BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BACTERIAL CONSORTIUM OF MUNICIPAL SOLID WASTES

Thesis Submitted for the degree of Doctor of Philosophy (Ph.D) in Science

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WEST BENGAL

INDIA

2012

DEDICATION

I DEDICATE THIS THESIS TO –

MY ONLY BROTHER

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&

MY BABA

(*ASIM KR. SAHA*)

MAA

(*MALA SAHA*)

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CERTIFICATE

TO WHOM IT MAY CONCERN

I have the pleasure to certify that **Ms. Amrita Saha** has completed her **Ph.D** work entitled "**BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BACTERIAL CONSORTIUM OF MUNICIPAL SOLID WASTES"** through repeated laboratory analysis in the department of Environmental Science, University of Kalyani, under my supervision. This work is based upon her original research work and I further certify that neither this thesis nor any part of it has been submitted for any degree / diploma or any other academic award anywhere before.

Date: ---------- ----------

Prof. S.C. Santra
(Supervisor)

“ If I have seen further than others, it

is by standing upon the shoulders of

giants” - Isaac Newton

ACKNOWLEDGEMENTS

First and foremost I would like to sincerely thank my supervisor Prof. S.C. Santra of Department of Environmental Science, University of Kalyani, for all his help, support and guidance throughout my Ph.D. This project would not have been possible without all his input. Thank you for allowing me to join your group and letting me work on this project. I am extremely grateful for having been given this opportunity to work in such a great laboratory.

I am indebted to Prof. Rintu Banerjee of Microbial Biotechnology and Downstream Processing Laboratory, Agriculatural and Food Engineering Department, I.I.T Kharagpur for design and statistical analysis of optimization of protease and lignin peroxidase production using response surface methodology part of this study. Thanks for your belief in my abilities.

I want to express my thankfulness and gratitude to Dr. Shaon Ray Chaudhuri of Biotechnology Department, West Bengal University of Technology for microbiological characterization and molecular characterization part of this project. Thanks for your words of encouragement.

I am also grateful to Dr. S. Mukherjee (Head of the Department), Dr. R. Bhattacharyya, Prof. D.K. Khan and Prof. D. Das of Department of Environmental Science, University of Kalyani for their support.

Special thanks to the research scholars of the Department of Environmental Science, University of Kalyani, who were always there to lend a helping hand. Many thanks to the staffs of the Department of Environmental Science, University of Kalyani, who helped me enormously throughout the years.

Thanks to all the scholars of Microbial Biotechnology and Downstream Processing Laboratory, Agriculatural and Food Engineering Department, I.I.T Kharagpur and also to the scholars of Biotechnology Department, West Bengal University of Technology for their acceptance and cooperation. You guys are the best bunch of people I have ever worked with and I hope we can all meet up again one day.

I would also like to thank all the staffs of Microbial Biotechnology and Downstream Processing Laboratory, Agriculatural and Food Engineering Department, I.I.T Kharagpur and Biotechnology Department, West Bengal University of Technology for their support.

I will always remain thankful to Prof. C.K. Mukherjee of Aquaculture Department, I.I.T Kharagpur and his wife, Mrs. Swagata Mukherjee for permitting me to stay in their house as their daughter during the major period of my work in I.I.T Kharagpur. Without their cooperation, hospitality and incessant support my stay and therefore my work in I.I.T Kharagpur would hardly have been possible.

My Ph.D work would remain incomplete without mentioning another name, Late. Mrs. Rekha Saha Chowdhury, my granny,

who blessed me with inspiration and strength to carry out my work throughout her life.

I sincerely thank Mrs. Minu Saha (monidi), one of my dear ones who has always been beside me in times of need.

To everyone else who I met along this journey and who provided me with valuable guidance and assistance, thank you!

Last but not the least a great big thanks to my family especially my only brother for keeping me company and always bringing a smile to my face and my parents to whom I will forever be indebted to. They protected me from floundering situations and guided me through. I have expressed my frustrations and harsh behavior towards them but they tolerated all of them with a smile. They gave me more what a person could ever expect. They emboldened my self confidence and made me realize that there is no shortcut for success. Especially, my brother was my source of inspiration and in any tough time, he was always beside me with a solution. I learnt a lot from him too. Thank you for all your love and support throughout the years during this thesis as in all stages of my life. Thanks for being with me and listening to me whenever I needed to vent or escape. Three of you are my source of inspiration and I dedicate this thesis to you.

Place: ---------- ----------

Date: ---------- ***Amrita Saha***

Preface

The use of biotechnology to solve environmental problems, according to William K. Reilly, former head of the Environmental Protection Agency, "could be - should be - an environmental breakthrough of staggering positive dimensions."

Everything under the sun degrades, or breaks down, into different materials. Fallen leaves become compost, iron rusts, milk turns sour, and food 'goes bad.' Just as light, heat, and moisture can degrade many materials, biotechnology relies on naturally occurring, living bacteria to perform a similar function. Some bacteria naturally 'feed' on chemicals and other wastes, including some hazardous materials. They consume those materials, digest them, and excrete harmless substances in their place.

For decades now, municipalities have used biological methods to treat their sewage, and industry has used secondary aerobic treatment to remove harmful materials from liquid wastes. Biological treatment is not a new idea. What is new is the expanded range of biotreatment capabilities offered by the science of biotechnology.

Bioremediation uses natural as well as recombinant microorganisms to break down toxic and hazardous substances already present in the environment. Biotreatment is a broader term, which refers to all biological treatment processes, including bioremediation. Biotreatment can be used to detoxify process waste streams at the source - before they contaminate the environment - rather than at the point of disposal. This approach involves carefully selecting organisms, known as biocatalysts, which are enzymes that degrade specific compounds, and define the conditions that accelerate the degradation process.

The degradation of organic wastes by the bacterial consortia is highly significant. It reduces the time span of degradation and produces no foul odour. Pretreatment of the organic solid wastes can also be for mineralization of garbage wastes and further as biomanure which is a novel approach. An Indian city produces about 0.8 to 1 kg solid wastes per capita per day. These wastes are collected and dumped into the landfills, causing major pollution. This results in loss of potentially valuable materials that can be processed as fertilizer, fuel and fodder. The bulk of organic kit comprising mainly carbohydrates, amino acids, peptides andproteins, volatile acids, fatty acids and their esters are easily biodegradable. The biological treatment of these wastes appears to be most cost effective and carry a less negative environmental impact. This process of

biological treatment of wastes is also known as Composting. It is a self-heating, aerobic solid phase biodegradative process of organic materials under controlled conditions, which distinguishes it from natural rotting. It has clearly been established that composts have the potential to protect the soil against erosion, to enhance the soil water retention, to reduce soil compactibility, to decrease soil acidity, to enhance soil biochemical and biological activity and to establish a sound soil ecological equilibrium. Additionally, composts can protect plants from soil or seed borne pathogens Hence, compost can be considered as a much-needed soil conditioner with generally positive crop yield effects. The exploitation of the metabolic versatility of microorganisms is advantageous in biological waste treatment but the actual number of degraders of a target compound in a mixed culture may only represent 5-10% of the microbial community. To understand how microorganisms may be manipulated and exploited to reduce the frequency of such breakdowns and shorten start-up times of biological waste treatment, the important bacterial strains actively involved in the degradation of food waste were isolated and screened.

Thus, the main aim of this study was to develop some successful bacterial consortium that can concomitantly degrade different components of the organic municipal solid wastes with the help of their enzymes in less span of time under natural conditions without producing any foul odour. In addition to that the other important aim was to characterize the enzyme wit high market value, produced in due course of this microbial degradation and enhance its production for future application in industry.

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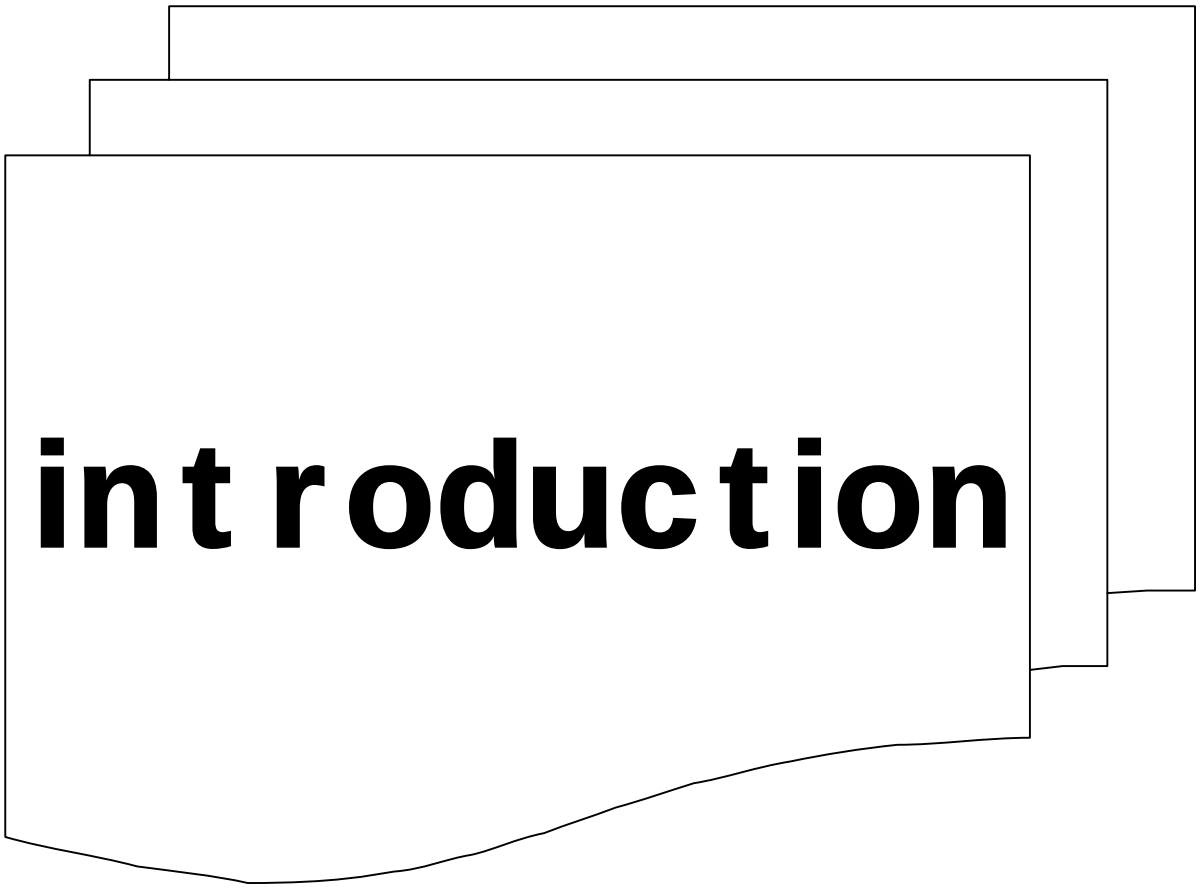

introduction

1.1. Municipal Solid Waste (MSW)

Municipal solid waste (MSW) generation is an issue of worldwide concern. The generators of municipal solid waste are broadly classified as residential, industrial, commercial, institutional, construction, demolition, municipal and agricultural types (Sehker and Beukering, 1998). Municipal solid waste is also generated by human and animal activities that are discarded as useless or unwanted waste. Municipal solid waste commonly known as trash or garbage (US), refuse or rubbish (UK) is a waste type consisting of everyday items we consume and discard. It predominantly includes food wastes, yard wastes, containers and product packaging, and other miscellaneous inorganic wastes from residential, commercial, institutional, and industrial sources. Examples of inorganic wastes are appliances, newspapers, clothing, food scrapes, boxes, disposable tableware, office and classroom paper, furniture, wood pallets, rubber tires, and cafeteria wastes. Municipal solid waste does not include industrial wastes, agricultural wastes, and sewage sludge.

Economic development, urbanization and improving living standard in cities of developing countries have lead to increase in the quantity and complex composition of municipal solid waste. Management of municipal solid waste resulting from rapid urbanization has become a serious concern for government departments, pollution control agencies, regulatory bodies and public in most of the developing countries (Glawe et al., 2005; Erdogan et al., 2008).

Several other factors like education standard and infrastructure of the country have significant effect on municipal solid waste generation. The estimation and prediction of municipal solid waste generation play an important role in municipal solid waste management. The quantity of municipal solid waste in developing countries has been consistently rising over the years (Kansal, 2002). The municipal solid waste composition varies from place to place and also bears a rather consistent correlation with the average standard of living (Visvanathan and Trankler, 2003). The waste generated in the developing countries is similar in

composition, the variation between regions being dictated by the climatic, cultural and industrial, infrastructural and legal factors.

Inefficient management and disposal of municipal solid waste is an obvious cause for degradation of environment in the developing countries. Ecological impacts such as land degradation, water and air pollution are related with improper management of municipal solid waste (Khajuria et al., 2008). In Asian developing countries, most of the municipal solid waste is dumped on land in more or less uncontrolled manner. Lack of sufficient awareness at the grassroots level of the waste generators add to the problem of littering. As a result there is a serious threat to public health due to environmental pollution.

Limited landfill space and resistance to siting such facilities has spurred consideration of new approaches to increase the longevity of landfills. Such efforts have included exploring methods to enhance degradation rates of municipal solid waste (MSW) and subsequently, to recover materials and landfill space.

1.2. Qualitative and Quantitative Analysis of MSW

There are many categories of MSW such as food waste, rubbish, commercial waste, institutional waste, street sweeping waste, industrial waste, construction and demolition waste, and sanitation waste. MSW contains recyclables (paper, plastic, glass, metals, etc.), toxic substances (paints, pesticides, used batteries, medicines), compostable organic matter (fruit and vegetable peels, food waste) and soiled waste (blood stained cotton, sanitary napkins, disposable syringes) (Jha et al., 2003; Reddy and Galab, 1998; Khan, 1994). The quantity of MSW generated depends on a number of factors such as food habits, standard of living, degree of commercial activities and seasons. Data on quantity variation and generation are useful in planning for collection and disposal systems. With increasing urbanization and changing life styles, Indian cities now generate eight times more MSW than they did in 1947. Presently, about 90 million tons of solid waste are generated annually as by-products of industrial, mining, municipal, agricultural and other processes. The amount of MSW generated per capita is estimated to increase at a rate of 1–1.33% annually (Pappu et al., 2007; Shekdar, 1999; Bhide and Shekdar, 1998).A host of researchers (Siddiqui et al., 2006; Sharholy et al.,

2005; CPCB, 2004; Kansal, 2002; Singh and Singh, 1998; Kansal et al., 1998; Bhide and Shekdar, 1998; Dayal, 1994; Khan, 1994; Rao and Shantaram, 1993) have reported that the MSW generation rates in small towns are lower than those of metrocities, and the per capita generation rate of MSW in India ranges from 0.2 to 0.5 kg/ day. The quantity of MSW generated (CPCB, 2000) and the per capita generation rate of MSW (CPCB, 2004) is shown in Table 1.1 and Figure 1.1, respectively.

Table 1.1. Municipal solid waste generation rates in different states in India

S. No.	Name of the state	No of cities	Municipal population	Municipal solid waste (t/day)	Per capita generated (Kg/day)
1	Andhra Pradesh	32	10,845,907	3943	0.364
2	Assam	4	878,310	196	0.223
3	Bihar	17	5,278,361	1479	0.280
4	Gujrat	21	8,443,962	3805	0.451
5	Haryana	12	2,254,353	623	0.276
6	Himachal Pradesh	1	80,054	35	0.427
7	Karnataka	21	8,283,498	3118	0.376
8	Kerala	146	3,107,358	1220	0.393
9	Madhya Pradesh	23	7,225,833	2286	0.316
10	Maharashtra	27	22,727,186	8589	0.378
11	Manipur	1	198,535	40	0.201
12	Meghalaya	1	223,366	35	0.157
13	Mizoram	1	155,240	46	0.296
14	Orissa	7	1,766,021	646	0.366
15	Punjab	10	3,209,903	1001	0.312
16	Rajasthan	14	4,979,301	1768	0.355
17	Tamil Nadu	25	10,745,773	5021	0.467
18	Tripura	1	157,358	33	0.210
19	Uttar Pradesh	41	14,480,479	5515	0.318
20	West Bengal	23	13,943,445	4475	0.321
21	Chandigarh	1	504,094	200	0.397
22	Delhi	1	8,419,084	4000	0.475
23	Pondicherry	1	203,065	60	0.295
		299	128,113,865	48,134	0.376

Source: Status of MSW generation, collection, treatment and disposal in class-I cities (CPCB, 2000).

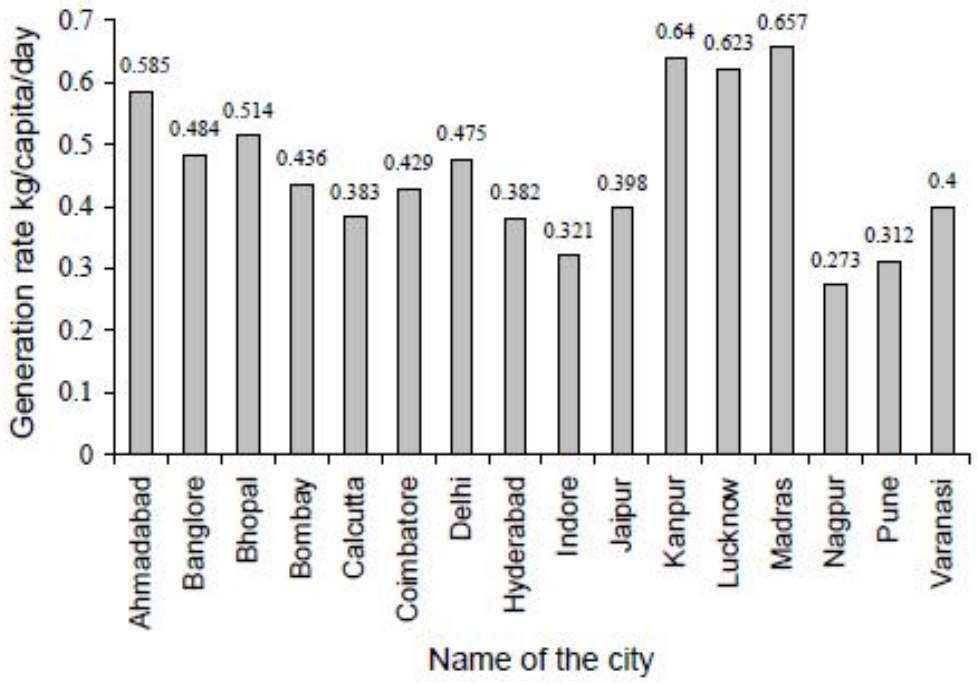


Figure 1.1. Per capita generation rate of MSW for Indian cities (CPCB, 2000)

It can be seen from Table 1 and Fig. 1 that the per capita generation rate is high in some states (Gujrat, Delhi and Tamil Nadu) and cities (Madras, Kanpur, Lucknow and Ahmedabad). This may be due to the high living standards, the rapid economic growth and the high level of urbanization in these states and cities. However, the per capita generation rate is observed to be low in other states (Meghalaya, Assam, Manipur and Tripura) and cities (Nagpur, Pune and Indore).

1.3. MSW Characteristics and Composition

The composition and the quantity of MSW generated form the basis on which the management system needs to be planned, designed and operated. In India, MSW differs greatly with regard to the composition and hazardous nature, when compared to MSW in the western countries (Gupta et al., 1998; Shannigrahi et al., 1997; Jalan and Srivastava, 1995). The composition of MSW at generation sources and collection points was determined on a wet weight basis and it consists mainly of a large organic fraction (40–60%), ash and fine earth (30–40%), paper (3–6%) and plastic, glass and metals (each less than 1%). The C/ N ratio ranges between 20 and 30, and the lower calorific value ranges between 800 and 1000 kcal/kg. The

physical characteristics of MSW in metrocities are presented in Table 2. It has been noticed that the physical and chemical characteristics of MSW change with population density From Table 2, it is observed that the differences in the MSW characteristics indicate the effect of urbanization and development. In urban areas, the major fraction of MSW is compostable materials (40–60%) and inerts (30–50%). The relative percentage of organic waste in MSW is generally increasing with the decreasing socio-economic status; so rural households generate more organic waste than urban households. For example, in south India the extensive use of banana leaves and stems in various functions results in a large organic content in the MSW.

Table 1.2. Physical characteristics of MSW in Indian metrocities

Characteristics (% by weight)								
Name of metrocity	Paper	Textile	Leather	Plastic	Metals	Glass	Ash, fine earth and others	Compostable matter
Ahmedabad	6.0	1.0	–	3.0	–	–	50.0	40.00
Banglore	8.0	5.0	–	6.0	3.0	6.0	27.0	45.00
Bhopal	10.0	5.0	2.0	2.0	–	1.0	35.0	45.00
Mumbai	10.0	3.6	0.2	2.0	–	0.2	44.0	40.00
Calcutta	10.0	3.0	1.0	8.0	–	3.0	35.0	40.00
Coimbatore	5.0	9.0	–	1.0	–	–	50.0	35.00
Delhi	6.6	4.0	0.6	1.5	2.5	1.2	51.5	31.78
Hyderabad	7.0	1.7	–	1.3	–	–	50.0	40.00
Indore	5.0	2.0	–	1.0	–	–	49.0	43.00
Jaipur	6.0	2.0	–	1.0	–	2.0	47.0	42.00
Kanpur	5.0	1.0	5.0	1.5	–	–	52.5	40.00
Kochi	4.9	–	–	1.1	–	–	36.0	58.00
Lucknow	4.0	2.0	–	4.0	1.0	–	49.0	40.00
Ludhiana	3.0	5.0	–	3.0	–	–	30.0	40.00
Madras	10.0	5.0	5.0	3.0	–	–	33.0	44.00
Madurai	5.0	1.0	–	3.0	–	–	46.0	45.00
Nagpur	4.5	7.0	1.9	1.25	0.35	1.2	53.4	30.40
Patna	4.0	5.0	2.0	6.0	1.0	2.0	35.0	45.00
Pune	5.0	–	–	5.0	–	10.0	15.0	55.00
Surat	4.0	5.0	–	3.0	–	3.0	45.0	40.00
Vadodara	4.0	–	–	7.0	–	–	49.0	40.00
Varanasi	3.0	4.0	–	10.0	–	–	35.0	48.00
Visakhapatnam	3.0	2.0	–	5.0	–	5.0	50	35.00
Average	5.7	3.5	0.8	3.9	1.9	2.1	40.3	41.80

Source: Status of solid waste generation, collection, treatment and disposal in metrocities, (CPCB, 2000).

Also, it has been noticed that the percentage of recyclables (paper, glass, plastic and metals) is very low, because of rag pickers who segregate and collect the materials at generation sources, collection points and disposal sites.

Solid wastes may seem to be the most ordinary forms of wastes, but they could be responsible for many problems such as spread of diseases and emission of green house gases. All these years, solid waste disposal was a neglected issue as these wastes were simply dumped on land in the outskirts of the city. This gave rise to problems like odors, flies, mosquitoes, groundwater pollution, emission of landfill gases etc.

A world which is on the rapid path of development has led to an increasing waste generating World. It has also posed a challenge not only with respect to treating and disposing waste properly but also to see this as an opportunity to derive useful products from it. With the severe energy crises in the World today, an attempt has been initiated to produce energy from solid wastes. This started with the concept of Biomethane production on the lines of the older practice of 'Gobar Gas' production. Later, newer concepts like fuel alcohol production, bio-hydrogen have also started coming up. Micro-organisms are the agents which bring about the conversion of these wastes into useful products like fuel gases, fuel alcohol and also compost which can be used as manure. The gases produced in landfills due to decomposition by anaerobic organisms also can be used as a source of energy. The major problem today in producing energy from waste is the cost factor. Efforts are being made to produce genetically modified organisms which will produce energy from wastes more efficiently and at least cost. We live in a world where Solid Waste is no more a waste, but a storehouse of precious potential products. We can look forward to major breakthroughs in the field of Solid waste management using microorganisms.

1.4. Applications of Microbiology in Solid Waste Management

Micro organisms are omnipresent and are responsible for many good as well as bad things in our biosphere. They are present even in waste materials. These micro organisms carry out various biochemical processes to degrade waste materials. This process may be aerobic or anaerobic. Solid waste decomposition is carried out by bacteria which decompose complex organic materials to simple water soluble organic compounds. These are then converted to CO_2 and H_2O aerobically , or to CH_4 anaerobically. Fungi are mostly aerobic and feed on decaying organic matter. Soil fungi play a vital role in stabilizing solid wastes in composting and landfilling

processes by decomposing plant tissues like cellulose and lignin. Protozoa are predators on bacteria. They are found wherever bacteria are prevalent. Thus they help to maintain the equilibria of microbial flora in solid waste disposal systems.

1.5. Bacteria in Decomposition of Solid Organic Waste

The microbial population of soils is made up of five major groups including bacteria, actinomycetes, fungi, algae and protozoa, and among these groups, bacteria are the most abundant group [Alexander, 1961] and the most important microbe for decomposing waste. Bacteria use wastes for their own metabolism and finally they produce some simple and useful compounds which are important for soil health, plant growing and over all to keep well balance of natural ecosystem. Composting is the controlled biodegradation or transformation of organic material, usually under aerobic conditions by which a material is transformed into an end product which is stable and soil like material called compost. Number of microbes along with rodents and insects play a vital role for solid waste degradation. Among them, bacteria play the most important role and therefore, the effective bacteria can be employed for planned decomposition of solid organic waste. In the due course of these decomposition bacteria produces an essential metabolite of great economic value, known as enzyme.

1.6. Bacterial Enzymes

Microbes live in an environment where the nutrients are mainly macromolecular in nature. These nutrients are not utilizable by the microbes unless cleaved into smaller molecules that they can absorb. The cleavage of macromolecular nutrients into smaller molecules is accomplished by the enzymes secreted by the microbes themselves. Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. More than 3000 different enzymes described to date the majority have been isolated from mesophilic organisms. These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known enzymes unrecommendable. Thus, the search for new microbial sources is a

continual exercise, where one must respect biodiversity. The microorganisms from diverse and exotic environments called as extremophiles, are an important source of enzymes, whose specific properties are expected to result in novel process applications (Kumar and Takagi, 1999). The role of enzymes in many processes has been known for a long time. Their existence was associated with the history of ancient Greece where they were using enzymes from microorganisms in baking, brewing, alcohol production, cheese making etc. With better knowledge and purification of enzymes the number of applications has increased many folds, and with the availability of engineered enzymes a number of new possibilities for industrial processes have emerged (Beg et al., 2003). The current estimated value of the worldwide sales of industrial enzymes is $1 billion.

1.7. Bacterial Proteases

Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of total worldwide enzyme sales. This dominance of proteases in the industrial market is expected to increase further by the year 2005 (Rao et al., 1998). Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. They are essential constituents of all forms of life on earth, including prokoryates, fungi, plants and animals. They can be cultured in large quantities in relatively short time by established fermentation methods and produce an abundant, regular supply of the desired product. In recent years there has been a phenomenal increase in the use of alkaline protease as industrial catalysts. Alkaline proteases (EC.3.4.21–24, 99) are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine center (serine protease) or are of metallo- type (metalloprotease); and the alkaline serine proteases are the most important group of enzymes so far exploited (Gupta et al., 2002b). These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons; for example they exhibit high catalytic activity, a high degree of substrate specificity can be produced in large amounts and are economically viable. Microbial alkaline proteases dominate the worldwide enzyme market, accounting for two-third of the share of the detergent industry. Although production is inherent property of all organisms, only those microbes that produce a substantial amount of extracellular

protease have been exploited commercially. Alkaline proteases of Bacillus sp. origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Agrawal et al.,2004).

1.8. Bacterial Lignin Peroxidases

Lignin is a complex, three-dimensional aromatic polymer consisting of dimethoxylated, monomethoxylated and non-methoxylated phenylpropanoid subunits (Martinez et al., 2005). It is found in the secondary cell wall of plants, where it fills the spaces between the cellulose, hemicellulose and pectin components, making the cell wall more rigid and hydrophobic. Lignin provides plants with compressive strength and protection from pathogens (Kumar et al., 2008; Rubin, 2008). Presently, millions of tons of lignin and lignin-related compounds are produced as waste effluent from the pulping and paper industries (De los Santos Ramos et al., 2009). These amounts are expected to further increase in the near future as a result of the recent developments aimed at replacing fossil feedstocks with lignocellulosic biomass for the production of fuels and chemicals. Generally, biorefinery processes only employ the (hemi-) cellulosic part; the lignin component remains as a low-value waste stream (Stewart et al., 2008) that is commonly incinerated to generate heat and power (Himmel et al., 2007; Ragauskas et al., 2006; Zaldivar et al., 2001). To expand on the range of products which can be obtained from lignocellulosic biomass, the lignin component should be utilized as feedstock for value-added chemicals such as substituted aromatics, instead of being incinerated for heat and energy. Enzymes could provide an effective means for lignin depolymerization into products of interest. Enzymes could provide a more specific and effective alternative for lignin depolymerization.

The white rot basidiomycetes are the most extensively studied natural lignin degrading microorganisms (Ahmad). These fungi produce an array of powerful ligninolytic enzymes such as laccases, lignin peroxidases (LiP’s) and manganese peroxidases (MnP’s) (Arantes et al., 2007; Shary et al., 2008). These oxidative enzyme systems commonly require low-molecular weight co-factors and mediators, such as manganese, organic acids, veratryl alcohol and substituted

aromatics (e.g. 4-hydroxybenzyl alcohol, aniline, 4-hydroxybenzioc acid) (Perez et al., 2002; Kunamneni et al., 2008). These mediators are the actual oxidants responsible for lignin degradation, and can penetrate deeply into the lignocellulosic matrix thanks to their limited size. Fungal lignin depolymerization usually results in a variety of low molecular weight aromatic compounds such as guaiacol, coniferyl alcohol, p-coumarate, ferulate, protocatechuate, p-hydroxybenzoate and vanillate (Harwood et al., 1996; Masai et al., 2007). Ligninolytic bacteria are less well studied, but several examples have been found among a-proteobacteria [e.g., Sphingomonas sp. (Wenzel et al., 2002; Masai et al., 2003; Masai et al., 1999)], g-proteobacteria [e.g., Pseudomonas sp. (Delalibera et al., 2007),] and actinomycetes [Rhodococcus, Nocardia and Streptomyces sp. (Zimmermann et al., 1990; Bugg et al., 2010)]. The enzymes reported to be involved in bacterial lignin degradation are laccases, glutathione S-transferases, ring cleaving dioxygenases (Masai et al., 2003; Allocati et al., 2009), monooxygenases and phenol oxidases (Perestelo et al., 1989). Such enzymes are also involved in degradation of polycyclic aromatic hydrocarbons (PAHs), which show similar structural properties and resistance to microbial degradation as lignin (Allocati et al., 2009; Canas et al., 2007). Thus, the bacterial ligninolytic potential is still largely unexplored and many novel ligninolytic enzymes may await discovery. These bacterial enzymes may be superior to their fungal counterparts with regard to specificity, thermostability and mediator dependency (Kumar et al., 2008; Masai et al., 2007; Ruijssenaars et al., 2004). They may also have specific advantages for the depolymerisation of the modified lignin residues typically encountered in waste streams from the pulping or 2nd generation biofuel/biobased chemicals industry.

1.9. Production of Enzyme in Solid State Fermentation

Among processes used for enzyme production, solid-state fermentation (SSF) is an attractive one because it presents higher productivity per reactor volume, lower capital and operating costs, lower space requirements, simpler equipment and easier downstream processing compared to that of submerged fermentation (SmF) (Acun˜a-Argu¨elles et al., 1995). In addition, it permits the use of agricultural and agro-industrial residues as substrates which are converted into bulk chemicals and fine products with high commercial value such as alcohol, organic acids, fats,

proteins, enzymes, etc. (Manonmani et al., 1987; Spagnuolo et al., 1997) . On the other hand, there is evidence that some enzymes are less affected by catabolic repression, are more thermo stable and their optimum temperature values are higher than those obtained by SmF (Lozano et al., 1988). Major problems in the exploitation of commercial enzymes are their yield, stability, specificity and the cost of production. New enzymes for use in commercial applications with desirable biochemical and physio-chemical characteristics and a low cost of production have been the focus of research. Application of agro-industrial wastes which are largely available in India as carbon sources in enzyme production reduces the cost of production and also helps in solving disposal problems (Pandey et al.,2000; Baladhandayutham et al., 2010).

1.10. Application of Bacterial Enzymes in Industry

Enzymes may be extracted from any living organism. Sources of commercial enzymes cover a wide range, from microorganisms to plants to animal sources. But for various reasons, microorganisms became the major source of enzymes. In commercial enzyme production, fungi and yeast contribute about 50%, bacteria 25%, animal 8% and plant 4% of the total. Microbes are preferred to plants and animals as they are cheap sources, their enzyme contents are predictable and growth substrates are obtained as standard raw materials. In addition, genetic engineering has opened a new era of advanced enzyme technology. Recombinant DNA technology has made it possible to obtain enzymes present in valued sources, to be synthesized in easy growing microorganisms and also to produce tailor-made enzyme proteins with desired properties as per customers' requirements. Enzymes retaining activity under extreme conditions of temperature, pH and salt concentrations, partially active in organic solvents are all becoming a reality. The prospects of the enzyme industry look very bright with increased market position for existing use, new use of known enzymes and new enzymes Large volumes of industrial enzymes are usually not purified and are marketed as concentrated liquid or granulated products with specified enzyme lives.The industrial enzyme market is frequently segmented on the basis of applications. Application sectors have been classified in different major sectors with respect to applications of enzymes.

i. Food enzymes

a. Enzymes for starch processing (amylases for production of glucose)

b. Sweetener production (glucose isomerase for fructose production)

c. Bakery products (xylanase, α -amylase, glucose oxidase for dough conditioning, dough quality, loaf volume)

d. Dairy product (rennin, lactase for milk coagulation and hydrolysis of lactose)

e. Fruit juice (pectinase, cellulase, xylanase for juice clarification, juice extraction)

f. Wine making (glucanase and papain for haze clearance)

ii. Enzymes for technological applications

a. Detergent enzymes (proteinase)

b. Enzyme for textile (cellulase and laccase for microfibril removal and for improving brightness of cloth)

c. Enzymes for leather processing (protease, lipase)

d. Enzymes for paper and pulp processing (xylanase)

e. Enzymes of analytical use such as:

i. Uric acid by uricase

ii. Ethyl alcohol by alcohol dehydrogenase

iii. Ammonia by L-glutamate dehydrogenase

iv. Cholesterol by cholesterol oxidase

v. Glucose by glucose oxidase

vi. Urea by urease.

iii. Enzymes for animal feed

a. Xylanase for fiber solubility

b. Phytase for removal of phosphate

iv. Enzymes for medical applications

a. Digestive enzymes: Pancreatic enzymes, mammalian protease (pepsin) plant proteases (Bromelain, papain), fungal amylases.

b. Enzymes with potential therapeutic applications

i. Asparaginase and glutaminase hydrolyzing L- aspargine and L-glutamine to aspartic and glutamic acids respectively in the treatment of leukemia.

ii. Urokinase and streptokinase (plasminogen to plasmin) for dissolving blood clot in heart attack.

iii. Penicillinase for hydrolyzing penicillin during acute penicillin allergy.

iv. Hyaluronidase for hydrolyzing hyaluronate in heart attack

v. Collaginase for hydrolyzing collagen in skin cancer

vi. Uricase for oxidizing uric acid in gout

v. Enzymes for clinical and diagnostic applications

a. Enzyme linked immunosorbent Assay (ELISA): Enzymes used are: peroxidase, alkaline phosphatase, ß- galactosidase

b. Enzyme multiplied immunoassay technique (EMIT): Enzymes used are lysozyme and malate dehydrogenase

c. Enzymatic analysis of blood constituents: Glucose, uric acid, urea, cholesterol, pyruvate, lactate, triglyceride etc.

Protease is the first largest group of enzyme used in industry. Lignin peroxiadse is another important enzyme though very few reports have been published till date.

1.11. Molecular Characterization of Potent Bacterial Isolates

Fast and reliable molecular techniques based essentially on rDNA amplified sequences analyses, have provided tools to determine microbial presence and diversity (Ranjard et al. 2000; Cardinale et al. 2004). The use of molecular techniques in microbial ecology has made possible the discovery of new microorganisms previously unknown (Macrae et al., 2000; Whitman et al., 1998). Identification of bacteria that exist in the environment has been based on the determination of 16S rRNA sequences of amplified and cloned genes derived from the soil microbiota's DNA (Borneman et al., 1996; Gelsomino et al., 1999; Kuske et al., 1997; Lee, 1996). Sequences of the gene currently have been used with success in studies of microbial ecology and phylogeny (Mota et al., 2005). This bacterial characterization of gene 16S rRNA allows the identification of several bacterial populations in the soil.

1.12. Application of Bacteria in Biocomposting

Bacteria use wastes for their own metabolism and finally they produce some simple and useful compounds which are important for soil health, plant growing and over all to keep well balance of natural ecosystem. The municipal solid waste in the urban centers is generated by domestic, commercial and industrial sources It contains mostly organic wastes that can be decomposed by composting. The bacterial conversion of the organics present in MSW in the presence of air under hot and moist conditions is called composting, and the final product obtained after bacterial activity is called compost (humus), which has very high agricultural value. It is used as fertilizer, and it is non odorous and free of pathogens (Ahsan, 1999; Khan, 1994). As a result of the composting process, the waste volume can be reduced to 50–85%. The composting methods may use either manual or mechanical means and are accordingly termed as a manual or mechanical process. Manual composting is carried out in smaller urban centers and mechanical composting plants have been set up in big Indian cities. (Bhide and Shekdar,1998; Chakrabarty et al.,1995). The main objectives of composting are to reduce the solid volume, weight and moisture content, minimize odor, decrease pathogens and the spread of disease and increase potential nutrients for agricultural applications. Therefore, composting is emerging as a popular waste management alternative both in developed and developing countries Implementation of composting technology has great potential for mitigating several problems related to an ecological imbalance due to loss of nutrients from ecosystems and the disposal of organic wastes that cause water, soil and air pollution and corresponding health hazards.

The scope of this thesis was to screen potent bacterial isolates from municipal solid waste, producing industrial enzymes with high titer value and to optimize the environmental conditions during fermentation, developing an industrial media formulation using response surface methodology and evaluating the characteristic properties of the crude alkaline protease enzyme produced from the isolates. The bacterial strains were also used for application in degradation of municipal organic waste.

Chapter - ii

Review of literature

2.0. Introduction

Modern times have plagued the humanity with new problems due to industrialization and simultaneous population explosion. Rapid industrialization and population explosion in India has led to the migration of people from villages to cities, which generate thousands of tons of Municipal Solid Waste (MSW) daily. Municipal solid waste consists of household waste, construction and demolition debris, sanitation residue and waste from streets. This garbage is generated mainly from residential and commercial complexes. The MSW amount is expected to increase significantly in the near future as the country strives to attain an industrialized nation status by the year 2020 (Sharma and Shah, 2005; CPCB, 2004; Shekdar *et al.*, 1992). Poor collection and inadequate transportation are responsible for the accumulation of MSW at every nook and corner. Solid waste generation in India was 229 million tons in 2001 and solid waste generation per capita per day in India ranged from 100 to 500 grams (Arrifa and Jayalakshmi, 2005). According to a study by The Energy and Resources Institute (TERI), the annual per capita municipal solid waste generation in India is projected to grow from 1 to 1.33 per cent, which would lead to a generation of over 260 million tones of waste by 2047 - a five fold increase over 1997 levels. It is further projected that an additional 1400 km^2 of land is needed to dispose this waste, most of it in urban areas. Modern urban living brings on the problem of waste, which increases in quantity, and changes in composition with each passing day (Singh and Shekhawat, 2000). It has been estimated that overall municipal waste generated in urban centers, anywhere between 45 to 75 per cent constituted organic matter. It is also important to note that waste consumption varied significantly across areas of different economic levels of residents. The per capital solid waste reaching disposal sites in Bombay, Calcutta, Chennai and New Delhi ranges from 0.45 to 0.6 kilo gram per person per day. While in other Indian cities it is from 0.15 to 0.53 kilo gram per person per day (Manimozhi *et al.*, 2006). It is established that about 500 grams of biodegradable kitchen waste is generated per day in a family consisting of four members. Each household produces solid wastes, which can be broadly classified as biodegradable (vegetable and fruit peels, leftover food etc)

and non biodegradable (plastic bags, metal containers and glass bottles and hazardous or toxic wastes) (Venkataratnam, 2001). The household wastes include seeds of fruits, fruit peels and remnants, waste vegetables, wasted flower, rotten food, used tea dust, remnants of eaten food, egg shells, bones, paper, garden waste, glass, metals, used cosmetics, medicine bottles, rubber, leather, plastics, textiles etc (Karpagam, 2005). When organic waste decomposes in landfills and uncontrolled dumps, it produces methane, one of the major greenhouse gases contributing to climate change. The management of MSW is going through a critical phase, due to the unavailability of suitable facilities to treat and dispose of the larger amount of MSW generated daily in metropolitan cities. Unscientific disposal causes an adverse impact on all components of the environment and human health (Rathi, 2006; Sharholy *et al.*, 2005; Ray *et al.*, 2005; Jha *et al.*, 2003; Kansal 2002; Kansal *et al.*, 1998; Singh and Singh 1998; Gupta *et al.*, 1998). Domestic waste from urban areas, without proper planning, is turning to be a problem unconquered (Lakshmanan, 2009). Organic wastes, which are produced in large quantities all over the world, create major environmental and disposal problems. These materials cause major unpleasant odour problems and use of large quantities of land for disposal and are often a source of contamination of ground water (Edwards and Bater, 1992; Kannaiyan and Lilly, 1999). Road side garbage from houses remains uncleared because its volume is more than what the corporation can handle. Rag pickers, stray animals and birds scatter the garbage, looking for items useful to them. This results in the familiar site around the street corners of most towns and cities (Mani, 1996). The waste accumulation has increased simultaneously with the rapid increase in residential colonies, fast food outlets, vegetable vendors, fruit shops and other customer outlets in the respective areas. Garbage is also dumped in huge plastic bags that obstruct the traffic. Viswanathan (2005) says that the amount of large solid refuse has been gradually increasing and its treatment and disposal has become a major social and environmental problem as well as a challenge. Open dumping of garbage facilitates the breeding of disease vectors such as flies, mosquitoes, cockroaches, rats, and other pests. Further, the poorly maintained landfill sites are prone to groundwater contamination because of leachate production (Maheswari, 2005). The waste generated is consequently released into the nearby environment. Consequently, the management of the MSW needs to be

revamped to accommodate the changes in the quantity and quality to ensure the longevity of the environment. Due to several legislative, environmental, economic and social constraints, the identification of most sustainable disposal route for MSW management remains an important issue in almost all industrialized countries (Adani *et al.*, 2000). Generally, MSW is disposed of in low lying areas without taking any precautions or operational controls. Therefore, MSW management is one of the major environmental problems of Indian megacities. It involves activities associated with generation, storage, collection, transfer and transport, processing and disposal of solid wastes. But, in most cities, the MSW management system comprises only four activities, i.e., waste generation, collection, transportation, and disposal. The management of MSW requires proper infrastructure, maintenance and upgrade for all activities. This becomes increasingly expensive and complex due to the continuous and unplanned growth of urban centers. The difficulties in providing the desired level of public service in the urban centers are often attributed to the poor financial status of the managing municipal corporations (Mor *et al.,* 2006; Siddiqui *et al.,* 2006; Raje *et al.,* 2001; MoEF, 2000; Ahsan, 1999). Agricultural application of MSW, as nutrient source for plants and as soil conditioner, is the most cost effective MSW disposal option because of its advantages over traditional means such as landfilling or incineration. According to Canellas *et al.,* (2001) the use of MSW in agricultural lands can be justified by the need of finding an appropriate destination for waste recycling. Organic waste is a potential resource of both nutrients and organic matter that can be used to replenish the soils under pressure from traditional agriculture. However, direct use of waste is impractical. Composting offers a method of biological stabilization that eliminates odour and pathogens and renders a product that is safe and pleasant to use (Divya, 2001). Compost acts as a natural fertilizer by providing nutrients to the soil, increasing beneficial soil organisms, and suppressing certain plant diseases, thereby reducing the need for chemical fertilizers and pesticides in landscaping and agricultural activities. The chief objective to compost organic wastes should not be for the disposal of solid organic wastes but to produce superior quality manure to feed our “nutrient-organic-matter-hungry” soils. Composting of wastes controls the pollution of soil and water and ensures the survivability and growth of fish, prawns and other organisms (Setua *et al.,* 2008).

2.1. Characterization of Municipal Solid Waste

Municipal solid waste contains organic materials such as paper, food and yard waste and plastics. To be specific, MSW can be divided into six major chemical compound classes: non-cellulosic carbohydrates (hemicellulose, starch, and mono- and oligosaccharides), cellulose, proteins, lipids, lignin, and plastics (Pichler and Kögel-Knabner, 2000). Paper and paperboard products make up the largest component of MSW. Of the total 229.9 millions of tons of MSW generated in the U.S. in 1999, 38.1% were paper products. Although 41.9% was recovered by recycling, there were still 50.8 million tons of papers that were deposited in landfills (US EPA, 1999). Newsprint is produced from mechanical pulp with some chemical wood pulp. Only 7% of the raw materials are lost during the production of newsprint. Therefore, most compounds of the wood, lignin, cellulose, hemicellulose are present in newsprint. Office paper is made from a 5chemical pulp of high purity. Most of the lignin is removed during chemical treatment, leaving cellulose as the major component. The percentage of the cellulose varies depending on specific chemicals used (Calkin, 1957). Food waste comprised 12.1% of MSW; the major organic components of food waste are carbohydrate, protein and lipids (US EPA, 1999).

2.1.1. Types of Municipal Solid Waste

Table 2.1. The sources of municipal solid waste

Sources	Examples
Residential	Single family homes, duplexes, town houses, apartments
Commercial	Office buildings, shopping malls, warehouses, hotels, airports, restaurants
Institutional	Schools, medical facilities, prisons
Industrial	Packaging of components, office wastes, lunchroom and restroom wastes (but not industrial process wastes)

Source: **(Tchobanoglous G, Kreith F, 2002)**

2.2. Organic Waste

Organic waste is produced wherever there is human habitation. The main forms of organic waste are household food waste, agricultural waste, human and animal

waste. The organic waste component is broken down by microorganisms to form a liquid leachate. This leachate presents serious hazards if it reaches water course or enters water table. In developing countries there are different approaches to deal with organic waste. In fact the word waste is an inappropriate term for organic matter which is often put to good use. The economies of most developing countries dictate that materials and resources must be used to their full potential and this has propagated a culture of reuse, repair and recycling.

2.2.1. Cellulose and Hemicellulose

Cellulose, a linear polymer made of glucose subunits linked by β-1, 4 glycosidic bonds, is the most abundant biopolymer on earth (Senior, 1990). Most native celluloses are composed of two different forms. The parallel oriented chains form highly ordered crystalline domains. The crystalline domain is interspersed by more disordered, amorphous regions. The native crystalline form of cellulose has a structure designated as type I, which can be converted into type II by alkali treatment. Depending on origin and pretreatment, the degree of crystallinity of cellulose can vary from 0% to 100% (Béguin and Aubert, 1994). Evans *et al*., (1995) studied the crystallinity change during Kraft pulping process using X-ray diffraction, infrared (IR) spectroscopy and NMR spectroscopy. All three methods indicated that the degree of crystallinity of the cellulose increased as Kraft pulping proceeded due to preferential removal of the less ordered carbohydrates. Hunt et al., (2001) also reported cellulose fibril distortions were partially relaxed by the pulping process, which led to significant cellulose crystallinity increase.

Water decreases the glass transition temperature (Tg) of dry cellulose from 25 °C to 45°C (Leboeuf *et al.,* 2000). In aqueous solution, HOC sorption to cellulose should therefore be dominated by partitioning mechanism. Pure cellulose has a fairly low sorption capacity for organic compounds, with 1.0 mg/g for benzene and 1.4 mg/g for tetrachloride phenol (Rutherford et al., 1992). The low uptake of cellulose is determined by its high organic polarity (O+N)/C (Rutherford *et al*., 1992) and lack of aromaticity (Xing *et al*., 1994). Cellulolytic microorganisms are found among diverse taxonomic groups. They usually occur in mixed populations comprising cellulolytic and non-cellulolytic species, which often interact synergistically (Béguin and Aubert, 1994). Cellulose is highly insoluble in water.

Cellulolytic microorganisms can hydrolyze the 1,4-glycosidic bonds of cellulose with cellulase, which can convert the complex cellulose to smaller cellobiose molecules (Pelczar Jr. and Reid, 1958). Cellulases form a multicomponent enzyme system; with endoglucanases (EGs) that hydrolyse cellulose chains randomly, cellobiohydrolases (CBHs) that hydrolyse cellobiose from the polymer ends and cellobiases that hydrolyse cellobiose to glucose (Cavaco-Paulo, 1998). Amorphous cellulose is preferentially hydrolyzed while crystalline cellulose is more resistant to hydrolysis (Senior, 1990). In both aerobic and anaerobic conditions, cellulose is substantially degraded by fungi and bacteria (Eleazer *et al.*, 1997; Pichler and Kögel-Knabner, 2000). In most natural environments, cellulose cannot be completely mineralized due to the protective effect of lignin, soil minerals and humus polymers. Cellulose can form chemical or physical linkages to these constituents to persist for a sufficient length of time to participate directly in humus formation (Bollag *et al*., 1998).

Enzymatic hydrolysis was used recently to investigate of the residual lignin and lignin-cellulose bonds in pulps. Karlsson *et al*., (2001) used cellulases and hemicelluases to study lignin-cellulose as well as lignin-hemicellulose bonds in Kraft pulps. It was found 40% of pine Kraft pulp was degraded by the cellulase treatment. The remaining cellulose was still of high molecular weight, which indicated a considerable portion of the residual lignin in the pine Kraft pulp was bonded to cellulose. The authors also reported the simultaneous removal of lignin and cellulose by the cellulase treatment. Hemicelluloses contain xylan, mannan, galactan and arabinan as the main heteropolymers (Dekker and Richards, 1976). The principal monomers present in most hemicelluloses are Dxylose, D-mannose, D-galactose, and L-arabinose (Wenzl, 1970). For native hemicelluloses, the most probable Tg is around 180 °C, and 30% moisture can lower the Tg to room temperature (Back and Salmen, 1982). Because of the much greater complexity of sugars and linkages in hemicelluloses, many more enzymes are involved in the complete hydrolysis of the backbone and the branches (Deobald and Crawford, 2000). Because xylan is a common hemicellulose backbone constituent, much of the research on hemicellulases has focused on xylanases. The xylanolytic enzyme system is composed of β-1,4-endoxylanase, β-xylosidase, α-Larabinofuranosidase,

α-glucuronidase, acetyl xylan esterase, and phenolic acid esterase. All these enzymes act cooperatively to convert xylan into its constituent sugars (Beg *et al*., 2001). Sugars produced can be readily detected colorimetrically (Sharrock, 1988) using method proposed by Miller *et al*., (1960); Lever, (1973); Mullings and Rarish, (1984).

2.2.2. Lignin

Lignin is, after cellulose and hemicellulose, the third most abundant biopolymer on earth. It is present in the primary cell wall, which provides mechanical strength. The polymer is composed of aromatic alcohols, particularly three p-hydroxycinnamyl alcohols. Lignin plays an important role in humification processes. One of the widely accepted theories concerning organic matter humification in soil is that lignin and its degradation products such as phenols, quinones and more complex compounds are the main precursors in the formation of humic substances; their polymerization and condensation with N-compounds such as protein, amino acids, nucleic acids is brought about by the soil microorganisms (Stevenson, 1994; Sánchez-Monedero *et al*., 1999). Lignin is a glassy polymer with a glass transition temperature of 70 °C (LeBoeuf *et al*., 2000). White-rot fungi are responsible for most of the lignin decomposition in nature and lignin degradation by white-rot fungi is faster than by any other organism (Tuomela *et al*., 2000). There are many genera of actinomycetes and eubacteria that can degrade extracted lignin and dehydrogenation polymer (synthetic lignin). Many bacterial strains, especially actinomycetes, can solubilize and modify the lignin structure extensively, but their ability to mineralize lignin is limited (Buswell and Odier, 1987). Aerobic microorganisms are the primary lignin degraders in most environments. Anaerobic degradation of lignin is either not observed (Micales and Skog, 1997; Pichler and Kögel-Knabner, 2000; Odier and Monties, 1983) or happens at very low speed under specified conditions. It was found that anaerobic rumen microorganisms are capable of degrading plant fiber cell wall (Kuhad *et al*., 1997). Colberg, (1988) investigated the anaerobic microbial degradation of lignin compounds and concluded that the intermediate metabolic products called oligolignols may be partially degraded to CO_2 and CH_4 by

anaerobic microorganisms. Benner and Hodson, (1985) found elevated temperature of 55°C could enhance the anaerobic degradation of lignin.

2.2.3. Poly (vinyl chloride) (PVC) and High Density Polyethylene (HDPE)

PVC is composed of repeating vinyl chloride monomers (-CH2-CH-Cl). In its unmodified form, PVC is a rigid polymer. Plasticizers are organic additives added to PVC compounds in order to improve their processing properties and make flexible products for a wide variety of purposes (Mersiowsky *et al*., 2001). PVC is a stable polymer in landfill environment. No biological or abiotic mechanisms for a depolymerisation process are known. Temperatures usually encountered in the landfill are in the range of 18-55°C, which are substantially lower than temperatures required for thermal destruction (Mersiowsky *et al*., 2001). Plasticizers are important in determining the glass transition temperature of PVC and consequently its sorptive behavior. Rigid PVC undergoes glass transition at temperatures between 60 and 80 °C, whereas for flexible PVC compounds this temperature range is reduced to around 0 °C at an average plasticizer content of approximately 30% (Mersiowsky *et al.*, 2001). Significant plasticizer loss can occur in landfill due to microbial transformation during the methanogenic landfill stage (Ejlertsson *et al*., 1997; Mersiowsky *et al*., 2001), which indicates that PVC containing plasticizers may be transformed from a rubbery to a glassy state in landfill environment. Polyethylene consists of repeating ethylene (CH2=CH2) monomers. The glass transition temperature of polyethylene is –68 °C (Brandup et al., 1989). HDPE contains interstitial spaces between the polymer segments through which small molecules can diffuse.

2.2.4. Protein and Lipid

Proteins are one of the major constituents of living organisms. Proteins are made up of one or more polypeptide chains, each consisting of many α-amino acid residues covalently linked by peptide bonds. The naturally occurring proteins are too large to enter the bacterial cell. The decomposition of protein occurs in two stages:

Enzymes

Protein + H_2O → polypeptides

Enzymes

Polypeptides + H_2O → mixture of individual amino acids

The amino acids taken into the cell may be used for the formation of proteins or subjected to further degradation to ammonia and CO_2. Analysis of organic matter of municipal solid waste showed no significant change in protein content during aerobic and anaerobic treatment (Pichler and Kögel-Knabner, 2000). One mechanism attributed to the persistence of protein or peptide is the microbial resynthesis of protein. Proteins may be preserved by encapsulation into refractory cell wall polymer of microorganisms, e.g., lipids, and become poorly degradable (Dinel *et al*., 1996; Lichtfouse *et al*.,1996) and may be incorporated into humic substances (Sánchez-Monedero *et al*., 1999).

Lipids include a variety of fat-like substances. Many bacteria are capable of decomposing fats through lipases. Lipases cause a hydrolytic breakdown through addition of water; the lipid molecule is split into glycerol and its respective fatty acid. González-Vila *et al*., (1995) studied the progressive transformation of lipids in landfills. Samples were taken from different depths in the landfill. The depth of the samples represented the disposal time. The amount of Sohxletextractable lipids showed irregular behaviors with depth, which might be due to the great heterogeneity of the samples and microbial resynthesis. Microbially synthesized long-chain lipids are resistant to attack by microorganisms (Dinel *et al*., 1996).

2.3. Organic Waste Treatment

Open dumping and simple landfills are the most common treatment methods for municipal solid waste in developing countries. The main reason is the low investment and operation costs. However, the environmental problems related to these treatment techniques are well known and, as mentioned in the introduction, many municipalities and communities are trying to introduce improved methods. Paradoxically, upgrading open dumps to landfills may contribute to increased emissions of GHG. Methane generation is higher in deep, compacted landfills than

in shallow, loosely packed open dumps because of the anaerobic conditions prevailing in the former. Hence, by introducing measures aimed at improving waste management, local authorities run the risk of shifting local problems related to health impacts, water pollution, odour and land shortage to the global problem of climate change. However, a number of alternative treatment methods exist and more experience in using these methods is gradually being gained.

2.3.1. Mechanical Biological Treatment

Mechanical-biological treatment (MBT) is a group of hybrid methods where unsorted waste undergoes pre-treatment before disposal in landfills. There are many possible designs, but a common MBT pre-treatment system includes (i) mechanical separation where recyclable materials such as ferrous metals and plastics are removed; and (ii) biological treatment where the organic fraction is partly degraded. The biological step can include both anaerobic and aerobic treatment, generating biogas which can be recovered. MBT can reduce the volume of the waste by up to 40% and lower the leakage and gas emissions from landfills significantly (Visvanathan *et al*., 2005). If the treated waste contains low levels of pollutants, it can be used for landscaping instead of being deposited in a landfill, but not for food production.

2.4. Role of Various Microbes in Biocycling

Biodegradation is a viable bioremediation technology. It has long known that microorganisms (bacteria. fungi, actinomycetes etc) degrade environmental pollutants in various matrices and environments. A goal of bioremediation is to transform organic pollutants into harmless metabolites or mineralize the pollutants into carbon dioxide and water (Alexander, 1999). The organic carbon compounds that eventually are deposited in the soil are degraded by the activities of microorganisms which are mainly the bacteria and fungi. The CO_2 is released into the air and soil.

2.4.1. Cellulose Decomposition

Cellulose is the most abundant organic material in plants. It is readily attacked by many species of fungi and bacteria. The process of cellulose decomposition to carbon dioxide can be summarized in the form of following reactions.

$$\text{i. Cellulose} \xrightarrow[\text{Cellulase}]{\text{Enzyme}} \text{Cellobiose}$$

$$\text{ii. Cellobiose} \xrightarrow[\text{B-glucosidase}]{\text{Enzyme}} \text{Glucose}$$

$$\text{iii. Glucose} \xrightarrow[\text{of many microbes}]{\text{Enzyme system}} CO_2 + H_2O + \text{and/or other end products}$$

The fungi which decompose cellulose in soil are mainly, viz., *Trichoderma, Aspergillus, Penicillium, Fusarium, Chaetomium, Ver illlium, Rhizoctonia, Myrothecium, Merulius, Pleurous,* xmes etc.

The bacteria that bring about cellulose decomposition in soil consist of mainly the species of *Clostridium, Cellulomonas, Streptomycin, Cytophaga, Bacillus, Pseudomonas, Nocardia, Micromonospora, Sporocytophaga, Polyangium, Cellfalcicula* etc.

2.4.2. Hemicellulose Decomposition

Hemicelluloses are the polymers of simple sugars such as pentoses, hexoses and uronic acid. The decomposition of hemicelluloses by microorganisms takes place through, the agency of extracellular enzymes called hemicellulases. The fungi that

degrade hemicelluloses in soil are exemplified by *Chaetomium, Aspergillus, Penicillium, Trichoderma, Fusarium, Humicola etc. Bacillus, Pseudomonas, Cytophaga, Vibrio, Erwinia, Streptomyces, Actinomyces* etc. are the bacteria that degrade hemicelluloses in soil.

2.4.3. Lignin Decomposition

Lignin is the third most abundant constituent, of the plants. It is highly resistant of microbial degradation. However, certain fungi (exemplified by *Aspergillus, Penicillium, Fusarium, Lenzites, Clavaria, Polyporus*etc.) and bacteria (exemplified by *Streptomyces, Nocardia, Flavobacterium, Xanthomonas, Pseudomonas, Micrococcus* etc.) are known to degrade lignin at slow rates

A feasible remedial technology requires microorganisms being capable of quick adaptation to and efficient uses of pollutants of interest in a particular case in a reasonable period of time. Many factors influence microorganisms to use pollutants as substrates or cometabolize them. Bacteria having better adaptation capability and quick doubling time have a age over other microorganisms with respect to degradation of waste.

2.5. Biodegradation of Organic Waste by Bacteria

It is the easily biodegradable fraction of immediate interest. The breakdown of the complex organic matter is accompanies by production of intermediates or end products. These processes are performed by bacteria efficiently and in the due course they produce several enzymes having application in diverse fields. The importance of knowing the process of decomposition carried out by bacteria in sewage solids digestion cannot be overemphasized. It has been recognized for several years that knowledge of changes taking place during decomposition would aid considerably in understanding digestion and might even change or improve existing methods. Such information may easily be the basis for improved solids decomposition by regulating conditions to favour the most desirable process. Further study on the product produced during the degradation of sewage solids might lead to some valuable byproduct recovery. Heterotrophic bacteria play a fundamental role in the biodegradation process. In spite of the increasing interest in

this type of organic waste processing, few, if any, reviews on this subject have been published. O'Shaughnessy, (1914) reported the presence of bacteria of the coli and proteus group as well as denitrifying, fat splitting and cellulose organisms in a skudge after active digestion. Hotchkiss and Murray, (1923) observed bacteria capable of attacking complex protein, yielding soluble compounds. Gaub, (1924) observed that under anaerobic conditions, colifirm was the largest group with, *Salmonell typhosa* and *B.subtilis* present in great numbers. Decomposition of litter can be largely performed by bacteria (Kominkova *et al*., 2000; Kuehn et al., 2000; Findlay *et al*., 2002; Anesio *et al*., 2003). In the process of decomposition bacteria produce different enzymes which play dominant role in the process of degradation as well as these enzymes have varied application and huge commercial values.

2.5.1. Activities of Bacterial Enzymes in Organic Litter Decomposition

The microbes need to produce extracellular enzymes to convert polymeric compounds—such as cellulose, hemicellulose, and lignin—into smaller molecules that can be assimilated (Chro´ st, 1991). The most relevant enzymes from this aspect involve those that break down the plant fibers (cellulases, hemicellulases, pectinases, phenol oxidases) as well as enzymes important for microbial acquisition of nitrogen and phosphorus (peptidases, ureases, and phosphatases) (Sinsabaugh *et al*., 2002). The enzymes enabling the degradation and utilization of chitin (b-glucosaminidases and chitinases) may also cause lysis of fungal cell walls (degradation of fungal cell wall for fungal growth and/or fungal lysis by bacterial action, Wohl and McArthur, 2001). Due to the close connection between enzyme activity and degradation of different fractions of organic matter, enzyme assays can be used to estimate degradation rates of particulate and dissolved organic carbon in freshwater systems (Sinsabaugh *et al*., 1994). Production of cellulolytic and xylanolytic (hemicellulolytic) enzymes has been reported to occur also in bacteria (Robb *et al*., 1979; Tanaka, 1993; Sala and Gu¨de, 2004). In a few cases, bacteria (particularly actinomycetes) have been shown to contribute to degradation of lignin, either as primary decomposers (Benner *et al*., 1984), or through mineralization of intermediate products released through fungal activity (Ru¨ttimann *et al*., 1991). However, it is generally assumed that bacteria mainly decompose polysaccharides and polymeric compounds after the previous

decomposition of high molecular and/or lignified compounds by fungi. Microorganisms surviving under extreme environments as well as enzymes (secreted from them in the process of this decomposition) stable and active under extreme conditions are both scientifically interesting and industrially significant for several useful applications. The biodegradation ability of bacteria reduces the waste by their effective utilization to produce useful enzymes.

2.5.2. Bacteria as Source of Industrial Enzymes

Bacteria are a rich source of enzyme. Bacteria have been extensively screened for industrial enzyme production and numerous processes and enzyme product has been developed over the years. The enzymes vary broadly in their properties particularly in regard to temperature and pH optima for activity and stability. For example, mesophilic *Bacillus* strains secrete alpha amylases active at 90-100^{0}C and find widespread application in textile desizing. *Bacillus* strains also secrete highly active alkaline proteases. Municipal waste dump being a combination of several substrates is the best habitat for diverse bacteria. This bacterial diversity can be screened for production of a number of economically important enzymes.

Among the industrial enzymes, proteases account for approximately 60% of the total enzyme sales in various industrial market sectors such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery (Godfrey and West, 1996). Bacterial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community.

2.5.3. Bacterial Lignin Peroxidases

Lignin is the most abundant renewable carbon source on earth (Bo Zhang et al., 2008). When plants die, it will rot and degrade in soil. Researches are widely done on fungi which degrade lignin. While bacteria grow and multiply faster, it is anticipated to be better in production of lignin degrading enzymes. There are many types of lignin degrading enzymes such as lignin peroxidase, manganase

peroxidase, laccase and glyoxal oxidase are produced by fungi as well as bacteria such as lignin peroxidase by the bacteria *Streptomyces viridosporus* (Ramachandra et al., 1987). Lignin peroxidase is an enzyme that is used to degrade lignin. It was first discovered in 1983. Lignin peroxidases are produced by many wood degrading fungi as a family of isoenzymes (Kirk et al., 1987). Recent researches also showed that it can be produced from bacteria such as *Streptomyces viridosporus.* These hemeproteins are similar to the more familiar plant peroxidases in structure and mechanism, and utilize hydrogen peroxide and organic peroxides to oxidize a variety of substrates (Tien et al., 1985, 1986). Some of the most important features distinguishing these enzymes from other oxidoreductases (such as horseradish peroxidase), for example, are their very low pH optima and much higher redox potentials.

The substrates of lignin peroxidase include both phenolic and non-phenolic aromatic compounds; the phenolic substrates are oxidized to yield products similar to those produced by classical peroxidases, while the oxidation of the non-phenolic methoxybenzenes are unique to the lignin peroxidases (Korsten et al., 1996), the oxidation of these substrates yield aryl cation radicals. Lignin peroxidase can catalyze the oxidation of substrates with a reduction potential greater than 1.3 volts. The enzyme is capable to oxidizing lignin monomers, dimers and trimers as well as polycyclic aromatic compounds such as benzopyrene (Haemmerli et al., 1996). The powerful and relatively non-specific nature of this enzyme has lead to the investigation of its potential use in diverse fields of biodegradation of toxic chemicals, pulp, paper processing, and in the textile industry.

2.5.4. Bacterial Proteases

Microorganisms elaborate a large array of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products (Kalisz, 1988). At the same time, these extracellular proteases have also been commercially

exploited to assist protein degradation in various industrial processes (Kumar and Takagi, 1999; Outtrup and Boyce, 1990).

The proteases from different bacterial sources differ widely not only in functions but also in properties, based on which they can be classified into different groups. Depending upon the optimum pH for activity they can be classified into acid, neutral and alkaline types. Microbial proteases can be classified into four groups, based on the essential catalyt~c residue at their active site. They are the serine, thiol, acid (carboxyl) and metallo proteases.

Serine Proteases

Serine proteases are characterized by a catalytically active serine residue in the active centre. They are inactivated by organic phosphate esters which acylate the active serine residue. Alkaline proteases from different microbial sources mainly belong to this group.

Thiol Proteases

Enzymes in this group have cysteine at their active site. In general they are activated by reducing compounds and inhibited by oxidising agents. They are susceptible to sulfhydryl (S H) reagents such as 4-hydroxy mercuri benzoic acid (p-CMB) and are activated by reducing agents such as hydrogen cyanide. The best known microbial thiol proteases are clostripain obtained from Closbdium histolyticum and the streptococcal protease.

Acid (Carboxyl) Proteases

Many of them contain aspartate residues at their active site. They are insensitive to sulfhydryl agents and chelating agents. They include microbial rennets and microbial acid proteases from moulds and yeasts.

Metallo Proteases

They have metal ion involved in the catalytic mechanism and are consequently sensitive to chelating agents such as EDTA. They are insensitive to sulfhydryl agents and organic phosphate esters. Neutral proteases from many microbial sources belong to this group.

However, the largest share of enzyme market has been held by alkaline proteases, useful in the detergent industry contribute to more than 30% of the total global enzyme market estimated at several billion US dollars annually. The bioindustrial viewpoints of bacterial alkaline proteases from sources to cellular role, production, downstream processing, characterization and commercial applications have also been reviewed (Anwar et al., 1998; Kumar et al., 1999).

2.6. Alkaline Proteases

Alkaline proteases (EC.3.4.21-24, 99) are defined as those proteases, which are active in a neutral to alkaline pH range. They either have a serine centre (serine protease) or are of metallo-type (metalloprotease); and they are the most important group of enzymes exploited commercially. Alkaline proteases are most active at pH values of about pH 10. They are sensitive to DFP and a potato inhibitor but not to TLCK or tosyl-L-phenylalanine chloromethyl ketone (TPCK). They are all specific against aromatic or hydrophobic amino acid residues at the carboxyl side of the splitting point.

In recent years there has been a phenomenal increase in the use of alkaline proteases as industrial catalysts. These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons, for example they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts and are economically viable. The commercial superiority of alkaline proteases is due to their suitability for use in the field of detergent industry, where they are required in large quantities. A major trend in the detergent industry is the development of non phosphate based products. The alkaline proteases widely used as detergent enzymes are subtilisins, produced by different *Bacillus sp.*

2.6.1. Application of Alkaline Proteases

Alkaline proteases account for a major share of the enzyme market all over the world (Godfrey and West, 1996; Kalisz, 1988). Alkaline proteases from bacteria find numerous applications in various industrial sectors and different companies worldwide have successfully launched several products based on alkaline proteases. The success of detergent enzymes has led to the discovery of a series of

detergent proteases with specific uses. Alkazym (Novodan, Copenhagen, Denmark) is an important enzyme for the cleaning of membrane systems. Other enzymes used for membrane cleaning are Terga-zyme (Alconox, New York, USA), Ultrasil (Henkel, Dusseldorf, Germany) and P3-pardigm (Henkel-Ecolab, Dusseldorf, Germany). Pronod 153L, a protease enzymebased cleaner is used to clean surgical instruments fouled by blood proteins. Subtilopeptidase A is an enzymebased optical cleaner, presently marketed in India (Kumar *et al*., 1998). Sakiyama *et al*., (1998) reported the use of a protease solution for cleaning the packed columns of stainless steel particles fouled with gelatin and β-lactoglobulin. In addition to these major applications, alkaline proteases are also used to a lesser extent for other applications, such as contact lens cleaning (Nakagawa, 1994), molecular biology for the isolation of nucleic acid (Kyon *et al*., 1994), pest control (Kim *et al*., 1999), degumming of silk (Kanehisa, 2000; Puri, 2001) and selective delignification of hemp (Dorado *et al*., 2001), which all may be technically interesting, but have not reached commercial success in terms of impressive sales figures.

2.7. Extracellular Alkaline Protease Production by Bacteria

Screening of effective bacteria for extracellular alkaline protease production and optimization of the same is of prime research importance.

2.7.1. Screening and Selection of Strains

The proteolytic bacteria when grown on casein agar or milk agar can produce a zone of clearance around their colonies and hence can be easily distinguished from the non-proteolytic ones. But no significant correlation has been reported between the size of clearance zone and the quantity of protease produced under fermentation conditions. Aunstrup, (1974) reported that Bacillus lichenifomis could produce very narrow zones of hydrolysis on casein agar despite giving very good protease production in submerged cultures.

Methods of Production

Submerged fermentation is the commonly employed method for the production of commercially important alkaline proteases from bacterial sources. Solid

state fermentation for the production of bacterial alkaline proteases has been reported only by a few workers.

2.7.2. Optimization of Cultural Conditions for the Production of Alkaline Proteases by the Sub-merged and Solid State Fermentation Methods.

Among bacteria, Bacillus spp. are specific producers of extracellular alkaline proteases (Godfray, 1986). Bacillus being the most prominent and serve as an ideal source of these enzymes because of their rapid growth and limited space required for their cultivation. An ease with which can be genetically manipulated to generate new enzymes accounting with altered properties that is desirable for their diverse applications. Studies on other strains of Bacillus showed that nutritional, chemical and physical factors can influence protease production. Nutritional factors include the sources of carbon, nitrogen and metal ions (Adinarayana, 2003; Thangam, 2002). Besides that, physical factors such as inoculums concentration, aeration, temperature, pH and incubation time also significantly affect protease production (Votruba *et al*, 1991; Rahman *et al*, 2005; Chen, 2002). It is well established that extracellular protease production in microorganisms is greatly influenced by media components. Therefore, the effect of various carbon and nitrogen substrates, divalent metal ions, environmental and fermentation parameters are required to be evaluated (Adinarayana, 2002).

Alkaline proteases are generally produced using sub-merged fermentation due to its apparent advantages in consistent enzyme production characteristics with defined medium and process conditions and advantages in downstream inspite of the cost-intensiveness for medium components. In this context, solid state fermentation has gained renewed interest and fresh attention from researchers owing to its importance in recent developments in biomass energy conservation in solid waste treatment and in its application to produce secondary metabolites. Production of these biocatalysts using agro biotech substrates under solid state fermentation conditions provide several advantages in productivity, cost effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization etc (Pandey *et al*., 2000). There are several reports describing the use of agro industrial residues for

the production of alkaline protease, for example, nugmeal and *Bacillus sp*. AR 009 (Gessesse, 1997), pigeon pea and *Bacillus* sp. (Johnvesly *et al.,* 2002) etc.

2.7.3. Response Surface Method, an Efficient Tool for Optimization of Protease Production

Response surface methodology is a procedure for fitting a series of regression models to the output variable of a simulation model (by evaluating it at several input variable values) and optimizing the resulting regression function. Response Surface Methodology (RSM) is concerned with the modeling of one or more responses to the settings of several explanatory variables. The nature of the function relating the responses to the variables is assumed to be unknown and the function or surface is modeled empirically using a first-or a second-order polynomial model. The broad aims of RSM are to investigate the nature of the response surface over a region of interest and to identify operating conditions associated with maximum or minimum responses. RSM is generally conducted in three phases, as emphasized in Myers and Montgomery, (2002). Phase 0 involves the screening of explanatory variables to identify those which have a significant effect on the responses, phase 1 is concerned with the location of optimum operating conditions by conducting a sequence of suitable experiments and phase 2 involves the fitting of an appropriate empirical model, usually a second-order polynomial model, in order to examine the nature of the response surface in the vicinity of the optimum. The fundamentals of RSM are set out in the seminal papers of Box and Wilson (1951) and Box and Draper (1959) and in the books by Box and Draper (1987), Khuri and Cornell (1996) and Myers and Montgomery (2002). Further developments are drawn together in three key review articles, namely those of Hill and Hunter (1966), Myers *et al.,* (1989) and Myers *et al*., (1989, 2004). It is clear from these articles that research into RSM within academia continues to flourish and that the associated techniques are used extensively in industry. RSM is a design that has been described as the most powerful statistical techniques in technological research. Tang *et al*., 2004 also reported 1.4 times higher production of alkaline protease using RSM in the production optimization.

2.7.4. Purification of Alkaline Proteases.

Studies on the cost effective purification method is very much essential for the industrially important enzyme like protease. To characterize an alkaline protease and determine its potential commercial application it is necessary to purify the enzyme thus produced. Salt precipitation and chromatographic separation ensures purification of the enzyme to some extent. Protein gel electrophoresis reveals the degree of purification and accordingly several other techniques are employed for purification of the same to further extent. Kunamneni adinarayana *et al*, (2003) have isolated, purified and partially characterized thermostable serine alkaline protease enzyme from a Bacillus subtilis PE ll. They have extracted proteases in bulk amount, checked its molecular weight is 15kDa, optimum pH at 10, optimum temperature is 60°C with casein as substrate. This enzyme is activated by metal ions such as ca^{2+} Mg^{2+} and Mn^{2+} but its activity is strongly inhihbited by phenyl methyl sulphonylthoride (pmsf) and disopropyl thorophosphates (DFP).

2.8. Studies on the Properties of Purified Alkaline Proteases.

Purification followed by characterization of the properties of alkaline protease is pf prime importance prior its commercial application. Hyo-Kyung *et al.,* (2003) have purified and characterized the 33kDa serine protease from *Acanthamoeba lugdunensis* KA/E2 isolated from a Korean keratitis patient. The purified 33kDa protease had a pH optimum of 8.5 and a temperature optimum of 55°C. Phenylmethylsulfonylfluoride and 4-(2-Aminoethyl)-benzenesulfonyl-fluoride, both serine protease specific inhibitors, inhibited almost completely the activity of the 33 kDa protease whereas other classes of inhibitors did not affect its activity. The 33 kDa enzyme degraded various extracellular matrix proteins and serum proteins. Their results strongly suggest that the 33kDa serine protease secreted from this keratopathogenic Acanthamoeba play important roles in the pathogenesis of amoebic keratitis, such as in corneal tissue invasion, immune evasion and nutrient uptake.

2.8.1. Optimum Temperature and Thermostability of Alkaline Proteases

The heat stability of enzymes is affected by at least two factors alone or in combination. The first one is the primary structure of the enzyme. A high content of hydrophobic amino acids in the enzyme molecule provides a compact structure, which is not denatured easily by a change in the external environment. In addition, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation. Secondly, specific components such as polysaccharides and divalent cations, if any, can stabilize the molecule (Öztürk, 2001). Even though there is no firm evidence to suggest that thermostable enzymes are necessarily derived from thermophilic organisms, nevertheless there is a greater chance of finding thermostable proteins from thermophilic bacteria (Rahman *et al*., 1994). Therefore, a wide range of microbial proteases from thermophilic species has been extensively purified and characterized. These include Thermus sp., Desulfurococcus strain Tok12S1 and Bacillus sp. Among them alkaline proteases derived from alkaliphilic bacilli, are known to be active and stable in highly alkaline conditions (Rahman *et al*., 1994). The earliest thermophilic and alkaliphilic Bacillus sp. was B. stearothermophilus strain F1 isolated by Salleh and friends in 1977, which was stable at 60ºC (Haki and Rakshit, 2003). Further studies on microbial alkaline proteases have been done in view of their structural-function relationship and industrial applications, as they needed stable biocatalysts capable of withstanding harsh conditions of operation (Rahman *et al*., 1994). Generally alkaline proteases produced from alkaliphilic Bacillus are known to be active over a wide range of temperature. The optimum temperatures of alkaline proteases range from 40 to 80ºC. In addition, the enzyme from an obligatory alkaliphilic Bacillus P-2 showed an exceptionally high optimum temperature of 90ºC. The protease has also good thermostability at high temperatures, being thermostable at 90ºC for more than 1 h and retained 95 and 37% of its activity at 99ºC (boiling) and 121ºC (autoclaving temperature), respectively. Bacillus P-2 was the only mesophile reported until 2001, which produced a proteolytic enzyme that was stable for so long even at autoclaving (121ºC) and boiling temperatures (Kaur et al., 2001). In some studies it has also been observed that the addition of Ca2+

further enhanced enzyme thermostability (Takami et al., 1989; Gessesse, 1997; Rahman et al., 1994).

2.8.2. Optimum pH of Alkaline Proteases

Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly located on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This will affect the total net charge of the enzymes and the distribution of charges on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighborhood of the active sites, which will overall affect the activity, structural stability and solubility of the enzyme (Chaplin and Bucke, 1990). In general, all currently used detergent-compatible proteases are alkaline in nature with a high pH optimum; therefore they fit the pH of laundry detergents, which is generally in the range of 8 to 12. Therefore, most of the commercially available subtilisin-type proteases are also active in the pH range of 8-12 (Gupta *et al.*, 2002a). A good example for this is the well-known detergent enzymes, subtilisin Carlsberg and subtilsin Novo or BPN' which show maximum activity at pH 10.5 (Banerjee *et al.*, 1999). Alkaline proteases of the genus Bacillus show an optimal activity and a good stability at high alkaline pH values (Margesin *et al.*, 1992). The optimum pH range of Bacillus alkaline proteases is generally between pH 9 and 11, with a few exceptions of higher pH optima of 11.5 (Fujiwara and Yamamoto, 1987), 11-12 (Kumar *et al*., 1999), 12-13 (Takami *et al*., 1989, Fujiwara *et al*., 1991, Ferrero *et al*., 1996) (Kumar and Takagi, 1999).

2.8.3. The Isoelectric Point

The pH referred as isoelectric point (pI) at which the net charge on the molecule is zero, is a characteristic of each enzyme, where solubility in aqueous solutions is generally minimum. In aqueous solution, charged groups interact with polar water molecules and stabilize the protein, which is intrinsically hydrophobic. A low number of charged groups and a high number of aliphatic or aromatic side chains characterize a protein that is less soluble in water. As one move farther from pI, the number of ionized groupsincreases therefore the solubility tends to increase. Hence

the isoelectric point is important as it affects the solubility of proteins as well as interaction between them (Öztürk, 2001).

2.8.4. The Molecular Weight

The molecular weights of alkaline proteases generally range from 15 to 30kDa (Kumar and Takagi, 1999) with few reports of higher molecular weights of 32.0 kDa (Huang *et al*., 2003), 33.5kDa (Rahman *et al*., 1994), 36.0kDa (Durham *et al*., 1987).

2.8.5. Metal Ion Requirement and Inhibitors of Alkaline Proteases

Alkaline proteases require a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations, for maximum activity. These cations were also found to enhance the thermal stability of a Bacillus alkaline protease. It is believed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Kumar and Takagi, 1999). Inhibition studies give insight into the nature of the enzyme, its cofactor requirements, and the nature of the active site. Alkaline proteases are completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP). In this regard, PMSF sulfonates the essential serine residue in the active site, results in the complete loss of activity. This inhibition profile classifies these proteases as serine hydrolases. In addition, some of the alkaline proteases were found to be metal ion dependent in view of their sensitivity to metal chelating agents, such as EDTA. Thiol inhibitors have little effect on alkaline proteases of Bacillus spp., although they do affect the alkaline enzymes produced by Streptomyces sp. (Kumar and Takagi, 1999).

2.9. Testing the Suitability of the Proteases for Commercial Application.

Screening of alkaline proteases producing bacillus spp. from different ecological environments can result in isolation of new alkaline proteases with unique physio-chemical characteristics (Shumi *et al*., 2004; Singh *et al*., 1999). One of the most important characteristics that determine the industrial suitability of proteases is their requirement of high pH for optimum enzyme activity. The characterization will render valuable information on the thermostability of the enzyme, the pH

range of the stability of the enzyme and other necessary information. The characterization of the protease produce will determine the application of the enzyme. The application specially industrial application of alkaline protease is manifold.

2.9.1. Food Industry

Alkaline proteases can hydrolyse proteins from plants and animals to produce hydrolysates of well-defined peptide profiles. These protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations specific therapeutic dietary products and the fortification of fruit juices and soft drinks. In recent years there has been substantial interest in developing enzymatic methods for the hydrolysis of soya protein, gelatin, casein, whey and other proteins in order to prepare protein hydrolysates of high nutritional value. In developing commercial products from these proteins, emphasis is placed on achieving a consistent product in high yields, having desirable flavour, nutritional and/or functional properties (Ward, 1991). Alkaline protease from B. licheniformis is used for the production of highly functional protein hydrolysates (Ward, 1991). This commercial alkaline protease, Alcalase, has a broad specificity with some preference for terminal hydrophobic amino acids. Using this enzyme, a less bitter hydrolysate and a debittered enzymatic whey protein hydrolysate were produced (Kumar and Takagi, 1999). Soluble meat hydrolysate can also be derived from lean meat wastes and from bone residues after mechanical deboning by solubilization with proteolytic enzymes. Alcalase has been found to be the most appropriate enzyme in terms of cost, solubilization, and other relevant factors. In an optimized process with Alcalase at a pH of 8.5 and temperature of 55-60ºC, a solubilization of 94 % was achieved. The resulting meat slurry was further pasteurised to inactivate the enzyme and found wide application in canned meat production, soups and seasoning (Kumar and Takagi, 1999). Very recently, another alkaline protease from *B. amyloliquefaciens* resulted in the production of a methionine-rich protein hydrolysate from chickenpea and soy protein, which found major application in hypoallergenic infant food formulations (Kumar and Takagi, 1999). In another study, Rebecca *et al*., (1991) reported the production of fish hydrolysate of high nutritional value, using B. subtilis proteases. Perea *et al*.,

(1993) on the other hand used alkaline protease for the production of whey protein hydrolysate, using cheese whey in an industrial whey bioconversion process.

2.9.2. Detergent Industry

Ideally alkaline proteases used in detergent formulations should have high activity and stability over a broad range of pH and temperature, should be effective at low levels (0.4-0.8%) and should also be compatible with various detergent components along with oxidizing and sequestering agents. They must also have a long shelf life (Kumar and Takagi, 1999). Alkaliphilic Bacillus strains are good sources of alkaline proteases with the properties that fulfil the essential requirements to be used in detergents; therefore the main industrial application of alkaliphilic proteases has been in the detergent industry since their introduction in 1914 as detergent additives. (Ito *et al.*, 1998; Horikoshi, 1996). The major use of detergent–compatible alkaline proteases is in laundry detergent formulations. Detergents available in the international market such as Dynamo®, Eraplus® (Procter & Gamble), Tide® (Colgate Palmolive), contain proteolytic enzymes, the majority of which are produced by members of the genus Bacillus (Anwar et al., 1998). Banerjee and his colleagues (1999) have studied on an alkaline protease from a facultative thermophilic and alkalophilic strain of Bacillus brevis. The alkaline protease from B. brevis having maximum activity at pH 10.5, showed a high level of thermostability at 60ºC. The enzyme showed compatibility at 60ºC with all of the commercial detergents tested. It could also remove blood strains completely when used with detergents. All the tests were studied in the presence of Ca2+ and glycine and the data obtained in this study implies that the protease of B. brevis has most of the properties to be used as a detergent enzyme.

2.9.3. Leather Industry

The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental pollution and safety hazards. Thus, for environmental reasons, the biotreatment of leather using enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction. Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable

pigments, increases the skin area and thereby clean hide is produced (Gupta *et al.*, 2002b). Alkaline proteases with elastolytic and keratinolytic activity can be used in leather processing industries. During bating, the hide is softened by partial degradation of the interfibrillar matrix proteins (elastin & keratin). Therefore enzyme preparations with low levels of elastase and keratinase activity but no collagenase activity are particularly applicable for this process. (Cowan, 1994). Bating is traditionally an enzymatic process involving pancreatic proteases. However, recently, the use of microbial alkaline proteases has become popular. The substitution of chemical depilatory agents in the leather industry by proteolytic enzymes produced by Bacillus sp. could have important economical and environmental impacts (Anwar *et al.*, 1998) where the dehairing process is accelerated by the use of alkaline proteases.

2.9.4. Medical usage

Alkaline proteases are also used for developing products of medical importance. It was stated in Gupta *et al.*, (2002b) that Kudrya and Simonenko (1994) exploited the elastolytic activity of *B. subtilis* 316M for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses. Kim *et al.*, (2001) reported the use of alkaline protease from Bacillus *sp*. strain CK 11-4 as a thrombolytic agent having fibrinolytic activity (Gupta *et al.*, 2002b). Furthermore, Bacillus sp. has been recognized as being safe to human. (Kumar and Takagi, 1999).

2.9.5. Management of Industrial and Household Waste

Alkaline proteases provide potential application for the management of wastes from various food processing industries and household activities. Feather is composed of over 90% protein, the main component being keratin, a fibrous and insoluble protein. Worldwide several million tons of feather is generated annually as waste by poultry-processing industries. Feathers constitute approximately 5% of the body weight of poultry and can be considered a high protein source for food and feed, provided their rigid keratin structure is completely destroyed. Pretreatment with NaOH, mechanical disintegration, and enzymatic hydrolysis results in total solubilization of the feathers. The end product is a heavy, greyish

powder with a very high protein content, which could be used as a feed additive (Kumar and Takagi, 1999). Considering its high protein content, this waste could have a great potential as a source of protein and amino acids for animal feed as well as for many other applications. In some countries, feather is used as animal feed supplement in the form of feather meal. Development of enzymatic and/or microbial methods for the hydrolysis of feather to soluble proteins and amino acids is extremely attractive, as it offers a cheap and mild reaction condition for the production of valuable products (Gessesse *et al*., 2003). Gessesse *et al*., (2003) isolated an organism from an alkaline soda lake in the Ethiopian Rift Valley Area and identified as *Bacillus pseudofirmus. B. pseudofirmus* AL-89 and the protease it produces offers an interesting potential for the enzymatic and/or microbiological hydrolysis of feather to be used as animal feed supplement (Gessesse *et al*., 2003). Dalev (1994) reported also an enzymatic process using a B. subtilis alkaline protease in the processing of waste feathers from poultrys laughterhouses. A formulation containing proteolytic enzymes from *B.substilis, B. amyloliquefaciens* and *Streptomyces sp*. and a disulfide reducing agent (thioglycolate) that enhances hair degradation, helps in clearing pipes clogged with hair-containing deposits and is currently available in the market. It was prepared and patented by Genex (Gupta *et al*., 2002b).

2.9.6. Photographic Industry

Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery. These waste films contain 1.5-2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Conventionally, this silver is recovered by burning the films, which causes undesirable environmental pollution. Furthermore, base film made of polyester cannot be recovered using this method. Since the silver is bound to gelatin, it is possible to extract silver from the protein layer by proteolytic treatments. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also in obtaining polyester film base that can be recycled (Gupta *et al*., 2002b). Fujiwara and co-workers studied on this interesting application of alkaline proteases. They reported the use of an alkaline protease to decompose the gelatinous coating of X-ray films, from which silver was recovered (Horikoshi,

1999). Protease B18' had a higher optimum pH and temperature, around 13.0 and 85ºC. The enzyme was most active toward gelatin on film at pH 10 (Fujiwara *et al.*, 1991). Singh *et al.*, (1999) isolated an obligate alkaliphilic Bacillus *sphaericus* strain from alkaline soils in the Himalayas, which produced an extracellular alkaline protease. The alkaline protease of this strain efficiently hydrolysed the gelatin layer of used X-ray films within 12 min at 50ºC and at pH 11.0 (Singh *et al.*, 1999). Enzymatic decomposition of gelatin layers on X-ray films and repeated utilization of enzyme for potential industrialization were also investigated using thermostable alkaline protease from the alkaliphilic *Bacillus sp.* B21-2 by Masui and his co-workers (1999). They concluded that the decomposition of gelatin layers at 50ºC with the mutant enzyme (Ala187Pro) was higher than those of the wild-type and the mutant enzyme could also be satisfactorily used five times.

2.9.7. Peptide Synthesis

Amino acids are of increasing importance as dietary supplements for both humans and domestic animals. Only the L-amino acids can be assimilated by living organisms, since the chemical synthesis of amino acids produces a racemic mixture, it is necessary to separate the isomers before commercial use. Alcalase is a proteolytic enzyme isolated from a selected strain of *B. licheniformis*, its major component being subtilisin Carslberg. It was determined that Alcalase was stable in organic solvents and could be of use as a catalyst in the resolution of N-protected amino acids having unusual side chains (Anwar *et al.*, 1998).

2.9.8. Silk Degumming

One of the least explored areas for the use of proteases is in the silk industry where only a few patents have been filed describing the use of proteases for the degumming process of silk. The conventional silk degumming process is generally expensive and therefore an alternative method suggested, is the use of protease preparations for degumming the silk prior to dyeing. In a recent study, the silk degumming efficiency of an alkaline protease from Bacillus sp. RGR-14 was studied. After 5h of incubation of silk fiber with protease from Bacillus sp., the weight loss of silk fiber was 7.5%. Scanning electron microscopy of the fibers

revealed that clusters of silk fibers had fallen apart as compared with the smooth and compacted structure of untreated fiber (Gupta *et al.*, 2002b).

2.10. Molecular Characterization of Bacterial Strains

The use of molecular techniques in microbial ecology has made possible the discovery of new microorganisms previously unknown. Identification of bacteria has been based on the determination of 16S rRNA sequences of amplified and cloned genes derived from the isolates's DNA. The 16 S rDNA is the most widely accepted gene employed for bacterial classification and identification. Goto et al. suggested that 5' end region (~275 bp) is the hypervariant (HV) region in the gene, highly specific for each type strain, and considered as an useful index for identification or grouping of *Bacillus* sp. Gupta et al. emphasized that use of molecular markers like 16 S rDNA as species-specific identification tool have provided with a truly "microscopic" sensitivity down to single-cell detection. Bacteria which grow well in pH range from 9 to 11 on the pH scale are of ecological, industrial and basic bioenergetics interests (Horikoshi, 1999). Patent literature showed a number of Subtilisins from *Bacillus pumilus* species to be appropriate for detergents. In fact, Damodaran and Han described an interesting detergent-stable alkaline protease (termed Protease Q) from *Bacillus pumilus* strain ATCC 202073. Vetter *et al.*, (2003) demonstrated a suitable use of two alkaline proteases, named Protease P46 and Protease P415, from *Bacillus pumilus* DSM 5777, in composition for cleaning and washing purposes. Recently, Merkel et al. reported a novel Subtilisin-type alkaline protease from *Bacillus pumilus*, with potential for use in washing and cleaning agents. Even though a relatively high number of *Bacillus pumilus* proteases have already been reported for detergent, few of these have a high optimum pH (>10) and thermoactivity/thermostability (>65 °C) as well as an important compatibility and stability against oxidants, bleaches and denaturing agents.

Alkaliphiles are reported to be rich sources of alkaline active enzymes such as proteases with numerous applications. With increasing industrial demands for the biocatalysts that can cope with industrial processes at harsh conditions, the isolation and characterization of new promising strains is a recent approach to

increase the yield of such enzymes (Gupta *et al.,* 2002). The present exploitations from enzymes are still not sufficient to meet all the demands. A major cause for this is the fact that many available enzymes do not withstand industrial reaction conditions. As a result, the characterizations of microorganisms that are able to thrive in extreme environments have received a great deal of attention. Being the most important sources for enzyme production, the selection of suitable microorganism plays a key role in high yield of desirable enzyme.

Thus, the above-accumulated evidence provides a window into a world of microbial diversity that is astonishing in its magnitude and breadth and is open to tap the vast genetic and biochemical potential of microorganisms to obtain products and processes of biotechnological value. The metagenome approach thus provides a means to view both the structural and the functional genomics of microbial diversity and pave a pathway to search out and discover novel genes for obtaining newer and useful industrial alkaline proteolytic enzymes with better properties.

Chapter - iii

Aims And objectives

aims and objectives

Everyday, a huge quantity of waste generate in all the developed and developing countries. In rapidly growing cities of the developing world, urban solid waste management is currently regarded as one of the most immediate problem. Various types of waste are causing adverse effect on living organisms and environment. As a result, human and animal diseases occur, the air and soil environment are spoiled and the entire natural ecosystem balance is disturbed.

Municipal solid waste has a very high organic content (70- 85 %) that is biodegradable. Under proper conditions this biodegradable fraction could be composed or co-composted for beneficial use. Bacteria are the most abundant group and the most important microbe for decomposing waste.

Bacteria are found in literally every single habitat on the face of the earth and are one of the most essential forms of life on our planet. With seriously important roles to play and in composting; they cope with all kinds of conditions that threaten their survival, from extreme temperatures to lack of food supply, while coordinating between themselves a wide range of activities as if they were one collective being. Composting would not work without bacteria, not even vermicomposting would work without them, because without bacteria, there would not even be life on the planet as we know it. Bacteria use wastes for their own metabolism and by means of composting finally they produce some simple and useful compounds which are important for soil health, plant growing and over all to keep well balance of natural ecosystem. Therefore, the effective bacteria can be employed for planned decomposition of solid organic waste.

In the due course of decomposing waste, bacteria produces several metabolically active enzymes / metabolites of high commercial value. These hyperactive strains if properly screened can be of high economic importance. Enzyme derived from the isolates can be optimized for increasing activity and thus marketed to fetch foreign dollars. The production of enzyme is central to the modern biotechnology industry. Commercial enzyme production has grown during the past centuries in volume and number of products in response to expanding markets and increasing

demand for novel biocatalysts. Bacteria due to their easy adaptability and fast reproducibility constitute the major source of enzymes.

Considering tremendous importance of municipal solid organic waste decomposition and screening of hyperactive strains producing important novel biocatalysts, this investigation was undertaken in order to screen potent bacterial isolates from municipal solid waste dump site to apply both the isolates and their cosortium for biodegradation of organic waste and to trap their potential of producing enzymes. The optimization of enzyme production and purification for marketing it to achieve economical benefits was also the part of this goal. The successfully and effective degradation of the organic wastes would produce useful components and the optimization and purification of the enzyme derived would have great market potential.

In order to address the aims of this study, the following objectives were taken into consideration.

► Sample Collection from municipal waste dump sites.

► Isolation of bacteria from waste samples collected.

► Microbiological characterization of the strains isolated.

- Colony Margin Characterization
- Surface Characterization
- Strain Morphology Analysis

► Biochemical characterization of the isolates.

► Antagonism test between the strains for application as consortium.

► Heavy Metal Tolerance Assay of the Strains.

► Antibiotic Sensitivity Assay of the isolated strains.

► Antimicrobial Activity Assay of the Isolates.

► Enzymological characterization of the strains.

- Qualitative assay of different enzymes
- Quantitative assay of enzymes produced
- Selection of substrate for production of enzyme
- Optimization of enzyme production
- Purification of enzyme
- Characterization of purified enzyme

► Application of individual isolates and bacterial consortium in waste degradation.

► Molecular characterization of the potent strains by direct sequencing of polymerase chain reaction (PCR)-amplified 16S rRNA gene products

► Application of purified enzyme

These objectives were met through the employment of different methodologies in different experiments.

Chapter - iv

Materials And methods

In order to achieve the objectives outlined in chapter III, different experiments were carried out following the methodologies mentioned below.

4.1. Study Area

Soil sample was collected from waste disposal site of Barrackpore Municipality in Barrackpore (latitude $22^0$44' N and longitude $88^0$30'E) in North 24 Parganas District of West Bengal State in Eastern India and from Dhapa situated at $88^0$20'E-$88^0$35'E and $20^0$25'N-$20^0$35'N at Kolkata District in West Bengal.

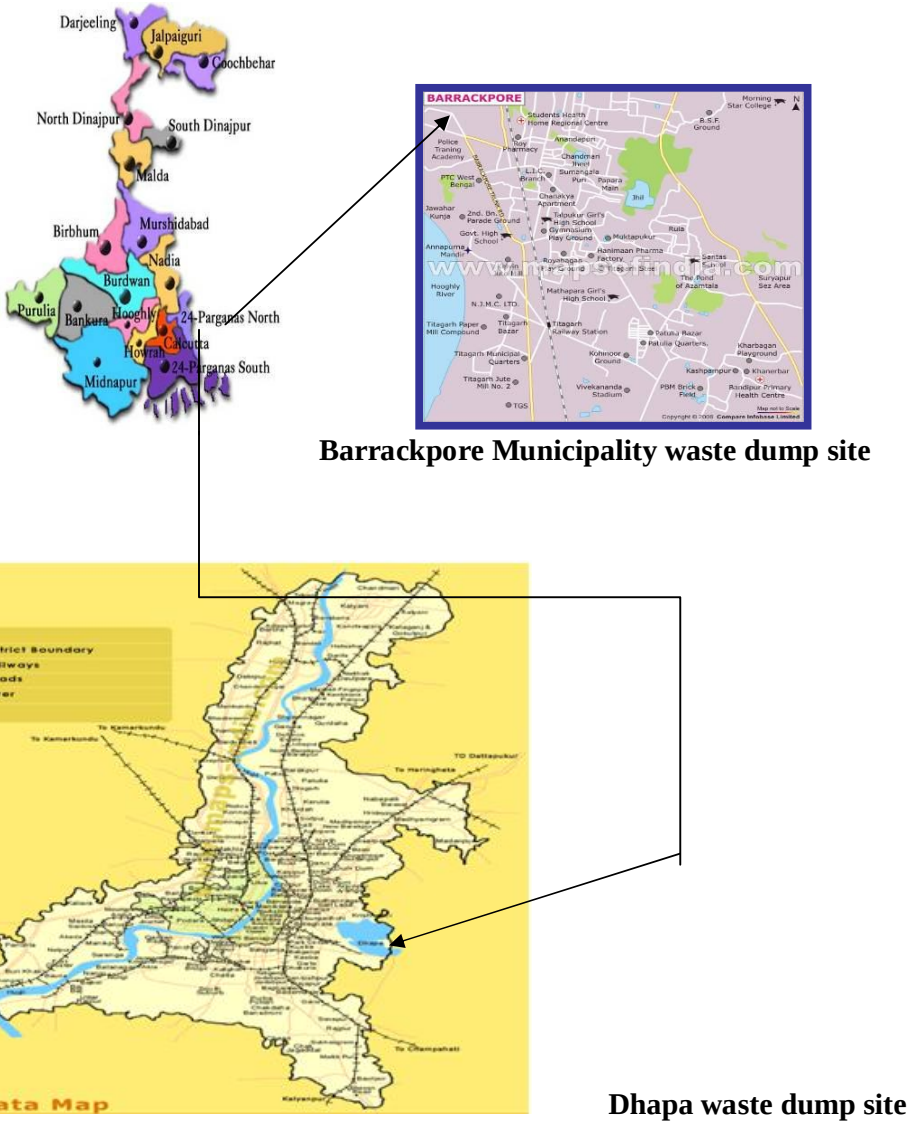


Fig 4.1. Map showing the sampling locations

Dhapa waste dump site **Barrackpore municipality waste dump site**

Fig 4.2. Dhapa and Barrackpore Municipality waste dumping ground

4.2. Chemicals.

Casein for protease assay was from Sigma (St. Louis, MO, USA). All other analytical reagents and media components used were of highest purity grade available commercially in India.

4.3. Collection of samples

A total of 10 waste samples were collected 5 from waste disposal site of Barrackpore Municipality and rest 5 from Dhapa, landfill site of city of Kolkata. Sample (soil mixed with waste) was collected in sterile zip-lock plastic maintaining aseptical condition, stored at 4^0C and marked accordingly to their source and location. The collected samples were brought to the laboratory for isolation of soil bacteria and the moisture content and pH of sample were documented.

4.4. Determination of moisture content (%) and pH of waste samples

Freshly collected samples were kept in filter paper and the initial weight was recorded. After that these samples were kept inside a hot air incubator at 110^0C. The samples were weighed several times until a constant weight was achieved. Samples moisture content was calculated employing the following formula provided by AWPA (AWPA, 1986).

$$MC\ (\%) = \frac{W-w}{w} * 100$$

Where, MC is moisture content, W is the original weight and w is the constant weight after oven drying.

pH was determined in Electrometric method with the help of a pH meter using combination glass electrode.

4.5. Chemical analysis of waste

Chemical characteristic of sample were analyzed for the following properties, Organic matter (%), total N (%), P(%) and K (%). Organic carbon was determined following a rapid titration method Walkey and Black (Walkey and Black 1934). Determination of total Nitrogen content was performed by digestion of the waste with a mixture of acids ($HClO_4$, HNO_3, H_2SO_4) and then using Kjeldahl procedure according to the method described by Bremner (Bremner 1960). Total Phosphorous was estimated by Colorimetric method using ammonium molybdate and stannous Chloride (APHA, 1992) .Total Potassium was estimated by Flame photometric method ((Jackson, 1973).

4.6. Isolation of bacteria from waste Samples

Serial dilution techniques were used for the isolation of bacteria. In this technique sample suspension was prepared by adding soil mixed with waste (1g) was added to 10 ml of sterile water (the stock) and shaken vigorously for atleast 1 minute. The dilute was then sedimented for a short period. Sterile dilution blanks were marked sequentially starting from stock and10^{-1} to 10^{-4}. One ml from the stock was transferred to the 10^{-1} dilution blank using a fresh sterile pipette. One ml from the 10^{-1} dilution was transferred to the 10^{-2} tube for each succeeding step then from the 10^{-2} to the 10^{-3}, then from the $^{10-3}$ to the 10^{-4}. From each dilution tube 0.1 ml of dilution fluid was transferred into Nutrient Agar culture media (composition in [g/l] 10.0 g Bacteriological peptone; 1.0 g Glucose; 5.0 g NaCl; 10.0 g Beef Extract, pH 7-7.5) and incubated at 37^0C for 24 hours. Nutrient Agar (NA) culture

media contained 0.5% Peptone, 0.3% Yeast extract, 0.5% Nacl, 0.25% Glucose, 1.5% Agar, distilled water and pH was adjusted to 7 at room temperature. After successful growth of microorganisms the pure cultures of bacteria were sub-cultured in NA slants; incubated at 37^0C to achieve vigorous growth and then preserved in 20% glycerol vials at -80^0C (Williams and Cross 1971).

4.7. Microbiological and biochemical characteristic of isolated bacteria

After successful growth of microorganisms, each colony morphology e.g., size, shape, margin elevation, consistency, colour, transparency was determined. Gram stain was performed to observe the cellular morphology and gram nature of the bacteria and biochemical characterization of the strains were also carried out. The biochemical tests of sugar utilization; amino acid decarboxylation; nitrate reduction; hydrogen sulfide production; starch, casein, and urea hydrolysis; and IMVIC tests were performed (Pacarynuk et al., 2004; Collins et al. 1989).

4.8. Optimization of growth conditions of the isolates

Three semi-solid media as NA (Nutrient Agar), BCDA (Czapeck Dox Agar medium [Basic]) and ACDA (Czapeck Dox Agar medium [Acidic]) were used to optimize the cultural media of isolated bacteria. The pH were adjusted to 5.2, 6.5, 7.2, 8.9 and 10.2 in NA medium; 2.2, 3.2, 4.0, 5.5 and 6.9 in BCDA medium; and 7.1, 7.6, 9.1, 10.06 and 12.10 in ACDA medium. For optimization of incubation period and temperature the culture plates were incubated at 25, 29, 33, 37 and 41^0C for 6 – 72 hours (Zaved et al., 2008).

4.9. Antagonism of bacterial strains

Each strain was grown at room temperature and subsequently tested by the cross-streaking method at room temperature and at 37°C. The cross-streaking method was performed as described by (Gillies et al., 1966) and (Govan et al., 1969) with two modifications. The strain to be tested was inoculated as a 1.5-cm-wide streak (instead of 1 cm) diametrically across duplicate nutrient agar plates. The plates were incubated overnight at either room temperature or 37°C. A wider streak of the original inoculum was used because the inhibitory zones produced were larger and clearer. After overnight incubation, the inoculum was removed with a glass slide,

and remaining viable growth was killed by exposure to UV light for 30 min. The indicator strains were streaked singly at right angles to the original inoculum by using a wire loop (8 strains per plate). The plates were incubated at room temperature or 37°C overnight, and inhibition was recorded where the indicator strains crossed the original inoculum. This procedure was followed until all of the strains had been tested against each other.

4.10. Metal tolerance assay

Study of MIC (Minimum Inhibitory Concentration) of heavy metals viz. Arsenic (As), Zinc (Zn), Lead (Pb), Cadmium (Cd), Mercury (Hg) was carried out for the bacterial strains through Cup Assay method (Smânia et al., 1999). The sterilized agar medium (Beef extract-10 gm, Peptone- 10 gm, Sodium chloride- 5 gm, Glucose-1 gm, Agar-20 gm per litre of distilled water, pH-7.5) was prepared for bacterial growth. The plates were inoculated with bacterial suspension through spread plate method. On each plate cups were made by sterilized cup borer .Various concentrations of each metal compounds poured on cups of pre-inoculated plates. For each metal concentration separate Petri plate with pre-inoculated bacteria were used. All the plates were incubated at 37 ± 2^0C for 48 hours. The diameters (milimetre) of inhibition zone around each cup were measured against each concentration and also against each bacterium. Plates without any metal concentration were treated as reference plates.

4.11. Antibiotic sensitivity assay

The cup assay method (Cooper 1955) was used for antibiotic sensitivity testing. The sterilized agar medium (Beef extract-10 gm, Peptone- 10 gm, Sodium chloride- 5 gm, Glucose-1 gm, Agar-20 gm per litre of distilled water, pH-7.5) was prepared for bacterial growth. The plates were inoculated with bacterial suspension through spread plate method. On each plate cups were made by sterilized cup borer. A constant concentration of 100 ppm of each antibiotic (Gentamycin, Oxytetracyclin, Penicillin, Streptomycin) was poured on cups of pre-inoculated plates. For each antibiotic concentration separate petri plate with pre-inoculated bacteria were used. All the plates were incubated at 37 ± 2^0C for 48 hours. The inhibition zones around each cup were observed against each antibiotic and also

against each bacterium. Plates without any antibiotic concentration were treated as reference plates.

4.12. Antimicrobial activity

The search for new antimicrobial agents is a field of utmost importance. The prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide. All the 9 isolates were screened for antibacterial and antifungal activity by cross streak method (Nakano et al., 1990). In the cross streak method, the soil isolates were streaked on modified nutrient agar as a straight line in the left side corner of the Petri plate and the plates were incubated overnight at either room temperature or 37°C. After incubation, the test human bacterial pathogens (*Klebsiella pneumoniae, Staphylococcus aureus, Salmonella sp.*) and fungal pathogens (*Fusarium sp., Alternaria sp., Helminthosporium sp.*) were streaked at right angle to the original streak of the bacterial isolates. The zone of inhibition against human bacterial pathogens and fungal pathogens were observed after 48hrs of incubation. Plates with the same medium without inoculation of bacterial isolates but with simultaneous streaking of test organisms were maintained for controls.

4.13. Enzymological characterization of the strains

The bacterial isolates were screened for enzyme production and optimization, purification and characterization of produced enzyme was carried out applying following procedures.

4.13.1. Qualitative assay of extracellular enzyme production

All the isolated bacterial strains were screened qualitatively for the production of eight important enzymes such as protease, lecithinase, DNase, lipase, cellulase, amylase, catalase, and oxidase. Each bacterial strain was streaked on the four corners of the respective substrates such as milk, egg yolk, toluidine, tributyrin, casein, tween 80 amended agar plates separately and for catalase and oxidase discs were used. The Petri plates were incubated overnight at either room temperature or 37°C. Then the plates were flooded with indicator solution and the development of

clear zone around the growth of organism was considered positive for enzyme activity (Peterson et al., 1994; Pointing, 1999).

4.13.2. Quantitative assay of enzyme produced

The isolates were screened for quantitative production of enzymes to select the hyperactive enzyme producers.

4.13.2.1. Assay of protease

Protease activity was determined by caseinolytic method (Mohawed et al.,1986). One unit (U) of enzyme is defined as the amount of protease that liberates peptide fragments equivalent to one mg of bovine serum albumin (BSA) per unit time (min-1) under the assay conditions (Patil et al., 1981).

4.13.2.2. Assay of lignin peroxidase

LiP activity was determined spectrophotometrically by veratryl alcohol oxidation according to Tien and Kirk (Tien et al., 1984). One unit (U) of enzyme was defined as 1 micromol of veratryl alcohol oxidized in 1 min, determined at 310 nm.

4.13.3. Selection of the potent producer and study of its growth characteristics

All of the bacterial isolates were screened for selecting the bacteria with the highest protease activity and also selecting the isolate having lignin peroxidise activity. The growth characteristics of the potent strain were studied by measuring the optical density (of the medium where bacteria is grown) per hour depicting the bacterial growth.

4.13.4. Optimization of protease and lignin peroxidise enzyme production in sub-mergerd fermentation

To enhance the production of both the enzymes, the process parameters (viz, media, pH, inoculums volume, incubation period and temperature) was optimized (Vidyalakshmi et al., 2009).

Effect of Media

Enzyme activity was estimated in the four fermentation medium (Czapeck Dox broth, Nutrient broth, Nutrient broth + Yeast Extract [0.5%] and Nutrient broth + Peptone [0.5%]) to select the best media (Vidyalakshmi et al., 2009).

Effect of pH

The fermentation medium was prepared by varying the pH values (6, 7, 8 and 9) for the production of enzyme (Vidyalakshmi et al., 2009).

Effect of Inoculum Volume

The effect of inoculums volume on enzyme production was studied by carrying out the fermentation process applying different inoculums volumes (1%, 2%, 3%, 4% and 5%) (Vidyalakshmi et al., 2009).

Effect of Incubation Period

The incubation period was varied (8 hours, 10 hours, 12 hours, 14 hours, 16 hours, 18 hours, 20 hours, 22 hoyrs and 24 hours) for studying the effect of incubation period on enzyme production (Vidyalakshmi et al., 2009).

Effect of Temperature

To study the effect of temperature on enzyme production, the sub merged fermentation was carried out at different temperatures (30°C, 32°C, 34°C, 37°C and 40°C) (Vidyalakshmi et al., 2009).

4.13.5. Optimization of enzyme production in solid state fermentation

Solid state fermentation (SSF) holds tremendous potential for the production of enzyme especially where the raw fermented product may be used directly as the enzyme source (Tengerdy, 1998). This system offers numerous advantages over submerged fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipments, etc (Pandey, 1994).

4.13.5.1. Selection of substrare for enzyme production

Saw dust, cabbage waste, orange peel waste, jack fruit seed waste, banana waste, potato waste, soyawhey and rice straw were purchased from the grain market of Kharagpur in West Bengal and the substrates were dried and grounded to powder form (40 mm mesh size). The substrate was then stored in plastic jar for subsequent use in a fermentation medium (Mehboob et al., 2011).

4.13.5.2. Fermentative microorganism and inoculum preparation

The pure culture of the hyperactive bacterial strain *Paenibacillus mucilaginosus* S4 isolated from the waste dumping site of Barrackpore municipality (north 24 pgs) was screened for high yield of extracellular alkaline protease and lignin peroxidise production The bacteria was cultured on minimal media (Gradisar et al., 2005) and the culture obtained was preserved at 4°C in the refrigerator.

The 12-h-old culture prepared in minimal media (pH 8) (with the composition of : sucrose-2%, lactose-1%,peptone-1%, sodium nitrate-0.25%, potassium chloride-0.05%, manganese sulphate-0.05%,sodium chloride-0.05%,ferric chloride-0.05%) at 37°C under shaking (200 rpm) was used to inoculate production flasks.

4.13.5.3. Enzyme production in solid state fermentation

For the production of protease and lignin peroxidise in solid state fermentation flasks (250 ml Erlenmeyer flasks) were prepared in triplicate each containing 5g of the substrate moistened with the minimal medium (pH-8) in the ratio of 1:4 and autocalved at 121°C for 15 minutes. Ongradual cooling to room temperature, the inoculum was aseptically added to each flask in laminar air flow. Then the inoculated flasks were incubated for 13 h at 37°C, under shaking [200 rpm] (Mehboob et al., 2011).

4.13.5.4. Extraction of enzyme and sample harvesting

After incubation, triplicate flasks were harvested after every 13 h. The fermented biomass was soaked in extraction media comprising of deionized water (pH 7) for two hours and extracted in the tincture press. The extracted enzyme was centrifuged 10,000 rpm for 10 min. to remove the particulate matters and bacterial

cells. The supernatants were collected and stored at 4^0C for further use and analysis (Mehboob et al., 2011).

4.13.5.5. Optimization of process parameters

The various parameters that may influence the production of protease and lignin peroxidase during SSF process were optimized. The classical method was adopted for optimization of fermentation parameters by varying one parameter in an experiment and to incorporate it at a standardized level before optimizing the next parameter. Different process parameters that were standardized include the effect of incubation period, inoculum volume, temperature, pH, and substrate to media ratio (Mehboob et al., 2011).

pH

Triplicate flasks containing 5 g of rice straw were moistened with 20 ml of minimal medium of varying pH (6.0, 7.0, 8.0, 9.0 and 10.0). Prior to inoculation, all flasks were autoclaved and then were incubated in an incubator at 37°C. After 12 hours of incubation, samples were harvested; the contents of the flasks were diluted five times with distilled water and were analyzed to determine the protease and lignin peroxidase activity (Mohawed et al., 1986; Kirk et al., 1988).

Incubation period

For the optimization of incubation period, the culture was grown in a set of ten flasks. All flasks were autoclaved before inoculation with the bacterial suspension. All flasks were incubated at 37°C. Triplicate flasks were harvested after every 12 hours, and culture supernatants were subjected to protease and LiP assay (Mohawed et al., 1986; Kirk et al., 1988).

Incubation temperature

In order to determine the most suitable incubation temperature for the efficient production of protease and lignin peroxidase, the media was adjusted to pH 8.0. After autoclaving, all flasks were inoculated and incubated at varying temperatures (30, 32, 34, 37, 38 and 40°C) for 13 hours. The samples were removed after 12

hours of incubation and were diluted five times to measure the enzyme activity (Mohawed et al., 1986; Kirk et al., 1988).

Inoculum volume

To investigate the effect of inoculum volume on the production of protease and lignin peroxidase, four different levels of inoculum (1, 2, 3, 4, 5, 7 and 10%) were tested in triplicate and compared with a control without inoculum. The culture was harvested on the 13th hour of incubation and subjected to enzyme assay. Each 1 ml-1 of bacterial suspension contained (7´ 103 cell/ml-1).

Substrate to media ratio

For the optimization of substrate to water ratio, 5 g substrate was moistened with varying volumes (5, 10, 15, 20, 25 and 30) of minimal medium. The flasks were autoclaved and then incubated at 35°C. After four days of incubation (optimum incubation period) samples were removed and assayed for lignin peroxidase (Mohawed et al., 1986; Kirk et al., 1988).

4.13.5.6. Response surface methodology in production optimization

Response surface methodology (RSM) is an empirical modeling technique used to obtain an optimal response of a sequence of designed experiments. It explores the relationship between explanatory variables and response variables. This optimization process consists of three major steps: (i) performing statistically designed experiments, (ii) estimating the coefficients in a mathematical model and (iii) predicting the response and checking the adequacy of the model (Box et al., 1960). For determining the optimal levels of five crucial variables, viz., pH (A), incubation time (B), temperature (C), inoculum volume (D) and solid to liquid ratio (E) on protease and lignin peroixidase production along with enhancements of its total production, the RSM was adopted. The statistical software package used a set of experimental design (FCCCD:Face Centered Central Composite Design) using multiple factors each studied at two different levels (-1,+1). The five parameters of ., pH (A), incubation time (B), temperature (C), inoculums volume (D) and solid to liquid ratio (E) were chosen to be crucial based on the results from one factor at a time approach in optimizing the enzyme production. The boundary

conditions for each parameter are as follows: pH (6 and 10), incubation time (8 and 24), temperature (30^0C and 40^0C), inoculums volume (1% and 10%), and solid to liquid ratio (1 and 6).

4.13.6. Purification of protease enzyme

a. Enzyme purification

The protease purification steps were described as previously mentioned by El-Safey, (1994). This included the following steps:

Step 1. Enzyme production and preparation of cell free filtrate

Paenibacillus mucilaginosus S4 was grown under optimized conditions. The filtrate broth (crude protease) was collected and centrifuged at 10,000 rpm for 15 min at 4ºC in order to obtain a cell free filtrate (cff). After performing a test for sterility, 200 ml of the cell free filtrate (CFF) containing protease were collected and their proteolytic activities and protein content were determined.

Step 2. Ammonium sulfate fractionation

200 ml-1 of the crude protease enzyme were first brought to 20% (w/v) saturation with solid ammonium sulfate (enzyme grade) according to the chart of Gomori (1955) as mentioned as Dixon et al., 1964. The precipitated proteins were regimented by centrifugation for 15 min at 500 min-1. The resulted pellet was dissolved in 5 ml of Tris-Hcl buffer at (pH 8.5). The left supernatant was applied again with ammonium sulfate to achieve 20, 30, 40, 50, 60, 70, 80, 90 and 100% (w/v) saturation. Both enzyme activity and protein content were determined for each separate fraction. The 70 and 80% fraction precipitate was collected and pooled together. This precipitate was subjected to dialysis.

Step 3. Dialysis against distilled water and buffer

The obtained ammonium sulfate precipitate (in solution) was introduced into special plastic bag (dialysis membrane) for dialysis against distilled water for 3hrs, followed by dialysis against Tris-Hcl buffer at pH 8.5. The obtained protease enzyme preparation was concentrated against crystals of sucrose and kept in the refrigerator at 4ºC for further purification.

Step 4. Application on column chromatographic technique

Preparation of the gel column and the fractionation procedures was determined as previously mentioned by Ammar (1975). For this purpose, a Pharmacia column (column length-56cm; column radius-1.5cm; column volume-98.91cm^3) has been used. Sephadex G-200 (Pharmacia, Upsulla, Sweden) "practical size 200 μ" was also used. 20 mM Tris-Hcl buffer was used at pH 8.5 and the slurry was allowed to swell for 3 d at room temperature (approximately, 22 ±1ºC). Sodium azide (0.02%) was added to prevent any microbial growth. Applying a mixture of blue dextran 2000 and bromophenol blue determined the void volume. One ml-1 of the enzyme preparation sample was applied carefully to the top of the gel. It was allowed to pass into the gel by running the column. Buffer was added without disturbing the gel surface and to the reservoir. 120 fractions were collected (each of 2 ml-1 at the flow rate of 1.2ml/min.).

Proteolytic activity and protein content assay (at 280nm) were carried out for each individual fraction. Sharp peaks of fractions obtained after applying Sephadex G 200 column were collected and investigated for the properties of the partially purified protease enzyme. The active fractions justified by high protease activity coupled with low protein content were pooled and concentrated by ultrafiltration (Centriprep YM-30, Millipore) and then stored at -20^{o}C prior to further study on the enzyme properties.

b. Enzyme activity

The protease enzyme activity was determined as previously mentioned by El-Safey and Ammar, (2003).

c. Protein determination

The protein content of protease enzyme was determined by the method of Biuret as mentioned in Chykin, (1966).

d. Determination of the specific activity of protease enzyme

The specific activity of the protease enzyme protein was expressed in terms of units/mg protein/ml-1 according the following equation:

Specific activity = enzyme activity / protein content (mg/ml-1)

e. Molecular Weight Determination

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% acrylamide gel by the method of Laemmili (Laemmili, 1970; Yossana et al., 2006). Proteins were stained with Coomassie brilliant blue R-250 (Sigma). The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins consisting of Phosphorylase b, 97 kDa; Albumin, 66 kDa; Ovalbumin, 45 kDa; Carbonic anhydrase, 30 kDa; Trypsin inhibitor, 20.1 kDa; Alpha lactalbumin, 14.4 kDa.

4.13.7. Characterization of purified protease enzyme

The purified enzyme thus obtained was subjected to assay for its pH optimization, temperature optimization and determination of substrate specificity.

4.13.7.1. Study on Optimal pH for enzyme activity

The purified protease was determined for an enzyme activity by using 1% casein as a substrate. The pH-activity profile was studied in a pH range of 6.0–12.0 at 60°C using 50 mM buffers of different pHs [6.0–6.5, sodium acetate; 7.0–8.0, Tris–HCl and 8.5–12.0, NaOH:glycine] (Banerjee et al., 1999).

4.13.7.2. Study on Optimal temperature for enzyme activity

The purified protease was determined for an enzyme activity by using 1% casein as a substrate. The temperature-activity profile was studied by assaying protease activity in a temperature range of 25–75°C at pH 10.5 (Banerjee et al., 1999).

4.13.7.3. Study on substrate specificity of the enzyme

The purified protease was assayed at 60^{o}C for substrate specificity by using different substrates dissolved in 50mM NaOH: glycine buffer (pH 10.5) at the concentration of 1 mg/ml. The substrates tested were casein, gelatin, albumin (egg), soybean protein isolate, gluten, albumin (bovine) and cytochrome C (Yossana et al., 2006).

4.14. Application of individual isolates and bacterial consortium in biodegradation of municipal solid waste

4.14.1. Inoculum Preparation

The bacterial strains used in this study were individual isolates and consortium (between isolates having no antagonism). 24 hours of old bacterial culture in tube 10 ml of autoclaved distilled water was added and then mixed well for making a suspension. After that the sterilized garbage was inoculated with 5ml of this bacterial suspension and mixed well. Control treatments were also performed with inoculation (Elango et al., 2009).

4.14.2. Biodegradation of municipal solid waste

The Waste Degradation Potential of Bacteria was studied by weight loss method. Litter sample was collected in sterile litter bags under aseptical condition. Collected samples were brought to the laboratory and then cut into small pieces, and 5 g of each was aliquoted into petri plates, which were then wrapped by using polythene bags. The plates containingmunicipal solid waste were then autoclaved at 121◦C for 15 min, After sterilization, the inoculum was inoculated. Moisture content was maintained at 50–60% throughout the active biodegradation in the plates. The pH and temperature were also measured periodically after 10 days intervals. Turning of the organic waste was provided once in every week to ensure aerobic condition in the plates. Changes in odour and weight loss of the decomposed organic solid waste were observed at 10-day intervals upto 30 days (Gautam et al., 2011). For measurement of weight loss (%), the following formula was used:

$$\text{Weight loss (\%)} = W \frac{W1}{W} * 100$$

Where W is initial weight, and W1 is final weight.

4.15. Molecular characterization of potent bacterial strains

The molecular characterization was done on the basis of 16S rDNA sequence analysis. This

analysis was performed by Chromous Biotech Pvt. Ltd. (Bangalore - 92, India). DNA was

isolated using the Bacterial Genomic DNA Isolation Kit (RKT09) and evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16s Forward (5'-AGAGTRTGATCMTYGCTWAC-3') and 16s Reverse (5'-CGYTAMCTTWTTACGRCT-3') primers using Big Dye Terminator version 3.1" Cycle sequencing kit on ABI 3500 XL Genetic Analyzer. Consensus sequence of 1398 bp rDNA gene was generated from forward and reverse sequence data using Seq Scape_ v 5.2 software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using Jukes-Cantor corrected distance model and the phylogenetic tree was constructed using weighbor with alphabet size 4 and length size 1000 (William et al., 2000; Wiley et al., 1991). Among the sequence obtained, the sequence of the most potent isolate was submitted to MTCC GenBank.

4.16. Application of protease from *Paenibacillus mucilaginosus; s-4,* in removing stains

A clean piece of cloth was soaked in tea and blood stains. The cloth was then dried and soaked in 2% formaldehyde and washed with water to remove the excess formaldehyde. The partially purified protease was dropped on the cloth and incubated at 40°C. After incubation, each piece of cloth was washed and dried. Controls were put up without enzyme (Rai and Mukherjee, 2010).

4.17. Statical procedures

In all experiments, the measurements were carried out with duplicated parallel cultures. The values reported are means ± S.D. calculated as described by Snedecor and Cochran (1980).

Chapter-v

Results And discussion

Results and discussion

The isolation and characterization of bacterial strains from waste dumping site of Dhapa and Barrackpore municipality was undertaken in this study. Search of strains with novel characteristics of industrially important enzyme production was also considered as a major part of this investigation.

5.1. Physical and chemical characteristics of municipal solid waste

Bacteria can grow in a wide range of moisture level. In this study, it was found that the moisture content of the collected sample from Barrackpore Minicupality and Dhapa waste dump site was about 65.32% and 66.45% respectively. Bacterial population of various soils is closely correlated with their moisture content. The maximum bacterial density is found in regions of fairly high moisture content and the optimum level for the activities of aerobic bacteria often is a 50%-75% of the soil moisture holding capacity (Alexander, 1977). Numbers of the genera *Pseudomonas, Achromobacter* and *Bacillus* are found in most aerobic soils; where conditions are anaerobic and moist *Clostridium* will occur. *Actinomycetes* showed a similar quantitative increase under such conditions (Berkeley et al., 1972).

In this study the pH of the two selected media (BCDA and NA) was optimized for culturing bacterial strains. The ph of the collected sample was 7.79 in both of the samples. The pH is a key factor for growing bacteria in artificial media. Optimization of pH was carried out in two selected media viz. Nutrient Agar (NA) And Basic Czapek Dox Agar (BCDA). NA and BCDA at pH 7.2 and 7.6 were found to be suitable for the maximum growth of the bacterial strains. From the results, it was found that the pH of the sample was about 7.79 and possible for this reason, these strains were also found to grow well in in vitro condition at pH 7-8 in BCDA and NA. Bacteria can tolerate a soil reaction between pH levels 4 and 10, but the most favourable pH for the majority is just an alkaline side to neutrality. Bacteria such as *Thiobacillus thiooxidans* and *Acetobacter sp.* Are capable of growing at the very low pH values between pH level 0 and 2 and some Bacillus sp. Can grow at pH 11 (Berkeley and Campbelt 1972). Optimal growth of

Thermoactinomycetes occurs at pH 8 or 9 and is greatly depressed by reactions of around pH 5 (Krishnamourthy and Vajranabhiah 1986).*Vibrio, Streptococcus faecalis* and *Escherichia coli* also tolerate an alkaline reaction (pH 8-9) (Krishnamourthy et al., 1986).

The NPK content of the sample was studied initially. The organic matter content was found to be 27.84% (Barrackpore Municipality) and 29.32% (Dhapa), Nitrogen Content in % was 0.165 (Barrackpore Municipality) and 0.179 (Dhapa), Phosphorous Content in % was 0.502 (Barrackpore Municipality) and 0.545 (Dhapa) and Potassium Content in % was 18.29 (Barrackpore Municipality) and 19.21 (Dhapa).

All these analysis gave a clear understanding of the native environment of the bacteria and thus was the determining factors in the isolation and culture of the strains.

5.2. Cultural characteristics of bacterial isolates

In our study, BM1, BM2, BM3, D1, D2, D3, D4, D5 and D6, – these 9 bacterial strains were isolated in culture media. Czapeck dox agar and Nutrient agar were selected to determine the best suitable media for ensuring massive growth of the isolated strains. Czapeck dox agar (BCDA) was suitable for massive growth of BM3, D1,D5 and Nutrient agar (NA) medium was suitable for massive growth of BM1, BM2, D2, D3, D4, D6. It was seen that yeast extract xylan containing media was suitable for maximum growth of bacteria but *Pseudomonas spp*., *Bacillus spp*., *Aeromonas spp.*grow well in nutrient agar media.

Visual and microscopic observation was used to characterize the selected strains. Details of the colony features of the bacteria are noted (table no. 5.1).

Table 5.1. Colony and cell features of isolated bacteria

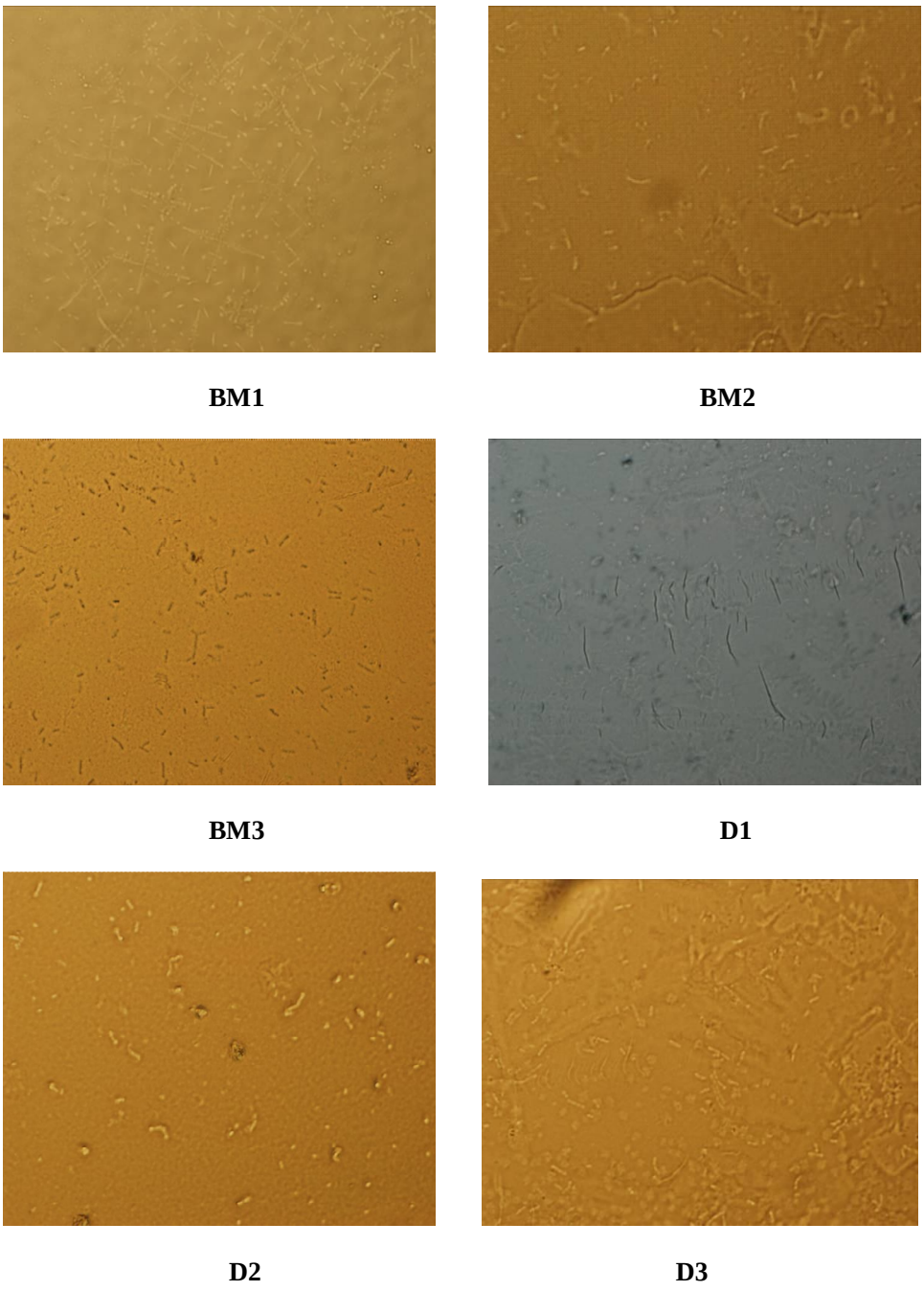
Strain No	Colony Features		Cell Features	
	Colour of Colony	**Nature of Colony**	**Gram Nature**	**Shape**
BM1	White	Irregular, boil like, having secretion within	Gram Positive	Bacilli
BM2	Cream	round shaped, transparent	Gram positive	Short bacilli
BM3	Cream	Irregular, transparent	Gram positive	Bacilli
D1	Cream	Round shaped, shiny	Gram positive	Bacilli
D2	White	Irregular, boil like, shrinked	Gram positive	Diplobacilli
D3	Cream	Roumd shaped, slimy growth	Gram negative	Bacilli
D4	Cream	Irregular, boil like, having secretion within	Gram negative	Short bacilli
D5	Cream	Round shaped, transparent, shiny	Gram negative	Bacilli
D6	Cream	Round shaped, shiny	Gram positive	Coccus

Gram staining is an old and reliable method for observing the bacteria. Gram negative bacteria were decolourized by alcohol, losing the purple colour of crystal violet. Gram positive bacteria did not decolourized and remained purple (Harold, 2002).

In the present investigation, BM1, BM2, BM3, D1, D2, D3, D4, D5 and D6, – these 9 bacterial strains were isolated and the microbiological characterization was carried out. The results showed that BM1, BM3, D1 are gram Positive bacilli, BM_2 is gram positive short bacilli, D2 is gram positive diplobacilli, D3, D5 are

gram negative bacilli, D4 is gram negative short bacilli and D6 is gram positive coccus.

The bacterial isolates as observed under the microscope in bright field (60X) could be depicted in figure no 5.1.a – 5.1.b.

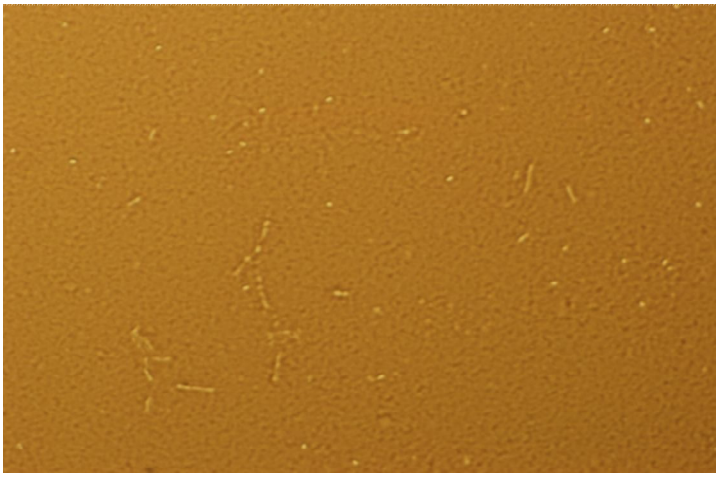


BM1 **BM2**

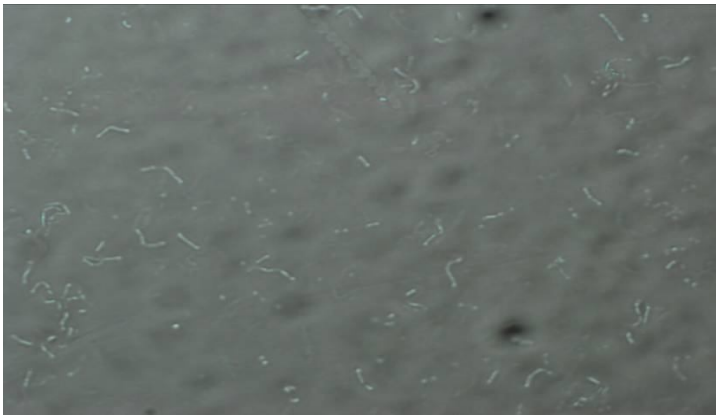
BM3 **D1**

D2 **D3**

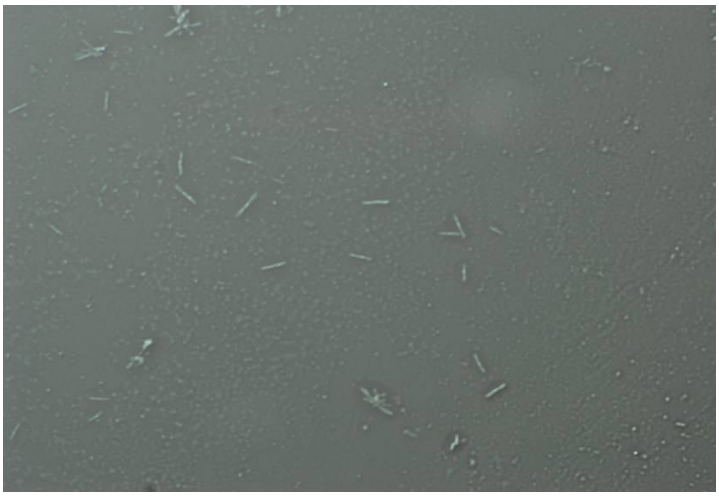
Figure 5.1.a. Bacterial isolates as observed under the microscope in bright field (60X)



D4



D5



D6

Figure 5.1.b. Bacterial isolates as observed under the microscope in bright field (60X)

Different biochemical tests were also performed for the 9 isolates to know their biochemical characteristics. Details of the biochemical characters of the bacteria are noted in (Table no. 5.2).

Table 5.2. Biochemical characteristics of isolated bacteria

	Biochemical Tests								
Strain No	**Catalase**	**Indole**	**Starch**	**Ammonia**	**Eijkman**	**Urease**	**Carbohydrate**	**Amylase**	**VP**
BM1	+	+	+	+	+	-	+	+	-
BM2	+	-	-	+	+	-	-	+	+
BM3	+	+	+	-	-	+	+	-	+
D1	+	+	+	+	+	+	+	-	+
D2	+	+	+	+	+	-	+	+	-
D3	+	+	+	+	+	+	-	+	-
D4	+	+	+	-	+	+	+	+	-
D5	+	-	+	+	+	+	-	+	-
D6	+	+	+	+	+	+	-	-	-

+ = Positive; - = Negative

The above results gave an idea of the morphology, colony characteristics and biochemical nature of the isolated strains which helped in deriving an idea about the genus of the strains. The biochemical nature of the strains suggested that the genus of the isolated strains were Bacillus, Paenebacillus (Collins et al., 1987). These results would aid in the identification and characterization of the isolated bacterial strains in future.

5.3. Optimization of growth conditions

In the present investigation, the growths of isolated strains were observed in various growth media like NA, ACDA and BCDA. It was seen that the basic Czapeck dox agar (BCDA) was suitable for massive growth of BM3, D1, D5 and Nutrient agar (NA) medium was suitable for massive growth of BM1, BM2, D2, D3, D4, D6.

In this experiment, the bacterial cultures of 9 strains were incubated at different temperatures like 25, 29, 34, 37 and 40^{o}C. The optimum growths of all the strains were found in 37^{0}C. The optimum temperature range for bacteria is from about 25-36^{0}C. A great number of bacteria may grow quite well over the temperature 10-40^{0}C (Williams 1971). Sultana (1997) observed that 33 ± 4^{0}C temperatures was ideal for the growth of bacteria (Sultana 1997).Certain bacteria develop most vigorously at temperatures below 20^{0}C. Thermophiles grow well at temperatures 45-65^{0}C and some thermophiles are incapable of multiplying below 40^{0}C (Alexander, 1977).

The strains obtained in this study were incubated for different incubation periods (6, 12, 24, 36, 48 and 72 h). Incubation period of 24 h was suitable for BM1, BM2, D2, D3, D4, D6while BM3, D1 and D5 was found to be suitable with incubation period of 36 h. Coliform bacteria grow in the incubation period of 24 ± 2 h and at 32^{0}C and it shows good growth at 37^{0}c for 48 h of incubation. In visual observation, it was found that after 24 h of incubation, the colour of BM2 was light orange, BM1 was white and BM3 was creamy white in their preferred medium (BCDA and NA). After 48-72 h of incubation, the colour of BM2 was orange, BM1, D1 were yellow, D5 was red and BM3, D2, D3, D4, D6 remained creamy white. The colony types of BM1, BM2, D2 and D4 strains were wet and the rest were creamy. Staphylococci and Micrococci produce golden brown, yellow or white colony on ordinary media. Some Enterococci, Coryneforms and Enterobacteria may produce black colonies on ordinary media (Collins et al 1989).

5.4. Antagonism assay

Cross streaking method was employed to determine the antagonism among the bacterial strains for their future application in different aspects. Details of the antagonism within the bacterial isolates are described (Table no.5.3).

Table 5.3. Antagonism within the isolated bacteria

	Antagonism with								
Strain No	**BM1**	**BM2**	**BM3**	**D1**	**D2**	**D3**	**D4**	**C2**	**C3**
BM1	×	+	-	-	-	+	-	-	-
BM2	+	×	+	+	+	+	+	+	+
BM3	-	+	×	-	-	-	-	-	-
D1	-	+	-	×	+	+	+	+	+
D2	-	+	-	+	×	-	-	-	-
D3	+	+	-	+	-	×	+	+	-
D4	-	+	-	+	-	+	×	-	-
D5	-	+	-	+	-	+	-	×	+
D6	-	+	-	+	-	-	-	+	×

BM2 has antagonism with all the other strains so it is not possible to develop a consortium using this strain as one of the isolates. D1, D3 also have antagonism with most of the other strains. BM1 is the most potent strain as it has antagonism with none of the other isolates. BM3, D2, D4, D5 and D6 have antagonism with a few isolates and these strains along with BM1 can be tested in different combinations for preparing consortium.

5.5. Heavy metal tolerance assay

Five heavy metals (As, Zn, Pb, Hg, Cd) were selected for determination of metal tolerance capability of the isolated bacterial strains (BM1, BM2, BM3, DF1, D2, D3, D4, D5, D6). The tolerance test indicated that among five experimented heavy metals, maximum tolerance was shown to Pb showing the growth of microorganisms up to 4000 ppm and minimum tolerance to Cd showing no growth above 30 ppm . MIC was noted when the isolates failed to grow on plates even after 10 days of incubation. The result shows that for all the nine bacteria the MIC

ranged from 250 ppm to 350 ppm for As, Cd (10 -30 ppm), Zn (200-300 ppm), Hg (200-300 ppm) and Pb (3000-4000 ppm) (Table.4). In the present study, highest tolerance of As and Cd found in BM_1 while highest Zn tolerance was observed in BM_2 and BM_3 showed maximum Hg and Pb accumulation. In our study the most toxic metal (with the lowest MIC) is cd whereas the least toxic metal tested is Pb (Table. 5.4).

Table 5.4. Metal tolerance of isolated bacterial strains

	Inhibitory Concentration (ppm)					
Strain No	As^{3+}	As^{5+}	Zn	Pb	Hg	Cd
BM1	285	350	200	3750	200	30
BM2	260	300	300	3000	285	15
BM3	250	320	280	4000	300	20
D1	255	345	270	3650	220	15
D2	270	325	260	3500	250	10
D3	265	300	275	3550	280	20
D4	260	315	215	3450	275	25
D5	275	340	225	3700	270	10
D6	280	330	210	3650	260	15

MIC was noted when the isolates failed to grow on plates even after 10 days of incubation (Shakoori et al. 1998). Mergeay et al. (1985) tested the minimal inhibitory concentrations (MICs) of several different metals and foumd the most toxic metal (with the lowestMIC) was mercury whereas the least toxic metal was manganese (Mergeay et al.1985). The microbial tolerance at each concentration of heavy metal was depicted by the cup assay test.The diameter of inhibition zone around each cup increased with the increase in concentration of heavy metals indicating toxic effect of the heavy metals on the growth of microorganisms. The Barrackpore Municipality and Dhapa waste dumping site collects all the domestic as well as industrial solid waste of the Barrackpore city, Kolkata city, respectively and its surrounding areas. The waste coming from domestic and industrial sources

is the appropriate environment where the microorganisms can develop resistance to heavy metals. The presence of small amount of heavy metals in the solid waste can induce the emergence of heavy metal resistant microorganisms. The microbial resistance to heavy metal is attributed to a variety of detoxifying mechanism developed by resistant microorganisms such as complexation by exopolysaccharides, binding with bacterial cell envelopes, metal reduction, metal efflux etc. These mechanisms are sometime encoded in plasmid genes facilitating the transfer of toxic metal resistance from one cell to another (Silver, 1996).The heavy metal resistant organism could be a potential agent for bioremediation of heavy metals pollution. Since heavy metals are all similar in their toxic mechanism, multiple metal tolerances are common phenomena among heavy metal resistant bacteria (Rajbanshi 2008).

5.6. Antibiotic sensitivity assay

Antibiotic sensitivity test helps to determine how effective an antibiotic is against the test organism. The 9 isolates were screened for its sensitivity towards four antibiotics and the result is noted (Table. 5.5).

Table 5.5. Antibiotic sensitivity of bacterial strains

	Antibiotics concentration(100ppm)			
Strain No	**Gentamycin**	**Oxytetracyclin**	**Penicillin**	**Streptomycin**
BM1	+	-	-	+
BM2	+	+	+	+
BM3	+	-	+	+
D1	+	+	+	+
D2	+	+	+	+
D3	+	+	-	+
D4	+	+	-	+
D5	+	-	+	+
D6	+	+	+	+

+ = growth inhibited; - = growth not inhibited

Antimicrobial compounds are produced by most of the isolates which can serve medical science. D1, D3 and D5 showed no antimicrobial activity.

5.7. Antimicrobial activity assay

Production of antimicrobial compounds seems to be a general phenomenon for most bacteria. In the present study 3 isolates showed antibacterial activity and 5 isolates showed antifungal activity, but 3 isolates showed neither antibacterial nor antifungal activity against the pathogens. The result has been depicted (table. 5.6).

Table 5.6. Antimicrobial activity of the bacterial strains

	Bacterial and Fungal Pathogens					
Strian No	***Klebsiella pneumoniae***	***Staphylococcus aureus***	***Salmonella sp.***	***Fusarium sp.***	***Alternaria sp.***	***Helminthosporium sp.***
BM1	+	+	-	-	+	-
BM2	-	-	-	-	-	-
BM3	+	+	+	+	+	-
D1	-	-	-	-	-	-
D2	-	-	-	+	+	+
D3	-	-	-	-	-	-
D4	+	+	-	-	+	-
D5	-	-	-	-	-	-
D6	-	-	+	+	-	-

+ = antimicrobial activity present; - = antimicrobial activity absent

Similar study was reported by Subramani et.al (2009). A variety of antimicrobial compounds are produced by members of the genus Bacillus, many of these identified as peptides, lipopeptides and phenolic derivatives. Search for novel secondary metabolites with diverse biological activity in assorted environment has gained greater attention in recent years.

5.8. Screening for extracellular enzyme production

With the growing awareness on environmental protection, the use of enzymes, particularly from extremophiles, gained considerable attention in many industrial processes. In recent years, the microbial enzymes have been replacing chemical catalysts in manufacturing chemicals, textiles, pharmaceuticals, paper, food agricultural chemicals.Enzyme based industrial bioprocess now directly competes with established chemical based process. However in this study, the 9 isolates were subjected to qualitative assay for production of seven different enzymes such as protease, lignin peroxidase, lecithinase, DNase, lipase, catalase, and oxidase. The result is noted (Table. 5.7).

Table 5.7. Qualitative assay for enzyme production

Strain No	Enzyme						
	Protease	**Lignin Peroxidase**	**Lecithinase**	**DNase**	**Lipase**	**Catalase**	**Oxidase**
BM1	+	+	+	-	-	+	+
BM2	No growth	-	-	-	-	+	+
BM3	+	-	+	-	-	+	+
D1	+	-	-	-	-	+	+
D2	No growth	-	-	-	-	+	+
D3	+	-	No growth	+	-	+	+
D4	No growth	-	No growth	No growth	No growth	+	+
D5	+	-	-	-	-	+	+
D6	+	-	-	-	-	+	+

Similar study was reported by Subramani et.al (2009). Interestingly in our study 6 of them showed production of protease enzyme and 1 produced lignin peroxidase enzyme both of which have high market values. All the 9 strains produced catalase and oxidase enzyme. Figure 5.2.a – 5.2.d depicis the qualitative assay of the production of some of the enzymes.

Qualitative assay of enzyme production

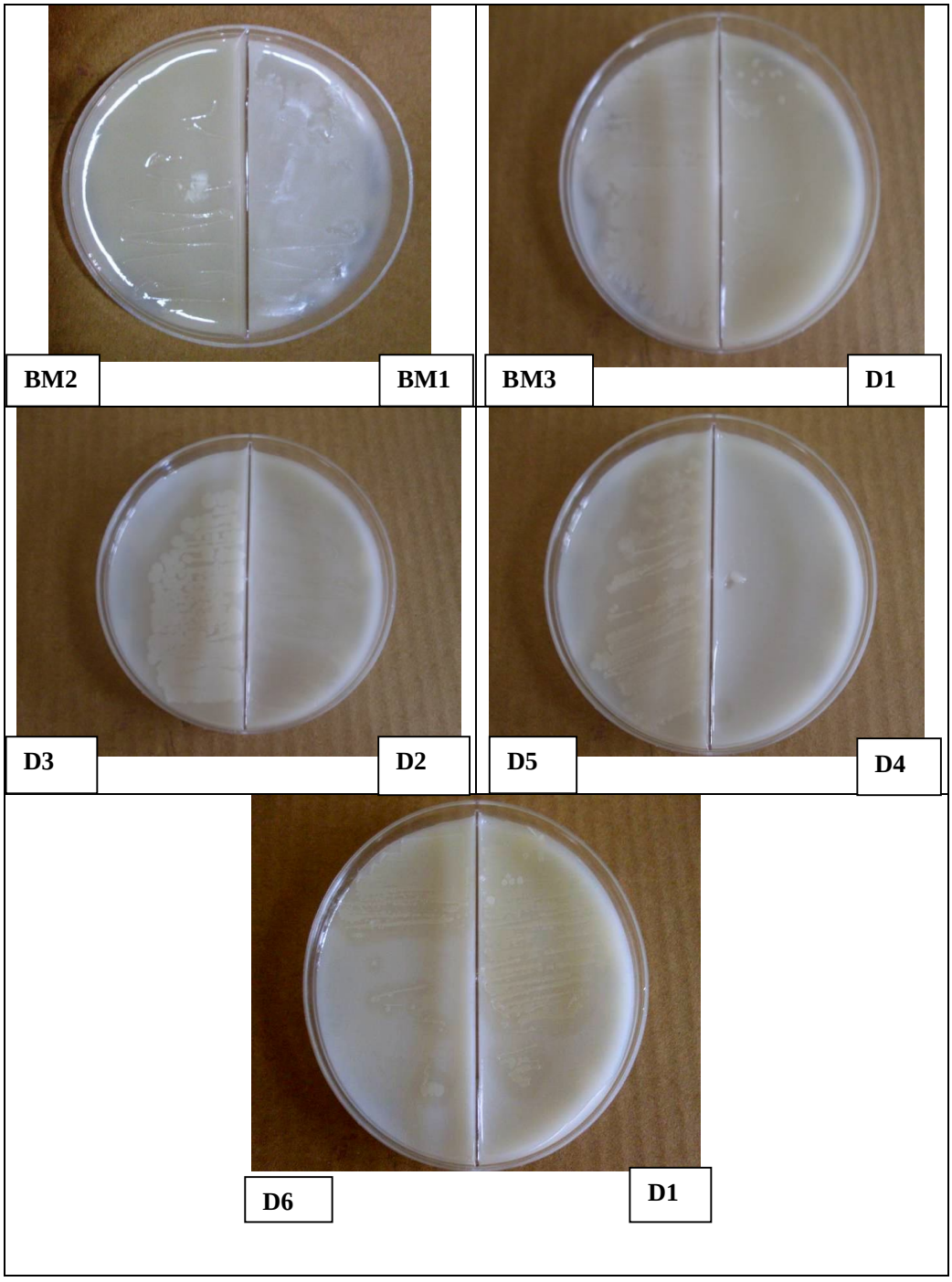


Figure 5.2.a. Bacterial isolates subjected to qualitative assay of protease

Figure 5.2.b.Positive test for qualitative assay of catalase production

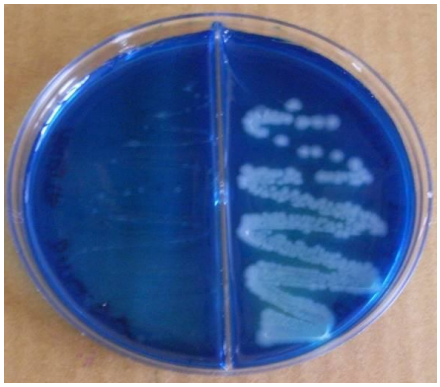


Figure 5.2.c Positive Test for quantitive assay of DNase production

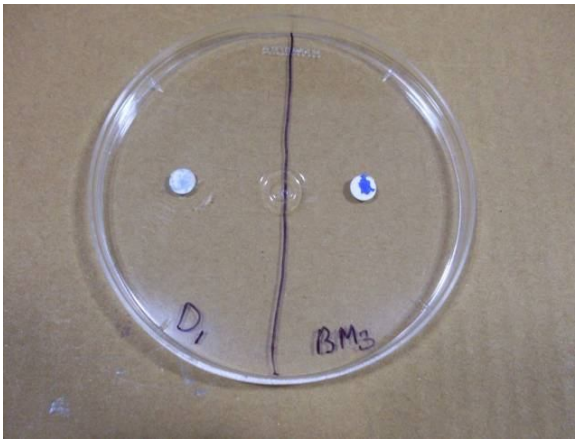


Figure 5.2.d. Positive test for qualitative assay of oxidase production

5.9. Selection of potent protease producer

6 strains (BM1, BM3, D1, D3, D5,D6) among the 9 isolates exhibited protease production. Protease having a wide spread application in food industry, detergent industry, pharmaceuticals as well as in solid waste degradation, quantitative assay of the protease produced was carried out. All of the bacterial isolates were screened for selecting the bacteria with the highest protease activity. The activity of the protease enzyme thus produced was determined and the result was expressed in U/ml. The result is depicted (table no.5.8).

Table 5.8. Activity of protease produced by the bacterial strains

Strain No	Activity in U/ ml
BM1	17233.40
BM3	3693.29
D1	796.21
D3	126.35
D5	366.21
D6	401.20

The quantitative assay of protease invariably proved that among the 6 strains, BM1 is producing protease with high titer value and Gupta et al (2002) similarly reported alkaline protease production from bacterial species and its industrial application.

5.10. Study of growth characteristics of the potent isolate

Study revealed among the 6 strains BM1is producing highest protease activity and simultaneously it is also producing lignin peroxidase enzyme which is reported in very few bacteria till date. BM1 is a potent bacteria which might prove to have industrially value and thus its growth characteristics was studied by measuring the optical density (of the medium where bacteria is grown) per hour depicting the bacterial growth.

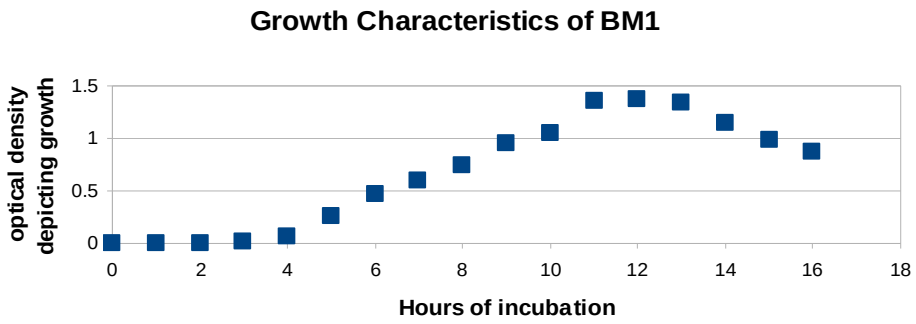


Figure 5.3. Growth curve for BM1

The figure 5.3 depicts the growth phases of the bacteria. From 6 to 12 hours of incubation it has the exponential phase of growth and then it enters into the stationary phase.

Municipal solid waste is a combination of different substrates thus it is an ideal enrichment media for cultivation of numerous microorganisms. Bacteria in this environment are metabolically active which leads to the production of various enzymes and bioactive compounds compared to other environmental condition. Therefore, it is important to understand the waste derived bacteria in ecological terms and also as a resource for biotechnology. Our present study evidently revealed that municipal waste dump site is a potential source for wide spectrum of antimicrobial and industrial enzyme producing bacteria. Moreover it can be an imperative resource for bio prospecting novel / rare species which could yield valuable bioactive molecules necessary for eco friendly degradation of waste and can also act as a good substitute in chemical processes in industry. BM1 is a potent strain producing two valuable enzymes viz. protease and lignin peroxidase, thus optimization of the process parameters would aid to increase the production of both of the extracellular enzymes from the strain.

5.11. Optimization of protease and lignin Peroxidase production of BM1 in sub-merged fermentation

The process parameters were optimized to increase the enzyme production of the bacterial strain. The classical method was adopted for optimization of fermentation parameters by varying one parameter in an experiment and to incorporate it at a

standardized level before optimizing the next parameter. Different process parameters that were standardized include the effect of incubation period, inoculum volume, temperature, pH, and media.

5.11.1. Effect of media

It is known that the medium has a profound influence on growth and the production of products by microorganism (Babu et al., 1996). Elevated enzyme levels (15,233.4 U/ml and 9.16 U/ml for protease and lignin peroxidase respectively) were obtained using Nutrient Broth + Peptone (0.5%) as medium (table no.5.9).

Table 5.9. Effect of media composition on enzyme production

Media	Activity in U/ml	
	Protease	**Lignin Peroxidase**
Czapeck Dox broth	813.99	0.70
Nutrient broth	1163.76	5.64
Nutrient broth + Yeast Extract [0.5%]	348.816	6.407
Nutrient broth + Peptone [0.5%]	15,223.40	9.16

There was a decline in enzyme production in other medium studied here. Nutrient Broth with 0.5% peptone served as the standard medium for obtaining high yields of both of the enzymes at the same time.

5.11.2. Effect of pH

In our study the protease and lignin peroxidase production by BM1, was found maximum at 8.0 (Table no.5.10).

Table 5.10. Effect of varying pH of the medium on enzyme production

pH	Activity in U/ml	
	Protease	**Lignin Peroxidase**
6	13,721.56	15.91
7	17,907.798	14.921
8	18,605.504	16.211
9	18,184.252	15.501

Further increase in the pH resulted decrease in the activity. However, the pH of the fermentation medium was found to be optimum at 8. When pH is altered below or above the optimum the activity is decreased or becomes denatured. Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth (Ramesh et al., 1991).

5.11.3. Effect of inoculum volume

The inoculum level was also an important factor for the production of protease and lignin peroxidase. Various inoculum levels (1%, 2%, 3%, 4% and 5%) were used to study their effect on both of the enzyme production. The higher enzyme production (17,897.333 U/ml and 18.565 U/ml for protease and lignin peroxidise, respectively) was obtained at 4% (v/w) inoculums level (table no.5.11).

Table 5.11. Effect of inoculum volume on enzyme production

Inoculum Volume	Activity in U/ml	
	Protease	Lignin Peroxidase
1	12,606.652	15.824
2	12,792.202	16.039
3	13,792.027	16.426
4	17,897.333	18.565
5	16,250.702	17.200

However Sen reported a 10% inoculum level for the production of alkaline protease by *Bacillus licheniformis* S40 (Sen, 1995). With the increase in inoculum level, the production of enzyme declined due to exhaustion of nutrients in the fermentation mash.

5.11.4. Effect of incubation period

The incubation time is governed by characteristics of the culture and is based on growth rate and enzyme production. In submerged fermentation the production of protease and lignin peroxidase was reached maximum of 26,905.325 U/ml and 22.796 U/ml, respectively at 14 h of incubation period (table no.5.12).

Table 5.12. Enzyme production in various incubation periods

Hours of Incubation	Activity in U/ml	
	Protease	Lignin Peroxidase
8th	22,089.895	19.258
10th	24,967.723	20.129
12th	26,105.625	21.897
14th	26,905.325	22.796
16th	24,987.562	20.778
18th	23,569.285	20.120
20th	21,598.232	19.452
22nd	20,200.235	18.215
24th	18,920.416	15.356

Further increase in incubation period did not show any significant increase in enzyme production rather it was decreased. Thus optimum time of enzyme synthesis was to be 14 h after inoculation. Ramesh et al., (1991) reported the enzyme production was initiated at about 6 h.

5.11.5. Effect of temperature

The effect of different incubation temperature on the production of protease and lignin peroxidase by BM1is summarized (table no.5.13).

Table 5.13. Effect of varying incubation temperature on enzyme production

Temperature (^{0}C)	Activity in U/ml	
	Protease	Lignin Peroxidase
30 ^{0}C	14,651.38	12.62
32 ^{0}C	19,725.656	14.38
34 ^{0}C	22,201.212	16.039
37 ^{0}C	26,957.325	22.825
40 ^{0}C	22,791.743	16.426

The maximum production of both protease and lignin peroxidase was obtained at 37°C. Increase in incubation temperature, decreased the production of enzyme. The production of the enzyme was greatly inhibited at 40°C. It might be due to that at high temperature, the growth of the bacteria was greatly inhibited and hence, enzyme formation was also prohibited (Pandey et al., 2000; Radley, 1976).

The effects of optimum conditions were investigated for the simultaneous production of protease and lignin peroxidase by BM1 strain. Maximum protease and lignin peroxidase activity (26,957.325 U/ml and 22.825 U/ml respectively) was found in the growth medium (Nutrient Broth + Peptone [0.5%]) 14 h incubation period, inoculums volume 4%, pH 8 and temperature 37^0C. Results indicate the excellent scope of producing both protease and lignin peroxidase with very high activity simultaneously in the same media composition in sub merged fermentation. Further optimization of the production process in solid state fermentation is expected to give much better yield which would be both economically viable and industrially important.

5.12. Optimization of protease and lignin Peroxidase production of BM1 in solid state fermentation

In the present study, environmentally isolated bacterium BM1 was screened for having high activity of protease and lignin peroxidase enzyme. The production was optimized further in solid state fermentation to get much higher activity. A substrate was selected and the enzyme was optimized for better yield using that substrate in solid state fermentation.

5.12.1. Selection of media for mixing with substrate for Production of Enzyme in Solid State Fermentation

It is necessary to maintain a perfect solid and liquid ratio (the ratio) between substrate and media used to enhance the production of enzyme in solid state fermentation and in the present study, the isolate was subjected to enzyme production in different media and the ideal media was chosen as noted in table 5.14.

Table 5.14. Selection of media

Substrate	Activity in U/gds	
	Protease	Lignin Peroxidase
Minimal Media	23,373.165	24.338
Minimal Media+2%Casein	25,582.045	41.796
Minimal Media+2%Soyawhey	20,000.917	31.734
Minimal Media+2%Soybean	18628.440	28.734
MinimalMedia+2%Casein+1%Urea	34,991.852	50.912
Minimal Media+1%Urea	28,582.363	31.304
Minimal Media+1%Sodium nitrate	11,628.630	9.073
Minimal Media+1%Amonium	15,235.102	18.877
Minimal Media+1%Tryptone	12,333.795	12.556

The table depicts that Minimal Media+2%Casein+1%Urea is the best media for production of enzyme in combination with a solid substrate in solid state fermentation.

5.12.2. Selection of Substrate for Production of Enzyme in Solid State Fermentation

Among the various substrates studied BM1 produced maximum protease in rice straw (1,06,652.25 U/gds) followed by cabbage waste (86,674.948 U/gds) and wheat bran (53,061.411 U/gds) whereas it produced maximum lignin peroxidase in rice straw (1002.3856 U/gds) followed by saw dust (958.5724 U/gds) and cabbage waste(925.3265 U/gds) as summarized in Table 5.15, when optimum conditions were 12 hours incubation period, 37^0C temperature, 4% inoculums volume, pH 8, solid and liquid ratio 1:3 and basal medial with 2%casein and 0.5%peptone under shaking (200 rpm).

Table 5.15. Selection of substrate for production of protease and lignin Peroxidase Enzyme in solid state fermentation

Substrate	Activity in U/gds	
	Protease	Lignin Peroxidase
Rice Straw	1,06,652.25	1002.3856
Wheat Bran	53,061.411	845.2568
Saw Dust	33,253.632	958.5724
Cabbage Waste	86,674.948	925.3265
Orange Peel Waste	37,524.401	902.3258
Jack Fruit Seed Waste	30,376.335	898.2398
Banana Waste	31,365.669	725.3105
Potato Waste	18,223.210	345.6958
Soyawhey	22,456.659	545.6958

5.12.3. Optimization of pH for production of enzyme in solid state fermentation

BM1 produced both maximum protease activity (1,03,660.42 U/gds) and lignin peroxidase activity (1,012.3586 U/gds) at pH 8, as noted in table 5.16, when optimum conditions were 12 hours incubation period, 37^0C temperature, 4% inoculums volume, rice straw as substrate, solid and liquid ratio 1:3 and basal medial with 2%casein and 0.5%peptone under shaking (200 rpm).

Table 5.16. Optimization of pH

pH	Activity in U/gds	
	Protease	Lignin Peroxidase
6	85,241.258	925.3589
7	97,856.329	1000.2569
8	1,03,660.42	1012.3856
9	98,325.215	978.2584
10	83,569.925	847.5402

The table suggests that activity in case of both the enzyme above and below pH 8 decreases leading to decreasing microbial growth and enzyme production.

Similar study on *Bacillus subtili*s (Das et al., 2010) showed maximum production at pH 8.0 and 9.0. Growth of *Bacillus* GPA4 was high at pH 7.0 but enzyme production was higher in alkaline pH which coincided with the study by *Bacillus amovivorus* (Sharmin et al., 2005).

5.12.4. Optimization of incubation period (in hours) for production of enzyme in solid state fermentation

Incubation period has a profound influence on microbial enzyme production as summarized in table 5.17.

Table 5.17. Optimization of incubation period (in hours)

Incubation Period	Activity in U/gds	
	Protease	Lignin Peroxidase
8	55,512.984	482.5689
10	72,358.589	624.2039
12	89,312.925	842.5246
13	1,02,660.10	1000.3149
14	90,256.252	987.2565
16	80,985.256	812.5625
18	72,456.985	800.2563
20	69,325.235	705.5565
22	45,658.569	524.3521
24	32,897.562	411.3625

Maximum protease activity (1,02,660.10 U/gds) and maximum lignin peroxidase activity (1000.3149 U/gds) was obtained at 13th hour of incubation when optimum conditions were 37^{0}C temperature, 4% inoculums volume, pH 8, solid and liquid ratio 1:3, substrate as rice straw and basal medial with 2%casein and 0.5%peptone under shaking (200 rpm). Since 8th hour of incubation the activity of both the enzyme increased upto 13th hour, increase of incubation period further abruptly decreased the activity.

The fermentation time is mandatory for the optimum protease production by bacteria or fungus may vary from 48 hrs to 9 days depending upon the strain and substrate used as reported in many cases (Prakasham, 2005; Puri et al., 2002).

Prakasham (2005) found that protease synthesis occurred at exponential phase of bacterial growth which is associated with sporulation of *B. subtilis*. But according to Dercova et al., the secretions of enzyme occurs mostly between the ends of the exponential phase to an early stationary phase so maximum production occurs after cell population reached its peak.

5.12.5. Optimization of incubation temperature for production of enzyme in solid state fermentation

The effects of different incubation temperatures on protease and lignin peroxidase production were evaluated. It is obvious from the results in table 5.18, that 37 ºC was generally more favorable for production of both the enzymes. Protease showed an activity of 1,01,660.21U/gds and that for lignin peroxidise it was1022.2585 U/gds when optimum conditions were 12 hours incubation period, 4% inoculums volume, pH 8, solid and liquid ratio 1:3, substrate as rice straw and basal medial with 2%casein and 0.5%peptone under shaking (200 rpm).

Table 5.18. Optimization of incubation temperature

Incubation Temperature	Activity in U/gds	
	Protease	Lignin Peroxidase
30	40,569.897	560.2569
32	69,875.201	672.8021
34	89,582.302	898.5602
37	1,01,660.21	1022.2585
38	91,598.658	970.2589
40	84,236.258	740.2125

However, the temperature below or above 37 ºC caused a sharp decrease in protease yield as compared to the optimal temperature. Similar observations were suggested by Hindhumathi et al., 2011.

5.12.6. Optimization of inoculums volume for production of enzyme in solid state fermentation

Eight different inoculums size represented in table 5.19 were investigated for their effect on productivity of the protease and lignin peroxidise enzyme by BM1. Our

results indicated that the use of 4% (7.0 × 103 cell/ml-1) gave the highest yield of both the enzymes (1,04,660.89 U/gds for protease and 1003.1092 U/gds for lignin peroxidise) when optimum conditions were 12 hours incubation period, 37^0C temperature, pH 8, solid and liquid ratio 1:3, substrate as rice straw and basal medial with 2%casein and 0.5%peptone under shaking (200 rpm).

Table 5.19. Optimization of inoculums volume

Inoculum Volume	Activity in U/gds	
	Protease	**Lignin Peroxidase**
1	65,259.321	745.2358
2	73,569.256	845.5897
3	87,905.859	912.2502
4	1,04,660.89	1003.1092
5	90,259.30	987.3251
7	88,995.95	812.0241
10	69,365.23	689.5021

Higher or lower inoculum size resulted in a significant decrease in enzyme productivity.

The inoculum used is basically suspension of the particular bacteria being used. The inoculum size is affected by the density of spores in the suspension. The reported inoculum size is variable due to density of spores in the suspension. The appropriate concentration of the spores (10^6/ml) has been reported by Vahabzadeh et al. *(*2004).

An increase in inoculum size from 1-4% showed a progressive increase in enzyme activity reaching the highest at 4% level while further increase caused overcrowding of spore that decreased the enzyme activity (Gajju et al., 1996; Ahmed et al., 2010). Sen and Satyanarayana (1993) observed that the inoculum size of 2% was favorable for enzyme production by *B. licheniformis*. This variation with our findings might be due to difference in the nature of species.

5.12.7. Optimization of solid and liquid ratio for production of enzyme in solid state fermentation

Table 5.20 shows the results of different solid and liquid ratio (substrate:media) in relation to protease production by BM1 strain.

Table 5.20. Optimization of solid and liquid ratio

Solid(substrate):Liquid(media)	Activity in U/gds	
	Protease	Lignin Peroxidase
1:1	46,5851.246	862.3294
1:2	79,276.441	898.7254
1:3	1,06,650.75	1023.501
1:4	1,65,478.30	1264.8293
1:5	1,02,546.25	1142.2182
1:6	90,087.693	1002.3185

The highest level of protease (1,65,478.30 U/gds) and lignin peroxidase (1264.8293 U/gds) production was obtained when 1:4 (solid :liquid) ratio was used for production of both the enzymes. The other constant conditions maintained during this production were when optimum conditions were 12 hours incubation period, 37^0C temperature, pH 8, inoculums volume 4%, substrate as rice straw and basal medial with 2%casein and 0.5%peptone under shaking (200 rpm).

SSF processes are distinct from submerged fermentation (SmF) culturing, since microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents. Thus, it is crucial to provide optimized water content, and control the water activity (aw) of the fermenting substrate for the availability of water in lower or higher concentrations affects microbial activity adversely.

5.12.8. Response Surface Methodology in optimization of protease and lignin peroxidase enzyme production

Design of experiments

The effect of different parameters on protease and lignin peroxidase production from a locally isolated hyperactive strain BM1 was investigated and their interactions were studied. Box–Behnken design was adapted which considered low (-) and high (+) levels and central points (0) for the selected parameters. The experimental conditions for all experiments and the corresponding results obtained have been summarized in Table 5.21. The experimental data were first analyzed to determine second-order equations including term of interaction between the experimental variables. The equation given below is based on the statistical analysis of the experimental data shown in Table 5.21.

Table 5.21. Experimental design (conditions and responses) for protease production in terms of uncoded level of variables according to a 2^5 factorial central composite design.

Run order	pH	Temperature (^oC)	Solid to liquid ratio	Inoculum volume (%)	Incubation time (h)	Protease (U/gds)		Lip (U/gds)	
						Experimental	Predicted	Experimental	Predicted
1	8	37.5	4	4	11	88531.661	88443.043	1970.927	1920.067
2	8	31.55	4	4	11	11453.4492	14772.001	489.235	474.374
3	9	40	3	5	8	99483.342	101249.864	1924.023	1926.652
4	8	37.5	4	4	11	88298.388	88443.043	1893.415	1920.067
5	9	40	3	3	14	98125.674	99925.412	1194.217	1273.711
6	8	37.5	4	6.39	11	35298.388	37396.553	1574.585	1614.121
7	9	35	3	5	8	28452.62	36368.848	1221.679	1244.468
8	7	35	5	3	14	67241.32	73920.752	1300.201	1312.766
9	5.62	37.5	4	4	11	46187.51	38606.385	1344.487	1302.229
10	8	37.5	4	4	11	89928.838	88443.043	1893.977	1920.067
11	8	37.5	4	4	3.86	39772.824	31730.207	1250.610	1258.160
12	8	37.5	4	1.62	11	62343.588	54750.764	1651.366	1582.731
13	7	35	5	5	8	69465.921	76031.535	1026.238	979.677

Run order	pH	Temperature (^{o}C)	Solid to liquid ratio	Inoculum volume (%)	Incubation time (h)	Protease (U/gds)		Lip (U/gds)	
						Experimental	Predicted	Experimental	Predicted
14	8	37.5	4	4	11	88023.214	88443.043	1901.810	1920.067
15	7	40	5	5	8	45045.114	47432.955	1296.201	1288.722
16	7	35	3	5	8	19812.214	20398.332	1042.218	977.352
17	7	35	3	3	8	31791.824	18168.984	1102.194	1135.503
18	9	35	3	5	14	20813.912	17684.834	1162.165	1142.978
19	9	40	5	3	14	54621.252	59956.765	1372.254	1389.154
20	7	35	3	3	14	20928.914	21784.265	1380.186	1419.424
21	8	37.5	4	4	11	88975.983	88443.043	1954.127	1920.067
22	9	35	3	3	14	28542.026	35296.456	1124.962	1150.279
23	9	40	5	5	14	44241.62	43134.322	1549.278	1573.701
24	8	37.5	4	4	11	88918.927	88443.043	1902.987	1920.067
25	9	40	5	5	8	59792.254	64777.933	2221.203	2151.378
26	9	35	5	5	14	66521.034	48908.437	1243.921	1177.425
27	8	37.5	4	4	11	91351.612	88443.043	1972.919	1920.067
28	9	35	5	3	14	32163.213	29694.464	1234.240	1266.329
29	8	37.5	4	4	18.14	7742.831	10290.789	945.472	908.823
30	7	40	5	3	8	59236.127	44414.428	1243.910	1255.025
31	9	40	3	5	14	40124.291	46277.375	1597.916	1593.860
32	7	40	5	5	14	78124.272	84101.256	1041.401	1071.739
33	8	37.5	4	4	11	87124.912	88443.043	1889.488	1920.067
34	9	35	3	3	8	88452.611	89993.087	1261.279	1227.053
35	8	43.45	4	4	11	66432.792	57619.581	1002.917	988.678
36	7	35	5	5	14	150912.279	150912.279	1067.911	1048.298
37	7	40	3	3	8	10123.117	10123.117	1112.169	1171.704
38	7	40	5	3	14	43924.671	43924.671	1050.271	1062.759
39	9	35	5	5	8	36976.172	36976.172	1362.193	1469.501
40	9	40	5	3	8	111979.912	111979.912	1944.031	1942.114
41	7	35	5	3	8	34915.901	34915.901	1162.920	1219.431
42	10.3	37.5	4	4	11	73215.483	73215.483	1994.878	2008.036

Run order	pH	Temperature (^{o}C)	Solid to liquid ratio	Inoculum volume (%)	Incubation time (h)	Protease (U/gds)		Lip (U/gds)	
						Experimental	Predicted	Experimental	Predicted
	8								
43	7	40	3	5	14	27256.843	27256.843	1212.732	1260.607
44	8	37.5	6.39	4	11	122795.7	122795.700	1254.293	1260.549
45	7	35	3	5	14	54921.213	54921.213	1172.725	1236.557
46	9	40	3	3	8	206241.012	206241.012	1622.200	1636.086
47	7	40	3	3	14	40165.712	40165.712	1256.421	1170.023
48	7	40	3	5	8	39356.291	39356.291	1280.743	1287.004
49	8	37.5	1.62	4	11	113330.834	113330.834	1155.854	1120.498
50	9	35	5	3	8	46541.679	46541.679	1592.709	1533.688

The main effects of parameters on protease and lignin peroxidase production were estimated by subtracting the mean responses of parameters at their lower levels from their corresponding higher levels and divided by the total number of experimental runs. The adequacy of the model was tested and the parameters with statistically significant effects were identified using Fisher's test for the analysis of variance (ANOVA) (Table 5.22 &5. 23).

Table 5.22. Analysis of variance (ANOVA) analysis of RSM quadratic model for protease production

Source	DF[a]	Seq SS	Adj SS	Adj MS	F	P
Regression	20	66807964307	66807964307	3340398215	21.13	0.000
Linear	5	8212577652	13458603852	2691720770	17.03	0.000
Square	5	16778657023	16778657023	3355731405	21.23	0.000
Interaction	10	41816729631	41816729631	4181672963	26.45	0.000
Residual Error	29	4584431700	4584431700	158083852		
Lack-of-Fit	22	4572939628	4572939628	207860892	126.61	0.000
Pure Error	7	11492073	11492073	1641725		
Total	49	71392396007				
$R^2 = 0.9358$	R^2 (adj)= 0.8915		R^2 (predicted)= 0.7789			

Table 5.23. Analysis of variance (ANOVA) analysis of RSM quadratic model for lignin peroxidase production

Source	DF[a]	Seq SS	Adj SS	Adj MS	F	P
Regression	20	6542305	6542305	327115	104.16	0.000
Linear	5	1732953	2735210	547042	174.19	0.000
Square	5	3710425	3710425	740285	236.29	0.000
Interaction	10	1098927	1098927	109893	34.99	0.000
Residual Error	29	91075	91075	3141		
Lack-of-Fit	22	81629	81629	3710	2.75	0.086
Pure Error	7	9446	9446	1349		
Total	49	6633380				
$R^2 = 0.9863$,	R^2 (adj)= 0.9768		R^2 (predicted)= 0.9636			

Statistical analysis of RSM data for protease and lignin peroxidase production

All investigational conditions and the consequent outcome obtained are summarized in Table 5.21.

The experimental records were first analyzed for the determination of second-order equations including the expression of interaction between the investigational variables.

The mathematical expression of relationship of protease activity (Y) with different variables A, B, C, D and E (pH, incubation time, temperature, inoculums volume, and solid to liquid ratio) are given below in terms of uncoded factors.

$$Y1 = -3447831 + 42149\,A + 88758\,B + 108613\,C + 233278\,D + 142887\,E - 5566\,A^2 - 1325\,B^2 - 1478\,C^2 - 7490\,D^2 + 4751\,E^2 - 4859\,AB + 5911\,AC - 13963\,AD - 14435\,AE - 1210\,BC + 3001\,BD + 2777\,BE - 3604\,CD - 3437\,CE + 9206\,DE \quad (i)$$

$$Y2 = -45220.5 - 568A + 1006.3B + 2261.8C - 810.8D + 874.3E - 46.8A^2 - 16.4B^2 - 33.6C^2 - 56.9D^2 - 129E^2 - 30.1AB + 37.3AC + 43.9AD + 55.7AE - 9.5BC - 2.1BD - 15.9BE + 27.3CD - 0.1CE - 20.4DE \quad (ii)$$

Analysis of the Regression showed that all the five parameters i.e., pH, incubation time, temperature, inoculums volume, and solid to liquid ratio was found to have positive linear effect and inoculum volume shows highest impact on protease production. Factor like solid to liquid ratio showed significant positive quadratic effect on protease production. Enzyme production would boost up with enhance in the level of these factors primarily and reduce beyond certain specific values. Interactions between independent parameters also impart an important function in enzyme production. Interaction between pH and incubation time, pH and temperature, pH and inoculum volume, pH and solid to liquid ratio, incubation

time and temperature, incubation time and inoculum volume, incubation time and solid to liquid ratio, temperature and inoculum volume, temperature and solid liquid ratio and inoculum volume and solid to liquid ratio were significant because of their low P values (<0.05) in case of interactive terms.

RSM model for LiP production also show significant effect on the solid state production of the enzyme by using rice straw as a substrate. ANOVA table for lignin peroxidase was depicted in Table 23. It shows that incubation time, temperature and solid to liquid ratio have highest positive impact on the enzyme production. No factor shows positive quadratic effect on lignin peroxides production that means that if this factor was increased doubled than it herm enzyme production. Interaction effect of pH and temperature, pH and inoculum volume, pH and solid to liquid ratio, and temperature and inoculum volume shows positive effect on lignin peroxidase production. These all parameters of interaction effect have low P value (<0.05) so they all are significant in case of interactive terms.

An analysis of variance (ANNOVA) of the protease and lignin peroxidase enzyme production was performed to authenticate the regression coefficient (Table 5.22 and Table 5.23).

The values (Table 5.22) of the model F, model P > F and lack of fit, were found to be 21.13, <0.001 and 126.61 respectively, demonstrating that model was significant for protease production. Lack of fit (126.61) was found to be not significant, indicating a high-quality correlation between the experimental and predicted values of the protease producing system. The model had an R^2 value of 0.9358 confirming a good quality correlation between experiential and predicted responses. For a superior statistical model R^2 value should be close to 1.0, and a value >0.75 signifies the accuracy of the model. The present model indicated that the predicted R^2 value of 0.7789 was in reasonable agreement with the adjusted R^2 value of 0.8915. Three dimensional response contour curves were generated and analyzed using Minitab 15 software for determining the optimum levels of the independent variables and major interaction effects (Figure 5.4).

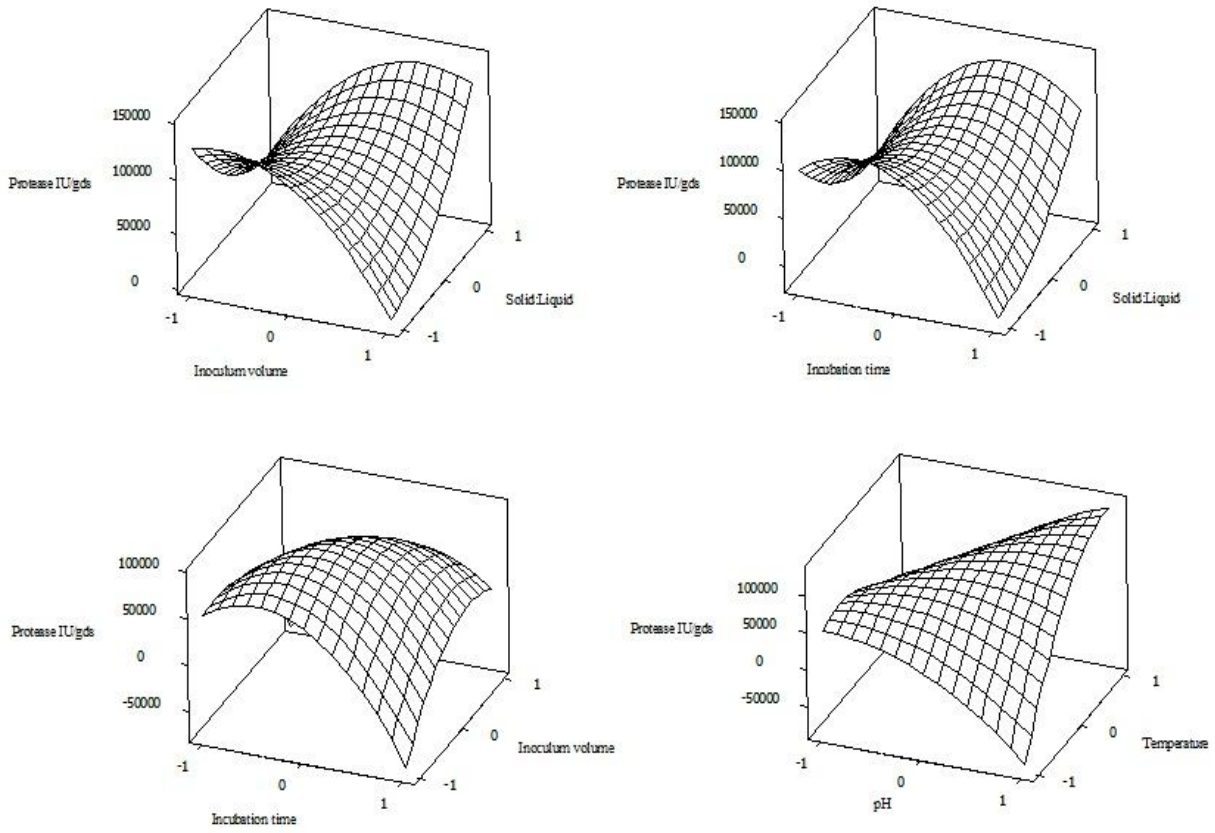


Figure 5.4. Response surface for protease production by BM1 according to the experimental design and model in Table 5.21. The three-dimensional plot shows the influence of different parameters on protease production. Protease activity is expressed in U/gds.

Table 5.23 values indicated the model F, model P > F and lack of fit, were found to be 104.16, <0.001 and 2.75 respectively, demonstrating that model was significant for lignin peroxidase production. The model had an R^2 value of 0.9863 confirming a good quality correlation between experiential and predicted responses. The present model indicated that the predicted R^2 value of 0.9636 was in reasonable agreement with the adjusted R^2 value of 0.9768. Three dimensional response contour curves were generated and analyzed using Minitab 15 software for determining the optimum levels of the independent variables and major interaction effects (Figure 5.5).

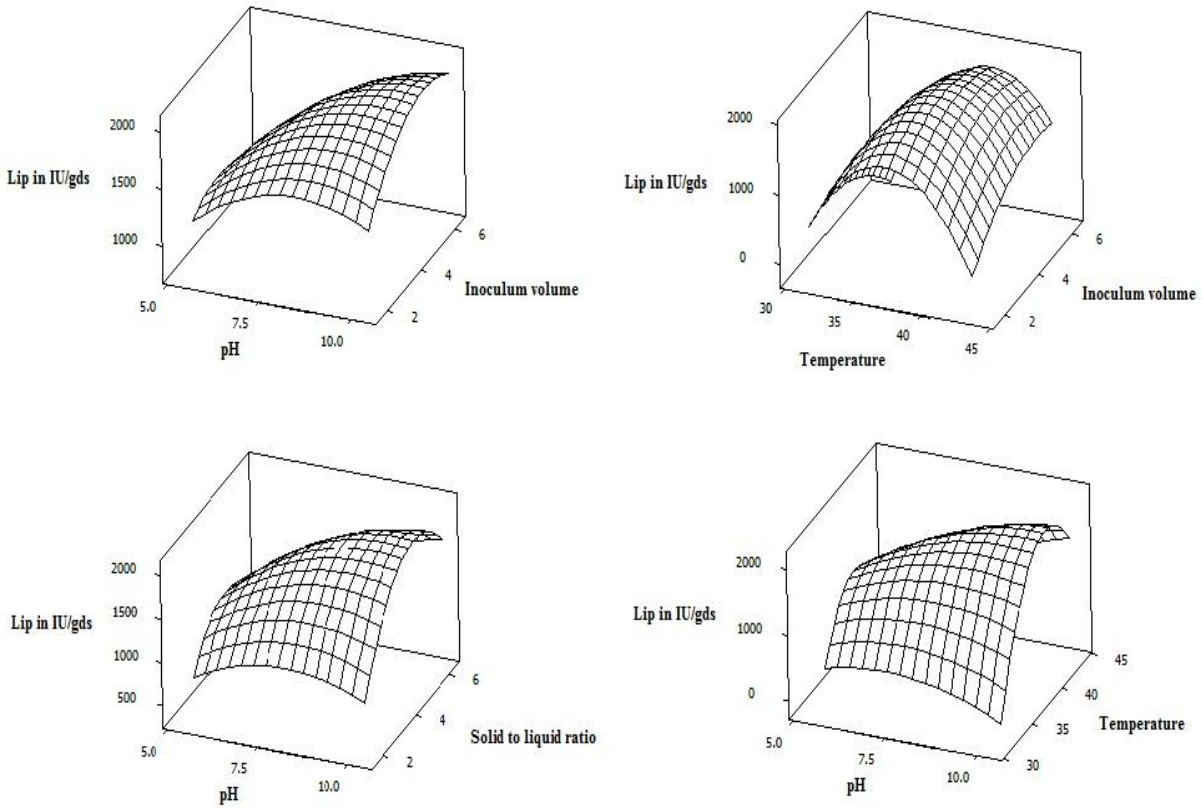


Figure 5.5. Response surface for lignin peroxidase production by BM1 according to the experimental design and model in Table 5.21. The three-dimensional plot shows the influence of different parameters on lignin peroxidase production. lignin peroxidase activity is expressed in IU/gds.

Validation of the optimized condition

Experiments were conducted to validate the optimal condition for the production of protease and lignin peroxidase. The ensuing quadratic model predicted that the maximum production of protease was 2,20,293 U/gds, when optimum conditions were pH 7.31, incubation time 13.8 h, temperature 37.5 ^{o}C, inoculum volume 4.83 ml and solid to liquid ratio is 1: 5.2 and the maximum production of lignin peroxidase was 2,219.35 U/gds, when optimum conditions were pH 10.38, incubation time 3.9 h, temperature 41.4 ^{o}C, inoculums volume 4.93 ml and solid to liquid ratio is 1: 6.2. To verify the predicted results, valedictory experiments were performed in triplicate tests. Under the optimized condition, the observed experimental titre of average protease was 2,18,726 U/gds and that of lignin peroxidase was 2,219.35 U/gds, suggesting that experimental and predicted values of protease and lignin peroxidase yield were in good agreement. This result therefore, correlates with the predicted values and the effectiveness of the model, indicating that the optimized medium favours the production of enzyme protease and lignin peroxidase.

RSM was successfully employed to study the modeling and interaction of the parameters and the regression equation obtained therein was coupled with differential evolution for the optimization of protease and lignin peroxidise production from a locally isolated hyperactive strain BM1. The models had a regression coefficient R^2 of 0.7789 whereas the adj R^2 was 0.8915 for protease and R^2 of 0.9636 whereas the adj R^2 was 0.9768 for lignin peroxidise indicating the adequacy of the model. An overall 2.07 folds increase in protease production and 2.22 folds increase in lignin peroxidise production was achieved compared with the mean observed response (1,06,652.25 U/gds for protease and 1002.3856 U/gds for lignin peroxidase respectively) in the basal medium (with all factors at their central levels) after optimization by using response surface methodology.

Such high enzyme yields for lignin peroxidase with such optimum conditions have not been reported earlier in bacterial batch fermentations. This work has demonstrated the use of a central composite design by determining conditions leading to the maximum enzyme production.

5.13. Purification of alkaline protease enzyme

The purification procedure is summarized in Table 5.24, showing that the enzyme was purified 5-fold with a specific activity of 80,568.479U/mg protein after Ammonium Sulphate precipitation and dialysis. The enzyme solution was further purified by gel filtration using Sephadex G-200 as column matrix.. This purification step showed 10-fold enzyme purification with a specific activity of 1,58,220.430U/mg protein. These results indicated the effectiveness of purification method. However, the yield of the enzyme after purification was found to be low (Chomsri, 2001). This might be due to the result of autolysis of the enzyme during purification. Chomsri suggested that the low enzyme yield obtained after enzyme purification was probably owing to partial autolysis by molecular unfolding of the enzyme (Ward, 1985).

Table 5.24. Summary of purification procedure

Purification step	Activity (U/ml)	Protein content (mg/ml)	Specefic activity (U/mg)	Purification (fold)
Crude enzyme (Initially)	16,744.954	1.1	15.222.685	1
Ammonium Sulphate precipitation followed by dialysis fraction	22,559.174	0.280	80,568.479	5
Gel filtration fraction (Sephadex G-200)	31,852.984	0.195	1,58,220.430	10

Protease enzyme was purified by ammonium sulfate precipitation and Sephadex G200 filtration as mentioned by El-Safey and Ammar, (2003). A trial for the purification of protease enzyme resulted in specific activity of 1,58,220.430 U/mg with purification folds 10 times. Similarly, ammonium sulphate pricipatation and applying sephadex G200 column chromatographic technique were applied for protease purification resulted in having two proteases (A) and (B) with specific activity of 229.6 and 286.46 units/mg prot/ml-1 corrospoding to purification folds of 55.7 and 69.5 times of the origin respectively (Abdul-Raouf, 1990).

The same method were used for purification of thermostable protease produced by *B. brevis geltinoamylolyticus* attacked fish wastes and poultry wastes. The thermostable protease were purified by applying ammonium sulphate fractionation and sephadex G200 and G100 column chromatography, where specific activity 44562.5 units/ml-1 protien/ml-1 with purification folds of 8.5 times for sehpadex G200 and 69017.5 units/ml-1 protien/ml-1 with purification folds 13.18 times for sephadex G100 (Ammar, *et al*, 2003).

Moreover, an extracellular protease produced from *Flavobacterium psychrophilum* (fish pathogen) was purified to electrophoretic homogeneity from the culture supernatant by using ammonium sulfate precipitation, ion-exchange chromatography, hydrophobic chromatography, and size exclusion chromatography (Secades, 2001).

In addition to that, a novel protease, hydrolyzing azocasein, was purified from the culture supernatant of *Yersinia ruckeri*.(fish pathogen) Exoprotease. The protease was purified in a simple two-step procedure involving ammonium sulphate precipitation and ion-exchange chromatography (Secades and Guijarro, 1999).

However, a protease (protease A) was successfully purified from the extracellular proteins of *Vibrio parahaemolyticus* no. 93, a clinical strain carrying neither tdh nor trh genes, using phenyl-Sepharose CL-4B hydrophobic interaction chromatography (Lee, 2002). Moreover, extracellular alkaline protease from the alkalophilic bacterium *Alcaligenes faecalis* was purified by a combination of ion-exchange and sizeexclusion chromatographic methods, and the purified enzyme had a specific activity of 563.8 *l*mol of tyrosine/min per mg of protein conditions (Berla and Suseela, 2002).

On the other hand, an extracellular proteinase (PSCP) produced by *Pseudomonas cepacia* was purified from culture supernatants by ammonium sulphate precipitation, anion exchange chromatography on DEAE-Sephacel, and G200 gel filtration chromatography (Mckevitt, *et al*, 1989). Moreover, Extracellular and membrane-bound proteases produced by *Bacillus subtilis* YY88 were purified by ammonium sulfate precipitation, dialyzed, and applied to a DEAE-cellulose column (Mantsala P. and Howard Z., 1980).

5.14. SDS-PAGE of the purified protease from BM1

The enzyme purity was confirmed by SDS-PAGE which demonstrated a single band (Figure. 5.6), indicating a homogeneous preparation (Adinarayana et al., 2003). The molecular weight of the enzyme under denaturing condition was estimated to be 27.5 kDa, similar to the molecular masses of alkaline proteases, which were in the ranges of 15 to 30 kDa(Fogarty et al., 1974).

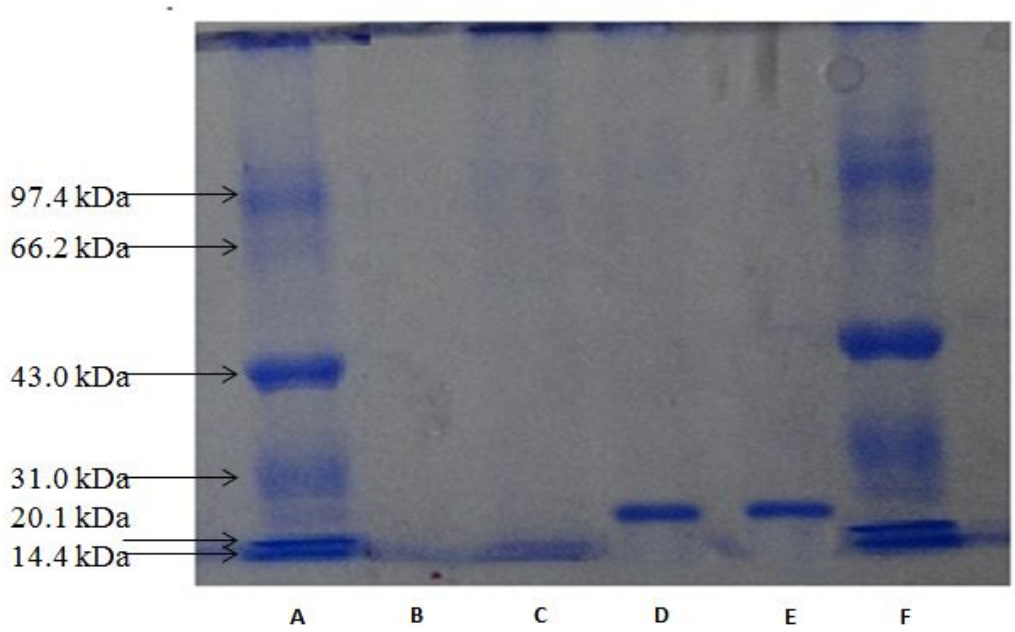


Figure 5.6. SDS-PAGE of the purified protease from BM1. Lane A and Lane F molecular markers (kDa) were as follows: Phosphorylase b, 97 kDa; Albumin, 66 kDa; Ovalbumin, 45 kDa; Carbonic anhydrase, 30 kDa; Trypsin inhibitor, 20.1 kDa; Alpha lactalbumin, 14.4 kDa. Lane D and Lane E purified enzyme.Lane B and Lane C blank.

The molecular masses of alkaline proteases produced by several bacteria including Bacillus ranged from 15 kDa to 30 kDa, with few reports of higher molecular masses of 33 kDa (Samal et al., 1991), 36 kDa (Tsujibo et al., 1990) and 45 kDa (Kwon et al., 1994).

5.15. Characterization of the purified alkaline protease enzyme

Characterization of the purified alkaline protease derived from the isolate BM1 was carried out in relation to the optimal temperature, optimal pH for the enzyme activity and the substrate specificity of the enzyme.

5.15.1. Optimal temperature for enzyme activity

Protease activity increased progressively with temperature and maximum activity was obtained at 60°C (Figure 5.7).

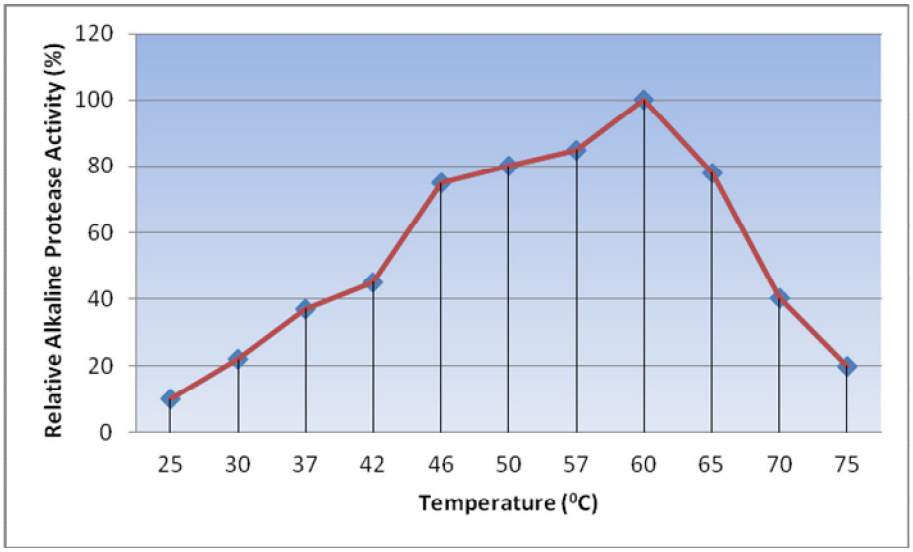


Figure 5.7. Effect of temperature on alkaline protease activity produced by BM1

Enzyme activity decreased at temperatures higher than 60°C. It is reported (Horikoshi ,1990; Durham et al., 1987) that a number of alkaline proteases isolated from *Bacillus* sp. have high optimal temperatures for their activity. This is an important characteristic for the use of alkaline proteases as detergent additives. Similar to our results, two important alkaline proteases of the detergent industry, subtilisin Carlberg, produced by *B. licheniformis* and subtilisin Novo or Bacterial Protease Nagase (BPN), produced by *B. amyloliquefaciens*, also showed optimum temperature of 60°C (Horikoshi ,1990).

5.15.2. Optimal pH for enzyme activity

The pH-activity profile of the enzyme is shown in Figure 5.8. At pH 6.5, only 22% of the maximum enzyme activity was obtained, increasing to 65 and 77% at pH 7.0 and 7.5, respectively. Maximum enzyme activity was observed at pH 10.5 and an increase in pH beyond 10.5 brought about a decline in protease activity resulting in 18% activity at pH 12.5.

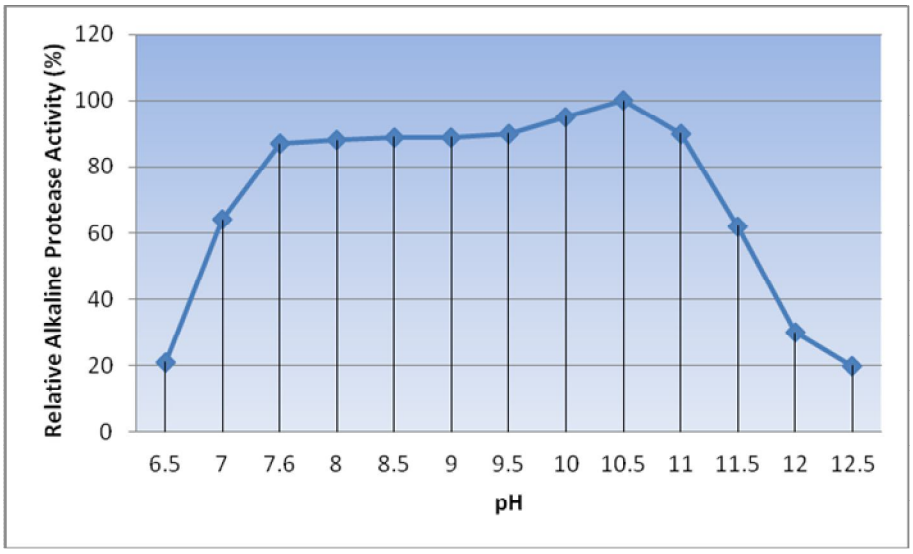


Figure 5.8. Effect of pH on alkaline protease activity produced by BM1

These findings are in accordance with several earlier reports showing pH optima of 10–10.5 for protease from *Bacillus* sp., *Thermus aquaticus*, *Xanthomonas maltophila* and *Vibrio metschniko*6*ii* (Durham et al., 1987; Matsuzawa et al., 1988; Matsuzawa et al., 1991; Kwon et al., 1994) The important detergent enzymes, substilisin Carlberg and substilisin Novo or BPN also showed maximum activity at pH 10.5 (Horikoshi ,1990).

5.15.3. Substrate specificity of the enzyme

The enzyme had a capability to effectively catalyze the hydrolysis of various proteins which were cytochrome C, soybean protein isolate, casein, human hemoglobin and wheat gluten with the relative activity of more than 70% . The best substrate specific to the enzyme was cytochrome C with the highest relative activity of 124% followed by soybean protein isolate (119% relative activity) and casein (110% relative activity). In contrast, gelatin, egg albumin and bovine albumin were less specific for protease with 50% or lower than 50% relative activity (table 5.25).

Table 5.25. Substrate specificity of the protease enzyme

Substrate	Relative activity (%)
Casein	110
Cytochrome C	124
Soybean protein isolate	119
Gluten (wheat)	75
Gelatin (fine powder)	50
Albumin (egg)	44
Albumin (bovine)	32

Note: The activity of casein, referred to 100% relative activity.

Aoyama (Aoyama et al., 2000) reported that cytochrome C, casein and soybean protein isolate were good substrates for serine protease enzyme from B. pumilus with the relative protease activity of 145, 130 and 100% respectively.

This research successfully purified alkaline protease from BM1strain. After final purification step, the enzyme was purified 10-fold with an increase in specific activity from 15.222.685 to 1,58,220.430 U/mg protein. The properties of the purified enzyme indicated that this purified protease enzyme functioned at alkaline pH. However, the study on amino acid sequences of this enzyme should be conducted to confirm our conclusion.

5.16. Application of individual isolates and bacterial consortium in bioconversion of municipal solid waste

Studies were conducted to select the best strain or consortium for municipal waste decomposition (Kaplovsky, 1952). As the waste is broken down by microorganisms (bacteria), the weight and volume of the matter decreases. In the present study of decomposition, we also observed that the weight and volume of treated garbage decreased because bacteria broke down the garbage and converted to simple molecules. Bacteria play an important role in the decomposition of organic waste and can be important contributors to optimal waste bioconversion. Suspension of individual bacterial isolates was applied in municipal solid waste and the isolates with better degradation ability were screened. For decomposition of organic solid waste by using bacterial suspension treatment of BM3, D5, BM2 and BM1 no bad smell was emitted after 30 days. It indicates the possible

complete degradation of organic waste in plates that contained 5 g organic waste (Table 5.26.). In case of the control and the other strains, the bad smell continued even after 30 days, and it indicates slow degradation. Data pre-sented in the table 26 showed that, after 30 days, the average weight loss in three trials (plates) was 33.25 for BM1, 32.75% for D5, 25.62% for BM2 and 25.32% for BM1.

Table 5.26. Degradation rate study of individual isolates

Strain no	Initial weight of petri (g)	Weight after 10 days (g)	Weight after 20 days (g)	Weight after 30 days (g)	Average weight loss in %
Control	55.180	55.152	55.101	55.085	9.51
BM1	59.470	59.379	59.311	59.217	25.32
BM2	58.679	58.562	58.499	58.423	25.62
BM3	57.412	57.389	57.211	57.079	33.25
D1	59.671	59.621	59.589	59.557	11.42
D2	58.420	58.375	58.321	58.293	12.62
D3	57.911	57.872	57.798	57.710	20.01
D4	59.241	59.201	59.092	58.998	24.32
D5	58.670	58.598	58.302	58.343	32.75
D6	59.071	59.049	59.002	58.949	12.15

BM3 has the maximum degradation ability followed by D5, BM2, BM1, D4, D3, D2, D6, D1 as summarized in table 5.26.

From table 5.3, it is evident that there is no antagonism between BM1, BM3 and D5. Thus consortium was formed using these three isolates to screen the best possible combination for faster and efficient conversion of municipal solid waste (table 5.27).

Table 5.27. Degradation rate study of consortium

Strain no	Initial weight of petri (g)	Weight after 10 days (g)	Weight after 20 days (g)	Weight after 30 days (g)	Average weight loss in %
BM1+BM3	55.270	55.091	54.879	54.908	36.20
BM1+D5	58.612	58.592	58.319	58.261	35.11
BM3+D5	55.702	55.653	55.171	55.249	45.21
BM1+BM3+D5	57.811	57.701	57.311	57.441	37.01

As depicted in table 5.27, the consortium formed in combination of BM3 and D5 exhibit the best potential of degradation and after 30 days there was no bad smell indicating possible complete degradation of organic waste (figure 5.9) as suggested earlier.

a. Municipal solid waste (day 0)

b. Municipal solid waste (day 15)

c. Municipal solid waste (day 30)

Figure 5.9. Bioconversion of municipal solid waste by using suspension of consortium of BM3 and D5 in petri plates.

During the bioconversion of organic waste, there was a shift in pH from the initial condition neutral (7.21 and 7.27) toward an alkaline condition in the piles. The occurrence of these conditions may be attributed to the bioconversion of the organic material into various intermediate types of organic acid and higher mineralization of the nitrogen and phosphorous into nitrites/nitrates and orthophosphate, respectively. This increase in pH during the biodegradation process could be due to the production of ammonium as a result of the ammonification process (Huang et al., 2004). In our experiment there was a similar trend with a fast pH increase during the first ten days of bioconversion and then stabilization with pH fluctuations between 7.33 and 7.25 after 30 days. At the beginning of the experiment, the fluctuations can be explained by the industrial process used, involving periodic turnings and consequently the volatilization of NH4 +. This acid production results from a lack of oxygen that can occur between two turnings. In such conditions, pH can reach values of about 6.0 Sundberg, 2004).. Our results show that the pH of composts decreased to a final, mature pH of approximately 7.40 (control) and 7.26, which meets the compost regulations of pH 5.0–8.0 for the US, and pH 5.5–8.0 for the Council of uropean Communities (CEC) (Felicita, 2003).

The initial average temperature of the turned compost in plates was 29 and 31◦C and rapidly rose to a peak of 55◦C after 10 days of decomposing. The high temperature continued until 20 days of decomposition, after which the temperature dropped to 36◦C by the 25th day of composting. Thereafter, the temperature varied within a narrow range (36–30◦C). The temperature levels in the compost in plates tended to increase and reach 40–55◦C due to the energy released from the biochemical reactions of the microorganisms in the compost piles, while the temperature levels in the compost piles tended to decrease after the thermophilic phase due to a loss of the substrate and a decrease in microbial activity (Bertoldi et al, 1983). It was previously reported that the compost material can be considered mature when an ambient temperature of 28◦C is reached (Bertoldi et al, 1997). Therefore, this parameter is considered as a good indicator for the end of the biooxidative phase in which the compost achieves some degree of maturity (Gautam et al, 2010; Gautam et al, 2010; Jimenez et al, 1989). After 30 days, the

mature compost was black in color, granular, and fibrous with a pleasant earthy smell. The appearance of black color indicated its maturity. In the case of the control (without culture), the biodegradation was very slow, and weight loss was also low. In order to assess the compost maturity, both the compost samples were placed separately in a sealed polythene bags for a week. After a week, the sealed bags were broken, and the odor was checked, which was found to have earthy smell in the compost produced by BM3and D5, indicating the quality of stable and mature compost and the control still produced bad smell after one week. These findings are in accordance with other previous studies where it was reported that mature compost produces an earthy smell after being sealed in the polythene bag for a week (Jilani, 2007).

5.17. Molecular characterization of potent bacterial isolates

From previous experimental results it is suggested that BM1 is a hyperactive protease and lignin peroxidase producer and thus has a great economical and industrial significance. On the other hand it is also a good decomposer.

Moreover the consortium formed in combination with BM3 and D5 had very good degradation ability, BM2 is also not far behind in this context. So, BM1, BM2, BM3 and D5 are the four potent strains among the nine initial isolates having significant importance. These four strains were subjected to molecular characterization for further detailing of the study.

The molecular characterization of the four strains including the following steps:

a. Genomic DNA was isolated from the pure culture pellet provided by the scientist

b. The ~1.4kb rDNA fragment was amplified using high –fidelity PCR polymerase.

c. The PCR product was sequenced bi-directionally using the forward, reverse and internal primer.

d. The sequence data was aligned and analyzed to identify the bacterium and its

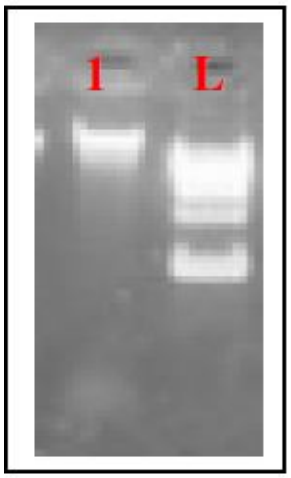
closest neighbors.

This total project was carried out by Chromous Biotech Ltd., India.

5.17.1. Molecular characterization of BM1 strain

The bacterial isolate BM1 bears maximum similarity 99% and identified as *Paenibacillus mucilaginosus; s-4 (GenBank entry: HM849729)*. The sequence obtained was submitted to GenBank and the GenBank accession no. is **MTCC 11281.**

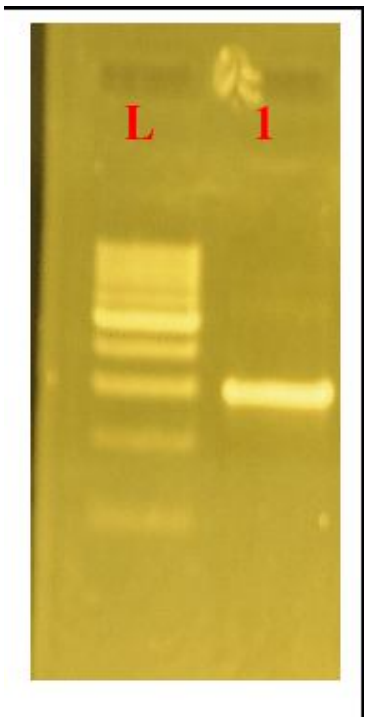
Genomic DNA isolation from BM1 strain



Lane description- L: 1kb DNA Ladder (Chromous Cat. No. LAD03); 1. Sample

Figure 5.10.a. Extraction of Genomic DNA from Bacterial sample (BM1) using the Bacterial Genomic DNA Isolation Kit (RKT09)

PCR amplification of isolated 16s rDNA fragment



Lane description- L: 500bp DNA ladder; 1. ~1.5kb 16srDNA fragment amplified

Figure 5.10.b. PCR amplification of ~1.5kb 16srDNA fragment from genomic DNA of the bacterial culture (BM1)

Alignment of the 16s rRNA gene sequences

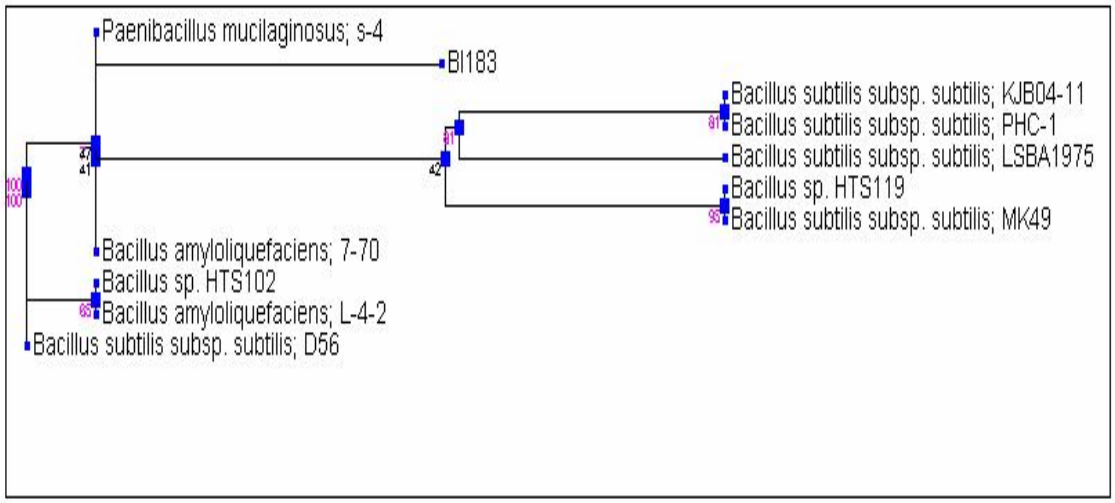
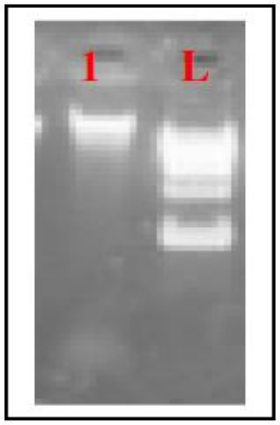


Figure 5.10.c. Phylogenetic tree based on the 16s rRNA gene sequence of 1497 bases length of the isolate BM1. Numbers at nodes indicate bootstrap values derived from 100 replications. genBank accession numbers of 16s rRNA gene sequences are indicated in parentheses.

5.17.2. Molecular characterization of BM2 strain

The microbe was found to be most similar to *Bacillus sp. enrichment culture clone CBMAI_975* (GenBank entry: GQ272679).

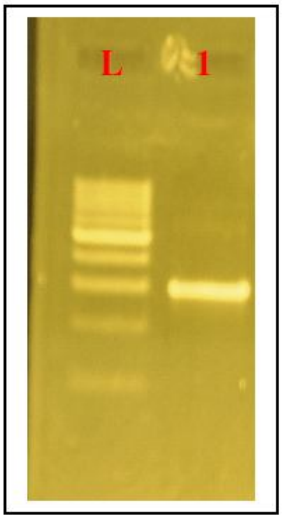
Genomic DNA isolation from BM2 strain



Lane description- L: 1kb DNA Ladder (Chromous Cat. No. LAD03); 1. Sample

Figure 5.11.a. Extraction of Genomic DNA from Bacterial sample (BM2) using the Bacterial Genomic DNA Isolation Kit (RKT09)

PCR amplification of isolated 16s rDNA fragment



Lane description- L: 500bp DNA ladder; 1. ~1.5kb 16srDNA fragment amplified

Figure 5.11.b. PCR amplification of ~1.5kb 16srDNA fragment from genomic DNA of the bacterial culture (BM2)

Alignment of the 16s rRNA gene sequences

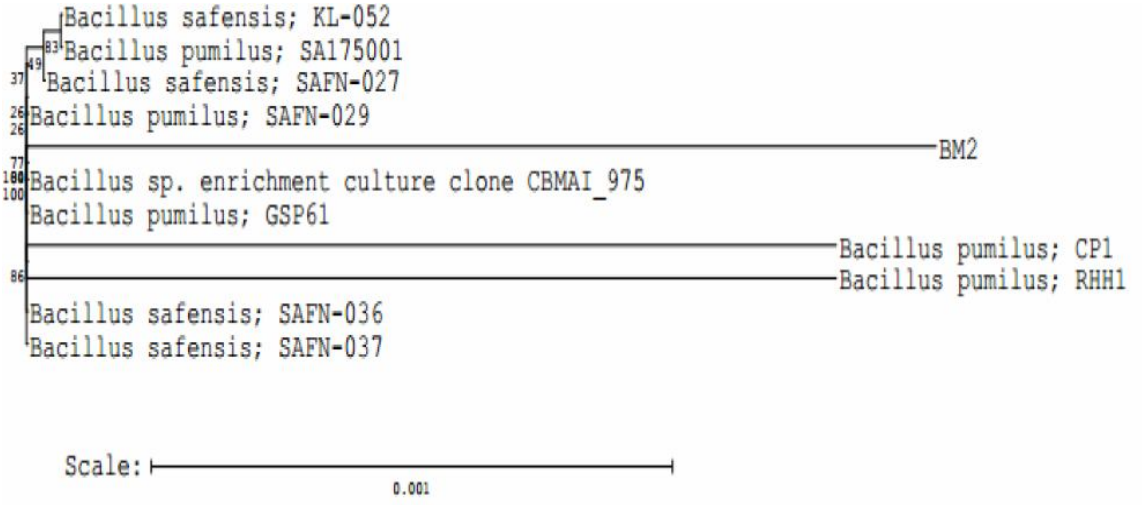
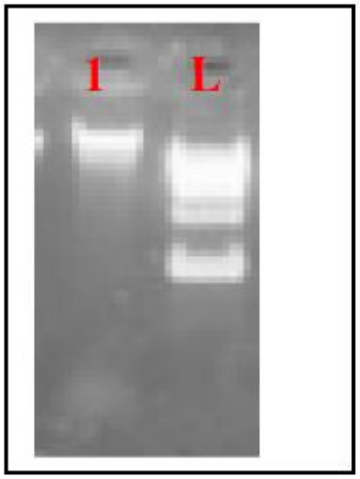


Figure 5.11.c. Phylogenetic tree based on the 16s rRNA gene sequence of 1263 bases length of the isolate BM2. Numbers at nodes indicate bootstrap values derived from 100 replications. genBank accession numbers of 16s rRNA gene sequences are indicated in parentheses.

5.17.3. Molecular characterization of BM3 strain

The microbe was found to be most similar to *Bacillus amyloliquefaciens; Kimura (GenBank entry: AB610803)* and identified as the same.

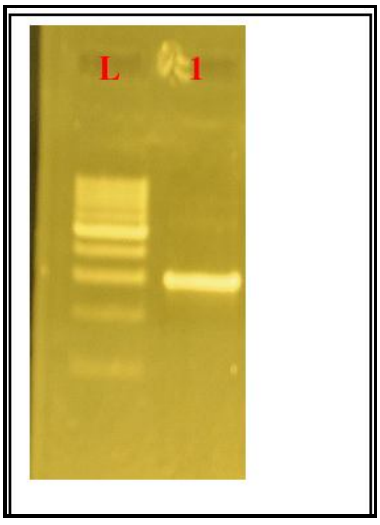
Genomic DNA isolation from BM3 strain



Lane description- L: 1kb DNA Ladder (Chromous Cat. No. LAD03); 1. Sample

Figure 5.12.a. Extraction of Genomic DNA from Bacterial sample (BM3) using the Bacterial Genomic DNA Isolation Kit (RKT09)

PCR amplification of isolated 16s rDNA fragment



Lane description- L: 500bp DNA ladder; 1. ~1.5kb 16srDNA fragment amplified

Figure 5.12.b. PCR amplification of ~1.5kb 16srDNA fragment from genomic DNA of the bacterial culture (BM3)

Alignment of the 16s rRNA gene sequences

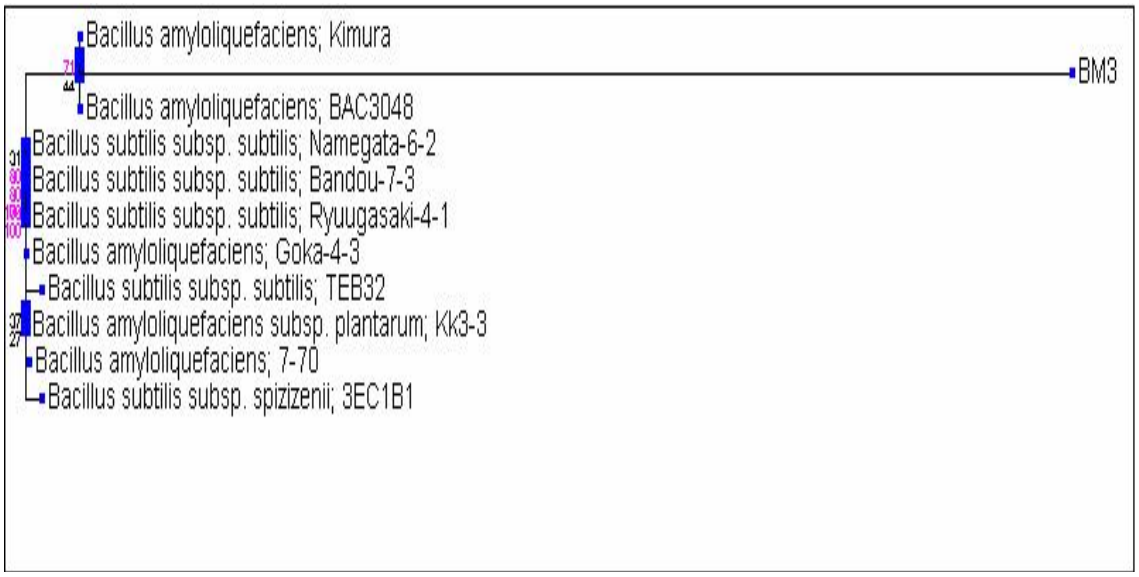
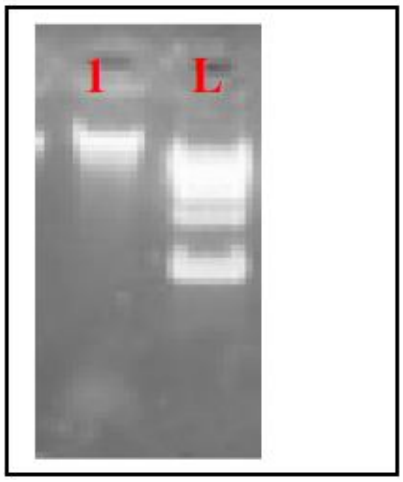


Figure 5.12.c. Phylogenetic tree based on the 16s rRNA gene sequence of 1475 bases length of the isolate BM3. Numbers at nodes indicate bootstrap values derived from 100 replications. genBank accession numbers of 16s rRNA gene sequences are indicated in parentheses.

5.17.4. Molecular characterization of D5 strain

The isolate D5 bear maximum similarity 99% and was identified as *Bacillus subtilis subsp. subtilis; PHB PTK1 (GenBank entry: HM061615).*

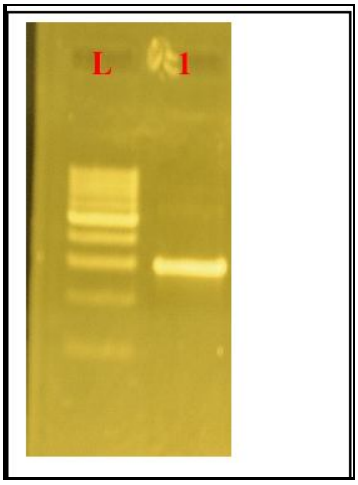
Genomic DNA isolation from D5 strain



Lane description- L: 1kb DNA Ladder (Chromous Cat. No. LAD03); 1. Sample

Figure 5.13.a. Extraction of Genomic DNA from Bacterial sample (D5) using the Bacterial Genomic DNA Isolation Kit (RKT09)

PCR amplification of isolated 16s rDNA fragment



Lane description- L: 500bp DNA ladder; 1. ~1.5kb 16srDNA fragment amplified

Figure 5.13.b. PCR amplification of ~1.5kb 16srDNA fragment from genomic DNA of the bacterial culture (D5)

Alignment of the 16s rRNA gene sequences

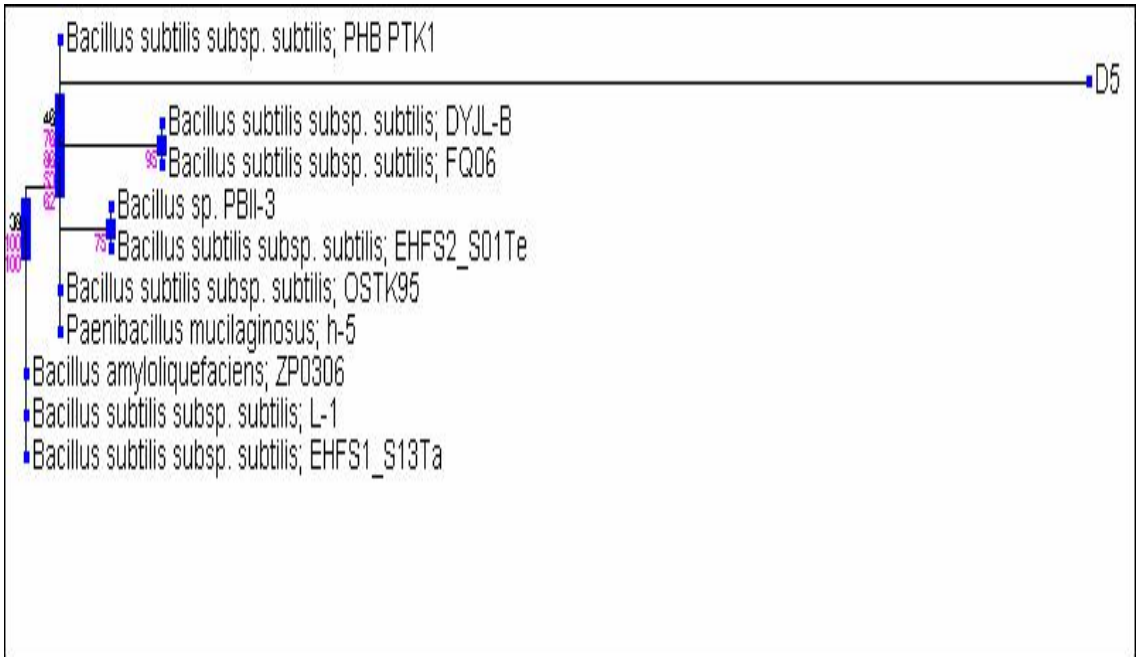


Figure 5.13.c. Phylogenetic tree based on the 16s rRNA gene sequence of 1450 bases length of the isolate D5. Numbers at nodes indicate bootstrap values derived from 100 replications. genBank accession numbers of 16s rRNA gene sequences are indicated in parentheses.

Bergey's classification of prokaryotes, well-recognized and widely used for bacterial identification, is based on the phylogeny of prokaryotes from the 16S rRNA gene. If the sequence of 16S rRNA gene of an unknown organism is >95% similar to those in the GenBank (Clarridge 2004), it is generally considered as the same genus. If the sequence of the 16S rRNA gene is >97% similar to those sequences of any cultures in the GenBack, it should be considered as the same species but may be a different strain (Embley and Stackebrandt 1994). In case of all the four isolates the sequence of the 16S rRNA gene is >97% similar to those sequences of cultures in the GenBack. So the strains have been aptly identified with its genus and species.

Molecular characterization helped in the identification of the four bacterial isolates bearing high market value. This identification would broaden the scope of further research on these microbes and its significant application.

5.18. Application of protease in stain removal

In removing blood and natural pigment stain from cloth, this protease showed greater efficiency. Due to its higher potential in removing stains from cloth it could be used in detergents as powder or solution. The washing efficiency of detergents was remarkably increased with addition of the enzyme (6 mg/ml). The protease enzyme was very effective compared to the enzymes reported earlier with respect to the short times required for complete removal of stains (Figure 5.14). Some have reported (Anwar and Saleemuddin., 1998) the effectiveness of protease on blood stain removal from cloth in the presence and absence of detergents.

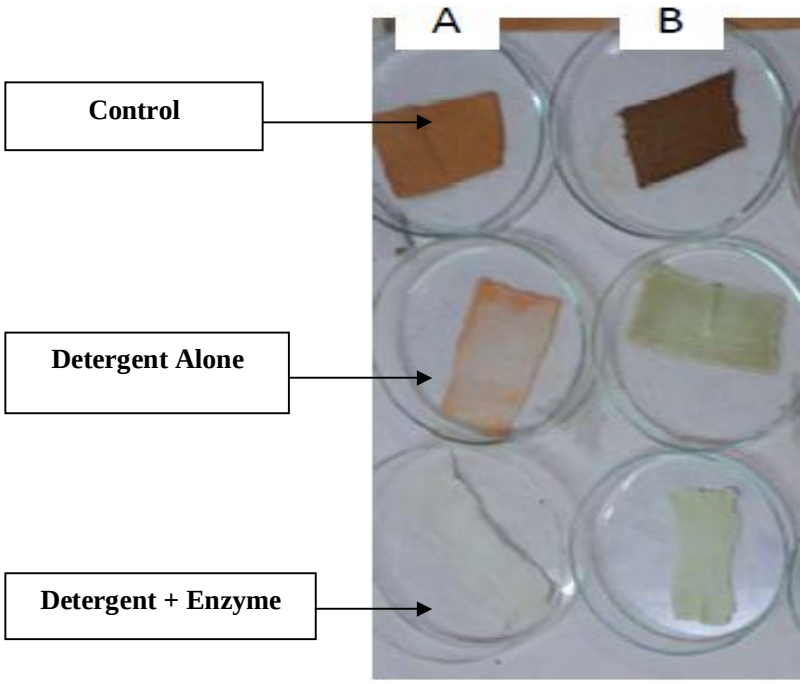
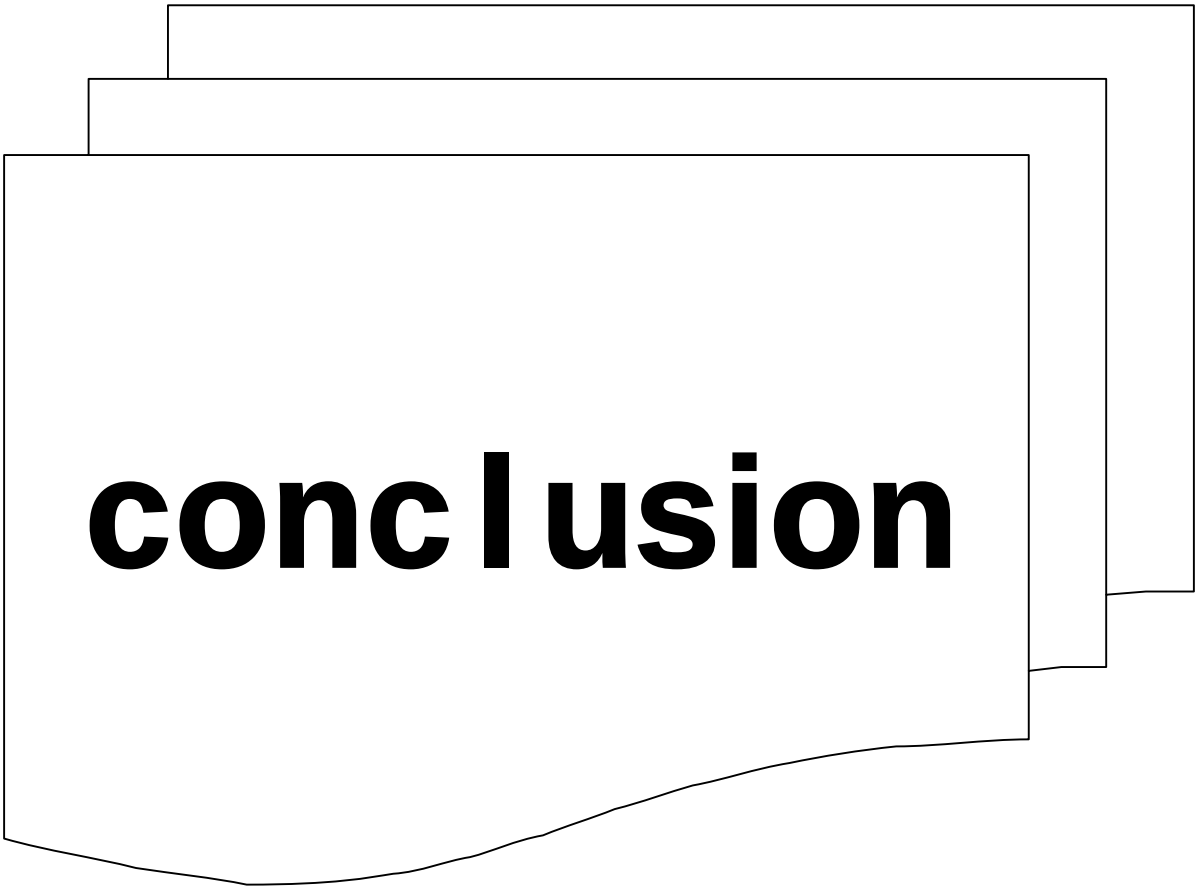


Figure 5.14. Stain (A- Blood Stain and B- Tea Stain) removal by Protease enzyme and combination of protease enzyme and detergent

Protease from *Paenibacillus mucilaginosus; s-4,*undoubtedly plays a significant role in removing tough stains. Further modified application as washing detergents and application in industrial processes through experimental trials will prove to have good market value and economical benefits.

Chapter – vi


conclusion

ConClusion

In recent times, much importance is given to the composting of municipal solid wastes because of its eco-compatibility and easy operational procedures. Successful conversion of organic matter into simpler units of organic carbon and nitrogen is the basic functional process of composting. Composting helps in managing large quantities of organic wastes in a sustainable manner. It is one of the technologies of integrated waste management strategies, used for the recycling of organic materials into a useful product. In a general solid waste stream, the overall efficiency of organic material break down depends on microbes and their activities. Microorganisms through different kinds of substrate based hydrolytic enzymes promote the degradation of organic materials. The enzymes released by the microorganisms during composting breakdown several organic compounds characterized by a complex structure, finally leading to the solubilisation of simple water soluble compounds.

Commercial enzyme production has grown during the past century in volume and number of products in response to expanding markets and increasing demand for novel biocatalysts. Microorganisms constitute the major source of enzymes. Production of a new microbial enzyme starts with screening of microorganisms for desirable activity using appropriate selection procedure. The harsh environment in which several enzymes are subjected during process applications has given impetus to screening of extremophiles for enzymes having desirable features of activity and stability. Both solid satte and sub merged fermentation are applied commercially, however the latter is preferred in many countries because of a better handle on aseptical conditions and process control. Isolation and purification, i.e. the downstream processing of the enzyme from the raw material constitute the subsequent stage of the productin process. The desired level of purification depends on the ultimate application of the purified product. The microorganisms used for enzyme production are grown in fermenters using an optimized growth medium.

Municipal solid waste being a admixture of several substrates is the most suitable habitat for diverse microbial flora with unique characteriatics. Potent and

prospective microbes could be isolated from such habitat with novel characteristics and these characteristics could be optimized for further application.

In view of the above, the present inestigation aimed of screening potent and hyperactive strains from municipal solid waste, their characterization, optimization of enzyme production and application of the isolates in degradation of the municipal solid organic waste.

From the two sample collection sites, viz., Barrackpore Municipality Waste Dumping Site and Dhapa Waste Dumping Site, samples (waste mixed with soil) were collected. Nine bacteria were screened out of this sample, transferred to pure culture and stored for future use. Physico-chemical characterization of the waste sample was undertaken. Microbiological and biochemical characterization of the bacterial isolates were carried out. Growth conditions were also optimized. Antagonism assay, heavy metal tolerance assay, antimicrobial activity assay and antibiotic sensitivity assay were also carried out for all the nine isolates. All the nine bacterial strains were then subjected to extra cellular enzyme production assay, interestingly six of them showed production of protease enzyme and 1 (BM1 strain) produced lignin peroxidase enzyme both of which have high market values. It is worth mentionable that very few bacteria exhibit the characteristics of extracellular lignin peroxidise production. Next, the six extracellular protease producer strains were subjected to qualitatitive assay of protease to screen the most efficient producer. The quantitative assay of protease invariably proved that among the six isolates, BM1 [*Paenibacillus mucilaginosus; s-4 (*GenBank accession no. is **MTCC 11281***)*] was producing the maximum protease. *Paenibacillus mucilaginosus; s-4* was the strain producing both protease and lignin peroxidise, so further study was conducted using the strain itself.

Growth characteristics of the strain was studied and then both the enzyme (protease and lignin peroxidise) production was optimized in sub merged and solid state fermentation. Appropriate media was optimized and a suitable agricultural residue (rice straw) was screened as substrate for enzyme production in solid state fermentation. Applying rice straw as substrate for enzyme production on one hand marked the cost effective production of the enzyme and also ensured utilization of waste in production of wealth. The highest level of protease (1,65,478.30 U/gds) and lignin peroxidase (1264.8293 U/gds) production was obtained when optimum

conditions were 12 hours incubation period, 37^0C temperature, pH 8, inoculums volume 4%, substrate as rice straw, 1:4 (solid :liquid) ratio and basal medial with 2%casein and 0.5%peptone under shaking (200 rpm). Response surface methodology was applied to enhance this enzyme production further. Experiments were conducted to validate the optimal condition for the production of protease and lignin peroxidase. The ensuing quadratic model predicted that the maximum production of protease was 2,20,293 U/gds, when optimum conditions were pH 7.31, incubation time 13.8 h, temperature 37.5 ^{o}C, inoculum volume 4.83 ml and solid to liquid ratio is 1: 5.2 and the maximum production of lignin peroxidase was 2,219.35 U/gds, when optimum conditions were pH 10.38, incubation time 3.9 h, temperature 41.4 ^{o}C, inoculums volume 4.93 ml and solid to liquid ratio is 1: 6.2. To verify the predicted results, valedictory experiments were performed in triplicate tests. Under the optimized condition, the observed experimental titre of average protease was 2,18,726 U/gds and that of lignin peroxidase was 2,219.35 U/gds, suggesting that experimental and predicted values of protease and lignin peroxidase yield were in good agreement. This result therefore, correlates with the predicted values and the effectiveness of the model, indicating that the optimized medium favours the production of enzyme protease and lignin peroxidase. RSM was successfully employed to achieve an overall 2.07 folds increase in protease production and 2.22 folds increase in lignin peroxidise production. The cost-effective technologies are needed for the production of enzyme and SSF is a suitable technology for economical production of proteases and lignin peroxidases using lignocellulosic residues as substrate. Major parameters affecting the fermentation process for enzyme production were studied and optimal levels were identified. It is concluded from the findings that the strategy to produce protease and lignin peroxidase from rice straw was successful as it resulted in a considerably good amount of this enzyme produced by the hyperactive bacterial strain under laboratory conditions. Furthermore, evolutionary operation factorial-design technique could be considerably effective in maximizing the yield of enzyme but all the parameter was optimized by one at a time method.

Such high enzyme yields for protease and lignin peroxidase with such optimum conditions have not been reported earlier in bacterial batch fermentations as far the

author's knowledge. This is really a novel search of enzyme activity for both protease and lignin peroxidise.

The protease enzyme was subjected to purification and characterization. The protease was revealed to be alkaline protease and The molecular weight of the enzyme under denaturing condition was estimated to be 27.5 kDa, similar to the molecular masses of alkaline proteases. Characterization of the purified alkaline protease derived from the isolate BM1 was carried out in relation to the optimal temperature, optimal pH for the enzyme activity and the substrate specificity of the enzyme. The optimum temperature and pH for the activity of the purified enzyme was 60°C and 10.5, respectively. Cytochrome C proved to be the best substrate for its activity.

This research successfully purified alkaline protease from *Paenibacillus mucilaginosus*; s-4 strain. After final purification step, the enzyme was purified 10-fold with an increase in specific activity from 15.222.685 to 1,58,220.430 U/mg protein.

Individual isolates and consortium of the selected strains were applied in suspension for bioconversion of municipal solid organic waste. Considering the results, it may be concluded that the consortium formed in combination of BM3 and D5 will be the potential one for degradation of municipal solid waste which will be used effectively to prepare compost within a short period to protect our natural environment and to get rid of solid wastes problem.

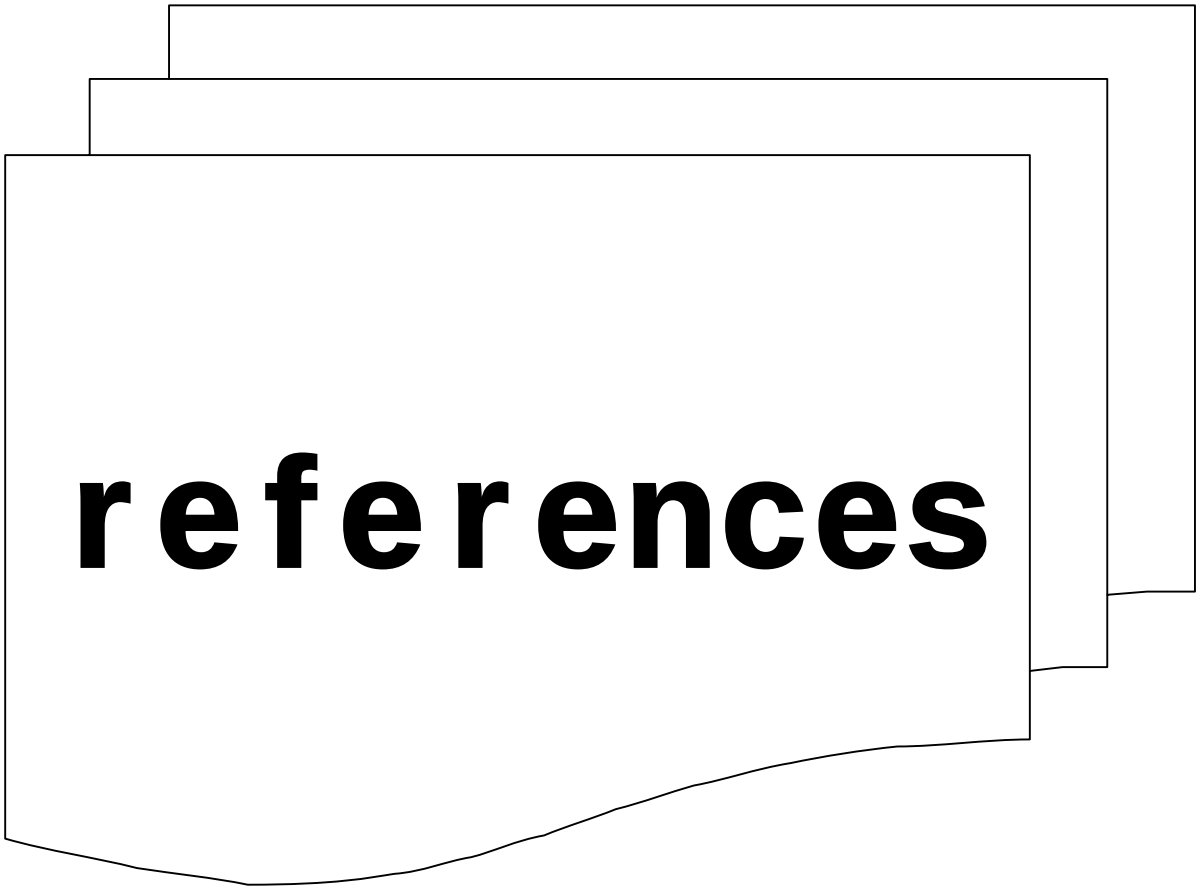
Considering the previous experimental results it was evident that BM1 is a hyperactive protease and lignin peroxidase producer with great market value, and consortium of BM3 and D5, as well as BM2 as an individual isolate have very good degradation ability. So, among the nine initial isolates, BM1, BM2, BM3 and D5 were the four potent strains which were subjected to molecular characterization for further information. The bacterial isolate BM1 bears maximum similarity 99% and identified as *Paenibacillus mucilaginosus; s-4 (*GenBank accession no. is **MTCC 11281***)*. BM2 was found to be most similar to *Bacillus sp. enrichment culture clone CBMAI_975* (GenBank entry: GQ272679). BM3 was found to be most similar to *Bacillus amyloliquefaciens; Kimura (GenBank entry: AB610803)* and identified as the same. The isolate D5 bear maximum similarity 99% and was identified as *Bacillus subtilis subsp. subtilis; PHB PTK1 (GenBank*

entry: HM061615). Molecular characterization of the potent strains would aid in successful application of the same in future.

The alkaline protease from *Paenibacillus mucilaginosus; s-4*. is significant for an industrial perspective because of its ability to function in broad pH and temperature ranges, moreover such high titer value of protease and lignin peroxidase from *Paenibacillus mucilaginosus; s-4,* has not been reported earlier elsewhere. As far the author's knowledge it is the first report of extracellular lignin peroxidase with such high titer value in any bacterial species so far reported. Thus this strain would surely serve to be an novel strain with high market potential for producing both the enzymes simultaneously in same media under same physical conditions. In addition rest of the strains characterized could be prospective for degradation of municipal solid organic waste in consortium form or asin the form of individual isolates.

Thus to conclude in a nutshell, this research invariably establishes some novel characteristics of isolates strains which are of high significance.

Chapter -vii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