CHAPTER - 1

Introduction

Biocatalysis is the center of attraction of intense research in the biological field. Enzymes are the catalytic foundation of metabolism. Since time immemorial, enzymes have played a vital role in many manufacturing processes, such as in the production of wine, cheese, bread, modification of starch, etc. In last half of the twentieth century, the awareness of using microorganisms, their metabolic products including enzymes in a broad area of basic research and their potential commercial applications was highlighted.

1.1 Problem statement

Lignocellulosic plant biomass is available plenty in nature. This biomass consists of 20-30% hemicellulose, a heterogeneous polysaccharides, along with cellulose (Timell, 1967). After cellulose, hemicellulose is the second most abundant renewable resources with a higher potential for degradation to useful end products. Monocotyls contain about 40% hemicellulose; whereas soft wood and hard wood contain 15-25% and 25-32% hemicelluloses respectively (Wong et al., 1988).

Xylan is the principal ingredient of hemicellulose. Huge amount of agro-industrial residues are produced every year which cause severe environmental pollution. One of the possible ways to use the agro-industrial residues is through fermentation to produce value added products. Therefore, it is most essential to develop the technologies based on hemicellulose degradation to value added end products such as enzymes in a cost effective level. In this context, microbial xylanases are the preferred catalysts for xylan degradation. Mild reaction conditions, high specificity, lower side-products generation, and negligible substrate loss make xylanase most preferable enzyme for degradation of hemicelluloses. Better xylanase production therefore is achieved by isolating novel fungal or bacterial strains, or by inducing mutant strains to obtain hyper producing, thermostable strains.

Xylanases are ubiquitous in nature and produced by plethora of organisms like bacteria, algae, fungi, protozoa, gastropods and arthropods. Most of the bacteria and fungi secret extracellular xylanases which act on the hemicellulosic material to
liberate xylose as end product allowing the organism to grow heterotrophically on xylan rich substrate.

Fungi are the most common source of hemicellulases like xylanases. A particular organism has unique potential for the synthesis of enzymes of unique characteristic. Over the years, a number of organisms including the strains of *Aspergillus niger*, *Trichoderma reesei* (Liu et al., 1999), *Aspergillus nidulans* (Pinaga et al., 1994; Ganga et al., 1998), *Aspergillus kawachii* (Ito et al., 2000), *Streptomyces sp.* (Patel et al., 1994; Kansoh and Gammel, 2001), *Bacillus pumilus* (Rashid et al., 1999), *Penicillium sp.* (Wakiyama et al., 2008) have been exploited for xylanase production. Recently *Aspergillus niger* (Chapla et al., 2010) has been stated as the most potent and the most effective organism for xylanase biosynthesis.

*Penicillium* species are also another potential organisms for xylanase production. *Penicillia* are typically saprophytic in nature, and numerous species are of particular value for human. Probably the best known is *Penicillium notatum*, the producer of the antibiotic *Penicillin*. Other important *Penicillium* spp. used in food industry are *P. roqueforti* and *P. camemberi*, associated with the production of particular types of cheese (Chavez, 2006). Most of the *Penicillia* are soil fungi, and develop on variety of organic substances, particularly dead plant materials. They produce extracellular hydrolases such as pectic enzymes, lipases, proteases, cellulases, and xylanases (Chen et al., 1990; Wang et al., 1998; Wu et al., 2000; Haq et al., 2002).

The production of xylanolytic enzymes by *Penicillia* has been explored in a number of species. Particularly noteworthy is the screening performed by Krogh et al. (2004). Twelve strains of different species of *Penicillia* belonging to the subgenera *Biverticillium* (found in plant products related to wood, paper and textiles) and *Furcatum* (present in grassland soils) were tested. They observed that the first subgenus strains produced more β-xylosidase, while no difference was detected for endoxylanase.

Techniques like solid-state fermentation (SSF) and submerged fermentation (SmF) have been employed for xylanase production. However, the culture conditions, type of strain, nature of substrate and availability of nutrients are taken into consideration for selecting a particular production technique, as they are the critical factors affecting the yield.

Cheaper hemicellulosic agricultural residues like corn cobs, wheat bran, rice bran, rice straw, corn stalk and bagasse have also been found to be the suitable solid substrate
for the production of xylanase using microorganisms such as *Aspergillus awamori*, *Penicillium purpurogenum* and alkalophilic *Bacillus* species NCIM 59. Among fungi, the maximum activity in SSF has been obtained from the fungus *Schizophyllum commune* (22700 IU/g). *Trichoderma hamatum* is reported to produce 7000 IU/g dry weight using wheat straw as substrate. Production of cellulase free xylanase has been reported in a few *Bacillus* sp. and fungi (Haltrich et al., 1993; Beg et al., 2000).

Selection of a suitable inducing substrate and the best possible medium composition are the two basic factors for efficient production of xylanolytic enzymes. The substrate not only serves as carbon and energy source but also provides the necessary inducing factors to the organisms for the production of desirable enzymes. Purified xylan is an excellent carbon source for xylanase production in small scale. Previous reports suggest that higher yield of xylanase with negligible cellulase can be obtained using pure xylan as carbon source by different organisms (Yu et al., 1987; Harmova et al., 1989; Biswas et al., 1990; Gilbert et al., 1992). However, pure xylan is not cost effective for the large scale production process. Therefore xylan rich agricultural residues can be used as substrate at industrial scale. Earlier report (Royer and Nakas, 1989) suggests that better xylanase production was achieved when organisms were grown on cellulose than on pure xylan at similar concentration (Haltrich and Steiner, 1994). It is reported in literature (Stewart et al., 1983; Smith and Wood, 1991; Gomes et al., 1992; Haltrich et al., 1993) that better yield of xylanase was achieved from selected lignocellulosic substrate than pure xylan or cellulose when various insoluble lignocellulosic substrates (barley husk, corn cobs, hay, sugarcane bagasse, wheat bran and straw) were compared for xylanase production. Certain problems such as viscous media, agitations, etc. arise while using these insoluble lignocellulosic materials as carbon sources. Therefore, most of the time along with soluble carbon source, insoluble lignocellulosic residues are preferably used for xylanase production (Purkarthofer, 1993).

In addition to the nature of the inducing substrate, a suitable pretreatment such as acid treatment, alkali treatment, organic solvent treatment, and steam treatment is required for higher production of xylanase (Haltrich, 1996).

Optimization of media and process conditions are the most important factors to reduce the production cost. Conventional method is lengthy and often does not reveal the effect of interaction of different parameters correctly. Response surface methodology (RSM) is the most commonly used statistical practice for bioprocess optimization.
RSM is a compilation of numerical and statistical techniques useful for analyzing the effect of several independent variables. This process consists of a low order polynomial equation in a predetermined region of independent variables. These independent variables are later analyzed to locate the optimum values of the independent variables for the best response. It can be used to evaluate the relationship between a set of controllable experimental factors and observed results. The interaction among the possible influencing parameters can be evaluated with limited number of experiments (Myers and Montgomery, 1995).

Although there are reports of optimization of xylanase using RSM with different fungi and bacteria, there are not many expert reports on optimization of xylanase production using *Penicillium citrinum* MTCC 9620 (Puri et al., 2002; Katapodi et al., 2007).

The importance of cellulase free xylanase system in the paper and pulp industry had originated research into the link between the production of xylanase and cellulase by microorganisms. Mostly, filamentous fungi are important producers of extracellular xylanases and yield of the enzymes are much higher than from yeast and bacteria (Stainer et al., 1987).

Commercial applications of xylanase involve conversion of xylan into xylose (Subramaniyan et al., 2002); clarification of juices and wines (Beily et al., 1991; Wong et al., 1998); extraction of coffee, plant oils, and starch (Wong et al., 1998; Harbak et al., 2002); improving the nutritional properties of agricultural silage and grain feed, and for the production of fuel and chemical feedstock (Wong et al., 1988; Biely, 1991). Recently, use of xylanolytic enzymes in pulp bleaching has been considered as one of the most important new biotechnological applications. Hydrolysis of xylan facilitates the release of lignin from paper pulp and reduces the dosage of chlorine as the bleaching agent. The bioconversion of lignocellulososes to fermentable sugars like xylose, xylbiose, and xylo-oligomers can be achieved by the enzymatic hydrolysis of xylan. Xylanases are used as bread-dough strengthener because they provide excellent tolerance to the dough towards variations in processing parameters and flour quality. Xylanases also significantly increase volume of the baked bread (Harbak et al., 2002) and can be used as anti-staling agent in bread manufacturing (Leon et al., 2002; Gavilighi et al., 2006).

Nowadays, consumers are very health conscious therefore, demand for chemical free processed food products are growing fast. Biotechnology can play a pivotal role to
meet the demands. Accordingly, fermentation industry has stepped forward and grew well in the recent past (Pandey et al., 2000).

Special precautions are taken while microbial enzymes are used in food processing. From consumer safety point of view, enzyme must be free from health hazard. Before human consumption, trial should be taken on animals. In addition of other enzymes, xylanase can be added to poultry feed to improve feed efficiency and meat quality. Earlier reports provided the evidences that application of xylanase on poultry feed is safe (Shah et al., 2005; Bababola et al., 2006). Therefore human trial can be carried out safely.

In recent years, the baking industry has focused its attention on the replacement of several chemical compounds by enzymes, since they are clean label compounds. Different enzymes are currently added to the bread making processes for improving dough handling, fresh bread quality, and also to enhance the shelf life.

Bakers throughout the world prefer manufacturing of bread from low fiber white wheat flour. Epidemiological observations revealed that diseases related to the reduction of blood serum cholesterol, cardiovascular, coronary heart, diabetes, colon cancer can be prevented by incorporation of dietary fibers in the diet (Qiang et al., 2009). These observations emphasize interest in addition of nonnutritive fibers in the diet that resist human digestive secretion and intestinal flora (Pomeranz et al., 1977). Bread can be manufactured using fiber rich whole wheat flour replacing white wheat flour. However, replacement of white wheat flour by whole wheat flour changes the quality of the final products. Adverse effect of flour replacement by whole wheat flour in bread cause dilution of gluten proteins and adverse quality attributes of bread (Park et al., 1997; Kerch et al., 2010). Fiber enriched flour disturbs the starch–gluten matrix, reduces starch availability for gelatinization, reduces swelling of starch granules, compels gas cells to expand in a particular dimension and adversely affects dough viscoelastic behaviour, constraining dough machinability and gassing power (Gan et al., 1992). Consequently poorly hydrated gluten results in lower bread volume due to reduced gas retention and produces hard bread of inferior quality with objectionable gritty texture, taste and grainy mouthfeel. These undesirable effects can be minimized with the addition of gluten, surfactants, surfactant/shortening blends and hemicellulase (Shogren et al., 1981, Haseborg and Himmelstein, 1988, Sosulski and Wu, 1988, Laurikainen, et al, 1998). In bread manufacturing, most important attributes is to make softer, smoother, better flavor, better color, more nutritious, and
longer shelf life bread. Longer shelf life bread can be achieved by preventing the key reactions crucial for staling and preventing microbial growth (Fernandez et al., 2006). Enzymes are added to improve dough handling, flexibility, machinability, stability of fresh bread quality, loaf volume, and enhanced shelf life of bread. Cell walls of cereal grains contain complex polysaccharides such as cellulose and hemicellulose (arabinogluconoxylans) and are regarded as potential source of mono- and oligosaccharides. Haseborg et al. (1988) reported that addition of cellulase and hemicellulase in dough resulted in bread of higher volume and uniform porous crumb. Free sugars such as pentose and hexoses are released due to hydrolytic action of hemicellulases/pentosanases, which are then used by the yeast. Xylanase, one of the most important hemicellulase enzymes, has an important role in bread quality due to its ability to degrade arabinoxylan, increasing water absorption as well as interaction and cross linking with gluten. It has been demonstrated that xylanase significantly improves manufacturing conditions making the dough slacker, softer, viscous, machine friendly, easily sheetable, and less stickier. In addition, xylanases act as an anti-staling agent in bread manufacturing (Harbak et al., 2002; Leon et al., 2002; Gavilighi et al., 2006).

Maheswari et al. (2000) reported that xylanases from *A. niger* var. *awamori* and *Thermotoga maritima* yielded wheat bread with improved specific volume. Moisture present in bread is an important parameter related to softness of bread crumb. Superior quality bread crumb contains 35-40% moisture (wet basis). Jianng et al. (2005) reported that when xylanases from *Thermomysis lanuginosus* CAU44 supplemented in wheat bread, moisture content increased to 34.5% compared to control (29.4%, without xylanase).

Starch undergoes gelatinization during baking and degree of gelatinization depends on baking temperature and time. The baked bread undergoes staling due to retrogradation of starch during storage. Retrogradation involves change of amorphous to crystalline nature of starch and is accompanied by gradual increase in rigidity and separation between bio-polymer and solvent (syneresis). Change in amylose is negligible as it is present in water soluble fraction, and amylopectin fraction controls the retrogradation. The extent of water soluble starch in bread decreases with aging as water become more bound or immobilized during staling (Leung et al., 1983; Kim-Shin et al., 1991).

Rate of staling (retrogradation) can be quantified and correlated with physicochemical and thermal properties. Change of moisture content, starch solubility, enzyme
digestibility, firmness, glass transition temperature and enthalpy have been correlated with crystallization of starch during staling (Gavilithi et al., 2006). Systematic studies on quality of bread manufactured using partially purified xylanases are however lacking.

Thermal analysis has been used extensively to study starch retrogradation as well as bread staling. Differential scanning calorimetry (DSC) has proven to be the most useful in providing basic information on starch retrogradation. When good bread samples are heated in a DSC pan, an endotherm is observed as amyllopectin reaches its glass transition and/or melting temperature and the enthalpy change associated with this transition can be measured. Because, the time scales for endotherm development and for the increase in crumb firmness are broadly similar in magnitude, DSC can be used to measure the rate of bread staling quantitatively. However, there are overlapping transitions over a wide range of temperature because of the variety of components and range of structure of component present, which cause difficulty in analysis. X-Ray crystallography has been used to examine bread staling, specifically the crystalline nature of the starch in the system, which can be related to the firmness of the product. Starch, in freshly baked bread is mostly amorphous, but slowly reorders during storage. The recrystallization is reflected in X-Ray Defraction (XRD) patterns. Therefore, X-Ray crystallography can be used to determine molecular organization of starch in bread. X-Ray crystallography can be compared with DSC for determining the increase in crystallinity during storage of bread and used in conjunction with DSC in the analysis of the effect of various anti-staling additives on whole wheat bread.

Systematic studies on the production of xylanases from microbial sources and its application in food processing are lacking. The major objective of this work was to produce xylanase enzyme by SmF and SSF and its application in bread manufacturing. Appropriate statistical techniques were used in data analysis and optimization of the process parameters. The specific objectives are:
1.2 Objectives

1. Screening, isolation and identification of a new xylanase producing strain from agricultural wastes, industrial effluents, or soil.

2. Optimization of medium components and process parameters to maximize xylanase production in shake flask.

3. Detailed studies in controlled environment to maximize xylanase production in laboratory-scale fermenter.

4. Comparison of xylanase production in submerged fermentation (SmF) with solid state fermentation (SSF).

5. Purification and biochemical characterization of xylanase.


7. Analysis of physicochemical, rheological, textural, thermal properties, microstructure, and spectral characteristics of bread dough and bread.