ISOLATION AND SCREENING OF AMYLASE AND ALKALINE PROTEASES PRODUCING BACTERIA FROM GODAVARI ESTUARY

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DEPARTMENT OF BIOTECHNOLOGY
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DECLARATION

I Sri G.Sam Babu, Head of the Department of Biotechnology, Sri Y.N.College (Autonomous), Narasapur, West Godavari District, declare that this Research Work entitled "**ISOLATION AND SCREENING OF AMYLASE AND ALKALINE PROTEASES PRODUCING BACTERIA FROM GODAVARI ESTUARY"** has been written by me under the scheme of S.T.C. Minor Research Project In Biotechnology, during the period 2019 -2020, which has not previously formed the basis for the award of any Degree, Diploma, associate-ship or Fellowship.

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ABSTRACT

Enzymes are among the most important products obtained for human needs mainly through microbial sources. The aim of the present study was isolation, screening and optimization of the enzyme production by using the estuarine bacterium. In the present study, the growth parameters showed the profound influence on the amylase production on the candidate species such as the maximum production of the enzyme obtained at 48 hrs of incubation and the optimum was pH 7. The C for higher production of the temperature also influenced on the maximum production of the amylase, the optimum was 35 enzymes. The optimum salinity of 2.0% showed to maximum production of the amylase. In the present study, the starch and peptone was found to the best carbon and nitrogen sources to maximum production of the amylase. With optimized parameters in the mass scale medium maximum growth of 2.89 OD and enzyme activity of 64U/ml/min was obtained at 48 hrs. of incubation.

CONTENTS

Abbreviation

Abs	absorbance
rpm	revolution per minute
mg	Milli gram
mM	Milli moles
Mg	microgram
Km	Michaels menton constant
nm	nanometer
%	Percentage
Fig.	Figure
et.al	Co-author
ASW	Artificial sea water
^{o}C	Degree Celsius
TEMED	NNN'N' Tetramethyl ethylenediamine.
APS	Ammonium persulphate
SDS	Sodium Dodecyl Sulphate.
BSA	Bovine Serum Albumin
PEG	Poly ethylene glycol
EDTA	Ethylenediaminetetraaceticacid
PMSF	Phenyl methane sulphonyl fluoride
IAA	Iodo Acetic Acid

DTT Dithiothreitol

ANOVA Analysis of variance

LSD Least Significant Difference

DFP Diisopropyl fluorophosphates

E64 L-trans- epoxysuccinyl- leucylamido (4-guanidino butane)

ONPG 0- Nitrophenyl-B-D Galactopyranoside

HPLC High Performance Liquid Chromatography

IEC lon Exchange Chromatography

INTRODUCTION

Enzymes are well known biocatalysts that perform a multitude of Chemical reactions and are characterized by their extraordinary specificity and reactivity. They occur in all living systems where they activate and regulate chemical reactions essential to the continued existence of the individual organism. Many microorganisms produce extracellular enzymes, which are chiefly hydrolases and are involved in the degradation of macromolecules to simpler units like peptides and amino acids. Over the past two decades considerable research has been undertaken to discover industrially useful enzymes and to increase their yield by environmental and genetic manipulations. Enzymes have now become very much a part of new industrial processes and appear to offer a great potential in a wide variety of as yet undeveloped applications.

The current estimated value of the worldwide sales of industrial enzymes is $ 1 billion (Godfrey and West, 1996). Of the industrial enzymes 75% are hydrolytic. Proteases represent one of the three largest groups of industrial enzymes.

Proteases belong to a class of enzymes, which occupies a pivotal position with respect to their application in both physiological and commercial fields. Proteolytic enzymes catalyse the cleavage of peptide bonds in proteins. Proteases are degradative enzymes, which catalyse the total hydrolysis of proteins. Advances in analytical techniques have demonstrated that proteases conduct highly specific and selective modifications of proteins such as,

activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots and processing and transport of secretory proteins across the membranes. Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level to produce cascade systems such as haemostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal path physiological conditions. The vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Poldermans, 1990; Fox et al., 1991).

Since proteases are physiologically necessary for living organisms they are ubiquitous, being found in a wide diversity of sources such as plants,animals and microorganisms. Important plant proteases are papain, obtained from the latex of Carica papaya fruits, bromelain, prepared from the stem and juice of pineapples, some keratinases and ficin. The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and rennins. The production and use of plant and animal proteases depend on several factors such as eavailability of land for cultivation, the suitability of climatic conditions for growth,

availability of livestock for slaughter which in turn, is governed by political and agricultural policies. The inability of plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases.

Microorganisms elaborate a large variety of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes. Extracellular proteases are significant for the hydrolysis of proteins in cell-free environments and they enable the cell to absorb and utilize hydrolytic products. At the same time, these extracellular proteases are of commercial value and exploited in various industrial sectors. Microorganisms serve as excellent sources of commercial enzymes as they can be cultured in large quantities, in a relatively short time by established methods of fermentation and they produce abundant regular supply of the desired product and also. Owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbes account for a two third share of commercial protease production in the world (Kumar and Takagi, 1999).

Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess all the characteristics desired for their biotechnological applications. Proteases are grossly subdivided into two major groups viz. exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxytermini of the substrate whereas, endopeptidases cleave peptide bondsdistant from the termini of the substrates. Microbial proteases are classified into three groups based on their pH optima of activity. They are acidic proteases, neutral proteases and alkaline proteases (Keay, 1971). A more rational system is now based on the comparison of active sites, mechanism of action and three-dimensional structure (Neurath, 1989). Four mechanistic classes of proteases are recognized by the International Union of Biochemistry, and within these classes, six families of proteases are recognized to date. The four classes are, serine proteases, aspartic proteases, cysteine proteases and metalloproteases

Many other proteolytic enzymes have been identified and isolated that do not fit into this c|assification(Lorand, 1976; Colowick and Lorand, 1980).

Proteases have a large variety of applications mainly in the detergent and food industries. In view of the recent trend of developing environment friendly technologies, proteases are envisaged to have extensive applications in leathe treatment and in several bioremediation processes. The worldwide requirement of enzymes for industrial applications varies considerably. Proteases are used extensively in pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations whereas, those that are used in medicines are produced in small amounts but require extensive purification before they can be used. Today proteases account for approximately 40% of the total enzyme sales in various industrial market sectors such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery. This dominance in the industrial market is expected to increase further by the year 2005 (Godfrey and West, 1996). However, until today the largest share of the enzyme market has been held by detergent alkaline proteases that are active and stable in the alkaline pH range.

Alkaline proteases

Alkaline proteases are defined as those proteases, which are active in a neutral to alkaline pH range (Gupta et al., 2002a). They are either serine proteases or metalloproteases, and the alkaline serine proteases are the most important group of enzymes exploited commercially.

Alkaline proteases are of considerable interest in view of their activity and stability at alkaline pH. Alkaline proteases belong to a physiologically and

commercially important group of enzymes used primarily as detergent additives. They play a specific catalytic role in the hydrolysis of proteins. The use of alkaline proteases in laundry industry accounts for approximately 25% of the total worldwide sale of enzymes. An upward trend in the use of alkaline protease is expected by the turn of the decade.

Although many microbial species are known to produce alkaline proteases, only a few are recognized as commercial producers. Most of them belong to the genus Bacillus (Rao et al., 1998). Bacterial alkaline proteases are characterized by their high activity at alkaline pH, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry. Looking into the vast diversity of microbial population, there is always a chance of finding microorganisms producing novel enzymes with better properties that are suitable for commercial applications. The multitude of physicochemical diverse habitats has challenged nature to develop equally numerous molecular adaptations in the microbial world. Microbial diversity is a major resource for biotechnological products and processes. Microbes have developed a wealth of physiological and molecular adaptations that enabled their survival in virtually every environmental niche, some so extreme and in hospitable that no other life form could coexist. The versatility and adaptive power of the prokaryotic design was such that with their evolutionary head start, the bacterial and archaeal

kingdom produced a degree of, organism and molecular diversity unparalleled in nature. Microbial biodiversity is our planets greatest. but least developed resource for biotechnological innovations. The biosphere is dominated by microorganisms, yet todate majority of microbes in nature have not been studied.

Microorganisms distributed in the marine and brackish environments play an important role in the decomposition of organic matter and mineralisation in the system (Seshadri and lgnacimuthu, 2002). Estuary is one of the most productive ecosystems, at the same time one among the least explored ecosystems on earth, which has immense potential as a source of potent microorganisms that produce valuable compounds particularly, enzymes such as proteases. In this scenario, it is very appropriate to embark on finding novel alkaline protease producers from the estuarine system. The area where the present investigation was carried out, is a part of the extensive estuarine system of South India viz. Godavari Estuary. There is meagre knowledge regarding the microbial composition, particularly the protease producers of Godavari Estuary. Hence, the present study has been undertaken with the objective of finding novel alkaline protease producing bacteria from Godavari Estuary.

Amylase

Amylase is an important and indispensable enzyme that plays a pivotal role in the field of biotechnology. It is produced mainly from microbial sources and is used in many industries. Industrial sectors with top-down and bottom-up approaches are currently focusing on improving microbial amylase production levels by implementing bioengineering technologies. The further support of energy consumption studies, such as those on thermodynamics, pinch technology, and environment-friendly technologies, has hastened the large-scale production of the enzyme. Herein, the importance of microbial (bacteria and fungi) amylase is discussed along with its production methods from the laboratory to industrial scales.

Objectives of the study

The major objectives of the present study are: -

- Isolation and identification of alkaline protease and amylase producing bacteria from Godavari Estuary.
- Selection of the most potent strains, capable of producing alkaline proteases.
- Purification and characterization of the selected proteases.
- Application of the selected proteases in detergent formulations

ISOLATION AND SCREENING OF AMYLASE AND PROTEASE PRODUCING BACTERIA

Aquatic environment is an ideal habitat for the growth of microorganisms. Bacteria are widely distributed in aquatic habitats, which are represented by almost all taxonomic groups. A sharp separation of them into soil bacteria and aquatic bacteria is not possible as the inland waters are constantly exposed to the flora of the soil. Interesting bacteriological conversions occur at the edges of huge aquatic ecosystems like the sea, the estuaries, marshlands, salt marshes etc. The particulate organic matter reaching the estuary finally finds its way into the sediment where they undergo transformation. Except a few millimeters in the upper layer the sediment hosts an anaerobic environment. The organic matter accumulating in the sediment as detritus contains mainly protein and carbohydrates, and lipids are found in small quantities. Ubiquitous nature of phosphates reaching the estuaries probably makes it the main cause of eutrophication. The bacteria harboured in the sediment water transitional zone play a key role in the degradation of organic matter reaching the sediment and thus the replenishment of the essential nutrients in the aquatic system. Bacteria have highly efficient extracellular enzyme systems such as proteases, amylases, lipases and phosphatases, in order to catabolize complex materials into simpler fractions. Hence bacteria and other microorganisms play a significant role in the ocean as they do in terrestrial environments but only meager information is available about their occurrence and activities.

Estuaries are considered as the most productive ecosystems on earth. The backwaters of Andhra Pradesh support as much biological productivity as the tropical rain forests. A Godavari backwater is one among them, which has been subjected to intense biological research. Godavari estuary.

The river Godavari which is the second largest in India, of about 1465 KMS. traverses the states of Maharastra and Andhra Pradesh and opens into the Bay of Bengal on the east coast. It has a catch1l1ent area of about 3,12,812 Sq. Kms. which lies in the States of Maharastra, Madhya Pradesh, Karnataka, Orissa and Andhra Pradesh. Raising at TrialTIbak which is about 110 Kms. north-cast of Bombay, it receives several small tributaries and assumes imposing proportions towards its lower reaches. At Rajahmundry, it is about 3.2 Kms. wide and further down at the head of the delta near Dhowleswaram, a masonary Dam and recently a barrage was constructed in four sections across the river. At Dhowleswaram which is about 63 Kms. in a straight line from the sea, the river divides into two principal branches namely the Vasistha Godavari to the west and Gautami Godavari to the cast. They flow through a wide delta into the sea. The Vasistha Godavari inturn branches at Gannavaram into two namely Vasistha Godavari to the west and Vaintheyanl Godavari to the east both opening into the Bay of Bengal independently. The Gautami Godavari flows south-east and opens into the Bay of Bengal at two places south of Yanam, namely Kottapalem and Bhairavapalem villages. The Gautami Godavari is also

connected to the big Kakinada Bay by two channels namely the Coringa arising at Yanam and the Gaderu arising at Bhairavapalem.

The Gautami Godavari estuary is a complex estuarine system due to its proximity and its connections to the vast Kakinada Bay into which the Coringa and Gaderu rivers (arising from Gautami Godavari) opens forming a part of the estuarine system. There are extensive mud flat and dense mangrove forests on the southern side of the Bay. The two rivers Coringa and Gaderu through which some water of the Gautami Godavari is drained into the Kakinada Bay during south west mansoon period. Dense mangrove vegetation is also noticed on the banks of both the rivers and in the extensive swampy areas surrounding between the two rivers. The swampy area is also traversed by a net work of creeks and canals connecting the two rivers. The Gautami Godavari estuary alongwith river Gadcru and Coringa forms a complex estuarine system as their hydrological conditions are affected by tidal influence from two sides. i.e. north through Kakinada Bay and South. through Godavari river. The opening of Gautami Godavari at Kottapalcm and near Bhairavapalem are without much mangrove vegetation and the estuarine part of this branch of Godavari normally extends upto village Kotipalli which about 20 Kms. Upstream from the mouth.

The remaining two branches of the Godavari i.e. middle one, Vaintheyanl branch opens at Vodalarevu (near Amalapuram) without forming any swampy-

mangrove habitats, while the southern most branch, Vasistha opens into the Bay of Bengal at Antervedi. ncar Narsapur town. Where also good mangrove formations not noticed. The estuarine waters of this branch extends upto about 15 Kms. upstream i.e. Narsapur. The estuarine ecosystem is a dynamic one, influenced by the daily tidal cycle of the sea on one side and the seasonal changes of the river flow during summer/rainy seasonal floods on the other; thereby influencing the qualitative nature of the estuarine fauna markedly. Therefore for the complete assessment of the faunal diversity of the Godavari Estuary, about 7 faunistic surveys were conducted in this area during premonsoon, monsoon and post monsoon periods of 1992-95. Since this study is aimed at faunal exploration, maximum collection sites were fixed at mount area of the estuaries and also at different localities along the estuarine course of three branches of the river Godavari, Coringa and Gaderu as well. From about 27 collection sites different faunistic constituents were collected during this study and at each collection 'centre some physico-chemical parameters viz. temperature, salinity, pH, sediment nature etc. were noted at the time of collection which gives an understanding of the nature of the habitat, ecology of the fauna concerned

Yanam

Near Kakinada, it is under the state of Pondicherry. It is a small town about 30 Kms. south of Kakinada town on the bank of river Gautami Godavari. There is a direct road from Kakinada to Yanam; River Godavari is very wide here. Beach is sandy with very little mud.Fauna is very less and there is no mangrove vegetation.

Bhairavapalem

This fishing village is about 10 Kms. from B. V. Palem (on the way of Yanam) by boat. The Gaderu river joins Godavari here. The mangrove swamps on either side of Gaderu are dense. This is actually the mouth of Gautami-Godavari branch of Godavari river opening into Bay of Bengal. This area is completely muddy and sandy towards the sea beach.

Gadimoga

This is also a fishing village about 3 Kms. from Bhairavapalem. Mangrove plants present on both sides of the Godavari river. Shore is muddy.

Dariyala Tippa

This is an island formation of sandy-clay in nature of about 3Kms. From Bhairdvapalem by boat towards the upstream of the river Godavari. Mangrove forest is scattered. Shore is a mixture of sand/mud. It is a good fishing area.

Boddu Venkatapalem (B. V. Palem) This is a small fishing village situated on the bank of Coringa river about 16 Kms. south of Kakinada town on the way to Yanam. Mangrove swamps are best approached from this place by boat.

Coringa

This is a small fishing village about 13 Kms. from Kakinada town on the way to Yanam. Coringa river passes by the side of this village and the vast mangrove-estuarine complex at this area is being protected as Coringa wild life sanctuary

Antervedi Ferry Point

"This is situated 12 Kms. from Narsapur and approachable from Narsapur Ferry point by boat. This is the largest fish landing station of Vasistha Godavari. This station is nearer to river mouth. Tidal influence is significant here. No significant mangrove vegetation is observed. The coast is sandy while the upstream sediments consist of a mixture of sand and mud.


THE GODAVARI RIVER COURSE
CHINA
PAKISTAN
NEPAL
BHUTAN
BANGLADESH
MYANMAR
INDIA
Arabian Sea
Bay of Bengal
INDIAN OCEAN
Yarlung (Brahmaputra)
Indus
Jhelum
Chenab
Ravi
Beas
Sutlej
Yamuna
Ganges
Ghaghara
Kosi
Teesta
Manas
Brahmaputra
Barak
Chambal
Banas
Betwa
Son
Parbati
Mahi
Narmada
Tapi
Mahanadi
Brahmani
Tel
Indravati
Godavari
Krishna
Bhima
Tungabhadra
Penner
Palar
Cauvery
Kolidam
Malegaon
Jalna
Chandrapur
Ahmadnagar
Parbhani
Nanded
Latur
Nizamabad
Karimnagar
Bidar
Warangal
Rajahmundry
Kakinada
Eluru
Machilipatnam
International Boundary
State Boundary
Rivers
Major Cities on Course
Map not to Scale
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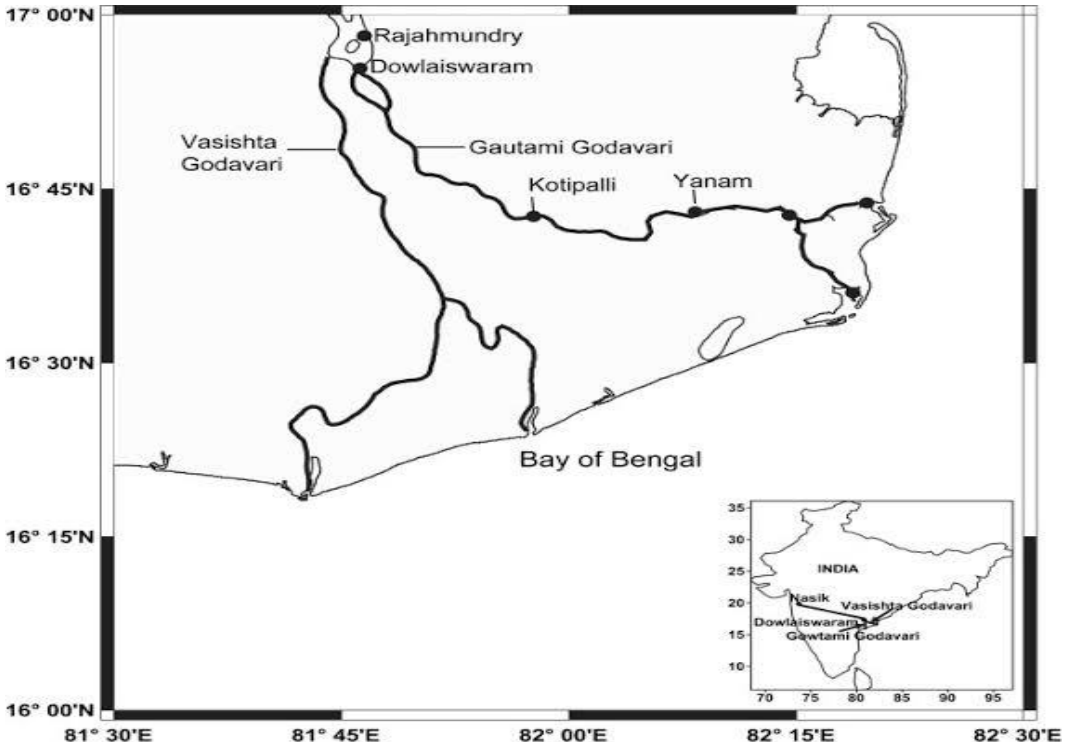
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Rajahmundry
Dowlaiswaram
Vasishta Godavari
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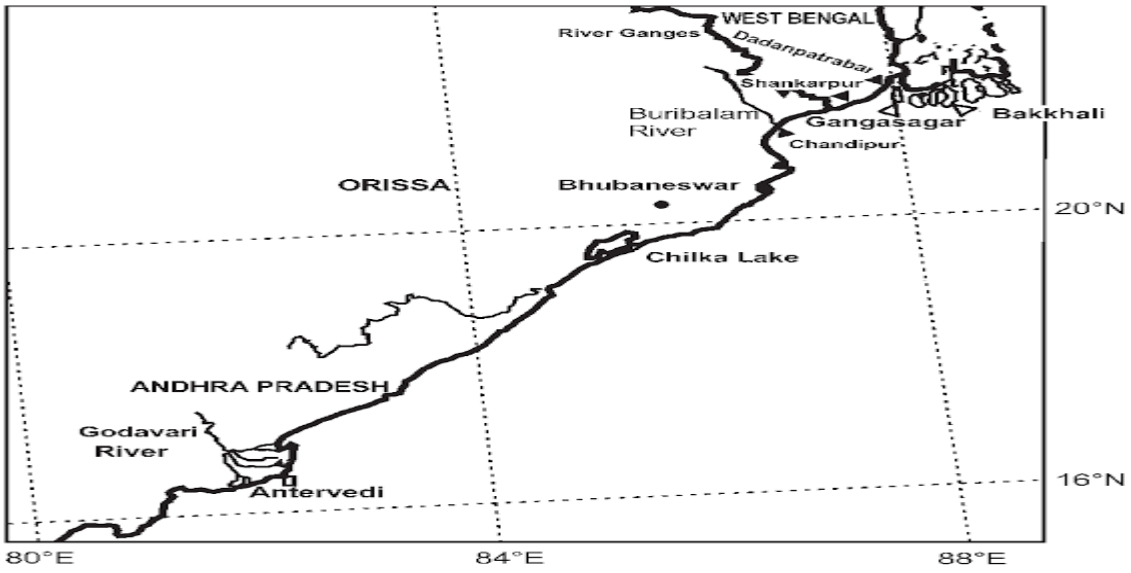
Bay of Bengal
INDIA
Nasik
Vasishta Godavari
Dowlaiswaram
Gautami Godavari


WEST BENGAL
River Ganges
Dadanpatrabar
Shankarpur
Buribalam River
Gangasagar
Bakkhali
Chandipur
ORISSA
Bhubaneswar
20°N
Chilka Lake
ANDHRA PRADESH
Godavari River
Antervedi
16°N
80°E
84°E
88°E

REVIEW OF LITERATURE

The main objective of the literature survey is to better understand the previous works on the present study area. It is also provides a good research design to carry out the study. In every research, survey of the literature plays a key role in understanding past researcher's thoughts and their deliberations. Scientific and systematic reviews provide a wide scope for constructing a good research design, planning and programming. Collection and study of literature on the relevant research area help to analyze the data with clarity and clear presentation of observations. Statistical computations give support in the analysis of the research work, scope for predictions and hypotheses and to achieve the objectives of the study. Selection of the study area and development of methodology are dependent on the suitable and authentic source of information. The technique of analysis depends on the gathered and generated data. So the most recent and advanced methods of statistical computations are essential for effective analysis of the data. Ecological studies on vegetation cover inventories and enumeration of plant species to reveal their diversity and distribution. The vegetation analysis varies with the study area, particularly it depends on the type of vegetation at different ecological zones i.e., forests, crop lands, waste lands, road side vegetation, sand dunes, coastal vegetation, estuarine vegetation, mangrove vegetation etc. The objective of vegetation

analysis varies with the utility as well as the identification of the diversity, dominance and frequency of different species and their significance in the particular ecological zone. The studies on the influence of topography over distribution revealed that the Avicennia, Aegiceras preferred low lying areas, the Ceriops –Dalbergia association preferred high land areas while Ceriops decandra and Excoecaria spread overall salinity ranges and topography types (Motilal and Mukherjee, 1984). Similar studies on the distribution and zonation of Coringa mangroves in Godavari delta were made by Azariah et al., (1992). As per the study, three dominant mangrove plants, Avicennia marina, Excoecaria agallocha and Sonneratia apetala were found to be present on the banks of a major channel of the Godavari river running through the forest. The area behind the belt consisting of Acanthus ilicifolius and Myriostachya wightiana is generally colonized by E. agallocha and A. marina. The zone has been called the Avicennia and Excoecariazone. Adjacent to this zone species like Aegiceras corniculatum and A. officinalis were the common species. In the flat clayey soil, Suaeda maritima was found to grow. In areas of high elevation, devoid of inundation of tidal seawater during the high tidal period, species such as M. wightiana and Acanthus were found to colonize both the banks of the channels. An analysis of species diversity indicated a definite trend in the distribution of mangrove from the mouth of the estuarine region of the inland waters. Similar variations are also studied elsewhere Umamaheswara Rao and

Narashimha Rao, 1988, Ghosh et al., 1990; Ammarsinghe and Balasubramanian, 1992; Anon, 1993. Mangroves are distributed circumtropically, occurring in 112 countries and territories. According to Scott (1991), globally an area of 14 million ha was covered by mangrove forests, which spread along the coasts consisting of third world nations. Indian Ocean and west pacific region together account for 20 percent of the world's total area of mangroves. Later, global coverage has been variously estimated by different authors - 10 million hectares (Bunt, 1999), 14-15 million hectares (Schwamborn and Saint-Paul 1996) and 24 million hectares (Twilley, et al., 1992). Spalding in 1997 had given an estimation of coverage of world mangroves over 18 million hectares, with 41.4% of it in south and southeast Asia and 23.5% of it in Indonesia. Krishnamurthy et al. (1987) observed that the Indian coastline mangroves are about 7% of the world mangroves while Untawale (1987) estimated it as 8%. The extent of mangrove forest cover in India is probably the third largest formation of the world after Indonesia and Australia. (Banerjee,1998). Earlier studies, Qureshi (1957), Sidhu (1963), Blasco (1975) and Chapman (1976) estimated the extents of areas covered by mangroves along the Indian coast. In those studies, remarkable variations with regard to percentage distribution of mangrove areas were observed. The mangroves of west coast constitute 12% of Indian coastal mangroves and the east coast (including Andaman and Nicobar Islands) mangroves form 88% of it. Sidhu

(1963) also reported that mangrove forests on east coast possessed relatively low lying alluvial flood plains with well preserved deltas as could be seen at Bitrakanika in the delta of Mahanadi and Coringa in the delta of Gautami Godavari. Blasco (1977) analysed that the distribution pattern of mangrove vegetation of the Indian subcontinent, extending over an area of 356,500 ha, revealed that more than 80% of Indian Mangals were confined to West Bengal and the islands of Bay of Bengal, whereas the remaining 20% had a sporadic appearance on either side of the Indian coasts. Blasco (1977) also observed that Indian mangal formations were distributed at the larger deltaic river mouths of Sunderbans of Ganges in Bengal, Mahanadi of Orissa, Godavari and Krishna mangroves of Andhra Pradesh, Cauvery delta of Tamilnadu and Andaman and Nicobar islands. The Krishna-Godavari deltaic mangroves hold the third position out of the four major Indian deltas. Ganapathy (2002) opined that Indian mangroves are bestowed with a coastline of 8,000 km, including certain stretches along which mangroves are distributed intermittently. The latest assessment of the Forest Survey of India (FSI) 2011 shows that mangrove cover in the country is 4639 Sq.km, which is 0.14% of the country's total geographic area. The very dense mangrove comprises 1405 Sq.km (30.29% of mangrove cover); moderately dense mangroves are 1659 Sq.km (35.76%), while open mangrove covers an area of 1575 Sq.km (33.95%). In recent years, the country has recorded an increase in mangrove cover by 58 Sq.km. It shows that West

Bengal has the highest share (46.4%), followed by Gujarat (22.5%), Andaman and Nicobar Islands (13.3%) and Andhra Pradesh (7.6%). The status of mangrove cover in rest of the states has been showing slight improvements particularly in Maharashtra, Orissa, and Tamilnadu. According to Thom (1982), Indian mangrove habitats are classified into three categories i.e., deltaic mangrove, coastal mangrove and island mangrove habitats. Distribution and the status of 12 mangrove habitats in India were estimated by Government of India based on the reports of Blasco (1977), Untavale and Jagtap (1992), Mandal (1996), Sandhya et al (1998). Tomlinson (1986) dealt with the ecology, floristic and distribution of the world mangroves and their associates. While Hogarth (1999) studied the biology of mangroves, Duke (1992 and 1995) worked on their evolution, distribution, systematics and phenology. From mid-20th century till now, floristics, vegetation ecology, silviculture and conservation aspects of Indian mangroves were dealt by several researchers such as Naskar (1996), Spalding et al. (1997), Banerjee and Ghosh (1998), Naskar and Mandal (1999), Untawale and Jagtap (1999), Kathiresan (2000, 2002 and 2004), Kathiresan and Bingham (2001), Nayak and Bahuguna (2001), Upadhyay et al. (2002), Selvam et al. (2003), Kathiresan and Rajendran (2005) and Kumaran et al. (2005). Banerjee et al. (1998) and Naskar (2004) studied the mangroves of India and prepared detailed manuals. The lack of systematic and ecological studies and availability of only fragmented information on Indian mangrove ecosystem

warrants a comprehensive research plan (Upadhyay et al., 2002). The present study is to identify the diversity of mangrove vegetation, to analyze the distribution, diversity, dominance of different mangrove species in the study regions. So far, studies on mangroves are concentrated in Gouthami Godavari mangroves (East Godavari district), Krishna mangroves (Krishna District) and other parts of India. However, there is less focus on the Guntur region with regard to the mangrove vegetation. In future perspective, the anthropogenic impacts lead to the possible threats for mangrove vegetation in Guntur region. Therefore, the fragile mangrove ecosystem needs conservation and appropriate management practices to restore and regenerate the mangrove vegetation. Hence, the present research is taken up on the mangrove vegetation in the two main Zones of the Southern fringes of Krishna estuary of Guntur District with 12 field stations viz. Gokarnamatam, Nizampatnam, Ex-military colony, Kallipalem, Palarevu, Konapalem, Parisevaripalem, Dindi, Jampanivaripalem, Sanjeeva nagar, Nakshtranagar and Saradhalabor colony. Mangroves comprise salt tolerant plant species that occur along inter-tidal zones of rivers and seas in the form of narrow strips or as extensive patches in estuarine habitats and river deltas of tropical and sub-tropical regions. These plants have special adaptations such as stilt roots, viviparous germination, salt-excreting leaves, breathing roots, knee roots by which these plants survive in water-logged, anaerobic saline soils of coastal environments. Rahaman (1990), Swaminathan (1991) and Moorthy &

Kathiresan (1996) observed that the mangrove plants have a great potential to adapt to the changes in climate, rise in sea levels and to solar ultraviolet–B radiation. Vegetation analysis and community structure of mangroves were studied by Natividad (2015) through cluster grouping. In this study, community structure was analyzed using multivariate analysis performing non-metric multi-dimensional scaling (MDS), cluster analysis and one way ANOSIM. The present study also correlated the results in the similar method. The concept of 'Important Value Index (IVI)' has been developed for expressing the dominance and ecological success of any species, with a single value (Mishra, 1968). This index utilizes three characteristics such as relative frequency, relative density and relative abundance. Studies on the ecology of mangrove vegetation and review reports of mangroves in Indian subcontinent appeared from time to time by different authors - Gamble, (1915- 35); Cornwell, (1937); Champion and Seth, (1968); Caratinal et al., (1973); Krishna Murthy et al. (1981); Gogate,(1984). Banerjee (2002) reported that the Krishna-Godavari delta of Andhra Pradesh is spread over an area of 585 Sq.km with a mangrove cover of 251 Sq.km. The major mangrove areas in this delta are Machilipatnam, Sorlagondi, Nachugunta, Yelichetladibba and the other important mangrove areas are Coringa, Kandikuppa, Salagondi, Yanam, Antharvedi, Repalle and Bandamuslanka. Later, in 2011 (FSI report), it is reported that Andhra Pradesh has a mangrove cover of 354 Sq.km, out of which Krishna- Godavari delta has

347 Sq.km. The remaining 7 Sq.km mangrove cover is present in Prakasam district. Floral composition of Godavari mangroves were studied by Venkateswarlu (1944) , Banerji (1957) Puri and Jain (1958) , Rao (1959), Sidhu (1963) , Raju (1968), Chapman (1976) and Rao and Rao (1992), and Brahmaji Rao (1998). They described that Godavari mangrove contained impressive grassy pastures with extensive mud banks, covered by Avicennia marina and Avicennia officinalis. Satyanarayana et al. (2002) studied the floristics and zonation pattern of Coringa mangroves. Occurrence of a new mangrove species, namely, Xylocarpus mekongensis was recorded in the Godavari estuarine region by Raju (2003). The delta was once dominated by Avicennia officinalis, Excoecaria agallocha and Lumnitzera racemosa constituting 90% of the vegetation cover, but subsequently, the same dwindled to 37% while Suaeda maritima and Suaeda monoica occupy 40% share (Selvam et al., 2003). Ramasubramanian et al. (2003) and Ravishankar et al. (2004) published a detailed account of the mangroves of Andhra Pradesh and reported the presence of 16 mangroves and 19 associates. Pioneering investigation of mangrove flora at Krishna delta was conducted by Cornwell (1937) and Venkateswarlu (1944). The delta was also studied by Rolla S. Rao (1959), Waheed Khan (1959), Rao T.A, et al (1974), Blasco (1975), Reddy C.S (1982). Maginnis and Elliott (2005) discovered that the forests in the area were relatively rich in mangrove species and that “these species-rich stands were considerably taller and denser than

stands elsewhere that were dominated by just a few species". Keith Forbes and Jeremy Broadhead (2007) opined that tree species diversity was an important factor in determining the degree of protection. According to WWF-India (2005), Krishna mangroves were completely unaffected by the tsunami, despite its vulnerable location near the mouth of Krishna river in Diviseema region. Mangrove vegetation comprises approximately 59 species 41 genera, of which 34 species 29 genera are present in India. This includes 25 species along the east coast and 25 species on the west coast as cited by Banerjee et al., (1989); Singh (1990); Deshmukh (1994). East coast mangroves represent 51 species, 41 genera belonging to 29 families. Venkateswarlu (1944), Mathauda (1957), Rao (1959), Sidhu (1963)]. Recent estimates by Mandal & Naskar (2008) reveal that 82 species of mangroves are distributed in 52 genera and 36 families in all the 12 habitats in India. Species composition has been reported from Godavari and Krishna Mangroves by Venkateswarlu (1944), Mathauda (1957), Rao (1959), Sidhu (1963), Blasco (1975), Lakshman (1984), Umamaheswara Rao and Narasimha Rao (1988). 26 species are observed in Coringa mangrove forest of Goutami Godavari deltaic region (Brahmaji Rao 1998). Phenomenon of zonation (composition of vegetation and distribution) is one of the characteristics of most of the mangrove forests. First comprehensive account on vegetation was given by Prain (1903), who divided Sunderban mangroves into three major regions viz., Southern, Northern and Central zones. Curtis (1933)

observed three zones in Sunderbans such as Fresh water forest, moderately salt water forest and salt water forest basing on salinity gradient. Champion and Seth (1968) classified the tidal forest of India into: (a) Low Mangrove forests, (b) Salt water-Hiretiera forest and (c) Fresh water Heriteria forest. Banerji (1957) identified five distinct zones in Coringa mangrove, on the basis of tidal inundation. Various workers classified the mangrove areas into different zones based on the dominance of a specific factor. Watson (1928) and Thanikaimoni (1980) followed inundation and salinity as a basis; Untawale (1987) pH, salinity of soil and salinity of water. Thanikaimoni (1980) stated that zonation in mangroves is disrupted mainly due to human interference and results in mixed zones. Bunt et al. (1982) noticed that some species common at the mouth of an estuary were not present near the fresh and more riverine head water region. Salient features of mangrove forests and their zonation pattern were well summarized by Smith (1992) in relation to succession and land building, Geomorphological control, physiological adaptations and inter specific interaction, while Rao and Rao (1992) divided entire Godavari deltaic mangroves into three major zones basing on floral composition studies. Coringa mangrove forest of Goutami Godavari deltaic region was classified into three zones for the studies of floral composition by Brahmaji Rao (1998). The zonation pattern in the study area is useful for the purpose of simplifying the field study. Accordingly the present work carried at two zones is classified into

four regions (main field stations) and each region is divided into three field stations. The studies of floral composition are compared among the field stations. Similar Zonation pattern is followed in the present study based on dense vegetation i.e. (Zone - I) and fragmented vegetation (Zone - II). Studies were mainly concentrated to estimate the distribution pattern, floristic composition, relative importance of each species rather than a quantitative analysis in relation to quality of mangrove habitats. The lacuna in quantification of mangrove vegetation with its environmental quality to conduct research studies, region-wise, for Better Management Practices (BMP). Mangrove forests and their long standing values have been recognized from ancient times (since 6500 B.C) as cited in Walsh (1977) from archaeological evidences and demonstrated by Sarma (1973). They were used as food, fuel, and medicine and tanning leather. Oviedo (1526) and Clausius (1601) stated that natives of the West Indies used the hypocotyls of Rhizophora seedlings as food in times of famine. Crossland (1903) describe the use of mangrove wood by Arab's for houses and furniture construction. Macnae (1968) observed that the sea going vessels from the shores of Gulf of Oman had keels of mangrove wood and the poles of Rhizophora mucronata, Bruguiera gymnorrhiza, were used for construction of buildings in Arabian cities. Chapman (1970) speculated that early man carried mangrove propagules from South America to Southern Pacific Oceans to serve as seed material for trees that produce tannins and

wood. The Indian subcontinent anecdotal studies are studied by Chatarjee (1958), Sidhu (1963), Ahmed (1964), Chapman (1976), Lakshman (1984), Untawale (1984) and Dagar (1988), Rao and Rao (1992). All these scientists recognized that the mangrove ecosystems had been an important source of livelihood, subsistence economy and were the most exploitable for the traditional use of aquaculture and agriculture practices. The values of mangrove habitats and its wildlife importance were evident from the studies of Macnae (1968); Saenger et al. (1983); Choudhury (1988); Prasad (1992) and Mittal (1993). A detailed account of review for different regions of the mangrove plant species composition and utilization pattern was evident from the studies of Watson (1928); Chatarjee (1958); Ahmed (1964); Walsh (1977); Chapman (1976) and Dagar et al. (1988). Utilization of mangrove habitats and its resource values were recognized and classified from many parts of the mangrove regions Soegierto (1980); Librero (1984); Uthoff (1996); Mastaller (1996); and Uibrig (1996). Kathiresan and Thangam (l987) stated that the sacred tree Excoecaria agallocha exudes an acrid latex that is injurious to human eyes. Reportedly the latex has a 'knockdown' effect on a variety of marine organisms under laboratory conditions at a concentration as low as 10-9 ppm. This was the basis for testing mangrove plants on mosquitoes, which transmit dreadful human diseases. Qasim (l998) observed that Avicennia species were cheap and nutritive feed for buffaloes, sheep, goats and camels. These animals are allowed

to graze in mangrove areas and camels are periodically taken to uninhabited islands with a good mangrove cover for grazing. This is very common in India, Pakistan, Persian Gulf region and Indonesia. As mangroves are rich in poly phenolic compounds, their leaves have been used in the preparation of a beverage that has the same qualities as black tea. The mangrove tea as a beverage was proved to have better quality with no mammalian toxicity (Kathiresan, 1995.) Several mangrove plants are used in indigenous medicine. For example, Bruguiera gymnorrhiza for diarrhea and blood pressure, Rhizophora mucronata for angina, Acanthus ilicifolius for asthma and rheumatism, Lumnitzera racemosa for herpes and itches and Excoecaria agallocha for leprosy (Kothari and Rao, 1995; Untawale, et al., 1995). Avicennia is largely used as fodder for camels and other cattle as it grows in arid regions of Gujarat as well as in Konkan and Goa. Similarly, Banerjee et al. (1998) observed that Avicennia is used as fodder for cattle in Andhra Pradesh. Dahdouh-Guebas et al. (2006) analyzed the ethno botanical and fishery-related importance of mangroves of the East Godavari Delta to promote conservation and management measures. The uses of mangroves are vividly described general list of the ecological benefits such as good natural shelterbelt and can reduce the disaster caused by a windstorm on the coasts, absorb CO2 and release O2 and purify the atmosphere and decrease the air pollution, promote the sedimentation of the matters and decrease the transgression of the rising Sea

level caused by the Green House Effect, concentrate the radio elements in seawater, such as Hg90 Sr etc. and can purify the seawater, acts as a good feeding ground, aqua cultural field, make the coasts green. According to Kathiresan & Bingham (2001), the role of Mangroves was known to remove CO2from the atmosphere through photosynthesis and that perhaps reduced the problems with the 'green house gases' and global warming. Mangroves play a very important role in peoples' lives and economy. SanitAksornkoae (2002) described that the value of mangroves was expressed in a common way as 'home 'for marine and terrestrial animals, as a 'kitchen' producing food for people and animals, as a 'water treatment plant' in purifying water, as a 'hospital' in providing medicines, as 'lung' in purifying air in coastal area, as 'carbon bank' to reduce global warming, as a 'coastal wall' in protecting soil erosion and wind stress, as a 'natural laboratory' for eco-biologists, and finally as a 'bridge' in connecting the land and sea. Available information from the above studies revealed species-wise usage and their economic values. However, they have not considered the dependency of local people on the mangrove forests in relation to utilization for traditional needs, subsistence and values of mangrove forest land, water and biological diversity (flora and fauna) of a particular region. Detailed scientific investigations in this orientation, on the basis of ecological principles and inventories are to be carried out. Kathiresan (2000) reported that extracts from mangroves had a potential for human, animal

and plant pathogens and for the treatment of incurable viral diseases like AIDS. Das (2005) said that the mangroves acted as good buffers against the hazards and played a crucial ecological role and their conservation was a good way of coping with natural hazards. Noronha and Nairy, in 2003, reported that several coastal marshes and mangrove areas had been reclaimed for agricultural purposes, particularly for growing paddy and vegetables. These low lying areas were locally called “Khazan lands”. Mangrove ecosystems perform various functions and do ecological services such as protection from waves and act as nurseries for commercially important fish – as stated by the authors -Spaninks and van Beukering, (1997); Dwight (2003); Moberg and Ronnback (2003); Crona and Ronnback (2007). Duke et al., (2007) said that the world mangrove experts were of the opinion that the long term survival of mangroves is at great risk due to fragmentation of the habitats and that the services offered by the mangroves may likely be totally lost within 100 years. Mangrove distribution within their ranges is strongly affected by temperature (Duke, 1992) and moisture (Saenger and Snadaker, 1993). Large-scale water currents may also influence the distribution by preventing propagules from reaching some areas (De Lange and De Lange, 1994). Individual mangrove species differ in the length of time of their propagules to remain viable, their establishment success, their growth rate and their tolerance limits. These factors, which appear quite consistent around the world, interact to produce characteristic distributional

ranges for most species (Duke et al., 1998a). Uma Maheswara Rao and Narasimha Rao, (1988); Ravishankaret al., (2004) described that the mangrove ecosystems were undergoing wide spread degradation due to variety of human induced stresses and factors such as changes in water quality, soil salinity, diversion of river water, sedimentation and conversion of mangrove lands to other land-use practices like agriculture, aquaculture and industrialization. Man-made problems are mostly in the reclamation of mangrove lands for agriculture and prawn culture practices, tree felling for firewood, house and boat constructions and developmental activities such as establishment of fertilizer factory nearer to the mangroves (Banerjee et al., l998). There is an over-exploitation of fishery resources, especially for the seeds of the tiger prawns. The gastropods like Cerithideacingulata and Telescopium telescopium and the bivalves such as Anadaragranosa and Meritrix species are exploited for lime preparation. Abdul Nabi (2012) described that the mangroves are excellent feed for cattle. The buffaloes, goats and cows are left among mangroves during the summer months and they graze Avicennia leaves and grasses (Porteresiacoarctata, Myriostachyawightiana, Aleuropuslagopoides). Local people collect the leaves in large quantities to feed their cattle. It is believed that the buffaloes when fed with Avicennia leaves produce more milk. The mangroves are used as firewood as they have high calorific value. A ton of mangrove firewood is approximately equivalent to 2 to 5 tons of Indian coal and

it burns with high heat without producing any smoke (Banerjee et al., l998). Other threats are due to salt manufacturing, replacement of mangroves by Casuarina plantation by farmers (Jayasundaramma et al., l987) and sea level rise (Prasad et al., l997). Mangroves are important ecosystems that suffer mainly from various types of threats, namely, natural, biological and anthropogenic. The major natural threats are cyclones, tidal waves and tsunamis. Mangroves in Andhra Pradesh have been destroyed due to the conversion of these habitats into aquaculture ponds, saltpans, human habitations and commercial plantations (Jayasundaramma et al., 1987). The decline of mangrove vegetation at Pichavaram, inspite of it being a Reserve Forest since 1980 was attributed to heavy anthropogenic pressures (Balasubramanian and Khan, 2002). The Sundarban mangrove forest was reported to be losing Heritierafomes due to decline in fresh water supply (Karim, 1988). Scott (1989) summarized the causes of damage to Sundarban mangroves and pointed out that freshwater inflow into estuaries is necessary for seed germination and seedling establishment. The conservation of mangrove forests and its environment has not received much attention till recently (Umali 1985). Status reports of mangrove forests and the possible threats to mangroves mainly from Asia and South East Asian countries appeared in the studies of Scott (1991); Uthoff (1996);Khan and Hussain (1996); Mastaller (1996) and Uibrig (1996). On the basis of present status of mangroves along Indian coasts, it is possible to prepare

a perspective plan for proper management and utilization of mangrove areas on sustainable yield basis. It is suggested to allocate certain areas for specific purpose like conservation, management, fishery and other activities according to the priorities (Untawale, 1984). The ecology of mangrove varies widely among coastal states of India. It is, therefore, necessary to consider each area distinctly for management, exploitation and other uses. Dagar et. al., (1991) formulated three suggestions, mentioned below, for the management of mangroves i.e. preservation of mangroves in its natural state (in situ), Utilization of mangroves on a sustainable basis and rational utilization for agriculture and aquaculture. The west coast mangroves have drawn much attention from the conservation point of view, according to the review reports made by Natarajan (1982); Untawale (1984); Deshmukh (1984); Dagar (1988); Ambio (1992) and Alagaraswamy (1995). Comparatively the east coast mangroves have been neglected and no serious attempts are made to study the ecological status in relation to developmental activities. An urgent need to undertake to implement management plans to conserve the mangroves. Soegiarto (1980) cautioned about the declining rate of the global mangrove forests. The traditional values of these mangrove forests have been recognized especially in South East Asian countries. The forests are used as sources of food, firewood and subsistence economy for livelihood rather than for large scale commercial exploitation. Mapping of various types of wetlands, including mangroves, could be used as a

baseline data for classification, preservation, conservation and utilization of the coastal zone, which forms the first step towards a rational mangrove ecosystem management (Nayak, 1993). Mangrove forests should be managed as an investment, where the earned interest remains analogous to the sustained productivity of the system (Clark, 1996). The Ministry of Environment and forests (MoEF), Central Government of India are constantly pursuing strengthen existing policies for protecting and improving the quality of the coastal environment. The legal system of coastal zone management in India came into force in 1991 (Anon, 1999). The Coastal Regulation Zone (CRZ) notification classifies the zones into four different categories for the purpose of regulation (Leelakrishnan, 1999). Singh & Odaki (2004) defined conservation as preservation of natural resources from destructive influences, natural decay or waste. Conservation of these habitats was meant for the maintenance of their rich biodiversity, sustainability of fishery, forestry and other products, and protection of coastal areas from fiery effects of natural calamity (Kathiresan & Qasim, 2005). Regeneration of mangrove forest could be divided into natural and artificial modes (Kathiresan & Qasim, 2005). Natural regeneration involves natural processess of establishment of seeds of mangrove. Artificial regeneration could be carried out by nursery development and transplanting seedling or mangroves in degraded or new areas. The United Nations (UN) conference on Environment and Development held at Rio De Janirio in 1992

strongly felt that the innovations of traditional life styles and the resource dependency of indigenous people are of prime importance for the conservation and sustainable utilization of any forest ecosystem. Mangrove wood is well known for being waterproof, resistant to borers, tough and effectively indestructible. This makes it highly prized and adds enormously to human pressure on the swamps throughout its range. In Malaysia, the wood is chipped for the manufacture of the pulp and rayon, and fragments of wood are made into charcoal in many places. The bark is high in tannins, and so has been used for tanning. It also yields black dye. (Kathiresan, 2004). Untawale (1992) a renowned scientist who made a distinct contribution to the knowledge on Indian Mangroves appreciates mangroves as the ecologically and economically important natural ecosystems. Ecological restoration of mangrove forests has started receiving proper attention, only recently (Lewis, 1999). It is important to understand that mangrove forests occur in a wide variety of hydrological and climatic conditions that promote a broad array of communities. Erftemeijer and Lewis (2000) commented that planting mangroves on mudflats would represent habitat conversion rather than habitat restoration and strongly cautioned on the ecological wisdom in doing so. Earlier, Stevenson et al. (1999) referred to this approach as “gardening” as planting of mangroves alone is considered important. Another common failure is to understand the natural processes of secondary succession and the value of utilizing nurse species such as smooth

cord grass in situations of high wave energy. Bosire et al. (2005) stated that the mangrove reforestation have enhanced litter degradation and concomitant nutrient demineralization, suggesting that other than species litter quality, tidal inundation and seasonal factors, specific stand management regimes play an important role in determining the efficiency of these ecological processes in mangrove ecosystems. Several aspects related to conservation and restoration of mangroves that received poor attention is geology, flora, degradation and sustainable utilization. An integrated approach is highly essential to formulate concrete policies and practices for better conservation and management of Indian mangroves. Misidentification of species is also noted in many of the earlier enumerations and communications. All these issues prompted a detailed study of the mangroves of Andhra Pradesh and the present contribution is the result of such an effort between 2004 and 2008. Falcaro and Pickles (2010) was performed to provide hierarchical clustering of observations applied to coordinate data or distance data. The dendrogram, which was used as a representation was used to graphically present the information concerning which observations were grouped together at various levels of similarity. The MDS is used to graphically represent relationship between objects in multidimensional space. The objects were represented on a plot with the new variables as axes and the relationship between the objects on the plot should represent their underlying similarity (Cox and Cox, 2001). Review of literature

is the important aspect to develop a research design and planning to select the study area and prepared to visit the field stations as per schedule to fulfill the objectives of the study such as identification of species, diversity, distribution and dominance. Besides community structure and socioeconomic aspects and factors are responsible for the loss of mangrove species in mind for the conservation and management of the present work.

Materials and methods

Collection of samples and isolation of bacteria

Sediment samples were collected from ten stations in Godavari estuary, using a Peterson's grab. Serial dilutions of samples were prepared using 50% sterile seawater and pour plated with Zobell's agar 2216e medium. The plates were incubated at 28° C for 24 hr. The colonies that developed were subcultured onto nutrient agar slants. These cultures were then streaked on nutrient agar plates and the separated colonies were isolated in pure culture and maintained in nutrient agar slants with periodic sub culturing.

Sampling sites

Bacteria were isolated from sediment samples from 02 stations in Godavari estuary including a mangrove station.

Name of the Estuary	Vasishta - Godavari
Name_of_the_river	Vasishta–Godavari
Place	Antarvedi
State	Andhra Pradesh
Latitude	16° 18’N
Longitude	81° 42’E
Important landmarks	Antarvedi close to Rajahmundry Bhairavapalem
Length	16 km
Average Depth	1.75 m
Nature of the substratum	Muddy–Sandy
Salinity	0.5–34.5 ppt
Dissolved oxygen	3.5–6.9 mg/l

Name of the Estuary	Gauthami Godavari
Name_of_the_river	Coringa
Place	Coringa
State	Andhra Pradesh
Latitude	16° -30 ‘ to 17°-00' N
Longitude	82°-14' to 82°-23'E
Important landmarks	Coringa close to Kakinada and Yanam
Length	18 km
Average Depth	1.75 m
Nature of the substratum	Muddy–Sandy

Name of the Estuary	Vasishta - Godavari
Name_of_the_river	Vasishta–Godavari
Place	Antarvedi
State	Andhra Pradesh
Latitude	16° 18’N
Longitude	81° 42’E
Important landmarks	Antarvedi close to Rajahmundry Bhairavapalem
Length	16 km
Average Depth	1.75 m
Nature of the substratum	Muddy–Sandy
Salinity	0.5–34.5 ppt
Dissolved oxygen	3.5–6.9 mg/l

Zobell's Agar 2216e medium

Peptone	5g
Yeast Extract	1.0g
Ferric phosphate	0. 02g
50 % seawater	1000 ml
pH	7.2

Identification of cultures

Identification of cultures was done, based on morphological, and biochemical characteristics, employing standard schemes based on Bergey's Manual of Determinative Bacteriology and Systematic Bacteriology. (Gordon et al., 1973; Breed et al., 1973; Kreig and Holt, 1984; Alsina and Blanch, 1994).

Primary screening for protease production

Screening for protease production was done using two substrates gelatin and casein. Thus, the bacteria were tested for their ability to produce both gelatinase and caseinase.

Gelatinase production

Frazier's gelatin agar medium (modified) of Harrigane and McCane (1972) of the following composition was used.

Peptone	5g
Beef extract	3g
NaC|	5g
Gelatin	10g
Agar	20g
50% Aged seawater	1000 ml
pH	7.2

Gelatin agar plates were spot inoculated with the cultures to be tested and incubated at 28°C for 24 hr. After incubation, the plates were overlaid with 15% HgC|2 in Con. HCI. (HgC|2-15 g, Con. HCI — 20 ml, distilled water—100 ml). Clear transparent zones around the colony indicated gelatinase production.

Caseinase production

Casein agar medium of Harrigane and McCane (1972) of the following composition was used.

Basal medium

Peptone	10g
Beef extract	3g

Agar	20g
50% Aged seawater	750 ml
pH	7.2

10g casein in 250 ml distilled water was sterilized separately and mixed with the basal medium just before pouring into plates. After incubation at 28°C for 24 hr. plates were overlaid with 15% HgC|2 in Con. HCI. Clear transparent zones around the colony indicated caseinase production.

Secondary screening for potent strains producing alkaline protease

Thirty isolates which had shown the potential to produce both gelatinase and caseinase in the primary screening were selected based on the diameter of the zone of clearance, and further screened to select the most potent strains from among them. This was done by the quantitative assay in liquid medium.

lnoculum preparation

The selected cultures were inoculated onto nutrient agar slants and incubated for 24 hr., and after incubation, the cells were harvested into sterile saline. A volume adequate to obtain an absorbance of 0.02 at 600 nm for the total medium was added to 50 ml nutrient broth in 250 ml conical flask and incubated at 28°C on a rotary shaker at 100 rpm. This was treated as the absorbance at 0 hr of incubation.

Measurement of growth

Bacterial growth was determined by measuring the absorbance of the culture spectrophotometrically at 600nm and was expressed in units of absorbance (Abs).

Preparation of crude enzyme

After incubation, the samples were drawn from the 50 ml culture to determine growth, and the remaining culture broth was centrifuged at 10000 rpm for 15 minutes at 4°C to remove the cells. The cell free supernatant obtained contained the enzyme. This was assayed for protease activity.

Assay of alkaline protease

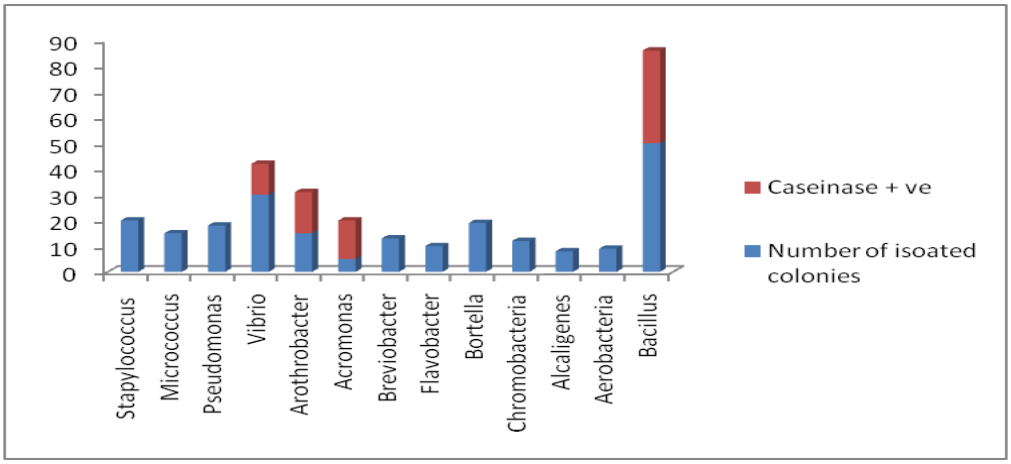
All the thirty potent strains selected after the primary screening were used for quantitative assay. The assay was performed at alkaline pH, to detect the alkaline proteases. Protease production was assayed in terms of protease activity exhibited by the culture supernatant in the enzyme assay. Protease assay was done by a modification of the casein digestion method of Kunitz (1947).To 3m| of 0.6% casein in an appropriate buffer of alkaline pH (phosphate buffer for pH 7-8, Glycine-NaOH buffer for pH 9-10), 0.5 ml of suitably diluted crude

enzyme was added and incubated for 30 minutes at 37°C after which 3m| of 5% trichloroacetic acid was added to stop the reaction and allowed to stand for 15 minutes at room temperature and the resultant mixture was filtered through Whatman no.1 filter paper. The absorbance of this filtrate was measured at 280 nm in a Hitachi 2000-20 UV Visible spectrophotometer. A suitable control was run simultaneously, in which TCA was added prior to the addition of enzyme solution. One unit of proteolytic activity was defined as that amount of enzyme, which liberated 1 ug of tyrosine per ml per minute under the specific conditions of assay. The absorbance at 280 nm (test — control) indicated the tyrosine content of the filtrate, which has been released by the hydrolysis of the protein substrate by the enzyme. The tyrosine content of the sample was read from the standard calibration curve prepared with pure tyrosine.

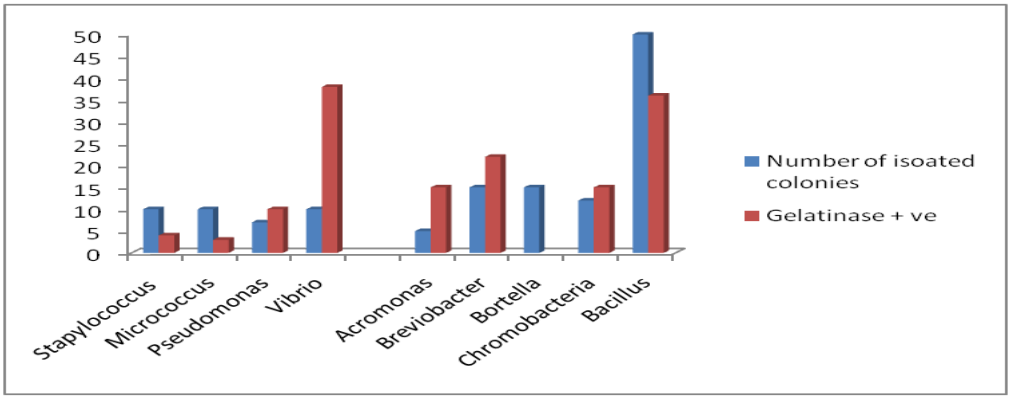
Results

A total of 50 bacteria were isolated from the sediment samples collected from Godavari estuary. Of this, 130 were gram-negative rods, 105 isolates were gram-positive rods and 16 gram positive cocci. Bacillus was clearly the single largest genus, which comprised of 70 isolates (28% of the total). The next abundant genus was Vibrio (18%) followed by Enterobacteriaceae group (11%). In most of the stations an abundance of Bacillus sp. was noted, followed by Vibrio sp. In Eloor, Bacillus predominated with 55% of the total isolates, followed by Vibrio. Other genera were Pseudomonas, Arthrobacter, Acinetobacter, Micrococcus and Staphylococcus. Samples from Kannamali station showed abundance of Bacillus (35%) and Vibrio. Pseudomonas, Micrococcus, Brevibacterium, Arthrobacter and Flavobacterium were the other genera isolated. Bacillus comprised of 40% of the total number of bacteria isolated from Varappuzha station. Vibrio, Enterobacteriacea group, Aeromonas, Bordetella and Acinetobacter, F/avobacterium, A/calignes and Achromobacter were isolated from this station. Vaduthala had 55% of its population as Bacillus. Vibrio, Aeromonas, Pseudomonas, Flavobacterium, Chromobacterium and Bordetella were the other genera found. Samples from Bolgatty station had Bacillus (31%) and Pseudomonas as the predominant genera. Vibrio,

Enterobacteriaceae, Staphylococcus and Aerobacter were also found here. Vibrio was the abundant genus with 35% of the totally isolated strains from the Barmouth station. Bacillus, Enterobacteriaceae, Pseudomonas, Aerococcus, Bordetella and Alcaligenes were the other genera found here. Mattanchery station had 55% Bacillus; Enterobactreiaceae group, Pseudomonas, Acinetobacter, Chromobacterium, Flavobacterium and Arthrobacter were the other genera found. In the samples from Thevara, Bacillus, Vibrio, Enterobacteriaceae, Pseudomonas, Alcaligenes, and Aerobacter were isolated. The predominant genus in Edakochi station was Vibrio. Bacillus, Aeromonas, Pseudomonas, Staphylococcus, Arthrobacter, Aerococcus and Achromobacter were the other genera found. The mangrove station, Puduvaippu, had an abundance of Bacillus (25%). Other genera were Vibrio, Pseudomonas, Brevibacterium, Alcaligenes, Chromobacterium, Bordetella, Achromobacter, Staphylococcus, Enterobacteriaceae group and Micrococcus. Of the 250 isolates obtained, 182 (73%) were found to be gelatinolytic and 73 (35%) caseinolytic. The percentage of proteolytic forms varied among the genera. The generic composition and the relative abundance of gelatinase and caseinase producing strains.



Generic distribution of isolates and caseinase producing forms



Generic distribution of isolates and gelatinase producing forms

After the primary screening, 30 strains were selected for secondary screening which were positive for both gelatinase and caseinase production. Finally three strains producing alkaline protease were selected after secondary screening. and were subjected to further study. Gelatin and casein hydrolysis are shown in Plates 1 and 2. Out of the three strains selected one belonged to the genus

Bacillus and two to the genus Vibrio. The selected strains were identified up to species level by further biochemical tests.

They were identified as

1. Bacillus circulans (B15)

2. Vibrio fluvialis (V10)

3. Vibrio sp. (V26)

The gram stained preparations of the selected strains are shown in plate The biochemical characters of the selected strains are given in the table.The sensitivity to Vibriostatic compound O/129 is shown in plate 4.

Morphological and Biochemical characteristics of B15

Culture	**B15**
Gram reaction	+
Spore	+.Oval
Motility	+
Catalase	+
O/F	+/-
MR	-
VP	-
pH in VP broth	5.9
Indole	-

Citrate	
Amylase	+
Phosphatase	+
Mannitol utilization	-
Arabinose utilization	-
Gas from glucose	-
Growth in 0% NaCl	+
Growth in 5% NaCl	+
Growth in 6% NaCl	+
Growth in 7% NaCl	-
Growth in 8% NaCl	-
Growth in 10% NaCl	-
Growth in 65%	-
Growth in 0%	-
Oxidase	+
NO_3 reduction	+
Oxidase	+
Phenylalanine deaminase	-
Identified as	Bacillus circulans

Morphological and Biochemical characteristics of V10 and V26

Culture	V10	V26
Gram reaction	-	-
Motility	+	+
Oxidase	+	+
O/F	+/+	+/+
Growth on TCBS Agar	+ Yellow colony	+ Yellow colony,become green afterwards
Arginine dihydrolase	+	-
Lysine decarboxylase	-	+
Ornithine decarboxylse	-	+
Indole	-	+
Citrate	+	+
MR	+	+
VP	-	+
Amylase	+	-
Phosphatase	+	+
Mannitol utilization	+	+
Arabinose utilization	+	-
Gas from glucose	-	-
Growth in 0% NaCl	+	+

Growth in 6% NaCl	+	+
Growth at 0ºC	-	-
NO_3 reduction	+	+
ONPG	-	+
Resistance to 0/129(150 ug)	+	
Catalase	+	+
Phenyalanine deaminase	-	-
Identified as	Vibrio fluvialis	Vibrio sp.

DISCUSSION

Biogeochemical turnover in the aquatic environment is mainly due to the metabolism of microbial population and this is performed through aerobic and anaerobic decomposition by which microbial cells are supplied with energy (Rajendran and Venugopalan, 1977). The sediment microflora play an important role in biodegradation of organic compounds and maintaining the ecological balance of the system. In the present study, microorganisms are found distributed widely in aquatic environments. Both gram-negative bacteria and gram-positive bacteria were found in almost equal proportions. i.e. 52% and 48% respectively. But in some of the ear1ier works on the bacterial population of water and sediment samples from Godavari estuary, gram-negative forms were reported to show predominance over gram positive forms (Philip, 1987; Chandrika and Ramachandran, 1994; Gopinath, 2002). The variation in the present observation could be due to the fact that in the present study only the sediment samples were analysed, which accounts for the occurrence of almost equal proportion of gram positive and gram-negative bacteria. As per the observation by Ramamoorthy and Jayabalan (1982) the most important heterotrophic genera present in estuaries were Alcaligenes, Vibrio, Pseudomonas, Aeromonas, Flavobacterium and Micrococcus. Bacillus was found mainly in the surface sediments. Philip (1987) found Vibrio and Pseudomonas to be the predominant genera in Cochin estuary. The abundance of Bacillus was also cited. According to Gopinath (2002), the predominant

genera were Pseudomonas, Vibrio, Bacillus and Staphylococcus. The predominant genus in the present observation was Bacillus, followed by Vibrio. Bacillus being a spore former can survive better in the sediment sample, when compared to non-spore forming bacteria. Amar (2001) obtained similar result, while studying the composition of microflora from Cochin estuary, where Bacillus was the dominant species, followed by Corynefonnes, Vibrios, Streptococcus, Pseudomonas and Acinetobacter. Veljo et al. (2002) reported that the same group of bacterial species dominated independently of the season investigated, based on his study conducted in Northern Baltic Sea. But according to Philip (1987), there was seasonal variation in the generic distribution as well as the occurrence of proteolytic forms in Godavari estuary. In the present study regarding the proteolytic potential of the strains, 73% of the total isolates were gelatinase positive and 35% were caseinase positive. It was to be noted that 94% of Bacillus strains isolated were gelatinolytic and 51% caseinolytic. Of the 30 strains selected after primary screening 11 were Bacillus, 10 Vibrio, 3 Pseudomonas, 3 Micrococci, and 1 belonging to Enterobacteriaceae group. In an attempt to understand the ecological implication of the extracellular protein degradation promoted by estuarine bacteria, Sizemore et al. (1973) studied the distribution and activity of the proteolytic bacteria in the sediments from the North Inlet Estuary near Georgetown, S. Carolina as well as the effects of various parameters on protein

hydrolysis by bacteria and observed that 56% of the isolates obtained from the sediment exhibited caseinolytic activity. While studying the physiological characteristics of 649 bacteria isolated from Long Island Sound water column, Murchelano and Brown (1970) observed that 63.9% were proteolytic. They indicated that although the generic composition of the bacterial flora changed with season there was no significant variation in the number of proteolytic. lipolytic and amylolytic flora. Reinheimer (1972) noted that most of the isolates from Arabian Sea of Northern Gulf of Oman were strongly proteolytic. Boeyo et al. (1975) reported that proteolytic forms were widely encountered in North Sea sediments. Observations on the microbial proteolysis in Lake Champlain revealed that the proteolysis was very much dependent upon water temperature. Pseudomonas and Flavobacterium were found to be the predominant proteolytic flora (Little et al., 1979). Observations on proteolytic and lipolytic bacterial counts in the sediments of marine environment of New York Bight Ape. Sandy Hook Bay and, Great Bay of New Jersy showed that proteolytic counts were 2-4 times higher in the polluted areas (Nitkowski et al., 1977).

The selected strains

The strains selected for detailed study are Bacillus circulans, Vibrio fluvialis and Vibrio sp. As widely reported, Bacillus is the most commercially exploited bacterial species for protease production (Gupta et al., 2002b) and they are considered as appropriate producers for commercial exploitation, being non

toxic and non pathogenic and thus safe to use. The genus Vibrio contains a number of species of marine origin (Baumann et al., 1980). The production of extracellular enzymes is common among marine members of this genus. But as yet enzyme secretion has been studied in detail in only very few marine species such as V. alginolyticus (Reid et al., 1978, 1980) and V.gazogenes (Ratcliffe et al.. 1982). Although it is known that many Vibrio sp. in particular Vibrio cholerae 01 can be responsible for disease state in humans, only a few Vibrio spp. have been investigated in clinical and food microbiology. These species include Vibrio paramaemolyticus, Vibrio cholerae and Vibrio vulnificus, which are known to be aetiological agents in several gastrointestinal pathologies linked to consumption of contaminated water and sea food products (Piersimoni et al., 1991; Chakraborthy et al., 1997; Jackson et al., 1997; Arias et al., 1999; Wright et al. 1999; Strom and Paranjpye 2000). However, in recent years the interest of microbiologists has turned to other halophilic Vibn'os also. Vibrio fluvialis, which is one among the autochthonous flora of aquatic ecosystems, has only rarely been associated with pathologies in humans and even then have not been identified as the cause of these pathologies (Baffone et al., 2001).

Mangroves - a unique ecological niche to be conserved One thing worth mentioning based on the present observation is the abundance of proteolytic forms obtained from the mangrove samples. 90% of the bacterial isolates from mangrove samples were producing gelatinase and

70% were positive for caseinase production. The abundance of Bacillus sp. also was noticed among the mangrove isolates. Also. There was a predominance of gram-positive forms over gram-negative forms. Of the 30 strains selected after primary screening 50% were mangrove isolates. The three strains finally selected after the two screening steps for the maximum production of alkaline protease were also from the mangroves. This shows that there is definitely some enhancing factor for the proteolytic forms, in the sediment samples of mangroves. The distribution of bacteria in sediments depends on several factors. The organic content of the sediment determines the abundance of heterotrophic bacteria, to a great extent. (Kuznetsov, 1968). Nevertheless, the role of other physical and chemical characteristics of the sediment as well as the surrounding environment influencing the bacterial proliferation and characterization of physiological groups cannot be underestimated (Sugahara et al., 1974). In this case we can see that all these factors are influential, as the mangrove sediment is rich in organic matter with the contribution from its indigenous flora and fauna. The literature regarding the proteolytic bacteria from mangroves is scanty. Mangrove ecosystem plays a key role in the nutrient and metal cycling (Harbison, 1981; Lacerda and Abrao et al., 1984) and it acts as a buffer between transitional near shore and Lagoonal / estuarine environments with respect to their influence on freshwater discharge, salinity regime and the

adjacent aquatic systems in general. The unique flora of mangrove with their specialized ecological characters afford suitable atmosphere for a set of peculiar flora and fauna- both living in their own highly specialized ecosystem. Man in his craving for supremacy over nature, has failed to understand the intricate ‘ecological niche‘of mangroves and started replacing the natural vegetation in the name of agriculture and industrial developments. The result is almost a total annihilation of the vast extent of mangrove vegetation of the Coastal area of Andhra Pradesh.

The mangrove where the present study was conducted is Anthervedi Estuary, which is the biggest mangrove area on the Coastal coast. undisturbed, the whole area will undoubtedly develop into a good mangrove forest (Basha, 1991).

The present study showed that proteolytic bacteria are widely distributed in the sediment samples of Godavari estuary, and Bacillus clearly showed abundance both in distribution and protease production, followed by Vibrio sp. The three strains selected for further study also belonged to Bacillus and Vibrio sp. The mangrove area was a potential habitat that is worth further exploration.

Materials and Methods

Sample collection

Sediment samples were collected from Godavari estuary (Latitude 16° 18'N, Longitude 81° 42'E, Latitude 16° 18'N, Longitude 81° 42'E), Andhra Pradesh. Samples were transported to the laboratory and kept at 4ºC until further analysis.

Isolation and screening of bacterial strains for amylase production

Isolation and primary screening for amylase producers was done by using starch agar (containing 1% starch and 2% agar) plate method. Sediment samples were serially diluted up to 10-4 with sterilized 50% aged sea water and 0.1 ml the diluted samples were spread over the surface of starch agar medium. Plates were incubated at 30oC for 24 hrs. Morphologically different colonies were selected for the secondary screening. In secondary screening, 50 µl of cell free culture was inoculated in the wells made in starch agar medium. The plates were incubated at 30oC for 48 hrs. After incubation, the plates were flooded with 1% of iodine solution for 5 min and washed with water to remove the excess color (Bahadure et al., 2010). Based on the highest size of zone of

clearance around the well the potential strain was selected and maintained on starch agar slant.

Identification of potential bacterial strain

The potential bacterial strain was biochemically identified using Bergey's manual of determinative bacteriology (Holt et al., 1994).

Optimization studies for amylase production

To study the effect of growth and enzyme production, submerged fermentation in a basal medium containing (g/100ml): peptone - 0.5g, yeast extract - 0.3g, NaCl - 0.3g, K2HPO4 - 0.1g, MgSO4 - 0.02 g and soluble starch - 1g (Madigan et al., 2011) was used. The medium was prepared by using 50% aged sea water and medium pH was maintained at 7. Different growth parameters like incubation period (0-72 hrs), pH (6, 7, 8, 9, 10 and 11),u temperature (25ºC, 30ºC, 35ºC and 40ºC), salinity (0.5%, 1.0%, 1.5%, 2.0% and 2.5%), different carbon (maltose, sucrose, glucose, starch and cellulose) and nitrogen sources (peptone, ammonium nitrate, beef extract and yeast extract). Enzyme activity was assessed for every 12 hrs and it was expressed as U/ml/min.

Amylase assay

Amylase activity was determined as described by Palanivelu et al., 2001. The reaction mixture consisting of 1.25 ml 1% (w/v) soluble starch (Merck)

solution, 0.25 ml, 0.1 M Sodium acetate buffer (pH 5.0), 0.25 ml of distilled water, and 0.25 ml of properly diluted crude enzyme extract was taken and after 10 minutes of incubation at 50ºC, the liberated reducing sugars (glucose equivalents) were estimated by the dinitro salicylic acid method (Miller, 1959). One unit (U) of α-amylase is defined as the amount of enzyme releasing 1µmol glucose equivalent per minute under the assay conditions. The amount of enzyme produced was expressed as U/ml/min.

Mass scale production

Based on the optimization results, the potential strain Bacillus subtilis was cultivated in the basal medium with the optimized parameters such as 48 hrs of incubation period, pH 7, temperature 35oC, 2% salinity, starch as the carbon source and peptone as the nitrogen source in a 1000ml of conical flasks. After sterilization at 121oC for 15 min. the medium was inoculated with full loop of Bacilllus subtilis and incubated at 35oC for 48 hrs. In the mass scale production medium biomass and the enzyme activity were assessed at the 48 hrs of incubation. Growth was assessed by measuring the OD at 600 nm with a UV spectrophotometer and the enzyme activity was assessed as previously.

Results and Discussion

The study has proved that amylase producers are abundantly present in the sediment sample collected from Godavari estuary. The observed density of amylase producers was 1.6 X 106 CFU/g .A total of 8 morphologically different strains were selected for amylase production screening. Based on the primary screening, only 5 isolates were selected for the secondary screening for amylase production. The secondary screening using well assay showed Strain 10 was the most potential strain and it was biochemically identified as Bacillus subtilis. This strain was used for further study.

S.No.	Number of the Strains	Zone of clearance
1	Strain 6	1.7cm
2	Strain 7	1.5cm
3	Strain 8	1.8cm
4	Strain 9	2.0cm
5	Strain 10	2.8cm

Screening of isolates for amylase production

Characteristics	Results
Colony shape	Irregular
Gram staining	+
Starch hydrolysis	+
Nitrate reduction	+
Citrate utilization	+
Indole	-
Glucose	+
Sucrose	+
Methyl red	+
Oxidase	-
Catalase	+

Morphological and biochemical characterization of the potential strain B.subtilis

EFFECT OF CULTURE CONDITIONS ON GROWTH AND PRODUCTION OF ALKALINE PROTEASES

Microorganisms show considerable variation with respect to their nutritional requirements for optimum growth and enzyme production. Very often the optimal conditions for growth may not be optimal for product formation. Their growth rate and metabolism depend very much on the composition of the medium and the prevalent physical and chemical factors. By understanding the specific requirements of a microbial species, it is possible to establish conditions in vitro to support the optimal growth and enzyme production of that organism.

In commercial practice. Medium composition is optimized continuously over the years so that the balance between the various components is maintained, thus maximizing productivity of microorganisms and minimizing the amount of unutilized components at the end of fennentations. This balance is highly dependent on the interaction between the particular strain of microorganism, the type of medium components and the process conditions. 30-40% of the production cost of industrial enzymes is estimated to be accounted for by the cost of growth medium (Joo et al, 2002). Alkaline proteases are generally

produced by submerged fermentation. In addition, solid-state fermentation process has been exploited to a lesser extent for production of these enzymes (Malathy and Chakraborthy, 1991;Chakraborthy and Srinivasan, 1993; George et al., 1995). With a view to developing an economically feasible technology, research efforts are mainly focused on the improvement in the yield of alkaline proteases and optimization of the fermentation medium and production conditions. For this, the parameters commonly standardized include (i) evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes. (ii) requirement of divalent metal ions in the medium; and (iii) optimization of environmental and fermentation parameters such as pH, temperature, aeration and agitation (Kumar and Takagi, 1999). No defined medium has been established for the best production of alkaline protease from different microbial sources. Each organism has its own special conditions for the optimum production. In the present study, an effort has been made to optimize the culture conditions for the maximum production of alkaline proteases from the three strains isolated from a mangrove area in Godavari estuary.

Review of literature

Numerous reports are available regarding protease production in Bacillus. Despite the long list of protease producing microorganisms only a few are considered as appropriate producers for commercial exploitation being 'generally regarded as safe' (GRAS), non toxic and non pathogenic. Bacteria are the most dominant group of alkaline protease producers with the genus Bacillus being the most prominent source. A myriad of Bacillus species from many different exotic environments have been explored for alkaline protease production but most potential alkaline protease producing bacilli are strains of B. megaterium, B. lichenifomris, B. subtilis, B. amyloliquifaciens and B. mojavensis. (Millet et al., 1969; Kalisz, 1988; Manachini and Fortina, 1998; Rao et al., 1998; Kumar and Takagi, 1999; Yang et al., 2000; Gupta et al., 2002b). Effect of glucose and amino acids on protease synthesis in Bacillus megaterium was reported by Votruba et al., (1987). Aderibigbe et al. (1990) studied the variability in extracellular protease production among the strains of B.subtilis group. The culture conditions were optimized for protease production by Bacillus acidophilus strain (Giesecke et al. 1991). Protease production in continuous suspension cultures of Bacillus flrmus was reported by Moon and Parulekar (1993). The protease production conditions were optimized for a

Bacillus sp. by Atalo and Gashe (1993). Kembhavi et al. (1993) reported the production of a thermostable alkaline protease from a Bacillus subtilis strain. Production of a therrno stable alkaline protease by a halotolerant strain of Bacillus lichenifonnis was reported by Manachini and Fortina (1998). Optimal production of Bacillus alkaline protease using a cheese whey medium was reported by Kumar et al. (1999). Sumandeep et al., (1999) isolated an alkalophilic Bacillus sp. from soil, which produced a thermostable alkaline protease. Madan et al (2000) optimized the culture conditions for protease production by a UV- mutant of Bacillus polymyxa.

Optimization of the production of protease from Bacillus horikoshii was reported by Joo et al. (2002).

Studies on the effects of cations on protease production of marine bacteria were conducted by Sakata et al., (1977). Another study of proteases of marine origin was by Sashihara et al. (1975) Protease of a Clostridium botulinum strain was studied by Nakane (1978). The effects of culture conditions were optimized for protease production were studied in Pseudomonas ma/tophi/a strain by Kobayashi et al. (1985). Effect of different culture conditions on protease production by Butyrivibrio flbrisolvens was studied by Cotta and Hespeli (1986). The role of casein on protease production by Iactobacilii was studied by Sakellaris and Gikas (1991). The effect of glucose on Thermoactinomyces sp. was reported by Sunitha et al. (1999). The effect of divalent cations on

Pseudomonas fluorescens protease production was studied by Liao and Mc Callus (1998). Another bacterial source known as potential producer is Pseudomonas sp. (Ogino et al., 1999; Bayoudh et al., 2000).

Though scarce, reports on protease production in Vibrio species are also available. Hare et al. (1981) studied the role of temperature and oxygen in the regulation of exoprotease production in Vibrio alginolyticus. Another study in V. alginolyticus was by Long et al. (1981) regarding the regulation of production of extracellular alkaline protease. The effect of growth media and temperature on a dairy strain of Aeromonas hydrophila was reported by Santos et al., (1996). The protease production aspects of an alklophilic bacteria isolated from an alkaline soda lake were reported (Gessesse and Gashe, 1997). Effect of culture conditions on production of an extracellular protease from the fish pathogen, Yersinis ruckeri was studied by Secades and Guijarro (1999).

There are a number of reports on alkaline proteases from fungi. The production of an alkaline serine protease from a mold, Monascus, which is a close relative of Aspergillus and Penici//ium, was reported (Aso et al., 1989). A comparison between two Arthrobotrys sp. regarding the nematode. degrading ability on the basis of protease production has been studied (Bedelu et al., 1998). Direct determination of the proteolytic activity and the inhibition profiles from the culture itself without the need of extraction or purification had been reported in a plant pathogenic fungus. Fusarium sp. (Girard and Michaud, 2002).

Ordas et al. (2001) studied proteolytic activity of a protozoan Pseudoperkinsus tapetis. The protease from an oyster protozoan parasite Perkinsus marinus was also reported (Maclntyre et al., 2003).

Materials And Methods

Selected strains

Three bacterial strains isolated from a mangrove area in Godavari estuary, which were potent producers of alkaline proteases were selected for further study. They were

Bacillus circulans

Vibrio fluvialis,

Vibrio sp.

Growth media

Nutrient broth supplemented with 0.2% gelatin and a mineral medium containing artificial seawater base (ASW) of Mac Leod (1968) and 0.2% sucrose as carbon source were used for the optimization studies.

Nutrient broth supplemented with gelatin

Peptone 5 g

Beef extract 3 g

NaCl 15 g

Water 1000 ml

pH 7.2

Mineral Medium

Composition of Artificial Seawater Base (ASW)

NaCl| 23.4 g

$MgSO_4.7H_2O$ 24 g

KCI 1.5 g

$CaCl_2.2H_2O$ 2.9 g

Salts were dissolved separately and combined

Basal medium

Tris hydroxymethyl-aminomethane

(Adjusted to pH 7.5 with HCI) 6.1 g

NH_4Cl 1.0 g

$K_2HPO_4.3H_2O$ 75 mg

$FeSO_4.7H_2O$ 23 mg

Sucrose 2 g

Yeast extract 10 g

Basal medium was mixed with half strength ASW lnoculum preparationInoculums preparation, Measurement of growth Growth section,

Preparation of crude enzyme Crude enzyme Assay of alkaline protease Alkaline protease

Effect of different physico chemical parameters on growth and alkaline protease production

Optimal conditions required for maximum protease production by the three test strains were determined by subjecting them to different levels of agitation, various incubation temperatures, periods of incubation, pH, NaCl concentrations, salts, carbon sources and nitrogen sources.

Effect of agitation speed on growth and protease production

The effect of aeration on the growth and protease production was determined by growing the strains, in nutrient broth supplemented with gelatin and incubating them at different rpm (0, 50,100,150 and 200) in a rotary shaker. Growth and enzyme production were determined after 24 hr. of incubation.

Effect of period of incubation on growth and protease production

The optimum period of incubation required for maximum growth and enzyme production was determined by inoculating the cultures in nutrient broth supplemented with gelatin. Growth and enzyme production were monitored at different time intervals. (2, 4, 6, 8, 10, 12, 14. 24, 36, 60 and 72 hr).

Effect of pH on growth and protease production

The effect of pH on growth and enzyme production was studied by inoculating the organisms in nutrient broth supplemented with gelatin, having varying pH (pH 4,5,6,7,8,9 and 10). The cultures were incubated at 28°C for 24 hr. and growth and enzyme production were measured.

Effect of temperature on growth and protease production

Effect of temperature on growth and enzyme production was determined by inoculating the cultures in nutrient broth supplemented with gelatin and incubating them at different temperatures (15°C, 20°C, 25°C, 30°C, 35°C and 40°C). Growth and enzyme production were determined after 24 hr. of incubation.

Effect of NaCl concentration on growth and protease production

The effect of NaCl concentration on growth and enzyme production was studied by inoculating the organisms in nutrient broth supplemented with gelatin having varying NaC| concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5%). The cultures were incubated at 28°C for 24 hr. and growth and enzyme production were measured.

Effect of metallic salts on growth and protease production

The effect of various ions on growth and enzyme production was studied by adding individually varying concentrations of the salts, $CaCl_2$, $MnSO_4$, $MgSO_4$,

KH_2PO_4, and $Fe(PO_4)_3$ as source of ions to nutrient broth and inoculating the media with the cultures. Growth and enzyme production were detected after 24 hr. of incubation at 28°C.

Effect of carbon sources on growth and protease production

Effect of carbon sources on growth and enzyme production was determined by inoculating the cultures in mineral medium containing different concentrations (0.1, 0.2, 0.4,and 0.6%) of glucose, mannose, maltose and sucrose, and different concentrations (0.05, 0.1, 0.2 and 0.4%) of molasses, as carbon sources. Growth and enzyme production were determined after incubation of 24 hr. at 28°C.

Effect of nitrogen sources on growth and protease production

Effect of nitrogen sources on growth and enzyme production was determined by inoculating the cultures in mineral medium containing one of the nitrogen sources at ‘l% concentration. The organic nitrogen sources used were beef extract, yeast extract, peptone, gelatin, casein, and tryptone, and the inorganic nitrogen sources were NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, NaNO3 and urea. Growth and enzyme production were determined after incubation of 24 hr. at 28°C.

Statistical analysis

The data obtained were subjected to two factor ANOVA (Analysis of Variance) with replication (for the effect of carbon sources), and Single factor ANOVA

(for all the other parameters and the Least Significant Difference (LSD) was calculated in each case.

Results

Results of the study are shown as graphs and tables.

Effect of shaking speed on growth and protease production

B. circulans showed considerable increase in enzyme production when the culture was agitated than when it was kept stationary. The production was maximal at a range of 50 to 150 rpm. At 200 rpm, production was significantly lowered. Growth was not much affected by shaking speed. However, there was decrease in growth rate in stationary condition

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