CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter reviews the literature related to marine microorganisms, fibrinolytic enzymes and their applications. Production of fibrinolytic enzymes from various microorganisms and substrates are discussed in detail. Optimization of process parameters and medium components are also reviewed. Literatures related to various methods of strain improvement used in bioprocess are presented. Literatures on application of ultrasonication and use of mathematical models in bioprocess are also included in this chapter.

2.2 ENZYMES

Enzymes are proteins evolved by the cells of living organisms. Their specific function is to catalyse chemical reactions. Enzymes increase the rate at which reactions approach equilibrium. Enzymes have found wide and diverse applications in different disciplines and fields of human endeavours. Enzymes play critical role in the metabolic activities of all living organisms, whether humans, animals, plants or microorganisms and are widely applied in agriculture, biochemistry, biotechnology, chemistry, genetics, industries, human and veterinary medicine, pharmacy, research, etc. Enzymes have been derived from various sources such as plants, animals and
microbes. Enzymes derived from animals e.g. rennet, bovine liver catalase, animal lipase, pancreatin, pepsin, trypsin and lysozyme. There are many different plant enzymes used in processing. Among them is pectinase, poly (1,4-α-D-galacturonide) glycanohydrolase, poly (1,4-α-galacturonide) lyase and pectylhydrolase (NOSB 1995). Plant-based enzymes, such as bromelain from pineapple and papain from papaya, have proteolytic activity. Advantages of microbe-derived enzymes are that they may be used at a lower dosage and possess a broader pH range of activity than animal-based counterparts. Varieties of lipase, amylase, protease, and lactase have been made from microbial species and have been used in the management of enzyme deficiencies. Enzyme therapy provides safe treatment for many disorders, such as exocrine pancreatic insufficiency and lactose intolerance. The growing study of plant-based and microbe-derived enzymes offers great promise in advancing the benefits of digestive enzyme therapy (Mario Roxas 2008).

2.3 BIOMEDICAL IMPORTANCE OF ENZYMES

Enzymes are biologic polymers that catalyze the multiple dynamic processes which makes life processes possible. Enzymes play central role in health and disease because they are determinants of the rates at which physiologic events take place. The breakdown of foods to supply energy and chemical building blocks, the assembly of these building blocks into proteins, membranes and the DNA that encodes genetic information and the harnessing of energy to produce cell movement are all made possible by the carefully coordinated actions of enzymes (Murray et al 2000). While in health, all biochemical and physiological processes occur in an ordered, regulated manner and homeostasis is maintained, homeostasis can be profoundly disturbed in pathologic states. For example, the severe tissue injury that
catalyses liver cirrhosis can profoundly impair the ability of cells to form the enzymes those catalyze a key metabolic process such as urea synthesis. The resultant inability to convert toxic ammonia to non toxic urea is then followed by ammonia intoxication and ultimately hepatic coma (Murray et al 2000). A series of rare but frequently debilitating and often fatal genetic diseases are additional examples of the drastic physiologic consequences that can follow impairment of the activity of a single enzyme. Following severe tissue injury (e.g. cardiac or lung infarct, crushed limb, liver damage or trauma to the liver) or uncontrolled cell growth (e.g. prostatic carcinoma), enzymes that may be unique to specific tissues are released into the blood. Measurement of these intracellular enzymes in blood serum therefore provides medical and veterinary doctors with invaluable information on the diagnosis and prognosis of diseases in the human and animal body/system.

2.4 APPLICATIONS OF ENZYMES

Many enzymes are used in biochemical systems. Some examples are aa-tRNA synthetase - used for charging a specific tRNA molecule with the appropriate amino acid and ß-galactosidase - splits lactose to the constituent monosaccharides glucose and galactose. It is the first step in lactose fermentation. Some of the enzymes such as alkaline phosphatase, Exonuclease and DNA polymerase 1 are employed in biotechnological applications. Enzymes are also used as markers for disease. For example, Lactose Dehydrogenase (LDH) has 2 different forms, called isozymes, in heart and skeletal muscle. The 2 forms differ slightly in amino acid composition and can be separated on the basis of charge as a result. Since LDH is a tetramer of four subunits, it too can exist in 5 different forms depending on the source of the subunits. An increase of any form of LDH in the blood indicates some kind of tissue damage. A heart attack can usually be
diagnosed with certainty if there is an increase of LDH from heart. Many enzymes such as Aspartate Aminotransferase and Alanine Aminotransferase are involved in the clinical diagnoses of various diseases in human and veterinary medicine. These enzymes facilitate or enhance rapid diagnoses of these diseases. The enzyme catalase may also play an important role in converting hydrogen peroxide to water and oxygen. Hence some enzymes can also be used as antioxidants. Enzymes may also be used as an alternative to radioisotopes as markers in immunoassays (Palmer 2001). Such procedures, known as enzyme-immunoassays, have been used for the determination of a variety of proteins and hormones. The role of enzymes in immunoassay procedures is a secondary role, they are used to replace radioisotopes as markers, since they are not hazardous to health and can be detected by techniques which are more generally available. Any enzyme with a sensitive and convenient assay procedure can be used for this purpose. Two common examples of Enzymeimmunoassay (EIA) procedures are Enzyme-Linked Immunosorbent Assay (ELISA) and Enzyme-Multiplied Immunoassay Test (EMIT). ELISA is a highly sensitive assay that can be used to detect either antigen or antibody. In a few cases enzymes have been used as drugs in the therapy of specific medical problems (Devlin 1986). Streptokinase is an enzyme mixture prepared from Streptococcus. It is useful in clearing blood clots that occur in the lower extremities. Streptokinase activates the fibrinolytic proenzyme plasminogen that is normally present in plasma. The activated enzyme is plasmin. Plasmin is a serine protease like trypsin that attacks fibrin, cleaving it into several soluble components (Devlin 1986). Another enzyme of therapeutic importance is asparaginase. Asparaginase therapy is used for some types of adult leukemia. Tumor cells have a nutritional requirement for asparagine and must scavenge it from the host’s plasma. By administering asparaginase the host’s plasma level of asparagine
is markedly depressed, which results in depressing the viability of the tumor (Devlin 1986). Enzyme replacements in individuals that are genetically deficient in a particular enzyme are also applications of enzymes as therapeutic agents. Also, enzymes such as u-plasminogen activator, formerly known as urokinase, extracted from human urine, can be infused into the blood stream of patients at risk from a pulmonary embolism (a fragment of a blood-clot lodging in the pulmonary artery): these enzymes stimulate a cascade system responsible for the production of active plasmin, a proteolytic enzyme which digests fibrin, the main structural component of blood-clots. Some enzymes may also be used to restrict the growth of cancer cells by depriving them of essential nutrients: for example, L-asparaginase may be used in the treatment of several types of leukaemia, since the tumour cells, in contrast to normal cells, have a requirement for exogenous L-asparagine.

2.5 THROMBOLYSIS OR FIBRINOLYSIS

The fibrinolytic enzyme, plasmin, like most other proteases, is capable of acting upon a number of different protein substrates in addition to its physiologic substrate fibrin like proteinaceous clotting-factors like fibrinogen, proaccelerin, antihemophilic factor, prothrombin and Christmas factor, and certain other blood proteins. But in the healthy blood circulation plasmin exist with its inhibitors antiplasmin as Plasmin- Antiplasmin complex which regulates the plasmin activity. Antiplasmin is 30 fold higher than plasmin in plasma concentration act as defensive factor for breakdown of other plasma protein (Ambrus et al 1962). The complete physiology of the fibrin–clot formation is relatively well understood. A blood clot or thrombus is consists of blood cells occluded in a matrix of the protein fibrin. Thrombolysis or fibrinolysis is enzyme regulated process for dissolution of clot. In mammalian circulation, the enzyme responsible for the fibrinolysis is
plasmin which is a trypsin-like serine protease (Back et al 1958). In the presence of activator the fibrinolytically active plasmin is produced from the inactive protein plasminogen which is present in systemic circulation. The biochemical conversion of the inactive plasminogen to fibrinolytic plasmin involves a limited proteolytic cleavage which is mediated by the various plasminogen activators (Castellino 1981). Generally two plasminogen activators that occur naturally in blood are the tissue type (t-PA) and the urokinase type (u-PA). The fibrinolytic activity in circulation is regulated by inhibitors of plasminogen activators (e.g., plasminogen activator inhibitor-1 (PAI-1), a fast-acting inhibitor of t-PA and u-PA) and plasmin (e.g., a1-antiplasmin, a2 macroglobulin.

2.6 THROMBOSIS AND THROMBOLYTIC AGENTS

Today, enzymes are used as anticoagulants, oncolytics, thrombolytics, anti-inflammatories, fibrinolytics, mucolytics, antimicrobials, and digestive aids. Enzymes, like their application in medicine, exert their effects in a multitude of ways. One primary focus of enzymatic action is on the protein fibrin. Fibrin is an insoluble protein involved in blood clotting. In the many steps of the clotting cascade, fibrin is the final product. It is derived from its soluble protein precursor, fibrinogen. Fibrin is laid down inside blood vessels that have been compromised by disease or injury. Fibrin forms minuscule strands that eventually dry and harden, capturing blood vessel components effectively. Certainly, fibrin occupies a vital role in health and healing; however, fibrin may also be responsible for an overzealous propensity to form inappropriate clots in the body. Inappropriate clotting, of course, is a major risk factor for myocardial infarction and stroke. When correctly balanced, deposition and removal of fibrin maintains avoidance of blood loss and adverse viscosity in the vascular system. A balance tipped in
favor of fibrin overproduction leads to dangerous clotting. Various types of thrombosis are responsible for an increasing number of deaths each year. In the USA alone, lung blood clots affect an estimated 1,000,000 patients annually. According to a report published by the World Health Organization (WHO) in 2001, 17 million people die every year of Cardiovascular Diseases (CVDs). The formation of a blood clot in a blood vessel (intravascular thrombosis) is one of the main causes of CVDs. The major protein component of blood clots, fibrin, is formed from fibrinogen via proteolysis by thrombin. Meanwhile, fibrin clots can be hydrolyzed by plasmin to avoid thrombosis in blood vessels. In an unbalanced situation due to some disorders, the clots are not hydrolyzed, and thus thrombosis occurs. So, several investigations are being pursued to enhance the efficacy and specificity of fibrinolytic therapy, and microbial fibrinolytic enzymes have attracted much more medical interest in recent decades (Goldhaber and Bounaumeaux 2001; Tough 2005). Currently, several thrombolytic agents such as streptokinase, urokinase, prourokinase, reteplase (r-PA), alteplase (t-PA), reptilase, brinase and Anisoylated Purified Streptokinase Activator Complex (APSAC) are available for clinical use (Cadroy and Haarkeer 1990). All these thrombolytic agents still suffer significant shortcomings, including requirement of large therapeutic dose, short plasma half-life, limited fibrin specificity, reocclusion and bleeding complications (Reddy 1998). Therefore, microbial fibrinolytic enzymes have also attracted much more medical interest during recent decades (Bode et al 1996).

2.7 SOURCES OF FIBRINOLYTIC ENZYMES

Over the years, more fibrinolytic enzymes from various sources have been discovered in succession. The studies reported on the enzyme from different sources are summarized in Table 2.1.
# Different Sources reported in the literature for the production of fibrinolytic enzymes

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<thead>
<tr>
<th>Fibrinolytic Enzyme</th>
<th>Microorganism</th>
<th>Remarks</th>
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<tr>
<td>Fibrinolytic enzyme</td>
<td><em>Cochliobolus lunatus</em></td>
<td>The enzyme was relatively more stable at pH 6.98 and 55-60 °C.</td>
<td>Abdel-Fattah and Ismail 1984</td>
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<td>Extracellular protease</td>
<td><em>Streptomyces spheroides M8-2</em></td>
<td>The organism is a mutant of <em>S. spheroides</em> spores strain 35. The mutant was characterized by the considerable increase (in 4-5 times) of the extracellular proteolytic enzymes which are able to dissolve fibrin and thrombs.</td>
<td>Egorov et al 1985</td>
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<td>Protease</td>
<td><em>Penicillium chrysogenum</em> H9</td>
<td>The highest fibrinolytic activity/caseinase activity ratio was obtained in immobilized cell cultures. The beads were affected by repeated exposure to phosphate ions after 12 cycles which was observed through semicontinuous production of the enzyme.</td>
<td>El-Aassar et al 1990</td>
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<td>Lumbrokinase</td>
<td><em>Lumbricus rubellus</em></td>
<td>The fibrinolytic enzymes in the earthworm, <em>Lumbricus rubellus</em>, were extracted and named lumbrokinase a collective name for six fibrinolytic iso-enzyme proteins having molecular weights of 25 to 32 KDa.</td>
<td>Mihara et al 1993</td>
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<tr>
<td>Fibrinolytic enzyme</td>
<td><em>Fusarium pallidoroenseum</em></td>
<td>Maximum activity was obtained at 25 °C and 50% moisture content. The pure enzyme was highly active on human fibrin and showed an optimum reaction temperature of 40 °C and pH 7. The enzyme was relatively sensitive to heat treatment at 55 °C and strongly inhibited by EDTA, it restored its activity by adding cobalt ions to the reaction.</td>
<td>El-Aassar, 1995</td>
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<td>Metalloprotease</td>
<td><em>Bacillus</em> sp. KA38</td>
<td>The bacterium was isolated from fermented fish. The specific activity was reported as above 1.41 U/mg under optimized pH 7 and 40 °C.</td>
<td>Kim et al 1997</td>
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<td><em>Codium intricatum</em> protease (CIP)</td>
<td><em>Codium intricatum</em> (Marine green alga)</td>
<td>From the extract of <em>C. intricatum</em>, two fibrinolytic enzymes, named CIP-I and CIP-II, were purified. Both enzymes hydrolyzed fibrinogen with preference to the $\alpha$ chain over $\beta$ or $\gamma$ chains. Maximum activity was observed at pH 8 – 9. The specific activities were 691 and 533 mU/mg for CIP-I and CIP-II, respectively.</td>
<td>Matsubara et al 1998</td>
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<td>Fibrinolytic protease</td>
<td><em>Pleurotus ostreatus</em></td>
<td>The maximum specific activity of the pure enzyme was 2062 U/mg. It was suggested that fibrinolytic protease in the oyster mushroom could be useful for fibrinolysis, since it is safe for ingestion and has high fibrinolytic activity.</td>
<td>Choi and Shin 1998</td>
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<td>Fibrinolytic enzyme</td>
<td><em>Fusarium oxysporum</em></td>
<td>The enzyme was produced through Solid State Fermentation (SSF) and the fermentation was continued for 31.5 days. The urokinase activity of the enzyme was found to be 154500 IU/L bulk volume. The optimum temperature range was found to be 30 °C – 32 °C.</td>
<td>Tao et al 1998</td>
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<td><em>C. latum</em> protease (CLP)</td>
<td><em>Codium latum</em> (Marine green alga)</td>
<td>The specific activity of the pure enzyme was 600 pkat/mg and proved it is a trypsin-like serine protease.</td>
<td>Matsubara et al 1999</td>
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<td>SW-1</td>
<td><em>Streptomyces</em> spp.</td>
<td>The specific activity was found as 2952.3 urokinase U/mg. The analysis of amino acid composition showed that SW-1 consisted of 262 amino acids. The fibrinolytic activity of SW-1 was entirely inhibited by 10 mmol/L PMSF, 1 mmol/L EDTA and 1 mol/L lysine, respectively, suggesting that SW-1 is a serine protease and metalloprotease, and that the lysine binding site might play a role in the activity. On plasminogen-free fibrin plates, SW-1 showed the same fibrinolytic activity as the mixture of SW-1 with plasminogen, indicating that SW-1 is a fibrinolytic enzyme which affects fibrin directly, but not a plasminogen activator which affects fibrin by activating plasminogen.</td>
<td>(Wang et al 1999)</td>
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<tr>
<td>Chymotrypsin-like serine peptidase</td>
<td><em>Streptomyces megasporus</em> strain SD5</td>
<td>The specific activity was reported as 4.2/µg. The enzyme resembled urokinase but directly acts on the fibrinolysis without activating the plasminogen. The enzyme, although produced by a prokaryote, was similar to urokinase, a eukaryotic protein which is important from the biochemical point of view.</td>
<td>(Chitte and Dey 2000)</td>
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<td><em>C. divaricatum</em> protease (CDP)</td>
<td><em>Codium divaricatum</em> (marine green alga)</td>
<td>The protease activity peaked at pH 9 and was completely inhibited by diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), identifying it as a serine protease. The specific activity of the enzyme was found to be 6.3 U/mg.</td>
<td>(Matsubara et al 2000)</td>
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<td>Extracellular proteinase (protein C activator type)</td>
<td><em>Aspergillus ochraceus</em> 513</td>
<td>The maximum fibrinolytic activity of the enzyme was 427 units/mL. A comparison of this enzyme with the protein C activator from the <em>Agkistrodon</em> snake venom showed that they are almost equally efficient in prolonging the thrombin formation time.</td>
<td>(Batomunkueva and Egorov 2001)</td>
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<td>31 kDa fibrinolytic enzyme</td>
<td><em>Bacillus subtilis</em> BK-17</td>
<td>Compared to the crude enzyme extract, the specific activity of the enzyme increased 929-fold with a recovery of 29%.</td>
<td>(Jeong et al 2001)</td>
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<td>Fibrinolytic metalloprotease</td>
<td><em>Bacillus subtilis</em> Strain A1</td>
<td>A fibrinolytic metalloprotease gene from <em>Bacillus subtilis</em> was cloned in <em>Escheridria coli</em> XL1-Blue and the bacterial expressed enzyme was purified. N-terminal amino acid sequencing of the fibrinolytic enzyme excreted from <em>E. coli</em> host cells revealed that the mature fibrinolytic enzyme consists of 288 amino acids.</td>
<td>(Jeong et al 2004)</td>
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<td>Earthworm fibrinolytic enzyme (EFE)</td>
<td>Earthworm</td>
<td>EFE-a possesses an S11 pocket, which is typical for an elastase-like enzyme, but it can still hydrolyze varieties of substrates, and it exhibits wide substrate specificity. It possesses multisubstrate-binding sites interacting with the substrates and significant conformation adjustment takes place at two loops binding to the N-terminal of the substrates, which may enhance the interaction between the enzyme and the substrates.</td>
<td>(Wang et al 2004)</td>
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<td>Nattokinase</td>
<td><em>Bacillus natto</em> NLSSE</td>
<td>The organism showed maximum activity of 1300 ± 60 units/mL.</td>
<td>(Liu et al 2005a)</td>
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<td>Vpr</td>
<td><em>Bacillus subtilis</em></td>
<td>In this study, to confirm the fibrinolytic activity of Vpr, the vpr gene was cloned and expressed it in <em>Escherichia coli</em>, where it is predominantly localized to inclusion bodies. After affinity purification and desalting steps, the expressed Vpr is auto-processed to an active form. Interestingly, after the desalting step, several additional bands with fibrinolytic activity were detected in zymography gel along with a mature form (68 kDa) of Vpr. MALDI-TOF analyses of these bands revealed that Vpr could exist in multiple forms.</td>
<td>(Kho et al 2005)</td>
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<td>Fibrinolytic enzyme</td>
<td><em>Rhizopus chinensis</em> 12</td>
<td>It was suggested that the activity center of the enzyme had hydrosulfuryl and metal through the biochemical characterization.</td>
<td>(Xiao-Lan et al 2005)</td>
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<td>Ussurenase</td>
<td>Snake venom of <em>Agkistrodon blomhoffii ussurensis</em></td>
<td>Fibrino(genol)ytic enzymes from snake venoms have been identified as high quality therapeutic agents for treatment of blood clots and strokes. They act on fibrinogen and fibrin, leading to defibrinogenation of blood, lysis of fibrin, and a consequent decrease in blood viscosity. Ussurenase reacts optimally with fibrin clots at pH 7.5–8.3 and a temperature of 33–41 °C. Ussurenase is a Ca$^{2+}$-containing protein with a molar ratio of 1:1 ([Ca$^{2+}$]:[enzyme]). Ca$^{2+}$ is crucial to the fibrin clot hydrolysis by ussurenase but also plays an important role in maintaining the structural integrity of the enzyme.</td>
<td>(Sun et al 2006)</td>
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<td>Thrombinase</td>
<td><em>Bacillus sphaericus</em></td>
<td>The production method yielded 64 mg/l of the crude enzyme and after purification it was 6.3 mg/l. The molecular weight of the compound was 18.6 kDa. The enzyme exhibited similar fibrinolytic activity as that of streptokinase, on fibrin plates that were devoid of plasminogen, suggesting that its fibrinolytic action is independent of plasminogen and it is not a plasminogen activator.</td>
<td>(Balaraman and Prabakaran 2007)</td>
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<td>Verticase</td>
<td><em>Verticillium</em> sp. Tj33 (Endophyte of <em>Trachelospermum jasminoides</em>)</td>
<td>The fibrinolytic activity of verticase was 3775 units/mg when determined with urokinase as the standard. It was reported that the fibrinolytic activity of verticase could be totally inhibited by serine protease inhibitors at low concentrations. This observation, along with its optimum pH range of 9–10, implied that it is a serine alkaline protease.</td>
<td>(Li et al 2007)</td>
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<td>N-V protease</td>
<td>Coelomic fluid of polychaete, <em>Nerites</em> (<em>Neanthus</em>) vireus (<em>Sars</em>)</td>
<td>The total activity of the enzyme was reported as 19767.6 U and the molecular weight was found to be 29 kDa. It was stable at 30 °C - 55 °C and pH 4-9.</td>
<td>(Zhang et al 2007)</td>
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<td>Fibrinolytic enzyme</td>
<td><em>Bacillus subtilis</em> DC-2</td>
<td>In this study, it was reported that the fibrinolytic activity in aqueous two-phase system (ATPS) is dependent mainly on Poly-ethylene glycol (PEG) 4000 concentration, ( \text{Na}_2\text{SO}_4 ) concentration and pH. Catabolic repressions in fermentation were reduced due to partitioning of substrate and product in the ATPS. The effect of PEG on the cell permeability leads to the higher secretion of the enzyme and the stabilizing effect on the enzyme made possible a higher enzyme activity in ATPS. The fibrinolytic activity of 1223.61 IU/ml was obtained in PEG phase, which was higher than homogeneous fermentation.</td>
<td>(Ashipala and He 2008)</td>
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<td>Nattokinase</td>
<td><em>Bacillus subtilis</em></td>
<td>The total activity of the enzyme was reported as 3194.25 U/mL.</td>
<td>(Deepak et al 2008)</td>
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<td>TKU015 Nattokinase</td>
<td><em>Pseudomonas sp.</em> TKU015</td>
<td>The specific activity of the enzyme was obtained as 28.9 U/mg. Maximum activity was obtained on 2(^\text{nd}) day. TKU015 nattokinase was inhibited completely by Phenylmethylsulfonyl fluoride (PMSF), indicating that the TKU015 nattokinase was serine protease.</td>
<td>(Wang et al 2009a)</td>
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<td>Nattokinase</td>
<td><em>Bacillus subtilis</em></td>
<td>Natto B-12</td>
<td>(Wang et al 2009b)</td>
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<td>The organism was isolated from fermented soybean food in Japan. The enzyme showed maximum urokinase activity of 903 IU/mL and was demonstrated to be homogeneous by SDS-PAGE and was identified as a monomer of 29000 Da. The optimal pH value and temperature were 8.0 and 40°C, respectively.</td>
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<td>Subtilisin-like protease</td>
<td><em>Pleurotus eryngii</em></td>
<td>In this study it has been reported that the fibrinolytic enzyme obtained from fruiting bodies of <em>P. eryngii</em> cultivated under solid-state conditions using corn cob shows a high degree of specificity toward fibrin. Hence, it could be useful in thrombolytic therapy as it is a directly acting thrombolytic agent. The optimum temperature and pH for the enzyme was 40 °C and 5, respectively.</td>
<td>(Cha et al 2010)</td>
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<td>Chymotrypsin-like serine metalloprotease</td>
<td><em>Cordyceps militaris</em></td>
<td>It was reported that the enzyme showed a specific activity of 499.6 U/mg. Optimal pH and temperature values of the enzyme were 7.0 and 40 °C, respectively. The enzyme activity was completely inhibited by phenylmethylsulfonyl fluoride (PMSF), TPCK, 1,10-phenanthroline, Cu^{2+} and Ba^{2+}.</td>
<td>(Choi et al 2011)</td>
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<td><em>P. tenuipes</em> fibrinolytic protease (PTEFP)</td>
<td><em>Paecilomyces tenuipes</em></td>
<td>PTEFP was purified from the entomopathogenic fungus <em>Paecilomyces tenuipes</em>. It was reported that the presence of Ca(^{2+}) provides the enzyme with structural stability. The specific activity obtained was 1431.81 U/mg.</td>
<td>(Kim et al 2011)</td>
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<td>Alkaline fibrinolytic protease</td>
<td><em>Bacillus</em> sp. strain AS-S20-I</td>
<td>The maximum fibrinolytic activity was reported as 749.0 × 10(^3) U L(^{-1}). The haloalkaline property of protease is of great interest for biotechnological application involving treatment of saline water containing protein residues.</td>
<td>(Mukherjee and Rai 2011)</td>
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<tr>
<td>Fibrinolytic serine protease</td>
<td><em>Streptomyces omiyaensis</em> (SOT)</td>
<td>Compared to plasmin, tissue-type plasminogen activator (t-PA), urokinase, and nattokinase, SOT showed higher hydrolytic activity toward mimic peptides of fibrin and plasminogen. The activity of SOT is about 18-fold higher than that of plasmin, and is comparable to that of t-PA. The specific activity was 136.2 ± 7.4 U/mg. SOT and nattokinase have very different fibrinolytic and fibrinogenolytic modes, engendering significant synergetic effects of SOT and nattokinase on fibrinolysis.</td>
<td>(Uesugi et al 2011)</td>
</tr>
<tr>
<td>Lumbrokinase</td>
<td><em>Eisenia andrei</em>, <em>Eisenia fetida</em> and <em>Lumbricus terrestris</em></td>
<td>Lumbrokinase exist as iso-enzyme in the intestine and tissue fluid and intestinal fluid of earthworm. The reason why earthworm is having such a potent protease as earthworm feed on the debris of plant and organic matter so digest all these probably it produce Lumbrokinase a serine protease.</td>
<td>(Verma and Pulicherla 2011)</td>
</tr>
</tbody>
</table>
2.8 ENZYME PRODUCTION FROM DAIRY AND TANNERY INDUSTRIAL WASTES

The use of residues from agroindustrial, forestry and urban sources in bioprocesses has aroused the interest of the scientific community lately. The utilization of such materials as substrates for microbial cultivation intended to produce cellular proteins, organic acids, mushrooms, biologically important secondary metabolites, enzymes, prebiotic oligosaccharides, and as sources of fermentable sugars in the second generation ethanol production has been reported (Sanchez 2009).

Gul et al (2012) investigated the production of extracellular cysteine alkaline protease from dairy industrial waste (cheese whey) using *Rhizopus oryzae*. It was also reported that industrial effluent spillage into sewage water can be efficiently reduced by converting this hazardous by-product into an industrially important metabolite by microbial cell factories. *Rhizopus oryzae* exhibited enormous potential of consuming lactose as sole carbon source in whey besides fats and proteins. Whey medium devoid of any nitrogen/carbon supplementation proved to be an attractive low cost medium for protease production hence lactose serving as a sole carbon source. Product separation from fungal mycelium was easily managed through a simple filtration process, which accounts for another reduction towards product costs and its promising application in textile or leather industry. Moreover as the whey is major contaminant of sewage waters worldwide, this strain can be effectively utilized for bioremediation purposes.

Radha et al (2011) analyzed the production of protease by *Aspergillus* spp using cheese whey and molasses. It was reported that cheese whey can be used as a nitrogen source for protease production.
Supplementation of whey and molasses to the medium improved the protease activity by *Aspergillus* spp with $0.4481 \text{ U mL}^{-1}$ from $0.2769 \text{ U mL}^{-1}$. A combination of 2.5 parts of whey and 1 part of molasses and produced higher amount of fungal biomass.

Ravindran et al (2012) studied the production of protease by *Selenomonas ruminantium* using Animal Fleshing (ANFL), an untanned tannery solid waste as the sole protein source. The production of enzyme was found to increase with the supplementation of animal fleshing along with the optimized medium comprising other mineral ions. The purified enzyme was stable at a pH of about 4 revealing its acid protease nature and was also found to be stable up to $40^\circ\text{C}$. The enzyme was activated by divalent cations like $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ and inhibited by Dithiothreitol (DTT), where the latter suggested its cysteine protease nature. The enzyme had good stability in the presence of non-ionic surfactants like tween 20, tween 40, tween 80 and triton X100 and also in the presence of solvents like methanol, ethanol and isopropanol. These characteristics revealed the potential of the enzyme for different industrial applications.

### 2.8.1 Fibrinolytic Enzyme from Various Substrates

Selection of medium components is usually critical for the fermentative production of fibrinolytic enzymes. Since different microbes possess diverse physiological characteristics, it is necessary to optimize nutrient components and environmental conditions for cell growth and fibrinolytic enzyme production (Lee et al 1999; Seo and Lee 2004). For instance, soluble starch or dextrin is the best carbon source for *B. amyloliquefaciens* DC-4 due to the strong amylase activity (Peng and Zhang 2002). In some cases, fibrin was found to enhance the enzyme
production, suggesting that fibrin, as a substrate of fibrinolytic enzyme, could activate or induce enzyme production during cultivation (Chitte and Dey 2002; Peng and Zhang 2002).

Kim et al (1996) used the medium containing Beef extract, peptone, soy peptone and milk casein for the production of fibrinolytic enzyme and found that the enzyme activity was increased when plasminogen was added to the substrate compared to the control i.e., without plasminogen.

Wei et al (2011) analyzed the production of fibrinolytic enzymes from chick peas using \textit{B. amyloliquefaciens} LSSE – 62. The results showed that SSF of chick peas with \textit{B. amyloliquefaciens} LSSE – 62 was effective for fibrinolytic enzyme production and the enzyme was biosynthesized along with the cell growth. The maximum activity was found to be 35.98 FU/g under optimum conditions of temperature, 34 °C and moisture content 50% (w/v).

Wang et al (2009b) optimized the medium components including carbon and nitrogen sources in the production of Nattokinase (NK). Moreover the cultivation conditions including incubation temperature, initial pH, volume of the resultant medium, rotated speed of flask shaker and incubation time were investigated. This study reported that the highest NK production was obtained when maltose was used. It was reported that maltose could decrease the catabolite repression and induce enzyme production. Wheat bran was most helpful to the enzyme yield. Bran is a biological complex, which is made of starch (12-18%), protein (15-18%), dietary fiber (35-50%), fat (3-5%), and ash (4-6%) (32). Therefore, the positive effect of wheat bran may be attributed to the fact that it enriches aminophenol, vitamins, mineral elements, and enzymes. The optimal temperature, pH, agitation and
incubation time for NK production were found to be 30 °C and pH 7, 180 rpm and 60 h respectively. In addition it was also revealed that NK activity obviously decreased with increasing medium volume when the medium volume was less than one-third of the total flask cubage (250 mL).

Liu et al (2005a) studied the optimization of nutritional conditions for nattokinase production by Bacillus natto NLSSE using statistical experimental methods. Effects of various nitrogen and carbon sources were investigated. Fractional Factorial Design (FFD) was applied to elucidate the key ingredients in the media and the results indicated that the soy peptone, yeast extract and calcium chloride have a significant effect on nattokinase production. Canonical analysis results showed that the response surface had a maximal point. According to the model, the predicted maximal nattokinase volumetric concentration was about 1273.8 units/mL and the corresponding concentration of soy peptone, calcium chloride and yeast extract were around 8.28, 0.64 and 0.74 g/L, respectively. The trial checking the developed medium showed a high nattokinase activity of 1300 ± 60 units/mL. The effect estimate of calcium chloride was positive, which suggested that the increase of its concentration in the medium will resulted in the promotion of nattokinase production, while the effect estimates of yeast extract and soy peptone were negative, which meant low level of the yeast extract content and soy peptone would benefit nattokinase production process.

Deepak et al (2008) employed Response surface methodology and Central Composite Rotary Design (CCRD) to optimize a fermentation medium for the production of Nattokinase by Bacillus subtilis at pH 7.5. The statistical analysis of the results showed that, in the range studied; only peptone had a significant effect on Nattokinase production. The optimized medium containing (%) Glucose: 1, Peptone: 5.5, MgSO₄: 0.2 and CaCl₂: 0.5
resulted in 2-fold increased level of Nattokinase (3194.25 U/mL) production compared to initial level (1599.09 U/mL) after 10 h of fermentation. It was also reported that central composite experimental design maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments required. Thus small and less time consuming experimental designs could generally suffice for the optimization of many fermentation processes. Therefore, with the increase in yield and productivity and simultaneous cost reduction, the industrial Nattokinase production by B. subtilis can be regarded as possible and economically attractive.

Wang et al (2009a) used shrimp shell wastes as the sole carbon/nitrogen source for the production of nattokinase by *Pseudomonas* sp. TKU015. The results showed that 1% shrimp shell powder was more suitable as an inducer for nattokinase production by TKU015.

Cha et al (2010) investigated the biochemical and enzymatic properties of a fibrinolytic enzyme purified from *Pleurotus eryngii* cultivated under solid-state conditions using corn cob as energy source. The specific activity was found to be 52.8 unit/mg and the results showed that among various waste material, corn cob is a promising agricultural resource for mushroom cultivation due to the extensive cultivation of corn and because corn cobs are rich in hemicelluloses.

Mukherjee and Rai (2011) studied the optimization of fibrinolytic alkaline protease from *Bacillus* sp. using Plackett-Burmann design. The results showed that among the tested factors, casein, ammonium sulphate and pH of the medium significantly (*p < 0.05*) enhanced the protease (fibrinolytic enzyme) yield in submerged fermentation. Further optimization of fibrinolytic protease production by *Bacillus* sp. strain AS-S20-I in submerged
fermentation by applying RSM was achieved as $749.0 \times 10^3 \text{ UL}^{-1}$ in the presence of 3.0% (w/v) casein and 0.12% (w/v) ammonium sulphate at pH 10.9 and 45 °C. This was a 4.0-fold increase in yield compared with that obtained before applying the Plackett–Burman and RSM experimental design. The protease preparation preferentially degraded the fibrin (specific activity $2408.0 \pm 70.0 \text{ Umg}^{-1}$) suggesting that its future application in pharmaceutical industry as thrombolytic and anticancer drugs is highly promising.

2.9 STUDIES ON STRAIN IMPROVEMENT

Biological production of chemicals and proteins starts with the identification of strains that are suitable for the production of desired products. The next stage is the optimization of bioprocess for economical production of desired product. Optimization of bioprocess can be achieved either at the process level or through strain improvement (Lee et al 2005). Since the improvement of strains that yield more desired products has greater impact on economics, strain improvement programs have attracted more interest recently (Lee et al 2005).

Hopwood and Ferguson (1970) insisted that auxotrophic mutants with resistance to analogues are widely used for commercial production of amino acids. Penicillin enrichment can be used for isolating auxotrophic mutants. This method can be modified to isolate a particular auxotroph. Moreover it was also reported that undesirable inhibition can be eliminated through such mutations to greatly increase the yield of the target product.

Madhuri Doss et al (2011) investigated the production of streptokinase by β-haemolytic Streptococci isolated from throat infected patients. The strain was improved through random mutagenesis by UV
irradiation and the enzyme activity was compared with the wild strain. The results revealed that the mutant showed an increase of 67% in the yield of streptokinase compared to the wild strain. It was also suggested that UV irradiation is an effective mutagen for strain improvement and enhanced streptokinase activity.

Abdelghani et al (2005) analyzed the effect of mutation on the production of streptokinase by β-haemolytic Streptococci isolated from throat infected patients. The organism was identified as *Streptococcus equisimilis*. The strain was mutagenized by UV and N-methyl-N’-nitro-N-Nitroso Guanidine (NTG). The streptokinase yield of the best UV mutant AUV10 was 120% higher than the wild strain. Also, the streptokinase yield of the best NTG mutant NUV7 was 146% higher than the wild strain. The results indicated that UV and NTG were effective mutagenic agents for strain improvement of *S. equisimilis* for enhanced SK productivity.

Wang et al (2008) reported a fibrinolytic enzyme producing strain from *Bacillus subtilis* LD-8547 from douche, a traditional Chinese soybean-fermented food and the strain was improved by mutation using different mutagenic agents i.e., UV, NTG and γ-radiation. After 72 h the strain mutated by UV showed 2760 U/mL fibrinolytic activity. The strain further mutated by NTG and the maximum activity was found to be 3280 U/mL. This strain was further mutated by γ-radiation and the highest fibrinolytic activity was found to be 3980 U/mL. The stability assay showed that the high production feature of the mutant LD-8547 was stable; indicating that mutagenesis for breeding was a useful and effective method for strain screening of high production.
Gohel et al (2004) investigated the strain improvement of chitinolytic enzyme producing isolate *Pantoea dispersa* for enhancing its biocontrol potential against fungal plant pathogens. Ultraviolet rays and gamma rays were used as mutagens separately for wild type strain of *Pantoea dispersa* and EMS was used for the further mutation of mutant obtained from the physical mutagenesis. The gamma mutant was found to produce higher chitinolytic enzyme activity than UV mutant. The EMS mutant was found to produce $13.97 \pm 0.25$ IU/mL which was proved to be the highest.

Gupta et al (2002) reported that Microbial strain improvement by either conventional mutagenesis (UV or chemical exposure) or recombinant DNA technology (rDNA) to selectively generate mutants exhibiting higher protease production is also used for improving protease production in microorganisms.

Feng et al (2001) revealed that rDNA technology was used to develop a newly engineered strain of *B. pumilis*, c172-14 (pBX96), by introducing the pBX 96 plasmid (carrying the $\alpha$-amylase *amy* gene) into the host strain of alkalophilic *B. pumilis* c172 by transformation. The level of alkaline protease production from this new strain was up to 43% improved, compared with the parent strain. The introduction of the pBX 96 plasmid not only changed the carbon source from glucose to starch, but also got rid of the inhibitory levels of high glucose concentrations on protease production.

In another study, Fleming et al (1995) showed that in vivo recombination can be used in *B. licheniformis* to prepare defined deletions and this deletion of the spoIIAC gene gives rise to a sporulation deficient strain exhibiting extracellular serine protease synthesis.
Shah et al (1986) introduced a phenotypic requirement for cysteine by UV-exposure in a strain of *B. licheniformis* to improve alkaline protease production.

Bierbaum et al (1994) used a classic mutagenesis approach for the mutation of two protease-producing strains of *Bacillus licheniformis* (strains 4a, 114) to make them insensitive to catabolite repression after UV-exposure, on the basis of increased resistance to the peptide antibiotic nisin. Two mutant strains of *B. licheniformis* insensitive to catabolite repression were selected by classical mutagenesis in connection with the development of a fed-batch procedure for protease production. *B. licheniformis* 4a produced up to 20 U (Anson-Units) subtilisin Carlsberg/ml in fed-batch experiments in the presence of up to 1.5 m glycerol, but was inhibited by excess ammonium. Formation of spores, excretion of agr-amylase and the biosynthesis of citrate synthase and isocitrate dehydrogenase were likewise not repressed by glycerol. The strain was characterized by unusually low activity of the agr-oxoglutarate dehydrogenase complex and increased biosynthesis of polyglutamic acid in the presence and excretion of agr-oxoglutarate in the absence of ammonium, respectively. The results are discussed in view of a possible connection between the defect in the agr-oxoglutarate dehydrogenase complex and insensitivity to catabolite repression. The second strain *B. licheniformis* 114 was able to synthesize 11.5 U protease/ml independently of the glycerol and ammonium concentration in the medium.

Jiang et al (2011) developed a mutant *Trichoderma viride* using Ethyl Methyl Sulphonate (EMS) followed by UV irradiation for cellulolytic enzyme production. The mutant EU2-77 proved to be the most promising extracellular cellulase producer. Soluble protein content, cellulase activity, β-glucosidase activity and endoglucanase activity of the fermentation broths of
the mutant strain were increased to 1.67, 2.49, 2.16, and 2.61 folds, respectively, compared with the wild strain. It was also proved that the crude enzymes prepared by *T. viride* EU2-77 showed much higher hydrolysis performance than that from the commercial strain Rut-C30 and demonstrated much comparable hydrolytic performance with the commercial enzyme mixtures. *T. viride* mutant EU2-77 produced high levels of extracellular cellulases as well as β-glucosidase, rendering the supplementation of β-glucosidase unnecessary in waste newspaper hydrolysis.

Fang et al (2009) reported that the mutant *Acremonium cellulolyticus* CF-2612 developed by using UV-irradiation and N-methyl-N'nitro-N-nitrosoguanidine (NTG) exhibited higher cellulose activity than the parental strain. Soluble protein production and β-glucosidase activity from strain CF-2612 were also significantly improved. Filter paperase activity, cellulose productivity and yield of CF-2612 using batch culture with 5% Solka Floc in a 2-l jar fermentor at 30 °C reached 18.0 U/mL, 150.0 FPU/L/h and 360.0 FPU/g carbohydrate, respectively; when fed-batch culture was used with Solka Floc, these values reached 34.6 U/mL, 240.3 FPU/L/h and 346.0 FPU/g carbohydrate, respectively. It was observed that more hydrolyzed glucose was released from pretreated eucalyptus with the enzyme of strain CF-2612, compared with that of the commercial cellulose GC-220.

Purohit et al (2006) investigated the strain improvement for tannase production from co-culture of *Aspergillus foetidus* and *Rhizopus oryzae*. Spores from the co-culture of *Aspergillus foetidus* and *Rhizopus oryzae* were subjected to UV, heat and NTG (3-nitro,5-methylguanidine) mutagenesis. Amongst all, the best mutant isolated from the heat treatment (60°C for 60 min) was SCPR 337. The maximum yield of gallic acid and tannase in case of mutant strain was 95.2% and 53.6 U/ml with an incubation period of
30 h as compared to wild strain where the incubation period was 48 h with an enzyme activity of 44.2 U/ml and gallic acid yield of 94%, respectively.

Adsul et al (2007) analysed the effect of strain improvement of *Penicillium janthinellum* NCIM 1171 on cellulose production. The strain was subjected to mutation involving treatment of EMS for 24 h followed by UV-irradiation for 3 min. Successive mutants showed enhanced cellulase production (EMS-UV-8). Mutants showed approximately two-fold increase in activity of both filter paperase and carboxy methyl cellulase in shake flask culture when grown on basal medium containing CP-123 (1%) and wheat bran (2.5%).

Bai et al (2004) reported an enhanced production of L(+)-lactic acid through strain improvement. A L(+)-lactic acid over-producing mutant, *Rhizopus oryzae* R1021, was isolated by mutagenizing the parent strain (R. oryzae R3017) with UV, diethyl sulfate (DES) and $^{60}$Co. Starting with a concentration of 120 g/L corn starch, mutant R1021 produced 79.4 g/L L(+)-lactic acid after 60 h in flasks, 52% higher than that produced by the parent strain. The L(+)-lactic acid purity was 99.05% by weight based on the amount of total lactic acid. The mutant R1021 was also morphologically different from the parent strain. The results of carbon flux analysis of the parent strain and mutants showed that the pyruvate node in the metabolic model was the principle and flexible node. The results showed that the key steps of the pathways in parent strain where most carbon is lost from lactic acid formation are the reactions form pyruvate to acetyl-CoA, oxaloacetate and ethanol. Although the fractions of the carbon from pyruvate to ethanol and acetyl-CoA were reduced in mutants, these two pathways are still the steps that are most likely to be further targeted to reroute the pyruvate metabolism to improve lactic acid production.
Pareek et al (2011) investigated the production of chitin deacetylase using mutant *Penicillium oxalicum* SAE₅⁻₅₁. Chitin Deacetylase (CDA) producing strain *P. oxalicum* ITCC 6965 was isolated from residual materials of seafood processing industries. Strain following mutagenesis using ethidium bromide (EtBr) and microwave irradiation had resulted into a mutant *P. oxalicum* SAE₅⁻₅₁ having improved levels of chitin deacetylase (210.71 ± 1.65 UL⁻¹) as compared to the wild type strain (108.26 ± 1.98UL⁻¹). Maximum enzyme production was achieved in submerged fermentation following 144 hours of incubation with notably improved productivity of 1.46 ± 0.82 UL⁻¹h⁻¹ as compared to the wild type strain (0.75 ± 0.53 UL⁻¹h⁻¹).

Tajima et al (2009) reported increased production of riboflavin by mutant *Ashbya gossypii*. This mutant was generated by treating the wild-type strain with N-methyl-N’-nitro-N-nitrosoguanidine. Riboflavin production was 10-fold higher in the mutant compared to the wild-type strain. The specific intracellular catalase activity after 3 d of culture was 6-fold higher in the mutant than in the wild-type strain. For the mutant, riboflavin production in the presence of 40 mM hydrogen peroxide was 16% less than that in the absence of hydrogen peroxide, whereas it was 56% less for the wild-type strain. The isocitrate lyase (ICL) activity of the mutant was 0.26 mU/mg of protein during the active riboflavin production phase, which was 2.6-fold higher than the wild-type strain. These data indicate that the mutant utilizes the carbon flux from the TCA cycle to the glyoxylate cycle more efficiently than the wild-type strain, resulting in enhanced riboflavin production.
2.9.1 Studies on Acclimatization

Varma et al (1976) revealed that the ability of a bacterium to metabolize a substrate depends on its genetic makeup in synthesizing the specific enzymes needed for this purpose. The bacteria unable to metabolize a certain substrate may acquire the metabolic potential by induction of needed enzyme(s) through molecular interaction with the substrate and the metabolic machinery of the microorganisms or selection and enrichment of the pre-existing bacterial mutants, “Adaptation” now is more often referred to as “induction”. In induction, bacteria have the genetic potential to produce the specific enzyme(s) needed for metabolism. The enzyme synthesis does not occur because of the steady repression of DNA transcription by a repressor molecule, however, until an inducer molecule inactivates the repression. A typical example of an inducible enzyme is $\beta$-galactosidase in *Escherichia coli*, which splits lactose into glucose and galactose. A culture of wild-type *E. coli* grown on a complex medium such as nutrient broth, when transferred to a minimal lactose medium passes through a short lag period before growth starts again. This transient inability of cells to grow in the new medium is caused by the absence of $\beta$-galactosidase in nutrient broth grown cells. During the lag period an inducer, which may be the substrate itself or a metabolite derived from it, inactivates the repressor and the transcription of the gene needed for the enzyme synthesis is resumed; the whole process takes 2 to 5 min. Moreover it was also reported in the acclimatization of wastewater bacteria by mutation that the acclimatization of wastewater is usually achieved in about 24 h.

Brown and Lester (1979) reported that acclimation may represent the period of time required to select species or strains of organisms that are tolerant to metals, or possess mechanisms that exclude metals, or can detoxify
metals. This may occur by mutation of existing strains. Alternatively, in a shorter time scale, acclimation may be the time required for synthesis of enzymes which function in excluding or detoxifying metals. Acclimated microorganisms can tolerate to the inhibitory quantities of toxic materials. The effect of metallic shock loads on unacclimated sludge was greater than that on acclimated sludge.

Arican and Yetis (2003) investigated the sorption of Ni\(^{2+}\) by acclimatized activated sludge treating Ni\(^{2+}\) bearing wastewater. The culture developed from sewage was acclimatized to 85.2 µmole/L Ni2+ influent concentration by stepwise increases; at a low dilution rate 0.11/h. Acclimation was found to enhance the sorptive capacity of the activated sludge. In fact, at all of the intermediate concentrations, percentage Ni2+ adsorbed by the biomass and also the sorptive capacity of the activated sludge drastically increased with an increase in the influent Ni\(^{2+}\) concentration. All influent Ni\(^{2+}\) concentrations were found to significantly stimulate the observed biomass yield of the culture over that observed in the base line. Experimental findings obtained at two other dilution rates; namely, 0.25/h and 0.45/h revealed that dilution rate is a significant operational parameter affecting the Ni\(^{2+}\) sorption characteristics of acclimatized activated sludge microorganisms. Considerable complexation of nickel and organic and inorganic ligands in the wastewater appeared to be responsible for a relatively lower Ni\(^{2+}\) sorption capacity.

Thomas et al (2012) reported that bacteria in the genus *Streptomyces* are soil-dwelling oligotrophs and important producers of secondary metabolites. Previously, it was showed that global messenger RNA expression was subject to a series of metabolic and regulatory switches during the lifetime of a fermentor batch culture of *Streptomyces coelicolor* M145. Here it was analyzed that the proteome from eight time points from the same
fermentor culture and, because phosphate availability is an important regulator of secondary metabolite production, compare this to the proteome of a similar time course from an *S. coelicolor* mutant, INB201 (ΔphoP), defective in the control of phosphate utilization. The proteomes provide a detailed view of enzymes involved in central carbon and nitrogen metabolism. Trends in protein expression over the time courses were deduced from a protein abundance index, which also revealed the importance of stress pathway proteins in both cultures. As expected, the ΔphoP mutant was deficient in expression of PhoP-dependent genes, and several putatively compensatory metabolic and regulatory pathways for phosphate scavenging were detected. Notably there is a succession of switches that co-ordinately induce the production of enzymes for five different secondary metabolite biosynthesis pathways over the course of the batch cultures.

### 2.10 ULTRASONICATION

Ultrasonic waves are generated by mechanical vibrations of an object at the same frequency as the frequency of ultrasound. Propagation of ultrasound in liquid media produces alternating compression and rarefaction cycles. During a rarefaction cycle, the rapid reduction in pressure leads to formation of gas and vapour bubbles in the liquid. These bubbles grow in the rarefaction phase and in the next compression phase the bubbles implode violently (Suslick 1988). During implosion, very high temperatures (approximately 5000 K) and pressures (estimated at 50000 kPa) can occur inside these bubbles (Gogate and Kabadi 2009). This formation, expansion and implosion of bubble is known as cavitation. Cavitation creates shockwave that propagates the liquid.
Gogate and Kabadi (2009) reported that cavitation in liquids generally causes rapid and complete degassing; may initiate various reactions by generating free chemical radicals; accelerates chemical reactions by facilitating the micromixing of reactants; breaks covalent and other bonds; disperses aggregates; increases emulsification rates; improves diffusion rates; micronizes dispersed solids and liquids; assists the extraction of substances from animal and plant tissue and microbial biomass; erodes and breakdown susceptible particles; including microorganisms.

2.11 USE OF ULTRASOUND IN BIOTECHNOLOGICAL PROCESSES

Ultrasound is widely used in laboratory protocols for breaking cell walls to release intracellular products (Chisti and Moo-Young 1986). Enzymes and other fragile macromolecules are known to be susceptible to damage by ultrasound (Potapovich et al 2005). Nevertheless, suitably applied ultrasound has the potential for enhancing the productivity of bioprocesses involving live cells and bioactive enzymes (Chu et al 2000; Shewale and Pandit 2009).

Pham et al (2009) investigated the effect of pretreatment of wastewater sludge by ultrasonication. Different wastewater sludge solids concentrations, ultrasonication intensities, and exposure times of pre-treatment were investigated for the optimization of ultrasonication treatment process. It was observed that the ultrasonication intensity and pre-treatment exposure time significantly affected the efficiency of the ultrasonication process followed by the solids concentration. The optimal conditions of ultrasonic pre-treatment were 0.75 W/cm² ultrasonication intensity, 60 min, and 23 g/L total solids concentration. The increases in soluble chemical
oxygen demand and biodegradability, by aerobic sludge digestion process, in
terms of total solids consumption increased by 45.5% and 56%, respectively.
The flowability of ultrasonicated sludge in terms of viscosity showed
exponential behavior at different total solids concentrations, and
pseudoplastic and thixotropic behaviour similar to rawsludge. Nevertheless,
the magnitude of viscosity values of ultrasonicated sludge was always lower
than the raw sludge.

So et al (2011) reported that ultrasonication is useful for inducing
amyloid nucleation and thus for forming fibrils, while the use of a microplate
reader with thioflavin T fluorescence is suitable for detecting fibrils in many
samples simultaneously. Amyloid fibrils produced by ultrasonication were
very short, with apparently similar lengths as determined by atomic force
microscopy, suggesting that short fibrils of homogeneous molecular sizes are
formed efficiently with the opposing effects of fragmentation and nucleation.
It was also revealed that the irradiation of an aqueous solution with ultrasonic
waves produces cavitation microbubbles, which repeatedly grow and collapse
in synchrony with the driving acoustic pressure (or ultrasonic amplitude).
When the microbubbles collapse, the temperature inside drastically increases
because of isothermal compression effects, providing hot spots and free
radical species. These decompose organic compounds in the solution (i.e., a
sonochemical reaction). There are two types of sonochemical reactions. The
first is pyrolysis, which occurs near hot spots. The other type is
decomposition by radical species, mainly OH and H radicals formed by the
dissociation of water. These sonochemical reactions occur most effectively in
the focused regions with a high-amplitude acoustic resonant mode, where
ultrasonic amplitude is much higher than the standard amplitude. However,
because β2-microglobulin is intact even after extensive ultrasonication,
sonochemical reactions do not seem to be the main mechanisms responsible for the breakdown and formation of the fibrils. On the other hand, the repeated growth and collapse of microbubbles and concomitant large shearing forces seem to be directly linked to the breakdown of preformed fibrils and the triggering of fibril formation.

Cho et al (2006) investigated the ultrasonication assisted extraction of resveratrol from grapes. When this method of extraction of resveratrol was applied to fruit stem of *Campbell* and *Gerbong* grapes, the recovery yield was increased by 24–30%, compared to that by the conventional solvent extraction with ethanol/water (80:20%, v/v) maintained at 60°C for 30 min. The ultrasonication-assisted extraction of resveratrol was modeled by the first-order reaction kinetics with reaction rate constants for extraction and degradation of resveratrol. For fruit stem of *Campbell* grape, the reaction rate constants $k_1$ and $k_2$ for extraction and degradation of resveratrol were 0.7797 min$^{-1}$ and 0.06481 min$^{-1}$, respectively, while the parameters for fruit stem of *Gerbong* grape were 0.4773 min$^{-1}$ and 0.03713 min$^{-1}$. Similarly, the maximum recovery of resveratrol for fruit stem of *Gerbong* grape and the amount of degraded compounds were 80.6% and 13.1%, respectively, of the total resveratrol, which were calculated with 194.8 µg/g-dry material and 31.6 µg/g-dry material.

Lin et al (2012) conducted experiments on sludge disruption by combined treatment of chlorine dioxide and ultrasonication. A lysis-cryptic growth system, which combined chlorine dioxide and ultrasonication for sludge disruption (ClO$_2$-ultrasonication disruption), was proposed to reduce the excess sludge in an activated sludge process. The optimal parameters of the ClO$_2$-ultrasonication disruption were determined by sequencing batch jar experiment and orthogonal jar experiments. Subsequently, a
ClO₂-ultrasonication disruption system was combined with a 1.8 m³/d pilot SBR for lysis-cryptic growth. The results indicated that most of the disintegrated sludge was inactivated and turned into biodegradable material at the optimal parameters of a ClO₂ dosage of 4 mg/g dry sludge, a ClO₂ treatment time of 60 min, an ultrasonic intensity of 0.5 W/mL and an ultrasonication time of 6 min. The enzymatic activity of the disrupted sludge decreased by 96.5% and the value of BOD/COD reached 0.4. The operating data from the pilot SBRs showed that the discharged excess sludge was reduced by 55% by returning 70% of the excess sludge pretreated by the ClO₂-ultrasonication disruption. Although lysis-cryptic growth resulted in an increase of 0.11 mg/L TP and 1.1 mg/L TN in the effluent, the impacts on the COD, ammonium and SS present in the effluent, as well as the sludge activity, were minimal.

Cho et al (2012) studied the effect of low strength ultrasonication on methanogenic granules in order to increase their activity and to enhance the performance of Upflow Anaerobic Sludge Blanker reactor (UASBr). Batch test results showed that 5 min of ultrasonication at 0.05 W/mL was found to be the optimal conditions, resulting in the increase of dehydrogenase activity and adenosine triphosphate content by 257%, and 374%, respectively, compared to the control. These increments were confirmed by specific methanogenic activity test. When ultrasonication (Ultrasonication Density (UD) 0.05 W/mL, Ultrasonication Time (UT) 5 min) was irradiated every 8 h during the continuous operation of UASBr, it caused a gradual drop of methanogenic activity, complete loss after 20 days. At further operation, UT was decreased to 1 s but irradiated every 1 min, which resulted in a 43% higher specific CH₄ production rate.
Elbeshbishy et al. (2012) investigated the effect of ultrasonication as a pretreatment method for pulp waste prior to anaerobic hydrogen production. Pre-treatment was conducted by sonicating a 100 mL of pulp waste at different sonication times varying from 0.5 min to 30 min. The ultimate hydrogen production increased with increasing sonication time. The highest ultimate hydrogen production was achieved at a sonication time of 30 min and reflected an 88% increase over the unsonicated food waste, of 80 mL/g VS<br>added. The highest final VFAs concentration after fermentation (corresponding to 70% increase over the unsonicated food waste) was also achieved at a sonication time of 30 min. There were no significant differences between the acetate-to-butyrate ratios (HAc/HBu) for the all sonication times. The maximum hydrogen production rate at sonication time of 30 min was about 145% higher than that the unsonicated food waste.

Koc and McKenzie (2010) determined the effects of high-intensity ultrasound irradiation and temperature on glycerin separation start time and separation rate during the transesterification of soybean oil. Response surfaces methodology was used to design the experiments. Reaction temperature, ultrasonication level and duration of ultrasonication at three levels were assigned as the control variables. The progressing transesterification reactions were monitored using a low-intensity ultrasound measurement system, which measures the ultrasound time of flight in glycerin as glycerin separates during the reaction. The effects of ultrasonication level, duration of ultrasonication and temperature on glycerin separation start time and separation rate were statistically significant at p <0.01 level. The effect of the interaction between temperature and duration of ultrasonication on glycerin separation start time was also statistically significant at p <0.05 level. The process conditions that provided the lowest starting times for glycerin separation were determined to
be the reaction temperature of 50 °C, ultrasonication of 5 min and ultrasonication rate of 90%. Low-intensity ultrasound measurement techniques and response surfaces experimental design are useful tools in determining the effects of various variables on the transesterification of vegetable oils.

Guo et al (2008) conducted experiments on hydrogen production using *Pseudomonas* sp. GZ1 (EF551040) by sterilization, microwave and ultrasonication pretreated waste sludge. The experimental results showed that different pretreated sludge had evident differences in the yield of hydrogen production and lag time. Sterilized sludge had the largest yield of hydrogen production, and the maximum yield was 15.02 ml/gTCOD. The lag time of using sterilized sludge was 15 h, longer than other two pretreated sludge. Using the ultrasonicated sludge, the hydrogen production yield was smallest and lag time was shortest in the three pretreated sludge. Protein and carbohydrate could be released from waste sludge by pretreatment. Protein was the main nutrient used for hydrogen production. The concentration of protein, carbohydrate and SCOD increased after pretreatment and fermentation.

Shah et al (2005) observed that use of ultrasonication as a pretreatment before aqueous oil extraction and aqueous enzymatic oil extraction as useful in the case of extraction of oil from the seeds of *Jatropha curcas* L. The use of ultrasonication for 10 min at pH 9.0 followed by aqueous oil extraction gave a yield of 67%. However, the maximum yield of 74% was obtained by ultrasonication for 5 min followed by aqueous enzymatic oil extraction using an alkaline protease at pH 9.0. Use of ultrasonication also resulted in reducing the process time from 18 to 6 h.
Bi et al (2011) examined Ionic liquid-based extraction of medicinal or useful compounds from plants as an alternative to supercritical fluid, cloud point and conventional organic solvent extractions. The method integrated extraction and preconcentration. Medicinal products were first extracted by an ionic liquid solution, part of which was then converted to a hydrophobic form by anion metathesis for preconcentration. The remaining soluble ionic liquid acted as a dispersive agent to enhance the efficiency of preconcentration. Protein in the extract was precipitated spontaneously without addition of further solvents. Ultrasonication assisted this method for extraction and preconcentration of cryptotanshinone, tanshinone I and tanshinone II A from *Salvia Miltiorrhiza* Bunge. 0.233 mg g\(^{-1}\), 0.695 mg g\(^{-1}\) and 0.682 mg g\(^{-1}\) of each, respectively, were extracted using [OMIM][Cl], and preconcentrated in a [OMIM][PF\(_6\)] phase at respective concentrations of 148.1, 507.1 and 486.1\(\mu\)g mL\(^{-1}\). The method exhibited potential applicability with other medicinal products.

Imai et al (2004) approached a practical high-performance enzymatic hydrolysis of cellulose using a ultrasonic irradiation as a pretreatment and a combination of cellulases from *Trichoderma viride* and *Aspergillus niger*. Their efficacy on enhancement of reactivity was discussed based on the kinetic parameters, i.e. Michaelis constant \(K_m\), maximum reaction rate \(V_{\text{max}}\) and initial reaction rate, correlating with ultrasonic conditions and enzyme combination, respectively. A relatively high-weight fraction of *A. niger* cellulase in the mixed-enzyme system afforded rapid initiation of the hydrolysis reaction, while a kinetic analysis revealed that a *T. viride* cellulase weight fraction of ca. 0.3 was optimal in enhancing \(V_{\text{max}}\) without increasing \(K_m\). Pretreatment of the cellulose fibers with ultrasonic irradiation prior to initiating the enzyme reaction further improved the
reaction rate. Enhancement of $V_{\text{max}}$ by ultrasonication was effective in greater weight fraction of *T. viride* used system. The correlation between the ultrasonic irradiation time required to obtain a desired initial reaction rate and the irradiation power employed was examined, and it was found that the time needed for effective degradation could be markedly reduced by increasing the irradiation power. This finding is considered to support the possibility of applying ultrasonication pretreatment in a practical cellulose saccharification process.

More and Ghangrekar, (2010) evaluated the effect of low-frequency ultrasound pre-treatment to inoculum on performance of microbial fuel cell (MFC). Mixed anaerobic sludge was pre-treated with ultrasonication duration of 2.5, 5, 7.5, and 15 min, and the performance of MFC using this inoculum was compared with the MFC inoculated without any pre-treatment to the sludge. The effect of combined pre-treatment, using ultrasonication and heating of the inoculum, on performance of the MFC was also evaluated. Maximum power density during polarization in a MFC inoculated with ultrasonication pre-treatment to the sludge for 5 min (40 kHz, 120 W) was 2.5 times higher than that obtained without any pre-treatment to the inoculum sludge. Substrate removal was higher in the MFC with ultrasonicated inoculum, than inoculum without any pretreatment and combined pre-treated with ultrasonication and heating. These results evidently demonstrated the advantages of ultrasonication pre-treatment to the inoculum for enhancing power and organic matter removal of the MFC.

Nabarlatz et al (2010) demonstrated the recovery of protease and lipase from the activated sludge by using stirring and ultrasonication, varying different parameters such as extraction time, concentration of additives (Triton X100, Cation Exchange Resin and Tris buffer), stirring velocity,
ultrasonic power and sludge source. Sludge was collected from two urban wastewater treatment plants located in Prague (Czech Republic) and Reus (Spain). It was found that stirring using 2% v/v Triton X100 for 1 h was enough to extract 57.4 protease units/g VSS, and that the same method using a combination of 10 mM Tris pH 7.5 + 0.48 g/mL CER + 0.5% TX100 as an additive allowed to extract 15.5 lipase units/g VSS from sludge collected from Reus Wastewater Treatment Plant. Ultrasonication allowed reducing the extraction time to 10 min for protease (using 2% v/v Triton X100 yielding 52.9 units/g VSS) and to 20 min for lipase (without any additive yielding nearly 21.4 units/g VSS), which makes this method appropriate for the extraction of enzymes from the activated sludge, and suitable to be scaled up for its application in the industry.

Chorvatovicova et al (1998) isolated Carboxymethyl-Chitin–Glucan (CMCG) from Aspergillus niger and subjected for ultrasonication to decrease its molecular weight. Ultrasonicated CMCG with molecular weight $0.19 \times 10^{-5}$ was administered either intraperitoneally or orally prior to Cyclophosphamide (CP) injection and its effect on the frequency of micronuclei in polychromatic erythrocytes of mouse bone marrow was evaluated. Both ways of CMCG administration significantly decreased the clastogenic effect of CP. The protective effect of CMCG was concentration dependent, with a higher decrease achieved by 200 mg/kg than by 100 mg/kg b.wt. Ultrasonic depolymerization of high molecular CMCG resulted in its anticlastogenic effect against CP not only on intraperitoneal, but also on oral administration, achieved by decreasing its molecular weight. Ultrasonication proved to be an efficient way to obtain molecules of CMCG able to pass through the cell walls of the gastrointestinal tract.
Pratap et al (2011) demonstrated the use of baker’s yeast as whole cell biocatalyst to accelerate the oxidative cyclization of 2-aminobenzenethiols and 1,3-dicarbonyl compounds in an organic medium, carried for obtaining 1,4-benzothiazines. The role of ultrasonication in expedition of the biocatalyzed cyclocondensation has also been mentioned. The principle of this strategy could be useful for another type of reactions. Further such types of investigations are underway in our laboratory and will be reported in due course.

Nguyen and Anema (2010) conducted experiments on the effect of ultrasonication on the properties of skim milk used in the formation of acid gels. Skim milk was ultrasonicated for times up to 30 min either with or without temperature control. Ultrasonication without temperature control resulted in the generation of considerable heat, with the milk reaching -95 °C within 15 min of treatment. The whey proteins were denatured. Changes to the casein micelle size were observed, with decreases during the early stages of US and increases (because of aggregation) on prolonged treatment. Significant κ-casein dissociated from the micelles. Acid gels prepared from these ultrasonicated samples increased in firmness (final G’) up to a maximum final G’ after ~15 min of ultrasonication, followed by a decrease from this maximum on prolonged treatment. Ultrasonication with temperature control demonstrated that the denaturation of the whey proteins was entirely due to the heat generated during ultrasonication, although the casein micelle size was still reduced. Acid gels prepared from ultrasonicated skim milk in which the temperature remained below the denaturation temperature of the whey proteins had low final G’, although a small increase was observed with increasing ultrasonication time.
Acid gels prepared from the samples that were ultrasonicated at temperatures above the denaturation temperature of the whey proteins had higher final $G'$, which could reach values similar to those obtained by the conventional heating of milk. The results of this study indicate that, in skim milk, most of the effect of ultrasonication can be related to the heat generated from the treatment, with ultrasonication itself having only a small effect on the milk when the temperatures are controlled. Industrial relevance: The control and the manipulation of the firmness of acid skim milk gels are important in many dairy food applications such as yoghurts and some types of cheese. Ultrasonication is an emerging technology that could be used to process skim milk for use in acid gelled products. This study has demonstrated that acid gel firmness can be substantially manipulated when skim milk is ultrasonically treated before acidification; however, most of the effect is due to the heat generated during ultrasonication treatment. As the effects of ultrasonication are similar to those obtained through conventional heating processes, and as ultrasonication can control spoilage microorganisms, using ultrasonication under controlled temperature conditions could be an alternative to conventional heating to give desired functional properties and storage stability to milk products. However, the temperature/denaturation/aggregation would need to be carefully controlled to minimize the detrimental effects of excessive heating.

Ciccolini et al (1997) studied the combined effect of low frequency ultrasound(20 kHz) with temperature on the survival of a strain of *Saccharomyces cerevisiae* suspended in water. The treatment temperature tested were 45°C, 50°C and 55°C; the actual ultrasonic powers tested were 50W, 100W and 180W. Application of ultrasonic waves at a non-lethal temperature did not display a deactivating action; a higher effect of ultrasound
at higher temperature was observed, and the synergy between ultrasound and temperature was confirmed. These results proved that the ultrasonic waves do not destroy the yeast’s cells; they damage them, thus increasing their sensitivity to heat. The existence of an optimal ultrasonic power for a maximal deactivating effect was shown, and was found to have a value of around 100W (actual power).

Khanal et al (2007) evaluated the use of high power ultrasonic energy to treat corn slurry in dry corn milling ethanol plants to enhance liquefaction and saccharification for ethanol production. Corn slurry samples obtained before and after jet cooking were subjected to ultrasonic pretreatment for 20 and 40 s at amplitudes of vibration ranging from 180 to 299 microm (peak to peak amplitude in microm). The resulting samples were then exposed to enzymes (alpha-amylase and glucoamylase) to convert cornstarch into glucose. A comparison of scanning electron micrographs of raw and sonicated samples showed the development of micropores and the disruption of cell walls in corn mash. The corn particle size declined nearly 20-fold following ultrasonic treatment at high power settings. The glucose release rate from sonicated samples increased as much as threefold compared to the control group. The efficiency of ultrasound exceeded 100% in terms of energy gain from the sugar released over the ultrasonic energy supplied. Enzymatic activity was enhanced when the corn slurry was sonicated with simultaneous addition of enzymes. This finding suggests that the ultrasonic energy did not degrade or denature the enzymes during the pretreatment. It was also reported that ultrasonic enhancement of bioprocesses has been attributed to improved interphase mass transfer as a consequence of microstreaming and turbulence induced in a fluid by sonication.
Suslick and Price (1999) revealed that the chemical effects of ultrasound derive primarily from acoustic cavitation. Bubble collapse in liquids results in an enormous concentration of energy from the conversion of the kinetic energy of the liquid motion into heating of the contents of the bubble. The high local temperatures and pressures, combined with extraordinarily rapid cooling, provide a unique means for driving chemical reactions under extreme conditions. A diverse set of applications of ultrasound to enhance chemical reactivity has been explored with important uses in synthetic materials chemistry. For example, the sonochemical decomposition of volatile organometallic precursors in low-volatility solvents produces nanostructured materials in various forms with high catalytic activities. Nanostructured metals, alloys, oxides, carbides and sulfides, nanometer colloids, and nanostructured supported catalysts can all be prepared by this general route. Another important application of sonochemistry in materials chemistry has been the preparation of biomaterials, most notably protein microspheres. Such microspheres have a wide range of biomedical applications, including their use in echo contrast agents for sonography, magnetic resonance imaging, contrast enhancement, and oxygen or drug delivery. Other applications include the modification of polymers and polymer surfaces. Moreover it was also reported that the improvement of bioprocesses by ultrasonication has been attributed to the extremely high local pressures and temperatures generated under high intensity sonication and permeability enhancement of microbial cell walls.

Radel et al (2000) suggested that the ultrasonic effect causes transmission of sonochemical forces into the cell to influence the biochemical processes and also enhances the intramolecular motion in macromolecules such as enzymes to improve enzyme-substrate interactions. No loss in cell
viability for cells treated with standing wave up to period of 2 h. Based on scanning electron micrographs (SEM) morphological changes occurred with the application of ultrasound compared with a control.

Neis (2007) reported that suitable applied ultrasound is known to enhance the productivity of at least some microbial fermentation but the causes of such enhancements are not entirely clear. Productivity enhancements by ultrasound may be brought about by some or all of the following factors:

1. Improved mass transfer to/from cells by ultrasound induced microturbulence in the broth.

2. Improved mass transfer to/from cells by disruption of clumps and flocs of cells without damage to individual cells.

3. Enhancement of gas-liquid, liquid-liquid and solid-liquid mass transfer by sonication in fermentations in which a limiting substrate is provided as a gas, solid or water immiscible liquid.

4. Ultrasound induced enhanced secretion of hydrolytic and other enzymes.

5. Possible induction of productivity-enhancing intracellular motions by ultrasound.

6. Ultrasound induced movement within and outside a cell to cause enhanced enzyme-substrate interactions.
Matsuura et al (1994) investigated the effects of ultrasonic wave irradiation on cell growth and the formation of ethanol and other volatile components in the fermentation process were investigated. The fermentation periods were reduced to 50–64% in wine, beer, and sake made from saccharified rice solution when weak ultrasonic waves were irradiated at 30 mW/cm²; the total intensity was 590 mW. YEPD medium fermented by a combination of optimal thermal trajectory control and continuous ultrasonic irradiation yielded a concentration of isoamylacetate about 2.5 times greater than the maximum concentration under isothermal conditions (20°C). Irradiation had virtually no effect when the dissolved Carbon Dioxide Concentration (DCO₂) was maintained at a level lower than the experimental value of Bunsen's CO₂ absorption coefficient. Therefore, it is suggested that irradiation accelerates the formation of ethanol and other components mainly by decreasing DCO₂.

Lateef et al (2007) studied the effect of ultrasonication on the release of fructosyltransferase from *Aureobasidium pullulans* CFR 77. The study examined the effect of acoustic power and sonication time on the efficient release of intracellular fructosyltransferase from *A. pullulans* CFR 77 for the production of fructooligosaccharides. It has been shown that acoustic power of 20W within 9 min of sonication yielded the best result. Furthermore, it was also reported that there was a considerable reduction in the reaction time as 9 h for the production of 59-60% fructooligosaccharides. The study demonstrated the potential role of ultrasonication in efficient release of the intracellular fructosyltransferase which can be used for the production of fructooligosaccharides, an industrially important prebiotic.
Lanchun et al (2003) studied about some vital physiological characteristics of *Saccharomyces cerevisiae* at logarithmic phase under ultrasonic stimulation including the stimulated conditions of the 24 kHz in frequency, 2 W in the power efficiency, 1 s for every 15 s in the stimulation time and 30 min in the duration cycle. After *S. cerevisiae* was inoculated and stimulated for passages, the effects of low ultrasound on the fermentation strength and proteinase activity of *S. cerevisiae* have been studied. Furthermore, cell flocculation and the ratio of ascospore production were detected in descendant *S. cerevisiae*. The results showed that ultrasonic stimulation specialized as above could enhanced the fermentation strength and proteinase activity of parents, but it had no influence on the fermentation strength and the ratio of ascospore production of the descendants while reduced the flocculation of the descendants. So, it can be concluded that appropriate low-intensity ultrasound will not affect the primary physiological characteristics of *S. cerevisiae*. Low intensity ultrasound changed the flocculation behavior of *S. cerevisiae* and improved the cell metabolism. It also changed the osmotic properties of membrane, improved transfer of substrate, drove up enzyme synthesis and enzyme activity.

Schlafer et al (2000) reported that increasing the energy input (0.3 - 12 W/L) did not increase the biological activity. At low frequency of ultrasonication, the ethanol concentration increased up to 67% during ethanol fermentation by *S. cerevisiae*. Ethanol production and cell growth were accelerated with ultrasound treatment. Moreover it was also revealed that discontinuous ultrasonic treatments were more beneficial for activating fermentation than continuous exposure.
Wang and Sakakibara (1997) observed that continuous sonication decreased cell viability. The maximum release of β-galactosidase during sonicated fermentation by *Lactobacillus* strains was in the exponential phase of culture. Lactose hydrolysis was effectively enhanced with the presence of β-galactosidase in the culture medium due to sonication.

Joyce et al (2003) revealed that flocs of fine particles e.g. clay can entrap bacteria which can also protect them against the biocides. It is because of problems such as these that alternative methods of disinfecting water are under active investigation. One such method is the use of power ultrasound, either alone or in combination with other methods. Ultrasound is able to inactivate bacteria and deagglomerate bacterial clusters or flocs through a number of physical, mechanical and chemical effects arising from acoustic cavitation. The effect of power ultrasound at different powers and frequencies on *Bacillus subtilis* was investigated. Viable plate count techniques were used as a measure of microbial activity. Results showed a significant increase in percent kill for *Bacillus* species with increasing duration of exposure and intensity of ultrasound in the low-kilohertz range (20 and 38 kHz). Results obtained at two higher frequencies (512 and 850 kHz) indicated a significant increase in bacteria count suggesting declumping. In assessing the bacterial kill with time under different sonication regimes three types of behaviour were characterized: High power ultrasound (lower frequencies) in low volumes of bacterial suspension results in a continuous reduction in bacterial cell numbers i.e. the kill rate predominates. High power ultrasound (lower frequencies) in larger volumes results in an initial rise in cell numbers suggesting declumping of the bacteria but this initial rise then falls as the declumping finishes and the kill rate becomes more important. Low intensity ultrasound (higher frequencies) gave an initial rise in cell numbers as a result
of declumping. The kill rate was low and so there was no significant subsequent decrease in bacterial cell numbers.

Herran et al (2008) reported that sonication at any power level tested, did not affect the biomass growth profiles. Medium and high intensity ultrasonication greatly reduced production of lovastatin by *Aspergillus terreus* and substantially altered the growth morphology. It was also revealed that sonication can be used to modify growth morphology and broth rheology without affecting growth of filamentous fungi. Sonication appears to influence the primary growth metabolism and secondary metabolism.

Chuanyun et al (2003) analyzed the production of riboflavin by *Ecemothecium ashbyii* with the effect of ultrasonication. The results revealed that the fermentation time was shortened by 36 h and the production rate of riboflavin was increased.

### 2.12 APPLICATIONS OF MATHEMATICAL MODELS

Gosling and ChemSim (2005) reported that simulation can be used at many levels, from simple mass balances for process audits, to complex descriptions of specific operations. There is now a range of tools available for process simulation. Selecting the appropriate tool depends on the level of detail required for the model and the questions being answered. Modeling complete flowsheets requires purpose-built software. Simulation can be used to tackle many problems, from initial design and proof-of-concept studies through operation and validation. It is now possible to link powerful software packages together to create unique solutions. Although a range of software tools exists, customization is usually required for tackling bioprocesses. Development of these tools is an ongoing process. However, the current tools
are sufficient to tackle real-world problems and, as cost pressures increase on biomanufacturers, there will be increased use of these tools to help reduce costs and streamline operations.

Gao et al (2008) suggested that process models can help us to understand the process in greater detail and to allow decisions to be made in a more effective manner. During the process development phase, such models can be used to guide decision-making in selecting the most efficient process sequences and the values of key operating variables. During product manufacture, process deviations are a key concern and tools allowing a swift response will be beneficial in ensuring the quality of products and the efficiency of manufacture are maintained.

Davies et al (2000) reported that developing bioprocess models is time consuming and there are several factors to be considered in order to achieve more efficient process descriptions. Firstly, a bioprocess contains multiple unit operations and there are strong interactions between the steps which need to be considered in order to investigate the whole process performance. Secondly, the time critical information such as the occurrence of an abnormal situation in the manufacturing process needs to be captured and to be responded to swiftly and effectively. This requires an evaluation of the whole process situation in a timely manner and a capacity to provide constructive decisions to cope with the unexpected situation. Various models so far used in bioprocess sector are listed in Table 2.2
Table 2.2 Biokinetic models used to evaluate different parameters during bioprocess

<table>
<thead>
<tr>
<th>S. No</th>
<th>Biokinetic model</th>
<th>Analysis</th>
<th>Equation number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>( X = \frac{X_m}{1 + \left( \frac{X_m}{X_0} \right) e^{-\mu_{max} t}} )</td>
<td>Growth kinetics</td>
<td>2.1</td>
<td>Hamidi-Esfahani et al (2004)</td>
</tr>
<tr>
<td>2.</td>
<td>( \text{OUR} = qO_2C_s = YOX\mu C_s \left( 1 - \frac{C_s}{C_{xm}} \right) + mO_2C_s )</td>
<td>Oxygen uptake rate</td>
<td>2.2</td>
<td>Santos et al (2006)</td>
</tr>
<tr>
<td>3.</td>
<td>( \mu = \frac{\mu_{max}[\text{dye}]}{K_s + [\text{dye}]} + \frac{[\text{dye}]}{K_i} )</td>
<td>Substrate inhibition</td>
<td>2.3</td>
<td>Adamo et al (1984)</td>
</tr>
<tr>
<td>4.</td>
<td>( \mu = \frac{\mu_{max}[\text{dye}]}{{K_s + [\text{dye}]* } + \frac{[\text{dye}]}{K_i} } } + \frac{[\text{dye}]}{K_i} )</td>
<td>Substrate inhibition</td>
<td>2.4</td>
<td>Adamo et al (1984)</td>
</tr>
<tr>
<td>5.</td>
<td>( \mu = \mu_{max} \left( e^{\frac{[\text{dye}]}{K_s} } - e^{\frac{[\text{dye}]}{K_i}} } \right) )</td>
<td>Substrate inhibition</td>
<td>2.5</td>
<td>Adamo et al (1984)</td>
</tr>
<tr>
<td>6.</td>
<td>( \mu = \frac{\mu_{max}[\text{dye}]}{K_s(1 + \frac{1}{K_i} + [\text{dye}]} )</td>
<td>Substrate inhibition</td>
<td>2.6</td>
<td>Shuler and Kargi (2002)</td>
</tr>
</tbody>
</table>

2.13 SCOPE AND OBJECTIVES OF THE PRESENT INVESTIGATION

The scope of the present study was to produce thrombinase using marine microorganism, and to improve the production through process optimization, strain improvement by random mutagenesis and application of low intensity ultrasonication. The main objectives of the present investigation include:

- To isolate the thrombinase producing marine actinomycetes and identify the isolate through colony morphology, biochemical and 16SrRNA analysis.
• To analyze the cell growth and thrombinase production using the marine isolate at various operational conditions.

• To formulate and optimize the medium components for the enhanced production of thrombinase.

• To study the influence of ultrasonication on cell concentration and thrombinase production.

• To develop improved microbial species by random mutagenesis (UV and chemical (EMS) mutagenesis) for the production of thrombinase.

• To compare the production of thrombinase using wild and mutant species.

• To purify the enzyme and analyze the stability of the enzyme at various operational conditions.