CHAPTER-1
INTRODUCTION AND REVIEW OF LITERATURE

The oceans cover more than 70% of our planet’s surface. The environment of the oceans involving high pressure, low temperature, high salinity and lighting conditions may contribute to the important variations of the enzymes generated by marine microorganisms. Oceans contain diverse microbial habitat. The total bacterial members on an average are high in coastal waters than in the open ocean (Sharma, 2004).

Microbial flora in marine environment forms an integral part of this unique ecosystem, but marine bacteria have remained unexplored (Colwell & Hill, 1992). An understanding of marine microbial biodiversity is of utmost importance for a variety of reasons. Bacteria play an integral role in the cycling of carbon, nitrogen, sulphur and phosphate. The role they play in the functioning of the ecosystem and to gauge the effects that anthropogenic forces (particularly pollution) exert on the diversity of marine microorganisms is very important. Indeed, it has already been shown that sewage sludge dumping off the coast had an impact on the microbial ecology at that site (Hill et al., 1993).

Numerous marine microorganisms secrete enzymes which can provide new insights and understanding of enzymes. Marine microorganisms have been attracting more attention as source for novel enzymes, because the microbial enzymes are relatively more stable and active than the enzymes derived from other sources (Bull et al., 2000; Kin, 2006). Bacteria have been regarded as treasure of many useful enzymes viz., amylases, proteases, lipases, hydrolases and reductases. Among them amylolytic enzymes have great biotechnological applications and economic exploitations.
Generally, the sea water has an average salinity of 35 ppm. The chief salts are chlorides, sulphates, bicarbonates, carbonates and bromides of sodium, magnesium, calcium and potassium, of which sodium chloride is present in maximum amount (Sharma, 2004). Microorganisms account for more than 90% of marine biomass and a majority of them remain unknown because it is difficult to isolate them on synthetic media.

The most important factor for the success of any fermentation industry is to use high yielding strain with stable biochemical characteristics. It should be easily cultivated on large-scale with cost effective media. However, it should not produce undesirable products like toxins, inhibitors etc.

Several enzymes are used industrially, half of them obtained from fungi and yeast, and one-third from bacteria and the remainder divided between plant (4%) and animal (8%) sources. Microorganisms are preferred to animals and plants as source of enzymes because of many reasons. Microorganisms have faster growth rate, short generation time and require less space for cultivation. Further, they require relatively cheap and inexpensive media to grow. Microorganisms can be genetically manipulated for better production. The enzymes produced by microorganisms can be controlled and more predictable. There are no seasonal fluctuations in enzyme production as in plants. Large quantity of the commercial product can be produced and the recovery of the product is easy. Use of inexpensive media makes the product more economic. Simple primary screening methods can be used to isolate potentially desired microorganisms. Strain improvement increases the quantity of the product many fold.
Classification of amylases

Amylases are divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases (Van der Maarel et al., 2002).

**Endoamylases** Enzymes that cleave α-1, 4 glycosidic bonds present in the inner part (endo) of the amylose e.g. α-amylase.

**Exoamylases** Enzymes that cleave α-1, 4 glycosidic bonds of amylose and amyllopectin. Beta-amylases and amyloglucosidases shorten the external side chains of amyllopectin by cleaving maltose and glucose molecules respectively.

**Debranching Enzymes** Enzymes exclusively hydrolyze α-1, 6-glycosidic bonds. Pullulanase and isoamylase hydrolyze α-1, 6-glycosidic bonds in amyllopectin (Israelides et al., 1999).

**Transferases** Enzymes that cleave α-1, 4-glycosidic bond of the donor molecule and formation of a glycosidic bond by transferring part of the donor molecule to a glycosidic acceptor.

**Alpha-amylase** (E.C:3.2.1.1, alternate names: 1, 4-α-D-glucan glucohydrolase): Alpha-amylases randomly break down amylose, yielding maltotriose and maltose and also break amyllopectin yielding maltose, glucose and limit dextrin. Alpha-amylases are metalloenzymes. They require calcium ions for their stability, activity and structural integrity. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes (Bordbar et al., 2005).
Figure showing the action of α-amylase on starch.

**Beta-amylase** (E.C: 3.2.1.2, alternate names: α-1, 4- D-glucan maltohydrolase; glycogenase; saccharogen amylase): It is synthesized by bacteria, fungi, and plants. It catalyzes the hydrolysis from the non-reducing end, of the second α-1, 4 glycosidic bond, cleaving off two glucose units (maltose) at a time.

**Gamma-amylase** (E.C: 3.2.1.3, alternative names: Glucan 1,4-α-glucosidase; amyloglucosidase; Exo-1,4-α-glucosidase; glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase): It cleaves α (1-6) glycosidic linkages in addition to cleaving the last α (1-4) glycosidic linkages at the nonreducing end of amyllose and amyllopectin, yielding glucose.

Marine bacteria have diverse range of novel enzymes, secondary metabolites and therapeutics (Das et al., 2006). The capability of amylase production widely occurs in various bacteria, fungi, plants and animals that have a major role in the utilization of polysaccharides (Shaw & Ou-Lee, 1984; Reddy et al., 1987; Tomita et al., 1990; Ilori et al., 1997; Ribeiro et al., 2000; Hagihara et al., 2001; Zoltowska, 2001; Bassinello et al., 2002; Haq et al., 2003). Amylases derived from bacterial sources have economically dominated applications in industrial sectors (Gupta et al., 2003). The literature survey revealed that bacterial amylases are widely used than fungal amylases.
Species of *Bacillus* are ubiquitous in terrestrial, fresh water and marine habitats (Ruger, 1989).

Screening is the detection and isolation of high-yielding species from the natural material such as soil, air and water containing heterogeneous microbial populations. Screening methods of bacteria include primary screening, secondary screening and strain improvement (Patel, 2003).

**Primary screening**

Primary screening consists of basic elementary tests required to detect and to isolate new bacterial species exhibiting the desired property. It serves to remove unwanted bacteria on the basis of relatively simple and fundamental criteria. Marine microorganisms are known to have a diverse range of enzymatic activity. Several authors reported α-amylase producing *Bacillus* spp. (Ramesh & Lonsane, 1990; Hawary, 1991; Castro et al., 1993; Lealem & Gashe, 1994; Tokoya et al., 1994; Machius et al., 1995; Babu & Satyanarayana, 1995; Horikoshi, 1996; Krishna & chandrasekaran, 1996; Mamo & Gessesse, 1997; Milner et al., 1997; Kunst et al., 1997; Ariga et al., 1997; Syu & Chen, 1997; Kelly et al., 1997; Omidiji et al., 1997; Dobrev et al., 1998; Machius et al., 1998a; Abate et al., 1999; Ben et al., 1999; Nielsen & Borchert, 2000; Lutz et al., 2001; Narang & Satyanarayana, 2001; Cordeiro et al., 2002; Burhan et al., 2003; Baysal et al., 2003; Reddy et al., 2003; Yumoto et al., 2003; Ray, 2004; Mishra et al., 2005; Demirkan et al., 2005; Anto et al., 2006; Gangadharan et al., 2006; Rasiah & Rehm, 2009; Vidilaseris et al., 2009).

Several reports of α-amylase producing bacteria include: *Thermoactinomyces* spp. (Saha & Bhattacharyya, 1990; Spreinat & Antranikian, 1990; Jensen & Olsen, 1992; Muramatsu et al., 1993; Leuschner & Antranikian, 1995; Busch et al., 1996; Liebl et
al., 1997), *Streptomyces* spp. (Ramakrishna et al., 1992; Rao & Satyanarayana, 2003; Swain et al., 2006; Mishra & Behera, 2008; Narayana & Vijayalakshmi, 2008; Kar & Ray, 2008), *Lactobacillus* spp. (Kim et al., 2008; Smita et al., 2008), *Micrococcus* sp. (Adeleye, 1990), *Geobacillus stearothermophilus* spp. (Rao & Satyanarayana, 2004; Ezeji et al., 2005; Zarcian et al., 2010), *Pyrococcus* spp. (Antranikian, 1990; Chung et al., 1995; De Souza et al., 1996), *Clostridium* spp. (Swamy & Seenayya 1996 a & b; Swamy & Seenayya, 1996; Reddy et al., 2000; Mrudula, 2010), *Alteromonas* sp. (Chessa et al., 1999), *Staphylothermus marinus* (Ostermeier et al., 1999), *Sofolobus sulfatafacus* (Worthington et al., 2003), *Arthrobacter psychrolactophilus* ATCC 700733 (Michael et al., 2005) and *Desulforcococcus fermentans* (Perevalova et al., 2005).

The potent α-amylase producing bacteria are selected by dinitrosalicylic acid (DNS) method. The 3,5-dinitrosalicylic acid is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm. It is mainly used in assay of alpha-amylase. It is preferred due to its specificity (Miller, 1959). This method tests the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group that is present. Simultaneously, 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions:

**Oxidation**

Aldehyde group --------> Carboxyl group

**Reduction**

3, 5-dinitrosalicylic acid --------> 3-amino, 5-nitrosalicylic acid
Dissolved oxygen can interfere with glucose oxidation, which itself is not necessary for the color reaction, sulfite is added in the reagent to absorb the dissolved oxygen.

The cultural, morphological and biochemical characteristics of bacteria help in identification and classification into taxonomic groups according to Bergey's Manual of Systematic Bacteriology. The potent $\alpha$-amylase producing \textit{Bacillus sp.} is further classified based on 16S rRNA gene sequence, mega BLAST score and BLASTN score. The 16S rRNA is a conservative component of the 30S small subunit of prokaryotic ribosome. It is approximately 1500 nucleotides in length. The genes coding for it are referred to as 16S rDNA and are used in constructing phylogenetic relationships (Weisburg \textit{et al.}, 1991). Multiple sequences of 16S rRNA can exist within a single bacterium (Case \textit{et al.}, 2007). The 16S rRNA is highly conserved between different species of bacteria (Coenye & Vandamme, 2003).

**Measurements of bacterial growth**

Growth is defined as an orderly increase of all cellular components, with multiplication as a consequence. Death is the irreversible loss of ability of organism to reproduce itself. The stages of growth curve are lag phase, log phase (exponential phase), stationary phase and death phase (decline phase) (Wulf Crueger & Anneliese Crueer, 2004).
There are four methods of growth measurements:

i) Direct microscopic count (direct cell count method) helps to count the total number of cells include both living and dead directly by direct microscopic counts using Petroff-Hauser chamber (nine squares, each 0.1 mm deep, volume of liquid over one square mm is 0.1 cubic mm).

ii) Viable cell count method is based on the principle that when material containing bacteria are cultured, every viable bacterium develops into a visible colony on a nutrient agar medium.

iii) Turbidity method helps to study the cloudiness of the suspension. Turbidity is directly proportional to the number of cells. It is quantified with the spectrophotometer, which measures the amount of light transmitted directly through a sample. The cells suspended in the culture interrupt the passage of light. The amount of light absorbed through the suspension is measured as optical density.

iv) Dry weight method helps to estimate the bacterial growth at regular time intervals during the incubation period. The growth of the bacteria is directly proportional to the dry weight harvested.

Fermentation

Fermentation is the technique of cultivation of microorganisms for biological conversion of complex substrates into simple compounds. Secondary metabolites (antibiotics, peptides, enzymes and growth factors) are bioactive compounds which have biological activity (Balakrishnan & Pandey, 1996; Robinson et al., 2001; Machado et al., 2004). Recently, researchers have proved that many of these secondary metabolites are industrially and economically useful.
The industrial-level production of bioactive compounds is made possible by the development of Solid State Fermentation (SSF) and Submerged Fermentation (SmF). These techniques based on substrates, various organisms used and the physical and chemical parameters applied.

**Solid State Fermentation (SSF)** utilizes solid substrates, like corn cob, rice bran, wheat bran, grains, soybean meal and paper pulp. The main advantage of using such substrates is that nutrient-rich materials can be easily recycled. In SSF technique, the substrates are utilized very slowly and steadily. It supports controlled release of nutrients. The SSF is best suited for fermentation techniques involving microorganisms that require less moisture content but cannot be used in fermentation processes involving organisms that require high water activity (Babu & Satyanarayana, 1996). The SSF is preferred by fungi, which require lesser water potential (Troller & Christian, 1978). The advantages of SSF are as follows: Enzyme yield per unit volume of incubator is high, power requirement is low, minimum control is necessary and extraction yields highly concentrated enzyme solution (Papagianni et al., 2001).

**Submerged Fermentation (SmF)/Liquid Fermentation (LF)** utilizes free flowing liquid substrates, such as molasses, whey, sulphite waste liquor and broths. The bioactive compounds are secreted into the fermentation medium. The substrates are utilized by microorganisms quite rapidly. This SmF is best suited for bacteria that require high moisture content. The advantage of this technique is that purification of products is easier. It is primarily used in the extraction of secondary metabolites that are in liquid form. The SmF is implemented in case of bacterial enzyme production, due to the requirement of higher water potential (Chahal, 1983). The influx of nutrients and efflux of waste materials need to be carried out based on metabolic parameters. Any deviation from the specified parameters results in an undesirable product. The
advantages of SmF are more. Industrial enzymes (75%) are produced using SmF. The SmF supports the utilization of genetically modified organisms to a greater extent than SSF (Subramaniyam & Vimala, 2012).

Secondary Screening

Secondary screening is strictly essential in any systematic screening programme which helps in detection of useful bacteria in fermentation processes. It gives an idea about the economic position of the fermentation process involving the use of a newly discovered culture. Thus, one may have a comparative study of the process providing information regarding the enzyme yield potentials of different isolates. Secondary screening also provides information pertaining to the effect of different components of the medium. This is valuable in designing the medium that may be attractive as far as economic consideration is concerned. It determines the optimum conditions for production of enzyme associated with culture (Patel, 2003; Wulf Crueger & Anneliese Crueer, 2004).

The production of α-amylases by fermentation had been thoroughly investigated and shown to be affected by a variety of physicochemical factors, such as the composition of the growth medium, the type of strain, cell growth, methods of cultivation, inoculum concentration, time of incubation, pH, temperature, salinity, carbon, nitrogen and mineral sources (Maksimova et al., 1973; Qirang & Zhao, 1994; Chang et al., 1995; Stanbury et al., 1995; Abate et al., 1999; Kumar & Das, 2000; Mevy, 2002; Haq et al., 2002 & 2005).

Physical parameters

The physical parameters include pH, temperature and salinity which influence the production of amylase. Slight change in temperature and pH has adverse effect on the
growth of microorganism as well as on the productivity of α-amylase (Anyangwa et al., 1993).

pH

Sudden variations in cytoplasmic pH can harm bacteria by disrupting the plasma membrane, inhibiting the activity of enzymes and membrane transport proteins. Changes in the external pH alter the ionization of nutrient molecules thereby reducing their availability to the bacteria. As the plasma membrane is relatively impermeable to protons, bacteria often adapt to environmental pH changes to survive. Potassium/proton and sodium/proton antiport systems may correct small variations of pH in bacteria. pH is one of the important factors that determines the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Earlier studies have revealed that fungi require slightly acidic pH and bacteria require neutral pH for optimum growth. pH is known to affect the synthesis, secretion and stability of α-amylase (Fogarty, 1983). The pH value also serves as a valuable indicator of the initiation, successful progression and end of enzyme synthesis (Gigras et al., 2002). Selection of a suitable fermentation medium and initial pH is very important for the enhanced alpha-amylase production (Hughes & Poole, 1991).

The available data suggest that most of the bacterial α-amylases optimally catalyze a reaction at acidic range, pH 2.0-6.5 (Feller et al., 1992; Laderman et al., 1993; Matzke et al., 1997; Jorgensen et al., 1997; Pujalte et al., 1999; Aguilar et al., 2000; Vieille & Zeikus, 2001; Mabrouk, 2008; Rajagopalan & Krishnan, 2008; Schwab et al., 2009; Asoodeh et al., 2010), neutral range pH 6.5-7.5 (Ramesh & Lonsane, 1991; Lealem & Gashe, 1994; Swamy & Seenayya, 1996b; Haseltine et al., 1996; Albayrak et al., 1996; Kelly et al., 1997; Syu & Chen, 1997; Coronado et al., 2000; Thaddeus et al., 2005; Chen et al., 2005; Asgher et al., 2007; Tanyildizi et al., 2007; Rao &
Satyanarayana, 2007; Konsoula & Liakopoulou-Kyriakides, 2007; Smita et al., 2008) and alkaline range pH 7.5-11 (Mc-Tigue et al., 1994; Horikoshi, 1996; Igarashi et al., 1998; Hagihara et al., 2001; Burhan et al., 2003; Amoozegar, et al., 2003; Gomes et al., 2003; Saxena et al., 2007).

**Temperature**

High temperatures damage bacteria by denaturing enzymes, transport carriers, and other proteins. Bacterial membranes are also disrupted by temperature extremes. The lipid bilayer simply melts, disintegrates and leaks. At very low temperatures, membranes solidify and enzymes do not work rapidly. The broad range of temperatures and the enzyme’s high activity at both moderate and lower temperatures make this enzyme highly attractive for both basic research studies and industrial processes. A modern trend among consumers is to use colder temperatures for laundry or dishwashing. The removal of starch from cloth and porcelain becomes more problematic at low temperatures. To overcome this problem, detergents with α-amylases at low and moderate temperatures can be used (Van der Maarel et al., 2002). Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable α-amylases were produced by mesophilic species of Bacillus (Teodoro & Martin, 2000; Burhan et al., 2003). Therefore, a high value is placed on extreme thermostability and thermoactivity of the enzymes.

Many authors reported α-amylase production by psychrophilic bacteria (Feller et al., 1999; Groudieva et al., 2004; Michael et al., 2005; D_Amico et al., 2006; Siddiqui et al., 2006) and mesophilic bacteria (Castro et al., 1992; Swamy & Seenayya, 1996; Lin et al., 1998; Coronado et al., 2000; Zlem kirani et al., 2005; Tanyildizi et al., 2007; Asgher et al., 2007; Smita et al., 2008; Rajagopalan & Krishnan, 2008).
Several reports are available on α-amylase production by thermophilic bacteria (Saha & Bhattacharyya, 1990; Lonsane & Ramesh, 1990; Fatma & El-Refai, 1991; El-Aasser et al., 1992; Padamanabhan et al., 1992; Ivanona et al., 1993; Anyangwa et al., 1993; Ara et al., 1993; Ramesh et al., 1994; Takasaki et al., 1994; Hendrickx et al., 1994; Wind et al., 1994; Swamy et al., 1994; Zhang et al., 1994; Kim et al., 1995; Chung et al., 1995; Bose & Das, 1996; Jana & Pati, 1997; Iqbal et al., 1997; Busch & Stutzenberger, 1997; Syu & Chen, 1997; Dobreva et al., 1998; Lin et al., 1998; Ganghofner et al., 1998; Olama, 1998; Reddy et al., 1998; Gessesse & Mamo, 1999; Mamo & Gessesse, 1999b; Ali et al., 1999; Aguilar et al., 2000; Amoozegar et al., 2003; Wanderley et al., 2004; Chen et al., 2005; Omemu et al., 2005; Saxena et al., 2007; Konsoula & Liakopoulou-Kyriakides, 2007; Mukherjee et al., 2009; Schwab et al., 2009; Asoodeh et al., 2010; Deeksha Gaur et al., 2012).

Extreme thermophiles like species of Pyrococcus (Brown et al., 1990; Koch et al., 1990 & 1991; Laderman et al., 1993; De-Almeida et al., 1997; Vieille & Zeikus, 2001), Thermococcus spp. (Chung et al., 1995; Mishra & Maheshwari, 1996), Rhodothermus marinus (Albayrak et al., 1996), Thermobifida fusca NTU22 (Yang & Liu, 2004) and Geobacillus thermodenitrificans HRO10 (Thaddeus et al., 2005; Rao & Satyanarayana, 2007) also produced α-amylase.

**Salinity**

Bacteria in marine habitat have modified the structure of enzymes, ribosomes, and transport proteins which require high levels of potassium for stability and activity. In addition, the plasma membrane and cell wall of *Halobacterium* are stabilized by high concentration of sodium ions and plasma membrane literally disintegrates if it is low. Halophilic bacteria are classified according to their salt requirement and growth pattern.
Slight halophiles show optimum growth at 2–5% NaCl, moderate halophiles at 5–20% NaCl and extreme halophiles at 20–30% NaCl (Bernhardsdotter et al., 2005; Dutta et al., 2006; Ashabil Aygan et al., 2008; Bal et al., 2009; Ikram-ul-haq et al., 2009; Mohammed Kuddus & Roohi, 2010). Many halophiles and halotolerant microorganisms grow over a wide range of salt concentrations, occasionally depending on environmental and nutritional factors for the growth and tolerance (Surajit Das et al., 2006).

Chemical parameters

The production medium must have suitable chemical composition and contain a source of carbon, nitrogen and mineral salts. The production of α-amylase by Bacillus spp. in natural and synthetic culture medium has been reported earlier by many workers (Maksimova, 1973; Yaronvenko et al., 1973; Markkanen & Bailey, 1974a; Hillier et al., 1996; Mei & Chen, 1997a). The supplementation of essential nutrients greatly affects the growth of bacteria and α-amylase production (Fogarty et al., 1999).

Carbon Sources: Natural and Synthetic

The addition of different carbon sources affects not only the mode of amylase production but also the rate of carbohydrates metabolized (Dubey et al., 2000; Abdullah et al., 2003). The addition of carbon source in the form of either monosaccharides or polysaccharides could influence the production of enzymes (Ajayi & Fagade, 2003; Anto et al., 2006; Sudharhsan et al., 2007). Fukunoto (1943) initiated a series of studies on economically important α-amylase biosynthesis by supplementing various carbon sources.

The activity of amylase was reported to be enhanced in presence of different carbon sources such as Oryza sativa (Haq et al., 2005), corn (Shigechi et al., 2004;
Nitrogen Sources: Natural and Synthetic

The addition of different nitrogen sources reported to be greatly affected the production of α-amylase by *Bacillus subtilis* (Birol et al., 1997). The role of amino compounds is considered to be neither as nitrogen nor as carbon source but as stimulators of amylase synthesis and excretion (Ikura & Horikoshi, 1987). Nitrogen sources that stimulated amylase production are - Yeast extract (Dercova et al., 1992; Mc-Tigue et al., 1994; Hamilton et al., 1999; Malhotra et al., 2000; Santos & Martins, 2003), beef extract (Thaddeus et al., 2005), peptone (Dettori et al., 1992; Yuguo et al., 1993; De-Souza & Martins, 2000; Ramachandran et al., 2004; Aiyer, 2004; Thaddeus et al., 2005), ammonium sulphate (Dercova et al., 1992), ammonium chloride (Das et al., 2004; Aditi et al., 2004; Saxena et al., 2007), casein (Pandey et al., 1994; Goto et al., 1998), cysteine (Ghosh & Chandra, 1984; Mahmoud & Hani, 2012), urea (Ramesh & Lonsane, 1990), potassium nitrate (Jana & Pati, 1997; Haq et al., 2002b) and ammonium nitrate (Suk et al., 1995b; Okolo et al., 1996).

Mineral Sources: Natural and Synthetic

The effects of metal ions on the activity of α-amylase were studied in *Bacillus sp.* strain KSM-1378 (Igarashi et al., 1998). Higher metal ion concentrations often inhibit microbial growth and enzyme production. Adequate concentrations of specific metal
ions are very essential for microbial growth in *Bacillus subtilis* (Kiuduliene et al., 1975; Linden et al., 2003). Chakraborty et al. (2000) reported inhibition of amylase by divalent metal ions. He also reported the effect of some mono and trivalent cations on the enzyme activity of *Bacillus stearothermophilus*.

The stimulatory effect of calcium chloride was reported in *Bacillus sp.* (Iwao et al., 1998), *Bacillus licheniformis* (Yuguo et al., 1994; Ednord & Dietrich, 1996; Allan et al., 1997), *Bacillus amyloliquefaciens* (Hewitt & Solomons, 1996), *Bacillus subtilis* (Suisheng et al., 1997; Gupta et al., 2003) and *Bacillus thermoleovorans* Np 54 (Noorwaz & Satyanarayana, 2000; Malhotra et al., 2000). The stimulatory effect of Magnesium ions was reported in *Bacillus spp.* (Zhang et al., 1994; Kim et al., 1995; Jana & Pati, 1997; Wu et al., 1999; Sodhi et al., 2005) and *Bacillus licheniformis* A-4041 (Yuguo et al., 1994; Ikram-ul-haq, 2009). Stimulatory effect of ferrous ions was reported in *Bacillus spp.* (Zhang et al., 1994; Kim et al., 1995; Pandey et al., 2000; Lo et al., 2001; Haq et al., 2002b; Dey et al., 2003; Goyal et al., 2005; Mohammed & Roohi, 2010), *Bacillus licheniformis* A-4041 (Yuguo et al., 1994), *Thermus sp.* (Shen et al., 1998) and *Bacillus halodurans* LBK 34 (Hashim et al., 2005).

The strong inhibitory effect of mercuric ions in *α*-amylase production was reported in *Bacillus spp.* (Shih & Labbé, 1995; Mamo & Gessesse, 1999b; Pandey et al., 2000; Aguilar et al., 2000; Lo et al., 2001; Cordeiro et al., 2002; Dey et al., 2003; Najafi & Kembhavi, 2005), *Bacillus licheniformis* (Ivanova et al., 1993), *Bacillus firmus* (Igarashi et al., 1998) and *Bacillus subtilis* (Konsula & Liakopoulos-kyriakides, 2004).

The inhibitory effect of zinc sulphate in *Bacillus spp.* (Zhang et al., 1994; Kim et al., 1995; Lin et al., 1998; Elif & Velittin, 2000; Sarikaya & Gürgün, 2000; Arik, 2008; Muhammad Hamad Ashraf, 2004; Bernhanrdsdotter et al., 2005), *Streptomyces*
aureofaciens 77 (Shatta et al., 1990), Bacillus cereus NY 14 (Kadrekar & Ramasarma, 1990), Bacillus subtilis JS-2004 (Laderman et al., 1993; Malhotra et al., 2000), Bacillus licheniformis (Ivanova et al., 1993; Yugu et al., 1994), Bacillus firmus (Igarashi et al., 1998), Thermophilic Bacillus spp. (Burhan et al., 2003; Zlem kiran et al., 2005), Gluconacetobacter diazotrophicus (Saravanan et al., 2007), Bradyrhizobium sp. strain INPA R-991 (Ashabil Aygan et al., 2008) and Bacillus ferdowsicus (Asoodeh et al., 2010) were reported.

**Immobilization of Cell and Enzyme**

Cell/enzyme immobilization is defined as confining the enzyme molecules to a distinct phase from the one in which the substrates and the products are present; this is achieved by fixing the enzyme molecules to or within some suitable material (Katchalski-Katzir, 1993). Immobilization of enzyme molecules does not necessarily render them immobile; the enzyme molecules move freely within their phase, while, in cases of adsorption and covalent binding they are in fact immobile. Material used for immobilization of cells/enzyme is called carrier matrices (inert polymers or inorganic materials).

In the past three or four decades, immobilization technology has developed rapidly and has increasingly become a matter of rational design but there is still the need for further development (Bickerstaff, 1997). Extension of the use of immobilized enzymes to other practical processes requires new methodologies and a better understanding of current techniques.

The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. Ideal support properties include physical resistance to compression, hydrophilicity, inertness toward enzymes, ease of
derivatization, biocompatibility, resistance to microbial attack, and availability at low cost (Treven, 1988; Brodelius & Mosbach, 1987; Buchholz & Klein, 1987).

There are four principal methods available for immobilizing enzymes: adsorption, covalent binding, entrapment and membrane confinement.

In **adsorption method** the enzyme molecules adhere to the surface of the carrier matrix due to combination of hydrophobic effects and the formation of several salt links per enzyme molecules. The binding of enzyme molecules to the carrier matrix is usually very strong, but it may be weakened during use of many factors like addition of substrate, pH and ionic strength. Examples of adsorption matrix are porous carbon, clay, hydrous metal oxides, glasses, polymeric aromatic resins and ion exchange matrices. Loading of about 1gm enzyme/gm matrix is possible.

In **covalent binding method** the enzyme molecules are attached to the carrier matrix by formation of covalent bonds. As a result the strength of binding is very strong and there is no enzyme loss during use. The covalent bond formation occurs with the side chains of amino acids of the enzyme, their degree of reactivity being dependent on their charged status. Lysine residues are the most useful in covalent binding of enzymes since they are usually exposed on the surface, are highly reactive and rarely occur at active sites of enzymes. Enzyme loading is quite low 0.02-0.3gm/gm of matrix.

The reactivity of the protein side-chain nucleophiles is determined by their state of protonation (i.e. charged status) and approximately follows the relationship \(-S^- > -SH > -O^- > -NH_2 > -COO^- > -OH > \) where the charges may be estimated from
the knowledge of the $pK_a$ values of the ionizing groups. Matrix used is agarose, cellulose, sepharose and other polysaccharides.

The possible effects of enzyme immobilization by covalent-binding on enzyme activity are shown in the figure.

(a) Immobilized enzyme (E) with its active site unchanged and ready to accept the substrate molecule (S), as shown in (b). (c) Enzyme bound in a non-productive mode due to the inaccessibility of the active site. (d) Distortion of the active site produces an inactive immobilized enzyme.

Non-productive modes are best prevented by the use of large molecules reversibly bound in or near the active site. Distortion can be prevented by use of molecules which can sit in the active site during the coupling process, or by the use of a freely reversible method for the coupling which encourages binding to the most energetically stable (i.e. native) form of the enzyme. Both (c) and (d) may be reduced by use of 'spacer' groups between the enzyme and support, effectively displacing the enzyme away from the steric influence of the surface.
In cross-linking, each biofunctional reagent (Glutaraldehyde) molecule binds to two enzyme molecules; ultimately, a network of enzyme molecules linked together. So it is used for producing immobilized enzyme membranes for biosensors, for example, lens tissue paper and nylon net fabric.

Immobilization may lead to loss in enzyme activity due to the involvement of active site in immobilization of the enzyme in an orientation which either distorts the active site or renders it unavailable.

Figure showing the intermolecular cross-linking of an enzyme.

Immobiliarization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups of an insoluble support matrix.

In entrapment method enzyme molecules are entrapped within suitable gels or fibers and there may or may not be covalent bond formation between the enzyme molecules and the matrix. A non-covalent entrapment may be viewed as putting the enzyme molecule in a molecular cage. When covalent binding is also to be generated, the enzyme molecules are usually treated with a suitable reagent. Acrylamide and bisacrylamide are mixed to form a gel containing the entrapped enzyme which may be
used to form small beads or a film on a solid support. An emulsion of the enzyme and cellulose acetate is prepared in methylene chloride. Entrapment in calcium alginate is the most widely used method. Enzyme loading is very high 1gm/gm of gel or fiber. The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through but retains the enzyme (O’Driscoll, 1976). This method differs from the coupling methods described above, in that the enzyme is not bound to the matrix or membrane. There are different approaches for entrapping enzymes such as gel (Bernfeld & Wan, 1963) or fiber entrapping (Dinelli et al., 1976) and micro-encapsulation (Wadiack & Carbonell, 1975).

**Figure showing the entrapment in beads and fiber.**

In **membrane confinement**, the enzyme molecules are usually in an aqueous solution may be confined within a semi permeable membrane which, ideally, allows a free movement in either direction to the substrate and products but does not permit the enzyme molecules to escape.
The entrapment method of immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. It is done in such a way as to retain protein while allowing penetration of substrate. It can be classified into lattice and microcapsule types. Lattice-Type entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. Some synthetic polymers such as polyacrylamide, polyvinyl alcohol and natural polymer (starch) have been used to immobilize enzymes using this technique. Microcapsule-Type entrapping involves enclosing the enzymes within semi permeable polymer membranes. The preparation of enzyme microcapsules requires extremely well-controlled conditions.

The use of immobilized cells offers several advantages over free cells, such as upon completion of the reaction the product can be easily recovered. The immobilized enzyme is easily removed from the reaction making it easy to recycle the biocatalyst, cost effective, prevent washout and reduce risk of contamination. Immobilized enzymes typically have greater thermal and operational stability than the soluble form of the enzyme, used in non-aqueous systems, support continuous production system, reduce
effluent handling problems during recovery and enzyme is used at much higher concentration.

The entrapment technique reported to improve productivity (Pandey et al., 2000; Goksungur & Zorlu, 2001; Rossi-Alva & Rocha-Leao, 2003). The decrease of the specific activity of amylases after the immobilization was reported by Gottschalk & Jaenicke (1991) and Roy et al. (1995). Many applications of immobilized cells/enzymes from bacteria in different matrices by various immobilization methods have been reported by Poulsen (1985), Klein et al. (1985), Peppler & Reed (1987), Suzuki et al. (1998) and Betancor et al. (2008). Stability of the enzyme on immobilization depends on the effect of the microenvironment.

Disadvantages of immobilized cells/enzymes are: increase in additional cost for immobilization, affects the stability and activity of the enzymes, immobilization is not possible when one of the substrates is insoluble and problems may arise in diffusion of the substrate to reach the enzyme.

Immobilization of amylase and cells were reported in agar and agarose (Matsunaga et al., 1980; Veelken & Pape, 1982; Kierstan & Coughlin, 1985; Trevan, 1988; Tayade & Kale, 2004), polyacrylamide (Treven & Grover, 1979; Freeman & Aharonowitz, 1981; Sadhukhan et al., 1993), sodium alginate (Smidsrod & Skjåk-Bræk, 1990; Kennedy & White, 1979; Kim & Lee, 1992; Stewart & Swaisgood, 1993; Rubio & Ghaly, 1994; Bickerstaff, 1997; Suzuki et al., 1998; Gomboz & Wee, 1998; Ramkrishna et al., 1992; E1-Kamal et al., 2003; Dey et al., 2003; Gargi et al., 2003; Bajpai & Sharma, 2004; Tayade & Kale, 2004; Zoc & Maria, 2006; Figen Ertan et al., 2007; Lily & Meyer, 2007; Borgio, 2011) and carrageenan (Chamy et al., 1990; Moon & Parulekar, 1991; Chao et al., 1986; Hugerth et al., 1997; Belyaeva et al., 2004;
Elnashar et al., 2005; Tanriseven & Dogan, 2002; Elnashar et al., 2008; Elnashar & Yasin, 2009a & 2009b; Elnashar et al., 2009; Danial et al., 2010).

**Strain Improvement**

Physical and chemical mutagens are promising and are used for screening of high yielding strains (Sidney & Nathan, 1975). Microorganisms usually produce commercially important metabolites in very low concentrations by their inherent control system. Although the yield may be increased by optimizing the cultural conditions, ultimately the productivity is controlled by the organism’s genome (Stanbury et al., 1995). In the last few decades, the exponential increase in the application of amylases in various fields has placed stress and demand in both qualitative improvement and quantitative enhancement through strain improvement. Such improved strains reduce the cost of the process. It creates new variations in the gene pool. The challenge is to isolate those strains which are true mutants that carry beneficial mutations (Parekh et al., 2000). Bacillus spp. such as B. subtilis and B. amyloliquefaciens are the most commonly used organisms of choice for amylase production (Qirang & Zhao, 1994; Haq et al., 2005; Sivaramakrishnan et al., 2006). Highly active α-amylase is preferred for the conversion of starch into oligosaccharides. So, it is worthwhile to select a potent microbial strain for α-amylase production.

**Physical mutagen**

Ultraviolet (UV) light exerts its mutagenic effect by exciting electrons in molecules. The excitation of electrons in DNA molecules often results in the formation of extra bonds between adjacent pyrimidines (specifically thymine) in DNA. When two pyrimidines bind together in this way, it is called a pyrimidine dimer. These dimers often change the shape of the DNA in the cell and cause problems during replication.
The cell often tries to repair pyrimidine dimers before replication, but the repair mechanism may also lead to mutation as well (Sambrook et al., 1989). Mutation is the primary source of all genetic variation and has been used extensively in industrial improvement of enzyme production (Ghisalba et al., 1984; Sidney & Nathan, 1975). The UV light is the best studied mutagenic agent in prokaryotic organisms (Meemu et al., 2000).

**Figures showing the effect of UV on DNA crosslinks; two neighboring pyrimidine bases.**

The UV mutant strains of *Bacillus* reported to show better ability to produce alpha amylase, which can be derived by mutagenesis and extensive screening (Konishi et al., 1990; Xuchi et al., 1991; Ito et al., 1991; Eugenia & Donia, 1992; Barnett et al., 1996; Sidu et al., 1997; Sidhu et al., 1997; Uguru et al., 1997; Rivera et al., 2003; Shiau et al., 2003; Dinu et al., 2001; Surendra Singh et al., 2011).

**Chemical mutagens** can be classified as base analogs (nitrous acid, bromouracil and aminopurine adenine), alkylating agents (nitrosoguanidine, methyl methanesulfonate and ethyl methanesulfonate) and intercalating agents (acridine orange, proflavin and ethidium bromide). Chemical mutagens are stronger mutagenic
agents and cause permanent changes in DNA sequence. They bring about transitions from G: C → A: T (Miller, 1972) and have preferential effect on DNA replication (Rowland et al., 1995; Wu et al., 2006; Haq et al., 2002; Joh et al., 2004; Paul et al., 2005; Besoain et al., 2007).

**Figures showing the chemical mutagens.**

![Chemical structures](image_url)

A. Nitrous acid   B. Acrylamide   C. 5'-fluorouracil   D. Ethidium bromide

**Nitrous acid** (HNO₂, Hydroxidooxidonitrogen, O=NO) causes interstrand cross-linking of DNA. The DNA strands fail to separate and there is no DNA duplication, which is lethal or deleterious. Nitrous acid is used to make diazides from amines; this occurs by nucleophilic attack of the amine onto the nitrite, reprotonation by the surrounding solvent and double-elimination of water. The diazide can then be liberated to give a carbene. Nitrous acid acts as a mutagen by deamination of the NH₂ group of adenine and cytosine to an ether group, thus altering their base pairing. It causes oxidative deamination of particular bases. Conversion of the amino groups to keto groups changes the hydrogen bonding potential of the bases. Nitrous acid alters a base directly to a "miscoding" form and thus does not require subsequent DNA synthesis for its effect. Since the deamination of adenine leads to AT.GC transition and the deamination of cytosine results in CC.AT transitions, nitrous acid induces transitions in both directions. Nitrous acid was reported to be a suitable mutagen for the improvement
Figure showing the effect of nitrous acid by deamination.

Ethyl Methane Sulfonate (EMS) is an alkylating agent which carries one, two or more alkyl groups in reactive form. They are capable of being transferred to other molecules where electron density is high. The transfer of methyl or ethyl groups to the bases such that their base-pairing potentials are altered and transitions result. Thus it induces all types of mutations (transitions, transversions, frame shifts and even chromosome aberrations) with various relative frequencies. Depending on the affected atom, the alkylated base may then degrade to yield a baseless site, which is mutagenic and mispair to result in mutations upon DNA replication. It produces random mutations in genetic material by nucleotide substitution, particularly by guanine alkylation. This typically produces only point mutations. It induces mutations at a rate of $5 \times 10^{-4}$ to $5 \times 10^{-2}$ per gene without substantial killing. Ethyl methane sulfonate was reported as
potent mutagen in amylase production in *Bacillus* spp. (Konishi et al., 1990; Sidhu et al., 1997; Haq et al., 2002; Sarikaya & Gurgun, 2000; Ikram-ul-haq et al., 2009).

**Figure showing alkylation with EMS or nitrosoguanidine.**

G $\rightarrow$ 6Eq pairs with T instead of C.

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**Mutagenesis by Ethyl Methane Sulfonate (EMS)**

Acrylamide (acrylic amide) is a chemical compound with the chemical formula C$_3$H$_5$NO. Its IUPAC name is prop-2-enamide. It is incompatible with acids, bases, oxidizing agents, iron and iron salts. It decomposes non-thermally to form ammonia and thermal decomposition produces carbon monoxide, carbon dioxide and oxides of nitrogen. It is effective in causing bacterial mutation. It shows direct and indirect modes of interaction with DNA results in alkylating DNA adducts (Ahmad & Gerd, 2005).

**Base analog mutagen (5-fluoro uracil)** has a structure similarity to the normal bases so that it can be metabolized and incorporated into DNA during replication, but by increasing the frequency of impairing. Structurally resembling purines and pyrimidines may be incorporated into DNA in place of the normal bases during DNA replication. It resembles thymine (has F atom instead of methyl group) and incorporate into DNA and pair with adenine like thymine. 5-Fluoro Uracil (5-FU) is a thymine
analog. It has a higher likelihood for tautomerization to the enol form (FU*). Methyl group in 5th position of thymine and fluorine at 5'-position in 5'-FU have similar effects. But probability of tautomeric shifts increase because of fluorine molecule. After tautomeric shift to its enol form, 5'-fluorouracil pairs with guanine and thus causes A. T to G. C transitions. Base analogs cause transitions in the bidirectional mode. Hence mutations induced by these substances can be reversed by using the same compounds.

**Ethidium bromide** (EtBr, Intercalating agent) acts as a mutagen because it intercalates double stranded DNA and deform the DNA (McCann & Ames, 1975). Ethidium bromide is a large, flat basic molecule that resembles a DNA base pair. This insertion causes "stretching" of the DNA duplex and the DNA polymerase is "fooled" into inserting an extra base opposite an intercalated molecule. This results in frameshift mutations. This affects DNA biological processes, like DNA replication and transcription.

**Alpha-amylase purification**

Ideal purification or recovery process (Downstream process) should optimize both the purity and the concentration of the metabolite. The desired product is a metabolite, which may be extracellular (amylases, proteases, alcohol, citric acid, penicillin and streptomycin), intracellular (nucleic acids, vitamins, sisomycin and griseofulvin) and both intra and extra cellular (vitamin-B12 and flavomycin) (Wulf & Anneliese, 2004).

There is no exact procedure that is sure to work for the purification of all proteins and each have steps that are specific to the properties of the protein. Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. The various steps in the purification process may free the protein
from a matrix that confines it, separate the protein and non-protein parts of the mixture and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps may exploit differences in protein size, physico-chemical properties, binding affinity and biological activity (Fisher & Stein, 1961).

The first step in product recovery is the separation of cell biomass and the insoluble nutrient ingredients from the supernatant by centrifugation method. A very common preliminary separation comes from adding ammonium sulfate in stages. Different proteins precipitate at different salt concentrations and this divides the enzymes and other proteins into fractions.

**Ammonium sulfate** is an inorganic salt. It is the best, first-choice salt for initial development of a salting-out program to precipitate sought-for proteins because of sulfate's kosmotropic and protein-molecule exclusionary powers. If precipitation of proteins does not occur quickly, it may be best to wait for few hours, to allow time for precipitation to form. This becomes necessary for dilute protein solutions if the overall concentration is less than 1%. Alpha-amylase purification by (NH₄)₂SO₄ precipitation is reported in *Bacillus* spp. (Kobayashi et al., 1992; Khoo et al., 1994; McTigue et al., 1995; Chung et al., 1995; Declan et al., 1997; Hamilton et al., 1999; Najafi & Kembhavi, 2005; Swain & Ray, 2007; Varalakshmi et al., 2008; Gangadharan et al., 2008).

**Dialysis** helps in separation of small molecules from larger ones by diffusion through a semi permeable membrane. It increases the volume of the enzyme solution, because of the initial osmotic effect of the (NH₄)₂SO₄. Dialysis in purification of α-amylase from the fermented broth is very essential for its stability as well as its activity.
(Jin et al., 1992; Fattah et al., 1992; Ying et al., 1997; Hamilton et al., 1999; Ali et al., 2001; Tan et al., 2003).

**Gelfiltration** is also known as molecular sieve or exclusion chromatography. Matrix is a crosslinked polymer which swells in water forming a gel of a three dimensional net work of pores. The size of pore is determined by degree of cross linking of polymeric chains. Large molecules are excluded through interstitial space; small molecules enter into the porous interior of the gel bead and are capable of diffusing in and out of the gel beads (Beedu sashidhar rao & Vijay deshpande, 2005).

**Ascending paper chromatography** is a process where the paper is saturated with water vapors to form a thin film of water around cellulose fibers of the paper acts as a stationary phase and the solvent as mobile phase. The separation of different components occurs on the basis of differences in their partition coefficients. The separated compounds are identified on the basis of their \( R_f \) values. Relative factor is equal to distance travelled by the component from the base line/distance travelled by the solvent from base line.

**Thin layer Chromatography** (TLC) helps in separation based on differential adsorption as well as partitioning of the analytes between the liquid stationary phase and mobile solvent phase. The \( R_f \) value of an analyte depends upon the solvent system, degree of saturation of the mobile phase, particle size of the adsorbent, type of adsorbent, temperature and humidity (Beedu sashidhar rao & Vijay deshpande, 2005). The reducing sugar released by the \( \alpha \)-amylase activity was maltose under assay conditions in TLC was reported by Hansen (1975), Gashaw Mamo & Amare Gessesse (1999a) and Ashabil Aygan et al. (2008). Vaseekaran et al. (2010) reported that the \( \alpha \)-amylase activity released glucose, under assay conditions.
Native Polyacrylamide gel electrophoresis (Native PAGE)

The separation of proteins in native polyacrylamide gel electrophoresis is based on both charge density and size of the molecules. In native PAGE, same buffer ions are present throughout the sample, gel and electrode vessel reservoirs at constant pH such that proteins retain their native conformation and biological activity and hence it is known as native PAGE. In the present study, native PAGE is used to determine the homogeneity of α-amylase (Sawhney & Randhir Singh, 2001).

Specific Staining (Iodine staining)

Alpha-amylase is an exoenzyme that hydrolysis starch. The ability to degrade starch is used as a criterion for the determination of α-amylase production. Starch test is used to determine the absence or presence of starch in the gel by using iodine solution as an indication. Starch in the presence of iodine produces a dark-blue colouration of the gel, and a halo zone around the band indicates amylolytic activity (Aneja, 2006).

Kinetics of α-amylase

Effect of temperature on α-amylase activity

The rate of an enzyme catalyzed reaction increases with rise in temperature till it reaches the maximum. The effect of temperature is due to thermal stability of enzyme, enzyme substrate affinity, alteration of pKₐs by the heat of ionization, affinity of an enzyme for the activators or inhibitors, different temperature coefficients and change in rate limiting functions (Sawhney & Randhir Singh, 2001). The α-amylase activity with optimum temperature range 30°C-50°C was reported in Bacillus spp. (Kim et al., 1992; Salva & Moraes, 1994; Kim et al., 1995; Macro et al., 1996; El-Safey & Ammar, 2002; Kusuda et al., 2003; Pimpa 2004; Elif Demirkan & Demirkan, 2011), Myxococcus
coralloides (Stevens et al., 1994), Streptococcus bovis (Freer, 1993), Clostridium perfringenes (Shih et al., 1995) and Bifidobacterium adolescentis (Lee et al., 1997).

The optimum temperature range, 55°C-70°C for alpha-amylase activity was reported in Bacillus spp. (Koch et al., 1990; Koch et al., 1991; Hawary, 1991; Schumann et al., 1991; Ming et al., 1992; El-Aassar et al., 1992; Laderman et al., 1993; Babu & Satyanarayana, 1993; Kim et al., 1995; Declan et al., 1997; Boltan et al., 1997; Lin et al., 1998; Hamilton et al., 1999; Malhotra et al., 2000; Deutch, 2002; Kariya et al., 2003; Duy & Fitter, 2005), Lactobacillus spp. (Adeleye et al., 1990; Burgess-cassler et al., 1991; Giraud et al., 1993; McTigue et al., 1994) and Streptomyces megasporus strain SD12 (Dey & Agarwal, 1999).

Extreme optimum temperature for amylase activity range 75°C-100°C was reported in Bacillus spp. (Stetter, 1992; Fengxie et al., 1992; Chunzhi et al., 1992; Ivanova et al., 1993; Zhang et al., 1994; Uguru et al., 1997; Lin et al., 1998; Akiba et al., 1999; Burhan et al., 2003), Pyrococcus spp. (Laderman et al., 1993; De souza et al., 1996) and Thermococcus spp. (Kobayashi et al., 1994; Chung et al., 1995; Egas et al., 1998).

Effect of pH on activity of α-amylase

The state of ionization of the components changes with pH, which may involve the free enzyme, the enzyme–substrate complex or the substrate itself. Distribution of the total enzyme among the various ionic forms depends on the pH and the ionization constants of the various groups. Since the catalytic activity is usually confined to a narrow range of pH, only one of the ionic forms of the enzyme is catalytically active. The optimal pH of α-amylase activity around acidic range 3.0-6.5 was reported in Bacillus spp. (Brown et al., 1990; Antranikian, 1990; Hawary, 1991; El-Aassar et
al., 1992; Ivanova et al., 1993; Zhang et al., 1994; McTigue et al., 1994; Salva Moraes, 1995; Chung et al., 1996; Macro et al., 1996; Ugaru et al., 1997; Declan et al., 1997; Boltan et al., 1997; Hamilton et al., 1999; El-Safey & Ammar, 2002; Gupta et al., 2003; Kariya et al., 2003; Pimpa 2004), Thermococcus spp. (Chung et al., 1996; Egas et al., 1998; Akiba et al., 1999), Lactobacillus spp. (Adeleye, 1990; Burgess-cassler et al., 1991; Giraud et al., 1993), Streptococcus bovis (Freer, 1993), Clostridium perfringenes (Shih et al., 1995) and Bifidobacterium adolescentis (Lee et al., 1997).

Several authors reported a neutral pH of 7.0 for amylase activity from Bacillus spp. (Ladermann et al., 1993; Syu & Chen, 1997; Tanyildizi et al., 2005; Haq et al., 2005), Bacillus subtilis GCBUCM-25 (Haq et al., 2002), Myxococcus varians (Loginova et al., 1970) and Bacillus thermoleovorans NP5 (Malhotra et al., 2000). The optimal pH of amylase activity at alkaline range of 8-12 was reported in Bacillus spp. (Kim et al., 1995; Lin et al., 1998; Burhan et al., 2003), Myxococcus coralloides (Stevens et al., 1994) and Natronococcus sp. strain Ah 36 (Kobayashi et al., 1992).

Effect of substrate concentration on α-amylase activity

The kinetics of amylase catalyzed reactions is studied by three theories. The Michaelis-Menten theory assumes that the enzyme first combines with the substrate to form the enzyme-substrate complex and latter breakdown to form free enzyme and the product. These reactions are assumed to be reversible, reactions catalyzed by enzymes have a single substrate and the rate of the reaction system is in steady state. The Michaelis-Menten constant is equal to the substrate concentration at which the initial reaction velocity is half maximal. The advantages of Michaelis-Menten equation are - it helps in mathematical description of enzyme kinetics, quantitative assay of enzyme activity in bacteria and enzyme purification. It is an analysis of some enzyme
regulatory mechanisms, describes the kinetic behavior of amylase, represents the standard catalytic efficiencies of amylases and used to determine the Km at various substrate concentrations and can be used in prediction rate-limiting steps. Line weaver-burk equation and the Eadie-Hofstee equation are the two transformations of the Michaelis-Menten equation.

The rate of an enzyme depends on the amount of substrate available. At relatively low substrate concentration, initial velocity increases linearly with an increase in the substrate. At higher substrate concentration, initial velocity increases smaller extent in response to increase in substrate. Finally a point is reached beyond which there is no increase in the velocity with increase in the concentration plateau is called the Vmax (Sawhney & Randhir Singh, 2001).

Several authors reported Km value of α-amylase in Bacillus spp. by using starch as substrate: Myxococcus varians (Loginova et al., 1970), Lactobacillus brevis (Adeleye, 1990), Streptococcus bovis (Freer, 1993), Lactobacillus plantarum (Giraud et al., 1993), Myxococcus coralloides (Stevens et al., 1994), Bacillus subtilis (Macro et al., 1996), Bacillus flavothermus (Bol 타인 et al., 1997), Bifidobacterium adolescentis (Lee et al., 1997), Thermococcus filiformis (Egas et al., 1998) and Bacillus cereus GUF8 (Aguilar et al., 2000).

Determination of the molecular weight of α-amylase by SDS PAGE

The molecular weight of α-amylase range 10-50 kDa was reported in Bacillus spp. (Chang et al., 1995; Anidyawati et al., 1998; Schokker & van Boekel, 1999; Rao et al., 2002; Duy & Fitter 2005; Alva et al., 2007), Bacillus caldolytyes (Ikram-ul-haq, 2010), Thermococcus profundus (Koch et al., 1991), Myxococcus coralloides (Stevens et al., 1994) and Alteromonas haloplanctis (Feller et al., 1992).
The molecular weight of α-amylase range 51-100 kDa was reported in *Bacillus spp.* (Kim et al., 1992; El-Aasser et al., 1992; Ivanova et al., 1993; Kong et al., 1993; Kim et al., 1995; Salva & Moraes, 1995; Macro et al., 1996; Boltan et al., 1997; Uguru et al., 1997; Hamilton et al., 1999; Aguilar et al., 2000; Pandey et al., 2000), *Microccocus luteus* (Fergus, 1969), *Bifidobacterium adolescentis* (Lee et al., 1997), *Thermococcus filiformis* (Egas et al., 1998), *Clostridium perfringenes* (Shih et al., 1995), *Streptococcus bovis* (Freer, 1993), *Pyrococcus spp.* (Koch et al., 1991; Laderman et al., 1993; De souza et al., 1996) and *Lactobacillus brevis* (Adeleye et al., 1990).

The molecular weight of α-amylase range 101-210kDa was reported in *Lactobacillus amylovorus* (Burgess-cassler et al., 1991) and *Chloroflexus aurantiacus* (Ikram-ul-haq et al., 2010).

**Effect of activators on α-amylase activity**

The stimulatory effect of CaCl$_2$ on amylase activity was reported by several investigators in *Bacillus spp.* (Brown et al., 1990; Ramesh & Lonsane 1990; Ming et al., 1992; El-Aasser et al., 1992; Chunzhi et al., 1992; Fengxie et al., 1992; Ivanova et al., 1993; Yuguo et al., 1994; Salva & Moraes, 1994; Takasaki et al., 1994; Goyal et al., 1995; Abou Zeid, 1997; Hori et al., 1997; Kelly et al., 1997; Iwao et al., 1998; Pandey et al., 2000; Savchenko et al., 2001; Haq et al., 2002; Gupta et al., 2003; Burhan et al., 2003; Tanaka & Hoshino, 2003; Sindhu 2005; Goyal et al., 2005; Swain et al., 2006; Polaina & MacCabe 2007; Sirima sukasesm, 2007; Carvalho et al., 2008; Ikram-ul-haq et al., 2009; Sun et al., 2010; Elif Demirkan & Demirkan, 2011). It was reported that the calcium ions stabilize the α-amylase activity in *Bacillus spp.* (Brown et al., 1990; El-Aasser et al., 1992; Chunzhi et al., 1992; Fengxie et al., 1992; Salva &
Moraes, 1994; Goyal et al., 1995; Jorgensen et al., 1997; Iwao et al., 1998; Haq et al., 2002; Burhan et al., 2003).

Magnesium sulphate reported to stimulate α-amylase in *Bacillus* spp. (Saito & Yamamoto, 1974; Roheena abdullah, 2005). In contrary to this result MgSO₄ reported to inhibit the enzyme activity. Probably this metal block binding sites of enzyme or enzyme contain number of metals and displacement of these ions by another metal ions, either with some change result in inhibition of enzyme activity (Abou Zeid, 1997; Ikram-ul-haq et al., 2010).

Stimulation of NaCl on activity of α-amylase was reported in *Bacillus* spp. (Good & Hartman, 1970; Velcheva & Galabova, 1984; EI-Aassar et al., 1992; Laderman et al., 1993; Shih & Labbé, 1995; Hori et al., 1997; Pandey et al., 2000; Sarikaya & Gürgün, 2000; Gupta et al., 2003; Bernhardsdotter et al., 2005; Najafi & Kembhavi, 2005; Sindhu, 2005; Roheena abdullah, 2005; Sun et al., 2010), *Helixdiaiptomus viâus* (Dutta et al., 2006) and *Pyrococcus woesei* (Laderman et al., 1993). Inhibitory effect of NaCl on the enzyme activity was also reported in *Bacillus* spp. (Chung et al., 1995; Burhan et al., 2003; Ikram-ul-haq et al., 2010).

**Effect of inhibitors on α-amylase**

Higher metal ion concentrations often inhibit microbial growth and enzyme production. Adequate concentrations of specific metal ions are very essential for microbial growth of *Bacillus subtilis* (Kiuduliene et al., 1975; Linden et al., 2003). Previous literature indicated that most amylase activities were inhibited in the presence of Ni²⁺, Cd²⁺, Cu²⁺, Ag⁺, Pb²⁺, Fe²⁺ and Zn²⁺. The α-amylases from *Bacillus* spp. were strongly inhibited by Ni²⁺, Cd²⁺, Zn²⁺ and Hg²⁺(Igarashi et al., 1998; Cordeiro et al., 2002). Zinc chloride has inhibitory effect on the activity of enzyme in *Aspergillus*
flavus (Abou zeid, 1997). The α-amylase from *Thermus* sp. was strongly inhibited by 
Cu$^{2+}$ and Fe$^{2+}$ (Shen *et al.*, 1998) and the α-amylase from *Bacillus subtilis* was strongly 
inhibited by Zn$^{2+}$, Ag$^+$, Cu$^{2+}$ and Fe$^{2+}$ (Elif & Velittin, 2000). However, the activity of 
*Nocardiopsis* sp.7326 amylase was not affected by Zn$^{2+}$, Ni$^{2+}$ and Fe$^{2+}$ but activated by 
Cd$^{2+}$ and Cu$^{2+}$.

Alpha-amylase was strongly inhibited by Ag$^{2+}$ in *Bacillus* spp. (Fengxie *et al.*, 1992; Chunzhi *et al.*, 1992; Ming *et al.*, 1992; El-Aassar *et al.*, 1992; Hn *et al.*, 1992; 
Kong *et al.*, 1993; Zhang *et al.*, 1994; Chung *et al.*, 1996; Malhotra *et al.*, 2000; Pandey 
*et al.*, 2000; Elif & Velittin, 2000; Cordeiro *et al.*, 2002; Gupta *et al.*, 2003; Sun *et al.*, 
2010; Elif Demirkan & Demirkan, 2011) and α-amylase was inhibited by Hg$^{2+}$ in 
*Bacillus* spp. (Hawary 1991; El-Aassar *et al.*, 1992; Babu & Satyanarayana, 1993b; Lin 
et al., 1998; Igarashi *et al.*, 1998; Pandey *et al.*, 2000; Gupta *et al.*, 2003; Ramirez 

The activity is inhibited in the presence of zinc ions in *Bacillus* spp. (Brown *et al.*, 
1998; Elif & Velittin 2000; Cordeiro *et al.*, 2002; Burhan *et al.*, 2003; Roheena 
Abdullah, 2005), *Bacillus dipsosauri* (Deutch, 2002), *Bacillus stearothermophilus* 
(Chakraborty *et al.*, 2000), *Bifidobacterium adolescentis* (Lee *et al.*, 1997) and *Bacillus 
puhiius* (Ming *et al.*, 1992).

Ethylendiamine tetraacetic acid exhibited no effect on amylase activity in 
*Bacillus* spp. (Ramesh & Lonsane, 1990; Ivanova *et al.*, 1993; Salva & Moraes, 1994; 
1998; Burhan *et al.*, 2003) and *Bifidobacterium adolescentis* (Lee *et al.*, 1997), but
stimulated the activity of amylase in *Bacillus* spp. (Babu & Satyanarayana, 1993b; Yuguo et al., 1994; Pandey et al., 2000; Gupta et al., 2003; Sindhu, 2005; Sun et al., 2010). Stimulatory effect of Cu$^{2+}$ was reported by Krishnan & Chandra (1983).

**Effect of temperature on stability of α-amylase activity**

All the enzymes have a narrow temperature range for their efficient functioning. The enzyme activity declines at temperature beyond optimum temperature. It is important to understand the thermostability of the enzyme. It was reported that the amylase was optimally active with a half-life of 3hrs at 100°C in *Bacillus thermooleovorans* NP5 (Malhotra et al., 2000). The enzyme was stable retaining more than 90% of its original activity after 60min exposure at 90°C and 20min exposure at 95°C in *Bacillus licheniformis* mutant 7902 (Kong et al., 1993; Xianlang et al., 1993). The enzyme was reported to be stable at 90°C for 10min in *Bacillus* sp. (Goyal et al., 1995). The optimum temperature for α-amylase activity was 40°C and 71% of the activity was still maintained until 30 min after heating at 80°C in *Bacillus* sp. (Chung et al., 1996). The thermal stability of amylase in bacteria was studied by Salva & Moraes (1994), Schokker & Van Bockel (1999), El-Safey & Ammar (2002), Pimpa (2004) and Alva et al. (2007).

**Applications**

Alpha-amylase obtained from the *Bacillus* spp.: *B. subtilis, B. stearothermophilus, B. licheniformis* and *B. amyloliquifaciens* are widely used for various applications. Today in the global enzyme market, α-amylases account for about 30% (Sidhu et al., 1997; Van der Maarel et al., 2002; Sivaramakrishnan et al., 2006). These enzymes have applications in starch processing, desizing of textiles, paper sizing, detergent additive, bread improvement, ethanol production, sewage treatment, effluent
treatment and other fermentation processes (Pandey et al., 2000; Maarel et al., 2002; Lowe, 2002; Haki & Rakshit, 2003; Gomes et al., 2005).

Food industry

Couto & Sanromán (2006) described how amylases were employed in processed-food industry such as fruit juices, starch syrups, baking, production of cakes, brewing, and preparation of digestive aids.

Bakery industry

Alpha-amylases have been used in the bakery industry for over more than hundred years. Commercially, a thermostable maltogenic amylase of Bacillus stearothermophilus is being used in the bakery industry (Van der Maarel et al., 2002). Amylases added to the dough of bread to degrade the starch in the flour into smaller dextrins, which are subsequently fermented by the yeast. The addition of α-amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, for the improvement in the volume and texture of the product. Moreover, it generates additional sugar in the dough, which improves the taste, toasting qualities of the bread and crust colour. In addition to generating fermentable compounds, α-amylases improve the moisture retention of the crumb, enhancing softness, anti-staling effect, shelf life and freshness (Dragsdorf & Varriano, 1980; Ottrup & Norman, 1984; Cauvain & Chamberlain, 1988; Akers & Hoseney, 1994; Leon et al., 1997; Duedahl-Olesen et al., 1999; Nielsen & Borchert, 2000; Van der Maarel et al., 2002; Gupta et al., 2003).

The heat stable bacterial α-amylases have been useful in anti-staling additives by delaying the starch retrogradation (Hebeda et al., 1991). Stickiness can be solved by
using maltogenic amylases to produce linear oligosaccharides of 2-6 glucose residues. Alpha-amylase induces dextrinization of starch granules reducing its ability to immobilize water as free water increases dough mobility (Miranda-Lopez, 1999). Mesophilic bacterial amylases have been confirmed to be useful in anti-staling by retarding the starch retrogradation. A thermostable maltogenic amylase of *Bacillus stearothermophilus* has been used commercially in the bakery industry (Hebeda *et al.*, 1991; Van Damme *et al.*, 1992; Hamer, 1995; Qi Si, 1996; Sahlstrom & Brathen, 1997; Christophersen *et al.*, 1998; Dauter *et al.*, 1999). Bacterial α-amylases have higher thermal stability and survive the baking temperatures (Armero & Collar, 1996; Martinez *et al.*, 1999).

**Laundry industry and Automation Dishwashing (Restaurants)**

Detergent industries are the primary consumers of enzymes, in terms of volume and value. Enzymes used in laundry detergent must be inexpensive, stable, and safe to use. The use of enzymes in detergent formulations enhances the detergent’s ability to remove tough stains and make the detergents environmentally safe. Automation dishwashing in restaurants and house hold usage is common. The demand is high for α-amylase detergents use in automatic dishwashing. Amylases are used in the formulation of enzymatic detergents and 90% of all liquid detergents contain amylase enzymes (Olsen & Falholt, 1998; Kirk *et al.*, 2002; Maarel *et al.*, 2002; Gupta *et al.*, 2003; Ito *et al.*, 2005; Thippswamy *et al.*, 2006; Mitidieri *et al.*, 2006; Arikan, 2008; Carvalho *et al.*, 2008; Shaw *et al.*, 2009; Ghorai *et al.*, 2009; Chi *et al.*, 2009; Hmidet *et al.*, 2009; Mukherjee *et al.*, 2009 Hoffman, *et al.*, 2010).

The enzymes in detergent formulation used in laundry and automatic dishwashing degrade the residues of starchy foods such as potatoes, custard, chocolate, gravies, etc. to dextrins and other smaller oligosaccharides (Olsen & Falholt, 1998;
Kirk et al., 2002; Chi et al., 2009; Mukherjee et al., 2009). Amylases used in the detergent industry are produced from Bacillus or Aspergillus spp. (Mitidieri et al., 2006).

**Ethyl alcohol dual fermentation**

For ethyl alcohol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world (Chi et al., 2009). In beer industries, microbial amylases are used to aid cereal amylases in the production of fermentable sugar (Kirk et al., 2002). The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar by using an amylolytic microorganism or enzymes such as α-amylase, subsequently by fermentation, sugar is converted into ethanol using an ethanol forming microorganism such as yeast Saccharomyces cerevisiae (De Moraes et al., 1995). Among bacteria, α-amylase obtained from thermoresistant bacteria, Bacillus licheniformis, engineered strains of Escherichia coli and Bacillus subtilis were used during the first step of hydrolysis of starch suspensions (Sanchez & Cardona, 2008). Still, further studies are needed to reduce the cost by dual fermentation method for ethanol production.

Ethanol formed by the bioconversion of starchy materials is commonly known as biofuel. In the present scenario, however, production cost is very high. Even though methods are developed to minimize cost by fermentation of starch to ethanol in one step using two different co-culture strains and low temperature cooking fermentation systems that succeeded in reducing energy consumption by approximately 50%, it is still necessary to add large amounts of amylolytic enzymes to hydrolyze the starchy materials to glucose (Matsumoto et al., 1982 & 1985).
Sewage water treatment and fodder production

In any treatment system, food processing wastewater offers a unique challenge; often containing multiple types of contaminants that pose serious threat to the ability of standard sewage treatment. By biotechnological effluent treatment of food processing starch waste water can produce valuable products such as microbial biomass protein and purified effluent (Aiyer, 2005).

Textile industry

Amylases are used in textile industry for desizing process. Prior to weaving of yarn into fabric, the warp yarns are coated with a removable sizing agent to lubricate and protect the yarn from abrasion during weaving. Historically, the main sizing agent used for cotton fabric has been starch because of its excellent film forming capacity. Before the fabric is dyed, the applied sizing agent must be removed. Before the discovery of amylase enzymes, the only method to remove the starch-based sizing was extended treatment with caustic soda at high temperature. However such chemical treatment does not totally effective in removing the starch, appropriate desizing of starch is now possible by application of alpha-amylase. Recently, α-amylase from Bacillus strain was employed in textile industries as desizing agent (Kumar et al., 1990; Chengyi et al., 1999; Tsurikova et al., 2002; Maarel et al., 2002; Feitkenhauer, 2003; Gupta et al., 2003; Ahlawat et al., 2009; Mohammed & Roohi, 2010).

Starch Industry

Nielsen & Borchert (2000) used α-amylases for starch hydrolysis in the starch industry. In starch liquefaction process starch converts into fructose and glucose syrups. Process requires α-amylase which is more active at high temperature. Gupta et al.
(2003) and Prakash & Jaiswal (2009) reported the involvement of α-amylases in enzymatic conversion of starch (includes gelatinization, liquefaction and saccharification) in to maltose and glucose.

Paper industry

The starch coating enhances the stiffness and strength of paper. The viscosity of the natural starch is too high for paper sizing and that can be altered by partially degrading the starch with α-amylases. The use of α-amylases in the pulp and paper industry is for the modification of starch of coated paper (Gupta et al., 2003). Alpha-amylase digests starch, protecting paper against mechanical force during finishing process (Tolan, 1996). Starch is a good sizing agent for the finishing of paper, improving the quality and eraseability, besides being a good coating for the paper. The starch can be partially degraded with α-amylases in a batch or continuous processes (Bruinenberg et al., 1996, Van der Maarel, et al., 2002; Gupta et al., 2003; Thippeswamy et al., 2006; Hoff et al., 2010). Examples of amylases obtained from microorganisms used in paper industry include Amizyme®, Termamyl®, Fungamyl, BAN® and α-amylase G9995®.

Glucose Industry

Bloom et al. (1952), Takasaki et al. (1994), Bajpi & Bajpi (1989), Shetty & Crab (1990) and Akiba et al. (1999) reported that many industries use α-amylases for the production of simple sugar glucose. The enzymes hydrolyze α-1, 4 glycoside linkages in the starch polymer in a random manner to yield maltose and glucose. Therefore alpha-amylase is extensively used in industries for the production of glucose.
Sugar industry

Alpha-amylase plays an important role in the synthesis of starch conversion products. The presence of polysaccharides in sugarcane creates problem throughout the sugar manufacturing process, which was eliminated by the action of amylase. The high quality products depend upon the efficiency of the amylase which leads to reduction of cost (Hamilton et al., 1998).

Chocolate Syrup industry

Amylases are used in the preparation of chocolate syrups, in which the chocolate starch dextrizes and thus syrup does not become thick (Imam et al., 1991).

Feed Industry

The feed of poultry usually constitutes many starches or barley material; The addition or pre-treatment of the feed with α-amylases improves the nutritional value and the digestibility of fiber in the feed (Ismail et al., 1992; Uhlig, 1998; Van der Maarel et al., 2002; Gavrilescu & Chisti, 2005; Ghorai, et al., 2009).

Building product industry

The starch modified with the enzyme is used in the manufacture of gypsum board for dry wall construction (Schwartz et al., 2000).

Unmalted cereal liquefaction industry

Amylase is used for the liquefaction which is responsible for inducing solubility of unmalted cereals. They have the capacity to liquefy the unmalted cereal in shortest available time and with minimum enzyme dosage (Denault & Underkofler, 1963; Antranikian, 1992).
Manufacture of maltose

Maltose has a great value in food industries since it is a disaccharide, non-hygroscopic, does not easily crystallize and made up of glucose units. It is commonly used as sweetener, as an intravenous sugar supplement and as a main component of maltosugar syrup. The manufacturing of maltose requires cassava, potato, sweet potato, and corn starches. The concentration of starch slurry is adjusted to 10-20% maltose for medical grade production and 20-40% maltose for food grade production (Sugimoto et al., 1974).

Manufacture of high fructose containing syrups

The starch is first converted to glucose by enzyme liquefaction and saccharification. The high fructose containing syrups 42 F (Fructose content - 42%) are prepared by enzymatic isomerization of glucose with glucose isomerase (Pandey et al., 2000; Maarel et al., 2002).

Manufacture of maltottielease syrup

Maltotetraose syrup (G4 syrup) is manufactured by hydrolysis of starch into maltotetraose by the action of amylase enzyme. The sweetness of the G4 syrup is as same as 20% of sucrose. Therefore, it can be used in place of sucrose in foods which reduces the sweetness without altering taste and flavor. It has high moisture retention power which maintains integrity of starch granules and retains suitable moisture in foods. It improves the food texture because of its high viscosity than sucrose. It lowers down the freezing point of water than high fructose syrup, so that it can be used to control the freezing points of frozen foods. This G4 syrup imparts gloss and that property can be used as paper sizer in paper industry (Outtrup & Norman, 1984).
Manufacture of high molecular weight branched dextrins

High molecular weight branched dextrins produced by the action of α-amylase on corn starch are used as extenders for production of powdery foods and a glozing agent for rice cakes. Degree of starch hydrolysis depends on the type of starch and the physical properties desired. High molecular weight branched dextrins can be collected as powder after spray drying (Outtrup & Norman, 1984).

Hydrolysis of starch to maltodextrins

Starch is hydrolyzed using a combination of α-amylases and glucoamylases. The first stage in this process is to solubilize the starch granules gelatinized by subjecting in a jet cooker using a combination of injected steam and mechanical shear. Temperatures of 100 to 150°C are typically used. Viscous solution containing about 30% dry solids are produced in this process. This is then thinned by limited hydrolysis with α-amylase (Inglett, 1987).

Other applications

Alpha-amylase has applications in clinical, medical and analytical chemistry (Giri et al., 1990; Becks, et al., 1995; Chiu & Chandler, 1995; Lepp et al., 1996; Kolb et al., 1996; Chelly et al., 1996; Menzel et al., 1998; Sutton et al., 1999; Strandberg et al., 1999; Pandey et al., 2000; Haq et al., 2003; Kandra, 2003; Cherry et al., 2004; Reilly, 2007; Samrot & Vijay, 2009).

Alpha-amylase, the first industrially produced enzyme in China, plays an important role in the nation’s enzyme industry. There are three categories of α-amylases in the market: high temperature-resistant α-amylase, medium temperature α-amylase and fungal α-amylase. From 2004 to 2010, the total output of α-amylase in China increased from 17,000 tonnes/year to 26,000 tonnes/year.

Novozymes’-enzymes produced by genetically modified microorganisms. Novozymes market a range of enzymes for various industrial purposes. The following is a list of Novozymes’ brand names produced by genetically modified microorganisms (GMMs) used in different industries - alcohol industry (SAN Super 360L®), animal feed industry (RONOZYMES RumiStar), detergent industry (Stainzyme, Termamyl® and Duramyl®), leather industry (NovoCor® AX), pet food industry (Extruzyme® Pro), textile industry (Aquazym AT-L, Aquazym® LT-L, Aquazym® 120 L, Aquazym® 240 L, Aquazym® Ultra, Aquazym Prime®, Aquazym® SP, Aquazym® SD, Aquazym® MT, Novoprim® D 615 and Novoprim® D 659), brewery industry (Ceremix® Plus, Cerezyme Sorghum® and Liquozyme®) and starch industry (Liquozyme®, Termamyl®, All except Termamyl® Classic, Sweetase and BAN - only BAN LS).

National α-amylase manufacturers are as follows:

Amylases used in baking industry are produced by Aumgene Biosciences Pvt. Ltd., (Surat, Gujarat), Meteoric Life Sciences (Ahmadabad, Gujarat), Advance Enzyme Technologies Ltd., (Mumbai, Maharashtra), Nature Biochem. (Bangalore, Karnataka),
Noor Creations (Kolkata, West Bengal), Gulf Exports Pvt. Ltd., (Mumbai, Maharashtra), Vet Biochem. India Pvt. Ltd., (Pune, Maharashtra) and Enzymes Naveen (Bangalore, Karnataka).


Amylases used in Brewery industry are produced by Varuna Biocell Private Ltd., (Varanasi, Uttar Pradesh) and Enzymes Naveen (Bangalore, Karnataka).

Amylases used in starch and paper industry are manufactured by Varuna Biocell private Ltd., (Varanasi, Uttar Pradesh).
AIMS AND OBJECTIVES

The present study is aimed to screen the potent $\alpha$-amylase producing bacteria from the coastal waters of Bay of Bengal, Visakhapatnam. As Bacillus species proved efficient in many industrial applications, our study is mainly focused on isolation, characterization of Bacillus species by primary and secondary screenings and strain improvement techniques for increase in $\alpha$-amylase yield. An emphasis is on the partial purification, kinetic studies of $\alpha$-amylase activity. The enzyme ability in starch processing, textile industry, improving shelf life of bread, ethanol production, sewage treatment and effluent treatment of industries is studied.

The work is mainly planned under five following objectives:

I. Screening of $\alpha$-amylase producing bacteria from the waters of Bay of Bengal, Coastal area of Visakhapatnam

To isolate and characterize amylase producing bacteria from marine coastal waters of Bay of Bengal, Visakhapatnam, as marine organisms are known to have a diverse range of enzymatic activity.

To carry out primary screening of $\alpha$-amylase producing bacteria by culturing on enriched starch agar by spread plate method.

To isolate pure culture colonies of $\alpha$-amylase potent bacteria by streak plate method on nutrient agar.

To select potent $\alpha$-amylase producing bacteria by DNS method.

To study colony, morphological and biochemical characteristics of the high yield $\alpha$-amylase producing isolates.
To identify and classify the high yielding α-amylase producing bacteria by referring Bergey’s Determinative Bacteriology.

To classify *Brevibacillus borstelensis* R1 based on 16S rRNA gene sequence and mega BLAST score. BLASTN score to construct phylogenetic tree.

II Optimization of α-amylase production by *Brevibacillus borstelensis* R1 in SmF (Submerged fermentation)

To optimize the medium best suited from different culture media.

To optimize the α-amylase production at varying physical parameters such as incubation period, inoculum size, temperature, pH and salinity.

To use natural and synthetic carbon sources, nitrogen sources and mineral sources as supplements to medium at optimal physical conditions.

Immobilization of α-amylases and *B. borstelensis* R1 cells in different supporting media at different temperatures.

III Strain Improvement by using physical and chemical mutagens

To study the survival of *Brevibacillus borstelensis* R1 by using survival curve.

To isolate the mutant strains of *Brevibacillus borstelensis* R1 by using physical mutagen and different concentrations of chemical mutagens.

IV Partial purification of α-amylase

Partial purification of α-amylase by using ammonium sulfate precipitation and gel filtration.
To determine the α-amylase activity by using paper and thin layer chromatography.

To study the homogeneity of α-amylase by using Native-PAGE.

To identify the enzyme by specific staining.

Kinetics of partially purified α-amylase produced by *Brevibacillus borstelensis* R1.

V Applications

To test the application of partially purified α-amylase in increasing the shelf life of bread and in preparations of puri and chapatti.

To study the effect of α-amylase on the stains of starch, starch gravies, chocolate and jaggery. To observe the effect of α-amylase in dish cleaning automation of dishwashing machines in restaurants.

To study the effect of *B. borstelensis* R1 along with *Saccharomyces cerevisiae* in ethyl alcohol dual fermentation.

To study the potency of *B. borstelensis* strain R1 on the sago starch in the sago effluent of sago industry and rice starch in the effluent from rice boiler & rice flakes of rice industry.

To test the capability of partially purified α-amylase from *B. borstelensis* R1 in degrading starch from household waste water and for the production of *B. borstelensis* R1 biomass as a protein source in poultry feed.

To test the capabilities of partially purified α-amylase as desizing agent in textile industry.