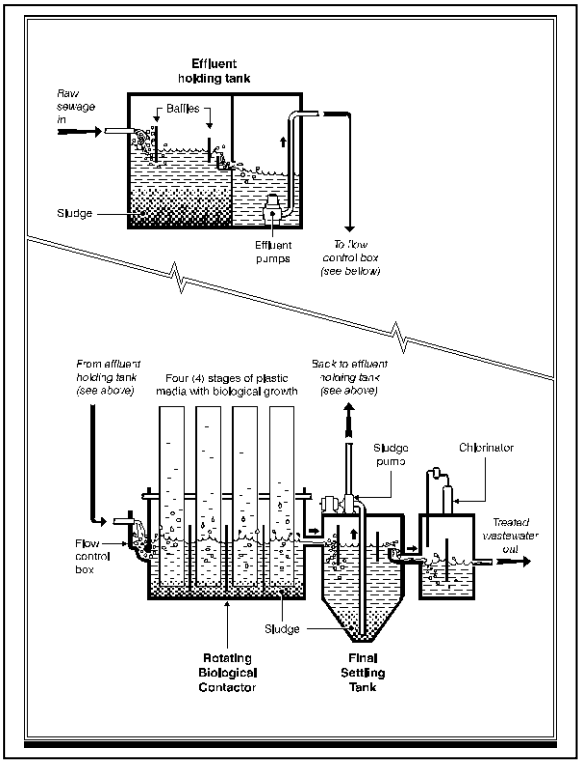

Figure 4: Rotating Biological Contactors (RBC)

Advantages of RBC:

(1) They are simple- to operate, have a low maintenance (only lubrication is required).
(2) It accommodates shock loadings.
(3) It does not have the channeling problem as in the percolators.
(4) It has reduced power costs (no sludge recycling) and less operational and maintenance costs, too.
(5) It requires less space than activated sludge plant.
(6) Fast startup, efficient mixing, little sloughing off of biomass.
(7) Effluent quality achieved is as good as after tertiary treatment.
(8) Foaming, aerosol, airstripping is reduced.
(9) Problems faced in trickling filters (percolators) like clogging, ponding, filter flies are eliminated.
(10) High waste-water temperature, oxygenation may be easier with RBC than with the activated sludge system.
(11) Lower head loss compared to the trickling filters.
(12) Process is self-regulating with respect to cell retention time and stability of process is a plus point as biomass neatly attaches to media support.

Fluidised Bed Reactors (FBR)-

It is combination of attached growth (percolating filter) and suspended growth (activated sludge) systems. Biological slime film is developed and maintained on a solid support medium consisting of particles small enough to be maintained in suspension by the upward flow of liquid being treated. Support medium particles neither sink nor outflow. Reactors are generally cylindrical with perforated distribution plates and tapered or conic," entry sections. Fluidization of support particles is allowed but clumping prevented (Figure 5).

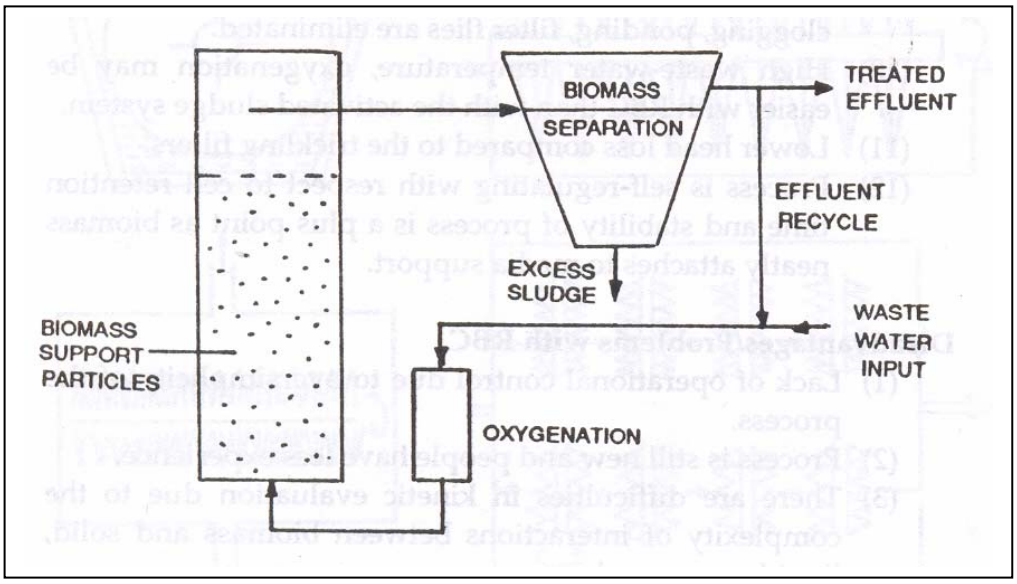

Figure 5 Fluidised Bed Reactors (FBR)

Inverse fluidised bed biofilm reactor (IFBBR)-

Fluidised bed reactors that are in use today are all operating with up flow systems, consisting of gas-solid, liquid-solid or gas-liquid-solid phases. Density of bio-particles is higher than the density of the medium. Upflow biofluidization is inconvenient for certain applications carrying aerobic processes. Collisions between the bioparticles and shear stress affects biofilm formation and then biofilm thickness. Inverse fluidization (Figure 6) removes the drawbacks of normal upflow FBR and gives a higher performance. Three advantages of inverse biofluidization are: (a) effective and simple control of biofilm thickness, (b) large specific support surface area, and (c) fast biofilm formation.

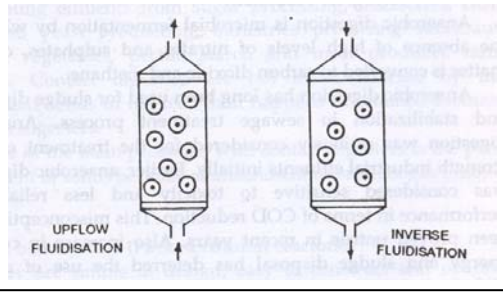

Figure 6 Upflow and inverse fluidisation bed biofilm reactor

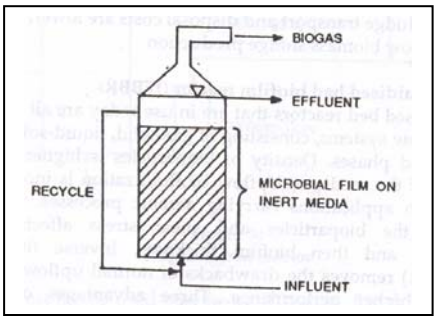

Figure7 Expanded bed reactor

Expanded Bed Reactor (EBR)-

The basic mode of operation is similar to the packed filters and fluidized bed processes. Fluidized processes were developed earlier and have diverse applications. The expanded bed system uses the operating mode of fluidized bed reactor. For biological applications, an expanded bed is a different process than fluidized bed in certain aspects. These are: (a) velocities or maintain the delicate attached living film, (b) separation; retardation and biocoagulation of fine suspended solids, (c) achievement of maximum biomass concentration (Figure 7).

Anaerobic Biological Treatment-

Anaerobic digestion is microbial fermentation by which in the absence of high levels of nitrates and sulphates, organic matter is converted to carbon dioxide and methane. Anaerobic digestion has long been used for sludge digestion and stabilization in sewage treatment process. Anaerobic digestion was seriously considered for the treatment of high strength industrial effluents initially. Earlier, anaerobic digestion was considered sensitive to toxicity and less reliable in performance in terms of COD reduction. This misconception has been proved untrue in recent years. Also increase in costs of energy and sludge

disposal has deterred the use of aerobic biological treatment procedures which are energy consuming sludge producing and acting towards net loss of material. As against this anaerobic digestion of effluents has several advantages over aerobic digestion. These are:

(1) Very low sludge production.
(2) Lower consumption of energy.
(3) Production of methane which has high calorific value.
(4) (4) Process can operate at high organic loading rate.
(5) No environmental nuisance of odour, aerosol as is the case with aerobic treatments.
(6) Microorganisms can remain dormant for several months and become operational within a week of start up. This is suitable for seasonally produced wastewaters.

Anaerobic contact digesters-

This is an aerobic equivalent of activated sludge process. It consists of a stirred tank and a tank under anaerobic conditions. The output of completely stirred tank (digester) is settled under anaerobic conditions and a part of settled sludge in returned to the digester. This results in the concentration of the sludge and longer retention time. This enables retaining of methanogenic organisms over a wide range of loading. Separation of bacteria is hindered by gassing of the effluent, hence the effluent is usually degassed before settling of the biomass.

Anaerobic contact digesters (Figure 8) are currently used for treating effluents from sugar processing, distilleries, citric acid and yeast production, industries producing saurkraut, canned vegetables, pectin, starch and meat products, farm I slurries. Contact digesters are not much affected by the suspended solids in the feed as it happens in retained biomass types of digesters.

One of the main problems with contact digesters is the poor settlement of solids because of attachment of product gas to solid particles.

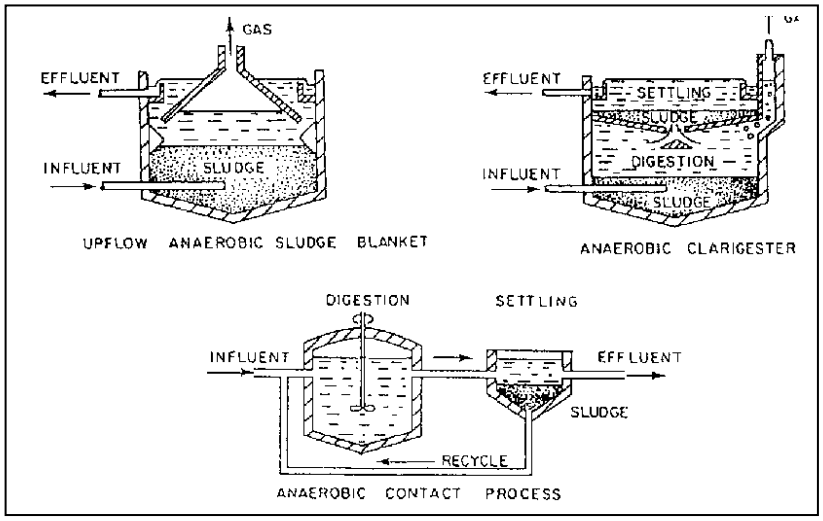

Figure 8 Anaerobic contact digesters

Packed Bed Reactors (Packed Column Reactors)

They are simple in design, easy to construct and operate (Figure 9). Organisms are contained within the packing medium in an enclosed vessel and liquid wastes pass upwards. Organisms do not form slime on packing. Regular backwashing will prevent clogging. This will also prevent high concentration of suspended solids sloughing continuously into the effluent. The treatment (removal) rate is directly related to surface area of packing.

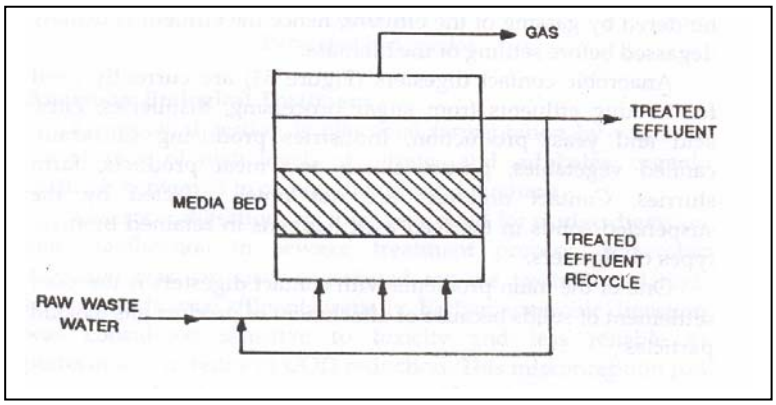

Figure 9 Anaerobic packed bed reactor

Polystyrene spheres in anaerobic filter or Neptune-Microfloc filter containing sand, silica, anthracite coal are used.

Anaerobic baffled digesters-

These reactors have walls across the tank built from the top to the bottom, so that effluent flowing along the tank has to go alternately under and over the baffles. The baffles tend to keep the bacteria in the tank and also help to prevent problems with the floating solids (Figure 10).

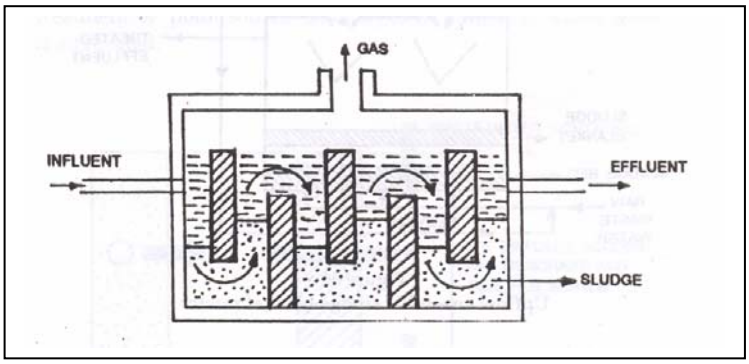

Figure 10 Anaerobic baffled digesters

Upflow anaerobic sludge blanket reactor-

These reactors require the active bacteria in the form of high density granular sludge which is retained in the digester tank despite gassing and upflow velocity of effluent (Figure 11). Sludge granulation is complex and not yet fully understood. Initially 10-15% inocula of granular sludge is required. Hydrodynamics of the digester created by feed distribution and the shape of digester are important 'in retaining the correct granular form. Well-adapted sludge may be sufficient in 1% volume only. The key element in the feed substrate for successful granule formation are calcium, phosphorus, magnesium, ammonia, aluminium, silicon. A large population of filamentous micro-organisms (e.g., *Methanothrix* spp) is also essential. Granular sludge develops with the dissolved wastes. Baffles in the digester, promote gas solid separation along with the shape of digester and upward liquid velocity. Baffles provide the surface area which reduces the upwards flow velocity and promotes biomass flocculation. Baffles are corrosion-resistant reinforced plastic. Long chain fatty acids prove toxic to methanogenic bacteria and addition of calcium chloride can reduce this toxicity and can help better granulation.

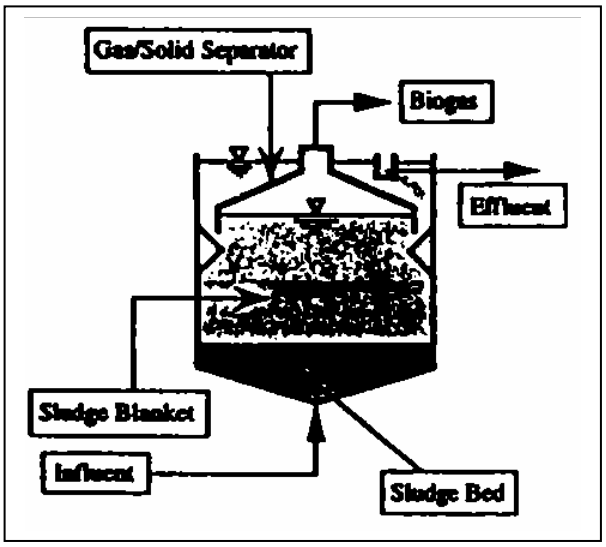

Figure 11 Upflow anaerobic sludge blanket reactor

Periodic biological reactors-

One of the most recent advances in. biological treatment processes is the use of Periodic biological reactors (Figure 12). These reactors markedly reduce the problems associated with effluent variations and eliminate sludge recycling.

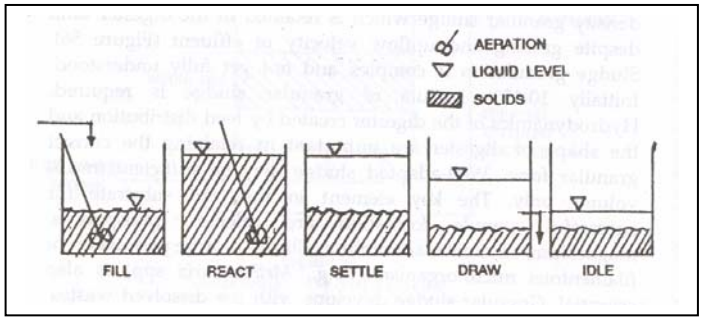

Figure 12 Periodic biological reactors

Membrane bioreactors-

Membrane separation is useful to remove, the inhibitory effects of biodegradable pollutants. Membrane bioreactor for volatile organic compounds (Figure 13) consists effluent inlet chamber, from where the effluents pass through membrane and reach over biofilm. Degradation occurs in biofilm. Aerating gas and waste water do not come in direct contact. The use of specialized cultures in conjunction with bioreactors dedicated to treatment of 'point source' discharges of particular waste water is advocated.

Use of immobilised enzymes or microbial cells for effluent treatment-

Suspended growth and fixed film systems are the two most common types of treatment used for effluents. Both are based on microbial growth, metabolic activities of micro-organisms, eventual death of organisms sludge production and bioconversions occurring during the whole process. Immobilisation of biocatalyst (Figure 13) cells or enzymes can add to the advantages of biological treatment system.

An immobilised biocatalyst can be defined as a biocatalyst for which movement in space in completely or severally restricted to form a distinct phase within the bulk phase in which substrate, effector, inhibitor molecules are dispersed and their exchange is possible.

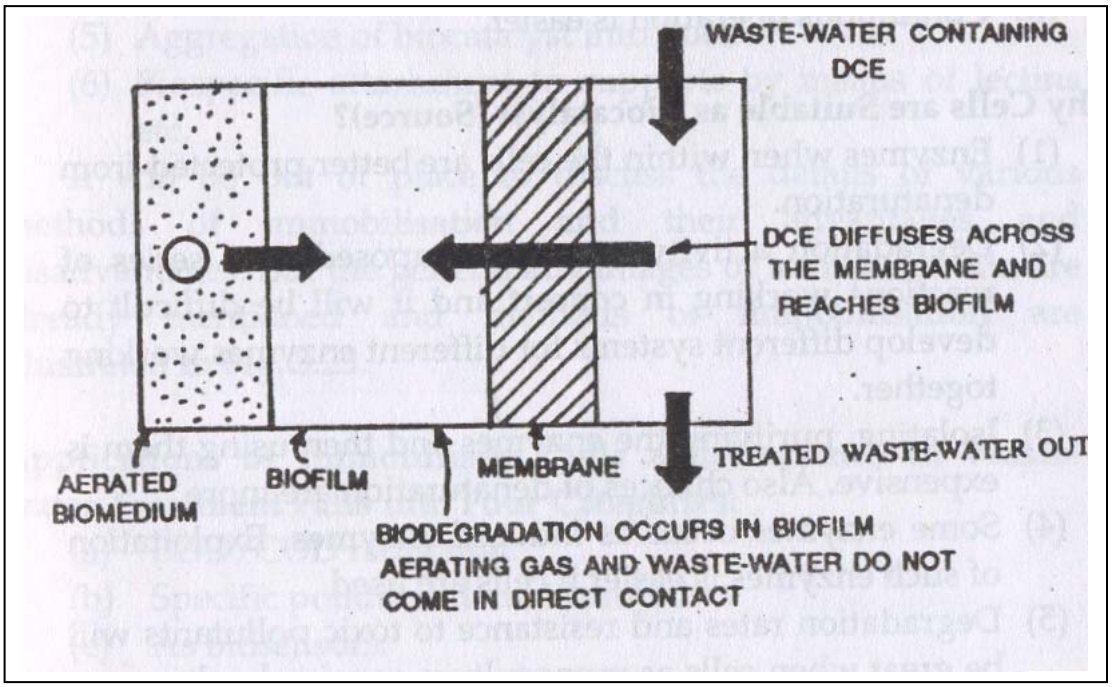

Figure 13 Immobilisation of biocatalyst

1.4 Biotechnology in Agriculture

The ability of modern biotechnology to change the characteristics of a plant or animal through the direct manipulation of genetic material is a remarkable scientific achievement. While scientists may not yet be able to accomplish the vision of Jules Verne, the tools of biotechnology developed over the last thirty years have clearly opened up dramatic opportunities to create new varieties of plants and animals.

The increasing use of modern biotechnology in agriculture has generated significant debate, much of which centers on the rapidly growing use of food crops that have been genetically modified to make them more resistant to pests or chemical herbicides. As a result, the debate has not usually addressed the potential products of agricultural biotechnology that are on the horizon. While technology developers believe that these new products will offer benefits in meeting needs for food, fuel d fiber, as well as for novel industrial and pharmaceutical uses, some of these future products are also likely to raise environmental and other concerns that will need to be addressed by the regulatory system.

In the last fifty years, since the discovery of the structure of deoxyribonucleic acid, or DNA, by American biochemist James Watson and British biophysicist Francis Crick, scientists have made enormous strides in understanding how genes work. Genes are segments of long DNA strings wrapped into chromosomes and present in most cells, whether plant, animal or human. Through a series of molecular “messengers”, genes make-

or "express"-the thousands of proteins responsible for virtually every living process. In general, each gene directs the production of a specific protein that has a specific function. For example, a single gene produces the human blood clotting protein known as Factor VIII. People with mutations in this single gene cannot make functional Factor VIII, which causes hemophilia. Gene expression is regulated by different DNA segments that cause genes to turn on or off, starting or stopping the protein production.

In the 1970s, scientists learned how to cut a specific gene out of a DNA string by using biochemical "scissors" called restriction enzymes. They were able to take the isolated gene and insert it into circular pieces of DNA known as plasmids that are found in bacteria. The bacteria rapidly reproduced, making thousands of copies of the inserted gene. Scientists developed several ways to insert the copies of the isolated gene into the DNA of a different bacteria, plant or animal. When successfully inserted into the new organism, the gene began to make the same protein it did in its original donor organism. The first successful effort of this "recombinant DNA" technology involved cutting a gene from a virus and inserting it into a bacterium, creating the first "transgenic" organism—that is, an organism that combined the DNA from two different species. Today, recombinant DNA technology is widely used to create transgenic bacteria that produce useful proteins, such as human insulin to treat diabetes, or chymosin, an enzyme widely used in making cheese. Scientists have been able to create transgenic plants in a similar way. First, they identify a gene in an organism that is responsible for a particular trait—for example, pest resistance. After isolating and making copies of the gene, scientists insert it into the target plant's DNA, generally through one of two techniques. One involves a "gene gun", which shoots microprojectiles coated with the isolated gene into the target plant's tissues (shown below). Another widely used method allows the isolated gene to hitchhike into a plant's chromosome on the back of a common soil bacterium that infects plants. In either case, scientists must test the plant to see whether the gene has been successfully inserted and whether it functions as expected. Once the gene has been inserted, the bioengineered plant cells are grown in a special culture that causes the cells to differentiate into the unique types of cells that make up the plant. The small plants are transferred from the laboratory culture to soil, where they are grown like conventional seedlings. The genetically engineered plants are then bred back with traditional crop varieties using conventional breeding techniques. Scientists test the resulting transgenic plant to make sure it continues to grow as well as the conventional variety and that the new trait works as expected.

How new is agricultural biotechnology?

Some scientists argue that modern biotechnology is just the next step in a progression of increasingly scientific efforts by humans to selectively breed better food crops and domesticated animals. Other experts, however, take the view that recombinant DNA technology is very different from anything we have done before. Changing the genes of plants and animals to better meet human needs is not a recent development. Farmers have known for centuries that they could gradually improve their crops by saving and replanting seeds from the best plants. Likewise, they knew, they could improve their animal stock by breeding the best pairs. Some also realized they could create new plant and

animal varieties with desirable traits by carefully selecting individual plants for subtle differences. It wasn't until Mendel's 19th Century work began to unravel the mechanisms of inheritance, however, that breeding became more scientific and deliberate. Over the centuries of crop cultivation and domestication of animals, the process of artificial (human) selection and selective breeding has created a diversity of food crops and animals with a wide variety of traits. For example, kale, cabbage, cauliflower, broccoli and Brussels sprouts are all vegetable varieties derived from a single species (Bailey and Bailey 1976). Hybridization—the process of breeding genetically different parents with contrasting characteristics to produce a hybrid offspring with the useful characteristics of both parents—has resulted in higher yields and more disease resistant crops. For example, improved varieties of rice with significantly higher yields than traditional varieties have helped meet the developing world's food needs. A consequence of selecting for traits through conventional breeding has been the gradual change in the genes of domesticated plants and animals. The genes of an Angus cow, for example, differ from the genes of a Holstein, just as they both differ from a common undomesticated ancestor. As a consequence, most of the food we eat today comes from plants and animals that are genetically different from their early ancestors. While modern biotechnology follows in the same tradition of improving crops and animals for human uses, its approach and techniques are quite different. In the past, breeders selected for traits without knowing which genes were responsible for the trait; the transfer of genetic material was controlled, for the most part, by the usual mechanics of sexual reproduction. The breeders had little power over, or even knowledge of, which genes were actually transferred. In contrast, biotechnology transfers only selected genetic materials, such as the gene for the specific trait, and other genetic materials to help track the gene and make it work effectively in the target plant or animal. Some scientists argue that this precision makes the effect of creating new varieties through biotechnology more predictable than conventional breeding, where genes with unknown and possibly undesirable functions can also be transferred with the genes responsible for the desired trait (Pueppke, 2001). Others disagree. They argue that most significant traits are likely to be affected by a complex interaction among numerous genes, about which there is limited knowledge. According to these scientists, conventional breeding using sexual reproduction is more likely to pass on all of the genetic material needed for a trait to work successfully in a plant or animal than recombinant DNA techniques (Palumbi, 2001). Further, since recombinant DNA techniques insert genetic material through direct manipulation rather than through sexual reproduction, scientists are not limited to moving genes between members of the same species. They can take a gene from one plant or animal species, directly insert it into the genes of a different plant or animal species, and find it expressing the same protein in the second organism as it did in the first. In this way, for example, it has become possible to take the gene from a bacterium that makes a protein toxic to insect pests and insert that gene in corn, so the corn now makes the same insect-killing protein in its tissues. While modern biotechnology falls within the long tradition of the human manipulation of the genetic materials of plants and animals, it also greatly expands the ability of scientists to move traits across species lines, and makes possible for the first time the ability to move genes across distant species, phylas or even kingdoms. It is precisely because the technology is so potentially powerful and capable of novel uses that a number of issues have been raised. These include concerns

about the safety of food made from genetically modified plants and animals and concerns about the impact on the environment, as well as the ethical and moral implications of the technology.

Is the ability to cross species lines new?

The ability to cross the species boundary is not entirely new. In the wild, tree species such as poplar and oak have been known to naturally create hybrids. Scientists using conventional hybrid breeding techniques have also been able to cross species. For example, a German experimenter in the 19th century developed a hybrid of rye and wheat, two different species. In addition, grafting—physically fusing two plants together so they grow as one—often involves the joining of different species. However, most conventional breeding is done within a species. Modern biotechnology, through its ability to directly transfer selected genetic material, greatly increases the potential to move genes between species and creates new possibilities to move genes across very distant species, phylas, or kingdoms.

1.4.1 Genetically Modifying Food

Pest and Disease Resistance:

Nationally, pests destroy about one-third of our crops and are an increasingly serious constraint to production, in spite of the advances in pest control technology over the last half century (Rosenzweig et al. 2000). A wide range of diseases and pests, including viruses, fungi, bacteria, insects, mites and plant nematodes are involved in significant crop losses each year. Some types of worms cause an estimated $7 billion in annual crop losses in the U.S.; the damage from insects is even more severe (National Research Council, 2000). Farmers have been trying to minimize losses from crop pests for hundreds of years. In the past, they have used conventional breeding practices, such as hybridization, to develop crops with better pest resistance, or chemical insecticides or biological control systems, such as predator insects that attack the targeted crop pests. Scientists can now make plants more pest resistant by inserting specific resistance genes from other plants or organisms. In some cases, recombinant DNA techniques were the first methods used to do this. This first generation of genetic engineering techniques for disease resistance relied mainly on affecting single gene traits. However, many resistance traits, such as those for fungal resistance, involve the interaction of several genes. Thus, future genetic engineering strategies are concentrating on means to control multiple gene transfers. Efforts to develop resistance to several pests or pathogens will require the use of many gene transformations. The development of pest resistance in plants remains an ongoing effort, however, as pests themselves acquire new invasive strategies and become resistant to control measures. While development of pest-resistant plants is underway in public and private laboratories, the time required to create resistant strains, breed them into stable varieties, perform field-testing and obtain regulatory approval has so far limited the number of genetically engineered varieties commercialized.

Efforts to build crops resistant to diseases are occurring on multiple fronts. One approach is to find varieties of plants that demonstrate resistance to a specific infection-causing organism, and then determine the genetic components responsible for this natural resistance. The responsible genes can then be transferred to plants that don't have them. Other strategies rely on identifying the genes within a plant responsible for generating substances that fight pathogens, and then learning how to enhance the plant's ability to make them. Still other transgenic manipulations may aim to destroy insects that damage crops and transmit pathogenic viruses and fungi.

Viruses:

Viruses are among the most ubiquitous pests in agriculture. Scientists are working to develop viral resistance in a variety of crops including squash, potato, sweet potato, wheat, papaya and raspberries. Viruses are studied widely because they not only cause disease in humans, plants, animals and insects, but also are used as tools in the study of molecular biology and, in some cases, in the development of vaccines to fight the diseases they can cause. Several techniques for virus resistance have been developed. These include viral coat protein technology and multiple gene transfers. A viral coat protein acts like a vaccine, causing the plant to develop resistance to the particular virus (illustration shown below). Transferring the gene for a viral coat protein, a part of the outer shell of a virus that does not cause disease, into a plant acts like a vaccine for the plant. The plant is then able to resist the virus, analogous to the way vaccines keep us from getting certain diseases like measles. The advantage of introducing only the coat protein is that it induces resistance without the introduction of the actual virus (Powell-Abel et al. 1986, Beachy et al. 1990**).** The technique has been used successfully in many plants against several different viruses.

Viral Coat Protein Technology:

The first genetically engineered virus-resistant food crop in the marketplace was yellow crookneck squash. Using the viral coat protein approach, this squash was engineered to resist the watermelon mosaic virus and the zucchini yellow mosaic virus (Animal Plant Health Inspection Service, 2000). Potatoes are highly susceptible to many viruses, including the potato mosaic virus and the potato leaf roll virus (shown right). A leaf roll virus epidemic in 1996 was responsible for heavy potato crop losses in Idaho. The virus, spread by aphids, damaged the potatoes to the point that they were unmarketable. Scientists in Mexico, in collaboration with researchers at Monsanto, have developed potatoes resistant to several forms of this virus. Research on disease-resistant potatoes is continuing at other laboratories. The feathery mottle virus has a damaging effect on sweet potatoes. In 1991, researchers began genetically engineering varieties of sweet potato grown in Africa, where it is an important subsistence crop. The sweet potato was engineered with coat protein from this virus and replicase genes. Replicase is an enzyme involved in the duplication of certain viral RNA molecules. Current field-testing has demonstrated successful gene transformations and the desired development of resistance to sweet potato feathery mottle virus. Although wheat is an important food source, development of genetically engineered varieties has been slower than in corn, soy and cotton. A major pest in wheat is barley yellow dwarf virus, which can cause damage in

major wheat-growing regions such as North Dakota, because no resistant strains are known. Work is in progress to engineer resistance to this disease using the viral coat protein technique.

The wheat genome is highly complex—ten to twenty times larger than that of cotton or rice—and carries an exceptionally large amount of repetitive DNA sequences. Thus, targeting particular genes is challenging, and transgenic wheat biotechnology has advanced more slowly than that of other crops. The papaya crop in Hawaii was nearly wiped out in the 1950s by the papaya ringspot virus (PRSV). Transmitted by aphids, this virus causes one of the most serious diseases of papaya worldwide (Gonsalves et al. 1998). Work to develop a transgenic virus-resistant variety began in the late 1980s. By 1992, resistant lines were field-tested; approvals for commercialization were granted in 1997. The transgenic-resistant papaya is now in wide use in Hawaii, and similar work is in progress in the Philippines, Malaysia, Thailand, Vietnam and Indonesia to enhance resistance in local papaya varieties where ringspot virus is a major pest. Researchers are also modifying other fruits for virus resistance. Field tests of transgenic raspberries engineered for resistance to the raspberry bushy dwarf virus began in Spring 2000 (Stelljes, 2000).

Fungi:

In fruit and vegetable crops, fungal diseases cause significant damage to plants and are characterized by wilting, moldy coatings, rusts, blotches, scabs and rotted tissue. The search for genetic engineering tactics to combat fungi has intensified with the need to find adequate substitutes for fungicides such as methyl bromide, widely used on fruit and vegetables but being phased out due to its links to ozone depletion. One emerging area is directed at a plant's production of defensins, a family of naturally occurring antimicrobial proteins which enhance the plant's tolerance to pathogens, especially bacteria (Garcia-Ollmedo et al. 1998). Certain defensins also demonstrate an ability to fight fungal infections. Defensins are found throughout nature in insects, mammals (including humans), crustaceans, fish and plants. Defensins from moths and butterflies, the fruit fly, pea seeds and alfalfa seeds all show potent antifungal activity (Landon et al. 1997, Lamberty et al. 1999, Almeida et al. 2000 and Gao et al. 2000). The first transgenic application of defensins was the incorporation into potatoes of the antifungal defensin from alfalfa (Gao et al. 2000). Laboratory and field trials showed that the transgenic potatoes were as resistant to the fungal pathogen *Verticillium dahliae* as non-transgenic potatoes treated with fungicide. Although studies are continuing, the chance that fungi will build resistance to defensins is thought unlikely. No known resistant strains of bacteria or fungi have yet evolved that can overcome these highly protective, pesticidal proteins.

Scientists are devising protection against the plant fungus *Botrytis cinerea,* a serious pathogen in wheat and barley. The strategy uses the gene for a natural plant defense compound named resveratrol (Lemaux and Qualset, 2000). Scientists have also introduced a gene from a wine grape into barley to confer resistance to *Botrytis cinerea*. Field trials are underway. Resistance to potato late blight (shown right), a disease caused by

Phytophthora infestans, receives high priority in potato research. Plant disease from this fungus can be destructive to crop production, as was dramatically illustrated in the Irish potato famine. In 1995, a U.S. late blight epidemic (caused by new aggressive strains of *Phytophthora infestans*) affected nearly 160,000 acres of potatoes, or about 20 percent of domestic production. Research is underway to genetically engineer potatoes that express the enzyme glucose oxidase and develop resistance to *Phytophthora* blights (Douches undated). At present, however, no products are close to commercialization. Potatoes are also being transformed using a soybean gene for a protein (beta-1, 3-endoglucanase) that confers resistance to infection by *Phytophthora* (Borkowska et al. 1998). Other studies report that transgenic potatoes expressing a protein called osmotin showed reduced damage from lesion growth in leaves inoculated with the *Phytophthora infestans* pathogen (Li et al. 1999). Still other research is attempting to boost fungal resistance in potatoes by transferring resistance genes from peas. Infection of these transgenic potatoes with the fungus triggers hormonelike signals in the potatoes that turn on the pea resistance genes. One substance that is produced, chitosan, stops fungal growth and activates the potato's own natural defense systems.

In rice, blast and sheath blight are major fungal diseases. Scientists created transgenic strains resistant to sheath blight that are currently being fieldtested. Other researchers are working on engineering rice strains for multiple resistances to both the fungus sheath blight and the stem borer, an insect pest.

Bacteria:

Most bacteria living in or on plants are not harmful to their hosts, and may, in fact, be beneficial. However, some bacteria will invade their hosts and cause disease. Most food crops are susceptible to bacterial diseases, but bacteria rarely attack certain plants, such as mosses, ferns and conifers. Bacterial infections in plants may cause leaf and fruit spots (lesions), soft rots, yellowing, wilting, stunting, tumors, scabs or blossom blights. When tissue damage occurs on the blossoms, fruit or roots of food crops, yields may be reduced. Potatoes are susceptible to blackleg and soft rot diseases caused by the bacterial pathogen *Erwinia carotovora*. To combat these bacteria, scientists have exploited the family of enzymes known as lysozymes that catalyze the breakdown of bacterial cell walls. Using cloned lysozyme genes and a promoter, transgenic potatoes were created that produced lysozyme. In laboratory tests, the transformed potatoes exhibited substantially enhanced resistance to *Erwinia carotovora*. Field tests and further development of resistant lines are in progress. A different transgenic strategy to combat *Erwinia carotovora* was demonstrated in tobacco engineered to overexpress a peptide that kills bacteria (Ohshima et al. 1999). The genetically engineered tobacco plants were resistant to both *Erwinia carotovora* and *Pseudomonas syringae pv tabaci,* the pathogen responsible for wild fire disease in rice. Scientists have also successfully transferred a bacterial resistance gene from wild rice to cultivated rice.

Insects and Mites:

Control of insect pests such as flies, aphids, borers and insect larvae is the aim of considerable research. There are several different combat tactics, including engineering for

the expression of toxins in plants that kill insects when they consume the plant material, but are non-toxic to other species that eat the plant. Other alterations focus on inducing sterility in the pest organism or affecting the digestion or metabolism of the pests. In addition, attempts to enhance a plant's natural ability to produce leaf wax could make the plant more difficult for insects to consume. The best known and most widely used transgenic pest-protected crops are those that express insecticidal proteins derived from genes cloned from the soil bacterium *Bacillus thuringiensis,* more commonly known as Bt. Crystal (Cry) proteins or delta-endotoxins formed by this bacterium are toxic to many insect species. Deltaendotoxins bind specifically in the insect gut to receptor proteins, destroying cells and killing the insect in several days (shown below). There are several different Bt strains containing many different toxins. Scientists have identified and isolated the genes for several toxin proteins from different Bt strains. In recent years, these genes have been introduced into several crop plants in an effort to protect them from insect attack and eliminate the need for spraying synthetic chemical pesticides. There are more than 100 patents for Bt Cry genes. Bt field corn, sweet corn, soy, potato and cotton are commercialized in the U.S., and one or more of these are commercialized in at least 11 other countries. Bt controls the larvae of butterflies and moths (*Lepidopteran* insects) that eat the plants. It is especially effective against the larvae of the European corn borer (shown left), a significant corn pest in the U.S., as well as the Southwestern corn borer and the lesser cornstalk borer. In sweet corn, Bt toxins effectively deter corn earworm and fall armyworm (Bhatia et al. 2000). Recently, a different strain of Bt, *Bacillus thuringiensis tenebrionis,* was used as a gene source to confer resistance to corn rootworm, another major pest in cornfields. The resistant corn is currently in field trials (Ferber, 2000). Bt hybrid rice is also undergoing field-testing and is showing considerable effectiveness in resisting major pests in Asia such as the leaf folder, yellow stem borer and striped stem borer. Bt canola is also under development (Tu et al. 2000). Borers also create a good environment for fungi to grow. Where *fusarium* fungi grow, they reduce plant quality and generate fumonisins—toxins that can be fatal to farm animals and have been linked to liver and esophageal cancer in African farmers (Marasas et al. 1988, Betz et al. 2000). Thus, one way to reduce fungal contamination is to control pests. Scientists have measured reductions in fumonisin levels in Bt corn of 90 percent or greater (Munkvold et al. 1997, Masoero et al. 1999). Bt works against insects that eat plant tissue. However, those pests that do not eat the leaves, but rather pierce and suck nutrients from the plant, require different defense strategies. These insects include aphids, white flies and stink bugs. White flies are a major pest in poinsettias, sweet potatoes and cotton. Because these insects do not consume large amounts of plant material, a leading way to combat them is the genetic expression of toxic proteins that are strong enough to kill the pest, yet safe for the plant and non-target organisms. Avidin in transgenic corn demonstrates a different approach. Avidin is a glycoprotein, an organic compound composed of both a protein and a carbohydrate, and is usually found in egg whites. Avidin is known for chemically tying up the vitamin biotin, making it unavailable as a nutrient. Insects eating transgenic corn modified to produce avidin die from biotin deficiency. Although this corn was not toxic to mice (Kramer et al. 2000), further evaluation of its potential for insect toxicity and safety for human consumption is awaited. Transgenic corn engineered to produce avidin for commercial uses is described in the Industrial Products section of this report. Plants

produce wax as a natural protective coating. Genetic modification can increase the expression of this inherent trait. Experiments to increase leaf wax are in the early stages, but scientists have already raised wax content by as much as 15-fold. This strategy is aimed at increasing the plant's resistance to both pests and fungal pathogens.

Improvement of Crops:

Improving field-crop production and soil management is another central aim of genetic engineering technology in commodity crops. Applications include crop resistance to herbicides; improved nitrogen utilization, reducing need for fertilizer; increased tolerance to stresses such as drought and frost; regulation of plant hormones, which are key to plant growth and development; attempts to increase yield, and a multitude of other, less widespread applications.

Herbicide tolerance and resistance:

There are many negative effects when weeds grow with crop plants, the most common being competition for sunlight, water, space and soil nutrients. If weeds grow with crops, they too use these growth factors, and may cause losses great enough to justify control measures.

The use of genetic modification techniques has created crops that are both tolerant and resistant to herbicides, or weed killers. This technology allows herbicides to be sprayed over resistant crops from emergence through flowering, thus making the applications more effective. To date, six categories of these crops have been engineered (Hager and McGlamery, 1997) to be resistant to the herbicides glyphosate, glufosinate ammonium, imidazolinone, sulfonylurea, sethoxydim and bromoxynil.

Improved nitrogen utilization:

There appear to be relatively few biotechnology applications specifically designed to enhance the characteristics of farm crops, such as size, yield, branching, seed size and number. Scientists have, however, created some enhancements. A recent example is the discovery of a gene in the alga *Chlorella sorokiniana* that has a unique enzyme not found in conventional crop plants. The enzyme, ammoniuminducible glutamate dehydrogenase, increases the efficiency of ammonium incorporation into proteins. In some plants, it increases the efficiency of nitrogen use. The practical implication is that less fertilizer would be necessary to grow these plants. When the gene was incorporated into wheat, biomass production, growth rate and kernel weight all increased, as did the number of spikes in the plant (Woods, 1999).

Plant hormone regulation:

There are five major classes of plant hormones: Auxin, cytokinins, gibberellins, abscisic acid and ethylene. Plant hormones have been targeted for genetic modification to influence plant growth and development; fruit development and ripening; stem elongation and leaf development; germination, dormancy and tolerance of adverse conditions. These hormone classes are highly interactive; the concentration of one affects the activity of

another. For example, the ratio of the hormone abscisic acid to gibberellin in a plant determines whether a seed will remain dormant or germinate.

The recent discovery of an enzyme involved in the production of the hormone auxin enabled researchers to investigate the effects of moderating auxin production in determining plant characteristics. When auxin is overproduced, branching is inhibited and leaves curl down as the plant elongates, a reaction typically related to reduced light exposure. The same gene that produces this enzyme is apparently related to a gene in mammals that governs enzymes that detoxify certain chemicals. In wheat, the hormone abscisic acid slows seed germination and improves the tolerance to cold and drought. Extending or enhancing the production of abscisic acid may also delay germination, a useful characteristic in climates where spring rain is sparse or falls late in the season. Production of abscisic acid is increased in response to environmental stress, and a family of enzymes called protein kinases stimulates its production. Selecting plant varieties high in abscisic acid, or engineering plants to produce more of the hormone, may confer greater drought and cold tolerance (Stelljes, 2001). Introduction of dwarfed, high-yielding wheat contributed to the 'Green Revolution' of the 1960s and 1970s, during which world wheat yields almost doubled. Shorter varieties of wheat grains, with a greater resistance to damage by wind, resulted from a reduced response to the hormone gibberellin. Scientists have since shown that the gene called *Rht* can cause "dwarfing" in a range of plants, opening up the possibility of quickly developing higher-yielding varieties in several crops. Researchers believe that this strategy could be applied to a still wider range of crops through genetic engineering (Peng et al. 1999). The plant hormone ethylene regulates ripening in fruits and vegetables. Controlling the amount and timing of ethylene production can initiate or delay ripening, which might reduce spoilage that can occur between the time produce is picked and brought to market. Transgenic techniques aim to regulate the enzyme that breaks down a precursor of ethylene production. By regulating the timing and rate of this degradation, ripening can be controlled. This technology has been applied and field-tested in tomatoes, raspberries, melons, strawberries, cauliflower and broccoli (Agritope, 2001), but has not yet been commercialized.

Increased Yield:

Often, increased yield—greater plant biomass, more numerous tubers, larger seeds and other characteristics—is an unexpected result of unrelated genetic modifications. For instance, transgenic potatoes with increased protein also produced more tubers and showed a 3 to 3.5 percent increase in yield (Chakraborty et al. 2000). Direct strategies to raise yields have focused on metabolic pathways such as photosynthesis that increase the activity of the plant. Other examples include transgenic rice with an antisense gene, which inhibits the formation of certain proteins and thus prolongs the grain-filling period of the plant. This rice, in its first field test, increased productivity by 40 percent (Finkel, 1999).

Application for improved product characteristics:

Genetic applications to alter product quality characteristics or "output" traits are aimed at increasing nutrition, modifying allergens and improving various functional

attributes for consumers. For example, rice, that has been genetically engineered to have increased iron and beta-carotene (the precursor of vitamin A), has received considerable publicity for its potential benefit to developing nations, where nutrient deficiencies are responsible for widespread health problems. While promising, research on these varieties remains at a relatively early stage.

Using bioengineering, scientists have added or modified nutrients in various crops, and created several nutritionally enhanced products. Although few have reached commercialization, examples include adding iron to rice, or increasing beta-carotene and vitamin E in vegetable oils to boost the nutritional value. Other genetic modifications have altered the fatty acid composition in oils from soy and canola to create healthier fats. Plants have also been engineered to increase phytonutrients—substances exclusive of nutrients that have benefits for improving health or preventing disease. These include isoflavones in soy and lycopene in tomatoes.

Two genetic modification strategies have also been devised to increase the iron levels in cereal crops. One is the introduction of the gene that encodes for ferritin, an iron-storage protein (Deak et al. 1999). Overexpression of this gene improves the storage capacity of plants by as much as three-fold (Goto et al. 1999). Using this and other genetic technologies, rice was engineered to contain beta-carotene, which it normally lacks, and enhanced iron content. This transgenic "golden rice" (shown right) has yet to be bred into hybrid and native strains, so field testing of modified local varieties, commercial production and acceptance are still years away. Another method for enhancing iron is reducing phytic acid content, which improves the degree and rate at which iron and other minerals are absorbed. In one experiment, corn genetically modified to be low in phytic acid was processed into tortillas. The iron absorption from these tortillas was 49 percent greater than from tortillas made with conventional corn (Mendoza et al. 1998). To further explore the effectiveness of iron absorption by reducing phytic acid, additional iron was added in the form of iron salt supplements and consumed with either strain of corn fed as porridge instead of tortillas. In this case, no absorption effect was observed. Although it is not clear why the phytic acid level had no effect, it is well known that when dietary iron levels increase, absorption decreases. Other substances in the diet may also have contributed to the reduced absorption. While plants are the primary dietary source of vitamin E, they contain relatively low concentrations of the vitamin. Recent genetic engineering technology has been able to increase the vitamin E content of oils (Shintani and DellaPenna, 1998). As it happens, many seeds have abundant levels—up to 20-fold more of gammatocopherol, the immediate precursor of alpha-tocopherol, the active form of the vitamin. However, little of the gamma form is converted to the active vitamin.

Researchers identified, isolated and cloned the gene responsible for expressing the enzyme that converts gamma-tocopherol to alpha-tocopherol. The gene was transferred to *Arabidopsis,* which subsequently exhibited a nine-fold increase in vitamin E. Incorporation of this gene to stimulate similar gamma-tocopherol to alphatocopherol conversion into soy, canola and corn is probably not far in the future. Seed oils—particularly mustard and canola—have also been developed to contain carotenoids, especially beta-carotene, a nutrient widely studied for its role in cancer prevention. But this project is still in the testing stage. Protein (or rather specific amounts of essential amino acids, the building blocks of protein) is needed to fulfill human nutritional requirements for growth, health

maintenance and muscle development. In regions of the world where cereal grains cannot be grown, people often rely upon starchy vegetables (roots, tubers or rhizomes) to supply most of their calories. While such crops often have high yields, the primary disadvantage is their very low protein content, less than one percent. Researchers are seeking to improve protein content and quality in vegetable staples such as cassava and plantain through changes in amino acid profiles. For example, a non-allergenic seed albumin gene was introduced into the potato to increase its protein content. Transgenic tubers had 35 to 45 percent more protein and enhanced levels of essential amino acids (Chakraborty et al. 2000). Moreover, transgenic plants produced more tubers and a yield increase of 3 to 3.5 percent. Scientists have also altered soybeans for higher protein in tofu (Protein Technologies, 2001). In an attempt to create healthier fats, researchers have modified the fatty acid composition of soy and canola in several ways. They have produced oils from soy and canola with reduced or zero levels of saturates; canola with medium chain fatty acids; high stearate canola oil free of trans-fatty acids; high oleic acid soybean oil, and canola with the long chain fatty acids gamma linolenic and stearidonic acid (Ursin, 2000). The latter is of interest as an indirect source of docosahexaenoic acid (DHA), one of two long chain Omega-3 fatty acids shown to be beneficial in protecting against heart attack. DHA is available almost exclusively from seafood, primarily fatty fish. The plant precursor of DHA, linolenic acid, is poorly converted to DHA. Transgenic high oleic acid soybean oil has 80 percent more oleic acid, one-third less saturated fatty acid than olive oil, and no trans-fatty acids. Researchers have also modified sunflower oil for high oleic acid content. Another type of modified soybean oil is low in saturated fatty acids (7 percent compared with 14 percent in commodity soybean oil) and richer in linoleic acid than commodity soybean oil (64 percent compared with 51 percent). Still another has reduced linolenic acid and no trans-fatty acids, increasing its stability for use as an ingredient in processed foods (Protein Technologies, 2001). Another seed unique for its high level of a single fatty acid is mangosteen (*Garcinia mangostana L.*). This tropical tree, grown in India, the East Indies and Southeast Asia produces seeds (shown right) with as much as 56 percent by weight of stearic acid, a saturated fatty acid widespread in foods. Stearic acid is noteworthy from a nutritional perspective for its stability and textural properties and because it is one of the few saturated fatty acids that does not appear to raise blood cholesterol levels. Thus, it is useful in fats for manufactured and processed foods. Enzymes cloned from mangosteen have also been expressed in canola with resulting increased levels of stearic acid. This research demonstrates the potential of the technology and the unusual sources of enzymes to alter fatty acid profiles in popular food oils such as canola (Facciotti et al. 1999). Biotechnology has also aimed at increasing phytonutrients—substances in plants— exclusive of nutrients, that have benefits for improving health or preventing disease. For example, new research in nutrition suggests lutein may support multiple lines of defense against eye disease, and that lycopene serves as a powerful antioxidant in cancer prevention. Also called “accessory health factors” phytonutrients include isoflavones in soy, lycopene in tomatoes and polyphenols in green tea. In the laboratory, scientists have engineered tomatoes with 2.5 times as much lycopene as traditional tomatoes (Weaver-Missick, 2000). At least one company is developing soy with more isoflavones (Protein Technologies, 2001), and canola with increased antioxidants and beta-carotenes, lutein and lycopene (Agri-Food Trade Service, 2001). There are major constraints on this research, in

part because there is still much about phytonutrients that is unknown. For example, some members of a class of phytonutrients may have deleterious effects while others are beneficial, as is the case with various flavonoids, water-soluble plant pigments that, while not considered essential, help maintain overall health as anti-inflammatory, antihistaminic and antiviral agents. In addition, scientists do not fully understand the biosynthetic pathways, or the succession of enzyme activities, for many phytonutrients. Another constraint is the limited scientific information about the safety and efficacy of potentially beneficial phytonutrients. However, there is considerable research activity on phytonutrients and further development and applications are anticipated.

Anti-nutritional factors:

Some plants, especially cereals and legumes, are nutritious foods and feeds but also contain varying amounts of substances that interfere with digestibility and nutrient absorption. In excess, these materials may even be toxic. Genetic modifications are being explored to reduce these anti-nutritional substances, including phytate in cereals and legumes; glycoalkaloids such as solanine and chaconine in potatoes; tomatine, solanine, lectins and oxalate in tomatoes and eggplant; gossypol in cottonseed; trypsin and other protease inhibitors in soy, and tannins and raffinose in legumes.

Phytate is widely distributed in cereals and legumes and reduces the absorption of iron, zinc, phosphorus and other minerals in humans and other animals. Phytate is indigestible for swine and poultry because their digestive tracts lack the enzyme phytase, which releases phosphorus from phytate. Studies have shown that including phytase in the food ration improves phosphorus absorption and reduces phosphorus excretion. In the food animal industry, particularly for swine and poultry, high phytate feeds are associated with high levels of phosphorus excretion. Excess phosphorus in animal manures can be washed into streams or leach into ground water and become a serious source of water pollution. Research has indicated that poultry have substantially reduced phosphorus excretion when fed phytase as a supplement alongside ordinary soybeans or alternatively, genetically transformed soybeans expressing the phytase enzyme (Denbow et al. 1998). Similarly, swine fed low-phytate corn showed increased phosphorus retention and reduced excretion (Spencer et al. 2000a). Genetically modified low-phytate corn contains at least five times as much available phosphorus as unmodified corn. Low-phytate corn feed was also associated with improved growth and finishing characteristics (Spencer et al. 2000b). In wheat engineered to expresses the enzyme phytase, seeds exhibited a two to four-fold increase in phytase activity (Brinch-Pedersen, 1999). This opens the possibility of improving the digestibility of wheat, especially among nonruminant animals. Scientists are also seeking ways to reduce toxic substances such as glycoalkaloids. Researchers inserted antisense genes into potatoes to block the activity of the enzyme UDP-glucose glucosyltransferase, key to the production of the glycoalkaloid alpha-chaconine. This toxic substance can, at high enough levels, cause irritation of the gastrointestinal tract or impairment of the nervous system.

Allergens:

Some people have an abnormally high sensitivity to certain substances, such as pollens, foods or microorganisms. These substances, known as allergens, exist in both food and nonfood plants. One out of every five Americans suffers from allergies, asthma or both, according to the National Institute of Allergy and Infectious Diseases. Common indications of allergy may include sneezing, itching and skin rashes. Food allergies and sensitivities cause a wide variety of conditions, symptoms and diseases, a few of which can be life threatening. A food allergy or hypersensitivity is one that provokes an immune response, while a food intolerance incites an abnormal physiological reaction (Sampson, 1997). Experts estimate that 2 percent of adults, and from 2 to 8 percent of children, are truly allergic to certain foods. Food intolerance is a much more common problem than allergy. Unlike allergies, intolerances generally intensify with age (U.S. Food and Drug Administration 1994). The eight most commonly allergenic foods are milk, eggs, peanuts, soybeans, fish, crustaceans, tree nuts and wheat. There are also significant allergies to non-food plants, such as ryegrass and other plants with airborne pollens that may cause hay fever or other seasonal allergic symptoms. Most known allergens in food are proteins, suggesting the possibility of modifying the structure, or possibly eliminating the allergenic protein from the food. In some cases, traditional plant breeding has identified hypoallergenic strains that are targets for further genetic modification to reduce allergenicity. Neutralizing the allergens in major food grains would have an enormous impact on millions of families, where one or more members cannot eat these foods that are household staples. Researchers have used this approach in rice, the first food crop with reduced allergenicity to be created through genetic engineering (Matsuda et al. 1993, Nakamura and Matsuda 1996). Further testing and development work continues to assure that people with known allergies to rice products can consume this genetically engineered food without developing their typical allergic reaction. In foods such as peanuts, however, which are highly allergenic to some sensitive individuals, the allergenic proteins constitute the majority of the plant's protein, so that elimination may not be possible (Wilkinson, 1998). Another example where genetic modification may be used to reduce allergenicity is in wheat, one of the "big eight" allergenic foods. Although not yet commercially available, scientists have genetically engineered wheat to overexpress the gene responsible for the synthesis of thioredoxin, an enzyme that catalyzes the reduction of disulfide bonds within protein molecules, thus reducing the protein's allergenic properties. When expressed in wheat, the enzyme reduced the bonds in the major allergenic proteins—the gliadens and glutenins—and to a lesser extent the minor ones, too, making them markedly less allergenic (Buchanan et al. 1997). At the same time, the functional characteristics of the wheat were not impaired. Scientists are also exploring the potential of recombinant DNA technology to reduce the allergenicity of non-food allergens. For example, ryegrass is a dominant source of airborne pollen in temperate climates, and using antisense technology, scientists engineered ryegrass with reduced Lol p 5 protein levels. As this is the major allergen in ryegrass, the modification reduced the plant's llergenicity (Bhalla et al. 1999).

Although genetic engineering has the potential to reduce allergenicity of foods, it also has the potential for unintentionally introducing new allergens. Scientists are working to establish methods to detect and assess allergenicity (Taylor and Nordlee 1996, Fuchs

and Astwood 1996), and this assessment is part of the review process for new transgenic foods.

Biopolymers and Plastic:

Petroleum-based chemicals are currently used in the manufacture of today's polymers and plastics. Scientists and researchers have been working to develop more renewable sources for these compounds that have less impact on the environment. Corn offers one solution. Until recently, the goal of synthesizing a plant-derived plastic, polyhydroxyalkanoate (PHA), directly in the plant remained elusive. In 1992, scientists isolated the genes necessary for plastic production, transferred them into *Arabidopsis,* and produced the first plant-synthesized PHA (Poirier et al. 1992). The technology was further adapted in corn so that the product would be expressed only in the leaves and stalk, leaving the ears free for food or feed. Scientists have had trouble refining the technology to make production feasible, in part because product synthesis occurs best in the chloroplast, the site of photosynthesis (Gerngross and Slater, 2000).

Inhibiting photosynthesis, the engine of plant metabolism, hinders plant growth and reduces yield. Subsequent purification and processing also require costly inputs of both chemicals and energy. To date, the energy cost of producing plastic in corn exceeds that of fossil fuel-based production by 300-fold. As a result, Monsanto, the primary commercial company producing PHA, abandoned the development of plant-based plastic. Other potential plant-based polymers, particularly for fiber applications, are being explored. Bacteria have been engineered to synthesize polymers that closely resemble the natural fibers silk, elastin, collagen and keratin (Somerville and Bonetta, 2001). Extending the technology, it may be possible to engineer plants to produce totally unique polymers similar to those of natural fibers. Production remains at low levels, however.

Biosensors:

Scientists are working to genetically modify plants to act as warning systems or biosensors that can detect or monitor hazardous materials. For instance, they engineered the bacteria, *Pseudomonas putida,* for sensitivity to trinitrotoluene (TNT), the material commonly used in explosives. The sensitive bacteria were then further modified to contain the protein from jellyfish that creates a green fluorescent color so that it would alter the bacterial color when triggered. When the bacteria were field tested, they detected all landmines without fail (Burlage, 1999). Although the use of bacteria as detective agents has many drawbacks, particularly with regard to handling and storage, the principle applied to plants would offer advantages. Scientists also showed that incorporating an enzyme derived from an explosive degrading bacterium into tobacco plants enabled the plants to grow in the presence of TNT (French et al. 1999). In this alternate use for tobacco plants, the addition of the fluorescent protein would enable plants to be readily monitored. Cultivating such transgenic plants in fields with land mines presents many logistical challenges, but it is possible that it could be accomplished with aerial seeding and care. A remote detection system based on laser-induced fluorescence spectroscopy could then be applied in the aerial detection of activated TNT-rich plants (Di Benedetto, 1999). Using similar technology, it is possible that transgenic plants altered for sensitivity to

radioisotopes or other organic toxins could be used to monitor radioactivity around nuclear plants or waste sites, or sites contaminated with organic toxicants.

1.4.2 Genetically engineered insects

Insect activity in agriculture can be both beneficial and adverse. Insects are essential for pollination, control of predators and food for other creatures. On the other hand, they transmit pathogens to plants and animals, consume plants and facilitate infestation by other organisms. Researchers are looking for alternatives to synthetic chemical pesticides, because of concern about the potential impact of pesticides on the environment and human health. More benign strategies to limit insect damage have evolved that use biological mechanisms already found in nature. Researchers are also genetically engineering insects in an effort to make them more effective predators, less virulent pests or ineffective carriers of disease. Efforts have centered on limiting insects' interaction with other pests, controlling their ability to survive in the wild and ensuring a high degree of target specificity, so that unwanted environmental effects can be minimized. This strategy has advanced slowly, in part because of environmental concerns and the importance of determining whether or not transgenic insects introduce unintended damage. Genetic engineering techniques are also being applied to insects for other purposes, such as developing insect viruses for use as biopesticides and controlling the transmission of diseases like malaria.

Control of Insect Populations:

In 1995, researchers at the University of Florida created a transgenic mite, the Western orchard predatory mite (*Metaseiulus occidentalis Nesbitt*), by adding a marker gene to it (Hoy, 2000). The mites were not viable in Florida, although they were effective predators of the spotted spider mites that infest strawberries and ornamentals. Mites genetically improved through selective breeding techniques had previously been shown to be more effective in controlling pests in California almonds, where their use resulted in fewer pesticide applications. Thus, transgenic pesticide-resistant mites could become more highly effective predators. The pink bollworm *Pectinophora gossypiella* is a destructive cotton pest that currently infests much of the Southwestern U.S. Pink bollworm larvae feed inside the growing cotton boll, destroying the cotton and costing farmers millions of dollars in crop losses and control costs. One existing pest management program involves releasing sterile bollworm moths to prevent the pest from reproducing successfully. In June 2000, the Animal and Plant Health Inspection Service (APHIS), a part of the U.S. Department of Agriculture, received an application for the field release of transgenic pink bollworm engineered to contain the green fluorescent protein from jellyfish. The purpose of this marker gene is to aid in monitoring and assessing the distribution of sterile pink bollworms used in insect control. This transformation is not intended to affect the behavior of the insect.

A 1996 application to the APHIS sought permission to test a transgenic nematode that occurs widely in nature, but is not a plant pest. The genetic transformations include

insertion of a gene from jellyfish for the green fluorescent protein to make successful transformations easily detectable, and another gene to modestly increase nematode tolerance to heat. The latter was designed to ensure better survival against unexpected temperature changes during transportation and storage, not to confer additional survival value. While designed mainly for research purposes, such transformations could lead to other strategies for effective pest control.

1.5 Biotechnology in Bioenergy Production

The idea that bioenergy will become the white knight of the 21st century is intuitively attractive, and receives much press, across a broad range of political and social agendas. However, on a detailed development level it remains unclear how bioenergy will allow a sustainable platform for continued world economic growth. Einstein said that "problems cannot be solved by the same level of thinking that created them "solutions to the energy crisis will require different ways of thinking. Shifting from a petro-driven economic base to a bio-based foundation is a significant challenge and success will require more than just "substitution" strategies. There is a need to clearly understand the magnitude of the problem, to accept that new breakthroughs in technology applications are required for any chance of success, and to acknowledge that acceptance of dramatic change is probably required before we can begin to build a more sustainable future.

The problem is straightforward and can be quantified with reasonable accuracy. The world currently utilizes 420 quads/year (quad = 10^{15} Btu) and the conservative case projection is that within 30 years the world requirement will be 650 quads/year, largely due to economic development in India and China. While energy demand is growing rapidly, fossil fuel reserves are finite. In addition, if the current global temperature elevation is even partly related to anthropogenic gas emissions then what will happen during the projected massive increase in the use of fossil fuels? A conceptually attractive feature of bioenergy is that carbon dioxide release will be at least neutral due to carbon recycling on a relatively short time-scale.

Currently, bioenergy and bio-based inputs account for less than 5% of all basic inputs to the existing Western economy. While several government-industry initiatives[1] have highlighted the issues and challenges, and some companies have also taken steps to embrace the emerging bio-industry, the pace of change may be too slow. Moving from 5% of inputs to >50% of inputs in less than 20 years is a "moon-shot" type of challenge.

What is Bioenergy?

Bioenergy is a type of renewable energy derived from biomass. Biomass is material produced by living organisms. It often refers to plant material produced through photosynthesis. However, some biomass can also be derived from animals and microorganisms.

Renewable energy is produced without depleting resources. Renewable energy sources include sun, wind, water, earth and biomass power, and energy from waste.

There are two main types of biomass:

- Raw Biomass is unprocessed material from living matter. Examples include forestry products, such as mature trees unsuitable for lumber or paper production, agricultural products, such as grasses, crops and animal manure, and aquatic products, such as algae and seaweed.
- Secondary Biomass is any material initially derived from raw biomass, but has undergone significant chemical and physical changes. Examples include paper, leather, cotton, hemp, natural rubber products, food processing by-products, and used cooking oils.

Biomass is then used to produce various forms of bioenergy, including motor fuels, heat, and electricity.

Types of Bioenergy:

Bioenergy has both traditional and modernised forms. Traditional forms of bioenergy products include burning wood for heat and cooking. Modernised forms of bioenergy products include the conversion of biomass to motor fuels and electricity. There are three main types of modern bioenergy products:

- **Bioethanol** is produced from starch and cellulose components in biomass, which emit fewer greenhouse gases. Gasoline is blended with bioethanol to minimize the harmful environmental impacts of motor fuels. These blended fuels are available at over one thousand filling stations in six provinces across central and western Canada. Car manufacturers are also developing engine systems that will accept motor fuels with higher levels of bioethanol in an effort to further reduce greenhouse gas emissions.

- **Biodiesel** is produced from new and recycled vegetable oils, including canola, corn, and flax, and tall oils produced from wood pulp, forestry and agricultural residues. Biodiesel can be used in diesel engines, however, it is not widely commercially available because it is more expensive to produce than conventional diesel.

- **Biogas** is produced by certain strains of bacteria. In the absence of oxygen, these bacteria break down biomass, such as animal manure and landfill waste, to produce a combustible gas made of methane and carbon dioxide. Biogas may be used as a natural gas alternative for heat and electricity.

The Science – How is Bioenergy Made?

Bioenergy products are being developed to be compatible with most of today's standard energy systems. Biotechnology will play a key role in developing bioenergy sources, like genetically modified oilseeds, and enhancing the production of bioenergy products, like enzymes used in bioethanol production. Bioethanol, biodiesel, and biogas are all produced through different methods. They are described in greater detail below:

- **Bioethanol:** There are two basic steps in bioethanol production: hydrolysis and fermentation. Hydrolysis is a chemical reaction, sped up by enzymes called cellulases, which break down long, complex-carbohydrate chains, such as cellulose, into smaller fermentable sugars. Fermentation further breaks down organic compounds, such as sugars, into alcohols like bioethanol.

- **Biodiesel:** Biodiesel is produced by extracting oils from oilseed crops, recycling used cooking oils, or converting biomass residues into tall oils using high temperatures. These oils then undergo a refining process similar to that used to refine petroleum products.

- **Biogas:** Biogas is produced by certain strains of bacteria that digest biomass in the absence of oxygen. The biogas produced is approximately 60 percent methane and 40 percent carbon dioxide. The leftover organic residue can be used as a nitrogen-rich fertilizer.

Biotechnology and Bioenergy:

Bioenergy products are often expensive to produce, and as a result, cannot compete with conventional energy products, such as fossil fuels. Biotechnology is providing scientists with the knowledge base and tools needed to develop more cost-effective methods of producing bioenergy products. Scientific studies are providing researchers with a better understanding of how biological systems and individual components of these systems work. When this knowledge is combined with tools like genetic engineering and fermentation technology, new and improved bioenergy products may be developed and produced.

Current Research Areas in Bioenergy:

Academic, private, and government laboratories are looking into new ways of making bioenergy a more substantial energy source. The main focus of this research involves making the production of bioethanol, biodiesel, and biogas more efficient. Biotechnology is playing a key role in this regard.

Bioethanol - Research is focussing on the enzymes involved in converting biomass to bioethanol. Cellulases are a family enzymes that convert biomass into higher quantities of fermentable sugars, and in turn, into bioethanol. More efficient cellulases are in development. New engine systems that burn fuels with higher levels of ethanol are currently being tested in North America.

Biodiesel – The main focus in biodiesel research involves the creation of plant matter with distinct characteristics, engineered specifically for biodiesel production. Plant matter being considered includes genetically modified trees and oilseed crops that yield higher amounts of oils.

Biogas – Researchers are studying anaerobic digestion systems that effectively break down biomass into methane and carbon dioxide.

First, it is important to define what "biomass" really means—while there are several meanings being associated with this word, for the purposes of this article biomass is taken as any output from primary production (i.e., plant materials). Traditional biomass can make a useful contribution to bioenergy production and, in recent years, biofuels have been on the leading edge of developments. For example, in 2004, approximately 3.4 billion gallons of ethanol fuel were produced in the US for blending as an oxygenate in gasoline. In this commercial case, the biomass used was largely maize starch (~95%), sorghum starch (~4%), and a small amount of other crop inputs. The application of new production technologies, conventional plant breeding, and early-stage biotechnology traits, have resulted in significant yield increases (at the same level of inputs) in maize. Hence, an increasing volume of grain has been made available for conversion into ethanol with no negative impact on the feed/food segments of the market (McLaren, 2005)

Lignocellulose biomass has been considered as a potential feedstock for biofuels and other bioenergy (Finkelstein et al., 2004) (e.g., gasification and the generation of electricity as well as steam). Lignocellulose is an abundant material created from solar energy in primary production. Theoretical calculations of conversion to ethanol indicate high potential to generate 25 to 50 billion gallons of ethanol per year. However, lignocellulose is a complex material (lignin, cellulose, pectin) and is not easily converted into biofuel in an economically viable manner. Consequently, progress over more than 20 years of research into conversion technologies has been disappointing in terms of creating an overall viable process for lignocellulose to ethanol.

The current use of biomass (for biofuels) is heavily focused on the development of complex conversion technologies, typically involving a fermentation step. It is only very recently that the first indications of change in the feedstock have appeared. For example, the major maize seed companies have screened their germplasm for hybrids that produce a higher fermentation yield in the dry-mill process (Monsanto, 2005). The results indicate that genetic components for higher ethanol do exist, but these have never been specifically targeted in the past. Major crop plants have been bred (genetically altered via recombination and recurrent selection) primarily for food or feed production, and when was there ever selection pressure to optimize for industrial biofuel traits in wild plants? It would seem there is a huge opportunity to optimize plants for use in bioenergy strategies.

Applications of biotechnology:

Biotechnology is a tool that provides an opportunity to design and optimize the feedstock materials, not just the microbial bioconversions in the process. For example, for corn-based ethanol, the particular traits now being explored for improved ethanol production include overall starch production (yield per unit impacts efficiency), starch types (amylose:amylopectin ratios), and compositional interactions. For lignocelluose, much progress has been made on enzymatic conversion of cellulose to ethanol, but the lignin and other components inhibit the overall process. Several research groups are now exploring the outcome when lignin biosynthesis is down-regulated—potentially a major breakthrough in moving lignocellulose into the commercial biofuel market.

The preceding comments are focused on ethanol only because it is currently the major biofuel. A very analogous situation exists for biodiesel (methyl esters of plant fatty acids, although recycled cooking oil and animal fats can be used) where the market potential is high but limited by the current overall economics. Strategies that focus on stacking industrial traits, for example, in specifically-designed non-feed soybeans, could open the door to directed design for improvements in subsequent bioenergy use.

There is much written about the future potential of a "hydrogen economy." Nevertheless, it is widely recognized that some inherent technical hurdles may take 10 – 15 years to resolve. Assuming success with those, there remains a need to have an energy source (hydrogen is an energy carrier, not a source) to drive the hydrogen economy. In schools of thought, the current assumption is that fossil fuels (reformulated natural gas) will be the main source, which seems to be a self-defeating achievement. Nuclear power appears to be a more logical choice. However, why would biomass not be a high priority, at least to be explored as a major energy source for a future hydrogen-based system. Research is ongoing into the use of ethanol to power bio-fuels cells. Biotechnology could also be a valuable tool to explore the possibilities of improved solar energy capture via plants with biosynthesis of material that facilitate energy transfer to hydrogen.

Currently, a number of bio-based products are made from various parts of different crops. The classic example is pulp/paper from lignocellulosic biomass. Others include specialty fibers, adhesives, boards, veggie-candles, crayons, and additives. However, to-date, and with the exception of paper, most have been small niche products due to difficulties in processing and/or product performance issues. Biotechnology really opens several new doors to creating "natural" bio-based products that are viable in contributing to a more sustainable future (Figure 14). For example, 1,3-propandiol (to be used for a polymer that replaces petro-derived polyester) can now be generated from a microbial bioconversion of starch-derived glucose, a process that required 18 genetic-driven changes in the biosynthetic pathway (Sanford et al, 2004) A large number of exciting opportunities exist to utilize natural polymers, rather than petro-polymers, for future needs with functional as well as resource advantages. The current well-known example is the spider silk protein that is very light but is stronger than steel. Since it is difficult to harvest spider webs, biotechnology is being used to express the protein in situations where high levels can be produced and harvested with relative ease. Biotechnology can make a significant difference to the success of a sustainable bio-system for the future, via specifically-designed improvements in several high impact areas.

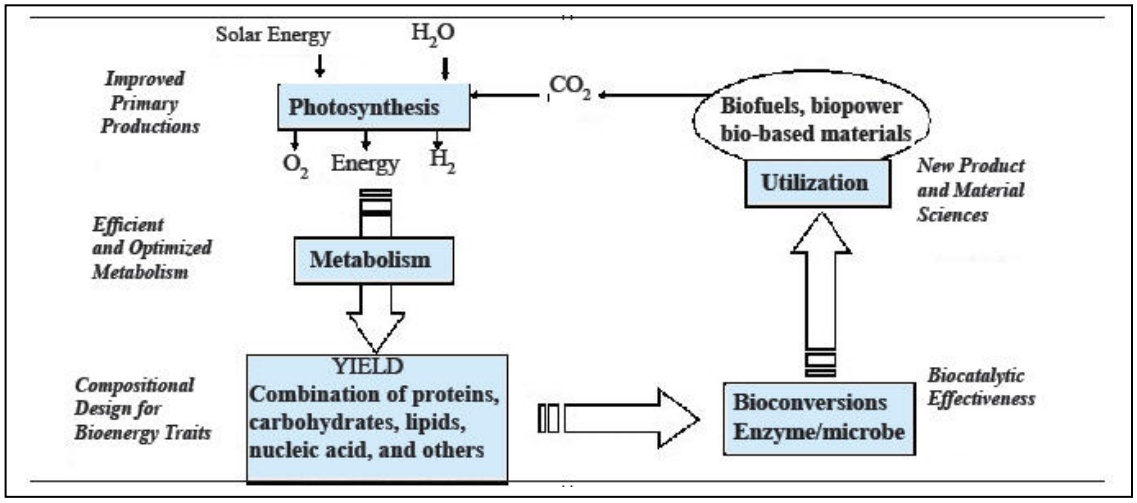

Figure 14 Flow diagram of bio power generation

In this discussion, the point has been made that biomass R&D must move beyond enhancing conversion technologies alone (analogous to petro-based chemical fractionations) and, for example, use biotechnology tools to re-design the feedstock for specific products. In addition, biotechnology opens the door for future success by being useful in an integrated product design strategy—for example, where feedstock and bioconversion can both be designed to allow optimal interaction in the system. Currently, such integrated approaches, requiring broad scientific coordination, managed teamwork, and complex intellectual property agreements, are not being given high enough priority for R&D support funding. Even in conventional starch to ethanol processes we see contradictory strategies: e.g., particular research to develop thermophilic enzymes, knowing that this requires more heat energy in the process, while practical research has focused on decreasing the temperature of the process to save energy. Biomass has potential as a feedstock and biotechnology has the potential to remove the decades-old hurdles, but we need a unified strategy if a white knight is to appear. An integrated cross-discipline strategy will be vital to making large enough technical and economic breakthroughs for biomass utilization to contribute to any future sustainable energy platform.

1.6 Nanobiotechnology

Indian nanotechnology efforts cover a wide spectrum of areas, including microelectromechanical systems (MEMS), nanostructure synthesis and characterization, DNA chips, quantum computing electronics, carbon nanotubes, nanoparticles, nanocomposites, and biomedical applications of nanotechnology. The Indian government

catalysed, through the Department of Science and Technology, the national nanotachnology Programme, which is funded with $10 million over 3 years. India has also created a Nanomaterials Science and Technology Initiative and a National Program on Smart Materials; the latter will receive $15 million over 5 years. This program, which is focused on materials that respond quickly to environmental stimuli, is jointly sponsored by five government agencies and involves 10 research centers. The Ministry of Defence is developing projects on nanostructured magnetic materials, thin films, magnetic sensors, nanomaterials, and semiconductor materials. India has also formed a joint nanotechnology initiative with the European Union (EU). Several academic institutions are pursuing nanotechnology R&D, among them the Institute of Smart Materials Structures and Systems of the Indian Institute of Science: the Indian Institute of Technology: the Shanmugha Arts, Science, Technology, and Research Academy; the Saha Institute of Nuclear Physics; and the University of Delhi, Pune and Hyderabad. The Council of Scientific and Industrial Research, India's premier R&D body, holds numerous nanotechnology related patents, including novel drug delivery systems, production of nanosized titanium carbide. In the Industrial sector, Nano Biotech Ltd. is doing research in nanotechnology for multiple diagnostic and therapeutic uses. Dabur Research Foundation is involved in developing nanoparticle delivery systems in anti cancer drugs. Similarly, Panacea Biotech has made advances in novel controlled-release systems, including nanoparticle drug delivery for eye disease, mucoadhesive nanoparticles, and transdermal drug delivery systems. CranesSci MEMS Lab, a privately funded research laboratory located at the Department of Mechanical Engineering of the Indian Institute of Science, is the first privately funded MEMS institution in India; it carries out product-driven research and creates intellectual property rights in MEMS and related fields with an emphasis on social obligations and education.

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Chapter-2

POLYHYDROXYALKANOATES IN CYANOBACTERIA: PROGRESS AND PROSPECTS

Nirupama Mallick

Abstract

In a world of shrinking petroleum reserves and increasing environmental issues, polyhydroxyalkanoates (PHAs) is receiving much attention as a material of biodegradable plastics. Amongst 150 different microbial PHAs known so far, poly-*β*-hydroxybutyrate (PHB) is the best-characterized PHA. Commercially available PHAs are not economically viable due to high costs incurred by expensive carbon sources and rich oxygen supply during bacterial fermentation. In order to reduce the cost of PHA, photoautotrophic production is thought to be a desired perspective.

Cyanobacteria, a group of oxygen-evolving photosynthetic bacteria with a short generation time, are the sole prokaryotes that accumulate PHA by oxygenic photosynthesis. During this investigation 21 different species of cyanobacteria were screened for the presence of PHA. Accumulation was found to be species-specific. In most of the species, homo-polymer of PHB was found, which was confirmed by FTIR, NMR and GC-MS. Highest accumulation was observed in *Nostoc muscorum,* i.e. up to 8.5% of dry weight followed by *Synechocystis* sp. PCC 6803 (4.5%) at the stationary phase of growth under batch mode.

PHB accumulation was found to be stimulated under P deficiency. Gas-exchange limitation under mixotrophy and chemoheterotrophy with acetate enhanced the accumulation to the level of 40% of dry wt. in both the test organisms. The test cyanobacteria were also found to accumulate P(3HB-*co*-3HV) co-polymer under propionate-supplemented condition. The prospects of using cyanobacteria as production hosts for bioplastics are discussed.

2.1 Introduction

In today's modern era of science and technology Plastics have become one of the most widely used products all over the globe. Their versatility, outstanding technical properties and relatively low cost (1 kg of polypropylene costs about US $ 1.0) caused their success. Today's applications are nearly universal: components in automobiles, home appliances, computer equipments, packages and even medical applications are areas, where plastics are clearly become indispensable. The credit for this invention goes to the famous scientist Dr. Alexander Parkes who had invented the first synthetic plastic, i.e. celluloid, way back in 1856. Since then the number as well as the types and qualities have greatly increased, producing superior materials such as epoxies, polycarbonates, teflon, silicones and polysulfones. How much ever we may applaud about the quality of plastics and its

uses in day-to-day life, they have long been vilified because they are environmentally unfriendly, i.e. they are not biologically degradable.

Durability and resistance to degradation are desirable properties when plastics are in use, but they pose problems for disposal when out of use. The mammoth scale per capita consumption of plastics to the tune of 80 and 60 kg, respectively in USA and Europe, and 4 kg in India (Kalia et al, 2000) poses a major threat to the solid waste management programme. In developed countries, most goods made of plastics end up after their useful life as discarded wastes. When discarded in nature, these conventional polymers can persist for many decades, at best a mere eyesore and at worst posing a threat to wildlife.

Incineration and recycling of plastics are practiced as the most sustainable options, but some critics agree that the environmental cost may be too high for solid waste recycling (Hendrickson et al, 1995). In any case, of all the uses to which plastic commodities are or can be put, some make the subsequent collection of wastes for incineration or recycling unlikely, impractical and impossible. For example, many packaging materials do not lend themselves for recycling owing to contamination with food, soil, etc., and the necessary cleaning prior to recycling is very expensive. These plastics neither can be recovered for incineration nor degraded. Moreover, certain plastics such as polyvinyl chloride (PVC) are potential source of highly toxic dioxins when burned in municipal incinerators or in accidental fires. Polystyrene foam products are often made with chlorofluorocarbons (CFCs) and hydrochlorofluorocarbons (HCFCs), both of which are ozone-destroying and greenhouse chemicals (Chiras, 1994).

The search for biodegradable plastics has led to a number of partially and completely biodegradable products. Partially biodegradable plastics are essentially blends of conventional plastics with biodegradable biopolymers like starch, cellulose and derivatives of these carbohydrates. On the other hand, completely biodegradable plastics like polylactides, polyhydroxyalkanoates, aliphatic polyesters, polymers of cellulose acetate, etc. are being developed. Amongst all, microbially-formed polyhydroxyalkanoates (PHAs) offer much potential for significant contributions as "bioplastics". It is not only the biodegradability that makes the PHAs so fascinating but they are also derived from renewable sources. Being the product of renewable carbon sources, its production and uses follows sustainable closed cycle (Braunegg et al, 1998).

The majority of PHAs are aliphatic polyesters of carbon, oxygen and hydrogen, and constitute a family of natural water insoluble stereo-specific polyesters of wide range of different hydroxyalkanoic acids (HAs), being characterized by the general chemical structure as presented in Figure 1, where ‘X’ can reach up to 30,000 and the R-pendant group includes H-atom or a large varieties of C-chains.

For example, when

n = 1	R = hydrogen methyl ethyl propyl pentyl nonyl	Poly(3-hydroxypropionate) Poly(3-hydroxybutyrtate) Poly(3-hydroxyvalerate) Poly(3-hydroxyhexanoate) Poly(3-hydroxyoctanoate) Poly(3-hydroxydodecanoate)
n = 2	R = hydrogen	Poly(4-hydroxybutyarte)
n = 3	R = hydrogen	Poly(5-hydroxyvalerate)

Polymers of HAs have been found in a wide variety of prokaryotes and some eukaryotic plants and animal cells, but only prokaryotes are capable of accumulating high molecular weight PHAs in the form of amorphous granules with negligible osmotic activity. More than 300 species of microorganisms are known to synthesize and accumulate PHAs (Lee et al, 1999).

Thus the most attractive features of these polymers are their biodegradability coupled with hydrophobicity; even if cheaper biodegradable plastics are commercially available, only PHAs have a good moisture resistance, so that their use in application for which these characteristics are needed, i.e. packaging of liquids and food, papers coating, etc. can be envisaged. The possible applications of PHAs as plastics, related to their more significant properties, and their actual commercial uses are summarized in Table 1.

The first observation of PHA granules in bacterial cell under the microscope was done by Beijernick (Chowdhury, 1963), and the first determination of composition of PHA was done in *Bacillus megaterium* by Lemoigne (Lemoigne, 1926), which was found to be a homopolymer of PHB. Lemoigne described PHB tentatively as a reserve material but the most convincing evidence for a functional role of PHB as carbon and energy reserve was known following the experiments of Macrae and Wilkinson (1958) in *B. megaterium*. The recognition of possible applications of PHAs as a biodegradable alternative to the conventional plastics was done in the first half of 1960s. During this period, patents related to production by fermentation (Baptist, 1962) and plasticization with additives (Baptist, 1965) were filed. The discovery of its biodegradability, biocompatibility and piezoelectric properties (Howells, 1982), and investigations of Imperial Chemical Industries (ICI, England) for profitable production of PHB (Senior, 1984), gained more impetus during early 1980s. Brittleness of pure PHB and low elongation to break (King, 1982) however, restricted its application. Detection of heteropolymeric PHA consisting of 3-hydroxybutyric acid (HB), 3-hydroxyvaleric acid (HV), 3-hydroxyhexanoic acid (HX) and 3-hydroxyheptanoic acid (HH) in isolates of activated sludge was another milestone in PHA research, owing to the more desirable properties of heteropolymers (Wallen and Davis, 1972; Wallen and Rohwedder, 1974). Numbers of patents were filed for production, extraction and blending processes during 1980s. Co-polymer of HB and HV is the best known PHA as commodity material owing to its properties of less crystalline, more ductile, easier to mold than pure PHB (Luzier, 1992), and has become the commercial

product. Co-polymer, P(3HB-*co*-3HV) produced from *Ralstonia eutropha* (more recently changed to *Wautersia eutropha*) was sold under the trademark BIOPOL® by Monsanto (Luzier, 1992), which has now been own by Metabolix (Massachusetts, USA). Other functional companies now engaged in production of bioplastics are BioMer (Germany) and Mitsubishi Gas Chemical (Japan) under the trade name of Biomer® and Biogreen®, respectively.

Until now, PHAs are being produced by heterotrophic bacteria with the help of fermentation technology. Best elucidation of the achievements, prospects and progresses in the advancement of technology for maximum production of bacterial PHAs can be seen in several extensive reviews (Lafferty et al, 1988; Anderson and Dawes, 1990; Fuller and Lenz, 1990; Sasikala and Ramana, 1996; Steinbüchel, 1996; Lee et al, 1999; Sudesh et al, 2000; Serafim et al, 2001; Reddy et al, 2003; Khanna and Srivastava, 2005a). Despite the 75 years, on and off, of research on PHAs and 20 years of intense industrial interest, PHAs still appear to be far reached from large scale production. The lack of commercialization of the initially promising bacterial copolymer, P(3HB-*co*-3HV), has been generally attributed to the high investment for the fermentation and product recovery processes on a large scale and to the cost of the substrates. To reduce the latter limitation, alternative substrates are receiving much attention, including starch and vegetable oils, but no major break-throughs in this area have been announced.

Synthesis of PHA in agricultural crops has been regarded as another promising alternative for production of this biodegradable polyester on large scale and at low cost. The first PHA produced in plants was the homopolymer of PHB. Expression of the last two enzymes, i.e. an acetoacetyl-CoA reductase and a PHA synthase, from the bacterium *W. eutropha* into the cytoplasm of *Arabidopsis thaliana* cells led to the production of PHB as intracellular inclusions up to 0.1% of the shoot dry weight (Table 2). Growth of this transgenic plant was severely reduced, presumably caused by the depletion of the acetyl-CoA pool available in the cytoplasm for isoprenoid and flavonoid biosynthesis (Poirier et al, 1992).

Much higher production levels were reached by targeting the PHB pathway to the plastid. In a study where three *W. eutropha* genes modified for plastid-targeting were expressed in *A. thaliana* from separate T-DNA vectors, PHB production in shoots were reported to reach up to 14% of shoot dry weight (Nawrath et al, 1994). More recently, a value up to 40% of shoot dry weight was achieved by expressing the same genes on a single T-DNA vector (Bohmert et al, 2000). However, plants accumulating PHB higher than approximately 5% of dry weight were chlorotic and showed a negative correlation between plant growth and PHB accumulation. These experiments concluded that while high level of PHB could be synthesized in the plastid, polymer accumulation beyond some critical point had a negative impact on chloroplast function (Rezzonico et al, 2002).

In an effort to bring this technology to the field, a group of scientist at Monsanto has shown that transgenic rape expressing the PHB pathway in leucoplasts of developing embryos accumulated PHB up to 8% of dry weight in mature seeds of heterozygous plants (Houmiel et al, 1999). Although the effects of PHB synthesis on triacylglycerol production has not been reported, no deleterious effects of PHB accumulation on seed development or germination have been detected. The same group has also expressed the PHB biosynthetic

pathway in the chloroplasts of stalks and leaves of corn, and reported a level of PHB only up to 6% (Mitsky et al, 2000). However, as with *A. thaliana*, accumulation of PHB in corn leaves was associated with chlorosis.

As presented in Table 2, expression of PHB biosynthetic pathway either in tobacco, *Linum* or maize has so far yielded only low amounts of polymer (Hahn et al, 1999; Wrobel et al, 2004; Lossl et al, 2003; Lossl et al, 2005). It is speculated that these plants may either not express the genes from *W. eutropha* very well, or that the bacterial proteins are poorly active due to instability or inactivation. PHB has been produced in cotton fiber cells in order to change the physical properties of the fibers (John and Keller, 1996; John, 1997). Accumulation of PHB to 0.3% of shoot dry weight of the fiber was reported. Thus, low expression level, long growth period and difficulties in isolating the PHB from other cellular components are the major disadvantages in plant-based PHB production.

The accumulation of polyhydroxyalkanaotes in cyanobacteria has attracted the attention since Prof. N. G. Carr, University of Warwick, UK, reported the presence of PHB in a N_2-fixing cyanobacterium, *Chlorogloea* fritschii (Carr, 1966). Cyanobacteria, however, are indigenously the sole prokaryotes that accumulate PHAs by oxygenic photosynthesis. Some are found to accumulate PHB under photoautotrophic conditions and others only in presence of acetate or other carbon sources (Vincenzini et al, 1990; De Philippis et al, 1992a; Lama et al, 1996).

Cyanobacteria are photoautotrophic gram-negative prokaryotes, also known as blue-green algae. They are the simplest known photoautotrophic organisms having characteristics of both bacteria as well as algae and are one of the oldest life forms on earth dating back 3.5 billion years. They are unified by the ability to carry out oxygenic photosynthesis using H_2O as electron donor with the possession of chlorophyll *a* and phycobiliproteins as photosynthetic pigments (Stal and Moezelaar, 1997). These organisms with a short generation time need some simple inorganic nutrients such as phosphate, nitrate (not in case of nitrogen-fixers), magnesium, sodium, potassium and calcium as macro-, and Fe, Mn, Zn, Mo, Co, B and Cu as micronutrients for their growth and multiplication. Further, these organisms can successfully be cultivated in wastewaters due to their ability to use inorganic nitrogen and phosphorus for their growth, and wastewaters such as effluents of farm-yards, fish-farms, rubber industries, sewage treatment plants, etc. are rich sources of N and P.

It is now clear that there are numbers of cyanobacterial strains with the inherent potential to accumulate PHB. However, the maximum accumulation reported so far under photoautotrophic condition is for *Spirulina platensis,* i.e. only 6% of dry wt. (Campbell et al, 1982). This was quite discouraging to consider cyanobacteria as production hosts for PHB. But report of Nishioka *et al.* (2001) demonstrated an accumulation up to 55% of dry wt. in *Synechococcus* sp. MA19, isolated from the volcanic rock of Japan under phosphate-limited conditions. This figure is quite encouraging and stimulates us to screen various cyanobacterial species for the presence of PHA. Thus, screening of new organisms with the help of acceptable method(s) of extraction and detection is certainly an important strategy for further exploration of the newly screened species on which the scope of optimization can be thought for the development of industrial organism(s) to produce desirable PHA at lowest possible cost.

2.2 Materials and Methods

2.2.1 Organisms and growth conditions

Several cyanobacterial species/ strains were isolated from different habitats of Orissa and West Bengal and few cyanobacterial species were obtained from the established culture collection centers of India (National Centre for Conservation and Utilization of Blue Green algae, IARI, New Delhi and Department of Botany, Utkal University, Bhubaneswar, Orissa). These cultures were maintained in the laboratory at 25 ± 2^0C under a photoperiod of 14:10 h at light intensity of 75µmol photon $m^{-2}s^{-1}$ PAR. For culturing the non-nitrogen-fixer forms, BG-11 medium with $NaNO_3$ and for nitrogen-fixers nitrogen-free media were used (Rippka et al, 1979). pH of the culture media were maintained at 8.0-8.5. *Spirulina platensis* was however, cultivated in CFTRI medium (Singh et al, 2002) at pH 9.5.

2.2.2 Determination of cell dry weight

Cell dry weight was determined gravimetrically according to Rai et al. (1991).

2.2.3 Extraction of poly-hydroxyalkanoates (PHA)

Solvent extraction method with hot chloroform was followed as per Yellore and Desia (1998) with certain modifications.

2.2.4 Detection and quantification of PHA

PHA was detected and quantified following the propanolysis method of Riis and Mai (1988) using a GC (Clarus 500, Perkin Elmer) in split mode (1:50, v/v), equipped with Elite-1 dimethylpolysiloxane capillary column (30 m x 0.25 mm x 0.25 µm) and flame ionization detector. Benzoic acid was used as the internal standard.

Confirmatory study of the extracted polymer was done with various spectroscopic techniques (UV-spectroscopy, crotonic acid assay method (Law and Slepecky, 1961), ^{1}H-NMR spectroscopy, Fourier-Transform Infrared spectroscopy (FT-IR) and GC-MS analysis). For crotonic acid assay, polymer extracted from cyanobacterial samples were digested with concentrated sulphuric acid and absorption spectra (200-1000 nm) were taken in Specord S 100 Spectrophotometer (Analytic Jena, Germany). For ^{1}H-NMR study polymer was dissolved in 1 ml of $CDCl_3$ and spectra was obtained using Bruker 200 Spectrometer. FT-IR spectrum of purified polymer was obtained in spectral range of 400-4000 cm^{-1} by taking the polymer with KBr block (NEXUS 870 FT-IR spectrophotometer, Thermo Nicolet Co., USA). The propanolysed sample prepared for GC was analyzed using a Perkin Elmer GC-MS system to confirm the monomer compositions of the polymer obtained from cyanobacterial sample.

2.2.5 Induction of P-deficiency

P-deficiency was induced by replacing K_2HPO_4 of the medium with equimolar concentrations of KCl.

2.2.6 Effect of gas-exchange limitation and chemoheterotrophy

Gas-exchange limitation in the culture vessels was imposed by wrapping the cotton plugs with aluminum foils followed by tightening with cellophane tapes. The exponentially growing cells were supplemented with various stimulatory concentrations of carbons and made limited of gas-exchange. For chemoheterotrophy, the stationary phase photoautotrophic cultures were supplemented with various concentrations of carbon doses and incubated under complete darkness. PHB content was analysed at stipulated time intervals.

2.2.7 Assay for P(3HB-co-3HV) co-polymer synthesis

Gas chromatography was performed for the assay of P(3HB-*co*-3HV) co-polymer synthesis. The esterified sample (0.2 µl) was injected in split mode (1:50) to the GC. The detection was made by comparing the retention time of the standard PHB and P(3HB-*co*-3HV) (Aldrich, USA) with the sample.

2.2.8 Statistical analysis

All the experiments were performed in triplicate to check the reproducibility. Results were statistically analyzed by Duncan's new multiple range test.

2.3 Results

2.3.1 Screening of cyanobacteria for PHA accumulation

A total 21 species of cyanobacteria, collected from different sources as mentioned in the Materials & Methods section, were screened under batch mode for accumulation of PHA by gas chromatographic analysis. Results showed that *Nostoc muscorum* was the best accumulator, and maximum accumulation was observed at the stationary phase of growth (Table 3). Gas chromatographic analysis also showed that the extracted polymers were homopolymer of *β*-hydroxybutyric acid (PHB) in all the species and in some species presence of PHA was not detected. Chemical proofs for this were obtained by analyzing the spectra of the sample and the standard by UV, IR, ^{1}H-NMR and GC-MS. Owing to the higher accumulation potential, *Nostoc muscorum* and *Synechocystis* sp. PCC 6803 were selected for further study.

2.3.2 Confirmatory study

The UV- spectroscopy, crotonic acid assay of the polymer extracted from *N. muscorum* and the standard PHB was done after sulphuric acid digestion, which highly resembled to the spectrum of crotonic acid (Figure 2).

The FT-IR spectra (Fig. 3) of the extracted polymer showed a strong absorption band at 1723 cm^{-1} corresponding to the carbonyl group, and the band at 1280 cm^{-1} corresponding to the ester group, which are the characteristics of the homopolymer of hydroxybutyric acid. The FT-IR spectra of the isolated polymer and the standard PHB depicted close similarity.

Analysis of H^1-NMR spectra (Fig. 4) of the polymer demonstrated that the polymer contained a single monomeric unit of HB. The resonance, as observed at 1.270, 2.535 and 5.252 δ were respectively for $-CH_3$ (HB side group), $-CH_2$ (HB bulk structures), -CH (HB bulk structures).

Mass spectrum of the propyl ester of the extracted polymer by GC-MS analysis (Fig. 5) showed the mass fragmentation pattern having distinct peaks at m/z 43 and 45, which represent the hydroxyl end and propyl end of the molecule. Peaks at m/z 60 and 87 were originated by the cleavage of C_3 and C_4 bond and ester bond.

2.3.3 Enhanced Production of PHB

2.3.3.1 PHB accumulation under P-deficiency

Impact of phosphorus deficiency on PHB accumulation is presented in Fig. 6. Cultivation of *Synechocystis* sp. PCC 6803 in P-deficient medium stimulated PHB accumulation up to 11% (w/w) of dry cells from the basal 4.5% of control culture. For *Nostoc muscorum* this rise was up to 23% from 8.5% of the control culture.

2.3.3.2 Chemoheterotrophy and PHB accumulation

Nostoc muscorum cultures grown for 21 days under 'light-dark' cycles depicted an increase in PHB accumulation up to 14% (dry wt) after 5 days of dark incubation, which was found to be the maximum for the control cultures (Table 4). Interestingly, carbon supplementation at the initiation of dark incubation boosted the PHB pool in a concentration-dependent manner. An accumulation up to 43% was recorded in *N. muscorum* under 0.4% acetate supplementation. For *Synechocystis* sp. PCC 6803 an accumulation up to 22% of dry wt. was recorded.

2.3.3.3 Impact of gas-exchange limitation (GEL) on PHB accumulation

PHB accumulation was studied under gas-exchange limitation (GEL) either alone or in combination with other parameters such as P-deficiency and in presence of carbons. GEL was found to raise the PHB pool significantly. For *Synechocystis* sp. PCC 6803, supplementation of acetate (0.4%) at the initiation of GEL boosted the accumulation up to 30% (dry wt.) on day 7 of incubation. Most significant rise in PHB content was however, recorded when the stationary phase cultures were incubated under simultaneous limitations of gas-exchange and phosphate in presence of carbons. A PHB pool of 38% (dry wt.) was recorded in fructose (0.4%) + acetate (0.4%)-supplemented cultures under the above condition. For *Nostoc muscorum* simultaneous impact of GEL and carbon supplementation was found to boost the accumulation of PHB profoundly, with a maxima of 40% (dry wt.) in glucose (0.2%) + acetate (0.2%)-supplemented culture.

2.3.4 Identification and quantification of P(3HB-co-3HV) co-polymer by gas chromatography

Figure 7 illustrates the gas chromatograms of the propanolysed *N. muscorum* sample grown under propionate-supplemented condition with the standard PHB and P(3HB-*co*-3HV). The peaks at retention time 4.8 and 6.3 min in the sample demonstrated the presence of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) units, when

compared with the standards. Under photoautotrophic as well as acetate-supplemented conditions this test cyanobacterium however, predominantly accumulated 3HB units only. Similar results were also recorded for *Synechocystis* sp. PCC 6803.

2.4 Discussion

Since Carr's publication (Carr, 1966) on the occurrence of PHB in the cyanobacterium, *Chlorogloea fritschii*, a small but unremitting flow of reports containing the presence of PHB in various cyanobacterial species followed (Stal, 1992). However, it must be stressed that in many cases the analytical methods used were only capable of identifying and quantifying the presence of PHB, as is the case for the methods based on the conversion of PHB to crotonic acid and subsequent spectrophotometric detection and quantification of this acid by taking the absorbance at 235 nm (Law and Slepecky, 1961) or HPLC analysis (De Philippis et al, 1992a). Thus some strains that have been referred to as not containing PHB could contain other PHAs, and alternatively the PHA concentration could have been under estimated, if the strain was synthesizing a co-polymer. On the other hand, some of the papers that reported the occurrence of PHAs in cyanobacterial strains on the basis of microscopic observations, not supported by chemical analysis, are difficult to interpret in order to establish the actual presence of the polyester, owing to the possibility of mistaking other cell inclusions as PHA granules. All these difficulties were annulled in the present study, where the screening test for PHAs was conducted with gas chromatographic analysis. Interestingly, all the test cyanobacteria were found to accumulate PHB only (Table 3), thus indicating that cyanobacteria accumulate primarily the homopolymer of HB. The non-occurrence of PHB in *Aphanothece* sp., *Cylindrospermum indicum*, *Nodularia* sp., *Nostoc commune* and *Plectonema* sp., and the discrepancy in PHB content even in the species belonging to same genus, e.g. *N. muscorum*, *N. linckia* and *N. commune* (Table3) indicate that accumulation of PHB in cyanobacteria is species-specific.

The chloroform extraction is now the most widely accepted method for extraction of PHA (Lee, 1996). The precipitation of polymer with diethyl ether and acetone in this method rules out the possibilities of any contaminating lipids, and therefore yields purified PHA. Our results also give testimony to the above view, where the absorption spectrum of acid-digested sample did not show the presence of any impurities, and when compared with the standard poly(3-hydroxybutyric acid) depicted the highest degree of similarity (Fig. 2). These spectra of acid-digested sample as well as the standard showed complete matching with the spectrum of crotonic acid (Fig. 2), thus again indicating the occurrence of PHB in the test cyanobacteria.

The observation of FT-IR spectrum also demonstrates the presence of homopolymer of HB units (Fig. 3). The absorption bands at 1723 cm^{-1} and 1280 cm^{-1} are characteristics of PHB and can be used to distinguish it from other PHAs (Hong et al, 1999). Further, the resonance at δ 1.270, 2.535 and 2.252 as observed from ^{1}H-NMR study (Fig. 4) and the mass fragmentation pattern (Fig. 5) also confirm the presence of PHB in the test cyanobactria.

PHB accumulation was found to increase under phosphate-deficient condition (Fig.

6). This affirms the earlier findings of Holmes (1985) and Pal *et al.* (1988), where PHB accumulation was enhanced when growth was restricted due to unavailability of phosphorus. De Philippis *et al*. (De Philippis et al, 1992a) opined that synthesis of PHB in cyanobacteria is to provide cells with a mechanism for removal of excess reducing equivalents, which results from a disruption of the balanced formation of ATP and NADPH from photosynthesis. Thus PHB biosynthesis is expected to increase under phosphate starvation, when reducing power may be in excess, because ATP production is known to decrease markedly with the onset of phosphate limitation, while the reduction of NADP through non-cyclic photosynthetic electron flow is not inhibited (Bottomley and Stewart, 1976; Konopka and Schnur, 1981).

An increase in PHB accumulation under dark aerobic condition was also observed (Table 4). This agrees well with the finding of Stal (Stal, 1992), where about 28% rise in PHB content of *Gloeothece* PCC 6909 was reported under dark incubation. Interestingly, addition of exogenous carbon at the initiation of dark incubation was found to promote PHB accumulation profoundly. The stimulatory effect of acetate on PHB accumulation could be due to direct utilization of acetate for polymer synthesis by means of the usual pathway operating in bacteria (Dawes, 1992). However, glucose might stimulate PHB accumulation through increased NADPH production as glucose utilization in cyanobacteria occurs via oxidative pentose phosphate pathway (Smith, 1982).

Gas-exchange limitations in presence of carbons were found to enhance PHB accumulation significantly (Table 5). One possible reason for this could be nitrogen limitation. Enhanced PHB production in mixotrophically-grown cyanobacteria was observed when nitrate become limited in the medium (Wu et al, 2002). Enhanced PHB accumulation under oxygen limitation was also observed. Oxygen limitation initiates a cascade of secondary metabolic events, including cessation of TCA cycle flux and an increase in acetyl-CoA/CoA ratio (Van Wegen et al, 2001). As acetyl-CoA is prerequisite for the activity of the enzyme ketothiolase, the first enzyme of PHB biosynthetic pathway, an increase in acetyl-CoA/CoA ratio might play an important role in the enhanced PHB accumulation during oxygen limitation. Thus, the observed rise in PHB pool under limitations of gas-exchange could possibly be due to the combined effects of limitations of nitrogen and oxygen, as in sealed cultures the possibility of limitations of both the gases could not be ruled out.

2.5 Concluding Remarks

Results from this study demonstrated about 5-8 fold rise in PHB pool in the test cyanobacteria by manipulating the nutrient status and culture condition. PHB content reached around 40% of cell dry weight. In heterotrophic bacteria, PHB accumulation up to 80% has been reported (Reddy et al, 2003). Although this seems profoundly higher as compared to the PHB synthesizing ability of cyanobacteria, the carbon requirement is about 10-20 fold higher in case of heterotrophic bacteria.

Cyanobacteria, thus, do have potential for PHB production. Using cyanobacteria is one of the most promising eco-friendly ways as the ‘green-house gas’ is photosynthetically converted into biodegradable plastics by utilizing sunlight as the energy source. Moreover,

cyanobacteria also have the ability to synthesize the co-polymer under appropriate carbon supplemented medium (Fig. 7). Therefore, the challenge would be to improve the expression level of PHA production in cyanobacteria either by metabolic engineering or by applying mutational approaches/ genetic engineering techniques. Further, cyanobacteria can successfully be cultivated in wastewaters. Thus research efforts should be expended to screen various N- and P-rich wastewaters for development of an integrated system for production of cyanobacterial biomass as well as PHB with simultaneous treatment of wastewaters, which may help in reduction of the production cost, and in turn successful replacement of the non-gradable plastics.

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Table 1: Actual and potential industrial applications of PHAs (Vincenzini and De Philippis, 1999)

Application field	**Use**	**Properties useful for specific use**	**Current status**
Disposables	Razors, tray for foods, utensils, etc.	Biodegradability, good mechanical properties	Commercial (Japan)
Packaging	Bottles	Biodegradability, good mechanical properties, moisture resistance	Commercial (Europe, Japan and USA)
	Films for food packaging	Biodegradability, hydrophobic, low oxygen permeability	Research
Agriculture	Controlled release of pesticides, plant growth regulators, herbicides, fertilizers	Biodegradability, retarding properties	Research
	Covering foils	Biodegradability	Field Experiment
	Seed encapsulation	Biodegradability	Conceptual
Medical	Absorbable sutures, surgical pins and staples	Biocompatibilty, biodegradability	Research
	Bone plates, film around bone fracture	Biocompatibility, piezoelectric properties	Conceptual
Pharmaceutical	Retarded drug release, drug carrier	Biodegradability, biocompatibility	Research
Chiral Chromatography	Stationary phase for columns	Chiral properties	Conceptual
Hygiene products	Diapers, feminine hygiene products	Moisture resistance, good water barrier,	Conceptual

		biodegradability	
Chiral synthesis/ cosmetics	Sources of chiral precursors	Stereoregularity, chiral properties	Research
Miscellaneous	Autoseparative air filters,	Biodegradability	Research
	Fibre-reinforced, biodegradable goods	Biodegradability, good mechanical properties	Research

Table 2: Summary of the production of PHAs in transgenic plants

Sub-cellular compartment	Species	Tissue	PHA type	PHA % (dcw)	Reference
Cytoplasm	*A. thaliana*	shoot	PHB	0.1	27
	Cotton	fiber	PHB	0.3	37
	Tobacco	shoot	PHB	0.01	64
Plastid	*A. thaliana*	shoot	PHB	14 40 8	28 29 65
	Cotton	fiber	PHB	0.05	38
	Rapeseed	seed	PHB	8	31
	Corn	stalks and leaves	PHB	6	32
	Tobacco	leaf	PHB	1.7	35
	Beta vulgaris	hairy root	PHB	5.5	66
	Linum usitatissimum	stem	PHB	0.0004*	34
	Tobacco	shoot	PHB	0.14	36
	A. thaliana	shoot	P(HB-HV)	1.6	67
	Rapeseed	seed	P(HB-HV)	2.3	67
	A. thaliana	seedling	MCL-PHA	0.6	68-69

Peroxisome	*A. thaliana*	seed	MCL-PHA	0.1	70
	Maize	cell suspension	PHB	2.0	33

***% Fresh weight**

Table 3: Poly-*β*-hydroxybutyrate (PHB) accumulation in some cyanobacterial species

Sl. No.	**Cyanobacterial species**	**PHB (% dry wt.) ---------- Incubation (day)**						
		0	7	14	21	28	35	42
1	*Anabaena cylindrica*	1.23	1.68	2.83	1.93	1.51	1.23	1.01
2	*Anabaena doliolum*	1.39	2.51	3.38	3.52	2.01	1.59	1.22
3	*Anabaena variabilis*	1.30	2.30	2.90	3.10	2.70	2.53	2.41
4	*Aphanocapsa* sp.	1.35	2.01	3.33	3.02	2.01	1.5	1.22
5	*Aphanothece* sp.	nd	nd	nd	nd	nd	nd	nd
6	*Cylindrospermum indicum*	nd	nd	nd	nd	nd	nd	nd
7	*Fischerella muscicola*	0.92	1.54	2.01	2.34	2.44	2.12	2.01
8	*Microcystis aeruginosa*	1.58	2.30	3.55	4.01	3.22	2.55	1.06
9	*Microcystis* sp.	1.79	1.92	2.38	3.61	2.96	2.38	2.10
10	*Nodularia* sp.	nd	nd	nd	nd	nd	nd	nd
11	*Nostoc commune*	nd	nd	nd	nd	nd	nd	nd
12	*Nostoc linckia*	2.10	3.10	3.50	3.60	2.80	2.63	2.59
13	*Nostoc muscorum*	4.05	5.61	6.72	8.52	7.45	6.18	5.07
14	*Oscillatoria limosa*	1.69	2.33	2.99	2.03	1.88	1.67	1.56
15	*Plectonema* sp.	nd	nd	nd	nd	nd	nd	nd
16	*Pleurocapsa* sp.	2.33	2.61	2.89	1.86	1.11	0.89	0.62
17	*Rivularia* sp.	1.58	2.01	2.53	2.65	1.89	1.60	1.25
18	*Spirulina platensis*	2.01	2.06	2.10	2.11	2.13	2.19	2.21
19	*Synechocystis* sp. PCC 6803	1.79	2.10	2.28	4.25	3.02	2.77	2.74

20	*Tolypothrix* sp.	1.42	1.46	1.85	1.96	1.97	1.94	1.95
21	*Westiellopsis prolifica*	2.30	1.14	1.41	1.63	1.96	2.98	2.33

Values are means of three replicates.

nd: not detected

Table 4: Chemoheterotrophic accumulation of PHB in *Nostoc muscorum* and *Synechocystis* sp. PCC 6803

Treatments	**PHB (% dry wt.)**	
	Nostoc muscorum	***Synechocystis* sp. PCC 6803**
Control (L/D Cycle)	8.56 ± 0.53^{a}	4.2 ± 0.26^{a}
Dark	13.85 ± 0.88^{b}	6.1 ± 0.35^{b}
Dark + A (0.2%)	20.35 ± 1.32^{c}	13.3 ± 0.57^{c}
Dark + A (0.4%)	42.82 ± 1.98^{e}	22.4 ± 0.66^{d}
Dark + G (0.2%)	18.17 ± 0.78^{c}	7.6 ± 0.28^{b}
Dark + G (0.4%)	25.33 ± 0.59^{d}	9.2 ± 0.36^{e}

Cells were grown for 21days in L/D cycles followed by dark incubation of 5 days.

All the values are mean ± SE, A: acetate, G: glucose

Values in the column superscripted by different letters are significantly ($P<0.05$) different from each other (Duncan's new multiple range test).

Separate analysis was done for each column.

Table 5: Accumulation of PHB in *Nostoc muscorum* and *Synechocystis* sp. PCC 6803 under gas-exchange limited condition

Treatment	**PHB (% dry wt.)**	
	***Synechocystis* sp. PCC 6803**	***Nostoc muscorum***
Control	2.95 ± 0.53^{a}	6.86 ± 0.51^{a}
Gas-Exchange limitation (GEL)	7.91 ± 0.61^{b}	9.86 ± 0.64^{b}
P-deficiency + GEL	14.5 ± 0.73^{c}	9.94 ± 0.83^{b}
GEL + A (0.4%)	22.19 ± 0.97^{d}	25.28 ± 1.36^{c}
P-deficiency + GEL + A (0.4%)	35.37 ± 1.21^{e}	25.32 ± 1.23^{c}
GEL + A (0.4%) + F (0.4%)	21.67 ± 0.89^{d}	NS
P-deficiency + GEL + A (0.4%) + F (0.4%)	38.54 ± 1.31^{f}	NS
GEL + G (0.2%) + A (0.2%)	NS	39.76 ± 1.45^{d}
P-deficiency + GEL + G (0.2%) + A (0.2%)	NS	40.04 ± 1.42^{d}

All values are mean ± SE, A: acetate, G: glucose, F: fructose, NS: not studied.

Values in the column superscripted by different letters are significantly ($P<0.05$) different from each other (Duncan's new multiple range test).

Separate analysis was done for each column.

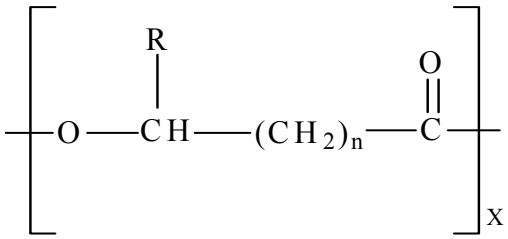


Fig. 1: **General structure of polyhydroxyalkanoates**

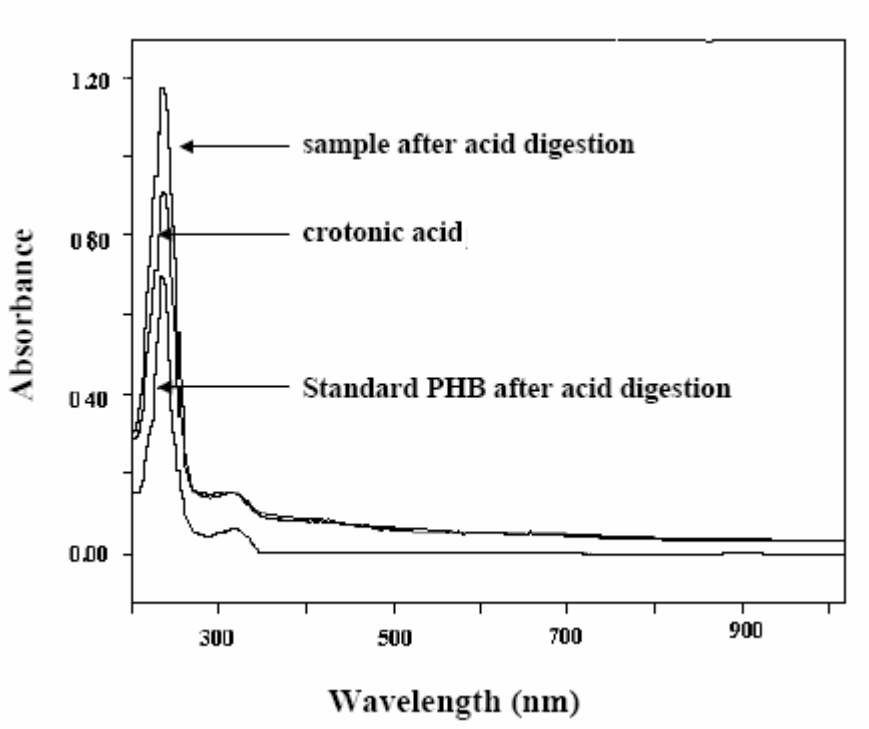


Fig. 2: **Comparison of absorption spectrum of crotonic acid with acid-digested polymer of *N. muscorum* sample and the standard poly(3-hydroxybutyric acid).**

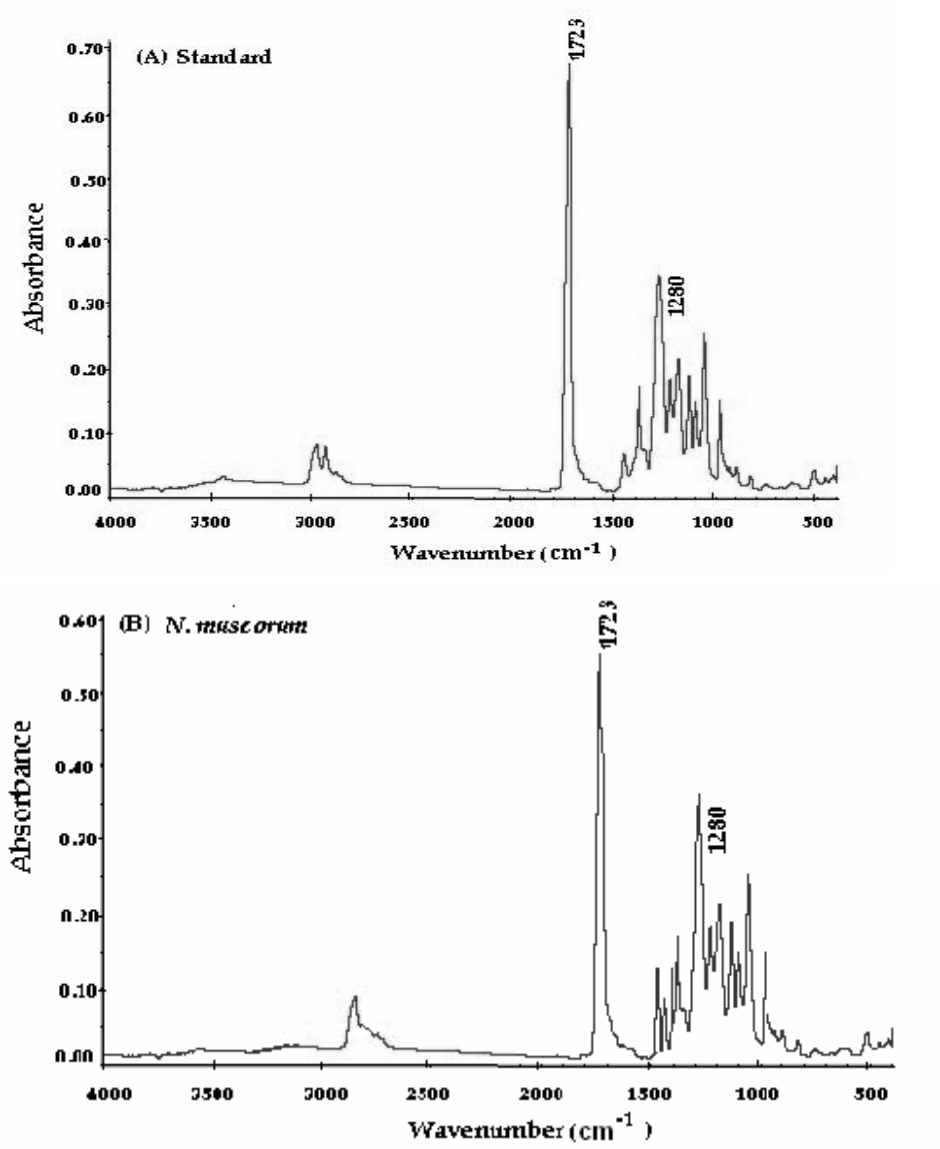
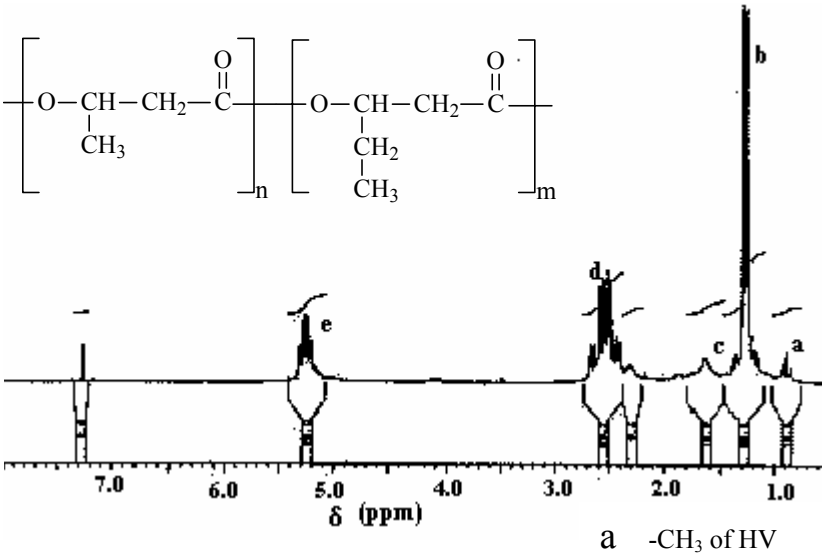


Fig. 3: FT-IR spectra of A) standard poly(3-hydroxybutyric acid) and B) isolated polymer from *N. muscorum.*

(A) *Standard P (3HB-co-3HV)*



a -CH_3 of HV
b -CH_3 of HB
c -CH_2 of HV
d -CH_2 of HB & HV
e -CH of HB & HV

(B) ***Extracted polymer from N. muscorum***

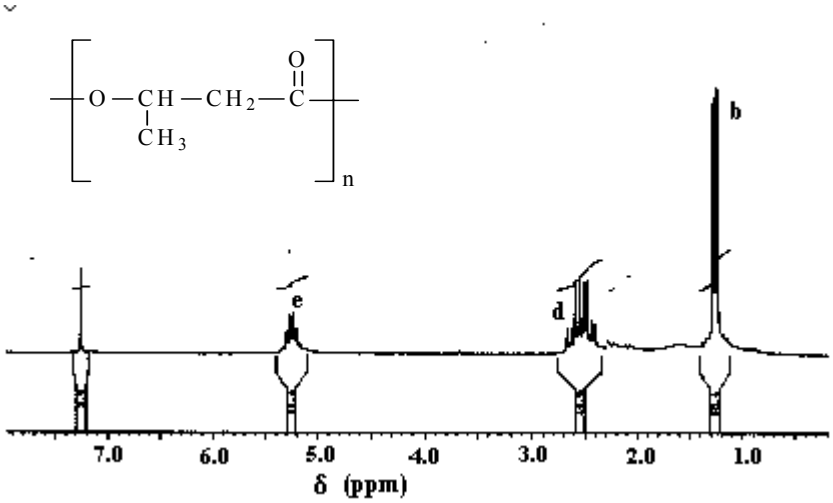


Fig. 4: ^{1}H-NMR spectra of A) standard poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) and B) *N. muscorum* sample.

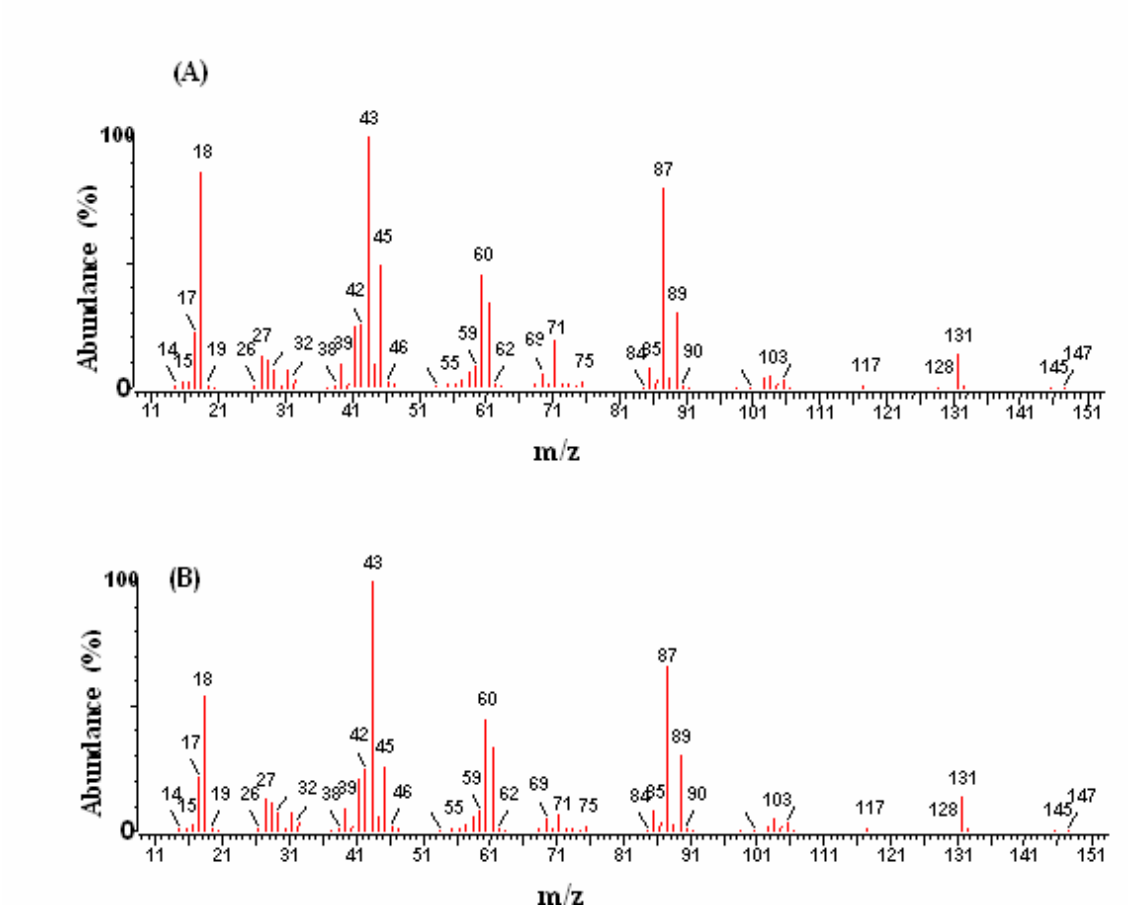


Fig. 5: Mass spectra of A) propyl ester of standard poly(3-hydroxybutyric acid) and B) polymer extracted from *N. muscorum.*

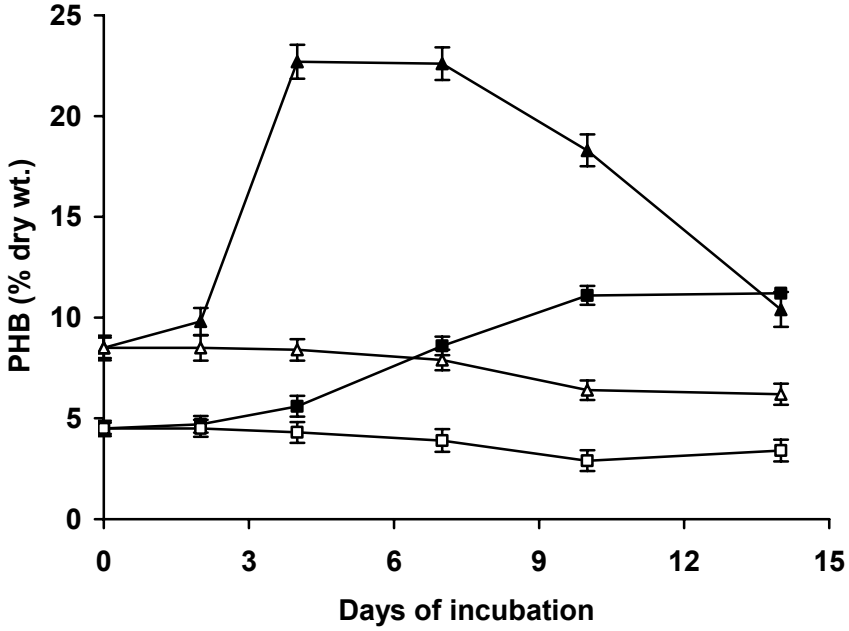
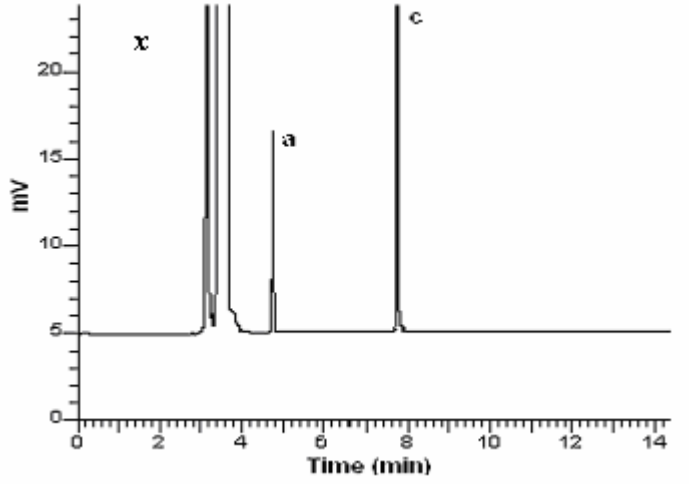


Fig. 6: Stimulaton of PHB accumulation in *N. muscorum* and *Synechocystis* sp. PCC 6803 under P deficiency. *N. muscorum*: control (∆), P deficiency (▲), *Synechocystis*: control (□), P deficiency (■).



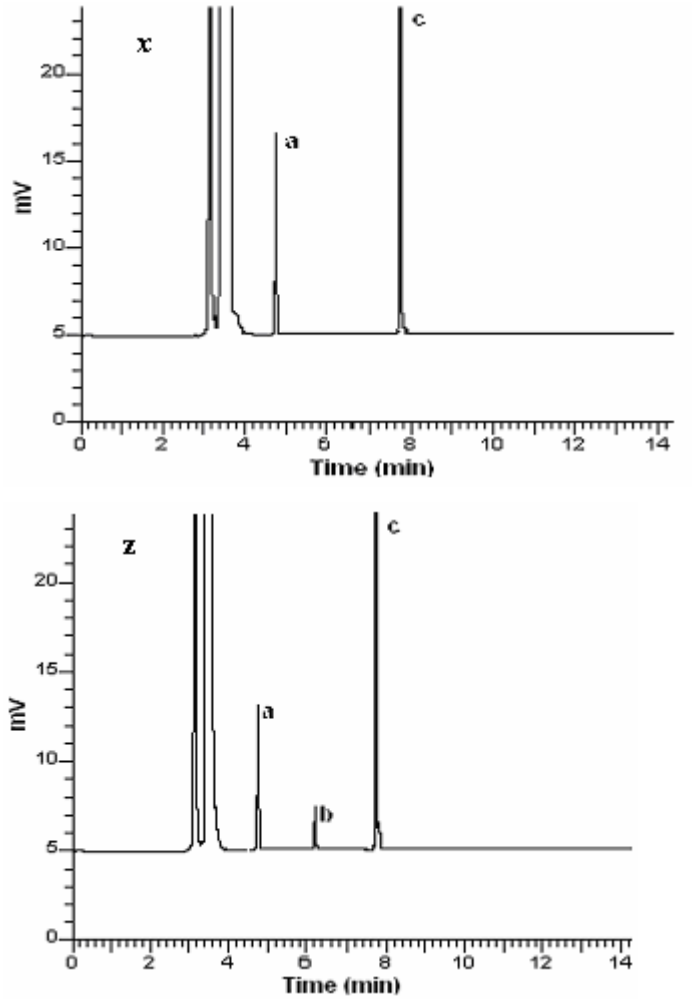


Fig. 7: Gas chromatograms of (x) standard PHB, (y) standard P(3HB-*co*-3HV), and (z) *Nostoc muscorum* sample. [a: 3-hydroxybutyric acid propylester, b: 3-hydroxyvaleric acid propylester, and c: benzoic acid propylester (internal standard)].

Chapter-3

MICROBIAL INTERACTIONS WITH HEAVY METALS AND THEIR APPLICATIONS IN BIOREMEDIATION OF WASTEWATER

S. Das and S. C. Santra

ABSTRACT

Bioremediation of metal containing wastewater is though a very new concept, still it is really advancing field of research. Different types of interactions like biotransformation, biosorption and binding of heavy metals with extracellular or intracellular organelles are effectively used to recover metals from wastewater. It is clear that the interactions between microbial cells and heavy metals or nuclides is extensive and varies in different species. Bioremediational processes for metal removal or recovery from liquid effluent are based on these interactions, specially biosorption and biotransformation. Many reports are also available revealing direct application oriented systems.

3.1 Introduction

With the advancement of human civilisation, there is increasing pressure or burden of over-exploitation of natural resources along with different degree of pollution. Pollution is the anthropogenic alteration of the natural sources in such a way that it causes ecological imbalance, harmful effects on living organisms as well as human being himself. The types of pollutants may be in all the three different forms of matter; solid, liquid or gaseous; and their sources are mainly domestic, agricultural and industrial. But industrialisation has the greatest effect on environment.

Since the industrialisation and human civilisation are intertwined and complementary to each other, it is not possible to go without industrialisation at this moment. In the contrary, more and more industries are being made and would be made. So, we have to think and act in a different way to achieve our goal without doing any harm to nature, at least as much as possible. The best possible solution is to treat industrial effluents or wastes to remove the pollutants to a safer degree prior to disposal or preferably, recycling.

The type and degree of treatment for removal of pollutants depend on the nature of pollutants, type of wastes, operational area, expenditure involved and surrounding ecosystem and most importantly on interests of the industry concerned. The different types of treatments include physical, chemical and biological processes, individually or in combination. The conventional method is physico-chemical method. However, accomplishment and utilisation of complete physico-chemical waste treatment plant is very

much cost-intensive and often itself is a source of pollution. While biological treatment is cost-effective as well as environment-friendly. That is why the waste-reclamation technologies have emphasised more on biological remediation of waste, solid or fluid. Biological systems apply some scientifically selected or introduced strains of micro-organisms, viz. bacteria including cyanobacteria, microalgae, microfungi etc. Wastes could be treated at the site of generation of waste i.e. *in situ* or at a site different from that of generation i.e. *ex situ.* Micro-organisms could be used for mitigation of different types of pollutants.

However, biological treatment is not a new concept in reclamation technology. Micro-organisms are in use for removal of organic matter and toxic chemicals from domestic and industrial sources of pollution. But only recently there has been a trend to categorically select certain individual strains or concerted communities for better treatment of waste.

3.2 Heavy metals in the environment

Heavy metals comprise a broad group of metals of atomic weight higher than that of sodium and having a specific gravity of more than 5.0. These metals are in the eye of environmental concern because when present in high amounts these elements are highly toxic and harmful for living organisms.

Heavy metal pollution is a wide spread problem in all parts of the world. Specially in the last few decades it has become more of a menace as more and more industries are coming up. The release of heavy metals from industries into the environment has resulted in severe problems for both human health and aquatic ecosystems. Toxic heavy metal contamination of industrial water is a significant and universal problem. Heavy metals accumulate in living tissues throughout the food chain which has humans at its top. These toxic metals can cause accumulative poisoning, cancer and brain damage when found above the tolerance levels. These metals, in fact, are ubiquitous in natural environment and always present in a certain trace concentration (Lindsay, 1979; Leppard, 1981). But when this concentration of metals rises due to anthropogenic causes, it may disrupt normal biogeochemical cycles and lead to environmental problems. Metal polluting industries include electroplating, chrome-tanning, textiles, photographic sensitizer manufacturing, nuclear processing and industries of batteries, paints, dyes, preservatives, insecticides etc. (Gadd and Griffith, 1978; Gadd, 1986; Hughes and Poole, 1989). But the most important is mining and other metallurgical activities (Down and Stocks, 1977). Heavy metals such as zinc, lead and chromium have a number of applications in basic engineering works, paper and pulp industries, leather tanning, organo-chemicals, petrochemicals, fertilisers etc. Major lead pollution comes from automobiles and battery manufacturers. For zinc and chromium the major sources are fertiliser industry and leather tanning respectively (Trivedi, 1989). These metals coming from different sources may exist in free metallic or compound form, either in solid or liquid form.

Elevated metal concentrations in an environment have severe impacts on all types of organisms including human. Different types of morphological and physiological changes were reported to occur in plants growing in metal contaminated soil (Brooks, 1983; Guruprasada Rao et al, 1989). These metals have severe effects like chlorophyll loss,

nutrient deficiency, root shortening etc. in plants (Canney, 1979; Carlisle, 1986). Metal translocation from plants to subsequent trophic levels of ecosystem affects all animals (Khasim, 1989).

Microbial growth is inhibited partially or completely by presence of excessive amounts of toxic metals and this may ultimately lead to imbalance of the total ecosystem (Duxbury, 1981; Baath, 1989). Physiological functions of microorganisms like those of different metal-dependent enzymes, ETS, nitrogen fixation etc. are often hampered by metal toxicity.

Such toxic heavy metals have been associated with different disorders in human including brain and bone structure damage, nervous system disorder etc. High levels of lead in drinking water may lead to Alzheimer's disease. Nriagu (1988) has suggested that over one billion people are currently exposed to elevated concentrations of toxic metals and metalloids in environment and several millions may be suffering from sub-clinical level of metal poisoning.

Fishes and other water inhabiting animals face several health irregularities due to presence of toxic metals and nuclides in water. Anaemia and blood deformities are reported in fishes grown in metal-contaminated water streams. From different sources like canals and rivers heavy-metals often reach coastal and estuarine waters and often found to be a serious threat to the ecologically very significant estuarine biota.

Several organisms, however, regularly being exposed to higher levels of metals have developed some resistance mechanisms. Micro-organisms are specially important in this aspect and their tolerance strategies include intracellular accumulation, precipitation, biotransformation etc. (Hughes and Poole, 1989; Francis, 1990).

Conventional methods for removing heavy metals from industrial effluents (*e.g.* precipitation and sludge separation, chemical oxido-reduction, ion exchange, reverse osmosis, electrochemical treatment and evaporation) are often ineffective and comparatively costly when applied to dilute and very dilute effluents (Aksu, 1998). Recently, biological removal processes has been attracting enormous attention for removing heavy metals from aqueous wastes and screening for microorganisms having higher potential for removing heavy metals from wastewater has been promising so far (Volesky, B. and Holan, 1995; Kratochvil et al, 1997, 1998; Pagnanelli, 2001; Singh, 2001; Padmavathy, et al, 2003). Microbial removal of heavy metals offers the advantages of low operating cost, minimizing secondary problems with metal-bearing sludge and high efficiency in detoxifying very dilute effluents.

3.3 Microbial interaction with heavy-metals

Though the biological treatment had been used for several decades for removal of specially organic compounds, only recently it has got attention for treatment of metal contaminated sites or wastewater (Summers, 1992). The first reports described how profuse biological resources could be used to remove, at a very low cost, even minute amounts of toxic heavy metals from industrial effluents. Metal-sequestering properties of non-viable biomass provide a basis for a new approach to remove heavy metals when they occur at low concentrations (Volesky, 1990). Metal uptake by microorganisms can occur actively (bioaccumulation) and/or passively (biosorption) (Ehrlich, 1997; Nies, 1999).

Feasibility studies for large-scale applications established that the biosorptive processes are more applicable than the bio-accumulative processes because living systems often need the supply of nutrients and hence raise biological oxygen demand (BOD) or chemical oxygen demand (COD) in the effluent, maintenance of a healthy microbial population is difficult due to metal toxicity and other unsuitable environmental factors and mathematical modelling of an undefined system is difficult (Sternberg and Dorn, 2002; Chandrasekhar et al, 2003).

Application of micro-organisms in bioremediation of of metals require vast knowledge of microbial interactions with metals. In spite of metal toxicity some microbes have developed very high degree of tolerance to certain metal or may be to some of these metals. These microbes have been shown to react with metals in different ways - intracellular or extracellular accumulation, biotransformation etc. Numerous chemical groups have been suggested to contribute to biosorption metal binding by either whole organisms such as algae and bacteria (Crist et al, 1981; Brierley, 1990). These groups comprise hydroxyl, carbonyl, carboxyl, sulfhydryl, thioether, sulfonate, amine, imine, amide, imidazole, phosphonate, and phosphodiester groups. The importance of any specified group for biosorption of a certain metal by a certain biomass depends on factors such as: the number of sites in the biosorbent material, the accessibility of the sites, the chemical state of the site (i.e. availability), and affinity between site and metal (i.e. binding strength). For covalent metal binding even an already occupied site is theoretically available. The level to which the site can be used by a given metal depends on its binding power and concentration as compared to the metal already occupying the site. For electrostatic metal binding, a site is only available if the metal is ionized.

3.4 Intracellular accumulation

Transport of the metal across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism. This means that this kind of biosorption may take place only with viable cells. It is often associated with an active defense system of the microorganism, which reacts in the presence of toxic metal.

Accumulation of metals inside the cell may be significant for the microbe for metabolic requirement, incorporation into enzymes or only for tolerance. Some essential metals such as iron and magnesium are actively taken up by micro-organisms from surrounding environment.

In some of the cases, the metal first binds with some surface or extracellular ligands. Then these ligands slowly transfer the metals inside cell through the cell surface. Once inside, these metals may become incorporated in enzymes, take part in biochemical pathways or just trapped in a inactive form by binding with another intracellular ligand (Wood and Wang, 1985; Sigg, 1987). Metal transport inside cells may occur either by passive diffusion along concentration or electrochemical gradient, facilitated diffusion along gradient but via a protein carrier molecule or by active transport via carrier with expenditure of metabolic energy (Brierley et al, 1989). Basically biosorption by living organisms comprises of two steps. First, a metabolism independent binding where the metals are bound to the cell walls and second, metabolism dependent intracellular uptake,

whereby metal ions are transported across the cell membrane (Gadd et al, 1988; Huang et al, 1990).

In general heavy metals are taken in by blue green algal cells by adsorption followed in sequence by metabolism-dependent intracellular cation intake as applicable to Zn, Cu, Cd or Al (Les and Walke, 1984; Singh and Yadava, 1985; Pettersson et al, 1986; Inthorna et al, 2002).

In some cases, there may be two or more transport systems operating for uptake of certain metals. As in case of technitium uptake by chlorophycean alga *Acetabularia acetabulum*, there are two different uptake systems viz. a reversible but high affinity system and a irreversible high affinity system with greater holding capacity (Bonotto et al, 2002).

Active transport systems generally uptake only specified metals, but in some cases exceptions were found. Cadmium can be transported via zinc transport system in *Escherichia coli* and manganese transport system in *Bacillus subtilis* . Nickel and cobalt are transported by the magnesium transport system in *E. coli* (Hughes, M.W. and Poole, 1989).

Citrobacter sp. produces a phosphatase enzyme which by cleaving hydrogen phosphate from organic phosphates, helps in accumulation of heavy metals like lead, cadmium and radionuclides like *uranium* as metal phosphates (Macaskie, 1990; Macaskie, et al, 1992). Bonthrone et al.(1996) reported bioaccumulation of nickel by intercalation into polycrystalline hydrogen uranyl phosphate deposited via an enzymatic mechanism. Strandberg et al. (1981) reported of *Saccharomyces cerevisae* and *Pseudomonas aeruginosa*, which could act as effective biosorbent for uranium. Apart from those, several bacteria, fungi and algae are reported to accumulate heavy metals (Gadd, 1990; Marques et al, 1990).

The biosorption kinetics of Ni(II) and Pb(II) by the resting cells of a lignolytic white-rot fungus, *Phanerochaete chrysosporium*, from a binary metal system showed slow intracellular diffusion after a rapid surface adsorption for the first 30 minutes.

3.5 Cell surface binding or adsorption of metals

Cell surface binding or rather sorption of heavy metals and of radionuclides by microbial cells is essentially a passive process and it does not require metabolic energy. Often a huge amount of metal is accumulated on cell surface and this phenomenon could easily be utilised for wastewater treatment. As the process is very passive, living cells as well as dead cells are capable of absorbing metals. The mechanism of biosorption is complex, mainly ion exchange, chelation, adsorption by physical forces, entrapment in inter and intrafibrilliar capillaries. This type of biosorption, i.e., non-metabolism dependent is relatively quick and can be reversible (Kuyucak and Volesky, 1988). There are several chemical groups that would draw and requisition the metals in biomass: acetamido groups of chitin, structural polysaccharides of fungi, amino and phosphate groups in nucleic acids, amido, amino, sulphhydryl and carboxyl groups in proteins, hydroxyls in polysaccharide and mainly carboxyls and sulphates in polysaccharides of marine algae. Different functional groups like amino, hydroxo, thio, phospho etc. groups on the surface of

phytoplankton can interact with metal ions (Xue et al, 1988). Physical adsorption takes place with the help of van der Waals' forces. Kuyucak and Volesky (1988) hypothesized that uranium, cadmium, zinc, copper and cobalt biosorption by dead biomasses of algae, fungi and yeasts takes place through electrostatic interactions between the metal ions in solutions and cell walls of microbial cells. Electrostatic interactions have been demonstrated to be responsible for copper biosorption by bacterium *Zoogloea ramigera* and alga *Chiarella vulgaris* (Aksu, 1992), for chromium biosorption by fungi *Ganoderma lucidum* and *Aspergillus niger*. Microorganisms may also generate organic acids (e.g., citric, oxalic, gluonic, fumaric, lactic and malic acids), which may chelate toxic metals resulting in the formation of metallo-organic molecules. These organic acids facilitate in the solubilisation of metal compounds and their leaching from their surfaces.

Unicellular yeast *Saccharomyses cerevisae* adsorb uranium on cell wall as needle-like fibrils in a 0.2μm thick layer (Standberg et al, 1981). Some chemical compounds of yeast cells can also act as ion exchangers with rapid reversible binding of cations. Volesky et al. working on cadmium biosorption by *Saccharomyces cerevisiae* demonstrated that this yeast is a potent biosorbent material for cadmium (Volesky et al, 1993).

According to Beveridge (Beveridge, 1986), bacteria make brilliant biosorbents because of their high surface-to-volume ratios and a high content of potentially active chemosorption sites such as on teichoic acid in their cell walls. The cellwalls of gram-positive bacteria are efficient metal chelators. The carboxylic group of glutamic acid and teichoic and teichuronic acids of peptidoglycan are metal binding sites (Gadd, 1990).

Filamentous bacteria *Thiothrix* sp. can adsorb metals in a very high rate on cell surface (Suttleworth and Unz, 1993). McEldowney et al. gave an elaborate account of biosorbtion of uranium by different bacteria (McEldowney, 1994). In India, several good works on this aspect have been done on bacteria (Asthana et al, 1995; Chatterjee et al, 1996; Ahuja et al, 1997; Ray et al, 2005).

In all the cases the cell-surface biosorbtion can be optimised by pH change, temperature, Biomass concentration, pre-treatment and presence of other heavy metals. pH seems to be the most significant parameter in the biosorptive process: it affects the solution chemistry of the metals, the action of the functional groups in the biomass and the competition of metallic ions (Galun, 1987). The presence of Fe^{2+} and Zn^{2+} was found to manipulate uranium uptake by *Rhizopus arrhizus* (Tsezos, M. and Volesky, 1981) and cobalt uptake by different microorganisms seemed to be entirely inhibited by the presence of uranium, lead, mercury and copper (Sakaguchi and Nakajima, 1991). Metal affinity to the biomass can be manipulated by pretreating the biomass with alkalies, acids, detergents and heat, which may amplify the amount of the metal sorbed (Galun, 1987).

3.6 Siderophore-metal binding

Siderophores are extracellular, low molecular weight metal chelating agents. These are often produced by bacteria in iron-limiting conditions to facilitate iron uptake. These are either catechol or hydroxamate derivatives and contain certain reactive groups like

dicarbxylic acids, polyhydroxy acids and phenolic compounds. In addition to iron, siderophores often bind to metal ions like aluminium, gallium, chromium, nickel etc. (Hausinger, 1987; Macaskie and Dean, 1990).

Specific extracellular metal-binding compounds can also be produced by micro-organisms in response to low levels of metals, in order to facilitate the uptake of essential metals. The most studied system is the production of siderophores in response to low environmental iron concentrations. Siderophores are low-molecular-mass Fe(III) coordination compounds (500±1000 Da) produced by many micro-organisms and act by complexing and solubilizing insoluble Fe(III) in a form which can be transported into the cell using specific transport mechanisms (Neilands, 1990). Although siderophores are iron(III)-binding compounds, they are also able to bind other metals such as magnesium, manganese, chromium(III), gallium(III) and radionuclides such as plutonium(IV) (Bulman, 1978; Birch and Bachofen, 1990).

The efficiencies of siderophores can be increased by chemical modification like substituting Cl^-, NO_2^- on benzene ring. Modified siderophores can be used for removal of metals like cadmium, mercury, copper and even radionuclides like strontium or cesium from mixed effluent (McEldowney et al, 1994; Sutherland, 1985).

3.7 Extra cellular biopolymer accumulation metals

A variety of extra cellular polysaccharidic polymer or metallothionein like proteins are able to bind to metal ions. The purpose is mainly to reduce metal toxicity. Metals are reported to bind with these biopolymers by following methods - firstly, metals bind to the electronegative functional groups present in biopolymer. These groups include pyruvate, suucinyl, hydroxyl, phosphates groups; secondly, some functional groups like thiol group may form co-ordination complexes with metal ions (Sutherland, 1985; Ford and Mitchell, 1990). Expression of metallothioneins (Pazirandeh, 1996; Romeyer et al, 1988; Romeyer, et al, 1990) or metallopeptides (Sousa, 1996; Chen and Wilson, 1997) to increase the affinity and biosorptive capabilities of bacterial cells for heavy metals is a promising technology for the development of bacterium-based biosorbents. Both naturally occurring metal-binding peptides such as metallothioneins (MTs) and synthetic peptides such as synthetic phytochelatins (ECs) have been expressed on the surface of bacterial cells for better uptake and biosorption of mercury (Sousa et al, 1998; Bae et al, 2000, 2001). Various bacteria develop resistance to heavy metals by inducing the expression of an array of resistance proteins (Nies, 1999).

Some microbially produced macromolecules can bind considerable amounts of potentially toxic metals. These include humic and fulvic acids arising from lignocellulose degradation and extracellular polymeric substances (EPS), a mixture of polysaccharides, mucopolysaccharides and proteins produced by bacteria, algae and fungi (Schreiber et al, 1990; Beech and Cheung, 1995).

Some microorganisms are known to produce extracellular, specific metal-binding compounds. Examples include *Sarcina urea*, which has been shown to produce metal-binding proteins (Beveridge, 1989), a *Pseudomonas* sp. which has been shown to produce cadmium-binding proteins in response to cadmium (Rayner and Sadler, 1989) and *Vibrio*

alginolyticus, which produces copper-binding proteins in response to high levels of copper (Harwood-Sears and Gordon, 1990).

Many bacteria produce polysaccharidic exopolymers which are either loosely associated with cell wall or present in a gelatinous matrix surrounding the cell. These acidic polymers are polar, hydrophilic and bind metals very effectively. Exopolymer of *Zooglea* is one such instance, used for metal removal by activated sludge method. Mittelman and Geesey (1985) reported high copper binding efficiency of exopolymer of a fresh water sediment bacterium. From fresh water system different bacteria were reported to immobilise metal ions by exopolymers in association with clay (Walker et al, 1989; Flemming et al, 1990). Cyanobacteria often produce polysacchiridic biofloculants when challenged with metals. Experiments by Bender *et al.* showed that microbial mats that are dominated by an *Oscillatoria* sp. are effective in removing lead, cadmium, zinc, cobalt, chromium etc. from water (Bender et al, 1994). Cysteine rich polypeptides i.e. metallothioneins bind essential and nonessential metals which are either stored as intracellular granules or transported out of the cell (Gadd, 1990). An extracellular rhamnolipid biosurfactant has been shown to complex metals like cadmium, lead and zinc (Miller, 1995). *Desulfococcus multivorans* was shown to have the excellent extracellular copper-binding capacity. It binds copper reversibly and zinc irreversibly (Bridge, 1999).

3.8 Ion exnhange mechanism

Cell walls of microorganisms contain polysaccharides and bivalent metal ions exchange with the counter ions of the polysaccharides. Ions present in cytosol like K+, Na+, Ca^2+, and Mg^2+ can exchange with counter ions such as Co^2+, Cu^2+, Cd^2+ and Zn^2+ resulting in the biosorptive uptake of heavy metals (Kuyucak and Volesky, 1988). The biosorption of copper by fungi *Ganoderma lucidium* and *Aspergillus niger* were also up taken by ion exchange mechanism (Muraleedharan and Venkobachar, 1990).

3.9 Oxidation - reduction reactions

Ferrous and manganese ions can be deposited by oxidation reactions of bacteria such as *Leptothrix*, *Sphaerotilus* and *Thiobacillus* (Hughes and Poole, 1989; Nealson et al, 1989; Ehrlich and Brierley, 1990). These reactions often solubilise certain metals, apparently increasing their biotoxicity. But however, it is only then possible for the bacteria to remove that metal from the surrounding environment. This process can be well utilised in recovery of metals like copper, cadmium, gold etc.

Micro-organisms can actively reduce toxicity or can even decrease bioavaillbility by reduction of metals. Such metals include mercury, iron, chromium, arsenic, selenium etc. Several bacteria like *Pseudomonas putida, Escherichia coli* are known to reduce hexavalent chromium to the non-toxic trivalent form (Ishabishi et al, 1990; Shen and Wang, 1993). Yeasts and fungi are recently reported to reduce chromium VI too (Sulzbacher et al, 1997; Ul Haq and Shakoori, 1998). Present authors have isolated several chromium tolerant fungi from tannery effluent of eastern Calcutta, capable of reducing

chromium VI very effectively (Das and Santra, 1999). Mercury and arsenic reduction by microbes are also well documented (Challenger, 1945; Schottel, 1978; Olson et al, 1982; Guangyong et al, 1989).

3.10 Alkylation reactions

Alkylation is another effective detixification mechanism often adapted by micro-organisms. Tin, lead, selenium etc. can be alkylated by incorporation of methyl groups which actually increases toxicity. But methylation facilitates diffusion of that metal away from the surroundings of the microbes (Hughes and Poole, 1989; Gilmour et al, 1987; Frankenberger and Krlson, 1995). Methylation of mercury leading to volatilisation can be a significant mechanism of removal of mercury from contaminated pond (Barkey et al, 1992; Saouter et al, 1994). However, it bears serious health implications as methylation, in all cases, leads to high toxicity.

3.11 Practical applications of microbial biotechnology

It is clear that the interactions between microbial cells and heavy metals or nuclides is extensive. Bioremediational processes for metal removal or recovery from liquid effluent are based on these interactions, specially biosorption and biotransformation. Though in most of the cases field based trials for verification of effectiveness of these processes under practical circumstances have not been done due to infrastructural and financial constraints. Still many reports are available revealing more direct application oriented systems. These systems may incorporate both living and nonliving micro-organisms.

The use of adsorbents of biological origin has emerged in the last two decade as one of the most promising alternatives to conventional heavy metal remediation strategies (Macaskie, 1990; Shumate and Strandberg, 1985; Fourest and Roux, 1992; Hussein et al, 2003). In many path-breaking studies, metal removal abilities of various species of bacteria, algae, fungi and yeasts were investigated (Guibal et al, 1992; Veglio and Beolchini, 1997; Yetis et al, 2000). Biosorption consists of several mechanisms as discussed above, mainly ion exchange, chelation, adsorption, and diffusion through cell walls and membranes, which differ depending on the species used, the source and processing of the biomass and solution chemistry (Kuyucak and Volesky, 1988; Churchill et al, 1995).

The exact mechanism by which micro-organisms take up metals may vary, but it has been demonstrated that both living and non-living fungal biomass may be utilised in biosorptive processes, as they often exhibit distinct tolerance towards metals and other adverse conditions such as low pH (Gadd, 1990; Standberg et al, 1981; Volesky et al, 1993). There is many environmental factors affecting the biosorption process, such as temperature, pH, agitation rate and metal concentration (Gadd, 1990; Veglio and Beolchini, 1997). Some of these factors (e.g. pH and metal concentration) have greater influence on metal removal by this process (Volesky and Holan, 1995; Veglio and Beolchini, 1997; Yetis et al, 2000).

In living systems, biocatalysts like bacteria, algae or fungi; specially selected, enriched or genetically engineered are applied in a controlled artificial ecosystem in scientifically designed meander channels or impoundments. This process is needed to be done at the site of production of such effluent *i.e. in situ.* Biological agents may be individual strains or a complex microbial community. Biomass can come from (i) industrial spent biomass; (ii) organisms easily available in large amounts in nature; and (iii) organisms of quick growth, especially and easily cultivated or propagated for biosorption purposes. Cost effectiveness is the main attraction of metal biosorption, and it should be kept in mind for biomass election. Most studies of biosorption for metal removal have involved the use of either laboratory-grown micro-organisms or biomass generated by the pharmaceutical and food processing industries or wastewater treatment units (Macaskie, 1990; Tsezos and Volesky, 1981; Rome and Gadd, 1987; Costa and Leite, 1991). If, for any reason, by-products of industrial fermentation processes would not be available, biosorbents could be produced by using relatively unsophisticated and low-cost culture propagation techniques. Nutrients from readily available and economical sources such as carbohydrate-rich industrial wastewaters, which often pose pollution/treatment problems, such as food, dairy and starch industries, might be conveniently used. On the contrary, the costs of biosorbents especially produced could be higher and affect negatively the overall economy of their application (Volesky, 1987).

Some chemical compounds of yeast cells can act as ion exchangers with rapid reversible binding of cations. Volesky et al. (1993) working on cadmium biosorption by *Saccharomyces cerevisiae* established that this yeast is a reasonably effective biosorbent for cadmium (Volesky et al, 1993). Some mucoralean fungi have shown fascinating metal biosorbent properties, particularly high for uranium and thorium whereby different metal deposition patterns could be clearly distinguished (Volesky, 1987).

Although microalgae are not unique in their bioremoval capabilities, selected microalgae strains, purposefully cultivated and processed for specific bioremoval applications, have the potential to provide significant improvements (Inthorna et al, 2002; Wilde and Benemann, 1993). The marine flagellate alga *Pavlova viridis* grown in an artificial seawater medium can uptake metals in the order Ni > Pb > Co > Hg > Cu > Cd > Ag at equilibrium (Chen et al, 1998). There is much evidence that algae could accumulate heavy metals in their tissues when grown in polluted waters, including the species *Ulva rigida* (Fe, Mn), *Padina gymnospora* (Zn), *Gracilaria tenuistipitata* (Cd), *Undaria pinnatifida* (Pb), *Cladophora* sp. (Cd), and *Cladophora glomerata* (Zn) (Lamaia et al, 2005). Bender *et al.* showed that microbial mats dominated by an *Oscillatoria* sp. are effective in removing heavymetals from wastewater (Bender et al, 1994).

The most relevant work on true bacterial biosorption has been done by the Brierley, who took the metal biosorption concept all the way to the commercial stage (Brierley et al, 1989). Bacterial cell walls are negatively charged under acidic pH conditions and the functional groups of cell wall display a high affinity for metal ions in solution (Collins and Stotzky, 1992). Churchill et al. used two Gram-negative strains *Escherichia coli* K-12 and *Pseudomonas aeruginosa* and a Gram-positive strain *Micrococcus luteus* to demonstrate biosorption of Cu2+, Cr3+, Co2+ and Ni2+ ions (Churchill et al, 1995). Their sorption binding constants suggested that *E. coli* cells were the most competent at binding copper, chromium and nickel and *M. luteus* sorbed cobalt most efficiently. Hu *et al.* worked with

Pseudomonas aeruginosa strain CSU, a genetically not altered bacterial strain known to bind dissolved hexavalent uranium (Hu et al, 1996).

In nonliving systems, only the biosorption capabilities of different micro-organisms have been used in specially designed bioreactors. The application of nonliving biomass for removal of metals by biosorption has several advantages. No nutrients or proper maintenance are required. Toxicity often cause loss of accumulation property in living cells, but in case of nonliving systems there is no scope of toxicity and hence no loss in biosorption rate. So we can use the same biomass for treatment purpose time and again. Hence , many works have been done to perfect the bioreactor system and to optimise efficiency.

The complete design of the system varies with the process application, sorption agent and capital expenditure. The main target is to achieve maximum surface contact between metal and biosorbent material. System may operate in batch, semi-continuous or continuous flow in stirred tank or column reactor (Summers, 1992; Brierley et al, 1989). Natural biomass has several disadvantages for use. So natural biomass of the selected strain is often modified in such a way so as to give it more mechanical strength, more hydrophilicity and uniformity in size and form. These modifications enable the biosorbent to be reused time and again. Immobilisation of biosorbent in an insoluble natural or synthetic polymer, is an effective solution. The materials used are polyurethane foam, silica gel, calcium alginate, polyacrylamide, polysulfone, polyethylenimine, formaldehyde and divinylsulfone (Sakaguchi, 1991; Costa and Leite, 1991; Holan et al, 1993; Peng and Koon, 1993; Macaskie et al, 1987; Jeffers, 1991; Brierley et al, 1993). Several immobilised biosorbents are available in commercial scale, eg. AMT-BIOCLAIM^{TM}, AlgaSORB^{TM}, BIO-FIX^{TM} etc. (Brierley, 1990).

AMT-BIOCLAIM^{TM} i.e. encapsulated *Bacillus* can simultaneously remove several heavy metals like U, Pb, Cd, Ni, Hg, Cr, Zn and Cu from effluent regardless of the initial low or high concentration of metals. An *Aspergillus oryzae* biosorbent is able to remove 90 % cadmium from wastewater in only five minutes time in column reactor (Kiff and Little, 1996). Algal biosorbent like that of *Chlorella vulgaris* is also in use (Beedell and Darnell, 1990).

Heavy metal recovery from biosorbents is of major importance in the assessment of competitiveness of biosorption processes. Understanding of the mechanisms of metal biosorption now allows the process to be scaled up and used in field applications, with packed-bed sorption columns being perhaps the most efficient for this purpose. Regenerating the biosorbents increases the process economy by allowing their reuse in multiple sorption cycles (Kratochvil and Volesky, 1998). Recovery allows metal recycling, leading to energy savings and materials conservation. Finally, biosorbent regeneration for use in multiple adsorption–desorption cycles, contributes to process cost effectiveness. The effectiveness of metal recovery depends on selection of eluant and elution conditions, as various eluants presenting different desorption mechanisms may be used (Tsezos, 1982). Lowering pH (e.g. with mineral acids) causes metal desorption,

resulting from competition between protons and metal ions for binding sites (Tam et al, 1998; Aldor et al, 1995). Mineral acids such as hydrochloric acid, sulphuric acid, acetic acid and nitric acid are proficient desorption agents (Holan, et al, 1993; Akthar et al, 1995; Pagnanelli et al, 2002). The strong chelating agent EDTA is another eluant commonly used (Aldor et al, 1995).

Despite several difficulties, living systems are also in use of metal removal in trickling filters or activated sludge. Living cells of bacteria, algae and fungi are also immobilised to form a fixed biofilm. Regeneration of living biomass in this case is very difficult as internal uptake of metals occur (Hutchins et al, 1986). The removal efficiencies of metals in trickling filters and activated sludge are comparable. But efficiency varies with metal type e.g. removal of Cu, Pb, Zn is far better than that of Ni or Co (Sterritt and Lester, 1986).

3.12 Conclusion

Bioremediation of metal containing wastewater is though a very new concept, still it is really advancing field of research. Different types of interactions like biotransformation, biosorption and binding of heavy metals with extracellular or intracellular organelles are effectively used and scientifically manipulated to perform with higher effectiveness. Designing of different types of bioreactors, within which microbes can accumulate or adsorb metals or radionuclides under controlled systems, is also being modernised. Several strains having the capacity to recover metals from waste were identified. However, more studies are needed to be done on application fields, so that we can better understand the effect of natural factors and of co-toxicants present in concerned effluent. Apart from choosing and developing such strains there remains a considerable amount of work required to be done on effective and easy desorbtion of metals and regeneration of the biomass for further use. Critical study reveals that not all metal-polluted wastewater generating industries have the concern or capability to treat effluents. Thus, most of the industries opt for just basic treatment to comply with the legalities. To attract more usage of biosorbent technology, certain strategies have to be formulated for reusing the used biosorbent to regenerate the biomass and then convert the recovered metal into usable form. This needs right interdisciplinary approach with integration of metallurgical skills along with sorption and wastewater treatment to develop biosorption technology for combating heavy metal pollution in aqueous solutions [135]. With so much to be done in this rapidly growing field of biotechnology, we hope that future promises us a better and healthier environment.

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Chapter-4

Cobalt-Resistant Microorganisms: Potentials for Environmental Biotechnology

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Abstract

Cobalt is an essential micronutrient for all living beings and has found wide degree of applications in industry, medial purposes and scientific research. Overexploitation and continuous discharge of cobalt containing wastes in the environment are causing deleterious effects on plant, animal and microbial communities. In view of the growing environmental awareness in public and industry and imposition of environmental legislation, there is an essential need for effective, low-cost technologies for remediation and removal of heavy metals including cobalt. Microorganisms resistant to cobalt have evolved in the metalliferous hostile environments and have been found to be more attractive in comparison with physico-chemical methods for removal of metals. The microbe-based technologies developed so far for removal of heavy metals from effluents and wastewaters have involved both living as well as non-living biomass as effective metal biosorbents. The uptake of toxic metal ions by algae, fungi and bacteria is mediated by bioaccumulation, the active mode of metal transport dependent on cellular metabolic activities or by passive biosorption by metabolically inactive cells or dead biomass. Following an effective accumulation, desorption of cobalt from metal-laden biomass has been experimentally established, which could find application in the recovery of cobalt from metal-contaminated aqueous systems.

4.1 Introduction

Environmental contamination due to heavy metals is a global concern because they are non-biodegradable and causes acute toxicity and threat to human life. Over-exploitation of metals in industries has lead to an alarming increase of metal pollution due to their release without proper treatment. Metal pollution due to anthropogenic activities has been aggravated due to their high dispersion, solubility, mobility in aquatic systems and biomagnification through food chain. Prolonged exposure to high levels of heavy metals is linked to birth defects, skin lesions, retardation of growth, lung infectious, liver disabilities, kidney damage and cancer (Hughes and Poole, 1989; Poole and Gadd, 1989).

Cobalt is one of the most important trace nutrients since it forms part of vitamin B_{12} and is required at nanomolar concentration. As an essential metal cobalt functions as catalysts for biochemical reactions, stabilizers of protein structures, cell walls and maintain osmotic balance in microorganisms (Ji and Silver, 1995). It is equally important in industry and is mainly employed for coloring porcelain and making super alloys. Disposal of industrial effluents laden with high levels of cobalt in to the soil and water bodies leads to cobalt pollution. Removal of heavy metals "at source" has been a long-term challenge.

Land filling, chemical immobilization and leaching are adapted along with flocculation, sedimentation, ion exchange, microfiltration, electrodialysis, evaporation, etc for decontaminating soil and waste water. However, chemical technologies use high energy inputs and are becoming expensive day by day.

During the recent era of environmental protection, the use of microorganisms for the removal and recovery of metals from contaminated systems has generated growing attention. The selective force in a metal-containing ecosystem has led to the development of various metal-resistant mechanisms in bacteria and fungi which are influenced of biotic and abiotic factors (Rouch *et al*., 1995).

Cobalt ion coming in contact with the microbial cell binds to the cell wall and subsequently enters the cell by virtue of uptake systems present on the cell membrane. Metal uptake processes are quick, unspecific, energy independent driven by a chemiosmotic gradient across the membrane (Ji and Silver, 1995). Bioremediation of cobalt ions or radionuclides may also occur through binding of metal ions to non-living biomass or microbial biopolymers. Biotransformation of cobalt also occurs following an alteration of their oxidation state and subsequent precipitation. Specific metabolic pathways leading to precipitation of cobalt as sulfides, phosphates, carbonates, etc. possess significance for possible biotechnological application. Moreover, the possibility of altering the properties of microbes used in bioremediation or constructing chimeric organisms possessing desirable features using genetic engineering is now under study in many laboratories (Kotrba and Ruml, 2000). The expression of metal-binding proteins or peptides in microorganisms in order to enhance metal accumulation also has great potential (Mejare and Bulow, 2001).

The present review attempts to discuss the occurrence and role of cobalt in our ecosystems, its toxicity, interactions with microbial diversity and population and also highlight the potentials of cobalt-resistant microorganisms for possible applications in the bioremediation of contaminated environments.

4.2 Cobalt in environment

The word cobalt comes from the German ‘kobolt’ or ‘kobold’, meaning evil spirit. Since 1450 B. C. cobalt salts have been used in Egypt and Babylon to produce brilliant blue colours in glass, pottery, clay in combination with nickel, chromium or manganese compounds. The lustrous, silvery-blue, hard metallic cobalt was discovered by George Brandt in 1753 in Sweden. It is found in the minerals like cobaltite, smaltite and erythrite, deposits of which occur in Zaire, Morocco, Canada, China and Australia.

Oxidized deposits of cobalt-rich ferromanganese crust occur throughout the global oceans on the flanks and summits of the seamounts, ridges and plateau. Seamounts in Pacific Ocean having rich deposits of the metal have been mapped and sampled in details. Cobalt-crusts are formed with the aid of bacterial activity at a depth of 400-4000 m below sea level comprise nearly1.7% of the ocean floor (equivalent to 1 billion tones of cobalt). The proportion of cobalt in crust is higher (1%) compared to land-based ores (0.1-0.2%).

On the earth’s crust the average concentration of cobalt is 23 ppm, whereas, total cobalt content of soils is usually within the range of 1 – 40 ppm. Soils derived from basic igneous rocks contain 20 – 100 ppm cobalt, but in normal soil it ranges between 8 – 10

ppm. In serpentine or ultramafic ecosystem the average cobalt concentration has been considered 100 ppm or more (Proctor and Woodell, 1975, Peterson and Girling, 1981). Mining areas in Zaire (now the Democratic Republic of Congo) was once recognized as the world's highest producer of cobalt. At present Zambia, Canada and Russia together accounted for more than 50% of the world's mine cobalt production.

4.3 Cobalt in living systems

Cobalt is a transition metal and in the periodic table occupies a position in between iron and nickel. It has two naturally occurring oxidation states: cobaltous [Co(II)] and cobaltic [Co(III), but it can exhibit oxidation states from -1 to +4. The cobaltic ion is a strong oxidizing agent and hence, its compounds are generally unstable. Cobalt is an essential element and forms part of the active site (corrin ring) of vitamin B_{12} (cobalamine). Vitamin B_{12} contains cobalt in a substituted corrin macrocycle (a porphyrin relative). Apart from this several other proteins containing noncorrin cobalt have also been isolated and identified. Kobayashi and Shimizu (1999) have extensively reviewed the structural and functional features of such novel cobalt containing enzymes and transporter proteins.

Cobalt is present in the enzyme methionine aminopeptidase, which catalyses the removal of N-terminal methionine from nascent polypeptide chains in all prokaryotes and eukaryotes. The enzyme has been isolated and characterized from wide variety of bacteria like, *Escherichia coli*, *Salmonella typhimurium*, *Bacillus subtilis* etc. (Roderick et al., 1993; Arfin et al., 1995). Other cobalt containing enzymes in bacteria include methylmalonyl-CoA-carboxytransferase from *Propiobacterium shermanii* which catalyzes the transfer of a carboxyl group from methylmalonyl-CoA to pyruvate to form propionyl-CoA and oxaloacetate (Ahmad et al., 1972); glucose isomerase from *Streptomyces albus* that catalyses the reversible isomerization of D-glucose to D-fructose. (Hemker et al., 1987); lysine-2, 3-aminomutase from *Clostridium subterminale* (Petrovich et al., 1991); proline dipeptidase from thermophilic archaea, *Pyrococcus furiosus* (Ghosh et al., 1998); low- and high molecular-mass nitrile hydratases (L-NHase and H-NHase) from *Rhodococcus rhodochrous* (Kobayashi et al., 1992). COT1, a membrane protein has been isolated from *Saccharomyces cereviseae* as inhibitor of cobalt toxicity, which is involved in cobalt transport, but is not solely responsible for it (Conklin et al., 1992). Similar cobalt uptake transporter has also been reported from the actinomycetes *Rhodococcus rhodochrous* J1 ATCC 12674 (Komeda et al., 1997).

Cobalt is actually a plant "bio-stimulant," similar to molybdenum, because it is required by nitrogen-fixing bacteria, especially on the root nodules of legumes. In root nodules, cobalt is associated with the production of cobamide compounds by the rhizobia. Cobamide coenzymes are required for metabolic processes of the bacteriods, especially for the normal functioning of the enzyme methylmalonyl-CoA-isomerase (Peterson and Girling, 1981).

Bacteria belonging to different taxonomic and physiological groups representing members of the genera *Pseudomonas*, *Brevibacterium*, *Rhodopseudomonas*, and *Lactococcus* have been reported to form intracellular cobalt- and chromium-containing magnetic inclusions. Ariskina et al. (2004) have described the structure, intracellular

localization and their similarity to the known non-crystalline iron-containing magnetic inclusions.

All ruminants can produce vitamin B_{12} if there is adequate cobalt in the diet and play a vital role in the food chain as producers of vitamin B_{12}. Monogastric ("one stomach") animals such as pigs and chickens are much more dependent upon the intake of actual B_{12}, "ready made" in the diet, since they do not have the advantage of an additional gut capable of synthesizing B_{12}. About 3-13% percent of the cobalt in the diet of a ruminant animal is incorporated by rumen microbes into vitamin B_{12}. However, deficiency in cobalt lead to loss of appetite; thiamine deficiency; reduced plasma levels of ascorbate, glucose and alkaline phosphatase, elevated plasma levels of pyruvate, pyruvate kinase, serum GOT forminino-glutamic acid and thyroxine, which affects the functioning of the hypothalamus. Cobalt deficiency is associated with the incidence of Johnne's disease, the ruminant analog of Crohn's disease in humans.

4.4 Uses of cobalt

Only in recent times cobalt has become an important industrial metal, especially in the production of alloys with nickel, iron and other metals used for jet engines and gas turbines, tool steels, magnets, turbine generators, in electroplating and as a catalyst (Kuyucak and Volesky, 1989). Cobalt in combination with nickel and manganese adds specific properties to the steel, like hardness, strength and resistance to corrosion. In industrial countries one-fourth to one-half of the total cobalt consumption is used by aerospace industry in super alloys. These metals are also employed in chemical and high-technology industries for such products as photovoltaic and solar cells, superconductors, advanced laser systems, fuel cells, etc. Organic and inorganic cobalt salts are also used in cobalt refining industry, diamond polishing, production of drying agents, paints, pigments, enamels, etc.

Radioactive cobalt (^{60}Co, ^{57}Co) is used for commercial and medical purposes. ^{60}Co is produced by thermal neutron bombardment of the natural isotope ^{59}Co, has a half-life of 5.3 years and decays by β- and γ-emission. It is used as a concentrated source of γ-radiation in cancer therapy, for sterilizing medical equipment and consumer products, manufacturing plastics, and irradiating food. It is a radioactive tracer in biological and industrial applications. ^{57}Co is used in medical and scientific research (Budavari et al., 1989).

Cobalt has also been used as a treatment for anemia with pregnant women, because it stimulates the production of red blood cells. In addition, cobalt salts in minor doses regulate mineral deficiencies in animals, growth of fresh water blue green algae and symbiotic nitrogen fixation (Barceloux and Barceloux, 1999).

4.5 Cobalt toxicity

The general population gets exposed to low levels of cobalt by breathing air, eating food, or drinking water. Food and drinking water are the largest sources of exposure to cobalt for all animals. The toxicity of cobalt is quite low compared to many other metals in soil and exposure to very high levels cause health hazards. Although the average level of cobalt in normal agricultural soils and water bodies is below 8ppm, it can exceed several hundred

times in rural and urban areas surrounding mines and in industrial places where workers are exposed to high concentrations of cobalt in the air.

The Occupational Safety and Health Administration (OSHA) have set a limit of 0.1 milligrams of nonradioactive cobalt per cubic meter of workplace air (0.1 mg/m^3). The Nuclear Regulatory Commission limits radioactive cobalt in workplace air to $1x10^{-5}$ microcurie per milliliter (μCi/mL) for ^{57}Co and $7x10^{-8}$ μCi/mL for ^{60}Co. Environmental Protection Agency (EPA), USA has set an average annual drinking water limit of 1000 picocurie per liter (pCi/L) for ^{57}Co or 100 pCi/L for ^{60}Co.

Workers in industries that make or use cutting or grinding tools; mine, smelt, refine, or process cobalt metal or ores; or that produce cobalt alloys or use cobalt are exposed to cobalt pollution. The general population is rarely exposed to radioactive cobalt unless a person is undergoing radiation therapy. However, workers at nuclear facilities, irradiation facilities, or nuclear waste storage sites may be exposed to radiation from these sources.

Exposure to high levels of cobalt can result in lung and heart effects and dermatitis. Effects on the lungs, including asthma, pneumonia, and wheezing, have been found in workers who breathed high levels of cobalt in the air. Liver and kidney effects have also been observed in animals exposed to high levels of cobalt. Acute exposure induces nausea, vomiting and other neurotoxicological symptoms, whereas, chronic exposure induce partial or complete loss of smell, gastrointestinal troubles and dilation of the heart. Exposure to large amounts of radiation from radioactive cobalt can damage cells. Acute radiation syndrome includes nausea, vomiting, diarrhea, bleeding, coma, and even death.

The International Agency for Research on Cancer has determined that cobalt is a possible carcinogen to humans. Studies in animals have shown that cobalt causes cancer when placed directly into the muscle or under the skin. Cobalt did not cause cancer in animals that were exposed to it in the air, in food, or in drinking water. Cobalt is often transported across biological membranes or through inhalation lading to significant toxicity (Moore, 1994).

There was evidence that soluble cobalt (II) cations exert a genotoxic and carcinogenic effect *in vitro* and *in vivo* in experimental animals but evidence in humans is lacking. Experimental data however, indicated a genotoxic potential for metallic cobalt *in vitro* in human lymphocytes. *In vitro* genotoxicity in mammalian cells occurs either by (a) a direct effect of Co(II) ions to damage DNA through a Fenton-like mechanism or (b) Co(II) ions affect the repair of DNA damage induced by other agents (Lison, 2001).

Cobalt toxicity on plants is unlikely to occur below soil cobalt concentrations of 40 ppm. Plant species vary in their sensitivity to cobalt depending on soil type and chemistry. Manganese oxide minerals play a key role in determining the availability of cobalt to plants. The more acidic the soil, the greater the potential for cobalt toxicity, at any concentration. However, soils with high cobalt concentrations usually also have high arsenic and nickel concentrations and these elements are generally more toxic to plants and humans than cobalt.

Cobalt at 5ppm inhibited seedling growth and chlorosis of young leaves of mung beans. Excess cobalt inhibited transport of iron to the shoot but stimulated uptake of excess sulfur (Liu et al., 2000). Cobalt was also found to inhibit growth and metabolism in tomato plants. At 0.05mM level, it showed visible chlorosis, followed by necrosis and withering.

Excess Co restricted growth, concentration of phosphorus, sulfur, and iron, chlorophyll a and b, DNA and RNA, sugars, starch, total soluble proteins, protein and non-protein nitrogen and catalase activity (Gopal et al., 2003). Although, cobalt is reported to produce toxic effects in plants, its accumulation in shoots and leaves of several plants growing in cobalt-containing soil is not uncommon. Several cobalt endemics like *Silene cobalticola* and *Crotolaria cobalticola* as well as cobaltophytes, *Anisopappus davyi* have been reported from cobalt-rich sites of Zaire. Similarly, *Pearsonia metallifera* from serpentine soils of Zimbabwe accumulate up to 3300 ppm cobalt (Peterson and Girling, 1981). These indicator species could be of use for biotechnological survey of cobalt ore deposits and phytoextraction of metals.

4.6 Cobalt-resistant microorganisms

Soil and fresh water habitats are often contaminated with cobalt by anthropogenic activities. Besides anthropogenic contamination, cobalt ions may also leak from naturally occurring minerals into soil or fresh water habitats. As an adaptive feature to this accumulated toxic metal ion concentrations, naturally occurring microorganisms have evolved resistance, which in many of the metal resistant bacteria have been found to be plasmid-encoded. Cobalt-resistant microorganisms have developed several mechanisms for transport and detoxification of metal ions. Cobalt ion transporters as well as small proteins and compound are involved in metal sequestration in prokaryotes (Silver et al., 1989). Glutathione related cadystins and cysteine-rich metallothionine proteins are involved in detoxification and storage of metals in eukaryotes (Mehra and Winge, 1991). Cobalt ions, which enter the cell following transport across the plasma membrane, are either sequestered by metal binding proteins or are compartmentalized in intracellular vacuoles (White and Gadd, 1986).

Metal resistance in *Alcaligenes eutrophus* CH34, the widely studied facultative chemolithotrophic bacterium is known to be determined by two plasmids, pMOL28 (163 kb) for resistance to cobalt, nickel, chromate and mercury and pMOL30 (238 kb) for resistance to cadmium, zinc and cobalt. Plasmid pMOL28-encoded nickel and cobalt resistances are inducible properties and are based on energy dependent cation efflux system (Nies and Silver, 1989a). On the contrary, plasmid free strain of *Alcaligenes eutrophus* AE 104 accumulates divalent metal cations including cobalt via the energy dependent magnesium transport system (Nies and Silver, 1989b).

Staphylococcus aureus is a common human pathogen associated with a number of diseases. Resistance to cadmium, mercury, antimony and arsenic in staphylococci is plasmid encoded, while staphylococcal strains without plasmid show resistance to nickel and cobalt. This implies that a plasmid-independent chromosomal determinant might encode resistance to heavy metals such as zinc and cobalt. Xiong and Jayaswal (1998) have reported the cloning, sequencing, and genetic analysis of a determinant located on the bacterial chromosome that codes for zinc and cobalt resistance in *S. aureus*.

Hafnia alvei 5-5, isolated from the rhizosphere soil of New Caledonian tree *Sebertia acuminata* (Sapotaceae), a nickel-hyperaccumulating plant was found to be highly nickel-resistant and moderately cobalt-resistant. Park et al (2003) demonstrated that the inducible nickel and cobalt-resistance determinants are located on plasmid pEJH501 of *H. alvei* 5-5, which hybridize with nickel-resistance genes from *Alcaligenes xylosoxydans*

31A and *Klebsiella oxytoca.* The structural genes for the nickel and cobalt resistance of the conjugative plasmid pEJH 501 of *Hafnia alvei* 5-5, contained on a SalI-EcoRI fragment of 4.8 kb, were cloned and sequenced. The DNA sequence included five genes in the following order: ncrA, ncrB, ncrC, ncrY, and ncrX (Park et al., 2004).

Inducible cobalt and nickel resistance in *Serratia marcescens* C-1 isolated from Cuban serpentine deposits have been investigated only recently (Marrero et al., 2006). Genes involved in metal resistance were identified by transposon mutagenesis followed by selection for cobalt- and nickel-sensitive derivatives. The transposon insertion causing the highest decrease in metal resistance was located in the ncrABC determinant. The predicted NcrA product was central for cobalt/nickel resistance in *S. marcescens* strain C-1. NcrB may be a regulatory protein. NcrC was a protein of the nickel-cobalt transport (NiCoT) protein family and necessary for full metal resistance in *E. coli*.

Amongst fungi, cobalt resistance and accumulation has been widely reported in yeast *Saccharomyces cerevisiae*. Joho et al., (1991) developed a mutant strain of *Saccharomyces cerevisiae* (NR 6) resistant to both Ni(II) and Co(II), which showed reduced Co(II) and Ni(II) uptake compared to the wild type strain (CMR-50). The inhibitory effects of Ni(II) and Co(II) on the growth of both mutant and wild strains was nullified by increasing the concentrations of Mg(II) in the medium and to a lower extent by the addition of Ca(II) which results in a decreased uptake of these metals. The resistant mechanisms of the mutant strain NR-6 was due to a reduction in the uptake of Co(II) and Ni(II) via a Mg(II) transport system.

Conklin et al. (1992) described the isolation of COT1 gene which when over expressed enables the wild type strain of *S. cerevisiae* to grow in a media containing up to 20.0 mM $CoCl_2$. Deletion of COT1 gene makes cells more sensitive to cobalt than are wild-type strains. The gene also affected cobalt transport in a dosage dependent manner and COT1 is involved in both accumulation and detoxification of Co(II) ions.

Cobalt resistance in *Neurospora crassa* cor, a cobalt-resistant strain was found to be associated with overproduction of a cobaltoprotein (Sajani and Maruthi Mohan, 1998). About 80% cobalt of cell-free extracts of this strain was protein bound while the same in wild type was only 25%. Cobalt content of the protein-bound fraction increased with time and cobalt concentration in the growth medium, and was not influenced by related metal ions. The cobaltoprotein was a glycoprotein and contained 70 μg cobalt / mg protein. Cysteine, glycine, glutamic acid and aspartic acid were the major amino acid constituents. While, the wall-less mutant of *Neurospora crassa* developed by Sri Rajyalaxmi et al., (2003) was ten-fold more resistant than the sensitive strain. About 70% of cobalt taken up by the resistant strain was bound to an inducible protein while only 2% was associated with the mitochondria. The comparison of cobalt-resistance and uptake of metal in the Co-resistant as well as Co-sensitive wall-less mutants of *N. crassa* suggested that both transport block and intracellular sequestration of metal play major role in cobalt resistance.
Microorganisms from serpentine soil are reported to show high degree of resistance to cobalt apart from nickel and chromium but the mechanisms of such resistance have not been studied so far. The occurrence and abundance of metal-resistant microflora in metalliferrous serpentine soil of New Caledonia (Amir and Pineau, 1998), Italy (Mengoni et al., 2001) and India (Pal et al., 2005) is well documented. Indian serpentines, which are exposed in patches in north as well as south Andaman Islands and are enriched with 93.2 –

533.4 mg Co / kg dry soil along with higher levels of Ni, Cr, Fe and Mg. Serpentine fungi belonging to *Aspergillus, Mortierella, Paecilomyces, Penicillium, Pythium, Rhizopus* and *Trichoderma* were found to be highly resistant to cobalt in comparison to bacterial isolates and 34.2% of serpentine fungi grew well at 4 mM Co. Among the bacterial isolates, only 7 tolerated 4 mM Co (Pal et al., 2003, 2004). These unique microbial resources from naturally metal-percolated ecosystem have not been effectively utilized in bioremediation of heavy metal pollutants. Evaluation of minimum inhibitory concentration of cobalt showed that majority (about 47%) of the fungi tested had MIC values ranging between 3.1 and 6.0 mM Co(II). However, only 29% of them were able to grow in presence of >6.0 mM Co(II) in the growth medium (Pal et al., 2005).

4.7 Bioaccumulation of cobalt

Bioaccumulation, the active mode of metal accumulation is dependent on the metabolic activity of the cell, which, in turn can be affected significantly by the presence of metallic ions. Bioaccumulation requires time for uptake by the microorganisms (Volesky, 1990).

Accumulation of cobalt (^{60}Co) by the estuarine microalga *Chlorella salina* has been characterized by Garnham et al, (1991). A significant amount of Co was bound within 1 min and was followed by a slower phase of uptake which was apparently active and inhibited by incubation in the dark or by the uncoupler dinitrophenol and the respiratory and photosynthetic inhibitor potassium cyanide in the light. Uptake of Co was affected by increasing concentrations of Na ions, decreasing pH, and the presence of variety of metal ions. Increasing cell density resulted in increased removal of Co from solution but decreased the specific amount of Co taken up by the cells.

The bioaccumulation of ^{57}Co (as an analog of ^{60}Co) by five phytoplankton species has been examined under controlled laboratory conditions using growing and non-growing cells. For growing cells, the uptake of ^{57}Co was highest in *Emiliania huxleyi*, for which it reached 1mBq / cell while, for non-growing cells, the uptake was highest in *Thalassiosira pseudonana* (Heldal et al., 2001). More recently, Adam and Garnier-Laplace (2003) have performed laboratory experiments to assess ^{60}Co uptake by two phytoplankton species, *Scenedesmus obliquus* and *Cyclotella meneghiana*. Cobalt along with Mn and Ag was more strongly accumulated by *C. meneghiana* than *S. obliquus* and vice versa for Cs.

Bacillus circulans strain EB1, a heavy metal resistant bacterium isolated from heavy-metal-contaminated soil in the southeast region of Turkey exhibited high MIC values for metals and a large spectrum of antibiotic resistance. Metal uptake by this isolate was detected during the course of growth. The strain EB1 was capable of removing 90% of Mn, 68% of Zn, 65% of Cu, 45% of Ni and 40% of Co during the active growth cycle with a specific accumulation capacity of 25, 22, 20, 13 and 12 mg/l, respectively. Since *Bacillus* can grow in the presence of significant concentrations of metals and due to high metal accumulation capacity in aerobic conditions, this bacterium may find application in *in situ* bioremediation of heavy-metal-contaminated aqueous systems (Yilmaz, 2003).

A novel cobalt uptake transporter from the actinomycetes *Rhodococcus rhodochrous* J1 ATCC 12674 has been studied in details. The gene (nhlF) product Nhl F showed significant similarity to nickel transporters from *Alcaligenes eutrophus*, *Bradyrhizobium japonicum*, and *Bacillus* sp. and is responsible for encoding low

molecular-mass nitrile hydratase. It functions as a specific cobalt transporter and the organism accumulated >15 pmol / Co(II) / mg dry cell after 25 min of incubation. The uptake phenomenon markedly decreased in presence of Ni(II) ions, whereas, presence of Mn(II), Fe(II) and Cu(II) did not affect cobalt uptake by the organism. Studies on the effect of various uncouplers on cobalt uptake by *R. rhodochrous* demonstrated that proton gradients are involved in cobalt transport (Komeda et al., 1997).

4.8 Biosorption of cobalt

Biosorption refers to the passive and non-metabolically active process of metal binding by living or dead biomass. The process is comparatively rapid and can be reversible. It involves physicochemical interaction between the metal ion and functional groups of the extracellular polymeric substances of the microbial biomass based on physical adsorption, ion exchange, complexation and precipitation (Kuyucak and Volesky, 1988). Microbial biomass derived from algae, fungi, yeasts and bacteria have the pronounced ability to bind and accumulate metal ions. Some investigators have examined the utility of dried; non-living microorganisms for the removal of metal ions from aqueous solutions, while others have used the "modified" microbial biomass to improve biosorption capacity (Gadd, 1988; Brierley, 1990; Akhter et al., 1996).

Metabolically inactive cells or dead biomass are capable of accumulating same or greater amount of cobalt from aqueous solution. Kuyucak and Volesky (1989) screened the marine algal biomass for sequestering of cobalt and demonstrated that non-living biomass of seaweed; *Ascophyllum nodosum* has a high cobalt uptake capacity from solutions. The process involves ion exchange and the alginates of the cell wall binds cobalt ions rapidly, whereas, penetration of cobalt into the cell occurs at a lower rate. Following an effective accumulation, desorption of cobalt from metal-laden biomass was experimentally established. The solution of $CaCl_2$ in HCl was the best eluent capable of desorbing more than 90% of the sequestered cobalt at pH 2-3. Similarly, *Sargassam asperifolium*, *Cytoseira trinode*, *Turbinaria decurrens* and *Laurencia obtusa* collected from the beach of Red Sea, at Hurghada in Egypt have also been screened for sequestering of Co along with other heavy metals like, Cr, Ni, Cu and Cd. Uptake of Co was maximum (86% of added metal) in *T. decurrens* (Hamdy, 2001).

Immobilized algal biomass from *Spirulina platensis* and *Chlorella* sp. has been used for the removal and recovery of cobalt, nickel and chromium from laterite heap leach liquors.The cyanobacterial biomass was immobilized on the highly porous surface of pumice stone and nearly 80% of cobalt biosorption was recorded from low concentration solutions at pH 6.0. Nickel removal was complete under the same conditions, whereas chromium sorption was ineffective. Desorption of the metal loaded columns was effected by dilute acid. The method could be applicable for recovery of cobalt and nickel from laterite heap leach solutions, such as heap wash waters, or for their removal from waste streams of hydrometallurgical processes (Agatzini-Leonardou and Zafiratos, 1997).

The non-growing biomass of the freshwater cyanobacterium, *Oscillatoria anguistissima* showed fast order kinetics of cobalt binding and could adsorb appreciable quantities of cobalt from aqueous solutions within 15 min of initial contact with metal solution (Ahuja et al., 1999). Biosorption of cobalt by this organism is an ion-exchange phenomenon and is strongly dependent on pH of the metal solution. The highest uptake of 468.75 mg Co(II) /

g dry weight was observed at 0.08 mg dry weight / ml of biomass at a residual cobalt concentration of 120 ppm. Amongst the various desorbing agents, Na_2CO_3 (1.0 mM) resulted in about 76% desorption of Co(II) from the loaded biomass.

Gram negative bacteria, particularly the constituents of their cell walls are known to bind strongly with metal ions (Beveridge and Fyfe, 1985). *Pseudomonas halodenitrificans*, a bacterium known to denitrify effluents containing high quantities of nitrates was used by Ginisty et al. (1998) to investigate the mechanisms of biosorption of cobalt. Studies on the influence of different cationic pretreatments of biomass on the biosorption of cobalt have indicated that Li was the least inhibitory of Co(II) biosorption and divalent ions were the most inhibitory. The metal accumulation properties of *Pseudomonas fluorescens* 4F39 were measured in terms of metal removed from aqueous solution by non-growing biomass. Accumulation of heavy metals by *P. fluorescens* 4F39 was rapid and found to be pH dependent. Unlike Ni, the accumulation of Co by the isolate was low, but it increased with increase in pH (Lopez et al., 2000).

The use of fungal biomass over other biosorbents could serve as an economical means for removal of metal ions because fungi can be grown in substantial amount without the use of sophisticated equipments and expensive chemicals. Moreover, continuous supply of fungal biomass as a byproduct is available from a variety of industrial fermentation processes (Kapoor et al., 1999). The fungal cell wall constituent, chitin and chitosan have been suggested as metal sequestering components, many other structural components have been implicated as well. Search for newer fungal biosorbents having specific priority for binding cobalt ions, therefore, remains a challenge for microbial biotechnologists.

Wilhelmi and Duncan (1995) investigated the binding of metal chlorides (Cu, Co, Zn, Cd, Ni and Cr) to immobilized *Saccharomyces cerevisiae* in packed-bed continuous-flow columns and determined recovery and concentration potential and the reusability of the biomass. The metal ions were effectively removed from solution by the yeast biomass until reaching a saturation threshold, following which uptake declined rapidly. The metals were desorbed from the biomass using 0.1 M HCI. Initial recovery of cobalt including copper and cadmium was 100 %. The high recovery by mild acid elution suggests accumulation by passive binding to the cell walls of *S. cerevisiae*.

Fungal biomass prepared and processed from *Neurospora crassa, Fusarium oxysporum, Penicillium* spp. and *Aspergillus niger* was examined for binding divalent metal ions including cobalt (Akhtar et al., 1996). The biosorbents were found to sequester metal ions efficiently both from dilute and concentrated solutions up to 10% of its weight (w/w). It was shown that the mechanism of metal binding by the biosorbent derived from *A. niger* was primarily, by exchange of divalent ions with resident Ca(II) and Mg(II) of mycelial biomass. They also established carboxylic groupings as the major determinants of metal binding by processed biomass.

The fungal-based biosorbent, PFB1 developed using the fungus *Rhizopus* spp. (Suhasini et al., 1999) exhibited removal of cobalt from aqueous solutions. The maximum uptake of cobalt by PFB1 was 190 mg / g occurred at an initial pH of 7.0 and at 30°C. During the sorption process cobalt may be exchanged with calcium and the process involves interaction with free radicals. The mechanism of cobalt sorption by PFB1 was suggested to be a combination of adsorption, ion-exchange and interaction with free

radicals. The adsorbed cobalt on to PFB1 was efficiently removed with 0.1 N HCl as the best desorbent. It was also emphasized that this fungal-based biosorbent exhibited about five times higher removal capacities for cobalt than the removal capacity of the fungal based biosorbent AMT^{TM}. The cobalt loading capacity of metal-removing agent (MRA) was lower at lower pH and the sequestering of Co was fast under ambient conditions. Desorption of Co from loaded MRA was performed by 0.1N HCl in a very short period of time. The desorbed MRA is recycled only after regeneration with 0.5N NaOH (Awadalla, 1992).

Comparative studies made by Habibi et al. (2002) on heavy metal biosorption by four different fungi, *Aspergillus terreus*, *Aspergillus niger*, *Trichoderma reesei* and *Phanerochaete chrysosporium*, showed different degrees of biosorption of Co, Ni, Pb and Mn. Biosorption of metals by these fungi was largely influenced by the growth media. *P. chrysosporium* grown on whey water had the highest biosorption capacity: Co(II), 10; Ni(II), 12.5; Pb(II), 27.6; and Mn(II), 35 mg / g was removed from the aqueous solution.

Cobalt-resistant fungi belonging to *Aspergillus*, *Mortierella*, *Paecilomyces*, *Penicillium*, *Pythium*, *Rhizopus* and *Trichoderma,* obtained from serpentines of Andaman were evaluated for cobalt biosorption using dried mycelial biomass (Pal et al., 2006). During long term (60 min) exposure, the selected Co(II)-resistant *Mortierella* SPS 403 showed significant biosorption of Co(II) at high initial metal concentration which is generally encountered in industrial effluents. However, in terms of rapid sorption of metal ions by the mycelial biomass during short-term (15 min) exposure, *Penicillium* SPS 106, CTS 402; *Aspergillus* SPS 202 and *Paecilomyces* SPS 107 were not inferior. Maximum Co(II)-loading (1036.5 µM / g, 60 min) was achieved with *Mortierella* SPS 403 biomass, which removed almost 50% of 4.0 mM cobalt from the aqueous solution. Co(II)-sorption kinetics of *Mortierella* SPS 403 biomass was fast and appreciable quantities of metal [562.5 µM / g] was adsorbed during first 10 min of incubation. The metal biosorption capacity of the isolate was accelerated with increasing cobalt concentration, while it was reverse with increase of initial biomass. The optimum pH and temperature for Co(II) removal were 7.0 and 30ºC, respectively. However, Co(II)-uptake was inhibited in presence of other metals (Pb, Cd, Cu, Ni, Cr, Zn). Freundlich adsorption isotherm appropriately describes *Mortierella* SPS 403 biomass as an efficient Co(II)-biosorbent.

Recently, Latha et al., (2005) in establishing the relevance of the fungal cell wall in metal ion transport, analyzed cobalt uptake in *Neurospora crassa*. Cobalt taken up was largely surface bound (>90%), resulting in a release of calcium and magnesium. Surface-bound cobalt could not enter intracellular locations upon further incubation of mycelia in a metal-free medium. Saturation of the surface with one metal augured subsequent dose-dependent entry of a different metal into intracellular locations. In comparison with the cobalt-resistant mutant, the cobalt-sensitive strain of *N. crassa* bound less cobalt on the surface but with significant intracellular accumulation. These findings demonstrated the importance of the cell wall in metal transport, toxicity, and resistance in fungi.

4.9 Bioreduction of cobalt

Microbial reduction of toxic metals and radionuclides has attracted special attention as these transformations can play crucial role in detoxification of a variety of heavy metals and may offer the basis of wide range of biotechnological applications including the

bioremediation of metal contaminated soil and water. In a recent review, Llyod (2003) focuses attention on the reduction of wide range of metals including toxic metals, metalloids and radionuclides.

Cobalt, specially Co(III) is stable and mobile when complexed with EDTA. Reduction of Co(III) to Co(II) decreases the stability of the radionuclide-chelate complex and can limit the transport of the ^{60}Co in subsurface environments. Co(III)EDTA is over 25 orders of magnitude more thermodynamically stable than Co(II)EDTA. The enzymatic reduction of Co(III)EDTA by the Fe(III)-reducing bacterium *Geobacter sulfurreducens* was reported earlier (Caccavo et al, 1994). This bacterium coupled the oxidation of acetate to the reduction of Co(III)EDTA in an anoxic, aqueous medium without metal oxide minerals. However, the product of the reaction was not identified. Later, Gorby et al (1989) have demonstrated that the metal-reducing bacterium *Shewanella alga* strain BrY enzymatically reduced Co(III)EDTA to Co(II)EDTA with a 1:1 stoichiometry. Manganese(IV) oxide minerals can also oxidize Co(II)EDTA to Co(III)EDTA, thereby enhancing the solubility and mobility of ^{60}Co. Thus, the oxidation and reduction of Co in the EDTA complex directly influences the stability and mobility of ^{60}Co near waste disposal sites.

4.10 Conclusion

Cobalt is an essential trace element and in the form of vitamin B_{12}, it plays a number of crucial roles in many biological functions. Cobalt salts in small doses have been found to be effective in correcting mineral deficiencies in certain animals. In addition recent studies have provided information on several protein-containing enzymes from both prokaryotes and eukaryotes. However, compared to other heavy metals, toxicity of cobalt is low. But exposures to high levels of cobalt are known to result variety of health hazards. Cobalt resistant microorganisms have been isolated from diverse natural and man-made ecosystems and their mechanisms of cobalt resistance have been elucidated quite extensively. The unique features like, bioaccumulation, biosorption and bioreduction of these cobalt-resistant microorganisms have been utilized for detoxification and removal of cobalt from the environment. Successful application of these potential microbiological methods will lead to the development of a green technology towards amelioration of cobalt contamination in the environment.

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Chapter-5

MICROBIAL DEGRADATION OF MELANOIDIN: A MAJOR ENVIRONMENTAL POLLUTANT OF DISTILLERY WASTE

Ram Chandra and Ram Naresh Bharagava

Abstract

Melanoidins are dark brown, complex natural biopolymer of amino-carbonyl compounds produced by Maillard reaction. Naturally melanoidins are widely distributed in all types of food and drinks having brown colour and widely discharged by various agro-based industries especially from sugar cane molasses based distilleries and fermentation industries as environmental pollutants. The structure of melanoidin is still not completely understood but it is assumed that it does not have a definite structure as its elemental composition and chemical structures largely depend on the nature and molar concentration of parent reacting compound and reaction conditions as pH, temperature, heating time and solvent system used.

Melanoidins have been shown to have commercial, nutritional and toxicological significance as these brown polymers have significant effect on the quality of food, since colour and flavours are an important food attributes and are key factor in consumer's acceptance. Food and drinks as bakery products, coffee and beer having brown coloured melanoidin pigment exhibited antioxidant, antiallergenic, antimicrobial and cytotoxic properties as in vitro studies have demonstrated that Maillard reaction products (MRPs) may offer substantial health promoting activity as they can act as reducing agents, metal chelators and radical scavengers. Besides these health-promoting properties, in vitro studies have also revealed some harmful effects of melanoidin polymer as it has mutagenic, carcinogenic and cytotoxic effects. Excessive glycation has also been stated to cause the destruction of essential amino acids, decreased digestibility, inactivation of enzymes, inhibition of regulatory molecule binding sites, cross linking of glycated extra cellular matrix, decreased susceptibility to proteolysis, abnormalities of nucleic acid function and altered macromolecular recognition etc. Due to antioxidant properties, melanoidins are toxic to aquatic macro and microorganisms. However, the wastewater released from distilleries and fermentation industries is a major source of aquatic and soil pollution due to presence of water-soluble recalcitrant colouring compound called melanoidin. Melanoidin's environmental toxicity is due to its high biological oxygen demand (BOD) (40,000 mg/L), chemical oxygen demand (COD) (90,000 mg/L) and other toxic components. Since, melanoidins are highly resistant to microbial attack, conventional biological processes such as activated sludge treatment are insufficient to decolourize the melanoidin containing wastewater released from distilleries and fermentation industries. Therefore, this dark coloured wastewater released from distilleries and fermentation

industries requires pretreatment before its safe disposal into the environment. Recently, decolourisation of molasses melanoidin has seen attempted, but with limited success. Decolourisation of melanoidin containing effluents by chemical methods, flocculation treatment and physicochemical treatment such as ozonation and activated carbon adsorption have been accomplished but these methods are not economically feasible on large scale due to cost limitation. Biological decolourisation of melanoidin pigment by using fungi such as *Coriolus*, *Aspergillus*, *Phanerochaete* and certain bacteria as *Bacillus* and *Lactobacillus* have been successfully achieved and thus can be applied as a bioremediation techniques. However, the biological decolourisation of melanoidin containing wastewater largely depends on pH, temperature, concentration of nutrients, oxygen and inoculum size while the enzymatic system responsible for melanoidin degradation consists mainly sugar oxidases and peroxidases as sarbos oxidase, glucose oxidase, manganese dependent and independent peroxidases (MnP and MIP). Since, MnP and MIP showed melanoidin decolourizing activity in presence of H_2O_2 and the melanoidin decolourizing activity of both sugar oxidases and peroxidases were found optimum at a particular pH, temperature and substrate specific. This book chapter contributes and summarizes the literature available on the structure, chemistry, properties, and enzymes involved in the degradation of molasses melanoidins in environment. The coverage of literature is extensive but by all means not complete. The biological transformation of melanoidin by fungal and bacterial system has been duly emphasized. Further, enzymatic induction in biological system and the effect of various environmental factors on enzymatic activity and melanoidin degradation has also been described. Therefore, this chapter has a major significance in the abatement of environmental pollution.

Key words: Melanoidin, structure, molasses, degradation, chemical, microorganism, enzymes

5.1 Introduction

Melanoidins are natural, dark brown, complex biopolymers produced by non-enzymatic browning reactions called as Maillard amino-carbonyl reaction taking place between the amino and carbonyl groups in organic substances (Wedzicha and Kaputo, 1992; Reynolds, 1968). It is considered that melanoidins extensively exist in food, drinks and wastewater released from distillery and fermentation industries. Maillard reaction is very common during food processing and the products are of great importance for the colour and flavour of foods. Hence, the Maillard reaction between amino and carbonyl compounds has gained increased attention in recent years. The main source of melanoidin compound in environment is industrial wastewater using raw material of plant origin. Thus, melanoidins exist not only in various foods but also in various industrial wastes e.g. distillery and sugar mill wastes using sugarcane molasses as raw material (Kumar and Chandra, 2006).

Melanoidins are closely related / analogous to humic substances of soil or melanin (Hayase et al, 1984) in regard to their chemical properties. Therefore, melanoidins are very important from nutritional, physiological and environmental aspects. Melanoidins owing to

their structural complexity, dark colour and offensive odour pose threat to aquatic and terrestrial ecosystem. Currently, the most visible environmental problem caused by contamination with melanoidin compounds is eutrophication in natural water bodies, reduction of sunlight penetration leading to decreased photosynthetic activity and dissolved oxygen concentration in lakes, rivers or lagoons (Kumar et al, 1997a; 1997b).

On land, it causes reduction in soil alkalinity and inhibition of seed germination (Kannabiran and Pragasam, 1993). Therefore, the degradation and decolourisation of molasses melanoidin by chemical (Kim et al, 1985) and biological means (Ohmomo et al, 1987; Kumar et al, 1997b) has been attempted in order to characterize the chemical structure and bioremediation aspects.

In the recent past / two-three decades, the intensive research in this area has lead to dramatic progress to understand the formation and degradation pathway of melanoidins as well as the microbial strategies and associated mechanisms for the decolourisation of melanoidins. These recent developments regarding synthesis, chemical structure and biodegradability of melanoidin polymers in the environment are the main focus of this book chapter.

5.2 Chemistry of melanoidins

(a) Melanoidin formation pathway

In general the browning reaction occurs when food is cooked. In foods, the amino compounds are mostly in free or protein bound amino acids and the reducing compounds are as reducing sugars. L.C. Maillard (1912a) who first observed the darkening accompanying the reaction of sugars with amino acids. In 1916, Maillard had shown that brown pigments and polymers are produced during the reaction of the amino groups of amino acids with the carbonyl groups of sugars. Since then, sugar and amino compounds the browning reaction between compounds is commonly called as non-enzymatic Maillard reaction (Fig. 1) produces a number of compounds.

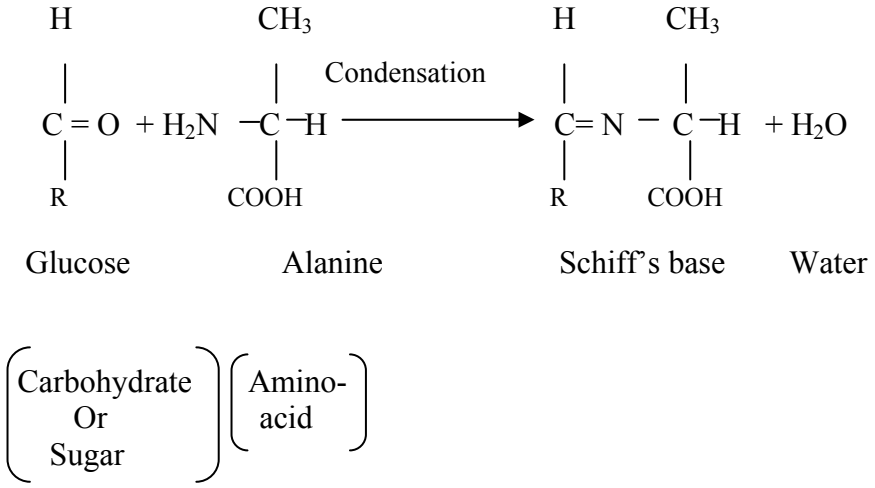

Fig.1: The browning reaction (Maillard's reaction) is believed to be due to the formation of Schiff's base between the amino groups of protein / amino acid and the Aldehyde /carbonyl groups of carbohydrates.

Since, Maillard first observed the darkening accompanying amino-carbonyl reaction. Subsequently numerous publications appeared discussing this phenomenon so-called as non-enzymatic browning of foods (Silvan et al, 2006). The mechanism proposed by Hodge (1953) for the amino-carbonyl reaction of sugar with amines or amino acids has been accepted as the most appropriate to give a browning polymer i.e. melanoidin pigment.

The main process of this pathway (Fig. 2) is initiated by the condensation of sugar with the amino compound to yield a glycosylamine followed by the Amadori rearrangement as a key step. Subsequently, the deamination of 1,2-enaminol form of this product gave 3-deoxy or 1-deoxyhexosones, which is considered to be an important intermediate in the formation of melanoidin. In this mechanism, no consideration has been given to sugar fragmentation as a source of reactive intermediates at an early stage and three carbon carbonyl products have been assumed only as minor active products from 3-deoxy or 1-deoxyhexosones in the middle stage of the reaction.

Later, Hayashi and Namiki (1986) proposed a new pathway for browning reactions involving cleavage of the sugar moiety of schiff base at initial stage of the amino-carbonyl reaction before Amadori rearrangement followed by formation of C_2 (glyoxal dialkylimine) product(s) and free radicals. They have also reported the presence of a C_3-compound as another fragmentation product in early stages of the sugar-amine reaction. Further, continuing their studies on browning mechanism, Hayashi and Namiki (Hayashi and Namiki, 1986; Hayase, 1982) have made identification of C_2-precursor and its role in the mechanism of radical formation through Maillard reaction. The precursor intermediate was found to be glyoxal dialkylamine, giving rise to free radicals. The ESR analysis finally led to assignment of an N, N^1-dialkylpyrazine cation structure to free radical.

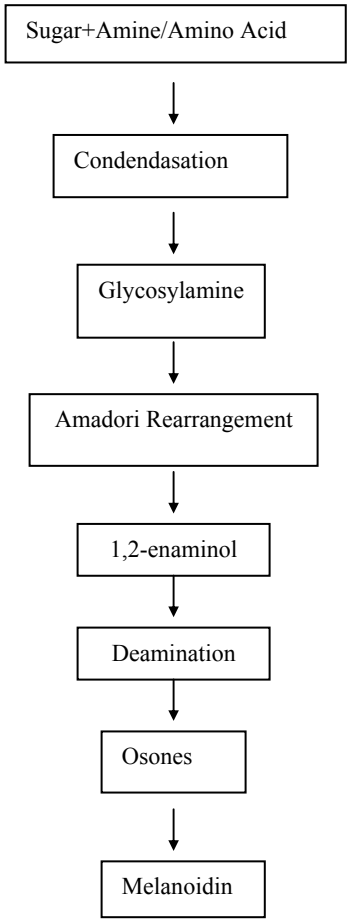

Fig. 2: Hodge's mechanism of melanoidin formation.

They also proposed the pathway for the formation of highly reactive radicals from the intermediate (Figure 3) and reported that the most important intermediate giving browning product and free radical is considered to be glycoaldehyde alkylimine and/or its enaminol (Figure 3).

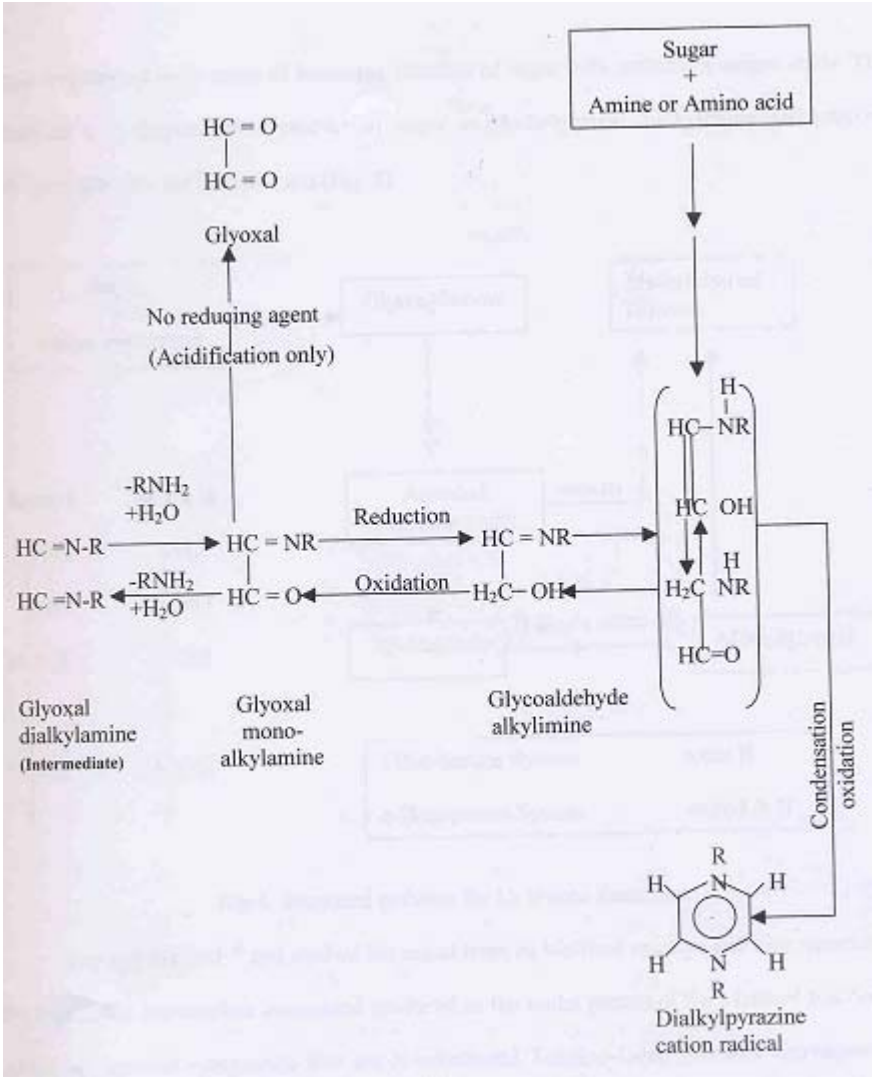

Fig. 3. Possible pathways of intermediate formation by the reaction of glucose with an amino compound and free radical formation from intermediate

Nevertheless, they have also suggested for more investigations on this product in order to determine the role of new pathway in browning as well as the detailed mechanism of radical formation. One year later, Hayashi and co-workers (Hayase and Kato, 1981; Hayase et al, 1989) proposed the formation of three-carbon sugar fragment at early stage of browning reaction of sugar with amines or amino acids. They identified a C_3-fragmentation

product of sugar as Methylglyoxal dialkylimine and proposed following pathway for its formation (Fig. 4).

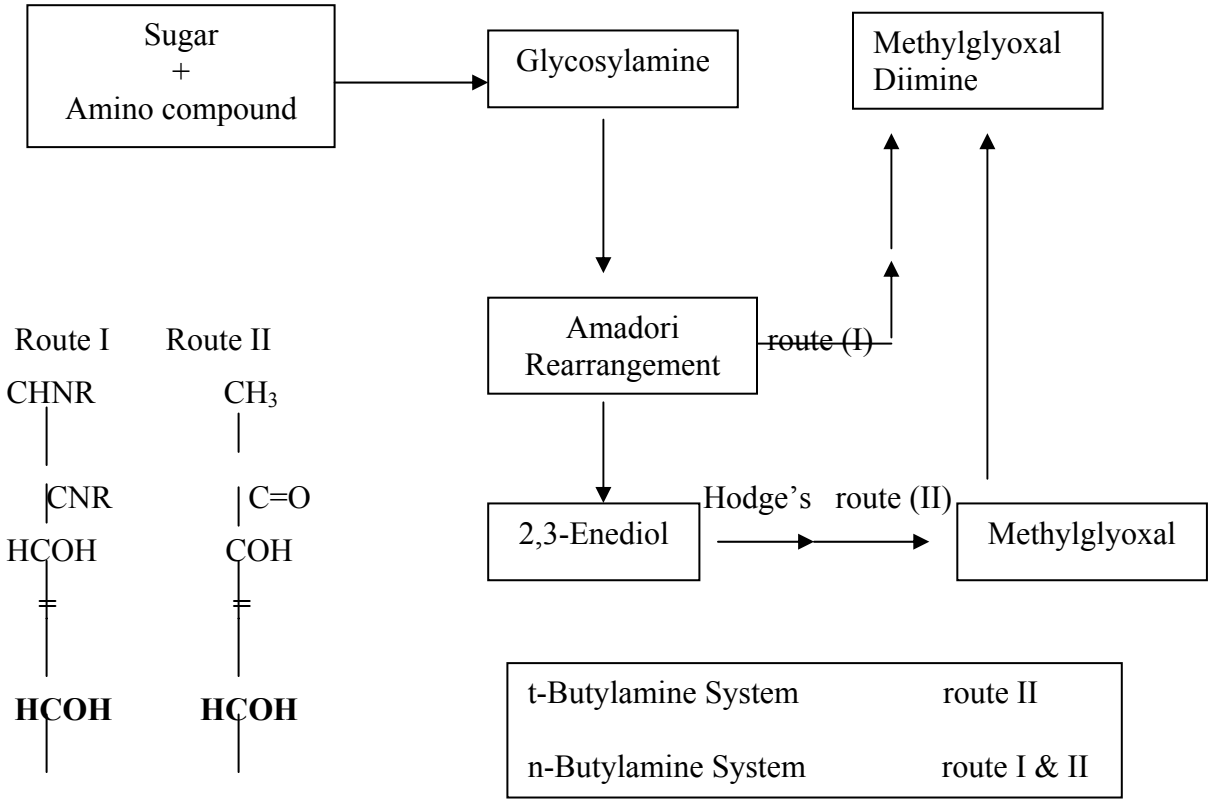

Fig. 4. Proposed pathway for C_3 Imines formation.

Fay and Brevard (Fay and Brevard, 2004) had studied the initial steps of Maillard reaction and they reported that the first stable intermediate compound produced in the initial phases of the Maillard reaction are called as Amadori compounds that are N-substituted 1-amino-1-deoxyketoses representing an important class of Maillard intermediates. These intermediates are produced during the initial phases of the Maillard reaction by Amadori rearrangement of the corresponding N-glycosyl amines. The later obtained by condensation of amino acids and aldoses such as glucose as shown in Figure 5.

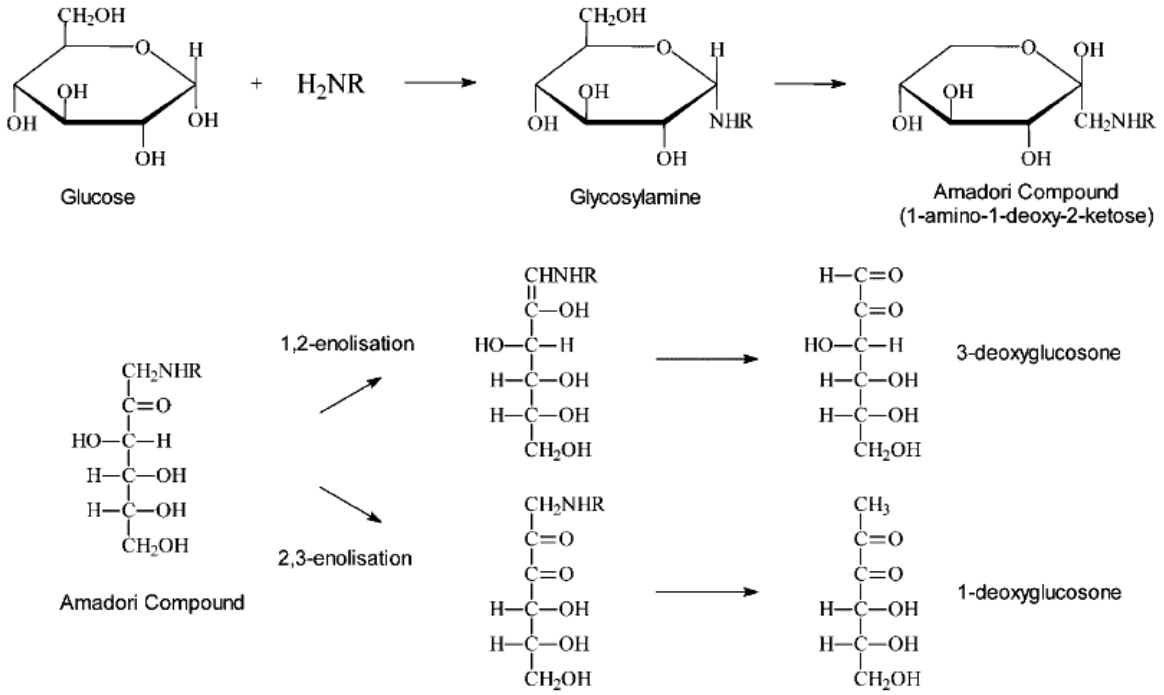

Fig. 5. Initial step of the Maillard reaction

This type of rearrangement was named after M. Amadori who was the first to demonstrate the condensation of d-glucose with an aromatic amine would yield two structurally different isomers, one N-substituted glycosyl-amine, which is more labile than the other towards hydrolysis. Hence, these intermediates of the Maillard reaction are termed as Amadori compounds.

Hayashi et al. (Hayase et al, 1982) have also observed that the course of C_3 imine formation followed the pattern of C_2 imine formation, and well correlated to decrease in the amount of glucosylamine and an increase in the formation of Amadori products. Reaction of Amadori products with n-butylamine rapidly produced C_3 compound in a manner similar to that of glucose-n-butylamine system. These results indicated the possibility of Amadori product participation in the formation of C_3 compound. Hayashi and Namiki (Hayashi and Namiki, 1986) studied the role of sugar fragmentation in early stage browning of amino-carbonyl compounds using glucose-β-alanine mixture and established that even if only a small amount of sugar fragmentation occurs browning by sugar fragmentation will make a major contribution to browning in the early stages especially at neutral pH or above.

In other words, they suggested that the browning observed at the early stages of the Maillard reaction at pH above neutral might be caused mainly due to the browning through fragmentation pathways. They also proposed that the browning products i.e. melanoidins produced at alkaline pH might be some what different in their chemical

composition to those produced at acidic pH due to difference in their precursor compounds.

Finally, they summarized the mechanisms of early browning reaction (**Fig. 6**) and concluded that in acidic conditions, the main pathways including melanoidin formation are naturally considered to be the route of osone formation while to a large extent the increase in browning by increasing the pH might be due to the pathway containing sugar formation caused mostly by amino-carbonyl reactions.

In spite of large amount of research done on the Maillard reaction, many parts as mechanism for the formation of brown pigment at later final stages of this complex reaction are still obscure. However, the proposed mechanisms discussed or reviewed above present a clear picture of melanoidin formation through Maillard amino-carbonyl reaction. Still, much is required to bring out the actual mechanisms involved in melanoidin formation.

(b) Structure of melanoidin polymer

As earlier mentioned, melanoidin is a brown polymer formed by an amino-carbonyl reaction during the food processing and preservation. Also, it is one of the polymers, which is widely distributed in nature through industrial process and is hardly biodegradable.

In general, separation of food/natural melanoidin from other food constituents is very difficult therefore the chemical and biological studies on melanoidins have been done on model melanoidin. Although the chemical structure of melanoidin is not yet clear, but some part of the chemical structure of model melanoidin has recently been elucidated by different spectral studies such as ^{1}H NMR, CP-MAS NMR etc (Hayase et al, 1986).

Some workers have analysed xylose-glycine (^{15}N) melanoidin by ^{15}N cross polarized-magnetic angle spinning (CP-MAS) NMR and reported that the nitrogen in melanoidin polymer was mainly in secondary amide form and some as pyrole and/or indole nitrogen. They also revealed that sterically hindered secondary amide bonds are very resistant to acid hydrolysis.

Hayase *et al.* (Hayase et al, 1984) during studies on decolourisation of model melanoidin prepared from glucose-glycine by hydrogen peroxide postulated a part of the chemical structure of melanoidin. According to them the melanoidin component seems to have a CH_3-COR moiety and C-terminal structures originated from glucose existing in melanoidin are suggested as follows:

1. CH_3-CO-R,
2. CH_3-C(H or OH)=C(H or OH)-CO-R”
3. R-CO-CO-R’
4. R-CO-CH(CH_3)-CO-R’
5. R-CO-CH_2-CO-R’
6. R-CO-CH_2-CH_2-CO-R’

7. CH_3-CH (OH)-CO-R and so on.

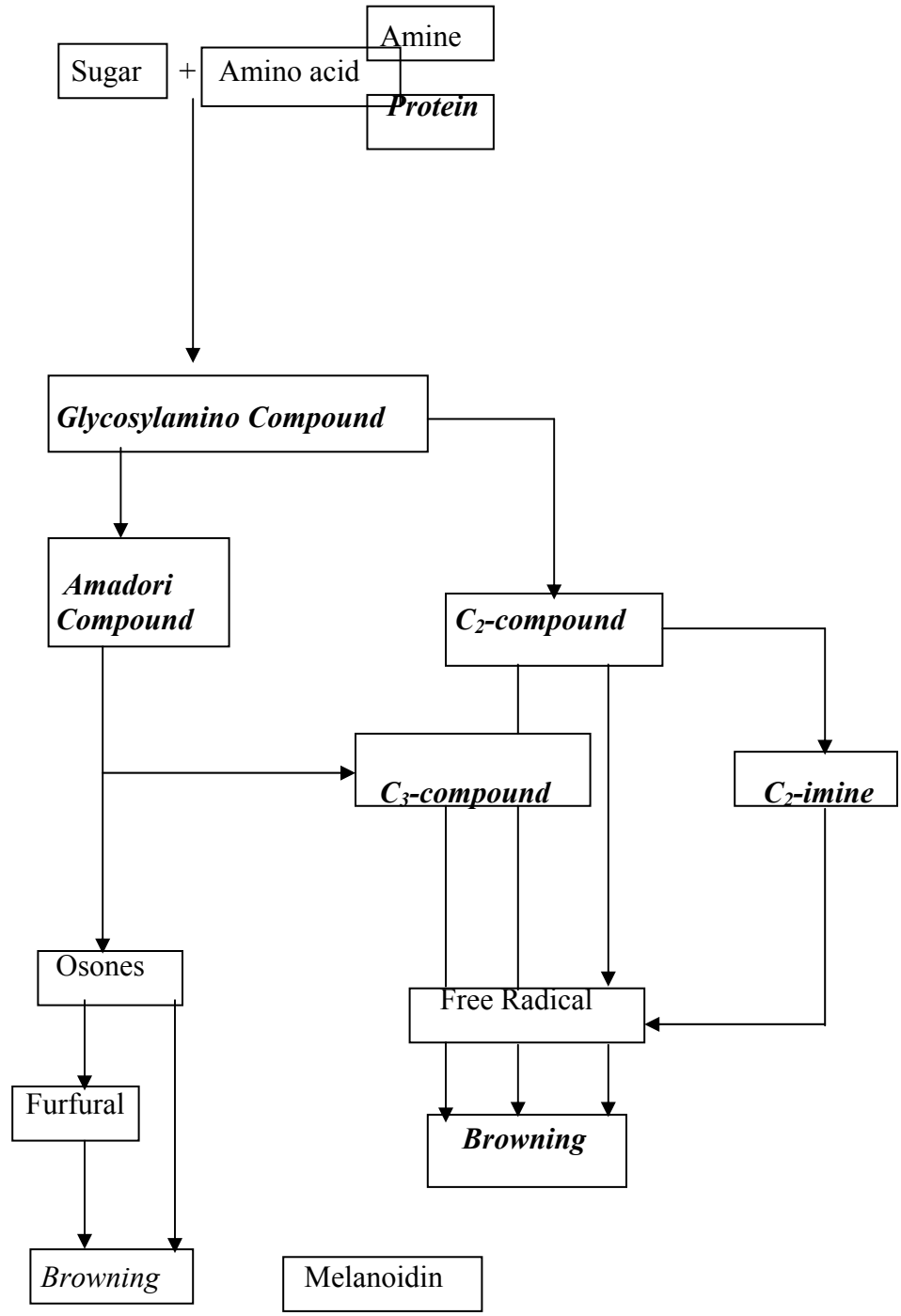

Fig. 6. pH dependent differnt pathways for melanoidin formation

The compound '1' (shown above) was estimated to exist as the main part of melanoidin polymer in which acetic acid was a major product in ether soluble acidic fraction of the degraded products. Similarly, Kim *et al.* (1985) while studying the degradation of model melanoidin from glucose-glycine by ozonolysis suggested the structures adjacent to carbon-carbon double bond originates from glucose existing in melanoidins to be as follows:

1. CH_2(OH)-C(H or OH)=C-R and
2. CH_3CH_2-CH(OH)C(H or OH)=C-R

In addition, based on the analyses of degraded products following structures are suggested as minor structures of aforementioned system:

1. $(CH_3)_2$C-(OH)C(H or OH)=R
2. $(CH_3)_2$C(OH)-CH_2C(H or OH)=R

Later, Hayase et al. (1986) have made study on the non-dialyzable melanoidins prepared from glucose-glycine system that were subjected to ^{13}C CP-MAS and ^{15}N CP-MAS NMR analyses before and after ozonolysis. They reported that carbon atoms adjacent to oxygen or nitrogen atom (peak around 70 ppm) were not affected by ozone treatment indicating a strong resistance to oxidation. The saturated and aliphatic carbon atoms are supposed to comprise the principal skeleton or backbone of melanoidin '1'.

Moreover, the ozone treatment is supposed to lead the cleavage of C=C or C=N bonds. These unsaturated bonds have been suggested to be important for the structure of chromophore. Many workers (Martins et al, 2001) have also reported similar results for non-dialyzable Maillard polymers prepared from glucose-glycine system reacted at pH 3.5 thus corroborating Kim *et al.* (1985) postulates. Studies using glycine (2-^{13}C) have revealed that most of the glycine was incorporated into melanoidins. Further, it was speculated that the nitrogen in melanoidins was mainly due to the conjugated enamine linkage and partly to amine linkage.

However, Kato and Hayase (Kato and Hayase, 2002) have isolated a blue melanoidin pigment (Blue-M1, $C_{27}H_{31}N_4O_{13}$) from the reaction mixture of D-xylose and glycine in 60% ethanol stored at 26.5^0C for 48 hrs or 2^0C for 96 hrs under nitrogen as the structure of this blue pigment given by these workers (Fig. 7).

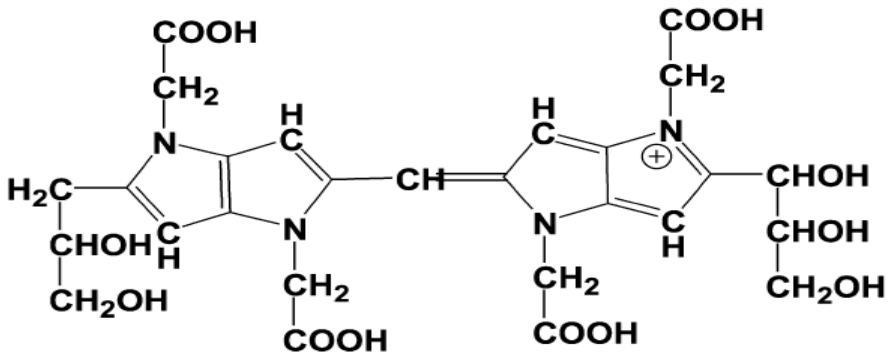

Fig.7. Chemical structure of Blue-M1

Which indicated the involvement of 3-deoxyxylosone, Xylosone and N-substituted pyrrole-2-aldehyde and has five hydroxyl groups belonging to 3-deoxyxylosone and Xylosone while Cammerer et al. (2002) have proposed the structure of basic melanoidin pigment (Fig.8) formed from 3-deoxyhexosuloses and Amadori reaction products.

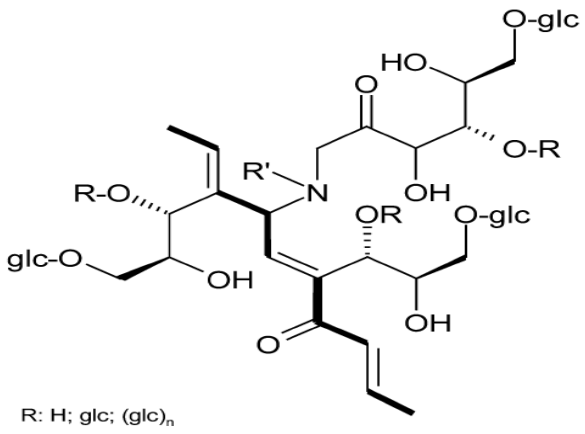

Fig. 8. Structure of basic melanoidin formed from 3-deoxyhexosuloses and ARP

In spite of these studies, the chromophore has not been yet identified. So the chemical structure of so-called melanoidin is still not clear but probably it does not have a definite one and there exists various types of melanoidins differing in structure depending on parent reactants and reaction conditions as pH, temperature and reaction time. Thus it can be assumed that in real systems the above mentioned structural proposals each partially supplement each other or that different structures co-exist. Therefore, it further needs intensive investigations with more refined recent and advanced techniques for the elucidation of chromophore structure to deduce the main skeleton of melanoidin polymer.

(c) Recent techniques available for the analysis of Maillard reaction products (MRPs)

I) Early stage MRPs

The first stable reaction products, which are formed during the early stages of the Maillard reaction, are the so-called Amadori compounds (Coca et al, 2004). These are the result of

the condensation between an amino group of amino acids, peptides or proteins and the carbonyl group of a reducing carbohydrate followed by a subsequent rearrangement. In the common case of foods containing proteins and glucose, the well-known Amadori compounds ε-fructosyl-lysine (Fig. 9) is formed. In heated milk samples, where the major carbohydrate is lactose, the formed Amadori compound is lactulosyl-lysine. This compound is often determined as processing indicator. Amadori compounds are precursors of numerous products important in the formation of characteristic flavours, aromas and brown polymers. They are also responsible for the loss of nutritional value of amino acids and proteins because their biological availability is reduced in the formation of Amadori compounds. However, the antioxidant role of the Amadori rearrangement product, fructosyl arginine, in a concentrated aged garlic extract has been described (Vinale et al, 1999).

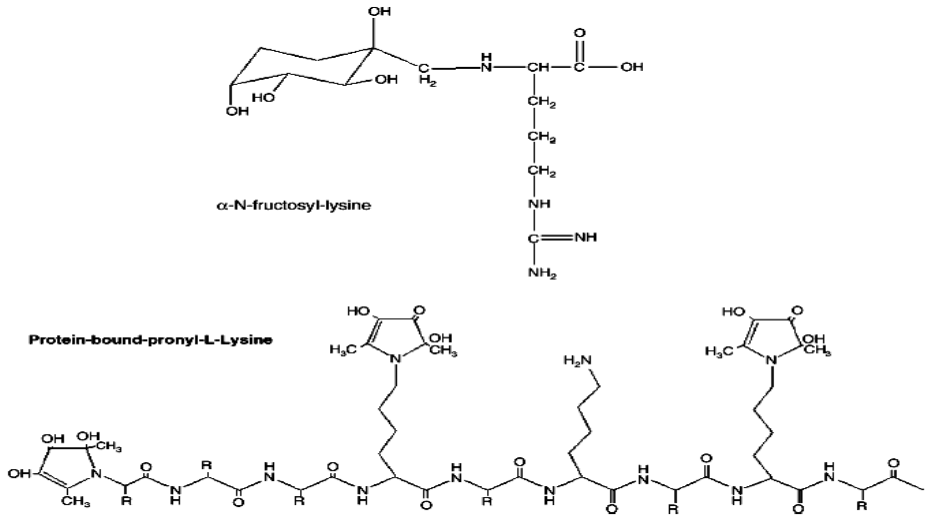

Fig. 9. MRPs with known structure and health promoting properties

Since they are formed before occurrence of sensory changes, their determination provide a very sensitive indicator for early detection of quality changes caused by Maillard reactions. The quantification of Amadori compounds in foods or physiological samples is difficult since they are converted during the acid hydrolysis of the proteins, thus making it impossible to detect them with routine amino acid analysis. Several direct and indirect methods as described below have been employed for the analysis of Amadori compounds.

(i) Direct analysis of Amadori compounds

Measurement of Amadori compounds was performed by column chromatography employing an automatic amino analyzer and a post-column reaction with ninhydrine. Alternatively, these compounds may be analysed by high-performance liquid chromatography (HPLC) with differential refractometry detection. However, the sensitivity of this method does not allow the detection of low Amadori compound concentrations. To improve sensitivity, HPLC methods involving derivatisation have also been proposed. A recent method using high-performance anion-exchange chromatography coupled with an electrochemical (ECD) and/or diode array detector (DAD) is regarded a powerful technique for the detection and monitoring of known Amadori compounds. Although gas chromatography (GC) shows better separation efficiency as compared to HPLC, the Amadori compounds need to be converted into volatile compounds prior to analysis. The necessity to derivatize and the ability of GC to separate tautomeric forms of Amadori compounds are the major drawbacks of this method. The detection of Amadori compounds can also be achieved by fast atom bombardment (FAB) tandem mass spectrometry (MS). The particular advantage of this method is its simplicity as the samples are directly introduced into the ion source. However, the simultaneous analysis of several Amadori compounds is difficult with this method. FAB has been almost completely replaced by electrospray ionisation (ESI) since this technique offers the advantage of a very soft ionisation and easy coupling to on-line separation techniques like HPLC and capillary electrophoresis (CE). The last technique (CE) is complementary to HPLC, with the advantage of a higher resolving power for the first dimension of the separation. In addition, the consumption of solvents and chemicals is low. CE coupled to MS is an alternative and powerful analytical method and allows a rapid separation and identification. Various analytical techniques have been developed for the analysis of lactulosyl-lysine in dairy products because of its usefulness as quality control indicator. Detection of lactulosyllysine has been conducted by spectroscopy techniques such as MALDI-TOF, electrospray and nuclear magnetic resonance (NMR) analysis. A selective liquid chromatography (LC)–MS procedure allowed the detection and quantification of lactulosyl-lysine after complete enzymatic hydrolysis in milk samples (Vinale et al, 1999). One of the main problems related to the direct quantification of lactulosyl-lysine and other Amadori compounds is the lack of a pure standard. The synthetic strategies proposed so far are rather time-consuming and do not yield a consistent rate of pure Amadori compound. A colorimetric method employed in foods for Amadori compounds direct detection is NBT is based on the reducing ability of fructosamines (glucose joined protein molecules) in alkaline solution, this procedure is fast, cheap and easily automated. Recently, immunological approaches have been developed to directly detect lactosylated proteins without using an hydrolysis step. Both competitive enzyme-linked immunosorbent assay (ELISA) and immunoblotting methods using specific antibodies against lactosylated proteins were employed.

(ii) Indirect analysis of Amadori compounds

Peptide-bound Amadori compounds of lysine (fructosyllysine, lactulosyl-lysine or maltulosyl-lysine) are mainly quantified indirectly. These indirect methods are either based on the quantification of reactive lysine or involves an acid hydrolysis to give 2-furoylmethyl amino acids (2-FM-AA). The latter can be directly measured by ion-pair RPHPLC or cation-exchange chromatography (CEC) both using UV-detection. HPLC–MS is a technique especially suitable for the identification of 2-FM-AA. 2-FM-AA are

common indicators used to monitor the Maillard reaction during heat treatment and storage of foods (juices, dairy products, dehydrated foods, honey and tomato products). 2-Furoylmethyl-lysine, also called furosine is a well-known indicator used to express the extend of damage in processed foods or stored foods with a long shelf life. Furosine is often determined by ion-pair RP-HPLC. The main drawbacks of this procedure are the time of analysis and the fact that only part of Amadori compound (~30%) is converted into 2-FM-AA. The Amadori compounds can also be estimated indirectly by rapid colorimetric or fluorimetric methods. These methods are based on the analysis of unreactive lysine, which can be measured through the reaction of a dye reacting with the free NH2 groups of lysine. Dyes such as o-phthalaldehyde, fluorescamine and FDNB are frequently employed. These methods have sometimes been criticized for their lack of specificity (Fay and Brevard, 2004; Hayase et al, 1986; Vinale et al, 1999).

(II) Advanced stage MRPs

Amadori compounds may undergo several degradation reactions during intense heating or prolonged storage. Depending on the incubation time and temperature, the initially formed Amadori compounds are degraded in the advanced stage to the highly reactive α-dicarbonyl compounds (e.g. glyoxal, methylglyoxal, 1-deoxyglucosulose or 3-deoxyglucosulose). The lysine and arginine side chains of proteins can react with these α-dicarbonyls to form stable peptide-bound amino acid derivates, the so-called advanced glycation end products (AGEs). To date, most AGEs have been isolated from model mixtures and are less frequently quantified in foods or biological systems. These compounds are rather important in terms of nutritional and biological aspects, moreover since AGEs can also be produced during pathophysiological processes in vivo. Diet derived AGE are major contributors to the total body AGE pool. Dietary AGEs may play an important role in the causation of chronic diseases associated with underlying inflammation. Excessive consumption of AGEs may represent an independent factor for inappropriate chronic oxidant stress and inflammatory factor surges during the healthy adult years, which over time may facilitate the emergence of complex diseases, such as diabetes and other disorders related to aging (Vagnarelli et al, 1991; Wang et al, 1987). The large effects of AGEs on human health demonstrate there is a need for good detection methods.

(III) General determination of AGEs

The fluorescence of advanced Maillard products and soluble tryptophan (FAST) method has been used to evaluate the formation of fluorescent advanced stage MRPs in milk. This method gives a rapid evaluation of the nutritional quality of milk proteins and is compatible with industrial requirements, since the method is cheap and easy to use. In model reactions, lactose was heated with primary amines and more than 12 amine-free or amine containing Maillard AGEs were separated, identified and quantified by HPLC/DAD (Silvan et al, 2006). Most of these products were specific for lactose since they were not detected in Maillard mixtures containing monosaccharides such as glucose.

(IV) Analysis of carboxymethyllysine (CML)

The first lysine derivative of the advanced Maillard reaction detected in foods was N-α-carboxymethyl lysine (CML). CML is formed from lysine and glyoxal during the

oxidative degradation of the Amadori compounds. This lysine derivate is a commonly used marker compound to evaluate the progress of the Maillard reaction in foods. CML has been evaluated in dairy products by RP-HPLC and modified RP-HPLC with o-phthalaldehyde pre-column derivatisation, which gave a high sensitivity and very good reproducibility. GC–MS analysis of the trifluoro acetyl methyl ester of CML or the silylated derivates has also been used to determine the CML content in foods. Derivatisation is required since CML is present in very low concentrations as compared to the native amino acids. Prior to GC analysis, samples are submitted to acid or enzymatic hydrolysis. ELISA using an antibody specific for carboxymethylated protein has also determined CML (Silvan et al, 2006). This method is a fast, specific and easy-to-handle procedure to evaluate CML formation in heated food products, and does not require sample work up. However, for certain samples (e.g. milk) proper control determinations are required because of background signals in the ELISA. SDS-PAGE immunoblotting can also be helpful to detect CML in insoluble food proteins.

(V) Pyrraline analysis

Another lysine derivate present in foods is pyrralin, which is formed from the amino group of lysine and 3-deoxyglucosulose, the degradation product of reducing sugars and Amadori compounds. Pyrraline can be found in food in either free or protein-bound form and can be a useful indicator to control the Maillard reaction in sterilized products that contain heat damage proteins. Pyrraline represents one of the dominating and few AGEs that have been quantified in foods such as milk, bakery products, enteral formulas and pasta. Its detection was done using either an amino acid analysis with photodiode array detection (PAD) or RP-HPLC (Coca et al, 2004) after the total enzymatic hydrolysis of the proteins.

(VI) Analysis of cross-linking products

Lysine dimmers (imidazolium compounds) result from the reaction between two lysine side chains and two molecules of glyoxal (GOLD), methylglyoxal (MOLD) or 3-deoxyglucosulose (DOLD). They were found in very low amounts in enzymatic hydrolysates of bakery products after analysis by LC–MS with ESI. The low concentrations detected indicated a minor role of lysine dimmers in protein cross-linking in foodstuffs.

Arginine–lysine cross-links such as GODIC (lysine–arginine cross-link + glyoxal), MODIC (lysine–arginine crosslink + methylglyoxal), DODIC (lysine–arginine cross-link + 3-deoxyglucosulose), glucosepan, pentosidine and imidazolinone can also be formed during the Maillard reaction. MODIC, GODIC, DODIC and glucosepan were identified and quantified in several foodstuffs using LC–MS with ESI after enzymatic hydrolysis. Quantitative results showed that MODIC were the most important Maillard cross-link. The other amino acid derivates were present in far lower concentrations. Pentosidine, resulting from the reaction of lysine, arginine and sugar derived carbonyl compounds, has been found in very low amounts in several processing foodstuffs (milk products, roasted coffee and bakery products) using ion-exchange chromatography with direct fluorescence detection and subsequent ninhydrin derivatisation. This method not only allowed the quantification of pentosidine but also the determination of all other amino acids of the acid

hydrolyzates. Imidazolinone is formed during the reaction between peptide-bound arginine and methylglyoxal and is regarded as one of the most abundant arginine crosslinking product in foods (Silvan et al, 2006; Coca et al, 2004). This compound has been quantified in alkali-treated bakery products and roasted coffee by FAB–MS.

(VII) Analysis of other amino acid derivatives

Argpyrimidine is formed from two molecules of methylglyoxal and the guanidino chain or arginine. It was identified and quantified as free acid in beer and malt by HPLC and coupled GC–MS. Oxalic acid monolysinylamide (OMA), a MRP of l-ascorbic acid, was detected in heat-treated milk products and commercial infant formulas using ELISA. In this qualitative assay, a polyclonal antibody, which binds with high specificity and affinity to OMA-modified proteins, was used. Propylimidazolinone-ornithine (PIO), resulting from the reaction between arginine with C5-dicarbonyls, represents a new type of post-translational protein modification formed during food processing. It might be responsible for the major part of arginine derivatisation in disaccharide containing foods like milk. PIO was isolated by semi-preparative RP-HPLC and identified by liquid chromatography–electrospray–time of flight–mass spectroscopy (LC–ESI–TOF–MS) and NMR. Peptide-bound pyrazinones are produced during the reaction between the N-terminal of peptides with glyoxal or methylglyoxal.

These compounds form a new class of fluorescent AGEs that may have quantitative importance in heated peptide containing foods. Using HPLC with UV and fluorescence detection, a rapid derivatisation of the peptide and the concomitant formation of well-defined products were observed. Lysine aminoreductone is another amino derivate which has been detected in heated or processed milk products by HPLC-DAD but has not yet been quantified (Silvan et al, 2006).

(VIII) Final stage MRPs

During the Maillard reaction brown coloured high molecular weight peptide-bound MRPs known as melanoidins are formed. Although not yet identified or quantified in browned foods, these compounds stand for a new class of MRPs. Melanoidins can be generated by a cross-linking reaction between a low molecular weight chromophore and a noncoloured high molecular weight biopolymer. Their composition strongly depends on the food composition and the technological conditions. Melanoidins appear in several heat treated foods and represent a significant part of our diet, with an average intake of grams per day. In spite of extensive studies, the exact chemical species responsible for this brown colouration still remains undefined. From the technological point of view, melanoidin formation is essential for food flavour, colour and texture. Different studies have also demonstrated the high antioxidant capacity of melanoidins, which substantially contributes to the shelf life of heat-treated foods and physiological processes in vivo. Melanoidins demonstrated antioxidant capacity through a chain breaking, oxygen scavenging and metal chelating mechanism without showing cytotoxic effects. Currently unidentified melanoidins, present, e.g. in coffee beverages, are hypothesized to act as antioxidants but also act in the activation of chemopreventive enzymes. In addition, several Maillard compounds identified as constituents of melanoidins were found to inhibit tumour cell growth. Several attempts have been undertaken to isolate and purify melanoidins from

foods such as coffee, dark beer and soya sauce. However, owing to the complexity of the melanoidin fraction in foodstuffs, it has been difficult to characterise a pure melanoidin. Identification of coloured structures after careful hydrolysis of the protein skeleton provided useful information regarding the chromophores, which are linked to it. After complete enzymatic digestion of the protein skeleton, two intense red-coloured chromophore substructures by HPLC have been identified (Silvan et al, 2006). These compounds were subsequently identified using several NMR techniques, MS, UV and IR spectroscopy. Melanoidins have also been analysed by MALDI-TOF mass spectrometry after separation by gel filtration chromatography. One melanoidin structure, called pronyl-l-lysine (Fig. 8) has been identified as a key antioxidant formed during heat treatment of Maillard-type reaction mixtures. This compound results from the reaction between lysine side chains and acetyl formoin and has been detected and quantified in the crust and crumb of bread by high-resolution GC–MS in the chemical ionisation mode (CI). It was confirmed that pronyl-lysine modulates a chemo preventive phase II enzyme, the glutathione S-transferase (GST). The induction of this enzyme by pronyl-lysine represents a promising strategy for cancer prevention. Recently, bread and coffee melanoidins have also been described as potential prebiotic ingredients (Silvan et al, 2006; Fay L.B. and Brevard, 2004; Coca et al, 2004). Hence, the study of melanodins in health is gaining interest due to their molecular complexity, lack of stability and biological functions.

Table.1: Analytical techniques for the analysis of Maillard reaction products (MRPs)

Maillard reaction products (MRPs)	Analytical techniques
Amadori compounds (Direct analysis)	Column chromatography
	HPLC differential refractometry detection
	HPLC involving derivatisation
	HPAEC coupled electrochimemical and/or DAD
	FAB–MS
	ESI coupled HPLC and EC
	EC coupled MS
	MALDI-TOF
	NMR
	LC–MS
	NBT
	ELISA

	Immunoblotting (lactosylated proteins)
Indirect analysis 2-FM-AA	Ion-pair RP-HPLC
	CEC UV-detection
	HPLC–MS
Unreactive lysine	Colorimetric and fluorimetric methods
Advanced Maillard products General AGEs	FAST
	HPLC-DAD
CML	RP-HPLC
	RP-HPLC *o*-phthalaldehyde pre-column derivatisation
	GC–MS
	ELISA
	Immunoblotting
Pyrraline	Amino acid analysis with PAD
	RP-HPLC
Crosslinking products Lysine dimmers	LC–MS with ESI
Arginine–lysine	LC–MS with ESI
	Ion-exchange chromatography
	FAB–MS
Other amino acid derivates	
Argypirimidine	HPLC-coupled GC–MS
OMA	ELISA
PIO	RP-HPLC/LC–ESI–TOF–MS/NMR
Pyrazinones	HPLC with UV and fluorescence detection
Lysine aminoreductone	HPLC-DAD
Final stage MRP's	

General melanoidins	HPLC, NMR, MS, UV, IR spectrometry
	MALDI-TOF mass spectrometry
Pronyl-l-lysine	GC–MS chemical ionisation

(d) Biological properties of the Maillard reaction products (MRPs)

The Maillard reaction can cause both deterioration and enhancement of food quality. In the past, many scientific works focused on the negative biological effects of the Maillard reaction. The formation of antinutritional and toxic Maillard reaction products (MRPs) has been reported frequently. In vitro studies revealed some harmful effects including mutagenic, carcinogenic and cytotoxic effects. Excessive glycation has also been stated to cause the destruction of essential amino acids, decreased digestibility, inactivation of enzymes, inhibition of regulatory molecule binding, cross-linking of the glycated extra-cellular matrix, decreased susceptibility to proteolysis, abnormalities of nucleic acid function, altered macromolecular recognition and endocytosis and increased immunogenicity. The formation of beneficial compounds during the Maillard reaction has also been found and is currently gaining a lot of attention. MRPs containing antioxidant, antiallergenic, antimicrobial and cytotoxic properties are amongst others mostly detected. Many studies focussed on the high antioxidant capacity of MRPs in model systems and foods such as beer, coffee and bakery products. In those studies it was shown that MRPs could contribute greatly to the shelf life of heat-treated foods. In vitro studies demonstrated that MRPs might offer substantial health-promoting activity as they can act as reducing agents, metal chelators and radical scavengers. It appears that especially low molecular weight MRPs exhibit antioxidant effects in the organism after they get absorbed by the small intestine. Both consumers and regulatory organizations demand high quality, healthy and safe food (ingredients) while food scientists attempt to develop new processes to obtain these. In addition, there is a great interest in novel healthy food ingredients with a large work done in this area. Several papers indicate that the Maillard reaction can be a good means of producing functional food ingredients also since they can be obtained without the use of harmful chemicals and tedious purification procedures. The determination of MRPs with their beneficial or harmful properties is thus of key importance for the production of safe foods and for the development of novel functional food ingredients. Most of the work focused on the biological properties of MRPs has demonstrated the formation of a large pool of compounds without knowing accurately which one is responsible for a particular biological activity. The analysis of known indicators can help understand at what stage of the Maillard reaction the health-promoting compounds are produced. Those indicators may then be employed to control the industrial production of these health-promoting MRPs, which are regarded as promising new functional food ingredients (Silvan et al, 2006; Vagnarelli et al, 1991; Wang et al, 1987; Castillo et al, 2002; Nicoli et al, 1997; Woffenden et al, 2001).

5.3 Degradation of melanoidin polymer

Melanoidins, the brown polymers are very recalcitrant in nature. It exists extensively not only in various foods but also in wastes released from many agro-based industries as sugarcane molasses based distillery and fermentation industries and keeping-in-view the hazardous nature of melanoidins, its chemical and biological (microbial) treatment has been attempted in order to reduce the pollution load and also to characterize its chemical structure so that better strategies could be made for its degradation. The noteworthy work done in this direction is the subject of this book chapter.

(a) Chemical degradation

Many workers had studied the chemical degradation and decolourisation of melanoidin pigment. It had been attempted on both model as well as natural melanoidins present in molasses spent wash, Cammerer et al (2002) have studied the chemical degradation of model melanoidin formed from oligomer and polymeric carbohydrates and amino acids and they reported that with increasing the degree of polymerization (dp) of carbohydrates used as starting material in the Maillard reaction, the release of monosaccharides was increased from about 3% (Glc) to 95% (Dex). Furthermore, investigations on lactose melanoidins indicated that the carbohydrates preferentially were released from the terminal end of saccharides incorporated in the melanoidin because galactose (Gal) is detected as main hydrolytic product from a lactose melanoidin. Subsequent hydrolytic reactions performed on melanoidins showed that the amount of intact monosaccharides released was dependent on reaction conditions used for melanoidin formation. For the release of Glc from a Glc/Gly melanoidin model formed under water-free reaction conditions, an explanation can be given by transglycosylation reactions that take place during Maillard reaction. A free Glc molecule might react via the formation of a glycosyl-cation either with another Glc or with α-dicarbonyl compounds like deoxyhexosulose to 1,6 branched saccharides (Kroh et al, 1996). Thus, Glc may be incorporated as side chain in the melanoidin skeleton and is easily split off by hydrolysis (Fig. 10).

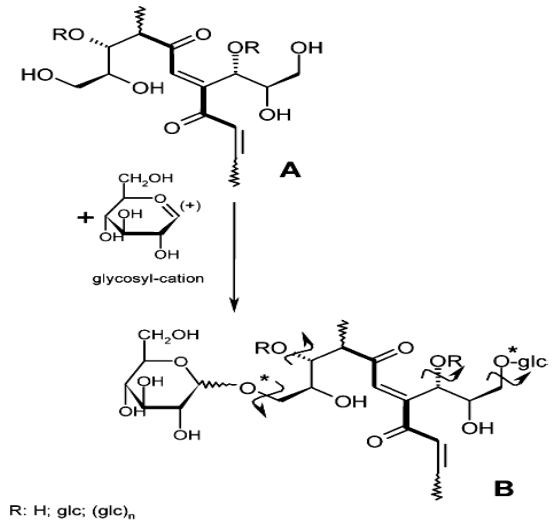

Fig. 10. A part of possible melanoidin structure formed from 3-deoxyhexosuloses (A) and schematic mechanism of 1,6-branched glucose side chain formation by Trans-glycolation (*) and possible hydrolytic scissons (⌐)

In contrast, in aqueous systems Glc mainly undergoes retro-aldol reactions to form high reactive C2, C3 and C4 dicarbonyl compounds that are involved in the Maillard reaction instead of the Glc molecule. Using di- and oligosaccharides as carbonyl components in the Maillard reaction, a significantly higher amount of sugars than in monosaccharide melanoidin models can be released by acid hydrolysis. These results confirm that under given Maillard reaction conditions the glycosidic bonds of di- and oligosaccharides mostly remain unchanged, which leads to the formation of a melanoidin skeleton with carbohydrate side chains. Additional carbohydrate side chains might be formed by transglycosylation reactions. The glycosyl-cation necessary for this reaction can be formed more easily from Mal than from Glc because Glc is a better leaving group than an OH-group (Kroh et al, 1996). With longer reaction time and increasing water content the stability of the glycosidic bond in di- and oligosaccharides is mainly responsible for the structure of melanoidin formed. Additional retro-aldol reactions had to be taken into account in all Maillard reaction mixtures. It causes a greater variety of possible reactive intermediates. Whereas changes in the basic melanoidin skeleton of di- and oligosaccharides might be only marginal, the number and structure of side chains can differ significantly. The possibility to release intact glucose molecules from melanoidins formed from di- or oligosaccharides indicates that during Maillard reaction of carbohydrate/amino acid model systems, a reaction involving the reducing OH-group of carbohydrate is the preferred mechanism. Dicarbonyl groups in intermediate products can be assumed as reactive centers. It is evident that the main formation mechanisms for melanoidins from mono- and disaccharides with amino acids are comparable with those of oligosaccharides.

However, Yaylayan and Keminsky (1998) have studied the pyrolytic degradation of nitrogenous and non-nitrogenous polymers produced in initial and later stages of Maillard reaction respectively. They reported that both nitrogenous and non-nitrogenous polymers of Maillard reaction produced a number of pyrolysis products (Table 2 and 3) during pyrolytic degradation

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Table 2. Pyrolysis product of nitrogenous

% Area	Compound
3.52	Acetic acid, methyl ester
25.23	Acetic acid
6.04	1-Hydroxy-2-propanone
2.77	1-Methyl-1H-pyrrole
2.69	2,3-Butanediol
1.96	Pyridine
0.43	4-Methyl-pyridine
3.43	Methy-pyrazine
2.02	2-Furancarboxaldehyde
0.96	1H-Pyrrole-2,4-dimethyl
2.87	Cyclopent-2-en-1,4-dione
0.76	1-(2-Furanyl)-ethanone
3.88	2,6-Dimethyl-pyrazine
0.88	2,3-Dimethyl-pyrazine
1.72	5-Methyl-2-furancarboxaldehyde
2.16	Trimethyl-pyrazine
0.67	1-(1H-pyrrol-2-yl)-ethanone
1.53	3-Hydroxy-2-methyl-4H-pyran-4-one (maltol)
1.89	5-Methyl-1H-pyrrole-2-carboxaldehyde
2.43	2,3-Dihydro-3,5-dihydroxy-2-methyl-4H-pyran-4-one

Table 3. Pyrolysis product of non-nitrogenous polymer

% Area			Compound
B1	B2	Sucrose	
2.18	2.36	1.71	Formic acid
7.47	5.30	5.33	Acetic acid
0.00	1.82	0.11	2-Methylfuran
1.60	4.30	0.85	1-Hydroxy-2-propanone
7.65	10.21	39.50	2-Furancarboxaldehyde
0.48	0.73	0.78	2-Furanmethanol
0.36	0.40	0.35	2(3H)-Furanone-5-methyl
2.67	1.24	0.0	Cyclopent-2-en-1,4-dione
0.60	0.70	0.57	1-(2-Furanyl)-ethanone
0.00	1.49	0.45	1,3-Cyclopentanedione
6.91	2.35	0.33	5-Methyl-2-furancarboxaldehyde
0.00	0.13	0.0	2,2′-Bifuran
0.00	0.25	0.0	2-Hydroxy-3-methyl 2-cyclo-penten-1-one
2.46	1.46	0.0	2-Furancarboxylic acid
0.00	0.84	1.43	3-Furancarboxylic acid, methyl ester
0.00	0.26	0.0	2H-Pyran-2-one
0.02	0.06	0.13	3-Hydroxy-2-methyl-4H-pyran-4-one (maltol)
8.64	4.32	2.07	2,3-Dihydro-3,5-dihydroxy-2-methyl-4H-pyran-4-one,
1.48	0.86	0.0	3,5-Dihydroxy-2-methyl-4H-pyran-4-one
34.5	35.59	33.57	5-(hydroxymethyl)-2-furancarboxalde-hyde
0.07	0.00	0.0	[2,2′-Bifuran]-3-carboxylic acid
0.48	0.00	0.30	5-[(5-Methyl-2-furanylmethyl)1-2-furan-carboxaldehyde

Hayase *et al.* (1984) had studied the chemical decolourisation of model melanoidin prepared from a glucose-glycine system by hydrogen peroxide treatment. They reported about 64% and 97% decolourization of melanoidin [6.72% (v/v)] using hydrogen peroxide in neutral (pH 7.0) and alkaline (pH 10.0) environment respectively under optimum conditions and the mean molecular weight of melanoidins, after H_2O_2 treatment decreased from 5,300 to 3,500. The major components in the ether soluble fraction obtained from melanoidins by oxidative degradation of alkaline H_2O_2 were identified as 2-methyl-2, 4-pentanediol, N,N-dimethylacetamide, phenol, acetic acid, oxalic acid, 2-furancarboxylic acid, furandicarboxylic acid and 5-(hydroxymethyl)-2-furancarboxylic acid.

Kim *et al.* (1985) had studied the decolourisation and degradation of non-dialyzable melanoidin prepared from glucose-glycine by ozone treatment. They reported that melanoidins were decolourised up to 84 and 97% after ozonolysis at -1^0C for 10 min and 90 min respectively. The mean molecular weight of melanoidins, after ozonolysis for 40 min, decreased from 7000 to 3000. They observed that in post ozonolysis IR measurements become disappear at 1620 cm^{-1} and appearance of new absorption at 1720 cm^{-1} respectively. The absorption at 1620 cm^{-1} disappeared on acid hydrolysis after ozonolysis. Furthermore, the major degradation products in the ether-soluble fractions obtained from O_3-treated melanoidin were identified as butanedioic acid, glycolic acid, 2-hydroxybutanoic acid and so on while glycine was one of the major products produced at the level of 1.05% per melanoidin on acid hydrolysis. They finally concluded that glycine was considerably incorporated into melanoidin molecules as amide form.

Chandra and Singh (Chandra and Singh, 1999) had investigated the decolourisation of colouring component of distillery effluent using aluminium sulphate and bleaching powder and they reported about 96.4% decolourisation using 0.3% (w/v) aluminium

sulphate and 0.5% (w/v) bleaching powder. Furthermore, they have also reported a remarkable decrease of 92% and 95% respectively in parameters like BOD, COD and heavy metals accompanying decolourisation of distillery effluent.

Many workers too have studied the decolourisation of spent wash melanoidin pigment by chemical method. They treated spent wash containing melanoidin pigment with hydrogen peroxide [(0.5% v/v)] and calcium oxide [CaO_2, 0.5% (w/v)] and obtained ~54% decolourisation. Furthermore, they reported a possible mechanism of decolourisation using CaO_2+H_2O_2 because due to calcium oxide (CaO_2) the solution (containing melanoidin) becomes alkaline. This solution has abundant perhydroxyl anion. The hydrogen peroxide reacts with hydroxyl anion, which gives mainly perhydroxyl anion (HOO^-), which has strong nucleophilic activity. Hence, the decolourisation might have been due to the hydrogen peroxide reaction.

Chandra and Pandey (Chandra and Pandey et al, 2001) reported ~99% decolourisation of anaerobically treated distillery effluent by adsorption on activated charcoal. The decrease in colour was accompanied with simultaneous reduction of 96.0%, 50.6%, 35% and 52% in BOD, COD, TS, TSS and TDS respectively. They also reported a remarkable decrease of about 56.0 to 81%, 42 to 89% and 12-10% in phenol, sulphate and heavy metals respectively along with removal of melanoidin.

(b) Biological/microbial degradation

Microorganisms (bacteria/fungi/actinomycetes) due to their inherent capacity to metabolize a variety of substrate have been utilized since long back for biodegradation of complex, toxic and recalcitrant compounds which cause severe damage to environment. Thus, these organisms have been exploited for biodegradation and decolourisation of melanoidin pigment present in industrial wastes especially from distillery and fermentation industry.

(i) Fungal Degradation of Melanoidin pigment

Fungi, due to their characteristic morphology (i.e. developed hyphae/mycelium) have excellent adsorption property. These also possess well developed enzymatic system to breakdown complex substrates to derive metabolic energy. Due to such unique features, fungi have been widely exploited for the degradation and decolourisation of melanoidin containing wastewater.

Watanabe *et al.* (1982) used *Coriolus* sp. among different basidiomycetes strains such as *Trametes, Pleurotus, Agaricus, Auricularia, Pholiota, Collybia, Cortirellus* etc. screened for melanoidin decolourising activity (MDA) to decolourise melanoidin pigment and characterize the enzyme(s) responsible for this activity. They reported a decrease of 77% in darkness of melanoidin solution [0.5% (v/v)] under the culture conditions at 30^0C for two weeks.

Similarly, Aoshima *et al.* (1985) had screened about 23 genera, 30 strains belonging to white and brown rot fungi for melanoidin decolourisation. They reported that melanoidin decolourization activity was found only in some genera of white-rot fungi (e.g. *Coriolus hirsutus, Coriolus versicolor* Ps4a, *Fomitopsis, Cystisina, Irpex lacteus* Ps8a,

Lenzites betulina L5b etc.) of which *Coriolus versicolor* Ps4a showed highest activity, a decolourization yield of approximately 80% under the optimal conditions. They also reported that production of MDA by *C. versicolor* was almost completely coincident with the growth of mycelia and was mainly due to intracellular enzymes and induced by the molasses melanoidin pigment.

Ohmomo *et al.* (1985b) had studied the continuous decolourisation of molasses waste treated by means of methane fermentation and activated sludge with the mycelia of *Coriolus versicolor* Ps4a under both free-cell and immobilized conditions. They attained a decolourization yield of approximately 75% in a bubbling column reactor and found that optimum decolourization with bare pellet-type mycelia in shaking flasks needed the addition of external carbon [glucose, 0.5%(w/v)] and nitrogen [peptone, 0.05% (w/v)] and aerobic conditions (1 ppm of dissolved oxygen).

Their studies on waste water decolourisation with mycelia immobilized within calcium alginate gel in a bubbling column reactor under the optimum conditions yielded constant decolourisation (65.7%) during continuous decolourisation for 16-days. They also stressed the need of added glucose and peptone to be necessary to maintain the MDA of mycelia.

Later, they have reported the products of enzymatic decolourisation of melanoidin using crude enzymes isolated and purified from mycelia of *Coriolus versicolor* Ps4a. They obtained some amino acids and organic acids from decolourised fraction of melanoidin. The organic acid fraction from glucose-glycine melanoidin (GGM) gave lactic acid as major acid and formate, oxalate etc. as minor acids. The amount of lactic acid was equivalent to 10.5% of the carbon in melanoidin.

Ohmomo *et al.* (1987) screened fungi that were found able to decolourise molasses melanoidin in the tropical zone and isolated some strains mainly of genus *Aspergillus*. They found that a strain no. G-2-6 most active, thermophilic and identical with *Aspergillus fumigatus*. *A. fumigatus* (G-2-6) decolourised about 75% of molasses melanoidin pigment when it was cultivated on a glycerol peptone medium at 45^0C for 3-days under shaking conditions. They also investigated the continuous decolourisation of molasses melanoidin pigment in a jar fermentor and found that it had an almost constant decolourisation yield of about 70%.

At the same time, they observed the decrease of about 51% in chemical oxygen demand (COD) and 56% removal of the total organic carbon (TOC) in initial solution. In contrast, they reported continuous decolourisation of non-dialyzed molasses melanoidin removed a little more COD and TOC than that of dialyzed molasses melanoidin but had a lower level of melanoidin decolourising activity (about 40%).

They (Ohmomo et al, 1987) have also studied the molasses melanoidin decolourisation by using the different strains of *Aspergillus oryzae* (eg. *A. oryzae* IAM 2731, *A. oryzae* IFO 5786 and *A. oryzae* Y-2-32) grown on glycerol-peptone medium and found that *A. oryzae* Y-2-32 was most active which decolourised about 75% melanoidin when cultivated at 35^0C for 4-days under shaking conditions. They observed that the type of sugars utilized for growth influenced the degree of adsorption. They also observed that

the melanoidin-adsorbing ability of mycelia was repressed by a high concentration of salt (e.g. NaCl or buffer).

Sirianuntapiboon *et al.* (2005) screened 228 strains of filamentous fungi belonging to classes Deuteromycetes and Basidiomycetes possessing the ability to decolourise molasses melanoidin pigment. They found that strain D-90 has shown the highest decolourisation potential (~93%) when it was cultivated at 30^0C for 8-days in molasses solution containing (w/v): glucose (2.5%), yeast extract (0.2%) and inorganic salts (KH_2PO_4 0.1% and $MgSO_4.7H_2O$ (0.05%). The strain D-90 was later found to be identical with the order *Mycelia sterilia.* Further, they carried out a detailed studies on the decolourisation of molasses wastewater by *Mycelia sterilia* D-90 and reported 90% decolourisation of molasses pigment in 10-days. They also demonstrated a simultaneous decrease of 80% in biological oxygen demand (BOD) by supplementing (w/v): glucose 2.5%, $NaNO_3$ 0.2%, KH_2PO_4 0.1% and $MgSO_4$ $7H_2O$ 0.05% as nutrient. They reported that decolourisation yield was 17.5% in absence of nutrient. Furthermore, *Mycelia sterilia* (D-90) showed the decolourisation yield of about 70% in 11 days and caused a decrease in BOD value of about 90% in 15 days under non-sterile conditions. In fed batch system the same strain showed constant decolourisation yield of about 80% and caused a decrease in BOD value of about 70% during three times replacement (24 days).

Fahy *et al.* (1997) have reported the decolourisation of molasses spent wash (MSW) by *Phanerochaete chrysosporium* ATCC 24725 and stated that *P. chrysosporium* decolourised molasses spent wash [6.25%, (v/v)] supplemented with glucose [2.5% (w/v)] about 85% after 10-days incubation. They observed that in presence of both carbon (glucose) and nitrogen (peptone) sources the decolourisation yield decreased upto 21%. They have also investigated MSW decolourisation under immobilized conditions using calcium alginate gel and reported that immobilized cells decolourised MSW more rapidly than free cells but there was an overall colour decrease (59%) after 10 days. Later, they reported the microbial decolourisation of melanoidin containing wastewater by using the fungus *Coriolus hirsutus* along with activated sludge. They found that pretreatment of heat treatment liquor (HTL) a melanoidin containing wastewater by activated sludge could enhance the fungal decolourisation of HTL by two to three folds. They also studied the role of Mn on HTL decolourisation as well as peroxidase production during HTL decolourisation.

(ii) Bacterial Decolourisation of Melanoidin pigment

The reports on the decolourisation of melanoidin polymer by bacterial strains are very recent. Due to versatility in the nature of nutrient utilisation, the bacteria are capable to degrade different xenobiotic compounds including melanoidin polymer.

Ohmomo *et al.* (1988b) screened some facultative anaerobes with melanoidin decolourising activity (MDA). They reported that strain W-NS showed high and stable MDA and was identical to *Lactobacillus hilgardii*. The decolourisation yield of this strain under optimum conditions was 28%. However, the immobilization of cells within Ca-alginate gels improved the decolourisation yield to 40%. They also observed that unlike Ascomycetes and Basidiomycetes, this strain decolourised smaller molecular weight fractions of melanoidins quickly. They also reported the MDA of this strain towards

various synthetic melanoidins (e.g. from glucose and glycine; glucose and valine etc.). The MDA of this strain was found quiet different from that of Basidiomycetes.

Investigations on the continuous decolourisation of molasses wastewater (MWW) by using the immobilized *Lactobacillus hilgardii* W-NS cells have shown the maximal decolourisation yield (90%) in presence of glucose [1% (w/v)] at 45^0C. Further, the successive decolourisation of MWW with the recycling of immobilized cells was recorded more than 90% of the maximal decolourisation that was maintained for one month when peptone (0.05%) was added to MWW. However, on adjusting the medium pH to neutral (pH 7.3) compared with the maximal pH value (5.0) has slowed down the decrease in the decolourisation yield.

Kumar *et al.* (1997a; 1997b) reported that two aerobic bacterial cultures LA1 and D-2 showed the highest decolourisation (36.5 and 32.5%) and COD reduction (41 and 39%) respectively under optimum conditions. They suggested that the decolourisation achieved might be due to the degradation of smaller molecular weight fractions of melanoidin. They ruled out the possible involvement of manganese dependent peroxidase and all other lignolytic peroxidases as suggested by previous workers in the decolourisation of melanoidin containing molasses spent wash. They also suggested that decolourisation may have occurred as a result of secondary metabolic reaction resulting from a secondary metabolite. Nevertheless, the actual mechanism of melanoidin degradation according to them remains yet to be confirmed. Later, they have reported that a facultative anaerobic bacterial strain L-2 belonging to genus *Lactobacillus* showed high decolourisation activity. It achieved 31% decolourisation and 57% COD reduction after 7-days incubation at 37^0C in 12.5% (v/v) diluted spent wash.

Nakajima Kambe *et al.* (1999) performed screening of various microorganisms for the assessment of ability to decolourize melanoidin containing molasses wastewater under thermophilic and anaerobic conditions and selected a strain (MD-32) of the genus *Bacillus*, most closely resembling with *B. smithii*. The strain decolourised molasses pigment upto 35.5% within 20-days at 55^0C under anaerobic conditions but showed no decolourisation activity when aerobically cultivated. They also reported that the decolourisation of molasses pigment by isolated strain was accompanied by a decrease in not only small (i.e. low molecular weight) molecules but also larger (i.e. high molecular weight) ones.

(iii) Actinomycetes decolourisation of Melanoidin

Until 1992, there had been hardly any report on the decolourisation of molasses melanoidin by actinomycetes. But Murata *et al.* (1992) have obtained a strain of *Streptomyces werraensis* TT14 after extensive screening of about 75 actinomycetes, which decolourized the model melanoidin pigment prepared from glucose and glycine. The strain TT14 showed the highest decolourizing activity 64% in optimum medium (2.0% starch, 1.0% yeast, 0.3% NaCl, and 0.3% $CaCO_3$) and 45% in a synthetic medium at pH 5.5 and 40^0C for 4-days.

They also tested the properties of decolourised melanoidin and reported that Cu-chelating activity of decolourised melanoidin was decreased to about half of the intact melanoidin. The electrofocussing patterns of melanoidin also differ from each other. A component of iso-electric point (PI) 2.45, not existing in intact melanoidin was formed in

microbial treated melanoidin. The melanoidin component of PI 2.5 was increased and that of PI 3.5 was reduced by the microbial treatment.

(iv) Melanoidin degradation pathway

Many workers have studied the chemical and microbial decolourisation and degradation of model as well as natural melanoidin present in sugarcane molasses based distillery effluent. But nobody has given the clear mechanism of melanoidin degradation so far. It is mainly due to the complexity of the Maillard reaction and uncertainty about the definite structure of melanoidin polymer because the elemental composition and chemical structure of melanoidin polymer mainly depend on the nature and molar concentration of parent reactants and reaction conditions as pH, temperature, heating time and water content during reaction.

5.4 Effect of environmental factors on melanoidin decolourisation

Environmental factors like pH, temperature and aeration etc. play vital role in any biological process. Thus, several studies have been made by various workers to understand the role played by different factors in melanoidin decolourisation and also to optimize the conditions to achieve maximum decolourisation by using different microorganisms.

However, the effect of different environmental factors on melanoidin decolourisation are stated as:

(a) Effect of pH

Hayase *et al.* (1984) while studying the melanoidin degradation by hydrogen peroxide has investigated the effect of pH on it. They studied the melanoidin decolourisation at different pH (pH 3 - pH 13). They found that melanoidin decolourisation in alkaline pH proceeds more rapidly than in acidic and neutral pH and it reached 94% at pH 10. Thus, the degree of decolourisation was found to be pH dependent.

Sirianuntapiboon *et al.* (2004) have studied the effect of initial medium pH on the decolourizing activity of various fungal isolates. All isolates were found to have high decolourizing activity in acidic medium (pH 6.0). Ohmomo *et al.* (1985b) later reported the effect of initial medium pH (2.8 to 7.2) on melanoidin decolourisation activity (MDA) of anaerobic bacterial isolate *Lactobacillus hilgardii* W-NS and they found that the degree of melanoidin decolourisation was pH dependent and the optimum pH for maximum MDA was ranged between 4.0 to 4.5. Furthermore, they have also examined the effect of initial pH (3.0 to 7.2) of the reaction mixture on MDA of immobilized bacterial cells. They reported that pH 5.0 was optimum for immobilized cells and the profile of pH curve of immobilized cells were similar to that of the free cells.

Kumar *et al.* (1997a) reported that media pH had a marked influence on the decolourisation of melanoidin containing anaerobically digested distillery spent wash with a facultative anaerobic bacterial strain with the highest percentage achieved between 7.0 to 7.5, which is the most preferred range for bacterial growth. Same year they have also

studied the effect of pH on decolourisation of anaerobically digested molasses spent wash containing molasses melanoidin by aerobic bacterial cultures and they found that selected bacterial strains showed a wide range of suitable pH (4.0 to 7.5) for melanoidin decolourisation with the highest activity at pH 7.0, which is typical of mesophilic bacterial cultures.

(b) Effect of temperature

Hayase *et al.* (1984) have examined the effect of temperature on the degree of melanoidin decolourisation by hydrogen peroxide at neutral (pH 7.0) and alkaline (pH 10.0) conditions at 37^0C and 90^0C respectively. The degree of decolourisation was predominantly influenced by the difference of temperature under neutral conditions but the differences were small under alkaline conditions in comparison to neutral conditions. They reported a high decolourisation degree (>95%) at alkaline rather than the neutral pH at higher temperature.

Ohmomo *et al.* (1985b) have demonstrated the effect of culture temperature on melanoidin decolourizing activity at different (25 to 45^0C) temperature. They reported that optimum temperature for anaerobic bacterial strain (*L. hilgardii*) to decolourise the maximum melanoidin pigment was 35 to 40^0C. They also reported the effect of reaction temperature (25 to 50^0C) on melanoidin decolourising activity of immobilized bacterial cells of *L. hilgardii*. They found that the maximum decolourisation yield increased in proportion to the temperature and the optimum temperature was 40 and 45^0C for free and immobilized cells respectively. Furthermore, the melanoidin decolourising activity of cells was lost on exposure to high temperature (50^0C) for a long time.

Kumar *et al.* (1997a) reported that the effect of temperature on spent wash melanoidin decolourisation by bacterial strains (LA1 and D-2) showed a wide range of suitable temperatures (25 to 40^0C) with the highest activity at 35^0C. Nakajima Kambe *et al.* (1999) have studied the effect of temperature ranged between 30 to 60^0C on decolourisation activity of thermophilic *Bacillus* strain MD-32 under anaerobic conditions and reported the maximum decolourisation yield at temperatures between 50 to 60^0C especially at 55^0C. The decolourizing activity of MD-32 was least at 30^0C.

(c) Effect of Oxygen

Ohmomo *et al.* (1985b) have also studied the influence of shaking rates on decolourisation yield of melanoidins. The shaking rate of more than 100-oscillation min^{-1} gave a sufficient decolourisation and the dissolved oxygen in that system was approximately 1 ppm. The maximum decolourisation was attained at 125 oscillation min^{-1}. They also reported the shaking rate of 80 or less oscillation min^{-1} gave only two third or less of the maximum decolourisation yield.

(d) Effect of Nutrients

Aoshima *et al.* (1985) examined the effect of various carbon (sugars) and nitrogen sources on the decolourisation of molasses melanoidin pigment by *C. versicolor* Ps4a and reported that organic nitrogen source as urea was unsuitable for high melanoidin decolourisation activity (MDA). Of the tested nitrogen source {e.g. corn steep liquor, meat extract, peptone, yeast extract, urea, $NaNO_3$, NH_4NO_3 and $(NH_4)_2$ SO_4 etc}, peptone was

most advantageous for the production of MDA. They also reported that decolourisation required essentially some specific sugars like glucose, sucrose and maltose giving especially high MDA. The glucose was the best source of carbon for the highest melanoidin decolourisation activity among the chosen carbon sources (e.g. Arabinose, Ribose, Fructose, Galactose, Maltose, Mannose, Xylose, Sucrose and Mannitol etc.). Furthermore, increasing the concentration of glucose to more than 5% resulted only increase in the mycelial mass and decolourisation yield but showed no correlation with the MDA.

Ohmomo *et al.* (1987) studied the effect of nitrogen and carbon sources (sugars) on the production of MDA by *Aspergillus fumigatus* G-2-6. They reported that organic nitrogen source (peptone) was effective in mycelial growth resulting a high MDA (58.8%) but inorganic sources as $NaNO_3$, NH_4Cl were found unsuitable for both mycelial growth and MDA. On the other hand, all the sugars as glucose, xylose, sorbose, maltose, sucrose, glycerol and mannitol were found effective in mycelial growth. However, only maltose, mannitol and glycerol were found suitable to obtain the high decolourisation yield (>65%).

Ohmomo *et al.* (1987) reported that in melanoidin decolourisation by *Aspergillus oryzae* Y-2-32, an organic source of nitrogen (peptone) resulted not so high level of MDA but it was effective for the mycelial growth while inorganic nitrogen sources as $NaNO_3$ and NH_4Cl were found unsuitable for the mycelial growth but $NaNO_3$ resulted a high level of decolourisation (~61%) whereas among different sugars (viz., glucose, arabinose, xylose, sorbose, maltose, sucrose, glycerol and mannitol) the use of mannitol, maltose and glycerol was effective to obtain the high decolourisation (>65%). However, the use of xylose was found unsuitable for high MDA inspite of the satisfactory mycelial growth.

Miyata *et al.* (1998; 2000) observed that the medium containing glucose required no nitrogen source for the decolourisation of synthetic melanoidin by fungus *Coriolus hirsutus.*. Furthermore, the study on the influence of inorganic nitrogen sources (NH_4^+ and NO_3^-) on the efficiency of melanoidin decolourisation revealed that NH_4^+addition caused a small decrease while addition of NO_3^- did not decrease the melanoidin decolourisation. Thus, an increase in melanoidin decolourisation efficiency appeared to occur under organic N-rich rather than inorganic N-rich condition.

(e) Effect of inoculum size

Ohmomo *et al.* **(1985b)** examined the effect of mycelial concentration on melanoidin decolourising activity of *Coriolus versicolor* Ps4a. They reported that a mycelial mass of 1.2 g dry mycelial weight per 100 ml medium was the most suitable concentration, which kept the dissolved oxygen (DO) in a flask at 1 ppm (approx.). But a mycelial mass of 1.7g dry mycelial weight per flask showed only $1/3^{rd}$ of the maximum decolourisation yield and the DO in a flask was almost 0 ppm. Later, they have investigated the relationship between inoculum size and culture period affording the maximum decolourisation yield. They reported that the thermophilic strain of *Aspergillus fumigatus* G-2-6 decolourised 70-75% of the melanoidin solution after 3-days of culture for an inoculum size of 10^8 spores per 100 ml of medium and 6-days of culture for an inoculum size of 10^4 spores per 100 ml of medium. The decrease in the inoculum size delayed the culture period affording the maximum decolourisation yield.

Sirianuntapiboon *et al.* (1988) reported that an inoculum size of more than 0.15 g mycelia per 100 ml of medium, strain D-90 (genus *Mycelia sterilia*) showed an almost constant decolourisation yield after 6-days. Ohmomo *et al.* reported the relationship between the inoculum size, nutrient supplementation and melanoidin decolourisation by *Lactobacillus hilgardii*. They found that low inoculum size (10^5 cells per tube) required carbon and nitrogen sources for good growth with high MDA. The high inoculum size (10^7 cells per tube) also required glucose for high MDA. However, the presence of organic sources of nitrogen rather repressed the production of MDA.

5.5 Enzymes involved in melanoidin decolourisation

Watanabe *et al.* (1982) reported the enzymatic decolourisation of melanoidin pigment by *Coriolus* sp. No.20. They reported that the decolourisation of melanoidin pigment occurred in presence of an intracellular enzyme, which required active oxygen molecules and sugars in the reaction mixture. This enzyme was later identified as sorbose oxidase having molecular weight of about 200,000. The enzymatic activity was found maximum at pH 4.5 and temperature 35^0C. The purified enzyme aerobically decolourised the melanoidin pigment in presence of glucose, sorbose, galactose, xylose and maltose. But the maximum melanoidin decolourising activity of enzyme was observed with sorbose as well as glucose.

Melanoidin pigment/polymer was suggested to be decolourised by the active oxygen (O_2^-, H_2O_2) species produced by the reactions catalyzed by enzymes probably oxidases because the reactions with the pure enzyme was accompanied by the oxidation of glucose into gluconic acid. According to them, the strain could probably produce sugar oxidases other than sorbose oxidase because the crude preparation utilized arabinose, fructose and mannitol while sorbose oxidase did not utilize these compounds. They finally concluded that almost all sugars contained in distillery wastewater might be oxidized by *Coriolus* sp. to form a large amount of highly active oxygen species for decolourisation.

Aoshima *et al.* (1984) reported that the melanoidin decolourizing activity (MDA) for molasses pigment by *Coriolus versicolor* Ps4a was mainly due to intracellular enzymes that are induced by the molasses pigment. The induced enzyme consisted of two components, namely a sugar dependant enzyme forming two-third part and other sugar independent part constituting one-third part of the system. The sugar dependent enzyme might be the same as that of sorbose oxidase as reported earlier by Watanabe *et al.* [33] from *Coriolus* sp. No. 20 or a same type of sugar oxidase. This was the first report about the existence of sugar independent enzyme decolourizing melanoidin polymer. They also suggested the presence of a constitutive enzyme that was almost completely sugar dependent and unstable. It decolourized MP solution to only quarter the level with the inducible enzyme.

Ohmomo *et al.* (1987) purified the melanoidin decolourizing enzyme (MDE) isolated from *C. versicolor* Ps4a and studied its properties. They found that MDE of this strain was an intracellular enzyme consisted a major P-fraction and a minor E-fraction. The P-fraction consisted at least five enzymes, which were made up of two types that required sugar or no sugar for the decolourizing activity. They took P-III and P-IV in the P-fraction as typical MDE for further studies of their enzymatic properties. Enzyme P-III had a

molecular weight of 48,400 – 50,000, an optimum pH of 5.5 and an optimum temperature of 30-35^{0}C. This enzyme required some particular types of sugars and oxygen for decolourizing activity and glucose was the most suitable substrate for this activity. P-III was inhibited weekly by p-CMB (p-chloromercuribenzoic acid), N-BSI (N-Bromosuccinylimide), Ag^+ (Silver cation), and O-phenanthroline. The decolourizing activity of P-III towards various melanoidins was almost same as that of PIV. P-III was found to be a type of sugar oxidase but differing from L-sorbose oxidase already reported by Watanabe *et al.* (1982) as mentioned above.

The enzyme P-IV had a molecular weight of 43,800-45,000, an optimum pH 4.0-4.5 and an optimum temperature of 30-35^{0}C. Unlike P-III, P-IV could decolourise the melanoidin pigment in absence of sugar or glucose and oxygen and was inhibited weakly by Ag^+, p-CMB and N-BSI. P-IV is the enzyme that attacks the melanoidin polymer directly in comparison of P-III, which attacks the melanoidin polymer indirectly as in sub reaction of sugar oxidase. Furthermore, a multiplicative effect between P-III and P-IV for decolourisation activity was observed. But the catalytic mechanism of these two enzymatic reactions remained to be resolved.

Ohmomo *et al.* (1987) reported that the melanoidin decolourizing activity (MDA) of a thermophilic strain of *Aspergillus fumigatus G-2-6* might be due to the presence of sugar oxidase and exopeptidase. Miyata *et al.* (2000) reported that melanoidin decolourisation by *Coriolus hirsutus* pellet was mainly due to the production of extracellular hydrogen peroxide (H_2O_2) and peroxidases. The culture fluid contained two major extracellular peroxidases, one manganese independent peroxidase (MIP) and other manganese dependent peroxidase (MnP) since both MIP and MnP showed melanoidin decolourizing activity in presence of H_2O_2. Hence, they concluded that melanoidin decolourisation by *C. hirsutus* cultures involved the production of extracellular H_2O_2 and peroxidases.

Later, Miyata *et al*. (2000) while investigating the decolourisation of heat treatment liquor (HTL), a melanoidin containing wastewater by the fungus *Coriolus hirsutus* reported that in pretreated HTL (i.e. before treatment with activated sludge) the fungus produced a large amount of manganese independent peroxidase (MIP). The addition of Mn (II) to pretreated HTL caused a further increase in decolourisation efficiency of fungus and a marked increase in manganese peroxidase (MnP) activity.

Consequently, they finally observed that the increase in MIP and MnP activities play an important role in enhancing the ability of *C. hirsutus* to decolourizing the heat-treated liquor. Further, they also reported that the peroxidase activity require hydrogen peroxide (H_2O_2), which was produced during glucose oxidation, thus establishing the necessity to add extra/external carbon source.

(i) Physicochemical properties of melanoidin decolourising enzymes (MnP)

Boer et al. (2006) have isolated and purified the melanoidin decolourising enzymes (MnP) from *L. edodes*. Two peaks of MnP as MnP1 and MnP2 were isolated by gel filtration (Fig. 11). They revealed that the purified enzyme yielded a single band after denaturing SDS-PAGE (Fig. 12). A molecular mass of 46 kDa was estimated after SDS-PAGE, and this molecular mass was confirmed by Sephadex G-100 gel filtration. The

carbohydrate content of MnP2 was estimated to be 17.8% by using the phenol–sulfuric acid method with D-manose as the standard. The molecular weight of the enzyme is well within the range of the MnP family, which is usually between 37 and 46 kDa.

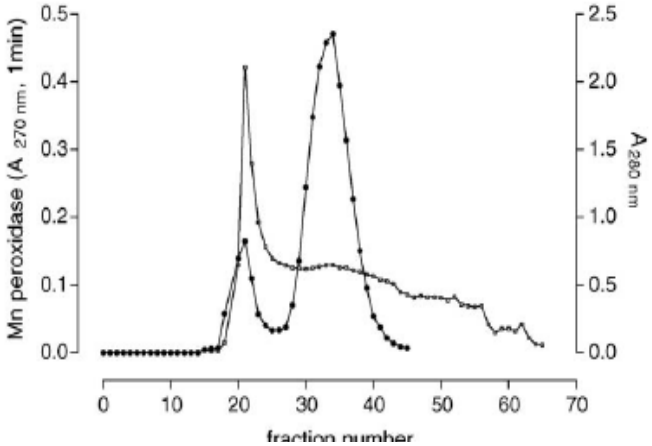

Fig. 11. Elution profile of MnP activity on sephadex G-100. $A_{280\ nm}$ (□), MnP activity (●).

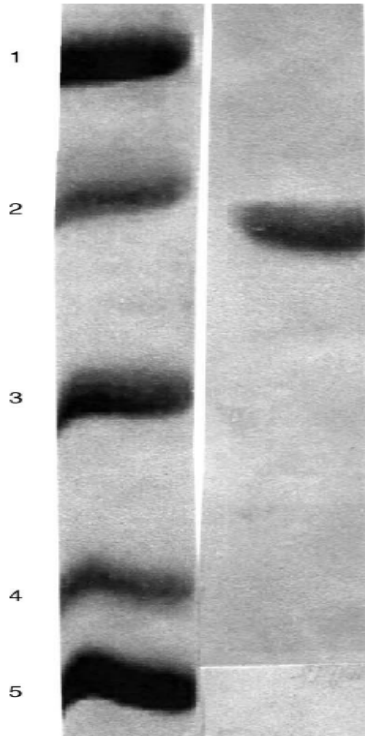

Fig. 12. Denaturing SDS-PAGE of *L. edodes* MnP_2 (Silver staining. {Lane 1: Standard protein, 1: Bovine serum albumin (66 KDa), 2: Ovaalbumin (45 KDa), 3: Pepsin (34.7 KDa), 4: Trypsinigen (24 KDa), 5: β-lactoglobulin (18.4 KDa). Lane 2: *L. edodes* MnP_2}

(ii) Temperature stability and pH optimum

The effect of pH on enzyme activity and stability was examined at pH values ranging from 3.0 to 8.0 using DMP as substrate. The enzyme was completely stable in a large pH range (4.0–6.0) and presented an optimum pH value of 4.5. The optimum temperature for the oxidation of substrate was 40°C. The purified enzyme was stable at temperature up to 40°C for several hours and presented a half-life of 20 min at 50°C. At 4°C, the enzyme was stable for several days. The optimum pH 4.5 for the enzyme was also usual for this family (Deguchi et al, 1998; Glenn, J.K. and Gold, 1985). MnP purified from L. edodes had relatively high thermostability when compared with other white-rot fungus MnP. It did not lose activity at 40°C for at least 24 h and retained about 80% of its original activity at 45°C for 2 h. However, the enzyme was not stable at 50°C. High thermostability is a desirable feature of an enzyme for various industrial applications.

(iii) Effect of some chemicals on MnP activity

The effects of several chemicals on MnP activity were determined (Table 4). MnP was strongly inhibited by Hg^{2+}, while Fe^{3+}, Ca^{2+} and Ni^{2+} did not cause any alteration in the activity. MnP was also resistant to presence of up to 5 mM H_2O_2, but it was inhibited in the presence of 10.0 mM peroxide. The enzyme showed a high percentage of activity in reaction mixtures containing 10% (v/v) of different organic solvents. The susceptibility of MnP to inactivation by H_2O_2 in the absence of substrate was also studied. It was found that the enzyme was very stable in the presence of up to 0.25 mM H_2O_2. However, in presence of higher amounts of H_2O_2, the MnP activity rapidly decreased (Fig. 13).

Table. 4. Effect of some chemicals on MnP activity

Reagent (mM)	Final concentration (%)	Residual activity
None	–	100
$CaCl_2$	10.0	105
$NiCl_2$	10.0	97
$CuSO_4$	1.0	108
$CuSO_4$	10.0	86
$FeCl_3$	10.0	95
$HgCl_2$	1.0	21
H_2O_2	0.1	97
H_2O_2	0.5	93
H_2O_2	1.0	92
H_2O_2	5.0	75
H_2O_2	10.0	17
Acetone	10	92
Isopropanol	10	107
Ethanol	10	86
Acetonitrile	10	71

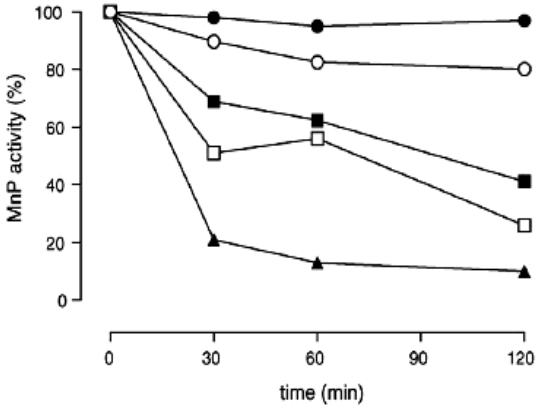

Fig. 13. Effect of H_2O_2 on the stability of *L. edodes* MnP. Control (•), H_2O_2 0.25 mM (o), 0.5 mM (■), 1.0 mM (□), 5.0 mM (▲)

In terms of industrial applications, the stability of MnP in the presence of high concentrations of H_2O_2 is a very important property. MnP requires H_2O_2 for its activity. However, H_2O_2 could also inactivate MnP quickly when it was applied in high concentrations (Bermek and Eriksson, 2002; Wariishi, 1988). When compared with reported MnPs from other white-rot fungi, the *L. edodes* MnP purified was appeared to be more resistant to presence of high H_2O_2 concentrations.

(iv) Substrate specificity

As the study revealed that the enzyme was dependent upon Mn^{2+} for its activity; i.e. the substrates DMP and ABTS were oxidized at a faster rate in the presence of Mn^{2+} than in the absence of Mn^{2+}. It appeared that MnP oxidized ABTS faster than DMP. Like MnPs from other white-rot fungi, this MnP was not able to oxidize veratryl alcohol, a non-phenolic lignin model compound. The Michaelis–Menten constants (KM) of MnP for H_2O_2 and Mn^{2+} were 20.8 and 22.2 $\times$ 10^{-3} mM respectively, which indicates high affinity of these enzymes for their substrates.

5.6 Proposed mechanisms of enzymatic decolourisation of melanoidin polymer

The structural complexity of melanoidins and very less knowledge about the enzymatic system involved in its degradation / decolourisation has created a lot of problems to elucidate the mechanism involved in melanoidin decolourisation. Nevertheless, workers have tried to explore it and built the concept about the same. Watanabe *et al*. (1982) suggested that melanoidin decolourization might be by the active oxygen (O_2^-, H_2O_2) produced by the reaction with pure enzyme obtained from fungal culture and accompanied with the oxidation of glucose in gluconic acid.

Hayase *et al*. (1984) suggested that the melanoidin decolourization by active oxygen species such as hydrogen peroxide (H_2O_2), which is secondarily produced by the enzymatic oxidation of glucose into gluconic acid by glucose-oxidase enzyme. They proposed that hydrogen peroxide reacts with hydroxyl anion to give perhydroxyl anion (HOO^-), which has a strong nucleophilic activity. The perhydroxyl anion was considered to attack the nucleophilically carbonyl groups of melanoidins.

Kim *et al.* (1985) proposed that decolourisation and decrease in molecular weight of melanoidins are mainly due to the cleavage of carbon-carbon double bonds by ozonolysis. Ozone attacks organic compounds electrophilically and is known to exist as a resonance hybrid of four canonical forms. Ozone especially as an amphoteric ion makes an electrophilic attack in an electron-rich π system such as a carbon-carbon double bonds and cause cleavage of bonds. Melanoidins have several carbon-carbon double bonds that are cleaved by ozonolysis leading to its degradation / decolourisation.

Ohmomo *et al*. (1988b) proposed that the microbial decolourisation of melanoidins might be due to two decomposition mechanisms that is an attack mainly against smaller molecular weight components of melanoidin and other against the larger molecular weight components of melanoidins. Further, they proposed that the facultative anaerobic bacteria use the former while Basidiomycetes and Actinomycetes use the latter. However, they could not elucidate the detail mechanism of enzymatic decolourisation of melanoidin.

Miyata *et al.* (1998) proposed that mangenese peroxidase (MnP) catalyzes H_2O_2-dependent oxidation of Mn (II) to Mn (III); Mn (III) then oxidizes phenolic compounds in similar manner as by lignin peroxidase (LIP). LiP catalyses one-electron oxidation of phenolic and non-phenolic compounds by H_2O_2 promoting the production of corresponding free radicals. The peroxidases require H_2O_2, which is generated by the oxidation of glucose/sugar.

However, finally they reported that a detailed mechanism of melanoidin decolourisation by MIP and MnP is still to be explored.

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Chapter-6

MOLECULAR PROBES FOR DETECTION OF *ENTAMOEBA HISTOLYTICA* AND ITS STRAIN VARIATIONS

Jaishree Paul

Abstract

Entamoeba histolytica and *Entamoeba dispar* are two morphologically similar and genetically different species. Among these only *E. histolytica* is considered pathogenic and the disease it causes is called amoebiasis. Among *E. histolytica* positive samples only a small proportion of individuals develop symptoms of disease and the others remain asymptomatic. In some cases *E. histolytica* become invasive and then cross the intestinal barrier causing extra intestinal diseases such as liver abscess and brain abscess. It has been speculated that the varied organ tropism and clinical presentation of infection might be related to genetic diversity of *E. histolytica*. Here we have discussed the development in this direction in order to detect and differentiate the two strains as well as the strain variations therein without cultivating the organism. Molecular probes were designed from the Hme region of the rDNA molecule to detect both *E. histolytica* as well as *E. dispar* positive samples. Subsequently to identify the species, primers were designed for PCR amplification from EhSINE2 element for *E. histolytica* and from ITS2 and 18S region of rDNA for *E. dispar.* The primers for strain differentiation were designed from Hmg region of rDNA and were validated with the experimental samples. Battery of primers that are discussed in the literature for distinguishing *E. histolytica* strains were also validated with the stool DNA samples collected from local area as well as hospitals located in and around Delhi.

6.1 Introduction

The detection of *Entamoeba histolytica*, the causative agent of amebiasis, is an important goal of the clinical microbiology laboratory. This is because amebiasis is presently one of the three most common causes of death from parasitic disease. The World Health Organization reported that *E. histolytica* causes approximately 50 million cases and 100,000 deaths annually (Anonymous, 1997; Walsh et al, 1986). The vast majority of these infections are acquired in the developing world. For example, it was observed that 39% of children from an urban slum in Dhaka, Bangladesh, had a new *E. histolytica* infection during a 1-year study. *E. histolytica* is a pathogen or invasive parasite, whereas *E. dispar* and *E. moshkovskii* are nonpathogenic and noninvasive parasites that are identical morphologically to *E. histolytica* (Clark, C. G., and Diamond, 1991; Edman et al, 1990; Tannich, E. and Burchard, 1991). There are at least eight amebas (*E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. coli*, *E. hartmanni*, *E. polecki*, *Iodamoeba butschlii*, and *Endolimax nana*) that live in the human intestinal lumen (Clark, 1998; Garcia and. Bruckner, 1999).

However, these are generally accepted as commensal organisms except for *E. histolytica* (Leber, A. L., and Novak, 1999).

E. histolytica is an enteric protozoan parasite which causes amoebic dysentery in humans (Guerrant, 1986; Anonymous, 1997) . The trophozoite of *E. histolytica* can convert to a precyst form with a nucleus (*E. coli* precysts have two nuclei), and this form matures into a tetranucleated cyst as it migrates down and out of the colon. The precyst contains aggregates of ribosomes, called chromatoid bodies, as well as food vacuoles that are extruded as the cell shrinks to become a mature cyst. It is the mature cyst that, when consumed in contaminated food or water, is infectious. In the process of becoming tetranucleated, the nucleus of the cyst divides twice. Chromatoid bodies and glycogen vacuoles cannot be seen at this stage (Clark et al, 2000; Katz et al, 1989). Cysts can remain alive outside the host for weeks or months, especially under damp conditions, but are rapidly destroyed at temperatures under -5°C and over 40°C (Ivey, 1980). Cysts are not invasive, but trophozoites can penetrate the gastrointestinal mucosa. From there, the trophozoites are able to migrate to other organs, causing extraintestinal infections.

The most common infection with *E. histolytica* is asymptomatic, in which the amoeboid forms of the parasite, called trophozoites, reside -in the lumen of the colon and transform to cysts, which are passed in the feces (Fig.1). Such asymptomatic infections with *E. histolytica* are frequent among homosexual men in Western countries (Allason-Jones et al, 1986). However, *E. histolytica* is a major cause of morbidity and mortality in developing countries such as Mexico and India, because the trophozoites may invade the colonic mucosa and cause dysentery or liver abscesses or both. In the light of earlier reports about the prevalence of amebiasis in such subjects, interpretation is very difficult because older data did not differentiate between morphologically identical species, one that also harbours in the human intestine but is noninvasive (*E. dispar*) and are that is invasive (*E. histolytica*), but they have a high degree of divergence genetically (Clark, C. G., and Diamond, 1991; Clark, C.G. and Diamond, 1993; Tannich et al, 1989).

It is very important to keep in mind that according to the older data, many *E. histolytica* infections were most probably confused with *E. dispar* due to limited data obtained from microscopic examinations. Microscopy, culture/zymodeme analysis, and molecular biology-based techniques are used for the diagnosis of *E. histolytica*. Each detection test has different advantages and disadvantages. The goals of this review are to describe the molecular based methods for detection *E. histolytica* and discuss what differentiates it from other *Entamoeba* species. The article is intended to highlight the recent developments in this direction that would help in accurate diagnosis and management of the disease.

6.2 Background

The early literature of *E. histolytica* research has been reviewed by Kean and Clark et al (1988; 2000). Milestones in the study (Saklatvala, 1993)of *E. histolytica* and amebiasis were its description by Losch in 1873, the delineation of amebic liver abscess and colitis by Osler and his colleagues in 1890, its axenic culture by Diamond in 1961 (Diamond, 1961), and differentiation of pathogenic (*E. histolytica* sensu strictu) from

nonpathogenic (*E. dispar*) *E. histolytica* in 1979. A clinical syndrome suggestive of intestinal disease was first widely recognized in the mid-1800s, although a parasitic etiology was not determined at that time. Suggestion of a parasitic etiology was first recorded in 1855 from a case where amebas were observed in a stool sample from a child with dysentery in Prague. In 1875, Fedor Losch isolated *E. histolytica* from the stool specimen of a patient with dysentery (Kean, 1988). Leonard Rogers designated emetine as the first effective treatment for amebiasis in 1912 (Rogers, 1912). In 1913, Walker and Sellards (Walker and Sellards, 1913) demonstrated the infective cyst form of *E. histolytica* and described the life cycle of *E. histolytica*. Brumpt (Brumpt, 1925) proposed that *E. histolytica* and *E. dispar* were identical morphologically but that only *E. histolytica* was pathogenic for humans. Diamond's first axenic culture of *E. histolytica* in 1961 was a major turning point in our understanding of the cell biology and biochemistry of *E. histolytica*.

In 1978, Sargeaunt and Williams (Sargeaunt et al, 1978) began to study the isoenzyme patterns of *E. histolytica* isolates using starch gel electrophoresis. It became apparent very quickly that the patterns they were observing fell into two categories that correlated with the sample origin. One group included all the isolates from individuals with amebic colitis and liver abscess while isolates from the other group were all from people without invasive disease. Initially called 'pathogenic' and 'nonpathogenic' *E.* histolytica (Sargeaunt, 1987), with the accumulation of additional evidence from antibody and DNA studies these two groups were eventually recognized as distinct species and are now known as *E. histolytica* and *E. dispar* (Diamond and Clark, 1993). The differentiation of the two Entamoeba species *E. histolytica* and *E. dispar* through molecular characterization in the last decade of the twentieth century led to the need to re-evaluate the epidemiology of amebiasis in terms of prevalence and morbidity of the infection in the world's population, particularly in those geographic regions with high endemic rates. However, it is now realized that those 'zymodeme' designations are problematic as most patterns are dependent on the bacterial flora, if not actually bacterial enzymes themselves. In fact, Jackson recognizes only three 'primary' zymodemes in *E. histolytica* and one in *E. dispar* that can be distinguished in starch gels (Jackson and Suparsad, 1997). Other separation systems (such as thin layer agarose gels) allow the detection of a few additional variants but the overall diversity is still very limited (Cunnick and Diamond, unpublished).

Presently in the diagnostic laboratories, infections with *E. histolytica* are still diagnosed by light microscopy identification of trophozoites or of cysts separated from stool by dilution, filtration, and flotation during centrifugation (Biagi and Portilla, 1957). These methods are time consuming and require extensive experience to assure accuracy of morphological identification. Past or present invasive amoebiasis may be suggested by the identification of antiamoebic antibodies in patient serum (Trissl, 1982). To circumvent the problems of microscopic or culture-based diagnosis and take advantage of the sensitivity, specificity, and simplicity of newer techniques, molecular biology-based technology has become commonly used. The investigation of diversity in *E. histolytica* is now fully dependent on the use of DNA variation detected using the polymerase chain reaction (PCR).

With the application of a number of new molecular biology based techniques, tremendous advances have been made in our knowledge of the diagnosis, natural history, and epidemiology of amebiasis. As more is discovered about the molecular and cell biology of *E. histolytica*, there is great potential for further understanding of the pathogenesis of amebiasis.

6.3 Pathogenesis

The pathogenesis of infection by *E. histolytica* is governed at several levels, chief among them are:

1. Adherence of trophozoite to the target cell,
2. Lysis of the target cell and
3. Phagocytes of target cell.

Several molecules which are involved in this process have been identified. Recently different technical advance like development of transfection system to introduce genes into trophozoites should also help to understand the mechanism of pathogenesis in amoebiasis. With the wealth of information from the ongoing genome sequencing efforts of *E. histolytica* by tiger and Sanger center, a genomics approach to identify novel virulence determinants in the parasite and their role in causing invasive desease has been undertaken. The interaction between the parasite and the host cells is multifactorial. In the first step the amoeba recognize the host cells by using a number of surface adhesins. In the second stage various effector molecules cause damage to the cells. Our knowledge about *E. histolytica* pathogenesis is still incomplete. Several factors contribute to the pathogenicity of *E. histolytica* are still unidentified. However three pathogenic factors have been extensively studied and characterized at molecular level.

A. GalNac lectin borne on surface mediates adherence of the trophozoite to human colonic glycoproteins (Chadee et al, 1987), human colonic epithelium (Ravdin et al, 1985), human neutrophils and erythrocytes (Ravdin and Guerrant, 1981; Guerrant et al, 1981; Burchard and Bilke, 1982), a variety of cell culture lines and to bacteria. The mucin layer of the human colon may be the first natural target for binding by trophozoites in the colon. The mucin layer may protect the host from contact dependent cytolysis by neutralizing the binding epitopes on the lectin, while simultaneously providing a site of attachment for the colonizing parasite. Penetration through the mucin layer permits the trophozoite to invade and attack host tissue. The Gal/GalNAc lectin can inhibit cytolysis of target cells adhered to amoebic trophozoites. The related avirulent *E. dispar* also expresses a similar lectin on its surface; however, there are differences in epitope specificity and the levels of Gal/GalNac inhibitable adherence appear to be lower.

B. **Amoebapore** are a family of small proteins contained in cytoplasmic granules in the trophozoite (Leippe et al, 1991). Trophozoites are able to depolarize target cells by the insertion of these poreforming proteins that form ion channels in their lipid membranes. In addition to eukaryotic cell targets, amoebapores are effective in forming pores in bacterial membranes.

C. Cysteine proteases are a key virulence factor of *E. histolytica* and play a role in intestinal invasion by degrading the extracellular matrix and circumventing the host immune response through cleavage of secretory immunoglobulin A (sIgA), IgG, and activation of complement. Cysteine proteinases are encoded by at least seven genes, several of which are found in *E. histolytica* but not *E. dispar*. Evidence supporting the role of the extracellular cysteine proteinases of *E. histolytica* as virulence factors include theproduction and extracellular release of 10- to 1,000-fold more cysteine proteinase from lysates of *E. histolytica* cells than from lysates of noninvasive *E. dispar* (Reed et al, 1989). Cysteine proteinases purified from axenized *E. histolytica* cleave collagen, elastin, fibrinogen and laminin, elements of the extracellular matrixthat trophozoites must penetrate to cause invasive disease (Keene et al, 1986; Luaces and Barrett, 1988). Cysteine proteinases are responsible for the detachment of tissue culture monolayers, the most widely used assay for amebic toxins and other virulence factors. The cytopathic effecton fibroblast monolayers as a result of supernatants of clinical *E. histolytica* strains are completely inhibited by Z-Phe-Arg-CH2F, a specific, irreversible cysteine proteinase inhibitor thatis not toxic to host cells (Reed et al, 1993). In vivo studies by Stanley's and Mirelman's groups demonstrated that inhibition of cysteine proteinase activity with inhibitors or an antisense construct significantly decreased liver abscess formation in SCID mice (Stanley et al, 1995) and hamsters (Ankri et al, 1999). Taken together, the data supporting a key role of cysteine proteinases in virulence are extremely strong.

One of our major interests now is the reassessment of epidemiology of amebiasis in India, in order to learn the real burden of disease and to evaluate the cost effectiveness of implementation of specific control measures in populations at risk. However, perhaps the most interesting question is whether all types of *E. histolytica* are equal, or are some more likely to cause disease than others. It is hoped that the implementation of the diversity detection methods described above will answer this and other questions in the near future.

6.4 Development of Molecular Probes

A number of DNA probes which hybridize to highly abundant DNA sequences of *Entamoeba histolytica* were developed by Garfinkelet al as early as 1989 (Garfinkel et al, 1989). Variations in the hybridization patterns of different *E. histolytica* strains were detected with selected probes. Four types of restriction fragment length patterns were obtained. Of these, the first class belonged to *E. invadens* (infecting reptiles only) and *E. histolytica*-like var. Laredo. The next two classes consisted of various strains of *E. histolytica* which were originally isolated from symptomatic patients and possessed pathogenic patterns of isoenzymes (zymodemes), whereas the fourth group contained *E. histolytica* strains with nonpathogenic zymodemes obtained from asymptomatic carriers. DNA probes, based on DNA sequences specific to *E. histolytica* isolates with pathogenic and nonpathogenic zymodemes were isolated, and their nucleotide sequences were determined. These probes (P145 and B133) hybridized selectively to DNA of isolates possessing either pathogenic or nonpathogenic isoenzyme patterns. The newly developed probes could be useful for diagnostic purposes but had several limitations as they some time cross reacted with non targeted DNA and therefore, search was on for more specific

probes that could serve as tools to investigate the molecular basis of pathogenicity and the genetic mechanism that regulate the variable aggressive behavior of the parasite.

DNA isolation technique from clinical samples

Although the distinction between these two species is of great clinical importance, the methods developed for this purpose either were very time-consuming or involve laborious procedures for isolation of the DNA. Troll et al (Troll et al, 1997) reported a simple PCR method starting with fresh stool specimen that allows for the sensitive and reliable distinction between *E. histolytica* and *E. dispar*. After initial concentration by the sodium acetate-acetic acid-formalin (SAF) method and digestion with proteinase K, a 0.88-kb sequence of the multicopy 16S rRNA gene served as a target for PCR amplification. The method starting with unpreserved specimens proved to be very sensitive and was not influenced by the quick exposure to SAF fixative during the initial concentration step. However, storage in SAF fixative prior to testing resulted in a decreased sensitivity within 2 days. The detection limit of the method was as low as one copy of the 16S rRNA gene. No cross-reactivity was observed with other common intestinal protozoa. Mixed infections involving both *E. histolytica* and *E. dispar* could easily be detected at a ratio of 1:10,000 by agarose gel electrophoresis or a DNA hybridization immunoassay.

Repetitive DNA in Entamoeba genome

Most of the *Entamoeba* genome is consist of repeat sequences. Several repetitive sequence has been identified and characterized. The rDNA plasmid constitute one of the major repeated DNA in *Entamoeba.* One of the rDNA EhR1 has been fully sequenced and characterized by Bhattacharya et al (1989). It is present in 200 copies per cell. Apart from ribosomal DNA many chromosomal repeats have also been studied in *Entamoeba.* There can be broadly two types of repeats in chromosomal DNA, tandom repeats and interspersed repeat elements. Second class of repeat sequences also include polyadenylated transcripts which does not have extensive ORF.

Protozoan parasites display a range of unusual molecular mechanisms that may be helpful for their survival in nature. *E. histolytica* transcribes different unusual transcripts, Tr, UEE1 and SINE1 that are polyadenylated but do nothave extensive ORF and are unlikely to code for proteins. These may function as RNA molecules as suggested by presence of extensive secondary structures. It has been speculated that these may be involved in regulation of transcription (Bhattacharya et al, 2003). As is seen in other parasites, some of the protein coding genes of *E. histolytica* also exhibit tandem repeats that tend to show intraspecies repeat variation. Polymorphic genes like SREHP (Stanley et al, 1999) and chitinase (De la Vega et al, 1997) encoding genes are one of them. Microsatellite loci are also present in *Entamoeba* genome (Zaki and Clark, 2001). Repetitive DNA have been used as a good markers to study polymorphism among species and strain variation in many organisms.

Extrachromosomal rDNA in Entamoeba

The *E. histolytica* ribosomal DNA are present in 200 copies per genome and are carried exclusively on circular DNA molecules. This was reported independently by two groups (Huber et al, 1989; Bhattacharya et al, 1989). There may be one or two copies of

rRNA transcription unit located on each circle. The nucleotide sequence of one such rDNA circle belonging to the strain HM1:IMSS, has been determined (Sehgal et al, 1994). It measures 24.5kb and carries two copies of the ribosomal RNA transcription units arranged as inverted repeats. This molecule is named as EhR1 and complete map with sequence details has been worked out (Fig. 2). According to EcoR1 restriction sites circle has been divided into HMe, HMd and HMg regions. The two transctiptoin units of EhR1 are separated by a 3.7 kb downstream intergenic spacer region and a 9.2 kb upstream spacer. The downstream spacer is composed of two families of short tandem repeats: the 170 bp Dra1 repeats and the 144 bp Sca1 repeats, eleven 145 bp Pvu1 repeats Hinf1 repeats which share regions of sequence identity with the Dra1 repeats of the downstream spacer, AvaII repeats and 74 bp repeats. The upstream spacer contains several unique sequences. One of these is transcribed into a polyadenylated 0.7 kb RNA detectable by northern blots. However, this RNA lacks ORF thus its function remains unknown. The rDNA circle for other strains (200:NIH, HK-9 and Rahman) of *E. histolytica* and Laredo strain of *E. moshkovskii* have also been characterized to some extent. The 200:NIH circle is very similar to EhR1 and differs only in the number of repeats in intergenic spacers. In contrast to HK-9, Rahman and Laredo circles are strikingly different in that they contain only one rRNA unit per circle. Not much is known about the origin and maintenance of these circles but they have been used extensively for diagnostics purposes as molecular markers. The molecular markers were designed from different segments of the rDNA molecule for screening Entamoeba positive samples from stool DNA (Fig.5 and 6) and they were further characterized by PCR reactions (Fig.3 and Fig.4). The location of the primers on rDNA molecule, specific for E. dispar is shown in boxes. Fig.4 represents the multiple alignment of EHSINE2 copies present in Entamoeba histolytica genome data and the conserved regions are chosen for designing primers.

EhLINEs and EHSINEs as molecular markers

Whole genome shotgun sequences of the protozoan parasite revealed three families of non- long terminal repeat (LTR) retrotransposon or long interspersed element (LINEs) (Van Dellen et al, 2002). The EhLINEs/SINEs together account for 6% of the *E. histolytica* genome as deduced from database analysis. Most copies of each element are truncated at the 5'- or 3'- end or at both ends. The 4.8 kb EhLINEs each have a single ORF with a putatitive nucleic acid binding motif (CCHC) and restriction enzyme like endonuclease domain located downstream of RT (reverse transcriptase) domain. EhLINE1 shared a common 3' end with a highly transcribed 0.55 kb short interspersed element (EhSINE1).

Similarily EhLINE2 shared a common 3' end with a highly transcribed 0.65kb EhSINE2. EhLINE1 and EhSINE1 probes showed that these elements reside on all chromosomal bands, do not seem to be telomeric, and might be dispersed in the *E. histolytica* genome (Bagchi et al). However, all the elements seemed to insert in AT-rich sequences, with a clear preponderance of T-residues in a 50-nt stretch upstream of the site of insertion of each element (Bakre et al, 2005). In a study of repetitive DNA

Sequence in *E. histolytica,* the first retotransposon like element EhRLE1 was identified in this organism (Sharma et al, 2001). Analysis of the consensus sequence showed that EhLINE1 had a length of 4804 bp and consisted of two easily identifiable

functional domains- the RT (nt 2605-3286), and the EN (nt4120-4477). It is present in about 140 copies per genome. All copies show sequence variation with respect to one another (2-4%). A complete ORF is missing in most copies.

EhSINEs are nonautonomous retrotransposons without ORFs and so lack the machinery necessary to replicate themselves. In *E. histolytica* genome there are three types of SINEs reported so far EhSINE1, EhSINE2 and EhSINE3 (Sharma et al, 2001). The 3' ends of SINEs are homologous to the 3' ends of their respective LINEs (Boeke, 1997; Okada et al, 1997).

A short repetitive sequence of 550bp, named EhSINE1or interspersed element (IE) or *E. histolytica* abundant polyadenylated transcript 2 (Ehapt2), has been reported by two groups (Cruz-Reyes et al, 1995; Willhoeft et al, 1999). It appears frequently in *E. histolytica* cDNA libraries, but all copies sequenced so far lack an ORF. The copy number of this element is estimated to be about 500 per genome. The sequence identity between different copies is of the order of 95%. A poly A+ transcript corresponding to the element is detected in Northern blots. The element is widely distributed in genome and frequently seen close to protein coding genes. Corresponding genomic copies of Eh SINE1 are absent in closely related but nonpathogenic species *E. dispar* (Willhoeft et al, 2002). It has also been observed in upstream of gene for poreforming peptide (Bruchhaus and Tannich, 1993) and as an insertion into an EhLINE1 family sequence (RLE5) described by Sharma et al (Bakre et al, 2005).

Polymorphic SREHP gene

The first DNA sequences shown to exhibit stable and significant polymorphism were the Serine Rich *Entamoeba histolytica* Protein (SREHP) gene (Clark and Diamond, 1993). SREHP has been used in a number of studies. The central region of this gene contains tandem repeats of 24 and 36 bases in length, encoding 8 and 12 amino acid repeats, respectively (Stanley et al, 1990; Ko¨hler and Tannich, 1993). When analyzed using Southern blots, SREHP appears to be a single copy gene. However, when the repeat region is amplified from a clonal line of an *E. histolytica* isolate, often two or three bands are observed, not always of equal intensity but usually quite close in size (Clark and Diamond, 1993). The data suggest that multiple alleles can be present and that the ploidy is likely to be four. The major way in which the alleles differ is in the number of 8 and 12 amino acid repeats that are present, but there are sequence differences between the DNA repeats also. This sequence variation has been exploited to increase the amount of diversity detected through the use of a restriction enzyme, AluI, that cuts in some variants of the repeat sequence but not others, leading to restriction fragment length polymorphism (RFLP) among isolates that might otherwise be difficult to distinguish (Clark and Diamond, 1993). To correlate the genetic differences with pathogenic potential of the parasite, Simonishvilli et al examined the SREHP gene polymorphisms among Georgian *E. histolytica* isolates (Simonishvili et al, 2005). Comparison of polymorphic patterns revealed the presence of several different genotypes of *E. histolytica*, thus preventing an association of a single genotype with hepatic disease, but supporting the previous finding of extensive genetic diversity among *E. histolytica* isolates from the same geographic origin. With the increased ease and decreased cost of DNA sequencing, the indirect approach to detecting variation in SREHP by RFLP is disappearing from use and is being

replaced with sequencing of the PCR product (Haghighi et al, 2003; Haghighi et al, 2002).

Polymorphic Chitinase gene

The chitinase gene of Entamoeba species encodes a protein with a repeat structure near the amino terminus (De la Vega et al, 1997). This degenerate 7 amino acid repeat region exhibits PCR product size polymorphism between isolates, again dependent on the repeat copy number. In *E. histolytica*, chitinase behaves like a single copy gene in Southern blotting, but in contrast to SREHP allelic variants appear to be rare or absent. Chitinase is also present and polymorphic in *E. dispar* (Ghosh et al, 2000). Most studies of chitinase have relied on DNA sequencing of the PCR products rather than on detection of variation in the product size, probably because size diversity is lower than in the case of SREHP.

tRNA-Linked Loci used as marker

In many eukaryotic pathogens, microsatellite loci have become the target of choice for detecting diversity, and so about 6 years ago an attempt was made to isolate such loci from *E. histolytica*. Although it failed and we now know that the *E. histolytica* genome is essentially devoid of microsatellites, the attempt did yield another type of polymorphic locus. These contained short-tandem repeats (STRs) but of a size and complexity much greater than is associated with 'traditional' microsatellites, as the repeats normally ranged between 8 and 36 bp in length and blocks of repeats often included two or more minor sequence variants (De la Vega et al, 1997). These STR-containing loci all proved to be linked to tRNA genes. As the *E. histolytica* genome project data started to emerge, analysis of tRNA sequences showed that this was a general phenomenon- almost all tRNA genes in this organism are flanked by STRs (Loftus et al, 2005). A comprehensive analysis of tRNA-linked STR polymorphism has recently been completed (Ali et al, 2006). The result has been the identification of six polymorphic sequences that can be amplified reliably from *E. histolytica* whether the DNA is extracted from culture, stool or liver abscess pus. Polymorphism is detected directly from the PCR product size without the need for further analyses. The primer pairs have also been designed to differentiate between *E. histolytica* and *E. dispar*.

In order to observe the strain variations in *Entamoeba histolytica* present in Indian population, these primer sets were tested in our laboratory with the sample DNA isolated from *E. histolytica* positive stool samples (Srivastava et al, 2005). The primers were validated successfully in the study samples. The results are represented in Fig. 7 and 8. The primer set R4/R10 showed two types of variation whereas R4/R9 exhibited three different types of population. Therefore, it was concluded that the strains of *Entamoeba histolytica* harbouring in our population are likely to be of diversified nature.

6.5 Real-time PCR

A closed-tube, real-time PCR assay was developed for sensitive and specific detection and differentiation of the two closely related intestinal protozoan parasites *Entamoeba histolytica* and *Entamoeba dispar* directly from human feces (Blessmann et al, 2002). The assay is performed with the LightCycler system using fluorescence-labeled detection probes and primers amplifying a 310-bp fragment from the high-copy-number,

ribosomal DNA-containing ameba episome. The assay was able to detect as little as 0.1 parasite per g of feces. The two pairs of primers used were specific for the respective ameba species, and results were not influenced by the presence of other Entamoeba species even when present in exceeding amounts. PCR was evaluated using several hundred stool samples from areas of amebiasis endemicity in Vietnam and South Africa, and results were compared with those of microscopy and ameba culture. PCR was found to be significantly more sensitive than microscopy or culture, as all samples positive by microscopy and 22 out of 25 (88%) samples positive by culture were also positive by PCR, but PCR revealed a considerable number of additional *E. histolytica*- or *E.dispar*-positive samples.

Comparison of different techniques:

Compared to culture and subsequent ameba differentiation by isoenzyme analysis, PCR was 100% specific for each of the two Entamoeba species. Interestingly, the comparison with PCR revealed that culture, in particular, underestimates *E. histolytica* infections. Given the high sensitivity and specificity of the developed PCR assay, the inability of microscopy to distinguish between the two ameba species, and the time it takes to culture and subsequently differentiate entamoebae by isoenzyme analysis, this assay is more suitable than microscopy or culture to correctly diagnose intestinal *E. histolytica* or *E. dispar* infection. When a study was was conducted to compare stool antigen detection with PCR for the diagnosis of *Entamoeba* sp. infection in asymptomatic cyst passers from Iran (Solaymani-Mohammadi et al, 2006). *Entamoeba dispar* and, in one case, *E. moshkovskii* were the *Entamoeba* spp. found in the amebic cyst passers. There was a 100% correlation between the results from the TechLab *E. histolytica* II stool antigen kit and those from nested PCR. It was concluded that *E. dispar* is much more common in asymptomatic cyst passers in Iran and that antigen detection and PCR are comparable diagnostic modalities. Now we are a in a position to develop a rapid assay that uses short (20-bp) synthetic oligonucleotide probes that can used as a tool to help in the diagnosis of amebic infections and to control the high worldwide incidence of amebiasis.

6.6 Conclusions

The studies conducted to date that have used methods capable of differentiating between the two species suggest that, in general, *E. dispar* is much more prevalent than *E. histolytica* and that only a small proportion of individuals specifically infected with *E. histolytica* will progress to having amebic disease. The necessity to identify and treat asymptomatic carriers of *E. histolytica* is emphasized by the observation that 10% of them develop invasive amebiasis in due course (Gathiram and Jackson, 1987). Additionally, asymptomatic carriers are more likely to spread the disease than symptomatic persons with invasive diseases, as the latter individuals seek medical attention. The ability to differentiate variants of *E. histolytica* has several potential applications. It can probably answer a pertinent epidemiological question-is there a structure to the population of *E. histolytica* in a given area and does this differ among regions? In the clinical setting the question of whether a post-treatment infection is the result of re-infection. However, perhaps the most interesting question is whether all types of *E. histolytica* are equal, or are some more likely to cause disease than others. It is hoped that the implementation of the diversity detection methods described above will answer this and other questions in the near future.

The advantage of either the DNA hybridization probe or the ELISA over microscopy is the brevity of specimen preparation: stool is directly spotted onto nylon filters or is solubilized and placed in microdilution wells, respectively. In addition, with the DNA probe as many as 96 specimens per sheet times 10 to 20 sheets can be hybridized in parallel. Because DNA hybridization is a simple and potentially inexpensive method for diagnosing *E. histolytica*, this technique might be used to survey large numbers of persons to estimate accurately the prevalence of *E. histolytica* infection and to evaluate the effects of control measures to limit infection with amoebae. Finally, methods to use the polymerase chain reaction to amplify target DNA and increase the sensitivity of the amoeba probe and to use non-radioactive detection systems to replace 32P-labeled probes are presently being developed.

One area of significant concern is the stability of the patterns observed at the different loci. In particular, for following transmission patterns in populations, stability is essential. Although rapid changes in repeat number have been observed in other regions of the rDNA episome (Bhattacharya et al, 1992). Hmg/Tr usually appears to be stable when present. However, deletion of the whole locus has been observed to occur, making its reliability suspicious (Ghosh et al, 2001). No such concerns have been raised about the other polymorphic loci being discussed. Indeed, where such observations have been possible it is clear from follow-up sampling that the PCR product sizes obtained remain stable over the course of the same infection (Ali, 2005; Zaki et al, 2003). It is also the case that the patterns remain stable over a period of years in culture. The degree of variation detected among the tRNA linked loci varies from moderate to substantial, and so some resemble more the level of diversity seen in the chitinase gene while others resemble SREHP. In combination the six loci can distinguish a remarkable number of distinct variants, almost 100 combinations have been observed in Bangladesh alone (Ali et al, 2005). This is quite puzzling. If the patterns are stable, where does the diversity come from? The long-term stability of the pattern in cultured trophozoites suggests that the cyst stage may be where variants are generated.

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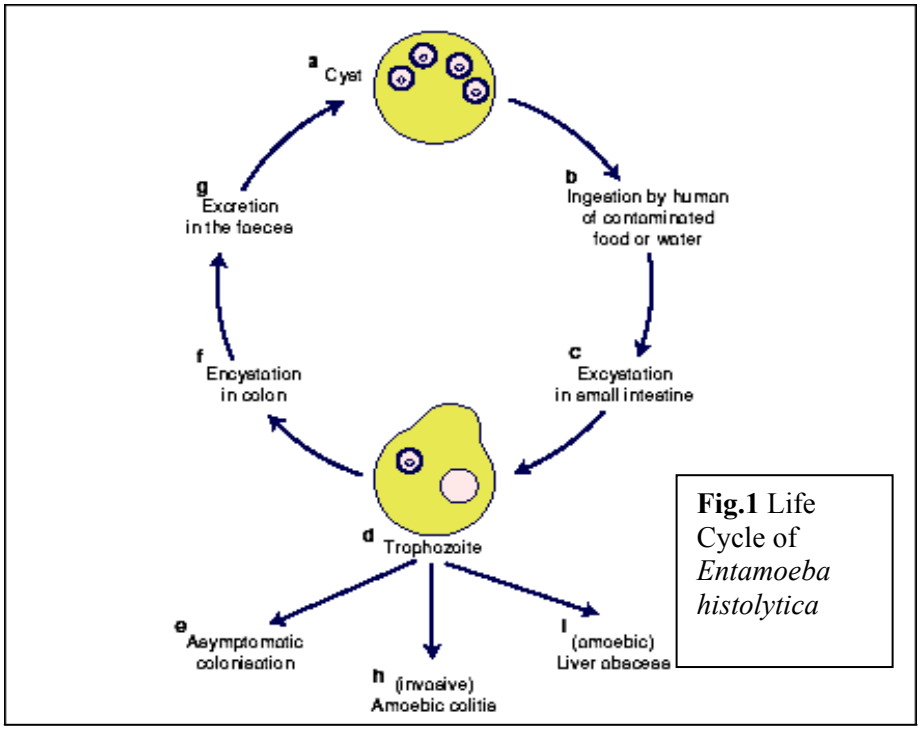


Fig.1 Life Cycle of *Entamoeba histolytica*

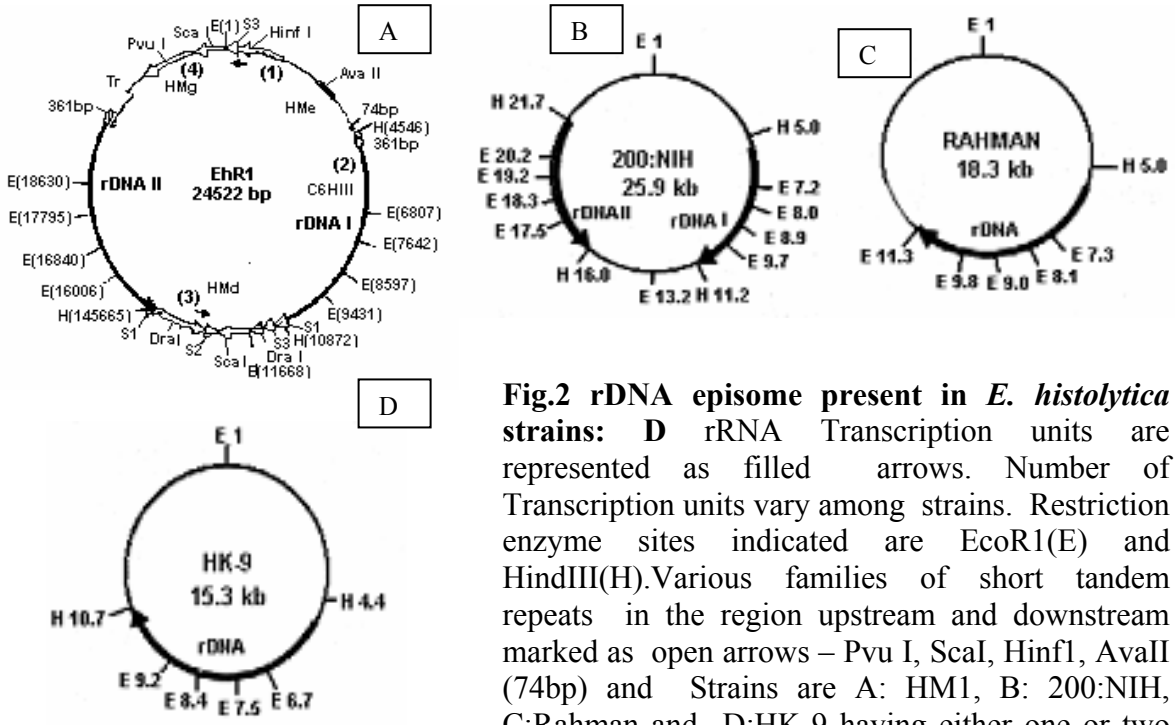


Fig.2 rDNA episome present in *E. histolytica* strains: D rRNA Transcription units are represented as filled arrows. Number of Transcription units vary among strains. Restriction enzyme sites indicated are EcoR1(E) and HindIII(H).Various families of short tandem repeats in the region upstream and downstream marked as open arrows – Pvu I, ScaI, Hinf1, AvaII (74bp) and Strains are A: HM1, B: 200:NIH, C:Rahman and D:HK-9 having either one or two transcription Units. Sizes of each rDNA is mentioned in the circle. Tr represents the polyadenylated RNA.(adopted from Sehgal et al[48])

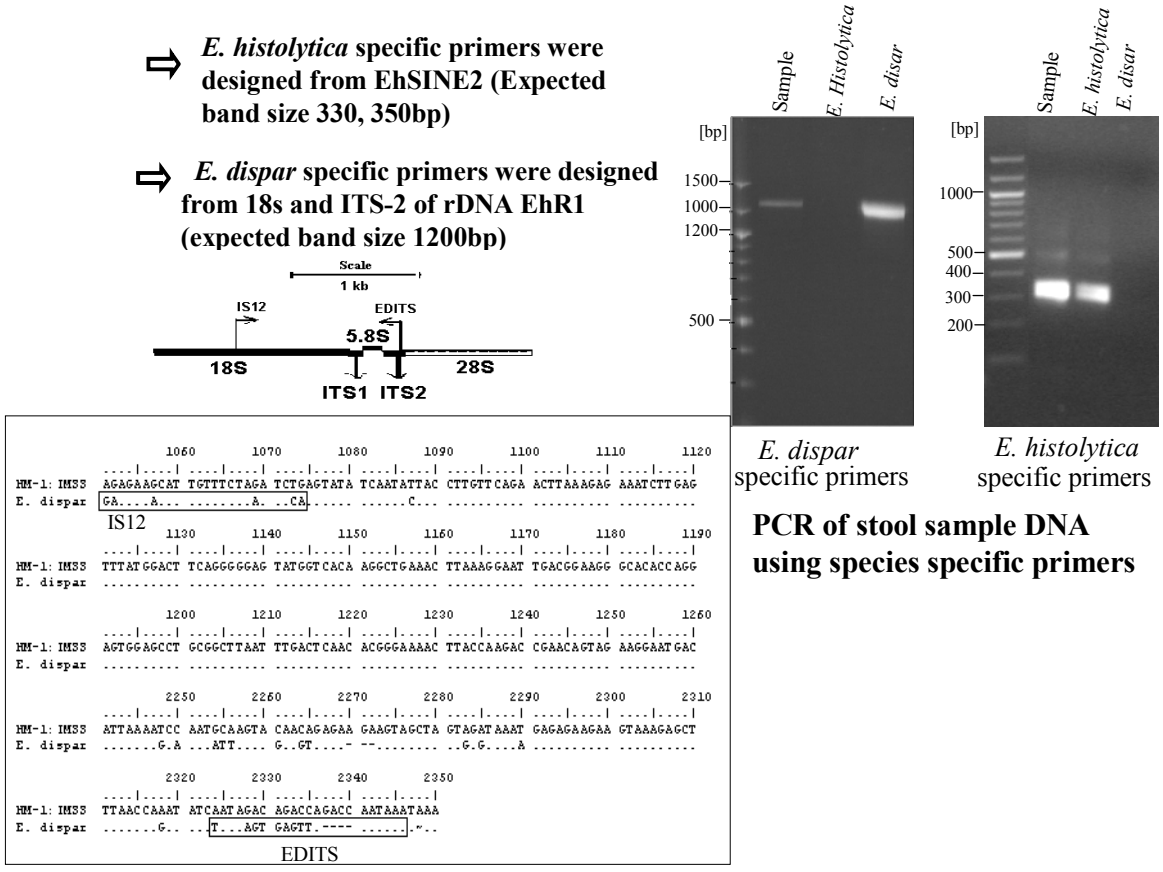


Fig.3: Sequence alignment of a segment of *E. histolytica* and *E. dispar* rDNA showing the primer location marked in boxes (left) and corresponding PCR on right (adopted from Srivastava et al.)

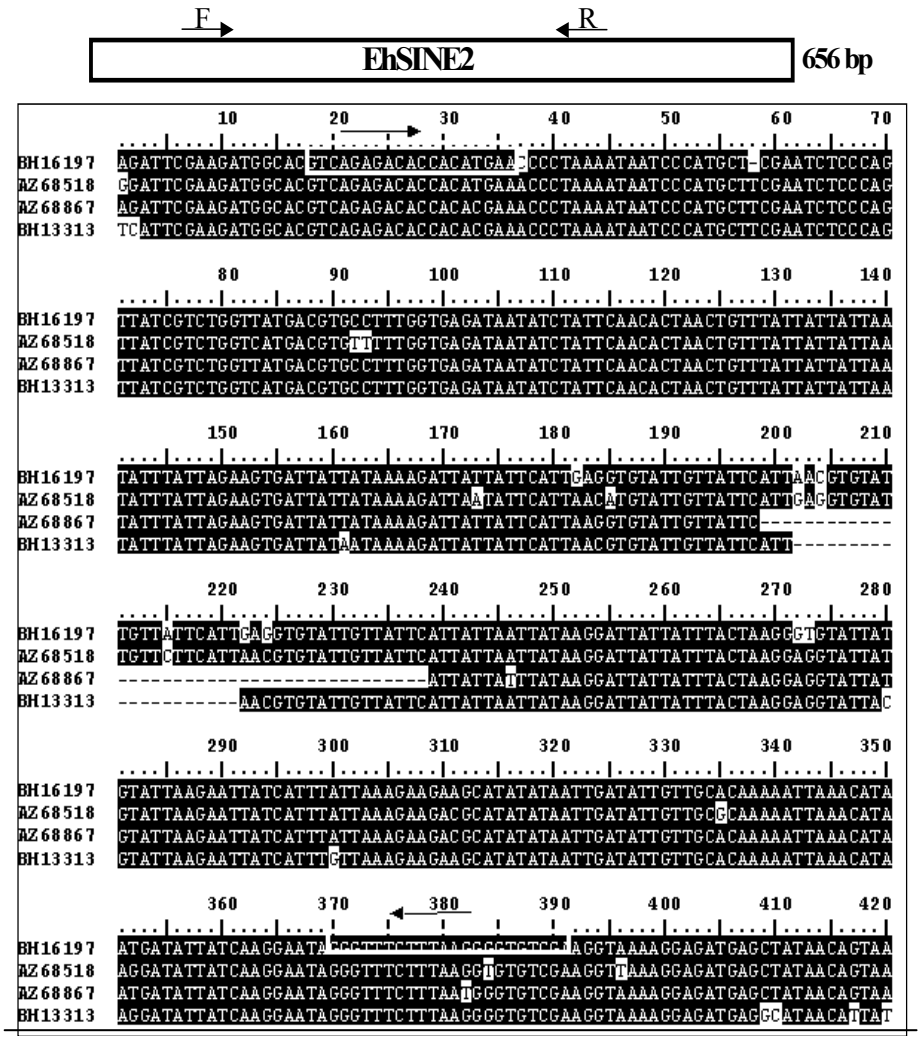
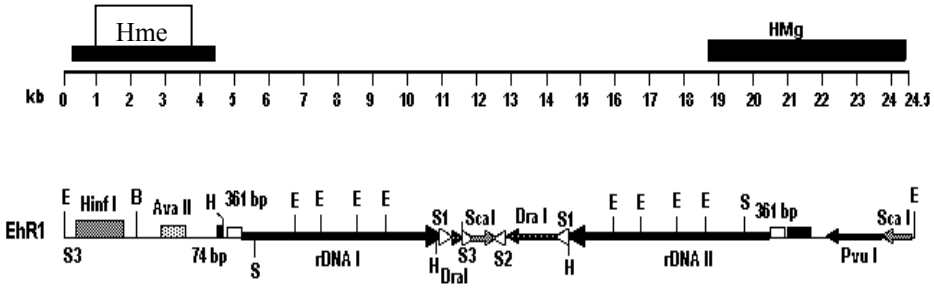
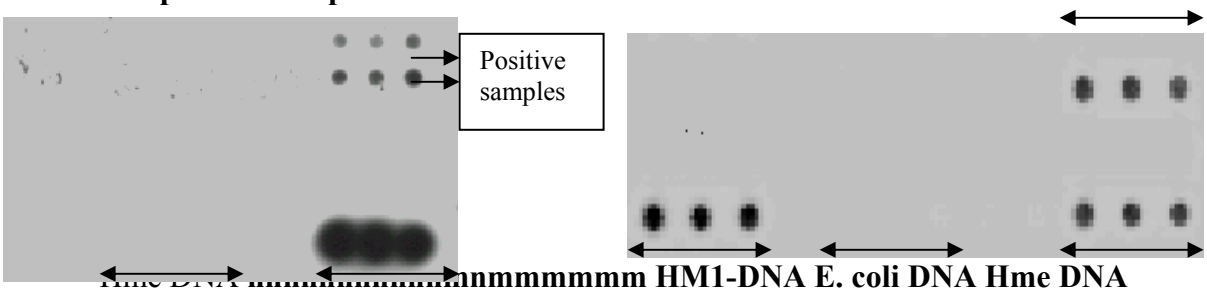


Fig. 4 Sequence alignment of EhSINE2 showing the location of *E. histolytica* specific primers. Highly abundant in *E. histolytica* genome, absent in *E. dispar.* Some of the copies have internal nucleotide deletions.

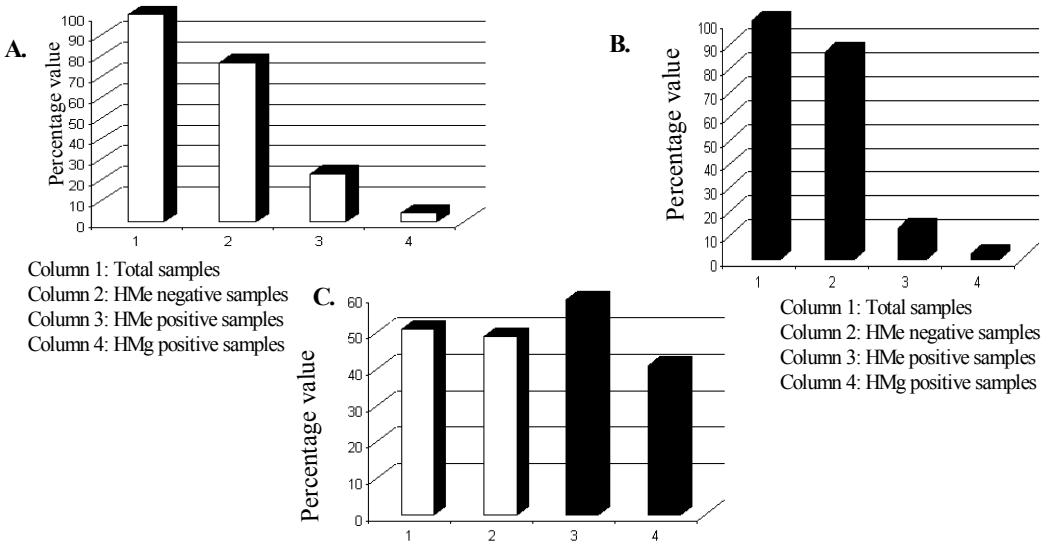


A B **positive sample**



Hme DNA Hmg DNA

Fig. 5 Identification of *Entamoeba histolytica* by dot blot method using Hmg (A) and Hme (B) probe from rDNA **(**adopted from Srivastava et al, 2005**)**



Column 1and column 3: *E. histolytica* positive samples in asymptomatic individuals and symptomatic individuals respectively;
Column 2 and column 4: *E. dispar* positive samples in asymptomatic individuals and symptomatic individuals respectively

D.

Asymptomatic volunteers	**Number of samples**	**Percentage value**
Total samples tested	268	100
HMe positive	63	23
HMg positive	11	4
E. histolytica positive	33	51*
E. dispar positive	30	49*

E.

Symptomatic individuals	**Number of samples**	**Percentage value**
Total samples tested	684	100
HMe positive	85	13
HMg positive	17	2.4
E. histolytica positive	50	59*
E. dispar positive	35	41*

Fig. 6: Dot-blot result of stool samples tested over a four year period (2000-2004): (A) Bar graph represents percentage distribution of *HMe and HMg* in asymptomatic population. (B) Bar graph represents percentage distribution of *HMe and HMg* in symptomatic population. (C) Bar graph indicates distribution of *E. histolytica* and *E. dispar* in symptomatic and asymptomatic people from total positive samples. Table (D) and (E) indicates overall study of asymptomatic and symptomatic individuals respectively. * Percentage out of total positive samples tested.

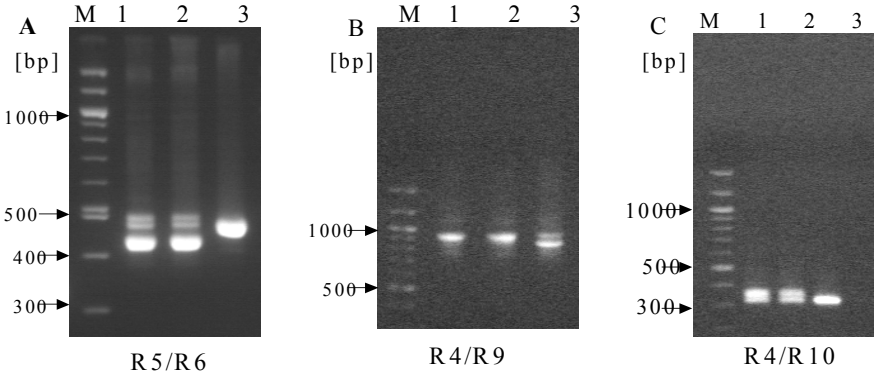


Fig.7 Strain differentiation in *Entamoeba histolytica* Cultures shown by PCR from polymorphic repeat region primers designed from tRNA gene linked tandem repeats. Lanes 1,2,3 are HM-1, HK-9 and Rahman strains of *E. histolytica* respectively.

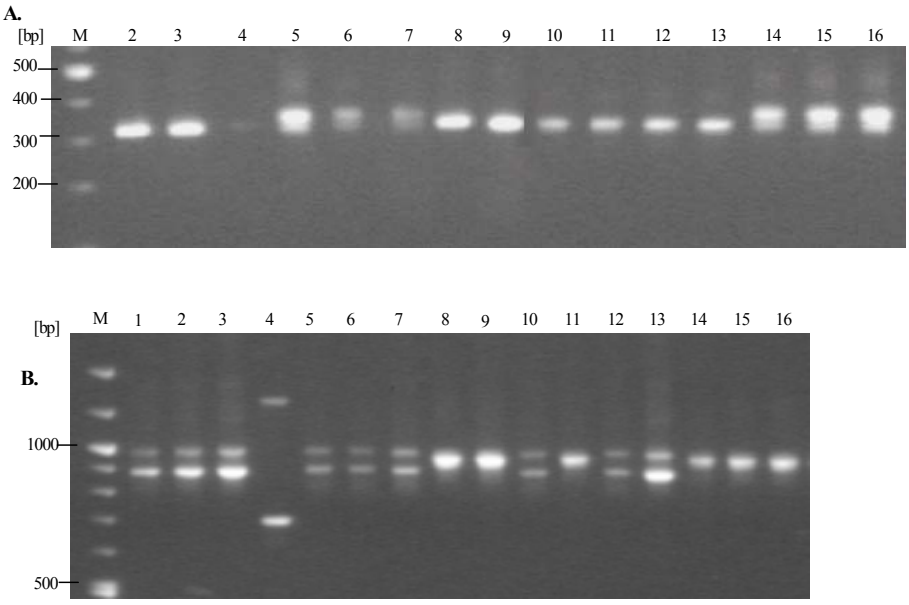


Fig. 8: Strain differentiation in *E. histolytica* positive stool samples using primers designed from tRNA gene linked tandem repeats: Primer set R4/R10 (A) and R4/R9 (B) were used for PCR with DNA isolated from stool samples. Gel picture of 16 representative samples are given. Primer set R4/R9 gave more than two type of band patterns. Variation in band patterns can be seen in the figure. Amplified products were run in 1.5% agarose gel

Chapter-7

INTEGRATED PROCESS BIOTECHNOLOGY SYSTEMS AID IN ENVIRONMENTAL BIOTECHNOLOGICAL APPLICATION FOR VALUE ADDED PRODUCTS

S. N. Mukhopadhyay

Abstract

India is emerging as a technologically advanced country. Along with its advancements currently it is facing problems of environmental pollution significantly. Waste water and solid wastes/refuges generated during human activities are posing health hazards to the inhabitats. Thus environmental pollution by solid, liquid and gaseous wastes must be abated for health care of the people. Using integrated process biotechnology systemic aid in environmental biotechnological application it is possible to take care of the problem of environmental crisis to a large extent and to produce value added products simultaneously. In this article a detailed approach with example is described.

7.1 Introduction

Indian Institute of Technology, Delhi (IITD) through its research and development (R&D) supported teaching and training and implementation activities in the Department of Biochemical Engineering and Biotechnology (DBEB) has always been involved to develop integrated process biotechnology (IPB). IPB systems aid in environmental process biotechnological application (EPBA) have been given one of the prime importances in DBEB's activities (Figure 1) (Mukhopadhayay, 1998, 2005). This is primarily to expand the scopes of biotechnology.

Scopes of biotechnology in daily life, R&D and in industry / commerce are well visible to all. Various arenas of biotechnology in our country is taking care in generating wealth from wastes as well as hightech areas with objectives of providing more comforts to masses.

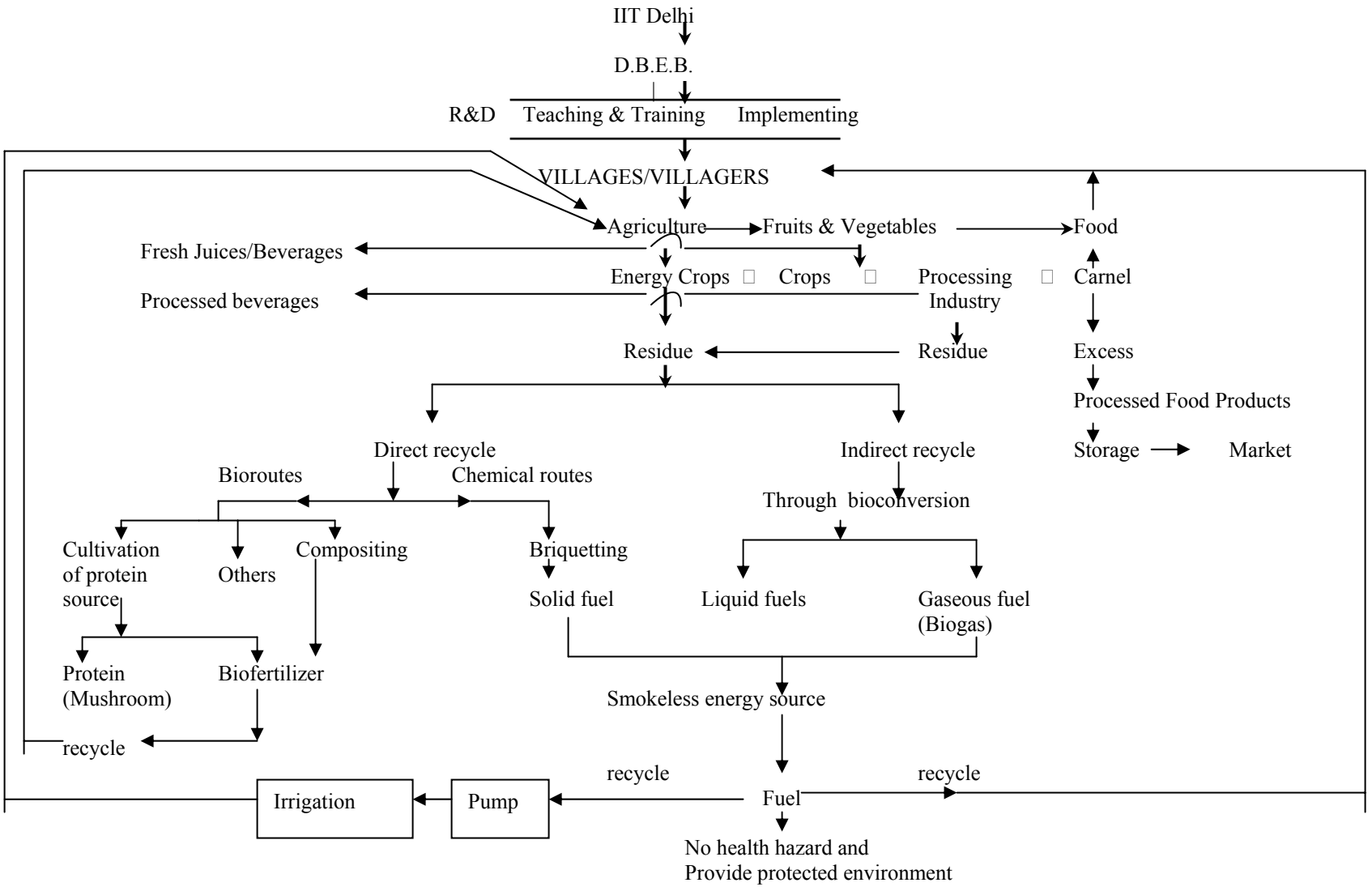


Fig. 1. Integration scope of process biotechnology to agriculture/food, energy and environment (Mukhopadhayay, 1998).

Some such objectives is in obtaining value added biotechnology products from rural industries and for cleaner environment using concepts of process integration. Both enzyme and cellular catalysis have been used to deliver useful value added products. Few examples include bioconversion of carbohydrate wastes/residue like starch, molasses (sugars), cellulose/hydrolysate etc. to food, biofertilizer/compost and monomeric L(+) homolactic acid using free cellular catalysis system of *Lactobacillus* sp. under most suitable cultivation conditions in process biotechnology followed by chemical technology of polycondensation of L(+) lactic acid in presence of high temperature and acid catalyst producing polylactide which is a biodegradable polymer. Another biodegradable polyamide based biopolymer that has been produced is from leather industry waste. This waste having high protein content has been enzymatically converted to hydrolysed protein (HP). This HP containing polyamides of aminoacids in turn could be condensed by polycondensation reaction to biodegradable polymer (BDP) which is water insoluble. The water insoluble BDP has been further treated with polyvinyl alcohol (PVA) to produce improved biodegradable polymer (IBDP) that is water soluble. These biodegradable polymers have great uses in daily life and for industrial growth. Likewise some of the bioactive surface generating biodegradable biopolymers has also been produced for health care industry scopes. Concerns of these bioproducts process technology have great relevance to agriculture, health care and pollution abatement and energy. Details are discussed.

1. NCMC Process Biotechnology in EPBA (Mukhopadhayay, 1998, 2004, 2005, 2006). Nonconventional mushroom cultivation (NCMC) process biotechnology aid has been taken in EPBA for integration of biomethanation and biofertilizer/composting. *Pleurotus sajor-caju* mushroom cultivation biotechnology is an example. India produces huge quantity of food through conventional cultivations on agricultural lands. Thus, in rural India availability of agri-residues are in abundance. Its blending with easily available other nitrogenous wastes/residues serves as a potential resource material having a good C/N ratio for solid state fermentative (SSF) cultivation of food from wastes. Edible NCMC process biotechnology may provide good scope for rural industrialization by development through integrated EPBA. NCMC process biotechnology for leafy mushroom (*Pleurotus sajor-caju*) production and utilizing the process residues/wastes in integrated biomethanation and biofertilizer/biomanure production has a great promise. Thus, R&D on integrated NCMC, biomethanation and composting through EPBA has a great scope to develop a village industry to create job opportunity for rural masses. An outline for integrated village industry development approach is shown in Figure 1.

Pleurotus sajo-caju was cultivated on various agroresidues and their blends using container blocks/bags or inside ventilated hut using 10% (w/w) inoculum (spawn). SSF at 30-35°C was carried out for 10-15 days. During this period mycelium of the mushroom spreaded and formed a bount compact mass. Mushroom mycelium on blocks was kept inside hut having relative humidity (r.h.) 80-90%. Young leafy mushroom started appearing after 5-7 days. Normal matured size attained in another 2-3 days. Fruit bodies needed harvesting at this stage (60% yield). Four flushes are harvested in nearly forty days. After four flushes harvesting the left over partially aerobically digested material is nearly 5-8 kg per block (dry basis) which has a C/N ratio nearly 19-28 and is well suited either for a) biomethanation by A.D. (anaerobic digestion) or b) subsequently through composting for biomanure.

a) Biomethanation of NCMC residue: The mixed blend residue obtained from above bioprocessing is a potential biomass for A.D. using adequate fresh cow dung inoculum and are charged into the digester. It is mixed with water sufficiently and allowed to digest anaerobically at suitable temperature (34-37°C) condition in an anaerobic digester of suitable design. For a typical pilot plant having capacity of 500 m^3 of biogas production per day requires an area of approximately 2400 m^2. It may consist of 12 cylindrical A.D. of fabricated shut construction, erected in two rows of 6 each. The digesters are to be connected to a gas holders of 300 m^3 capacity for storing biogas. Operational and miscellaneous machinery and other facilities are required on site.

Each digester is filled with the requisite charge of the NCMC residue and adequate volume of municipal sewage or bioliquid mixed with dung slurry, so that the charge has a moisture content 95%. A.D. is carried out for 12-15 days. The biogas production starts from nearly 12^{th} day of A.D. when the methane content of the gas reaches 60-65% in a few days time, the gas is then fed to the gas holder. After about 4-6 weeks when the gas production slows down the liquid from the digester is drained out.

b) Composting of Solids from biomethanation: The residual solids consisting of lean N:P:K is further treated for composting after mixing with aquatic biomass water hyacinath (W.H.) aerobically by blowing compressed air for nearly 6-8 days and then spreaded on the ground for drying. During this aerobic treatment the N:P:K value of the solids increased by 35-40% and becomes a potential biomanure. Using this biomanure typical pot cultivation of vegetables showed yield as given in **Table 1**. So integrated NCMC, biomethanation and composting technology is a suitable strategy in rural industrialization.

About 200 m^3 biogas is obtained from each tonne of biodegradable matter present in NCMC residue. This gas has a calorific value of nearly 5200 kCals/m^3. After biomethanation by A.D. the yield of biomanure from the residual solids by aerobic treatment is approx. 2 tonnes per tonne of dry organic residue. Its N:P:K value is nearly 2:1:2 which is comparable to farm yard biomanure.

Use of mushroom compost as biofertilizer in pot cultivation of vegetables like brinjal and lady finger was also encouraging. The yield of brinjal on compost was nearly 16% higher than on unsupplemented control.

The strengths and weaknesses of NCMC as an aid in EPBA has been listed in Table 2.

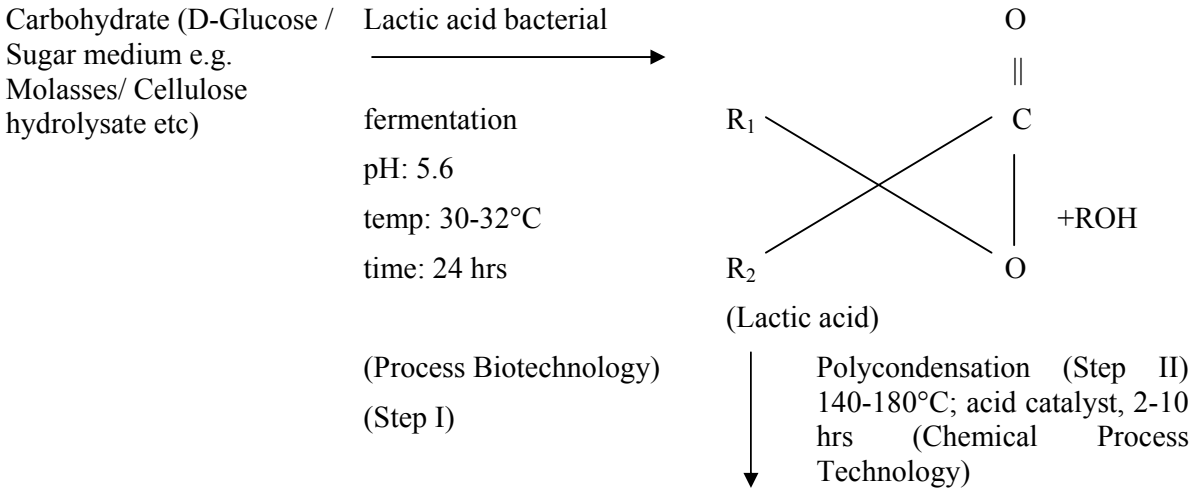
Table 1. Vegetable production data from mushroom compost in pot cultivation.

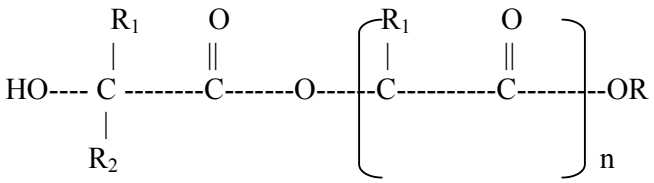
Pot No.	Brinjal			Lady Finger		
	FL(g)	Fmc(g)	Fc(g)	Fl(g)	Fmc(g)	Fc(g)
1.	853	790	660	202	165	120
2.	840	795	650	200	160	125
3.	830	785	645	210	170	122
4.	825	780	630	205	172	188
5.	810	780	632	204	168	115
6.	840	782	670	198	162	130
7.	820	770	688	195	160	125
8.	832	762	625	208	158	110
9.	845	750	630	202	155	115
10.	835	805	650	192	160	108
11.	830	810	645	205	165	115
12.	852	812	642	190	170	120
13.	815	775	678	212	162	125
14.	805	768	675	192	160	130
15.	840	778	668	195	165	122
16.	830	786	662	205	168	125
17.	858	792	642	208	170	120
18.	832	798	645	210	172	115

Table 2: Strengths and weakness of NCMC and Integrated EPBA in deliverying value added products.

Products	Strengths	Weakness
a. Mushroom	• Uncontentional • Processing conditions & preservation are favorable • B.V., PER, D.C., C.S and EEAP values are indicative of high nutritional value.	• Production & saleability in urban markets have not been surveyed. • Preservation for long duration is not well studied.
b. Biogas/Biomethane	• Highly useful in - lighting of gas mantle - cooking in stoves & burners - operation of fans, heaters, incubators - farm machinery operation - operation of I.C. engines - generation of electricity - running irrigation pumps	• filling technology in cylinders need improvement. • distribution in cylinders needs appropriate care.
c. Biomanure/Compost	• has suitable N:P:K ratio and C/N ratio	• Needs more evaluations with varieties of vegetable & fruit plants

b. BDP/IBDP: Soluble and insoluble biodegradable polymers are in great demand in recent years.





Polylactide

R_1 = H, R_2 = CH_3

Fig 2. Carbohydrate to polylactide conversion using Process Biotechnology and Chemical Technology

Using EPBA these are produced from waste/refuge waste materials. Polylactide polymers are produced involving microbial process biotechnology and chemical technology as shown in Figure 2. However, HP based biopolymers are produced by enzyme catalysis from leather industry chrome sludge waste as shown in Figure 3.

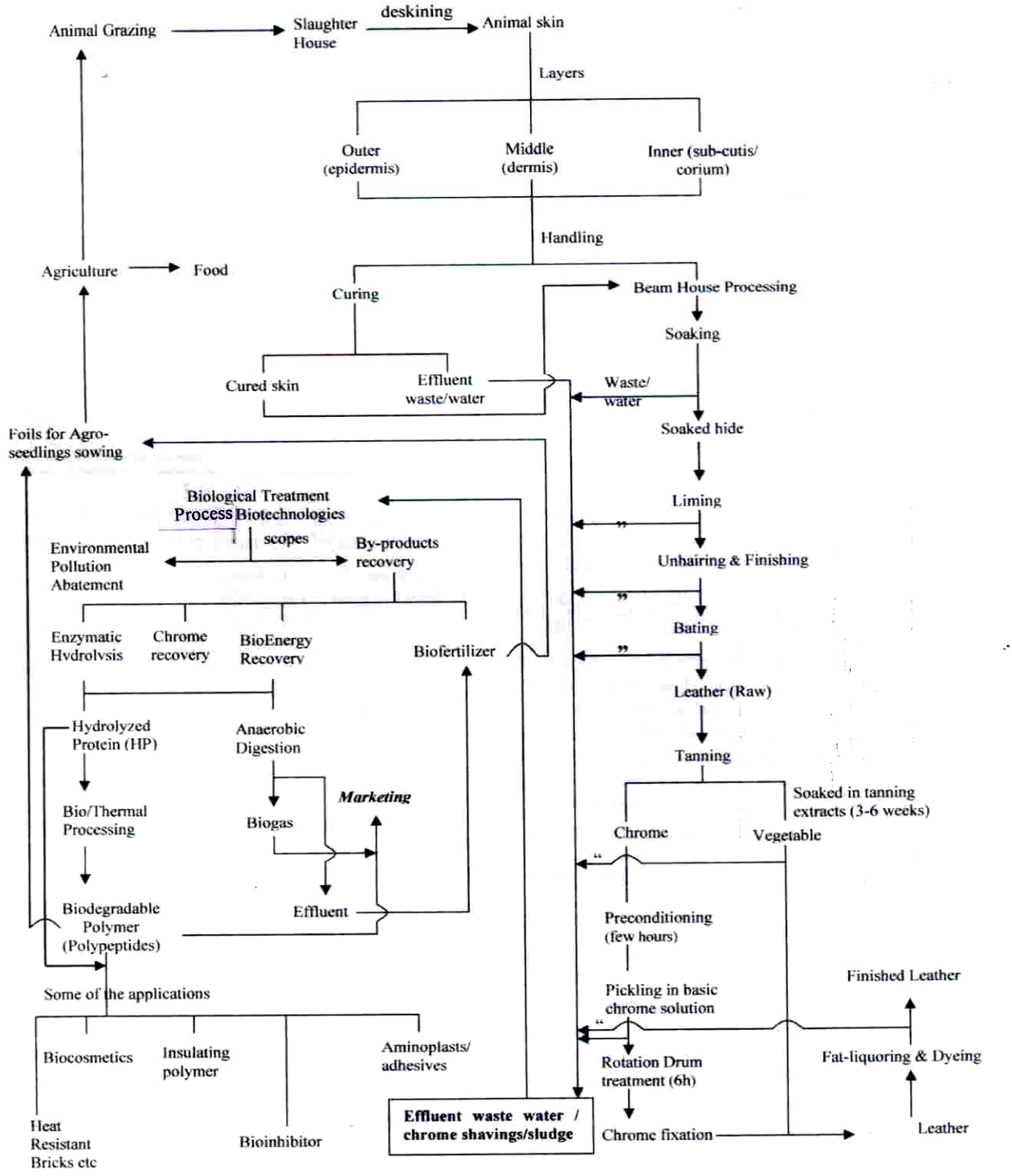


Fig 3. Total recycling process integration approach for economizing leather industry business and value creation opportunities (Mukhopadhayay, 2004a, 2006a**) showing EPBA.**

Industrial biodegradable polymers are produced in many ways. One of the ways is to use chrome sludge waste of tannery industry and its bioconversion to hydrolyzed protein by new enzyme catalysis. As chrome sludge waste resources have high protein content its protein content could be hydrolyzed economically by the enzyme alcalase (commercial grade) to produce HP as raw material. Subsequently this HP could be polymerized by polycondensation reaction with some aldehydes like gluteraldehyde to produce BDP. This BDP is water insoluble. Its further treatment with poly vinyl alcohol (PVA) transforms it into an improved biodegradable polymer (IBDP). IBDP is soluble in water. Both BDP and IBDP have been casted into membranes/films or other forms for various daily life and agricultural process biotechnology uses. Characterization of membrane / film in terms of pore dimension, action of acidic and alkaline soil solutions, biodegradability by mixed soil and sludge microbial flora, wettability and permeability have not been examined thoroughly. Based on the membrane/film porosity as determined from SEM photographs (**Figure 4 & 5**), important and interesting preliminary results were reported (**Pandey and Mukhopadhayay, 2006**). These results on characteristic properties of the film/membrane obtained from Tomas Bata University, Czech Republic and progress review on the usability of the BDP/IBDP membrane/film indicated that there are many possible uses and advantages of this BDP/IBDP as listed below.

Figure 4 Membrane Surface (324 X)

Figure 5 Membrane Porosity (28000 X)

7.2 Possible uses of BDP/IBDP

- Packaging industry, hospital laundry bags, sanitary products
- Containers of water-soluble substances, such as chemical agents for treatment of waste and potable water.
- Fertilizers, washing agents
- HP in blow extruded PVA makes it more economical – HP is 50% cheaper
- Contributing to solving a worldwide recognized disposal problem of the tannery industry.

7.3 Advantages

1. Play an important role towards environmental friendly materials and packaging and can improve the environmental impact of different produce.
2. Can solve some complex problems in agriculture, medicine, packaging industry and (bio) waste management.

3. Allow for cost reduction of the final items and are supposed to speed up the degradation process.
4. Indicate a possibly new lucrative option in Biodegradable Polymer Industry.

7.4 Conclusion

Integration of process biotechnology systems approach in concerned industry can provide multifacet advantages in terms of economic and technological feasibility. This approach would be a better solution towards cleaner environment protection and development of value added co products of commercial importance.

Acknowledgement:

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Chapter-8

UTILIZATION OF THE PERNICIOUS WEED TARO (*COLOCASIA ESCULENTA*) IN WASTEWATER TREATMENT AND AS A SOURCE OF METHANE AND MANURE

Bindu.T. and E. V. Ramasamy

Abstract

Aquatic plants cause immense harm to water resources by occupying lakes, ponds and canals and thus reducing the carrying capacity of these water bodies. In the absence of proper control measures, these plants emerge as harmful weeds and cause major nuisance to the environment. In this study we present our attempts on aquatic weed management through proper utilization. *Colocasia esculenta* – popularly known as *Taro* – a common semi-aquatic weed found in Kerala was first used as a bioagent in wastewater treatment, after the treatment the plants were harvested from the treatment system and subjected to anaerobic digestion for the recovery of energy.

Shallow raceways consisting 50 *Colocasia* plants in each, grown hydroponically in the wastewater were tested for their efficiency in removing the nutrients and stabilization of organic matter present in the wastewater. The weeds harvested from the raceways were anaerobically digested with solid feed anaerobic digesters (SOFADs). The fermentation of the weeds in the digester led to the formation of volatile fatty acids (VFAs) plus some biogas. The spent weed ensuing from the SOFADs was subsequently subjected to vermicomposting to obtain nutrient rich manure.

Key words**:** *Phytoremediation, biogas, earthworms, weed management, vermicomposting.*

8.1 Introduction

Biodegradable organics are the most common water pollutants. Sewage and night-soil are generated wherever human beings dwell, requiring processes to convert the organic pollutants in those waters to harmless inorganic substances such as carbondioxide, nitrogen etc.(Abbasi and Ramasamy, 1999). Developing methods which could rapidly, effectively and economically treat biodegradable wastes constitutes one of the greatest challenges to environmental and water resource engineers. Against this background, the emerging technology of using aquatic macrophytes – especially common weeds – for the effective treatment of biodegradable wastewaters, assumes greater significance.

The attention on vascular aquatic plants has been mainly directed towards their elimination from water bodies, since dense strands of these plants harm water resources in terms of quality as well as quantity (Abbasi and Nipaney, 1986). They also adversely

affect fisheries, impede navigation, hasten water loss, encourage insects, pests and disturb the fragile oxygen demand of water bodies through decay. As many as 90% of the approximately 2500 literature citations available on water hyacinth are related to their control (Abbasi and Ramasamy, 1999).

Colocasia esculenta, also known as Taro, the 'potato of the tropics', and 'elephant ears' is a wetland herbaceous perennial, found throughout the tropics and much of the sub tropics. An amphibious plant, it is fast emerging as one of the problematic aquatic weeds of Kerala. By now there is enough evidence, based on the experience from all over the world, that attempts to destroy a weed by chemical or biological means at best succeed in keeping that weed in check but at enormous direct costs (plus indirect costs to environment), and at worst facilitates the colonization of another equally or more harmful weed (Abbasi and Nipaney, 1986; Abbasi and Ramasamy, 1999; Bindu and Ramasamy, 2005; Kurien and Ramasamy, 2006). Alternatively, as learned from the survey of the past efforts, it is noted that the heavy initial clearance of the weeds from the water bodies/marshy lands followed by regular, periodic removal of the regrown weeds coupled with proper utilization of the harvested weeds appears to be a viable solution to the weed menace. By this means the high productivity of such weeds can be made as an asset, or else the weeds become a major nuisance to the environment (Gupta, 1979).

The authors have been trying to find ways and means of gainful utilization of *Colocasia* so that the cost of mechanically removing it from land / water can be partially or fully recovered from the gains, In the present study we describe our integrated approach of employing Taro as bioagents in domestic wastewater treatment followed by anaerobic digestion of the harvested weeds for the production of clean energy and finally bioconversion of the digested weed mass in to nutrient rich vermicasts through the action of earthworms.

Thin film technique (TFT) also known as nutrient film technique (NFT) has been used in the present study to treat the domestic wastewater. Among the various techniques of aquatic macrophytes based treatment systems (AMS) available, the TFT is the one which was rarely used despite the advantages such as high rate wastewater treatment and less land requirement are associated with TFT. In such systems the wastewater is made to flow through the aquatic plant roots at depths of only a few inches – as a 'thin film' just sufficiently deep to submerge the plant roots (Figure 1). This enables very intimate contact between the wastewater and the roots of the plans, enhancing the rate of treatment [1]. Anaerobic digestion of the harvested weeds was done with SOFADs, the advantage of solid feed process, as compared to the conventional anaerobic digestion process, is that the feed does not have to be liquefied. This has the potential of not only saving on expenses but also making the process simpler, hence more easily utilizable (Abbasi and Ramasamy, 1999; Bindu and Ramasamy, 2005).

Literature on the use of aquatic plants as bioagents in wastewater treatment systems and as feed stock in anaerobic digesters abounds with reports on these utilities with different plants, however, reports on the use of Taro (*Colocasia*) are scarce-perhaps none. An extensive survey of the literature carried out by the authors using computerised search methods reveal that the studies of the type described in this paper on the utilisation of Taro in wastewater treatment and subsequent conversion of it into methane and manure have not been reported so far.

8.2 Materials and Methods

Collection of plants

Colocasia esculenta plants were collected from marshy lands, banks of streams and canals of Kottayam, India. The stocks were maintained in plastic troughs.

Whole plants excluding the root portion harvested from raceways after completion of wastewater treatment were washed liberally with water to remove particles adsorbed on surfaces. After draining off the water, the plants were spread on a filter paper and air-dried for 30 minutes. The air–dried plants were chopped to pieces of approximately 2-3cm for charging into the digesters.

Collection and processing of domestic sewage

Fresh sewage was collected from nearby hostels, hotels and from a few houses. Then the wastewater was mixed well, and was allowed to stand for 30 minutes. The supernatant was decanted, also screened and sieved through coarse and fine sieves. The pH and COD of this screened wastewater was recorded. Based on the COD value, the wastewater was diluted with tap water to the desired level of COD.

Inoculum used in anaerobic digesters

The effluent slurry obtained from a mature plug-flow, cow dung-fed, floating-dome, biogas digester of KVIC (Khadi and Village Industries Commission) design was used as inoculum. The slurry was mixed with water in 1:4 (v/v) ratio, stirred well, and allowed to settle for about 4 hours in a glass column. The supernatant was used as inoculum after filtering it through a thin cotton cloth.

Digesters

Solid feed anaerobic digesters (SOFADs)

Two laboratory scale digesters were fabricated using PVC columns. Each digester had a total volume of 2.4 L and working volume of 2 L, the rest 0.4 L space has been left for fixing the leachate sprayer and empty headspace needed for biogas accumulation. The main body of the digester was partitioned in to two compartments using a perforated PVC disc (Figure 2). The upper chamber (solid phase) was used to load the solid feed and the lower (liquid phase), was allotted for the accumulation of bioleachate, which is dripping from the solid phase. The digesters were sealed airtight (Bindu and Ramasamy, 2005). The SOFADs were provided with the inlet and outlet ports as shown in figure 1. A separate methaniser (Methane phase reactor) unit was attached with each SOFAD. Continuously Stirred tank reactors (CSTRs) fabricated using one liter conical flask (Borosil glass) were used as methane phase reactors. Each CSTR was provided with two outlets – one for gas and another for effluent - and one inlet for the feed (Figure 2). The SOFADs and CSTRs will be referred as solid digesters and methanisers in the rest of the text, while referring to both SOFADs and CSTRs together it will be as digester system. All digesters and methanisers were connected individually to separate gas-collecting and measuring systems. The quantity of gas produced was measured daily by using a water displacement system.

Vermireactors

Circular, four plastic containers (diameter 24 cm, depth 9 cm) were filled from bottom up with successive layers of broken country bricks, river sand and garden soil of depth 2 cm, 2 cm and 4 cm respectively and used as vermireactors.

Analytical Methods

The wastewater (domestic sewage) was analysed periodically for the following parameters, COD, NO_3 -N, PO_4-P and DO (Dissolved Oxygen) as per the standard methods (APHA, 1998).

The dry solids (total solids - TS), volatile solids (VS), total nitrogen content of the plant material and inoculum were determined as per standard methods (APHA, 1998). Volatile fatty acids (VFA) content of the bioleachate was estimated by the method of Dilallo and Albertson (1961). Biogas was analyzed for its methane content by gas chromatography.

Experimental methods

Studies on wastewater treatment

The whole experiment was carried out in the laboratory with 14 hours photoperiod (7 am to 9 pm), with an average light intensity of 275 Lux and with a temperature range of 25 - 31^0 C.

In this experiment the wastewater (Sewage) was allowed to flow through shallow raceways (RW) consisting of plants with a low flow rate. 20 L of untreated wastewater with a COD concentration of. 250 mg O_2 / L was taken for treatment per raceway. Three parallel raceways were used in this experiment, two raceways were stocked with *C. esculenta* (50 plants per raceway) and one raceway was operated as control that is, without any plants in it. Each raceway was fed with the wastewater from a reservoir container (RC) through a flow – adjustable tap fitted at the bottom of the reservoir container. Each raceway had an individual RC. The RCs were plastic containers of 20 L capacity with lids. Each raceway is of half – cylindrical (a hollow cylinder cut horizontally into half) in shape, made out of poly vinyl chloride (PVC), having 2.5m length and 0.13m width (Figure 1).

The raceways (RW) were arranged with gentle slope so that the wastewater flows slowly from the head end through the root system of plants to the discharge end of the raceway and were finally collected in a collection container (CC). This operation was continued for 24 hours in two cycles. Each cycle represents 12 hours, thus, at the end of each 12 hour of continuous operation, the partially treated wastewaters collected in the CCs were recycled into the corresponding RCs. These experiments were operated in the same manner for 10 days. After 10 days, the entire wastewater from each RC and RW was completely drained and replaced with same quantity of fresh wastewater with COD of 250 mg O_2 / L. and the experiment was continued for the next 10 days in the same manner as described above, that is, as the second phase of the experiment starting from day 11 to day 20. However, the plant stock remained same in each RWs for the entire duration of 20 days. At the termination of each experiment (after 20 days), plants were removed from the raceways and the biomass was recorded.

The quality of wastewater being treated in the system was monitored through periodic sampling and analysis,

Studies on anaerobic digestion with SOFADs

To begin with, all digesters have been charged with 1.5 kg of fresh chopped *Colocasia* and 265 mL of inoculum providing total solids (TS) concentration of 10.7% in each of them. The bioleachate drawn from the outlet of the digesters were recirculated continuously for 20 minutes, thrice daily, using a peristaltic pump. After

one month of digester operation, as the VFA concentration in the bioleachate increased to the range of 4000 to 6000 mg /L a portion of the bioleachate was withdrawn from the digesters and was fed in to the methaniser (CSTR) units after appropriate dilution for further conversion into methane. The digesters were refilled with equal volume of distilled water in order to maintain the liquid volume constant in all the digesters till the end of the experiment. The start-up procedures of the CSTRs were adopted from Rosili and Ramasamy and Asha *et al* (2003; 2007). All digesters and methanisers were operated under ambient temperatures of 30 ± 2^0 C.

At the end of each month of operation, void created in each digester due to compaction of the feed was measured and the void volume was filled with new *Colocasia* (Bindu and Ramasamy, 2005).

Studies on vermicomposting

The spent (digested) weed withdrawn from SOFADs was air dried for one hour by spreading it over a tissue paper. After one hour, 150g of air-dried spent weed was weighed and spread over the vermibeds as feed. 20 healthy and adult earthworms of *Eudrilus eugeniae* Kinberg species were introduced in to each vermireactor. Water, 100 mL, was sprinkled over the bed on alternate days. Once in every 15 days, the castings were harvested, sieved to separate soil and other undigested matter. The casts and the remaining unutilized feed were air dried and quantified gravimetrically. Four vermireactors were operated in this study.

8.3 Results and Discussion

Studies on wastewater treatment

The performance of the raceways was assessed in terms of removal of NO_3-N, PO_4-P and stabilization of organic content in terms of COD removal. The change in the level of dissolved oxygen (DO) during the initial and final days of the experiment was also recorded.

A significant level of increase in the DO content of the treated water could be observed in all RWs (Figure1) however in control the increase was not significant. The COD removal was in the range of 7.20% to 95.36 % in the raceways with plants (Figure1). It was also observed that control raceway did not show any significant in removal. Among the nutrients, removal of NO_3-N was more prominent (Figure1).

The increased level of DO in the RWs demonstrates the ability of *C.esculenta* to pump more oxygen into the wastewater through its rhizosphere. The good oxygenation of the wastewater by the plants in their rhizosphere has lead to the 95.36% removal of COD. As it is the consortium of microorganisms residing in the vicinity of rhizosphere perform the oxidation of organic matter present in the wastewater and their action is dependent on the availability of DO. Thus the proper oxygenation of the wastewater by the plants has resulted in the significant level of COD removal. Similar observations are reported in the literature (Ignatius et al, 2006; Bindu and Ramasamy, 2007).

As far as nutrient removal is concerned nitrogen (nitrate – nitrogen) was the most preferred nutrient by the system. The PO_4- P uptake by plants in such shallow systems are generally reported to as poor (Ignatius et al, 2006).

When the removal percentage of nitrogen is examined, the removal seem to be

good during the first 10 days of the experiment, where the plants are fresh, the maximum removal of 98 – 100% could be noticed. Similar is the case with phosphate – phosphorus. All these observations are up to initial 7 days, after that, a slight increase in nitrate – nitrogen concentration was noticed. This might be due to the death and decay of plant parts such as roots, leaves etc, which in turn might be contributing nitrogen and phosphate to the wastewater. The nitrate – nitrogen removal is not that good in the second phase of the experiment, as the plants are old and their uptake might be less. Moreover, the change in the pH of the wastewater from neutral to acidic (due to the introduction of fresh sewage – which has a pH of 4.7 - 4.8 – from 10^{th} day noon onwards) might have caused little damage to the root system which in turn might have caused lesser uptake (Ansola et al, 2003; Dierberg et al, 2002). No significant removal of COD and nutrients was observed with the control raceway.

The results also indicate an increase in number as well in the phytomass of the plants used in this study. Apart from the number and phytomass increase, significant development in the root system was noticed in the plants towards the end of the experiment.

Studies on anaerobic digestion with SOFADs

The characteristics of *C.esculenta* and inoculum used in this study are presented vide Table1.

An increasing trend of biogas yield was observed with the digesters. The withdrawal of a portion of bioleachates from the SOFADs and replacing the loss with equal volume of distilled water once in every week might have has resulted in an enhanced performance of the digesters as evidenced by the biogas yield (Table 2), also the VFA concentration was maintained in the digesters with minor fluctuations. It may be noticed that the SOFADs used in this study along with CSTR methaniser units as whole digester system have performed with 4 to 5 fold higher gas yield than the gas yield reported in studies conducted only with high solid digesters without a methaniser unit (Asha et al, 2007). Further, the periodical removal of bioleachate from the digesters also helped in maintaining a constant level of VFAs in the digesters thus, avoiding the digesters becoming sour digesters due to the increasing concentration of VFAs if they are not removed periodically. Thus, this step has helped in operating the digesters continuously otherwise the accumulation of VFAs beyond certain concentrations would lead to the failure of the digesters (Hashimoto, 1986; Bjornsson, 1997).

The bioleachates, at the time of withdrawal were found to be having a VFA concentration ranging from 4000 to 6000 mg / L, thus diluting them to an appropriate concentration became essential before using them as feed in CSTRs. Accordingly, the bioleachates were diluted with enough distilled water to bring down the VFA concentration as 1500 ± 100 mg/L. With this concentration of VFAs in the feed the CSTRs have performed with a gas yield of 162 $L/m^3.d$ and with 54 and 39 % removal of VFAs and VS respectively (Table 3). The quantity of bioleachate withdrawn once in a week from the digesters was sufficient to feed the corresponding CSTR for 7 days with appropriate dilution. Thus the entire digester system consisting both of SOFADs and CSTRs could be operated sustainably.

The gas generated during initial two weeks was not combustible, only after third week of operation the gas was found combustible indicating the presence of methane.

Further analysis of the biogas with gas chromatograph has indicated that the biogas consisted 40-60% of methane.

Previous studies conducted by us and others have pointed out clearly that the efficiency of high-solid digesters could be enhanced with the increasing level of phase separation (Abbasi et al, 1992; Bhattacharya, 1996; Ramasamy and Abbasi, 2000; Hutnan et al, 2001). The findings of our earlier study (Bindu and Ramasamy, 2005) has indicated that the digesters designed with single compartment where all the three phases of anaerobic digestion taking place in the single digester unit have performed with lesser efficiency than the digesters designed with two compartments. The performance of digesters compartmentalized into solid and liquid phase were better because the liquefaction (hydrolysis) and acidification (acidogenesis) of the phytomass perhaps occurred in the solid phase compartment and the conversion of volatile acids into methane might have taken place in the liquid phase compartment. However, that design has not provided the complete phase separation as well any process control in terms of pH or VFA adjustment could be attained with. For instance, the lower chamber was not completely separated from the upper chamber, as the separation was with a perforated plate which allowed the bioleachate to continuously drip from the upper chamber. Such continuous dripping of bioleachate rich in VFAs would keep altering the pH of the liquid (bioleachate) present in the lower chamber, which in turn would have affected the methanogens, thus the overall process of methanogenesis might have been affected. Realising this limitation of the previous study an attempt was made in the present study to attain a complete phase separation. Thus in the digester system used in the present study the liquefaction (hydrolysis) and acidification (acidogenesis) of the phytomass was allowed to take place in the solid phase digester and the conversion of volatile fatty acids into methane was carried out in separate CSTR units.

Performance of vermireactors

The studies on the vermicomposting of spent weed indicate that the worms readily accept the feed and convert it into vermicasts. About 45-50% conversion of the feed in to vermicasts has been recorded in all four vermireactors operated in this study.

6.4 Conclusion

The findings of the study lead to the following significant conclusions:

1. The results indicate that *C. esculenta* has the potential of removing the nutrients and stabilizing the organic matter in the sewage when it is used as a bioagent in the thin film system (TFT) of wastewater treatment.
2. The harvested plants from the raceways could be utilized gainfully by subjecting them to anaerobic digestion for the generation of biogas – a clean fuel.
3. Solid feed anaerobic digesters (SOFADs) coupled with a methaniser appears to be a suitable system for the biomethanation of *C. esculenta*.
4. Bioconversion of the spent weed ensuing from the SOFADs in to vermicasts by the earthworms leads to the complete utilization of the pernicious weed.

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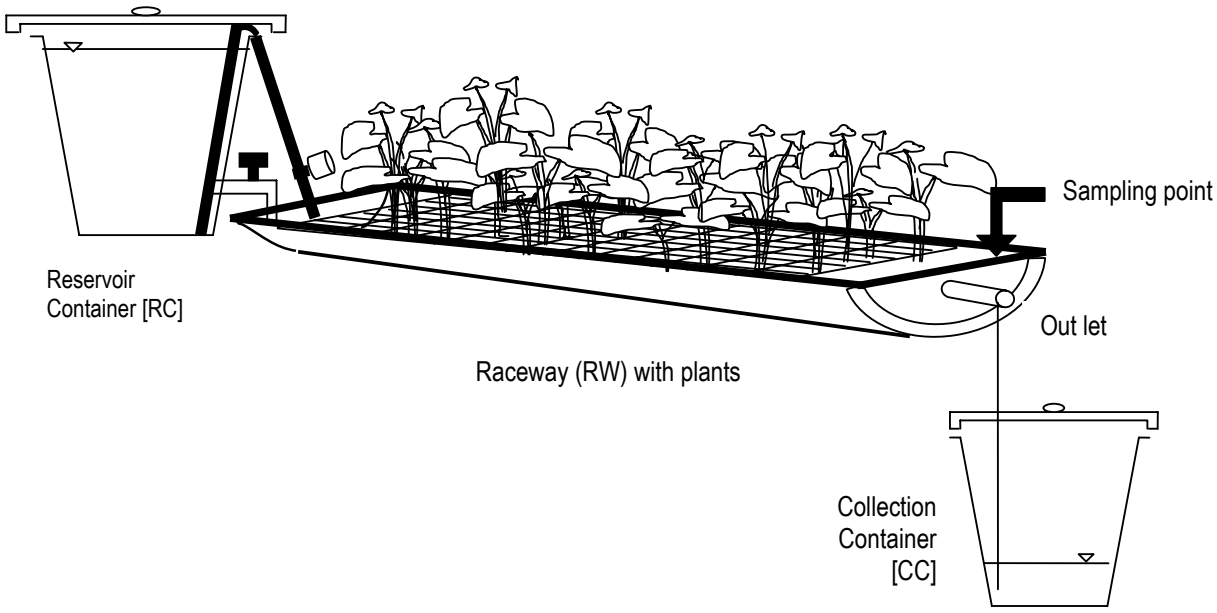

Figure 1 : Schematic diagram of the experimental set-up of thin film technique (TFT).

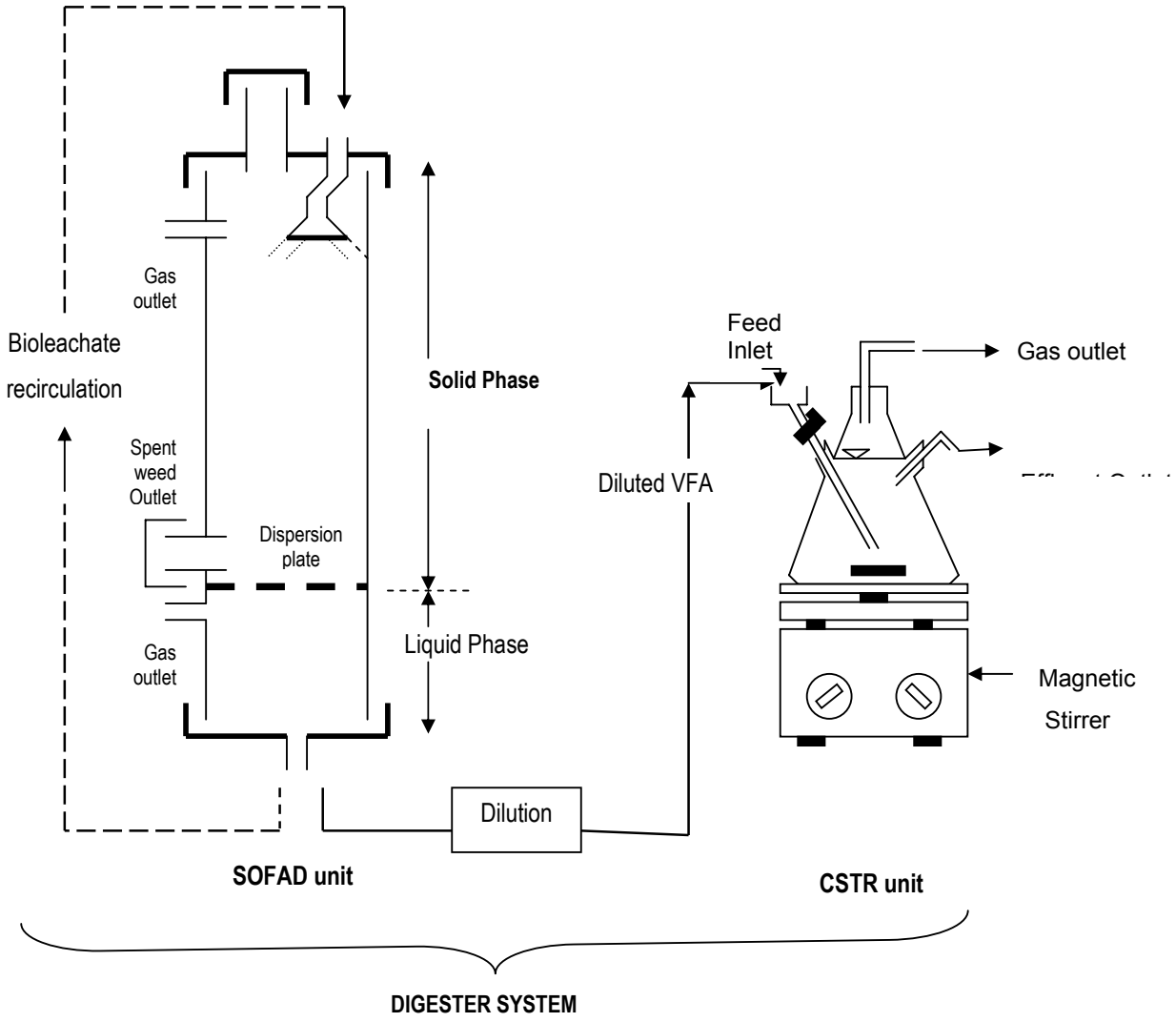

Figure 2: Schematic diagram of Digestion system used in this study

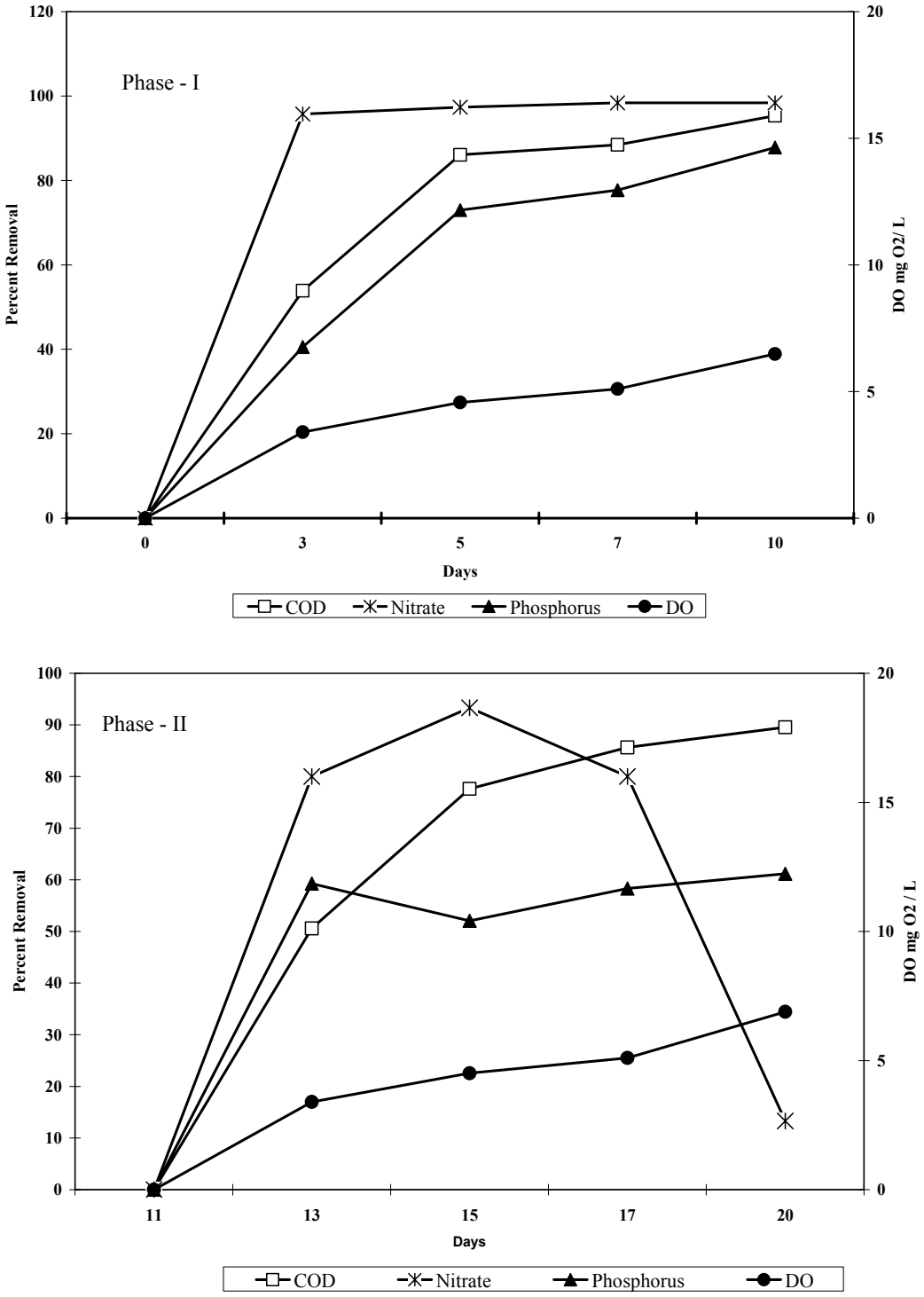

Figure 3 : Performance of TFT with C.esculenta in terms of removal of COD, Nitrate-Nitrogen, Phosphate-Phosphorus, and change in DO content at phase –I and phase-II of the experiments.

Table 1: Characteristics of feed stock, inocula used in SOFADs

Substrate	***T.S. (%)***	**VS(%)***	***TKN g/kg***	***Moisture content(%)***
Colocasia	8.32	80.55	-	91.68
Colocasia + inocula	10.7	41.3	28.98	89.3
Cow dung (inocula)	7.03	77.7	-	92.97

VS%*: Reported as percent of T.S.

Table 2 : Performance of SOFAD system in terms of biogas yield* (L / m^3.d)

Weeks	Biogas yield (L / m^3.d)		
	Solid digester unit (SOFAD)	**Methaniser unit (CSTR)**	**Total Digester system**
5	40	5.00	45.00
6	43	11.00	54.00
7	56	14.60	70.60
8	61	16.90	77.90
9	59	18.30	77.30
10	60	21.83	81.83
11	58	21.69	79.69
12	54	61.25	115.25
13	49	92.63	141.63
14	55	117.89	172.89
15	57	92.83	149.83
16	60	166.23	226.23
17	63	152.60	215.60
18	60	156.50	216.50
19	63	161.00	224.00
20	60	162.30	222.30

** : Biogas presented is the average (of duplicates) gas yield on the last day of the week*

Table 3 : Performance CSTR reactor in terms of biogas yield and VFA ,VS removal.

Period (Week)	Feed (Influent)		Effluent		Removal percentage of		Biogas yield
	VFA Concentration (mg /L)	VS Content (mg /L)	VFA Concentration (mg /L)	VS Content (mg /L)	VFA	VS	(L / m^3.d)
5	1400	23.0	950	16.7	32.1	27.3	5.00
10	1600	24.8	860	15.8	46.2	36.2	21.83
15	1450	23.7	720	14.7	50.3	37.9	92.83
20	1350	22.6	610	13.6	54.8	39.8	162.30

Chapter-9

Management of Urban Forestry in the Perspective of Biofiltering of Air Borne Contaminants

Sandeep Kar, A. C. Samal and S. C. Santra

Abstract

The forests and trees of urban areas are essential elements to both environment and the peoples of urban community, having great role in pollutant removal, climatic amelioration, engineering, architectural and aesthetic uses. Recent development in industrialisation and urbanization has resulted in a profound deterioration of urban environmental quality and also human health by producing large amount of pollutants specially air borne contaminants. To abate the impact of atmospheric pollutants, there is a need for a perennial green envelope requiring large-scale afforestation and green belt development in and around urban areas. Here comprehensive accounts of management system of urban forestry have been discussed to build up future guideline for development of urban forestry to clean the city environment.

9.1 Introduction

Most of the urban areas today have high concentration of different environmental pollutants from different sources coming from different anthropogenic activities resulting from rapid economic growth, industrialization, urbanization with associated increase in energy demands. These pollutants not only create a threat to human health and the urban environment but it can also contribute to serious regional and global atmospheric problem.

The air pollution problems are now affecting the developing world including India, which is witnessing rapid industrial and urban development. Indian mega cities are becoming the polluted ones with respect to particulate pollution (Samanta et al., 1998). The level of particulates is increasing beyond the recommended level of WHO. The focus has been made on major Indian cities like Delhi, Mumbai, and Kolkata etc., which constitute major pollution burden due to atmospheric pollutants, and shows salient issues in different kind of health risks (Ghosh et al., 2005). According to World Health organization, the top ten most polluted cities in the world include Kolkata as well as capital city New Delhi (WHO and UNEP, 1992). Sources of air pollution, come in several forms, including vehicular emissions, untreated industrial smoke, domestic combustion of fossil fuels, metallic workshop, wind blown dusts etc. The emission of air pollutants are gradually increasing while India's gross domestic product has increased 2.5 times over the past two decades, vehicular pollution has also increased eight times, while pollution from industries has also increased several times.

With the persistent efforts of monitoring agencies and research institutes a considerable data are now available with respect to urban air quality. All the components of the city environment showed a distinctive feature of maladies in urban environment and thus impose the necessity of appropriate management strategies for urban air environmental quality improvement. With regard to the mitigation of this severe problem, policy should be adopted to control the pollution burden by means of

monitoring, filtering and regulating the atmospheric pollutants and their proper management. One of the important technique is the green belt development with suitable plant species in appropriate manner which is highly imperative to biofilter the atmospheric pollutants as well as other economic value.

9.2 Status of atmospheric pollutants (Urban Scenario)

Among the air pollutants, gaseous elements like NOx, SOx, HC, CO and CO_2 and suspended particulates play a major role in urban atmospheric pollution. In addition some other harmful contaminants are PAHs, VOCs which cause detrimental impact if present in a substantial amount.

9.2.1 Gaseous elements in air

There are many substances in the air which may impair the health of plants and animals (including humans), or reduce visibility. These arise both from natural processes and human activity. Substances not naturally found in the air or at greater concentrations or in different locations from usual are referred to as 'pollutants'. Pollutants can be classified as either primary or secondary. Primary pollutants are substances directly produced by a process, such as ash from a volcanic eruption or the carbon monoxide gas from a motor vehicle exhaust. Secondary pollutants are not emitted. Rather, they form in the air when primary pollutants react or interact. An important example of a secondary pollutant is ozone—one of the many secondary pollutants that make up photochemical smog. Note that some pollutants may be both primary and secondary: that is, they are both emitted directly and formed from other primary pollutants.

Primary pollutants produced by human activity include:

- oxides of sulfur, nitrogen and carbon
- organic compounds, such as hydrocarbons (fuel vapour and solvents)
- particulate matter, such as smoke and dust
- metal oxides, especially those of lead, cadmium, copper and iron
- toxic substances.
-

Secondary pollutants include some particles formed from gaseous primary pollutants and compounds in photochemical smog, such as nitrogen dioxide, ozone and peroxyacetyl nitrate (PAN).

Sulfur dioxide-

Sulfur dioxide in the atmosphere arises from both natural and human activities. Natural processes which release sulfur compounds include decomposition and combustion of organic matter; spray from the sea; and volcanic eruptions. The main human activities producing sulfur dioxide are the smelting of mineral ores containing sulfur and the combustion of fossil fuels. Sulfur dioxide dissolves in water to form sulfuric acid. This is a corrosive substance that damages materials and the tissue of

plants and animals. Sulfur dioxide is a respiratory irritant and may worsen existing respiratory illness.

Oxides of nitrogen-

The main oxides of nitrogen present in the atmosphere are nitric oxide (NO), nitrogen dioxide (NO_2) and nitrous oxide (N_2O). Nitrous oxide occurs in much smaller quantities than the other two, but is of interest as it is a powerful greenhouse gas and thus contributes to global warming. The major human activity which generates oxides of nitrogen is fuel combustion, especially in motor vehicles. Oxides of nitrogen form in the air when fuel is burnt at high temperatures. This is mostly in the form of nitric oxide with usually less than 10% as nitrogen dioxide. Once emitted, nitric oxide combines with oxygen ('oxidises') to form nitrogen dioxide, especially in warm sunny conditions. These oxides of nitrogen may remain in the atmosphere for several days and during this time chemical processes may generate nitric acid, and nitrates and nitrites as particles. These oxides of nitrogen play a major role in the chemical reactions which generate photochemical smog. Nitrogen dioxide is also a respiratory irritant which may worsen the symptoms of existing respiratory illness.

Carbon monoxide-

Carbon monoxide is an odourless, colourless gas produced by incomplete oxidation (burning). As well as wildfires, carbon monoxide is produced naturally by oxidation in the oceans and air of methane produced from organic decomposition. In cities, the motor vehicle is by far the largest human source, although any combustion process may produce it. Carbon monoxide usually remains in the atmosphere for a month or two. It is removed by oxidation to form carbon dioxide, absorption by some plants and micro-organisms, and rain. When inhaled, carbon monoxide binds to the oxygen-carrying site on the blood's haemoglobin, which reduces oxygen transport in the body. At high concentrations it is very toxic, causing headaches, dizziness, reduced ability to think, and nausea.

Ozone-

Near the ground, ozone is a colourless, gaseous secondary pollutant. It is formed by chemical reactions between reactive organic gases and oxides of nitrogen in the presence of sunlight. Ozone is one of the irritant secondary pollutants in photochemical smog and is often used as a measure of it. Ozone is strongly oxidising and can irritate the eyes and the respiratory tract. It also damages plants. The formation of ozone in the upper levels of the atmosphere or 'stratosphere' is by a different process. Ozone there is not regarded as a pollutant because it is produced naturally. It is important in absorbing harmful ultraviolet radiation and preventing it from reaching the earth.

Lead-

The major source of lead in the air is leaded fuel used in motor vehicles. The introduction of unleaded fuel in 1985 has resulted in a substantial decrease in the concentration of lead in the air. Lead is a heavy metal and, when present in the body, can impair brain function, especially in children.

Air toxics-

In the past decade it has become known that many potentially toxic substances are present in measurable quantities in ambient air. New technology has enabled these to be measured at smaller concentrations than previously. These pollutants are collectively called 'air toxics'. Air toxics can enter the atmosphere from a variety of sources. They are present in motor vehicle exhaust, fuel vapour from service stations, the smoke and exhaust from wood-burning heaters, and the solvents used for dry cleaning and spray painting. Air toxics are also emitted from point sources such as chemical plants, oil refineries, electroplaters and incinerators. Air toxics are generally inhaled, but they may also contaminate water and soil and hence be ingested with food. They may also enter the food chain in this way. Some air toxics are naturally concentrated by animals, leading to high levels in those near the top of the food chain.

Like all other mega cities of the world, the Kolkata Metropolis (India) experiences a severe pollution problem over past couple of decades. The pollution of different categories arises out of industrial activities, vehicular emissions, domestic emission and other miscellaneous activities. Regarding air pollution, the situation is most dangerous as SPM, NOx levels showing high beyond their standards (Table-1).

Table-1: Annual average air quality of Kolkata city, 2005

Month	**Parameters (μg/m^3)**			
	SPM	**RSPM**	**NOx**	**SO_2**
January	345.09	210.08	92.80	17.16
February	347.31	203.74	92.34	16.22
March	285.39	143.30	61.59	7.41
April	223.44	81.93	39.80	5.47
May	180.81	64.01	37.55	4.74
June	164.31	59.43	36.54	4.53
July	121.25	50.06	36.99	3.63
August	135.01	52.52	40.98	4.33
September	133.04	49.78	37.66	3.84
October	132.54	53.55	49.02	5.92

November	239.13	122.71	78.71	11.72
December	346.78	207.80	101.83	18.29

Source: Vasundhara Annual Environment Survey Kolkata, 2005.

9.2.2 Air borne particulates

Atmospheric particulates include fine and coarse particles consisting various heavy metals and trace elements. Their presence in a higher level and consequent transformation to biological organism induce a variety of health effects which are currently considered as major problems in the highly urbanized regions of the world. Among the trace elements Pb, As, Ni, Cd, Zn, Cu etc. are more important with respect to their toxicity which causes adverse effects on urban population and on vegetation also. Total suspended particulate (TSP) pollution is reported (WHO & UNEP, 1992) as serious in major cities in Asia, including Mumbai, Kolkata and Delhi.

Source and occurrence of particulates-

The air quality in urban areas has a great influence, both on regional and local scale, on the transport of air pollutants and air transformation phenomena. Urban particulates are emitted directly to the atmosphere from various sources and remain suspended in the urban atmosphere. Sometimes they get dispersed to the surrounding areas due to meteorological factors specially wind flow. The PM_{10} mass is made up of a mixture of natural and anthropogenic sources whereas finer $PM_{2.5}$ component almost are from anthropogenic sources. However different source apportionment study highlights the importance of addressing several sources of atmospheric particulate pollution (Wang and Shooter, 2005; Samanta et al, 1998). In particular, fossil fuel use in industry and automobiles as well as for domestic heating can become a significant source of airborne fine particulate matter. It is considered that traffic is one of the main sources of particulate pollution. Thus traffic together with other particulate sources contributes to urban dust pollution. As much as 80% of emissions of these health damaging particles can come from road traffic. In Beijing, China particulate matters are carried over the city by dust storms especially in spring and in other seasons by common industrial and domestic activities (Kuang et al., 2003). In cities of NewZeland, mass concentration of PM_{10} exceeds 50 $\mu m/m^3$ 24 hr health guideline in winter to be caused by old fashioned domestic heating as reported (Krivacsy et al., 2006).

In India, the particulate has assumed serious proportion in the metropolitan cities because of the concentration of the individual and tertiary activities. They are steadily increasing because of spirally fossil fuel consumption and also in the transport sector (Varsney et al., 1997). The air pollutants of major concern in India are particulate matter (Singh et al, 1994) and traffic is the major concern for particulate pollution (Kar et al., 2006). The contribution of road dust can also be significant. Predictably the combined contribution of biomass and coal is the highest in winter presumably as a result of heating. Contributions from solid fuel combustion are also significant in non heating seasons. If diesel is attributed to mobile sources, vehicle exhaust becomes the largest contribution.

Trace elements especially heavy metals are most commonly associated with fine particles in contaminated atmosphere having major anthropogenic sources (Chandra Mouli et al., 2006). Investigation conducted in roadsides, industrial and urban environments have dramatically demonstrated the impressive burdens of particulate heavy metals (Tukoglu et al., 2004). In the case of lead in roadside ecosystem the increased lead burden of plants, largely due to surface deposition may be 5-200 time baseline lead levels for unwashed crops, grasses, trees respectively (Singh et al., 1995). Special attention has been focused to metals with highly toxic effects such as Pb whose predominant source in our country is still from road traffic leading to wide dispersal in city air (Kemp, 2002). The occurrence of arsenic contamination in particulate deposition on canopy leaves and street dust have also been studied in different city (Kar et al., 2006). The occurrences of other heavy metals such as Cd, Zn, Ni, and Fe have also been found in ambient air in different Indian cities (Chandra Mouli et al., 2006).

9.3 Health and Environmental Effects

Effects of gaseous pollutants-

Many studies have shown links between pollution and health effects. Increases in air pollution have been linked to decreases in lung function and increases in heart attacks. High levels of air pollution according to the EPA Air Quality Index directly affect people with asthma and other types of lung or heart disease. Overall air quality has improved in the last 20 years but urban areas are still a concern. The elderly and children are especially vulnerable to the effects of air pollution.

The level of risk depends on several factors:

- the amount of pollution in the air,
- the amount of air we breathe in a given time
- our overall health.

Other, less direct ways people are exposed to air pollutants are:

- eating food products contaminated by air toxins that have been deposited where they grow,
- drinking water contaminated by air pollutants,
- ingesting contaminated soil, and touching contaminated soil, dust or water.

Air pollutants usually act upon the respiratory tract, first. Air pollutants can enter a human body through several entranceways, such as the skin, mouth and lungs. Gaseous pollutants can deeply penetrate the respiratory system and absorb on wet body tissues on the way. This may cause the pH of the body fluids to change, causing irritations. Organic solvents can easily be absorbed by blood, transporting them through the entire body rapidly. The chances of penetration of a human body by contaminants mainly depend upon the size of the particles. Larger particles stay behind in the nose or are swallowed, but smaller particles can penetrate the lungs. Smaller particles absorb more toxic material, which they can take deeper into the body. When contaminants are water-soluble, they can immediately be absorbed by human blood.

The health effects that pollutants cause with animals are similar to the health effects they cause with humans. However, there are some effects that do not occur with humans. For example, acid rain can cause serious health effects for water organisms, such as reproduction failure, or even death.

Plants are much more susceptible for gaseous air pollutants than humans and animals. Examples of gaseous air pollutants are hydrogen fluoride (HF), ozone (O_3) and ethene. Air pollutants mainly penetrate a plant through skin pores. These are in connection with the intercellular spaces in the leaves of a plant. Through the skin pores gasses can penetrate cell walls, to be absorbed by the cell fluids. Some of the gaseous air pollutants directly damage plant leaves when they penetrate plant cells. This can cause the leaves of plants and trees to lose their colour, or even die off. It can also cause plant growth to stagnate. The leaves and stems may slacken and curl up. In some cases however, a plant can restore itself after fresh air is added to its location.

Health effects of air pollutants do not occur until a certain dose has penetrated a plant, animal or human body. This dose differs for each air pollutant. When the concentrations of air pollutants increase, the risk of health effects will be higher. Air pollutants can cause acute effects, such as coughing and throat pains, but there are air pollutants that only cause cronic effects, such as asbestos. Here, we will sum up some commonly known air pollutants and give a brief description of their health effects.

Carbon monoxide (CO)-

Carbon monoxide is a gas that is absorbed by haemoglobin in the blood after it has entered a human body, causing the blood to be less able to transport oxygen. When carbon monoxide is absorbed in low concentrations, people will experience tiredness. People that have heart conditions often experience chest pains. Higher concentrations of carbon monoxide cause bad vision, decreases in concentration, headaches, dizziness, nausea and confusion. When carbon monoxide concentrations are very high, it can be mortal.

Carbon monoxide is likely to fashion during incomplete combustion. To prevent the fashioning of carbon monoxide in your house, you may want to clean your chimneys and heaters once a year. It is also recommended to have water heaters checked out.

Sulphur dioxide (SO_2)-

The effects of sulphur dioxide mainly take place when it is converted to sulphuric acids. This acid has some negative effects on the respiratory tract. Sulphur dioxide is fashioned during fossil fuel combustion.

Ozone (O_3)

Ozone causes mucous membrane irritation, respiratory infections and lung infections. Ozone consists during thunderstorms, through electrolyses of oxygen. Ozone is generated through the same principle. Ozone is also shaped in traffic. It exists naturally in the atmosphere, under the influence of UV-radiation.

Nitrogen oxides (NO_x)

The health effects of nitrogen oxides are similar to those caused by ozone. However, health effects of nitrogen oxides only occur when higher concentrations are absorbed. Nitrogen oxides are fashioned during high-temperature combustion. Studies on human populations indicate that long-term exposure to NO_2 levels currently observed in Europe may decrease lung function and increase the risk of respiratory symptoms such as acute bronchitis and cough and phlegm, particularly in children. Even though some studies have shown associations between NO2 exposure and mortality, present evidence is not sufficient to conclude that effects on mortality can be attributed to long-term exposure to NO2 itself. NO_2 alone has been shown to cause acute health effects in controlled human exposure studies. Studies on human populations have not been able to isolate potential effects of NO_2, because of the complex link between concentrations in ambient air of NO_2, particulate matter, and ozone. Several studies have shown that NO_2 exposure increases allergic responses to inhaled pollens. People with asthma and children in general are considered to be more vulnerable to NO_2 exposure. There is no evidence for a threshold for exposure to NO_2 below which no effects on health are expected.

Asbestos

Asbestos has no acute health effects. Long-term exposure to asbestos can cause breast, peritoneum and lung cancer, along with various lung infections. Asbestos is a very persistent contaminant, it is hardly biologically degradable and it can withstand heat, acids and basics. This makes asbestos suitable for many different applications. Due to the fact that asbestos is a carcinogen, it is now banned from all applications in many countries.

Lead (Pb)

Cars that use lead-containing gasoline, will emit lead. High lead concentrations in air can cause disturbances in physical and mental development of children. Lead is commonly known to cause a decrease in coordination and mental capacities, as well as kidney damage, damage to the nervous system and a lack in red blood cells.

Other toxic elements:

Benzene is a carcinogenic contaminant. It causes leukaemia. PAH's are potentially carcinogenic contaminants. They are fashioned during several industrial processes and they are emitted by traffic. Cigarette smoke can cause mucous membrane irritation, eye, nose and respiratory infections, bronchitis and lung cancer. Hydrocarbons cause mucous membrane irritations, headaches, a loss of coordination, nausea and liver damage. Some hydrocarbons are potentially carcinogenic to humans, as well as animals. There are many more air pollutants that can cause toxic effects. Examples are acronytrile, carbon sulphide, dichloromethane, styrene, toluene, poly

vinyl chloride (PVC), hydrogen sulphide, arsenic, cadmium, chromium, manganese, mercury, nickel and vanadium.

The nature of an air toxic determines what sort of effects it may have. Some are irritants, especially to the eyes, nose, throat and lungs. Some are known to cause cancer, while others can lead to reproductive abnormalities or impair the nervous system. Air toxics containing chlorine are of special concern—many cause cancer and accumulate within the body. Particular examples are dioxins and furans. Others tend to accumulate within the food chain and have been blamed for causing a reduction in biodiversity and seal and dolphin populations.

Effects of particulates-

Atmospheric particulate pollution and its impact on the environment and particularly on human health, is an issue of significant public and government concern today. Exposure to particles can lead to variety of serious health effects. Continuous inhalation of toxic compounds present in the atmospheric particles may cause chronic pulmonary disease (Chakrabarty, 1988). High concentrations of PM_{10} have been linked with increased incidence and severity of respiratory illness and also plays important role in genesis and augmentation of human disorders and affect other respiratory system (Ghose et al., 2005). The smaller particles of the $PM_{2.5}$ size range (2.5 μm) are considered the most damaging to health, since they can more easily penetrate the respiratory tract. Pearce and Crowards (1996) focused on the relationship between particulate matter and human health. Their study suggests that as many as 12,000 deaths in the UK might be attributed to atmospheric particulate concentrations, or about 7,000 deaths if only PM_{10} is considered. Of the 3 million premature deaths in the world that occur each year due to outdoor and indoor air pollution, the highest number are assessed to occur in India (Energy Information Administration, 2005).

Coarse and fine particles pose the greatest problems because they can get deep, into the lungs, and some may even get in to the bloodstream. Long term exposures to PM such as those experienced by people living for many years in areas with large particulate concentration are associated with problems such as decreased lung function, development of chronic bronchitis and premature death. Short term exposures to particle pollution (hour/days) are associated with a range of effects, including decreased lung function, increased respiratory symptoms, cardiac arrhythmias, heat attacks. Sensitive groups at greatest risk include people with heart or lung disease, older adults and children.

Regarding the effects of atmospheric particulates on urban vegetation they shows different kind of toxicity (Nash and Sommerfield, 1981). Particulates cause direct injury to plants by interfering with light absorption, by contaminating stomata apertures, or by direct chemical reactions with plant tissue (Samal and Santra, 2002). However there is a lack of adequate knowledge on the impact of anthropogenic activities on chemical composition of urban particulates with respect to the concentration of the trace elements.

9.4 Existing control strategies

A number of cities in India are currently developing action plans to control and improve air quality specially the atmospheric particulates by different ways. The focus

would have been made to implement and develop better monitoring technique of suspended particulates and their proper characterization specially for trace elements and heavy metals. Understandingly, much policy attention has concentrated on to control vehicle exhaust in urban region by means of using unleaded fuel, adapting advance technology in combustion engine. With respect to other important source i.e. industry, specific point sources would be thoroughly monitored and regulated according to their pollutant emission. Sometimes legal approach has been made to control the particulate concentration.

Different organizations and researchers are currently investigating the biomonitoring processes by which trees can improve urban air quality by biofiltering the particulates. They focused on the removal of particulate pollution by urban trees and woodlands, with the aim of quantifying the effectiveness of different species and to provide a model of pollutant uptake for a variety of urban areas. Therefore green belt development could be an effective technique for the abatement of particulate pollution specifically in urban region with the help of planting suitable tolerant species.

9.5 Importance of Biofiltering techniques for pollutant remeriadiation

It is an established fact that vegetation plays an important role in cleaning the atmosphere by absorbing certain toxic air pollutants from its surroundings and also abatement of noise pollution. Thus Green belts are recommended for containment of air pollution in the human environment, especially in urban and industrial environment. NEERI has developed a model for green belt development in relation to pollution around industrial premises (NEERI, 1995). In addition, there were number of other benefits like aesthetic improvement, climatic amelioration, biomass generation, enhancement of biodiversity etc. are the bonus derived through the presence of greenery in the areas. In India, (CPCB, 1995) it has become mandatory for large scale polluting industry since mid nineties to plant appropriate green belts in and around its unit to protect surrounding ecology.

According to the CPCB report, green belts are thought to be effective in environments where green plants form a surface capable of absorbing air pollutants and forming sinks for these (Pandey, 2000). Therefore greenbelt can be a significant mitigatory option to reduce air pollution specifically for atmospheric particulates (Rao et al., 2004). Among the various air pollutants, particulates pollutants (size range 1-50 μm) including heavy metals are considerably removed from polluted air by vegetation cover (Zhuang and Wang, 2000). Research has shown that trees act as biological filters, removing large quantities of particles from the urban atmosphere as much as 234 tons per year in the Chicago, USA area, a recent study showed (Broad Meadow et al, 1998). This is predominantly due to their large leaf areas relative to the ground on which they stand, and the physiological properties of their surfaces i.e. the presence of trichomes or waxy cuticles on the leaves of some species.

9.6 Biofiltration of pollutants

Interest in the ability of plants to remove pollutants from the air has grown considerably in recent years (Agarwal and Agarwal, 2000) particularly in urban and suburban areas. Plants can reduce pollution by intercepting airborne particles. Various plants have differential degree of adsorption and assimilation capacity of such particulates consisting of metals without showing any toxicity. The capacity of plants to

act as a sink for air contaminants has been addressed by a variety of recent reviews (Harju et al, 2002; Pyatt and Heywood, 1989). These papers indicate that the surface of vegetation provide a major filtration and reaction surface to the atmosphere and also important function (Pandey, 1993). Plants with leaves having rough or hairy surfaces are most effective dust collector (ENVIS Newsletter, 2005). Some biological and environmental factors play an important role in the adhesion and subsequent potential accumulation of particulate pollutants.

Plants remove air pollutants primarily by uptake via leaf stomata and once inside the leaf, gases diffuse into intercellular spaces and absorbed by water films. Plants, grown in such a way as to function as pollutants sinks are collectively referred to as greenbelts, which have limits to their tolerance towards air pollutants. The capacity of taking up and accumulating environmental toxicants in tree foliages, the advantage big biomass and easy disposal makes it possible for plants to remove toxicants by phytoremediation in situ and consequently to reduce pollutant concentration in the urban environment. Therefore greening by plantation, which makes use of vegetation to remove, detoxify or stabilize persistent pollutants, is a green and environment friendly tool for clean environment.

9.7 Criteria selection of plant species for urban forestry

Green belts are thought to be effective in pollution abatement, where green plants form a surface capable of sorbing air pollutants and forming sinks for pollutants. Leaves with their vast area in a tree crown, sorbs pollutants on their surface, thus effectively reduce their concentrations in the ambient air, often such absorbed pollutants are incorporated in metabolic stream and thus the air purified. Plant grown in such a way as to function as pollutant sinks are collectively referred to as greenbelts. The effectivity of greenbelt towards pollution attenuation depends on type of plant species utilized in greenbelt and the architecture of plant species.

9.7.1 Selection of plant species for urban plantation

Air pollution tolerate index of plants species (trees & shrubs) are required to assessed at the initial stage. This is calculated by the formula proposed by Singh & Rao (**1983**).

$$APTI = \frac{A(T+P)+R}{10}$$

Where, A = Ascorbic acid content of leaf
T = Total chlorophyll of leaf (mg/gm fresh wt);
P = pH of leaf extract (cell sap); R = Relative water content of leaf (%).

On the basis of air pollution tolerance and some other relevant phytosocioeconomic characters, expected performance index (EPI) of plant species can be calculated. Finally trees were categorised as very good, good, moderate, poor and very poor sensitive categories. Species belonging to first four categories may be recommended for plantation. Gradation of plant species based on seven categories of characters. The details are given in Table 2 & 3.

Table–2: Gradation of trees species on the basis of air pollution tolerance index (APTI) and other phyto-socioeconomic characters

Grading character	Pattern of assessment	Grade allotted*
(a) Air pollution tolerance index (APTI)	7.0 - 8.5	+
	8.6 – 9.5	++
	9.6 – 10.5	+++
	10.6 – 11.5	++++
	11.6 – 12.5	+++++
(b) Tree habit	Small	-
	Medium	+
	Large	++
(c) Canopy structure	Sparse/Irregular/Globular	-
	Spreading crown/open/semi dense	+
	Spreading dense	++
(d) Type of tree	Deciduous	-
	Evergreen	+
(e) Lamnar Characters		
(i) Size	Small	-
	Medium	+
	Large	++
(ii) Texture	Smooth	-
	Coriaceous	+
	Delineate	-
	Hardy	+
(f) Hardiness	Hardy	+
(g) Economic value	Less than three uses	-
	Three of four used	+
	Five or more used	++

*Score value allocation as per '+' sign; maximum ++++ = 16 point

Table 3: Expected performance index (EPI) of plants species

EPI value number	Total score value, Percentage value	Assessment of plant species
0	Upto 30	Not recommendation for plantation

1	31-40	Very poor
2	41-50	Poor
3	51-60	Moderate
4	61-70	Good
5	71-80	Very good
6	81-90	Excellent
7	91-100	Best plus (+) plant

On the whole, selection of appropriate plant species is very important factor as they are the sink. In general, the plants should be fast growing with thick canopy cover to act as a mechanical and biological barrier, perennial and evergreen having large leaf area index (LAI), preferably indigenous, resistant to specific air pollutants and should maintain regional ecological balance for soil and ecological balance for soil and hydrological regimes of the region. A list of pollution tolerant plants thus selected for the better green belt development in and around urban region (Table–4).

Table–4: Checklist suitable for green belt development

Sl. No.	Local Name	Scientific Name	Criteria for selection of species
1.	Jarul	*Lagerstroemia flos-reginae*	Fast growing evergreen, medium size tree;
2.	Katbadam	*Terminalia catappa*	Evergreen tall tree
3.	Chatim	*Alstonia scholaria*	Evergreen tall tree with dense foliage;
4.	Kadam	*Anthocephalos chinensis*	Evergreen tall tree with large foliage;
5.	Am	*Mangifera indica*	Evergreen tall tree with dense foliage;
6.	Bandarlati	*Cassia fistula*	Evergreen, medium size tree with large leaves;
7.	Gulmohor	*Delonix regia*	Deciduous medium size tree with spreading canopy.
8.	Karanj	*Pongamia pinnata*	Evergreen, medium size tree with dense foliages;
9.	Arjun	*Terminalia arjuna*	evergreen tall trees with shiney leaves;
10.	Bahera	*Terminalia belerica*	Deciduous tall trees with coriaceous leaves;
11.	Palas	*Butea superba*	Deciduous medium size tree with coriaceous leaves
12.	Jamun	*Sizygium cumini*	Evergreen tall tree with large

			leaves;
13.	Kanchan	*Bahunia acuminate*	Deciduous medium size ornamental tree;
14.	Mehagini	*Swetania mohogini*	Evergreen tall tree with compact leaves;
15.	Neem	*Azadiracta indica*	Semievergreen tree with shiney leaves;
16.	Bat	*Ficus bengalenis*	Semievergreen tree with spreading canopy & compact leaves;
17.	Asatha	*Ficus religiosa*	Semievergreen trees with shiney leaves;
18.	Kanthal	*Artocarpus integrifolia*	Semievergreen tree with large leaves;
19.	Akasmoni	*Acacia auriculiformis*	Evergreen tree with compact leaves;
20.	Gamar	*Gmelina arborea*	Deciduous tree with hairy leaves;
21.	Putranjiba	*Putranjiba roxburghii*	Evergreen tree with shiney leaves.

However, tree plantation with objective of pollution attenuation is a very site-specific activity as it depends on the source strength, meteorological conditions of site and interaction with indigenous flora of the area. However, a few generalisations can be mad for green belt plantation as follows:

- Tall trees (Table-4) with height more than 8 to 10 meter should be planted around the source;
- Planting of trees should be in appropriate encircling rows, each rows alternating the previous one to prevent further fanning and horizontal pollution dispersion;
- Since tree trunks are normally devoid of foliage, it would be appropriate to have small shrubs in front and in between the tree spaces;
- The open areas between the process installations where trees can not be planted should be covered with lawn grasses for effective trapping and absorptions of air pollutants.
- Fast growing trees with thick canopy and perennial foliage should be selected so that the effective tree height with envisaged objective will be attained in minimum span of time.

9.7.2 Planting pattern

A standard horticultural practice involving planting of saplings in pits of substantial dimensions i.e., 1m x 1m x 1m for big trees and along half of these dimensions for smaller trees and shrubs. The bits are then filled with earth, sand, silt and manure in pre-determined proportions. Saplings planted in such pits are a watered liberally during dry months. The growing plants are then cared at least for the first two years under favorable conditions of climate and irrigation. Nutrients in pits are supplemented and the juveniles provided protection. Hence it is safe to assume that trees and bushes grown as green belt components.

For effective removal of pollutants, it is necessary that (i) plants should grow under conditions of adequate nutrient supply, (ii) absence of water stress and (iii) plants are well exposed to atmospheric conditions (light & breeze).

For roadside avenue plantation, both automobile pollution and pollutants from Power Plant need to be considered. Components of green belts on roadside hence, should be both absorbers of gases as well as of dust particles, including even lead particulates. Thus the choice of plants should include pollution tolerant shrubs of height 1 to 1.5 m and trees of 3 to 5m. The intermixing of trees and shrubs should be such that the foliage area density in vertical is almost uniform.

9.8 Future strategies for urban greening

There are three aspects to planning as related to the urban forest :
1) Comprehensive urban planning; 2) Land use planning and 3) Site Planning. Comprehensive urban planning considers all the social, economic, legal, political and physical factors of the urban environment and represents an attempt to positively influence the future of urban areas. Land use planning is predicted on urban area change and it must be goal oriented. Forestry's involvement with land use planning is generally extensive and concerns such inputs to the decision making process as technical recommendations concerning site and vegetation relationships, potential site impacts and the potential positive or negative impacts on the forest resources. Finally site planning and follow up involvement by urban foresters provide an excellent opportunity to prescribe the continuing management of not only landscape trees, but remaining natural stands of timbers as well. Intensive multiple use forestry is more applicable in these situations than any other areas of the nation (Gray and Deneke, 1986).

Information, education and training programmes are necessary for effective management of the urban forest. Municipal forestry departments have a responsibility to manage the total urban forest environment. Acceptance of the urban forestry programme by the public is absolutely necessary, both from the standpoint of existence of the department and the implementation of specific practices. Research in urban forestry is a dynamic need, because the charging face of the urban environment presents challenges requiring new trees and new technology.

In city like Kolkata, there is immediate need for raising urban forest plantation particularly on roadside as model plantation in which some suitable selected plant species which have long lasting value from economic, environmental and also aesthetic standpoint. To reduce air pollutants and noise from moderate speed car traffic, tree and shrub belts 20 to 50 feet wide is to be raised in future. Wherever possible use of taller varieties of trees should me made which have dense foliage and relatively uniform vertical foliage distribution. In case of the use of tall trees is restricted, combinations of shorter shrubs and tall grass can be used. Trees and shrubs should be planted as close together as practical to form a continuous, dense barrier. Evergreens or deciduous broad leaved species should be used as far as practicable.

9.9 Conclusion

Atmospheric pollution is now a growing concern for urban environment nowadays. Various toxic gases and Suspended fine and coarse particulates containing trace elements and heavy metals emanating from various man made sources results severe adverse impacts not only to urban environment, but also to human health and other biotic communities. Among with the anthropogenic sources, land use pattern also

somehow responsible for the dust pollution. This pollution problem generate different type of acute and chronic diseases to both population and vegetation by which the urban ecosystem going to be deteriorated at a tremendous rate. The capacity of taking up and accumulating environmental toxicants in tree foliages, the advantage big biomass and easy disposal makes it possible for plants to remove toxicants by phytoremediation in situ and consequently to reduce pollutant concentration in the urban environment. Therefore greening by plantation, which makes use of vegetation to remove, detoxify or stabilize persistent pollutants, is a green and environment friendly tool for clean urban environment. Thus in an urban region steps to be forward towards healthier living conditions would be to monitor the pollutants, to regulate them and ultimately to control them by taking appropriate measures. It is certainly a challenge for urban sustainable development to mitigate the negative consequences of such type of atmospheric pollution for living in urban region.

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ENVIS Centre on Environmental Biotechnology- A Brief Resume

Environmental Information System (ENVIS) is established in the year 1984 as a network of Information Centre. It is planned by the Ministry of Environment and Forest. Aim of this Network Centre is to provide information services related to all issues of environment and meeting the task of disseminating information on various environment related subject area. Now 78 centres are working under this network on various subject areas in the country. The focal point of this network is at the Ministry of Environment and Forest, Government of India, New Delhi.

Ministry of Environment and Forests has identified our ENVIS Centre on "Environmental Biotechnology", at Department of Environmental Science, University of Kalyani, West Bengal, India. With the emphasis on the management of natural resources and abatement of pollution as well as hazardous waste management, it is therefore proposed to set up an ENVIS Centre on 'Environmental Biotechnology'. Major emphasis of databank will focus on 'Pollutant Biodegradation' and its allied aspect.

Our center is also as one of the 20 selected ENVIS Centers (out of 78 Centers) in India, to work as partners under Sustainable Development Network Programme (SDNP) in collaboration with Indo- Canadian Environment Fund (ICEF). Our ENVIS Centre has been assigned an additional responsibility to work on the thematic area 'Environmental Biotechnology' to strengthen the ENVIS network for Sustainable Development. Under this SDNP-ENVIS link, the ENVIS Center envisages to collect, collate and disseminate the national level information on the assigned subject.

The primary objective of the centre are stated as follows:

The general objective of this centre is to collect data, related to the above mentioned subject, from different major libraries mainly in West Bengal and also from other states in India, consult with different journals, Annual reviews, Internet and to generate a database and create a website with this informations. View point of this journal abstract is to help the interested research workers, scientist, administrator and the public.

- Establishment of liaison with various Institutes of India and abroad, working on the field of Environmental Biotechnology
- Establishment of environmental data bank and documentation centre on the subject concerned
- Publication of regular "news letter" on the subject for wide dissemination of information to all concerned
- Establishment of advance training cum demonstration centre on some selected areas
- Development of a website in the field for accessing information through internet.

ENVIS Activities:

Our ENVIS center has created a website (www.kuenvbiotech.org) having related information on Environmental Biotechnology and regularly reformed and modified the website.

- Our ENVIS Centre has designed the website with several webpages with our brief Introduction, about our stuff and contact details, current news on biotechnology, current activities in environmental biotechnology in national and international laboratories, national and international organizations related to our theme area, experts on environmental biotechnology. Some other webpages linked to Database, Publication, Library, Photography, Query, SDNP have been included in our website.
- The database page contains thousands of recent abstracts published in different national and international journals on different aspects on environmental biotechnology. The abstracts were regularly uploaded into the webpage.
- A page on Microbes in Application has been included having different groups of microbes and their application. Different microbial culture centers have been included with their total number of microbial species present. Also links have been given with different national and international microbial culture centers.
- Our ENVIS Centre has published nine volume of abstracts and nine volume of newsletters till date with one annual volume of SDNP-ENVIS Newsletter.
- We have various library facilities consistes of Books, journals, news letters and also other periodicals. The books are mainly of Environmental Biotechnology and related subjects from national and international publication. We have several national level journals. Different Newsletters from other ENVIS Centres were received and displayed in our ENVIS Centre. In Envis-Library page, all information regarding no of books, their titles, newsletters, journals related to environmental biotechnology have been presented. Research publication of our center also presented in this webpage.
- Our SDNP-ENVIS thematic area center has develop a separate web page (http://envis.kuenvbiotech.org/sndp.htm) as SDNP-ENVIS Network with a linkage to our ENVIS website (www.kuenvbiotech.org). The web page contains ten key items (Emerging issues, Database, Organizations, Government Departments, Policy matters, Case Study, Publications, Agenda 21, News Clippings and Important Links) in the main page.
- We have a regional language interface on different major issues of biotechnology in our website.
- Our Centre has also organized three training cum workshop on techniques and applications of biotechnology in Department of Environmental Science, University of Kalyani. We have regularly put forthcoming informations on seminar/conference/symposia to be held related to biotechnology and environment.