Strain Improvement of *Penicillium janthinellum* for Cellulase Production and Biochemical Characterization of Xylanases from Yeast

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By

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Dedication

Dedicated to those research students who are excellent in scientific research but are lagged behind only because of lack of opportunities

“Each and every original research has a equal value, depends on preson how he exploits his own research”

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Mukund Gunwant Adsul

DECLARATION

This is to certify that the work incorporated in the thesis entitled **"Strain improvement of *Penicillium janthinellum* for cellulase production and Biochemical characterization of xylanases from yeast"** submitted by **Mukund Gunwant Adsul** was carried out under my supervision at NCIM Resource centre, National Chemical Laboratory, Pune 411008, Maharashtra, India. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. D. V. Gokhale
(Research Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the work of the thesis entitled **"Strain improvement of *Penicillium janthinellum* for cellulase production and Biochemical characterization of xylanases from yeast",** submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at NCIM Resource centre, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of Dr. D. V. Gokhale (Research supervisor). The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Mukund Gunwant Adsul

(Research Scholar)

ABSTRACT

Rationale of the study

Lignocelluloses are abundant sources of renewable biopolymer which has received a great importance, since sugars produced from hydrolysis of this raw material can serve as starting point to produce energy, food and chemicals. Cellulase enzymes have been reported for bioconversion of this biomass to useful products. The production of cellulase is a major factor in the hydrolysis of cellulose materials. Though much work has been carried out on the generation of improved strains, there is no process with commercial viability using improved enzymes, which can produce value added materials. Hence efforts are needed to produce cellulase complex hydrolyzing biomass to monomers with high economic potential. We also identified *P. janthinellum* NCIM 1171 as potent cellulase producer but not with desirable amount of cellulase activities in the broth. We would like to improve this strain with respect enhanced cellulase activities including β-glucosidase which could be exploited to convert biomass to monomers which then can be diverted to produce value added chemicals like lactic acid using SSF processes.

We also identified yeast strain which produces high levels of xylanase. This yeast was identified as new genus and hence we feel that the xylanase could possess novel characteristics. The purification and characterization of xylanase from this unique strain is necessary to understanding of novelty of this enzyme which may lead to the production of valuable product prom hemicelluloses.

Objective of the study

Considering the above background, the work entitled**, "Strain improvement of *Penicillium janthinellum* for cellulase production and Biochemical characterization of xylanases form yeast"** was taken up with following objectives.

- Mutagenesis of *Penicillium janthinellum* NCIM 1171 so as to generate a stable mutant possessing high activities of exo-glucanase, endo-glucanase, β-glucosidase and xylanase.

- Optimization of medium for the higher production of cellulase and xylanase by a mutant using natural biomass e.g. bagasse, wheat bran, etc.
- Production of value added products like lactic acid from bagasse cellulose using cellulases from improved strains.
- Purification of cellulase free xylanases from yeast strain in minimum one or two steps with maximum yield.
- Biochemical and biophysical characterization of purified xylanases.
- Active site characterization of both xylanases by chemical modification.

Chapter 1: General Introduction

This chapter describes the structure of cellulose, the enzymes involved in the hydrolysis of cellulose and their hydrolysis mechanism. It also covers the different organisms involved the cellulose production, the types of cellulase production methods. The *Penicillium* species which produces cellulases, their mutants and enzyme activities are also discussed here. The need for strain improvement and different methods for strain improvement such as conventional mutagenesis, genome shuffling and gene cloning are briefly described in this chapter. The few applications of cellulases in textile, detergent, pulp and paper, food , biofuels etc are described here.

Chapter 2: Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production

This chapter describes the detailed methodology of mutation, mutant isolation and production cellulases by selected mutants. The strain of *Penicillium janthinellum* NCIM 1171 was subjected to mutation involving treatment of Ethyl Methyl Sulfonate (EMS) for 24 h followed by UV-irradiation for 3 min. Successive mutants showed enhanced cellulase production (EMS-UV-8), clearance zone on Avicel containing plate (SM2) and rapid growth on Walseth cellulose agar plates containing 0.2% 2-deoxy-D-glucose (SM3). These mutants were transferred to Walseth cellulose plates containing higher concentration (1.5%) of 2-deoxy-D-glucose (SM4) in which only five mutants showed clearance zone on SM4. All these mutants showed approximately two-fold increase in activity of both FPase and CMCase in shake flask culture when grown on basal medium containing CP-123 (1%) and wheat bran (2.5%). The enzyme preparations from these

mutants were used to hydrolyze Avicel. Higher hydrolysis yields of Avicel were obtained with enzyme preparations of EU1. This is the first report on the isolation and selection of mutants based on hydrolysis of Avicel, which is the most crystalline substrate.

Chapter 3: Cellulose from sugarcane bagasse: Enzymatic hydrolysis and lactic acid production.

Here, hydrolysis of bagasse derived cellulose by cellulases form improved strain and production of L(+)lactic acid using simultaneous saccharification and fermentation was studied. The bagasse was chemically treated to obtain a purified bagasse cellulose sample, which is much more amenable to cellulase enzyme attack than bagasse itself. This sample, at high concentration (10%), was hydrolyzed by cellulase enzyme preparations (10 FPU/g cellulose) derived from mutants generated in our own laboratory. We obtained maximum hydrolysis (72%), yielding glucose and cellobiose as the main end products. Lactic acid was produced from this bagasse cellulose sample by simultaneous saccharification and fermentation (SSF) in a media containing a cellulase enzyme preparation derived from *Penicillium janthinellum* mutant EU1 and *Lactobacillus delbrueckii* mutant Uc-3. A maximum lactic acid concentration of 67 g/L was produced from a concentration of 80 g/L of bagasse cellulose, the highest productivity and yield being 0.93 g/L/h and 0.83 g/g, respectively. Considering that bagasse is a waste material available in abundance, we propose to valorise this biomass to produce cellulose and then sugars, which can be fermented to products such as ethanol and lactic acid.

Chapter 4: Production of Lactic Acid from Cellobiose and Cellotriose by *Lactobacillus delbrueckii* Mutant Uc-3.

In previous chapter we have described the enzymatic hydrolysis of bagasse cellulose and simultaneous saccharification and fermentation of cellulose to lactic acid. During hydrolysis the main end product produced are glucose and cellobiose. However, in SSF we didn't found the presence of cellobiose. It means that the cellobiose may be utilized by the *L. delbrueckii*. So we carry out the experiment to check the efficiency of *L. delbrueckii* for the conversion of lactic acid from cellobiose and other cellooligosaccharides. We also analyzed the enzymes involved in the

cellooligosaccharides utilizations and its localization. We found that *Lactobacillus delbrueckii* mutant Uc-3 utilizes both cellobiose and cellotriose efficiently, converting it into L(+) lactic acid. The enzyme activities of cellobiose and cellotriose utilization were determined for cell extracts, whole cells, and disrupted cells. Aryl-β glucosidase activity was detected only for whole cells and disrupted cells, suggesting that these activities are cell bound. The mutant produced 90 g/L of lactic acid from 100 g/L of cellobiose with 2.25 g/L/h productivity.

Chapter 5: Introduction -Xylanases

This chapter describes the structure of xylan, types of xylanases, their multiplicity, classification and different organisms producing xylanases. It is also discussed here the different yeast species which produces xylanases and their biochemical and biophysical characteristics. The application of xylanases in bleaching, pulp industries, food industries are described briefly. The application of xylanases in Xylooligosaccharide production and in probiotics is also discussed in detail.

Chapter 6: Xylooligosaccharide producing xylanases from *Pseudozyma hubeiensis*: Biochemical Characterization and analysis of catalytic amino acid residues.

Yeast strain, isolated from decaying sandal wood, is identified as *Pseudozyma hubeiensis* by rDNA D1/D2 sequence analysis (100% matches). This strain produced high levels of cellulase free xylanase. Two distinct xylanases were purified to homogeneity by DEAE cellulose chromatography followed by Sephadex G-50 column chromatography. Molecular masses of two native xylanases were 33.3 kDa (PhX33) and 20.1 kDa (PhX20) confirmed by MALDI-TOF mass spectrometry and also SDS-PAGE. The CD spectra analysis revealed that PhX33 is predominant with α-helix and PhX20 contained predominantly β-sheets. The chemical modification studies revealed the presence of three tryptophan and one carboxyl residues at the active site of PhX33. The active site of PhX20 is comprised of one residue each of tryptophan, carboxyl and histidine. Carboxyl residue is mainly involved in catalysis and tryptophane residues are solely involved in substrate binding. Histidine residue present at the active site of PhX20 appeared to have a role in substrate binding. PhX33 hydrolyzed xylan into xylotriose,

xylotetraose and xylopentaose and PhX20 hydrolyzed xylan into xylotriose, xylotetraose, xylopentaose, xylohexaose and xyloheptaose. No xylose and xylobiose were detected in the hydrolyzates. Both the xylanases produced only xylooligosaccharides (XOS) with degree of polymerization (DP) 3 to 7, which could be used in functional foods or as prebiotics. Lc ms-ms ion search of tryptic digestion of these xylanases revealed that there is no significant homology of peptides with known fungal xylanase sequences which indicate that these xylanases appear to be new.

Part-A

Strain Improvement for Cellulase Production and their Application

Chapter 1

Introduction to cellulases

CELLULOSE

Cellulose is the renewable biopolymer abundantly found on earth in the form of lignocelluloses. Cellulose was first discovered in 1838 by the French chemist Anselme Payen, who isolated it from plant matter and determined its chemical formula. It is a uniform, linear polymer of β-1, 4 linked β-D-glucopyranosyl units. The β-D-glycosidic bond causes alternate units to be positioned as shown in the structure (Fig. 1.1), so that the molecule is essentially a polymer of the disaccharides, cellobiose, actually the cellobiosyl unit. The molecule has a nonreducing end and an aldehyde end, although at times the latter may be oxidized to a carboxyl group. Chain length of native cellulose molecules varies from 7000 to 15,000 units, which are termed as degrees of polymerization (DP). Cellulose from wood pulp has typical chain lengths between 300 and 1700 units; cotton and other plant fibers as well as bacterial celluloses have chain lengths ranging from 800 to 10,000 units (Updegraff, 1969; Dieter et al., 2005).

Cellulose is a straight chain polymer with no coiling or branching, and the molecule adopts an extended and rather stiff rod-like conformation, aided by the equatorial conformation of the glucose residues. The multiple hydroxyl groups on the glucose from one chain form hydrogen bonds with oxygen molecules on the same or on a neighbor chain, holding the chains firmly together side-by-side and forming *microfibrils* with high tensile strength (Fig. 1.2). This strength is important in cell walls, where the microfibrils are meshed into a carbohydrate matrix, conferring rigidity to plant cells (Raymond, 1986; Perez et al., 2001).

Several different crystalline structures of cellulose are known, corresponding to the location of hydrogen bonds between and within strands. Natural cellulose is cellulose I, with structures Iα and Iβ. Cellulose produced by bacteria and algae is enriched in Iα while cellulose of higher plants consists mainly of Iβ. Cellulose in regenerated cellulose fibers is cellulose II. The conversion of cellulose I to cellulose II is irreversible,

suggesting that cellulose I is metastable and cellulose II is stable. With various chemical treatments it is possible to produce the structures of cellulose III and cellulose IV (Yoshiharu, 2002).

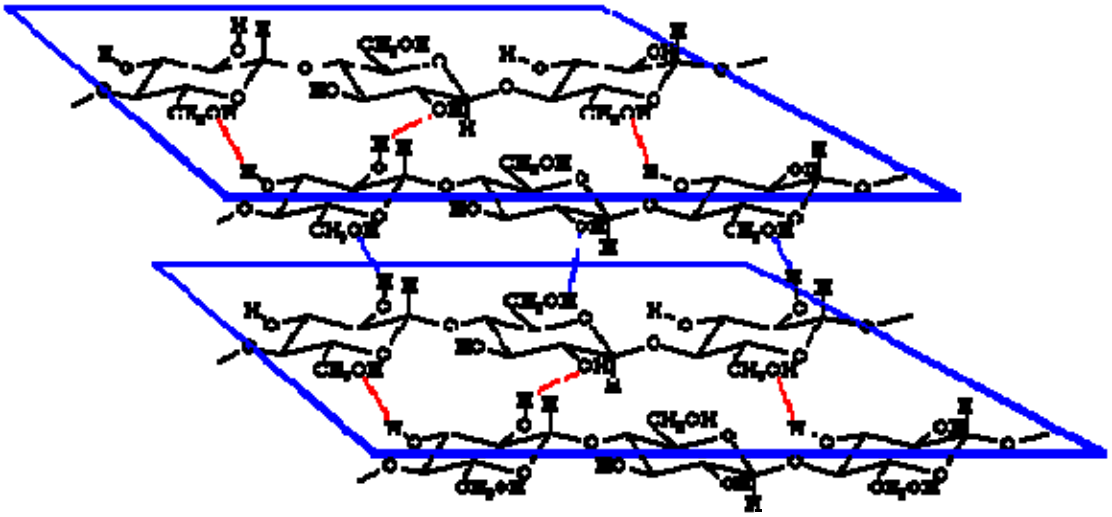

Fig 1.1 Structure of cellulose

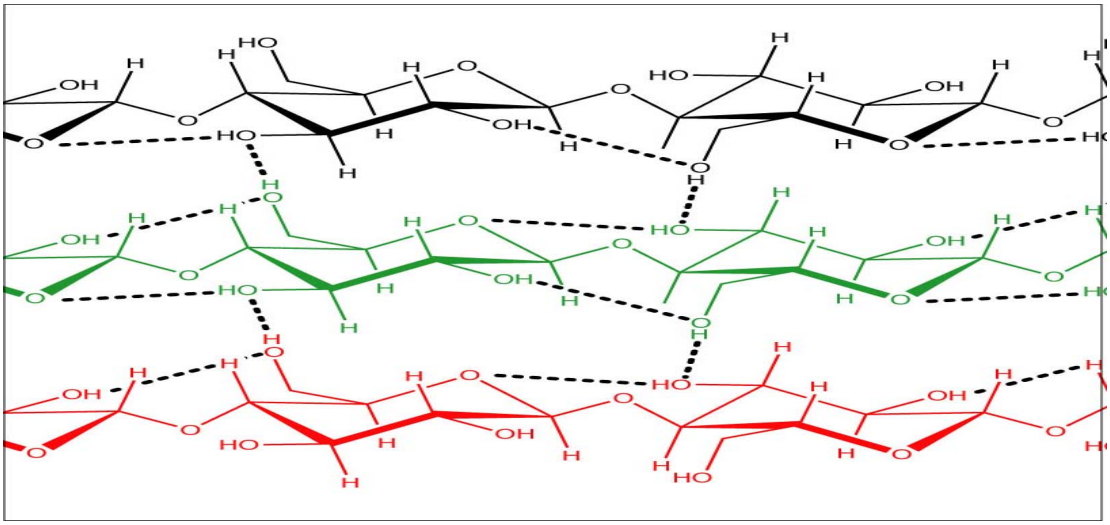

Fig. 1.2 Interactions in the cellulose Chains (dashed lines- hydrogen bonding)

In recent years much attention of researcher towards the cellulose is being paid because it contains valuable sugars which are basic carbon source for most of the microbial fermentation. Although it is the most promising renewable energy source to overcome the problems of energy resource, chemicals and food in the future, its

enormous potential would be recognized only when the cellulose is degraded in to the simple form. It is available in large amounts with low cost. To convert cellulose in to the simple sugars by biological route, it requires biocatalyst called cellulases.

CELLULASES

Cellulases refers to the class of hydrolytic enzymes (O-glycoside hydrolases, EC.3.2.1.-) which hydrolyze the β-1,4 linkage in the cellulose. Cellulases are mainly produced by Fungi and bacteria. Depending upon the type of reaction catalyzed and substrate specificity, the cellulases are classified in to three distinct classes.

i) The "endo-1, 4- β-D-glucanases" or 1,4- β -D-glucan 4-glucanohydrolases (EC 3.2.1.4), which act randomly on soluble and insoluble 1,4- β -glucan substrates and are commonly measured by detecting the reducing groups released from carboxymethylcellulose (CMC).

ii) The "exo-1,4-β-D-glucanases," including both the 1,4-β-D-glucan glucohydrolases (EC 3.2.1.74), which liberate D-glucose from 1,4- β -D-glucans and hydrolyze D-cellobiose slowly, and 1,4- β -D-glucan cellobiohydrolase (EC 3.2.1.91), which liberates D-cellobiose from 1,4- β -glucans.

iii) The "β -D-glucosidases" or β -D-glucoside glucohydrolases (EC 3.2.1.21), which release D-glucose units from cellobiose and soluble cellodextrins, as well as an array of glycosides.

The classification of glycoside hydrolases (GHs) and cellulases has changed several times. As new GHs were discovered during the 1980's and early 1990's, and as new activity experiments were performed, the older classification systems of the different GHs became more and more inefficient. The new way adopted for classification of GHs was hydrophobic cluster analysis (HCA), (Gaboriaud et al., 1987). In this system the different enzymes are classified into structurally related GH families based on similarities in the distribution of hydrophobic amino acids in their sequences. It was Bernard Henrissat who first applied the new HCA classification system to the GHs. He classified the then known 17 cellulases into different GH families named with a one letter codes such as A, B, C … (Henrissat, 1998; Henrissat et al., 1989; Henrissat et al., 1997). When the number of GH families grew, the one letter codes of the families had to be changed to

a number instead. Today there exist more than 2,500 known protein sequences of GHs, which have been classified into at least 90 different GH families. The classification of the different GHs can be monitored at the CAZy web page (URL: http://afmb.cnrsmrs.fr/~cazy/CAZY/index.html), (Coutinho et al., 1999). The cellulases are placed into a GH family according to the catalytic core of the enzyme. For example, *T. reesei* CBH I, has a catalytic core that has been classified as GH family 7. So the enzyme is called *T. reesei* Cel7A because it is a cellulase (Cel), and A because it is the first family 7 enzyme reported from this organism. To date cellulases have been assigned to 5,6,7,8,9,10,12,44,45,48,61 and 74 out of 97 GH families. This classification also sheds a light on the evolution suggesting convergent evolution of different folds resulting in the same substrate specificity. Some families are deeply rooted evolutionarily, such as family 9, which contains cellulases of bacteria, fungi, animals (protozoa and termites) and plants (Del Campillo et al., 1999; Watanabe et al., 2001). In contrast, family 7 contains only fungal hydrolases, whereas family 8 contains only bacterial hydrolases.

Most fungal cellulases are organized in two structurally independent domains; a catalytic core module, and a cellulose-binding module (CBM). These two domains are usually interconnected via a short flexible linker. The domain structure organization of *T. reesei* cellulases has been described in a review (Levy et al., 2002) (Fig.1.3).

The catalytic core module is the part of the cellulase where the hydrolysis of the cellulose chain takes place. This domain is the largest part of the enzyme. It varies greatly in size between different cellulases, e.g., the catalytic core modules of *T. reesei* cellulase vary from 166 to 430 amino acids in length.

The two domains in the enzyme are interconnected via a flexible linker. The length of the linker varies in size, from less than 20 to over 40 amino acids in the different enzymes. The linker is usually very rich in threonine, serine, and proline, and it is heavily glycosylated (Harrison et al., 1998). The role of the linker is probably to keep the two domains apart, and to restrict their movements with respect to one and another, so that the catalytic domain remains within close distance to the CBM, which binds on the surface of a cellulose fiber. The glycosylation of the linker probably makes it less flexible, and decreases its sensitivity to proteolytic enzymes (Harrison et al., 1998) that also are secreted by the microorganism. Deletion experiments on the linker of *T. reesei*

Cel7A have shown that if most of the linkers are removed, the rate of crystalline cellulose degradation is drastically reduced (Srisodsuk et al., 1993).

The Cellulose binding Module (CBM) is a small wedge-shaped domain consisting of approximately 35 amino acids. It can be connected via the flexible linker to either carboxy-terminus or the amino- terminus of the catalytic module. The function of the CBM is to bind on the surface of cellulose, and serve as an "anchor" for the enzyme, keeping it strongly adsorbed to the cellulose surface (Stahlberg et al., 1998; Reinikainen et al., 1992; Linder et al., 1996). This reduces the need for strong binding of the catalytic domain to the cellulose, and thereby enables the enzyme to have a higher rate of turnover (Stahlberg, 1991). There is no evidence that the fungal CBMs can penetrate into the cellulose fiber and disrupt the structure, or have any catalytic activity. Till today, more than 180 different CBM have been identified and classified into 13 families according to their amino acid sequence similatiries (Tomme et al., 1995). Most of the CBMs reported so far belong to families I, II, and III. CBMs of family are compact polypeptide of 32-36 amino acid residues, which are found only in fungi. The CBM of families II and III are specific to the bacterial enzymes and are much larger and containing 90-100 and 130-172 amino acid residues respectively. Besides different structures, CBMs also have quite diverse properties. In terms of substrate binding, they have different affinities and different specificities, with some binding to crystalline cellulose while others are restricted to the amorphous substrate. Again, in some cases the binding of isolated CBM follows a simple thermodynamic equilibrium (Linder et al., 1996) whereas, in other cases it does not and appears irreversible (Jervis et al, 1997; Carrard et al, 1999). In contrast to family I CBM, Family II CBM has been reported to enhance the physical disruption of cellulose fibre and to release small particle from cotton fibers (Tomme et al., 1995). However, few studies have compared the effect of different CBDs in stimulating the activity of a given cellulase (Tomme et al., 1995; Coutinho et al., 1999). The classification of CBMs can be monitored at the CAZy web page (URL:http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), (Coutinho et al., 1999).

MECHANISM OF CELLULOSE HYDROLYSIS

First Reese and his coworkers suggested the mode of cellulose hydrolysis involving a C_1 and Cx components (Reese et al., 1950). They reported that the conversion of native cellulose into soluble sugars is a two step process. The C1 component was

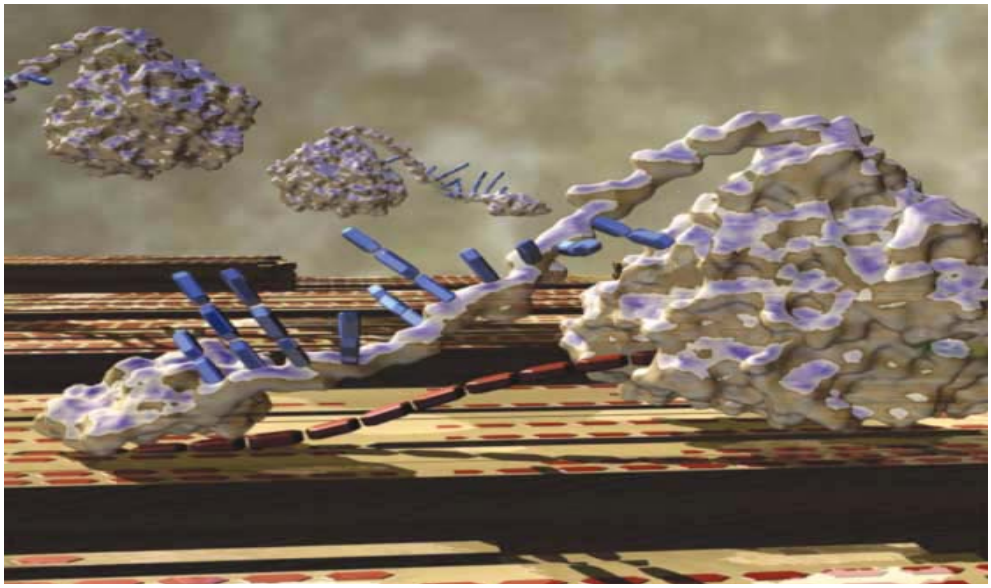

Fig. 1.3 *Hypothetical model of exoglucanase (the T. reesei cellobiohyrolase I) acting on crystalline cellulose. In this depiction, the carbohydrate-binding module (left) recognizes and binds to the cellulose surface. By a process not fully understood, a single chain of cellulose is "decrystallized" and directed into the active-site tunnel of the catalytic domain (right). This enzyme is thought to proceed along a cellulose chain cleaving one cellobiose unit per catalytic event until the chain ends or the enzyme becomes inactivated (Himmel et al, 2007; Rouvinen et al, 1990 and Vrsanska et al, 1992).*

believed to disaggregate or activate the cellulose chains so that the enzymes classified as Cx could carry out the depolymerization. They proposed that microorganisms that could grow, only on soluble forms of cellulose, such as carboxymethyl cellulose (CMC), synthesized only the Cx component, whereas microorganisms capable of growing on highly ordered forms of cellulose produced both C1 and Cx. The early studies were focused on the Cx components due to inability to produce culture filtrates active against crystalline cellulose. The discovery in 1964-1965 showed that, culture filtrates prepared

from of *T. viride* and *T. koningii* were capable of extensive hydrolysis of native cellulose was a turning point in the study of cellulases. In 1972, three independent research groups come up with an important discovery that the C1 component was, in fact, a hydrolytically active enzyme, cellobiosyl hydrolase (cellobiohydrolase), (Nisizawa et al., 1972; pettersson et al., 1972; Wood, 1972a; 1972b). Cellobiohydrolase was found to act synergistically with the Cx components to degrade crystalline cellulose. It was therefore proposed that Cx (CMC-ase) acts as an endoglucanase to produce available chain ends on cellulose which are substrates for cellobiohydrolase. It turned out to be the Cx component that initiates the cellulose breakdown rather than the C1 proposed by Reese et al, (Reese et al., 1950). Further, the widely accepted mode of enzymatic hydrolysis of cellulose involves synergistic actions of three enzymes i.e. endoglucanases, exoglucanases and β-glucosidase. Endoglucanases hydrolyse the intermolecular β-1, 4-glucoside bond of cellulose chain randomly to produce new chain ends, exoglucanases processively cleave cellulose chain ends to release soluble glucose and/or cellobiose and β-glucosidases hydrolyzes cellobiose to glucose. The feed back inhibition by cellobiose and glucose is also observed during cellulose hydrolysis. As compared to glucose, cellobiose cause severe feedback inhibition, i.e. it strongly inhibits the cellobiohydrolases.

There are two major mechanisms of enzymatic glycosidic bond hydrolysis first proposed by Koshland (Koshland, 1953). The retaining mechanism involves initial protonation of the glycosidic oxygen via the acid/base catalyst with concomitant formation of a glycosyl-enzyme intermediate through the nucleophile. The resulting glycosyl enzyme is hydrolyzed by a water molecule and this second nucleophilic substitution at the anomeric carbon generates a product with the same stereochemistry as the substrate. The nucleophile and acid/base are always found 5-6 Å apart on all systems studied so far. The inverting mechanism involves the single step protonation of the glycosidic oxygen via the acid/base catalyst and concomitant attack of a water molecule activated by the nucleophile. The consequent product shows a stereochemistry opposite to that of the substrate (Fig. 1.4). This mechanism is conserved within a given glycosyl hydrolase family and dictated by the active site architecture and atomic distance between the acid/base and nucleophilic residues (aspartic acid and/or glutamic acid). The acid and base are typically located about 7-13 Å apart in order to accommodate the nucleophilic water `below' the pyranoside ring. During

the hydrolysis of native cellulose, inverting type enzyme produces the α-sugar and a retaining enzyme produces a product in the β-configuration (Sinnott, 1990; Davies et al., 1995; Schulein, 2000; Zechel et al., 2000).

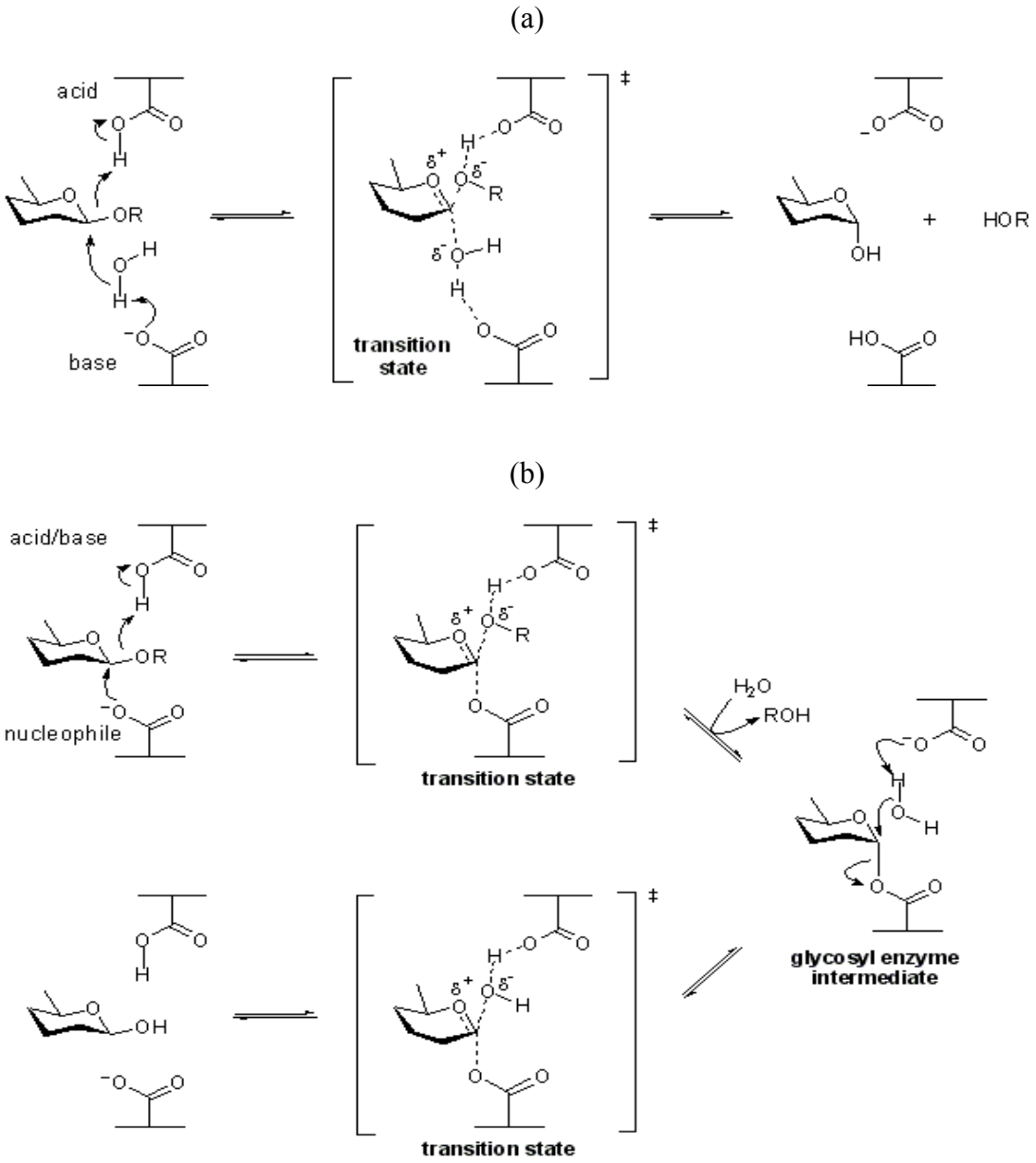

Fig.1.4 *Mechanism of cellulose hydrolysis by cellulases, a) Inverting mechanism of cellulose hydrolysis by cellulases, b) Retaining mechanism of cellulose hydrolysis by cellulases*

The recent concept of mode of cellulose hydrolysis by cellobiohydrolase is the concept of "processivity." Processive enzyme action can be defined as the sequential cleavage of a cellulose chain by an enzyme. In effect, exoglucanases /cellobiohydrolases are by nature and structure, processive enzymes. This topology allows these enzymes to release the product while remaining firmly bound to the cellulose chain, thereby creating the conditions for processivity. Their tunnel-like active site thus allows processive action on the cellulose chain (Fig. 5b). Another cleft or groove like 'open' structure (Fig. 5a) allows a random binding of several sugar units in polymeric substrates and is commonly found in endo-acting cellulases.

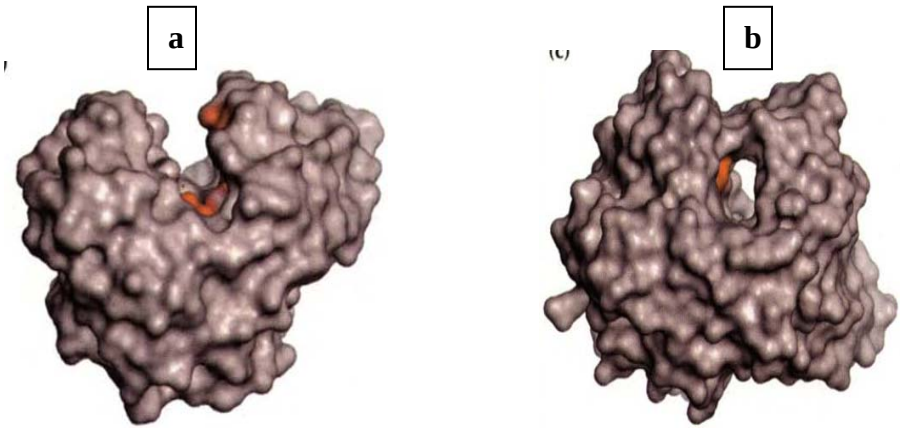

Fig.1.5 *a) The cleft type active site of endoglucanase E2 from T. fusca, b) The tunnel type active site cellobiohydrolase II from T. reesei creating the conditions for processivity.*

CELLULASE PRODUCTION

Organisms

Cellulases are widely distributed among prokaryotic and eukaryotic organisms. In case of animals, several cellulase studies were undertaken in molluscs, including snails, a sea slug, a periwinkle, and some bivalves. Some researchers have reported the possible endogenous enzyme sources such as the hepatopancreas, gastric teeth, and crystalline styles (Watanabe et al., 2001). Yokoe and Yasumasu (Yokoe and Yasumasu, 1964) investigated cellulase activity in 74 higher animals including several vertebrates and demonstrated that the activities of the possible endogenous cellulases in these animals were distributed according to phylogenetic relationships and not according to food habits. In fact, cellulase activities in insects are observed not only in strict xylophagous species

like termites and wood-eating cockroaches, but also in many other species with different food habits which do not have symbiotic relationships with cellulolytic protozoa or microorganisms, such as domestic omnivorous cockroaches (Yokoe and Yasumasu, 1964).

The order Actinomycetales comprises two genera which produce cellulases are *Microbispora* and *Thermornonospora.* Fractionation of *M. bispora* cellulases has identified six different enzymes. Two of these enzymes appear to be exocellulases and show synergism with each other and also with the endocellulases. The structural genes of five *M. bispora* cellulases have been cloned out of which one was sequenced. Fractionation of *T. fusca* cellulases has identified five different enzymes, all of which were purified to homogeneity and partially characterized (Wilson, 1992).

Both aerobic and anaerobic forms of bacteria are also involved in the cellulose degradation. The anaerobic bacteria are found in the soil, on decaying plant materials, in rumens, in sewage sludge, in termite gut, in wood-chip piles, in compost piles, and at paper mills and wood processing plants .Most of these bacteria occur in natural habitats such as soil and decaying plant materials, but some are enriched by human activities, such as in compost piles, in sewage plants, and at wood processing plants. Other natural habitats include the anaerobic rumen of various ruminants and the gut of termites, where they process plant materials for the host organism's nutrition. The examples of some anaerobic bacteria with active cellulolytic systems are, *Acetivibrio cellulolyticus, Bacteroides cellulosolvens, Butyrivibrio fibrisolvens, Clostridium acetobutylicum, Clostridium aldrichii, Clostridium cellobioparum, Clostridium cellulofermentans, Clostridium cellulolyticum, Clostridium cellulovorans, Clostridium herbivorans, Clostridium hungatei, Clostridium josui, Clostridium papyrosolvens, Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens* and *Clostridium thermocellum* (Maki et al., 2009).

The aerobic bacteria are usually found in the soil, in water, on plant materials, in humus, animal feces, sugar cane fields, and leaf litter. These are *Bacillus megaterium, Bacillus pumilus, Cellulomonas fimi, Cellulomonas flavigena, Cellulomonas gelida, Cellulomonas iranensis, Cellulomonas persica, Cellulomonas uda, Cellvibrio gilvus,*

Cellvibrio mixtus, Pseudomonas fluorescens, Streptomyces antibioticus, Streptomyces cellulolyticus, Streptomyces lividans and *Streptomyces reticuli (*Roy, 2008).

Compared to bacteria, large numbers of fungal strains are known to produce cellulases. A few examples of aerobic fungi, generally found in soil and decomposing wood and agricultural waste, are *Aspergillus niger, Phanerochaete chrysosporium, Piptoporus betulinus, Pycnoporus cinnabarinus, Rhizopus stolonifer, Serpula lacrymans, Sporotrichum pulverulentum, Trichoderma reesei (Hypocrea jecorina).* The anaerobic fungi which found in rumen and intestinal tract of large herbivorous animals are *Anaeromyces mucronatus, Caecomyces communis, Cyllamyces aberensis, Neocallimastix frontalis, Orpinomyces* sp., *Piromyces* sp., *Piromyces equi, Piromyces* sp. strain E247, *Neocallimastix patriciarum* etc.

Fungi are the most studied microorganisms for cellulases because of their higher enzyme yield and their ability to secrete complete cellulase complex. These are: *Trichoderma reesei*, *Penicillium pinophilum*, *Penicillium funiculosum*, *Fusarium oxysporum*, *Aspergillus niger*, *Sclerotium rolfsii* and *Humicola sp.*. However, other cellulolytic systems of *Phanerochaete chrysosporium*, *Taleromyces emersonii*, *Melanocarpus albomyces* and other anaerobic fungi belonging to genera *Neocallimastix*, *Cacomyces, Oprinomyces* have also been well characterized.

The cellulases have also been isolated from thermophillic fungi such as *Sporotrichum thermophile*, *Thermoascus auranticus*, *Chaetomium thermophile*, *Humicola grisea* and *Myceliopthora thermophila*. These fungi are of more interest because of their capacities to produce thermo-stable cellulases. These enzymes have shown stability at highly acidic or alkaline pH as well as temperatures up to 90°C. In order to overcome complexity of cellulosic substrates, fungi often produce a variety of cellulase components that differ in their molecular characteristics such as molecular weight, amino acid composition and sequence, isoelectric point and carbohydrate content etc. They also differ in their capacities to adsorb on to cellulose, substrate specificity and even in their catalytic activities (Vyas, 2004).

Though in environment, more cellulolytic organisms are present, majority of organisms can not be cultured by using available protocols. Consequently, the isolation and characterization of cellulases from uncultured organism from the nature has been

limited. Recent advances in molecular techniques like metagemomic approach made it possible to screen the large number of cellulolytic genes. Metagenomics is a cultivation independent analysis of the metagenome of a habitat and involves direct isolation of DNA from the environment followed by cloning and expression of the metagenome in a heterologous host (Handelsman et al., 1998). Although characterization of cellulases from uncultured microorganisms could reveal much information about cellulolytic diversity and industrial potential, there are few reports on metagenome-derived cellulases (Healy et al., 1995; Rees et al., 2003). Recently, a metagenomic approach was employed to produce ionic liquid stable endoglucanases. However, they were found to be moderately active and stable in 1-butyl-1-methyl-pyrrolidinium trifluoromethanesulfonate. (Pottkamper et al., 2009).

Production

Cellulase enzymes are produced in large scale by the species of fungi such as *Tricoderma, Penicillium* amd *Aspergillus*. Cellulases are gaining more importance because of their applications in textile industry, in detergent, paper and pulp industries, animal feed and food industries. Recently, researchers are engaged more in the cellulase production because they play a key role in the production of chemicals such as bioethanol, lactic acid, from cellulosic biomass. The fast growing concern about shortage of fossil fuels, the emission of greenhouse gases and air pollution by incomplete combustion of fossil fuel have also resulted in an increased focus on bioethanol production through hydrolysis of lignocellulosic materials by cellulases.

The laboratory scale and industrial production of cellulases were carried out by using two methods, i.e. submerged fermentation (SmF) and solid state fermentation (SSF). The submerged fermentation (SmF) method involves the cultivation of microorganisms in a nutrient rich aqueous medium. However, considerable expense is involved in concentrating and extracting enzymes from this large aqueous environment. An alternative to the traditional SmF method is solid state fermentation (SSF), which involves growth of microorganisms on solid materials in the absence of free liquid (Cannel et al., 1980). Since SSF requires relatively little liquid as compared to SmF, downstream processing of SSF derived enzymes is theoretically simpler and less

expensive. Cellulases are inducible enzymes, requiring the inducer substrate. Generally cellulose is the best inducer for cellulase production. The lignocellulosic materials used for cellulase production in solid state and submerged fermentations are wheat straw, rice straw, sugarcane bagasse, grasses, waste papers, cotton, corn cobs, soft and hard woods, diary manure, and other lignocellulosic materials (Howard et al., 2003). The lignocellulosic materials cannot be directly used for cellulase production because they are not directly accessible for cellulase production. These materials primarily need pretreatment by chemical (alkaline treatment to remove lignin) or thermal (steam explosion) methods. These pretreated lignocellulosic materials are utilized for cellulase production.

Cellulase gene expression is induced by oligosaccharides such as cellobiose (two β-1, 4-linked glucose units) or sophorose (two β-1, 2 –linked glucose units). Furthermore, cellulase genes are found to be induced when *T. reesei* was grown in the presence of several disaccharides, namely, laminaribiose, gentiobiose, lactose and xylobiose (Vaheri et al., 1979; Durand et al., 1988; Margolles Clark et al., 1997). The monosaccharide L-sorbose was also found to induce cellulase (Nogawa et al., 2001). *T. reesei* can use sorbitol and glycerol as the carbon source for its growth, but no cellulase gene transcription has been detected by Northern analysis (El-Gogary et al., 1989). These carbon sources are considered to be ineffective for cellulase expression (Mandels et al., 1962; Nisizawa et al., 1971; Ilmen, 1997). Sophorose is the most powerful inducer and is more specific to *T. reesei* since it does not induce cellulase expression in other fungi like *Phanerochaete janthinellum, Phanerochaete chrysosporium* and *Aspergilus. niger* (Bisaria et al., 1989; Hrmova et al., 1991; Gielkens et al., 1999). The cellulase production can reach high levels with slow feeding of cellobiose into growth medium (Vaheri et al., 1979).

Because cellulose is too large to be transported into cells, an inducer capable of passing through the cell wall needs to be formed when cellulose is used as the inducer for cellulase production. The expression of a very low level cellulase is considered to be related to the formation of an inducer from cellulose. The conidia of *T. reesei* have been shown to contain cellulases, mainly CBHII, on their surface. These conidia-bound cellulases are supposed to be responsible for the initial release of an inducer from

cellulose, resulting in cell growth on cellulose (Kubicek et al., 1988; Messner et al., 1991). However, cellulase transcripts are not detected from glucose-, sorbitol- and glycerol-grown mycelia in Northern blot analysis and no hybridization signals of *cbh1*, *cbh2* and *eg1* were detected in a slot blot analysis (Ilmen, 1997).

The presence of glucose, which is easy to metabolize and energetically favorable to the microbe, leads to the repression of other genes needed for the use of other carbon sources. The controlling mechanism is called glucose (carbon catabolite) repression. The cellulase production by *T. reesei* is under glucose repression. In filamentous fungi, glucose repression is manipulated by the transcription factor CREA and CREI, which are the proteins responsible for glucose repression (Dowzer et al., 1991; Drysdale et al., 1993; Ilmen et al., 1993). The expression of *cre1* in *T. reesei* is regulated by the carbon source, in which the expression is higher in presence of an inducing carbon source than in the presence of glucose (Ilmen et al., 1993; Strauss et al., 1995;), This indicates that the CREI protein function is regulated at the post-transcriptional level or by the presence of other factors. *T. reesei* Rut-C30 is a glucose de-repressed mutant strain, which contains a truncated *cre1* gene. Isolation of catabolite repressor mutants is a better choice for high titer cellulase production. The analog of glucose such as 2-Deoxy D-glucose is generally used for isolation of catabolite repressor mutants.

The general medium used for the cellulase production was developed by Mandels and Weber (1969). The lignocellulosic materials such as rice straw, wheat straw, sugarcane bagasse etc. are used as carbon source for cellulase production. Among nitrogen sources, ammonium salts, urea and peptone are the best sources. Besides carbon and nitrogen sources, several other factors have also been reported to be important in optimization of cultivation conditions. The morphological and physiological changes have an effect on cellulase production (Velkovska et al., 1997; Mcintyre et al., 1998). Magnesium sulfate and calcium chloride are very important for the enzyme function inside the cell. All of the trace-elements are essential to the cell growth and enzyme functions, however, the amounts are very small, otherwise it will cause cell death.

pH is an important parameter for the cell growth and cellulase production. A good cellulase production and cell growth were found at low pH 4.0 than at pH 7.0 (Bailey et al., 1993). The pH of the medium was influenced by the nitrogen source during the

fermentation process (Haapala et al., 1996). The pH of the culture broth dropped during the fermentation when ammonium salts were used as the nitrogen source. The temperature of the cultivation does not only affect the microbe growth, but also on the level of cellulase production. *T. reesei* Rut C-30 grew well at 17, 28 and 37°C when cultivated on lactose substrate, but cellulase production was reduced at higher temperature (Haltrich et al., 1996).

The agitation rate and aeration levels affect the fungal growth and enzyme production when the bioreactors are used. The significant effect of shear stress from the impellers on the morphology and productivity of filamentous fungi draw a more attention (Ilias et al., 1998; Gibbs et al., 2000). Too strong agitation and aeration has been shown to cause lower enzyme production. The enzyme production by *T. reesei* QM9414 has been affected by agitation seriously. The optimal agitation rate was 200 rpm when lactose was used as the substrate and inducer in a 15 liter bioreactor. When the cellulose powder was used, the effect of agitation rate was less obvious than with lactose (Lejeune et al., 1995). The influence of dissolved oxygen (DO) concentration has been investigated for *T. reesei* Rut C-30 grown on 1% cellulose. Enzyme production level was not affected by DO of 20% or above, however, was significantly reduced at 10% DO (Schaffner, et al, 1992).

Work on *Penicillium* species for cellulase production started in 1968. G. Pettersson reported, for the first time, cellulase production from *Penicillium notatum* in 1968. He isolated the cellulases and well characterized them biochemically (Pettersson, 1968). After knowing that the *Penicillium* species produced complete cellulolytic system, Deshpande and coworkers (1983) used *P.funiculosum* cellulases for ethanol production in simultaneous saccharification and fermentation using free or immobilized *S. uvarum* cells. During 1987, more work was carried out on *P. funiculosum* by a group in National Chemical laboratyory, India in relation to their induction, catabolite repression, stability, their efficiency of sacharification, fatty acid effect on production and also characterized multifunctional cellulase components (Rao et al., 1987). The maximal carboxymethyl cellulase, filter paper (FP) cellulase and β-glucosidase activities produced by *Penicillium janthinellum* grown in a fermenter were 60, 5 and 9 U/ml, respectively. Enzymic hydrolysis of 5M NaOH-pre-treated straw, cotton and FP was 57 to 58% in 48 h at 50°C,

with glucose as the major product (Keskar, 1992). A cellulolytic wild-type strain of *Penicillium purpurogenum* produced highest endoglucanase activity with Sigmacell as carbon source and corn steep liquor as nitrogen source (FPA-0.5, endo-glucanase-29.2 and beta-gluco- 1.5 IU/ml). Wheat bran enhanced the production of endoglucanase and beta-glucosidase (FPA-0.12, endo-glu- 43, beta-glu- 2.25 IU/ml), (Steiner et al, 1994). It was found that Cellulases in *Penicillium purpurogenum* were induced by gentiobiose, acetyl cellobiosee, cellobiose octaacetate, etc.

The control of gas phase, by periodic pressure oscillation coupled with forced aeration, was developed for improvement of cellulase production by *Penicillium decumbens* in solid-state culture (SSC).At 30°C, 4 atm, and an airflow rate of 7 l/min, the maximal FPA and β-glucosidase activities reached 17.7 IU/g substrate dry matter (SDM) and 52.8 IU /g substrate dry matter, respectively (Mo et al., 2004). The production of cellulases and hemicellulases by *Penicillium pinophilum* IBT 4186, *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 was studied during well-controlled batch cultivations using various polysaccharides as carbon source (Jorgensen et al., 2005). The cellulase was also produced by *Penicillium echinulatum* using lactose. A medium containing lactose only presented low FPAs (0.1 FPU/ml). The cultures associated with cellulose and lactose and those containing cellulose only presented similar enzymatic activities (1.5 FPU/ml), (Dillon et al., 2006). Response surface methodology (RSM) is employed to optimize the medium constituents (wheat straw, peptone and minerals) and fermentation conditions (pH, rev and temperature) for cellulase production using *Penicillium waksmanii* F10-2. The predicted maximal cellulase activity is about 5.64 U/mL (Han et al., 2009).

Nitrogen Sources were optimized for Cellulase Production by *Penicillium funiculosum*, using a sequential experimental design methodology and the desirability function. The optimal concentrations of urea and yeast extract predicted by the model were 0.97 and 0.36 g/L, respectively, which were validated experimentally. By the use of the desirability function, it was possible to maximize the three main enzyme activities simultaneously, which resulted in values for FPase of 227 U/L, for CMCase of 6,917 U/L, and for β-glucosidase of 1,375 U/L. These values corresponded to increases

of 3.3-, 3.2-, and 6.7-folds, respectively, when compared to those obtained in the first experimental design vs (Maeda et al., 2010).

STRAIN IMPROVEMENT

There are three main methods of strain improvement available to increase the extra cellular production of cellulases i.e. 1) Mutagenesis and selection 2) Genome shuffling and 3) gene cloning

Mutagenesis and selection

The random mutagenesis and selection is also called as classical strain improvement or non recombinant strain improvement procedure. In this method there is a permanent alteration of one or more nucleotides at specific site along the DNA strand. Mutation may be associated with the change of the single nucleotide (point mutation), through substitution deletion or rearrangement of one or more base pairs in chromosomes. In many cases mutations are harmful but sometimes it creates the strain more adaptable to environment and improves its biocatalytic performance. This method involves the three basic stapes 1) mutagenesis of population to induce the genetic variability 2) random selection and primary screening of surviving population to find improved strain 3) actual checking of desired improvement in fermentation or by assay. The improved mutant my again act as a parent strain for further mutagenesis. This is continued until we get superior mutant. An improvement, which is achieved by subjecting the cells or spores of microbial strain to variety of chemical or physical agents called mutagens. Table 1.1 shows some examples of mutagens.

Table 1.1 Chemical and physical mutagens used fro strain improvements

Mutagen	**Role**
Physical	
X-rays, gamma rays	Single or double strand breakage of DNA
UV rays	Pyrimidines diamerization
Chemical (base analog)	
5-chlolouracil	Result in wrong pairing
5-bromouracil	Result in wrong pairing
2-aminopurines deamination agent	DNA replication error
hydroxylamine	Deamination of C
Nitrous acid	Deamination of C, A and G
Alkylating agent	
N-nitrosoguanidine	Alkylation of C and A
Ethyl methane sulfonate	Alkylation of C and A
Intercalating agent	
Ethidium bromide, acridine dyes	Intercalation between two base pairs

The efficiency of random selection also depends on the several other factor such as type of culture used (spores or conidia), mutagen dose and time of exposure, the type of damage to DNA (deletion , addition transversion , substitution of bases or breakage of DNA), frequency of mutagen treatment etc. (Parekh et al., 2000).

In case of cellulase improvement, after mutagen treatment the spores or conidia, the desired mutants were screened on solid agar plate or they are enriched in a liquid media. On solid agar plate, the zone of hydrolysis of cellulosic materials around the colony is the measure of improved mutant for cellulase production. The cellulosic materials used for selection are soluble carboxymethylcellulose (CMC), insoluble solka flock, Avicel and acid swollen cellulose.

The fungal strain improvement for cellulase production was first carried out by Mandels and Weber (1969). They screened more than 100 wild-type strains of *Trichoderma* species to identify the best cellulolytic strain and best strain called *T.viride* QM6a was selected. The mutant, QM6a, was further mutated with UV-light and

chemicals, which resulted in the isolation of mutant, QM 9414, with higher filter paper activity (Mandel, 1971). The work was continued by other people to get more improved strain such as strains M7, NG14 (Montenecourt et al, 1977) and RUT-C30 (Montenecourt et al, 1979).

A mutant of *Penicillium funiculosum* was isolated using UV irradiation. This mutant showed an ability to metabolize inorganic nitrogen sources like urea and sodium nitrate both for growth and enzyme production (Joglekar et al, 1984). A number of mutant strains overproducing cellulase, β-glucosidase and xylanase enzyme were isolated from the cellulolytic fungus *Penicillium pinophilum* 87160iii by UV mutagenesis. Selection was carried out using either an agar-plate or an enrichment technique. Cellulase (filter paper-hydrolysing activity) production by some of the mutants in shake flask culture was approximately four-fold higher than the wild-type strain. Improvements in β-glucosidase production were of the order of eight-to-nine fold. The morphology of the mycelium of the mutants was quite different from that of the wild type. The mutants, for example, produced mycelium which was highly branched and thicker in cross section. In several of the mutants, synthesis of xylanase and β-glucosidase was completely derepressed in the presence of glycerol, which was a known repressor for the synthesis of these enzymes. Several mutants produced β-glucosidase enzyme which showed altered kinetics of hydrolysis in the presence of inhibitors. (Brown et al., 1987a**).** A mutant of *P. pinophilum* produced cellulase (FPA) activity of 9.8 units/ml at productivities of 137 units/L/h in submerged culture in a fermenter. β-Glucosidase levels were of the order of 35 units/ml. The productivity of cellulase by mutant NTG III/6 is comparable to that produced by the best mutant (C-30) isolated from the extensively studied *Trichoderma reesei*. Especially, the yield of β-glucosidase by *P. pinophilum* is much higher than that of *T. reesei* (Brown et al., 1987b).

The mutant Pol6 of *Penicillium occitanis* is an interesting strain that produced both cellulases and hemicellulases. When nitrate was used in Mandels and Weber's basal growth medium with a C/N ratio below 20.2, it resulted in more cellulase production than when urea or ammonium sulphate was used. Crude substrates such as wheat bran and wheat flour residues were used in combination with a local cellulose esparto grass paper pulp as an alternative nitrogen source with cellulose substrates. These combinations gave

high cellulase yields. Greatest cellulase yields and productivity were obtained by fed-batch cultivation (23 filter-paper activity units (FPU)/ml and 168 FPU/L/h), (Chaabouni et al., 1995).

Mutants of *Penicillium echinulatum* were isolated by treating conidia with hydrogen peroxide or 1,2,7,8-diepoxyoctane followed by incubating the conidia for 48 h in broth containing microcrystalline cellulose and 0.5% (w/v) aqueous to enrich the population grown in presence of 2-deoxyglucose. These germinated conidia were washed and then plated onto cellulose agar containing 1.5% (w/v) glucose. The colonies showed the fastest production of halos on cellulose hydrolysis. This approach resulted in the isolation of two new cellulase-secreting *P. echinulatum* mutants: strain 9A02S1 showing increased cellulase secretion (2 IU/ml FPA) in submerged culture in agitated flasks containing a mineral salts medium and 1% of cellulose, and strain 9A02D1, which proved more suitable for the production of cellulases in semisolid bran culture where it produced 23 IU of β-glucosidase per gram of wheat bran. (Dillon et al., 2006).

Genome shuffling

Protoplast fusion is an important approach and has been widely used in the fungal genetic modification since 1976, and it could induce DNA recombination between the two strains. Genome shuffling is established on the basis of protoplast fusion, but it is actually the recursive fusion of multiple parents with the combination of suitable screening method. Genome shuffling offers great potential for the improvement of industrially important micro-organisms through protoplast fusion. Genome shuffling is a process that could efficiently combine the advantage of multiparental crossing with the recombination of entire genomes normally with conventional breeding or through recursive protoplast fusion that greatly increases recombination, compared to the general protoplast fusion. Additionally, genome shuffling can accelerate directed evolution by facilitating recombination between members of a diversely selected population. Since this technique was first successfully used in bacterial system (Zhang et al, 2002) especially to improve acid tolerance in *Lactobacillus* sp. (Patnaik et al., 2002). It has been widely applied in improving some important phenotypes of micro-organisms, such as lipase production in *Penicillium expansum,* improvement of tylosin production in Streptomyces

fradiae, acceleration of screening and breeding of high taxol-producing *Nodulisporium sylviform,* pentachlorophenol degradation in *Sphingobium chlorophenolicum* etc. Genome shuffling accelerated combination of the advantages distributed in multiparents. Thus, it is more efficient and saves lots of energy and time consumption. Genome shuffling provides a new tool for cell and metabolic engineering and requires no sequence information or sophisticated genetic tools

Genome shuffling improves production of cellulase by *Penicillium decumbens* JU-A10. After two rounds of genome shuffling, three fusants, GS2-15, GS2-21 and GS2-22, were obtained, showing 100%, 109% and 94% increase in FPase activity than JU-A10 respectively. The cellulase production of the fusants on various substrates, such as corn stover, wheat straw, bagasse and the corncob residue, was studied. The maximum productivities of GS2-15, GS2-21 and GS2-22 were 92·15, 102·63 and 92·35 FPU l^{-1} h^{-1} on the corncob residue at 44 h respectively, which were 117%, 142% and 118% higher than that of JU-A10 (42·44 FPU l^{-1} h^{-1}, at 90 h). The improvements of cellulase production in the fusants could be due to their enhanced growth rates, earlier cellulase synthesis and higher secretion of extracellular proteins (Cheng et al, 2009).

rDNA /gene cloning

Using recombinant DNA technology, cloning the genes encoding the enzymes and heterologously expressing them in commonly used industrial strains has become a common practice. Such heterologous expression has become a powerful tool to improve yields and titers of enzymes. To develop robust fungal strain producing enhanced levels of cellulases, many fungal cellulases were cloned and expressed which produce higher titers of cellulase activities. β-glucosidase from *Taleromyces emersonii* was expressed in *T. reesei* RUT-C30 using strong cbh1 promotor which resulted in expression of highly thermostable β-glucosidase with high specific activity (Murray et al., 2004). Zhang et al (2010) have developed *T. reesei* strain by overexpression of β-glucosidase gene under the control of cellobiohydrolase 1 promotor. The resultant recombinants produce high levels of β-glucosidase and filter paper activities. Similarly, cellobiohydrolase I & II were over-expressed using additional copies of the the genes cloned under cbh1 promotor. This resulted in 1.5 fold increase in cellobiohydrolase I activity and 4 fold increase in

cellobiohydrolase II expression (Miettinen-Oinonen et al., 2005). In addition, chimeric proteins have also been developed, for example, the endoglucanase from *Acidothermus cellulolyticus* was fused with *T. reesei* cellobiohydrolase and expressed in *T. reesei*. This bifunctional cellulase (endo and exo acting) has been demonstrated to improve the saccharification yields (Bower et al., 2005). *Penicillium echinulatum* is effective for bioconversion processes. However, nothing is known about the molecular biology of its cellulolytic system. Rubini et al described, for the first time, the isolation, cloning and expression of a *P. echinulatum* cellulase cDNA (Pe-egl1) encoding a putative endoglucanase (Rubini et al, 2010).

Development of effective technologies based on biomass feedstock is challenging. Degradation of cellulosic substrates requires enzymes which hydrolyze completely these substrates to their respective monomers. This is possible only when new strains with high cellulase activity profiles will be developed using system biology, recombinant DNA technology, synthetic biology and metabolic engineering approaches. These strains/enzymes must be robust enough to tolerate extreme conditions employed during cellulose hydrolysis which may reduce the further down-streaming cost. There are 99% of the microbes that are uncultivable and hence remain untapped for their potential applications (Singh 2010). These untapped sources can be exploited for isolating efficient cellulase producers with desirable properties. This can be possible using metagenomic approach which is considered to be the most viable method to search for desirable enzymes such as cellulases.

APPLICATION OF CELLULASES

Cellulases have attracted great attention due to their variety of applications in various industries such as textile industry, detergent industry, food industry, deinking and for production of commodity chemicals such as ethanol, lactic acid , succinic acid and also biobutanol. Some of the applications are reported here.Cellulases are widely used in several industries, few applications are reported here (Bhat, 2000; Sukumaran et al, 2005; 2010; Soni et al., 2008)

Textile industry

Enzymes are the environment friendly alternative to the harsh chemicals. Cellulases have replaced the chemicals used in fabric finishing in textile industry. The successful application of cellulases in textile started with the process called ' Biostoning '. In this process, the cellulase enzyme is used in place of pumice stone to produce characteristic abraded, faded appearance of jeans. The cellulases break bonds between cotton fibres and insoluble, surface adhering particles of indigo pigment to produce the necessary faded effect. The enzyme process is more attractive as it overcomes several limitations of traditional process. Even small amount of enzyme dose could replace several kilogrammes of stones in the washing machines that indirectly help in higher processing capacities as well as high productivity. The cellulase treatment is fast and also avoids damage due to mechanical action of stones both on the garments and washing machines. Among the cellulases, the endoglucanases are important in the process of 'Biostoning' Cellulases that are active at neutral to alkaline pH range are in demands for ' Biostoning '. New cellulases from fungi Acrophialophora nainiana and Penicillium echinulatum were used in finishing of knitted cotton fabrics (Rau et al., 2009).

Cellulases are also useful in polishing of jute and its different blends. A hairy ball of fuzz protruding out of yarn is called as pill in textile trade. These pills present serious quality problem because they result in unattractive knotty fabric appearance. During the biopolishing process, cellulase cleaves the fibre ends protruding out from the fabric. The process is generally carried out in the presence of anionic or cationic detergents and different fabric softeners are also used during the process.

Detergent Industry

Generally the enzymes such as proteases, amylases, lipases are used as detergent additives. The incorporation of cellulases as detergent components improves washing performance of detergents. It is known that washing of cotton containing fabrics by normal detergents generally causes a pronounced unpleasant harshness to the fabric. Along with dirt removal and colour clarification, cellulase treatment also helps as fabric softener by reducing harshness of the cotton containing fabrics. As compared to fungal cellulases, bacterial cellulases can be more important from commercial point of view.

Many types of cellulases derived from fungi have pH optimum around 5. As the pH increases above 7, their activity is greatly reduced. Usually the pH of the detergent solution is 7.0-10.5. So the cellulases those are active at alkaline are important in this aspect.

Pulp and paper industries

The mechanical process to improve paper strength by enhancing fibrillation and inter fibre bonding is called beating or refining of pulp. Cellulases can be used for 'Biobeating' or Pulp refining. During recycling of mixed office waste papers, the deinking with cellulases play a key role in releasing thermally fused toners from fibre surfaces Alkaline active cellulases that are effective between pH 8-10 are preferred for this process. The exact role of cellulases in the deinking is still not clear. In the present studies it is observed that the alkaline active cellulases from alkalotolerant *Fusarium* sp. are useful for deinking of waste paper. The low molecular weight endoglucanase from alkalotolerant *Fusarium* sp. is more random in its action and has more access to the surfaces of cellulose fibres. The randomly acting endoglucanases are responsible for overall deinking process. When cellulases are used, the release of the ink suspension is generally attributed to the cellulose hydrolysis on the fiber/ink interbonding regions, which facilitates ink detachment. Fungal cellulases from Aspergillus sp., Aspergillus terreus and Myceliophthora fergusii showed significant deinking of composite waste paper as well as improve properties of recycled paper sheets (Soni et al., 2008). Cellulase C and cellulase H of Aspergillus niger were for deinking of laser printed waste paper at laboratory scale (Lee et al., 2006). Three commercial cellulase preparations were evaluated for deinking of both fresh and artificially aged old newsprint mixed with fresh old magazine. The results obtained showed that all three cellulase preparations were able to deink old news print and fresh old magazines (Zhang et al., 2008). Combined deinking technology using ultrasonic, UV-irradiation and enzymatic methods was developed by Zhenying et al. (2009) which was effective in deinking of HP laser printed paper.

Food industries

The various applications of cellulase in food industries are (1) for the improvement of the extraction process of fruit juices and oils from seeds, (2) in the clarification of fruit juices, (3) for better soaking efficiencies and homogeneous water absorption of cereals, (4) in removal of external soybean coat in production of fermented soybean foods such as Soya sauce, (5) in the isolation of proteins from soyabean and coconut, (6) for efficient isolation of starch from corn and sweet potato, (7) for the gelatinization of seaweeds to digest ball-milled lignocellulose which can be utilized as food additives.

Production of commodity chemicals

The production of commodity chemicals from lignocellulosic biomass using cellulases has been well reviewed (Shanmugan and Ingram, 2008; Dellomonaco et al., 2010; Adsul et al., 2011). Most of the commercial cellulase preparations are blends of biomass degrading enzymes produced by suitable microorganisms. These preparations are derived from strains of *Trichoderma, Aspergillus* species. There are other relevant microbes such as *Humicola, Talaromyces, Thermoascus, Bacillus* and *Penicillium* which produce cellulase enzymes. The economic viability of biomass derived chemicals depends on the enzyme cost, which triggered the search for high cellulase producing organisms, development of hypercellulolytic strains using classical mutagenesis, genetic engineering, enzyme engineering using advanced biotechnological techniques like directed evolution and rational design studies. These improved enzyme preparations are expected to possess desirable properties such as higher catalytic efficiencies, increased stabilities at elevated temperatures and higher tolerance to end product inhibition (Zhang et al., 2006). The improved low cost cellulase preparations for biomass conversion was developed in 2003 by collaborative efforts between NREL, USA, Genencore and Novozymes. This preparation succeeded in lowering the cost of enzymes to US$ 0.30 per gallon of ethanol produced from biomass. Considering the most optimistic scenario, enzyme cost has to be decreased to about 4 fold to meet the challenge of biomass ethanol production. Another approach to decrease the cellulase cost is “in house” enzyme production, which has been chosen by IOGEN company, Canada and Verenium Corporation, US. Due to importance of cellulosic ethanol, the cellulase market in US has

been estimated to be about US$ 400 million per year with an increase of 100% market by 2014.

Both simultaneous saccharification and fermentation (SSAF) and associated saccharification and fermentation (ASF) processes were evaluated for fermentation of biomass derived sugar syrups. The pretreatment followed by hydrolysis produces hexose and pentose sugars. Hexose fermentation is very straightforward with production of ethanol (yeast and *Zymomonas* strains), butanol (*Clostridium acetobutylicum*), lactic acid (*Lactobacillus* strains), succinic acid (*Actinobacillus succinogenes*). However, pentose fermentation poses a challenge. Lignocellulosic biomass requires intensive pretreatment and hydrolysis, which yields complex mixtures of sugars. For cost effective and efficient industrial processes, complete and fast conversion of all sugars in lignocellulosic hydrolysates is very important.

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Chapter 2

Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production

SUMMARY

The strain of *Penicillium janthinellum* NCIM 1171 was subjected to mutation involving treatment of Ethyl Methyl Sulfonate (EMS) for 24 h followed by UV-irradiation for 3 min. Successive mutants showed enhanced cellulase production (EMS-UV-8), clearance zone on Avicel containing plate (SM2) and rapid growth on Walseth cellulose agar plates containing 0.2% 2-deoxy-D-glucose (SM3). These mutants were transferred to Walseth cellulose plates containing higher concentration (1.5%) of 2-deoxy-D-glucose (SM4) in which only five mutants showed clearance zone on SM4. All these mutants showed approximately two-fold increase in activity of both FPase and CMCase in shake flask culture when grown on basal medium containing CP-123 (1%) and wheat bran (2.5%). The enzyme preparations from these mutants were used to hydrolyze Avicel. Higher hydrolysis yields of Avicel were obtained with enzyme preparations of EU1. This is the first report on the isolation and selection of mutants based on hydrolysis of Avicel, which is the most crystalline substrate.

INTRODUCTION

Lignocelluloses are abundant source of renewable biopolymer in the world, which is continually replenished by photosynthetic reduction of carbon dioxide with the help of sunlight as energy source (Fan et al., 1987). Degradation of this biopolymer to monomeric sugars has received a great importance, since sugars produced can serve as raw materials to produce energy, food and chemicals (Soloman et al., 2000). In fact, all lingo-cellulosic material can be converted to the products of commercial interest such as ethanol (Lawford and Rousseau, 2003), lactic acid (EI-Hawary et al., 2003), and single cell protein (Soloman et al., 2000). Cellulase enzymes have been reported for the bioconversion of lingo-cellulosic to useful products (Fan et al., 1987; Kansoh et al., 1999). The bioconversion of cellulosic materials has been receiving a great attention in recent years. Development of large-scale bioconversion process would alleviate shortages of food and animal feeds, solve modern waste disposal problems and also diminish the dependence on fossil fuels by providing an energy source in the form of glucose.

The production of cellulase is a major factor in the hydrolysis of cellulosic materials. Hence it is essential to make the process economically viable. Much work has been done on the production of cellulase from lignocellulosic materials (Depaula et al., 1999; Kansoh et al., 1999; Soloman et al., 2000). Among the cellulolytic fungi, *Trichoderma* and *Aspergillus* have been extensively studied particularly due to their ability to secrete cellulose-degrading enzymes. These strains produce extra-cellular endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.74) and β-glucosidase (EC 3.2.1.21), which act synergistically during conversion of cellulose to glucose (Eveleigh, 1987). *Aspergilli* are superior to several other fungal organisms with respect to β-glucosidase production. We have reported the hyper-production of β-glucosidase (Gokhale et al., 1984) and β-xylosidase (Gokhale et al., 1986) by *Aspergillus niger* NCIM 1207 isolated in our laboratory.

The strains have been subjected to mutagenesis and genetic modifications to obtain improved strains capable of producing high levels of cellulases (Anwar et al., 1996; Szengyel et al., 2000; Liming and Xueliang, 2004; Chand et al., 2005; Skomarovsky et al., 2005). Although these efforts were made with an objective to produce high levels of cellulase in order to degrade waste cellulose, no commercially

efficient enzyme complex has been produced. Also, the high cost of cellulase production hinders the use of enzymes at industrial scale for the production of soluble sugars. Additionally, *Trichoderma* cellulase is deficient in cellobiase, causing accumulation of cellobiose, which results in repression and end product inhibition of the enzymes. Thus, the conversion of waste cellulose to glucose is not yet commercially feasible.

Earlier Gokhale et al (1988) reported the isolation of mutants of *A. niger* NCIM 1207 capable of producing high levels of β-glucosidase. A strain of *Penicillium janthinellum* NCIM 1171 produced all enzymes, except CMCase with higher productivities than *Trichoderma viride* NCIM 1051 in a medium containing treated bagasse sample (Adsul et al., 2004). The present work describes the isolation of mutants of *P. Janthinellum* NCIM 1171 capable of producing enhanced levels of cellulases. The comparative enzyme production by some of these mutants and the wild type in shake flasks is also described.

MATERIAL AND METHODS

Chemicals

Cellulose-123 powder (CP-123) was obtained from Carl Schleicher and schull co. Dassel, FRG. *p*-Nitrophenyl β-Dglucopyranoside (*p*NPG), carboxymethylcellulose (CMC), xylan (oat spelts), 3,5-dinitrosalysilic acid, methane sulfonic acid ethyl ester (ethyl methyl sulfonate), 2-deoxy-Dglucose were obtained from Sigma-Aldrich co. St. Louis, USA. Avicel PH 101 was obtained from Fluka AG, Switzerland. Triton X-100 from Qualigens fine chemicals, Mumbai, India. Phosphoric acid swollen cellulose (Walseth cellulose) was prepared according to the method described by Walseth (1952). Yeast extract and peptone were from Hi-Media, Mumbai, India. All the other chemicals were of analytical grade and were obtained locally.

Microorganism and culture media

P. janthinellum NCIM 1171 was obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune, India. The wild type and the mutants were maintained on Potato Dextrose Agar (PDA) and sub-cultured once in every three months. PDA contained (g/l): extract from 200 g of potatoes; glucose 20;

yeast extract 1; and agar 20. Basal medium (BM) described by Mandels and Weber (1969) and *Aspergillus* minimal medium (AMM) (Gokhale et al., 1988) were used as a fermentation medium for the enzyme production. The selection media was the basal medium with (%) 0.5 Triton X-100, 2 agar and 0.5 phosphoric acid swollen cellulose (SM1) or 0.5 Avicel (SM2), 0.5% CMC with 0.2% 2-deoxy-D-glucose (SM3) or 0.5 phosphoric acid swollen cellulose with 1.5% 2-deoxy-D-glucose (SM4).

Enzyme production in shake flask

Shake flask experiments were carried out in 250 ml Erlenmeyer flask with 70 ml of fermentation medium containing respective amount of substrates. The flasks were inoculated with spores (approximately 10^7) from 15-days old culture grown on PDA slant and incubated at 30^0C with shaking at 180 rpm. The samples were removed at various time intervals and centrifuged at 3000 rpm for 10 min. The supernatant was analyzed for extra cellular enzyme activities and soluble protein.

Enzyme assay

Filter paper cellulase (FPase), endoglucanase, xylanase and β-glucosidase activities were determined as reported earlier (Gokhale et al., 1988). Filter paper activity was assayed by incubating the suitable diluted enzyme (0.1 ml) with 1.9 ml citrate buffer (50 mM, pH 4.5) containing filter paper Whatman no. 1 (50 mg, 1 · 6 cm). The reaction mixture was incubated at 50^0C for 60 min. Endoglucanase (CMCase, Endo-1, 4-β-D-glucanase; EC 3.2.1.4) activity was carried out in the total reaction mixture of 1 ml containing 0.5 ml of suitably diluted enzyme and 0.5 ml of 1% (w/v) CMC solution in citrate buffer (50 mM, pH 4.5). This mixture was incubated at 50^0C for 30 min. Xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) activity was determined under similar conditions as described above, except that 1% xylan solution was used as substrate in place of CMC. β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) activity was estimated using *p*NPG as substrate. The total of assay mixture (1 ml) consisting of 0.9 ml of pNPG (1 mg/ml) and 0.1 ml of suitably diluted enzyme was incubated at 50^0C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm after developing the colour with 2 ml of sodium carbonate (2%). One unit (IU) of enzyme activity was defined as the

amount of enzyme required to liberate 1 µmol of glucose, xylose or *p*-nitrophenol from the appropriate substrates/ min of crude filtrate under the assay conditions.

Analytical methods

Mutants were grown in basal medium with 1% CP-123 for biomass determination. Protein was estimated according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. The reducing sugar was estimated as either xylose or glucose equivalent by dinitrosalicylic acid (DNS) method (Fischer and Stein, 1961).

Isolation and selection of mutants

P. janthinellum NCIM 1171 (wild strain) was grown on PDA for 15 days at 30^0C. Conidia generated on slant are transferred into10 ml saline with 0.1% Tween-80 and count was adjusted to 10^7 spores/ml. EMS (20 mg) were added to 10 ml suspension containing 10^8 spores. This suspension was kept at room temperature for 24 h followed by UV irradiation for 3 min (11 cm apart from lamp), which resulted in 99% killing of spores. This treated suspension (100 µl) was spread on different screening plates. The plates were incubated at 37^0C temperature for 5 days and the mutants were selected on the basis of clearance zones appeared on SM1 and SM2 media. Mutants resistant to 2-deoxy-D-glucose were selected first as the fast growing colonies on SM3 medium followed by selection on SM4 medium exhibiting bigger zone of hydrolysis. The scheme of obtaining mutants is shown in the fig. 2.1.

Enzymatic hydrolysis

The enzyme preparations for hydrolysis of Avicel were derived from the growth of mutants and wild type in shake flask culture containing basal medium with CP-123 (1%) and wheat bran (2.5%) as mentioned in materials and methods above. The hydrolysis of the Avicel was carried out in 100 ml conical flask containing 50 ml citrate buffer (pH 4.5, 50 mM),2.5 g Avicel, 5 mg sodium azide and crude enzyme preparation. The enzyme preparation contains 20 units of FPase of each mutant, 560, 455, 360, 450 units of CMCase and 34, 23, 13, 100 units of β-glucosidase from EMS-UV-8, EU1,

EU2D21, parent strain, respectively. This mixture was incubated at 50^0C with shaking at 150 rpm. The samples were analyzed for the reducing sugars after suitable time intervals.

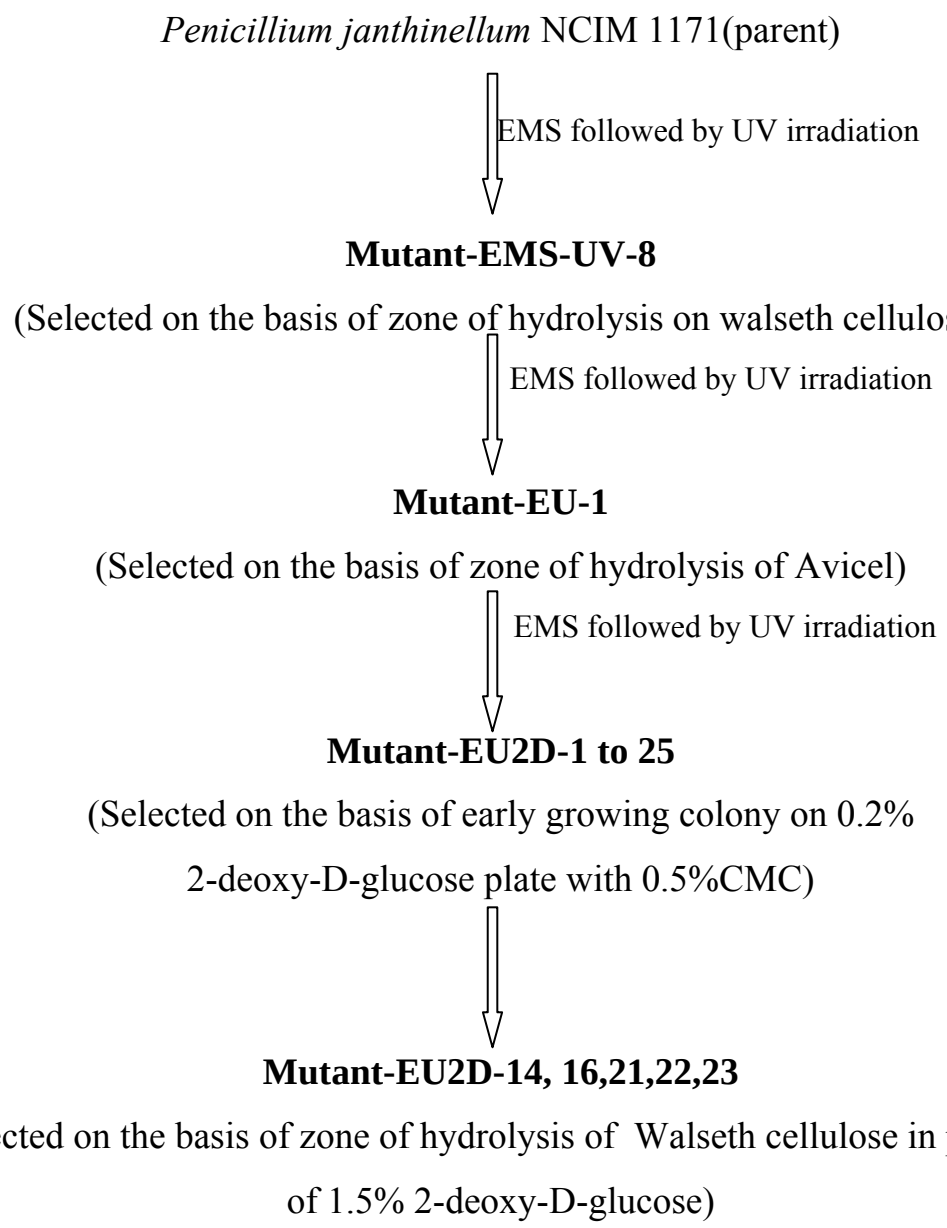

Fig. 2.1 *Diagrammatic Representation of Mutant's isolation*

RESULTS

Mutation of *P. Janthinellum*

P. janthinellum NCIM 1171 was subjected to successive mutagenic treatment with EMS followed by UV-irradiation as shown in the scheme. The screening media gave fairly reliable indication of increased cellulolytic activities. After each mutagenic treatment, enzyme production by the selected mutants was assessed in shake flask cultures and the most promising strain was further subjected to next mutagenic treatment. Mutants resistant to 2-deoxy-D-glucose were also isolated on SM4 plates containing 1.5% 2- deoxy-D-glucose.

In the first mutation experiment, 35 colonies were selected on the basis of zone of hydrolysis of Walseth cellulose in the basal medium (SM1). These colonies were again transferred to SM1 and seven mutants were selected on the basis of larger clearance zone surrounding the colony. These mutants were further assessed for cellulase and xylanase production in shake flasks containing the basal medium with 1% CP-123. One of the mutants, EMS-UV-8 showed three times higher FPase (1.2 IU/ml) and two times higher CMCase (29.8 IU/ml) activities than the parent strain (Table 2.1). However, it secretes extremely low levels of β-glucosidase activity. This mutant showed larger zone of hydrolysis on SM1 but did not show clearance zone on SM2 containing Avicel. Mutant EMS-UV-8 was further compared with parent for enzyme production using two media with different substrates or their combinations. The combination of wheat bran (2.5%) and CP-123 (1%) in basal medium was found to be better for production of both cellulases and xylanases (Table 2.1). It was also found that the enzyme yields were improved when the pH remained between 4 and 6 during fermentation. The EMS-UV- 8 mutant was further subjected to mutation and the mutant selection was based on hydrolysis of Avicel (SM2). One mutant (EU1) showing clearance zone on SM2 containing Avicel around colony was isolated (Fig.2.3), which showed poor growth on SM3 medium containing 0.2% 2- deoxy-D-glucose.

The further mutation of EU1 strain led to isolation of 25 mutants capable of growing faster on SM3 medium followed by selection on SM4 medium. Five mutants (EU2D14, EU2D16, EU2D21, EU2D22 and EU2D23) were selected which were capable

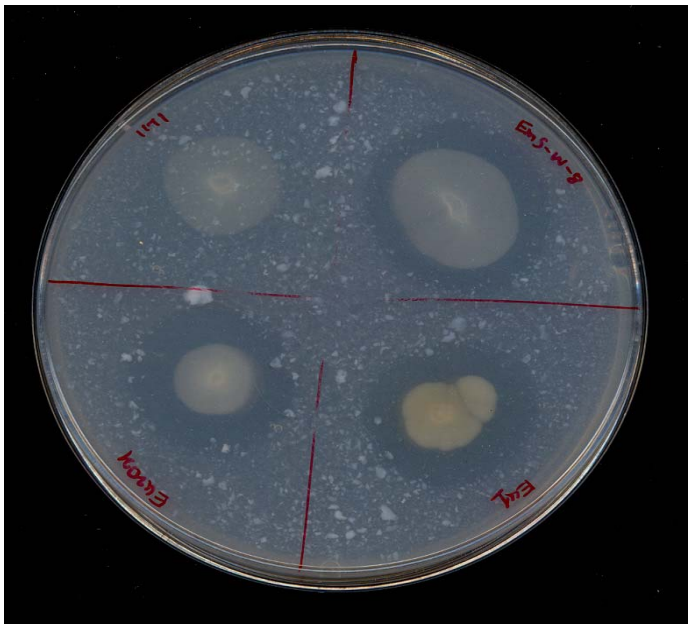

Fig. 2.2 *Mutants (EMS-UV-8, EU1, and EU2D21) and parent (NCIM 1171) shows Zone of hydrolysis on plate containing 0.5% Walseth cellulose.*

Fig 2.3 *Mutants (EMS-UV-8, EU1, and EU2D21) and parent (NCIM 1171) shows Zone of hydrolysis on plate containing 0.5% Avicel. (position of mutant is same as above)*

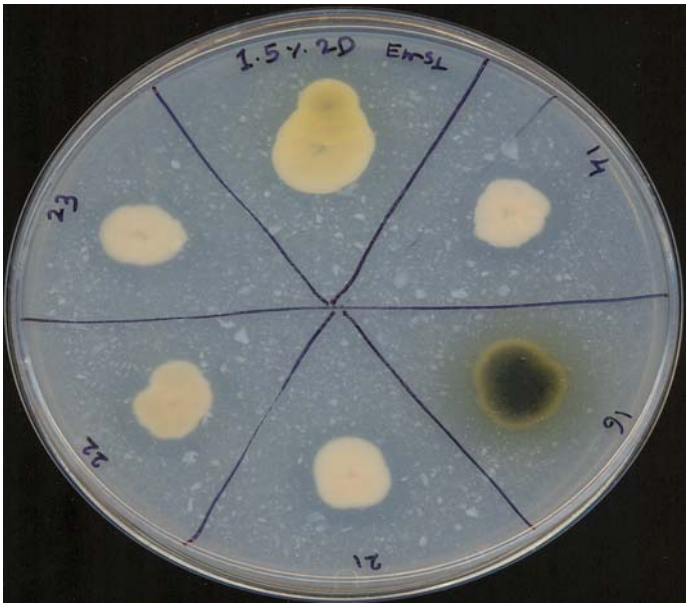

Fig 2.4 *Mutants (EMS-UV-8[EMS1], EU2D14, 16, 21, 22, 23) shows Zone of hydrolysis on plate containing 0.5% walseth cellulose with 1.5% 2-deoxy-D-glucose*

of growing and exhibiting hydrolysis zone on SM4. One of the mutants, EU2D21 gave larger clearance zone on Walseth cellulose agar medium with 1.5% 2-deoxy-D-glucose (Fig.2.4).

Production of enzymes by selected mutants

The selected mutants were evaluated for extra-cellular production of cellulases and xylanases in shake flasks containing optimized Basal medium with CP-123 (1%) and wheat bran (2.5%) as substrate (Table 2.2). All the mutants showed highest activities of FPase and CMCase. FPase activity of mutant strains was two times higher than the parent strain. No mutant produced as high β-glucosidase activity as that of parental strain. The mutant EU2D-21 produced highest FPase (3.4 IU/ml), CMCase (97 IU/ml) and xylanase activity (225 IU/ml). Unfortunately, EU1 did not give higher activities in shake flask containing basal medium with CP-123 (1%) and wheat bran (2.5%). Since it showed clearance zone on selection medium with Avicel, which is more crystalline substrate, it was further subjected to mutation as shown in the scheme.

Table. 2.1 Comparison of enzyme activities of *P. janthinellum* NCIM 1171 and its mutant cultivated in different fermentation media as described in materials and methods.

Medium + carbon source	Strain	Enzyme activities (IU/ml)				
		FPase	CMCase	Xylanase	β-Glucosidase	pH
BM + CP-123 (1%)	Parent	0.45 ± 0.027	15.0 ± 1.3	25.0 ± 2.1	0.90 ± 0.07	2.8 ± 0.1
	EMS-UV-8	1.20 ± 0.1	30.0 ± 2.4	21.5 ± 1.7	0.06 ± 0.01	3.0 ± 0.2
AMM + CP-123 (1%)	Parent	0.15 ± 0.01	3.6 ± 0.3	7.0 ± 0.5	0.45 ± 0.03	7.5 ± 0.3
	EMS-UV-8	2.30 ± 0.2	55.0 ± 4.8	30.0 ± 2.5	1.70 ± 0.14	6.0 ± 0.2
BM + CP-123 (2.5%)	Parent	0.40 ± 0.03	8.6 ± 0.7	11.0 ± 0.9	0.24 ± 0.02	2.7 ± 0.2
	EMS-UV-8	0.27 ± 0.02	8.3 ± 0.6	1.10 ± 0.1	0.09 ± 0.01	3.0 ± 0.2
AMM + CP-123 (2.5%)	Parent	0.26 ± 0.02	15.3 ± 1.3	18.5 ± 1.6	0.52 ± 0.04	5.6 ± 0.3
	EMS-UV-8	0.73 ± 0.04	30.0 ± 2.7	9.2 ± 0.7	0.83 ± 0.05	6.5 ± 0.2
AMM + CP-123 (1%) +WB (2.5%)	Parent	ND	ND	ND	ND	8.0 ± 0.3
	EMS-UV-8	1.60 ± 0.13	32.5 ± 2.8	27.5 ± 2.4	0.90 ± 0.07	7.5 ± 0.2
BM + CP-123 (1%) +WB (2.5%)	Parent	1.50 ± 0.13	38.5 ± 3.1	168 ± 13.0	7.0 ± 0.6	5.0 ± 0.2
	EMS-UV-8	3.20 ± 0.25	80.0 ± 6.8	160 ± 12.0	6.0 ± 0.4	3.5 ± 0.1
BM (2x) + CP-123 (1%) + WB (2.5%)	Parent	1.60 ± 0.1	42.0 ± 2.4	64.2 ± 5.1	8.0 ± 0.6	5.0 ± 0.3
	EMS-UV-8	4.0 ± 0.35	88.5 ± 4.8	120 ± 9.5	5.5 ± 0.4	4.0 ± 0.2

The values of enzyme activities given in table are the average of three independent experiments.

Table 2.2 Enzyme activities of all selected mutants grown in shake flasks

Strain	Enzyme activities (IU/ml)			
	FPAse	CMCase	Xylanase	β-Glucosidase
parent	1.5 ± 0.12	38.5 ± 1.8	168.0 ± 7.5	7.0 ± 0.5
EMS-UV-8	3.2 ± 0.25	80.0 ± 3.7	160.0 ± 6.8	6.0 ± 0.4
EU-1	2.0 ± 0.15	60.3 ± 3.0	96.1 ± 3.7	2.3 ± 0.2
EU2D-14	3.0 ± 0.20	64.5 ± 3.8	141.0 ± 7.5	6.1 ± 0.3
EU2D-16	2.7 ± 0.17	55.6 ± 2.0	79.0 ± 6.0	3.8 ± 0.2
EU2D-21	3.4 ± 0.20	97.0 ± 5.0	225.0 ± 15.0	3.8 ± 0.2
EU2D-22	3.0 ± 0.15	81.3 ± 3.6	121.5 ± 6.4	4.6 ± 0.3
EU2D-23	3.2 ± 0.18	82.6 ± 6.2	181.5 ± 9.6	4.4 ± 0.3

The cultures were grown in basal medium containing CP-123 (1%) and wheat bran (2.5%) as described in materials and methods. The values of enzyme activities given in table are the average of three independent experiments with 4–8% variations.

The time course of enzyme production by mutants and the wild strain in shake flask culture is shown Fig. 2.1(a)–(c). Compared to the wild strain, all mutants were found to secrete more FPase and CMCase at all intervals of time with concomitant increase in protein levels also. However, there was no increase in biomass production by mutant strains compared to wild strain (4.2–4.5 g/l, dry weight). Mutants EMS-UV-8 and EU2D-21 produced two times more extra-cellular protein than the wild strain. Both the mutants secreted maximum FPase, CMCase and β-glucosidase activities on 10th day of fermentation. The pH of the fermentation medium remained acidic (pH < 5.0) in case of mutants up to 8 days. Maximum xylanase activity was found on 4th day followed by abrupt decline in activity after 6th day.

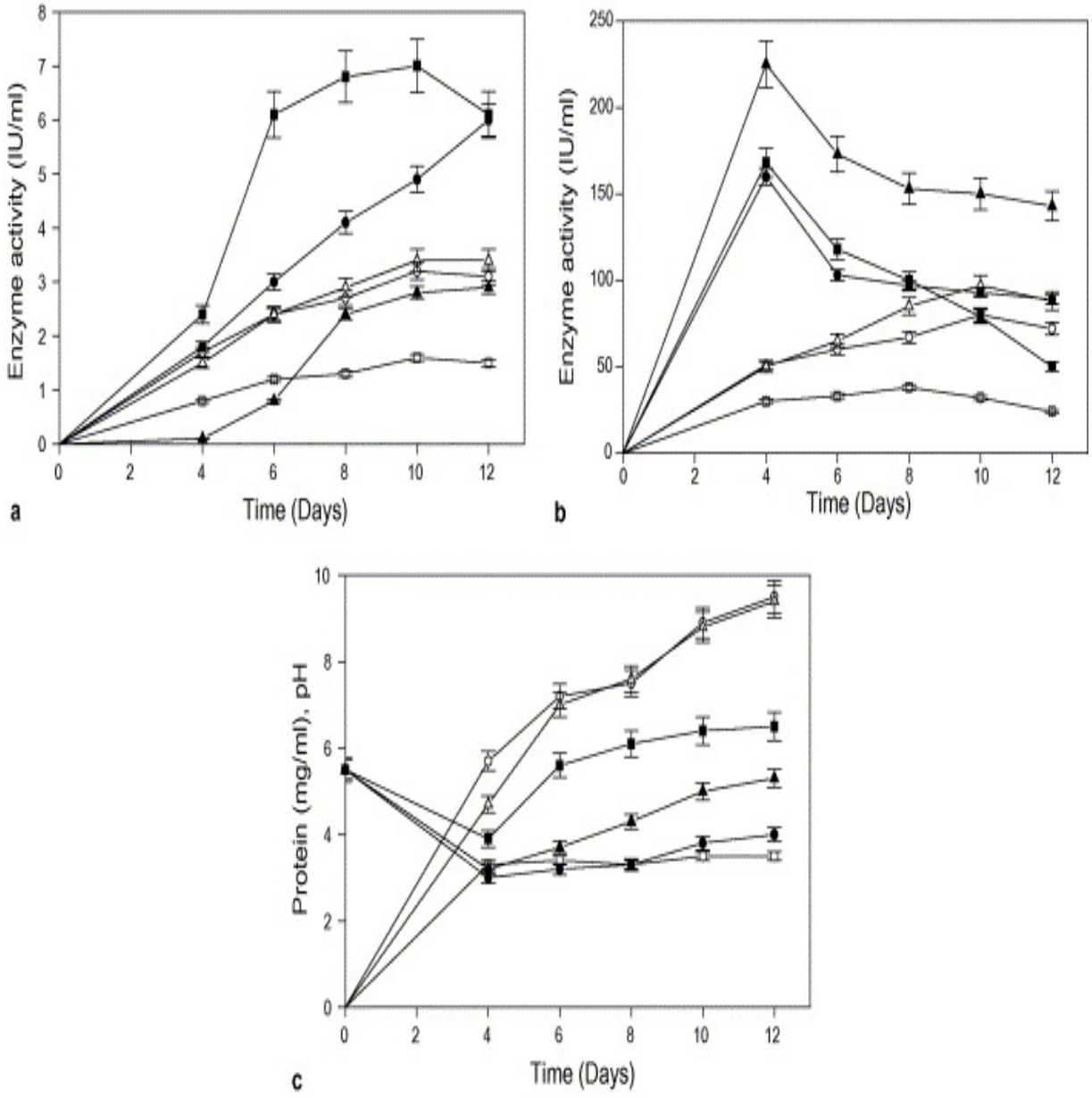

Fig. 2.5*. Profile of enzyme activities, extra cellular protein and pH during growth of strains on basal medium containing CP-123 (1%) and wheat bran (2.5%) in shake flask culture. (a) Fpase activity (□, Parent; ○, EMS-UV-8; Δ, EU2D-21) and β-glucosidase activity (■, Parent; ●, EMS-UV-8; ▲, EU2D-21). (b) CMCase activity (□, Parent; ○, EMS-UV-8; Δ, EU2D-21) and xylanase activity (■, Parent; ●, EMS-UV-8; ▲, EU2D-21). (c) Protein (□, Parent; ○, EMS-UV-8; Δ, EU2D-21) and pH (■, Parent; ●, EMS-UV-8; ▲, EU2D-21).*

Hydrolysis of Avicel

The enzyme preparations produced by mutants have been used to hydrolyze Avicel at 5% concentration. Yield of enzymatic hydrolysis was higher (61%) with enzyme preparations derived from mutant EU1 (Fig. 2.6). Enzyme preparations derived from mutants EU2D-21 and EMSUV-8 gave comparatively less hydrolysis yield. The increased yield of hydrolysis by EU1 mutant could be due to the fact that it was selected on the basis of Avicel hydrolysis. No other mutant showed clearance zone as large as shown by EU1 on SM2 containing Avicel. The Avicel hydrolysis was lowest using parent enzyme preparation even though it contained higher CMCase and β-glucosidase activities than the mutant preparations used.

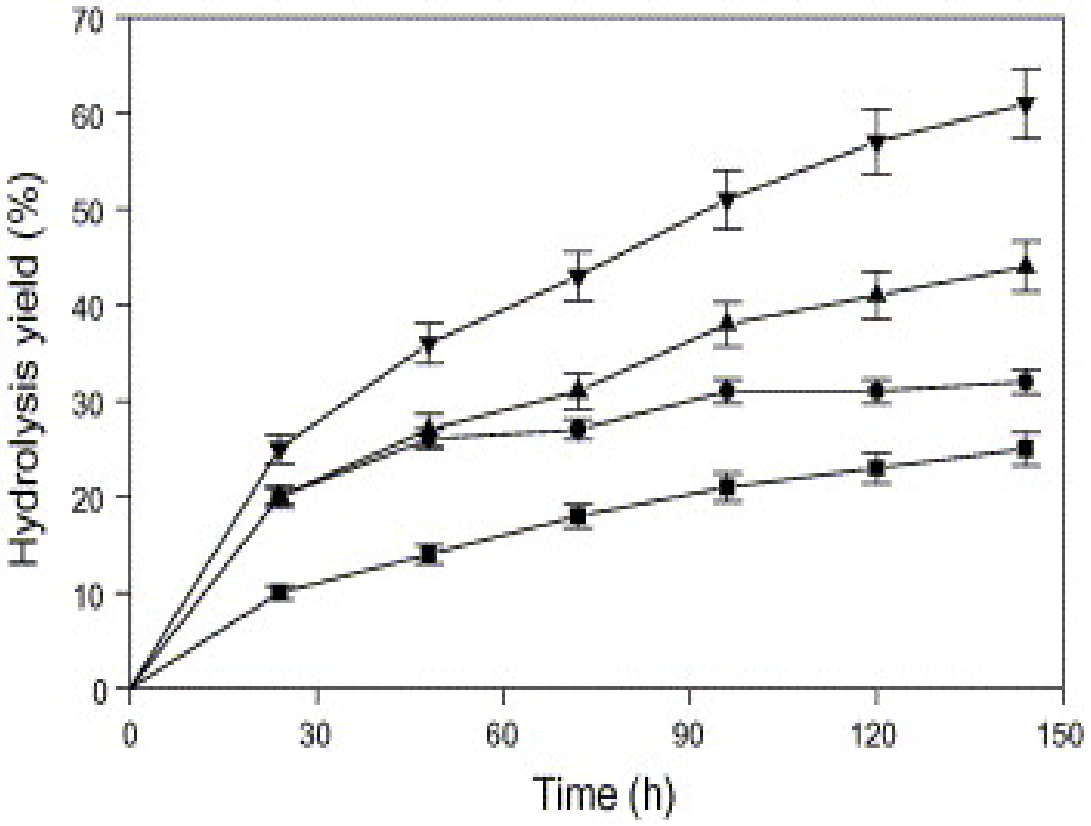

Fig. 2.6 *The hydrolysis of Avicel (5%) using enzyme preparations of Parent (■), EMS-UV-8 (●), EU2D-21 (▲), EU1 (▼). (20 FPase units from each mutant).*

DISCUSSION

The enzymatic degradation of cellulosic materials by fungal enzyme systems has been suggested as feasible alternative to produce fermentable sugars and fuel ethanol from lignocellulosics. (Oksanen et al., 2000; Shin et al., 2000). Many fungal strains have been improved using extensive mutagenesis because of their ability to produce high levels of cellulose degrading enzymes. *Trichoderma reesei* and *T. viride* have been most extensively studied for their ability to produce extracellular cellulose degrading enzymes. More than hundred wild type strains of *Trichoderma* have been screened by Mandels and Weber (1969) and the best one, *Trichoderma reesei* QM6a, was selected. Significant contributions were made further by several laboratories to improve T. Ressei strains leading to development of mutants strains such as M7. NG-14 (Montenecourt and Eveleigh, 1977) and RUT-C30 (Montenecourt and Eveleigh, 1979). These strains have been mutated and also genetically modified to obtain improved strains like RUT C 30, CL-847 and VTT-D, which have been used for the commercial production of cellulases (Mandels and Andreotti, 1978; Esterbauer et al., 1991; Szengyel et al., 2000). A wild type strain of Trichoderma atroviride was subjected to mutagenesis using N-methyl-N'-nitro-N-nitrosiguanidine (NTG) which resulted in generation of mutant, TUB F-1724. This mutant *T. atrioviride* TUB F-1724 produces high levels of cellulases and β-glucosidase (Kovacs et al., 2008). However, attempts to use these enzymatic systems from *Trichoderma* mutants for the degradation of cellulosic waste have not been successful for several reasons such as low enzymatic yields, low specific activities and end product inhibition. Most of the *Trichoderma* strains reported in the literature are deficient in the β-glucosidase production. *P. janthinellum* NCIM 1171 produces high levels of extra-cellular β-glucosidase (7.0 IU/ml) when grown in basal medium containing CP-123 and wheat bran and hence it was selected for further improvement.

Strain improvement by mutation is a traditional method used with great success for isolating mutants producing enhanced levels of enzymes. We tried to isolate the mutants by subjecting the spores of *P. janthinellum* to UV-irradiation but not a single mutant (after screening about 800 colonies) was found to exhibit enhanced clearance zone of Walseth cellulose or Avicel hydrolysis. Therefore we adopted the mutation procedure involving treatment of spores with EMS for 24 h followed UV-irradiation for 3

min where we observed 99% killing. We were successful in getting one mutant, EMS-UV-8, which exhibited larger zone of Walseth cellulose hydrolysis. Same thing was observed when strain of *Trichoderma reesei* Rut C-30 was subjected to mutation after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) for 6 h followed by UV irradiation for 15 min. Successive mutants showed two-fold enhanced cellulase production, clear hydrolysis zone and rapid growth on Avicel-containing plate (He Jun et al., 2009). We further investigated the production of cellulolytic enzymes by EMSUV-8 using two different media with different combination of substrates. It was observed that combination of CP-123 (1%) and wheat bran (2.5%) in basal medium was suitable for producing enhanced levels of FPase, CMCase activities (Table 1). The cheap raw material such as corn stover, a byproduct of agricultural maize production, has been reported to be most suitable for cellulase production (Juhasz et al., 2004, 2005).

In most studies, mutants were selected based on the resistance to 2-deoxy-D-glucose or to catabolite repression by glycerol (Gallo et al., 1978; Barredo et al., 1988; Anwar et al., 1996). Two UV induced mutants, M2-1 and M3-1 of *Trichoderma reesei* (*Hypocrea jecorina*), ATCC66589, produce cellulases in media containing both cellulose and glucose (Masakazu et al., 2010). Also mutant strain of *Trichoderma citrinoviride* produced 0.63, 3.12, 8.22 and 1.94 IU ml-1 FPase, endoglucanase, β-glucosidase and cellobiase, respectively. These levels were, respectively, 2.14, 2.10, 4.09 and 1.73 fold higher than those in parent strain. Glucose (upto 20 mM) did not repress enzyme production by the mutant under submerged fermentation conditions (Chandra et al., 2009). In the present study, mutants were isolated based on the rapid growth on Walseth cellulose containing agar plates with 0.2% 2-deoxy-D-glucose followed by selection on the basis of clearance zones appeared on SM4 medium. All these mutants were further studied for cellulase and xylanase production in the basal medium containing CP-123 (1%) and wheat bran (2.5%). One of the mutants, EU2D-21 produced highest levels of FPase, xylanase and CMCase activities. The enhanced production of cellulases and xylanases by mutants was due to the secretion of increased extra-cellular proteins. Such increased extra-cellular protein production by mutants of *Penicillium verruculosum* was correlated to enhanced cellulase production (Solov'eva et al., 2005).

The cellulase enzyme preparations of the mutants were used to hydrolyze Avicel (5%) in which 61% hydrolysis was obtained with EU1 mutant. Improved hydrolysis by EU1 preparations could be due to the fact that EU1 was selected on the basis of Avicel hydrolysis. EMS-UV-8 was selected on the basis of WC hydrolysis and hence its cellulase preparation hydrolyzed Avicel to only 30%. From these results, it could be concluded that the mutant selection based on hydrolysis of highly crystalline cellulose like Avicel is essential to get improved hydrolysis yields. This work may be meaningful in the conversion of renewable biomass to sugars, which can be fermented to commodity chemicals like ethanol and lactic acid.

CONCLUSION

Cellulose degrading fungal strains have attracted a great deal of interest as biomass degraders since they produce large amounts of extracellular lignocellulolytic enzymes. Attempts have been made by many researchers to improve the cellulolytic activity of fungal strains using traditional methods such as mutagenesis and also by recombinant techniques. Mutants were isolated by subjecting *P. Janthinellum* NCIM 1207 to mutation involving treatment of EMS followed by UV-irradiation. One of the mutants, EMS-UV-8, showed enhanced zone of Walseth cellulose hydrolysis. Successive mutation and selection resulted in isolation of two promising mutants, one selected on the basis of Avicel hydrolysis (EU1) and other on the basis of hydrolysis of Walseth cellulose in presence of 2-deoxy-D-glucose (EU2D-21). All these mutants produced two times FPase and CMCase activities than the parent strain. Enzyme preparation derived from EU1 mutant hydrolyzed Avicel to greater extent. These mutants could be the potential sources of cellulases to be used for the hydrolysis of lignocellulosic materials to produce monomeric sugars which can be converted to commodity chemicals such as ethanol or lactic acid. The next chapter deals with the production of L-lactic acid from sugarcane derived bagasse cellulose using mutants enzyme preparations and *Lactobacillus delbrueckii* mutant, Uc-3.

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Chapter 3

Cellulose from sugarcane bagasse: Enzymatic hydrolysis and lactic acid production

SUMMARY

Production of L(+)lactic acid from sugarcane bagasse cellulose, one of the abundant biomass materials available in India, was studied. The bagasse was chemically treated to obtain a purified bagasse cellulose sample, which is much more amenable to cellulase enzyme attack than bagasse itself. This sample, at high concentration (10%), was hydrolyzed by cellulase enzyme preparations (10 FPU/g of cellulose) derived from mutants generated in our own laboratory. We obtained maximum hydrolysis (72%), yielding glucose and cellobiose as the main end products. Lactic acid was produced from this bagasse cellulose sample by simultaneous saccharification and fermentation (SSF) in a media containing a cellulase enzyme preparation derived from *Penicillium janthinellum* mutant EU1 and cellobiose utilizing *Lactobacillus delbrueckii* mutant Uc-3 (Fig 3.1). A maximum lactic acid concentration of 67 g/L was produced from a concentration of 80 g/L of bagasse cellulose, the highest productivity and yield being 0.93 g/L/h and 0.83 g/g, respectively. Considering that bagasse is a waste material available in abundance, we propose to valorise this biomass to produce cellulose and then sugars, which can be fermented to products such as ethanol and lactic acid.

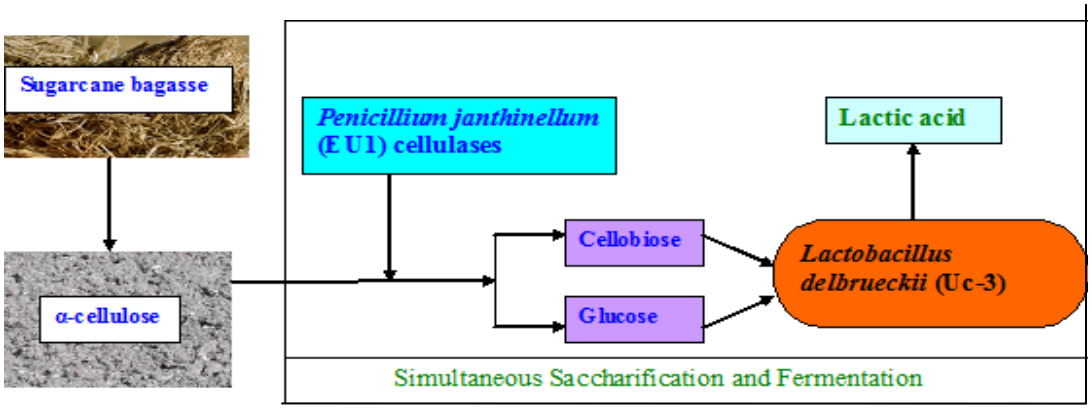


Fig 3.1 *Diagrammatic representation of overall process of SSF of bagasse cellulose to lactic acid.*

INTRODUCTION

Lactic acid and its derivatives have been widely used in food, pharmaceutical, cosmetic and industrial applications. It has a potential to become a commodity chemical for production of biodegradable polymers, oxygenated chemicals, plant growth regulators and special chemical intermediates. It has been receiving great attention as a feedstock for manufacture of polylactic acid (PLA), a biodegradable polymer used as environmentally friendly biodegradable plastic. Being highly reactive due to the presence of carboxylic and hydroxyl groups, lactic acid can undergo a variety of chemical conversions into potentially useful chemicals such as propylene oxide, propylene glycol, acrylic acid, 2,3 pentanedione and lactate esters (Datta et al., 1995; Vardarajan and Miller, 1999). Lactic acid is manufactured either chemically or by microbial fermentation. Chemical synthesis always results in racemic mixture of lactic acid, which is a major disadvantage. Microbial fermentation offers the advantage in both utilization of renewable carbohydrates and production of pure L- or D-lactic acid depending on the strain selected. The physical properties of PLA depend on the isomeric composition of lactic acid. Poly (L-lactide) (PLLA) obtained by polymerization of L-lactic acid or L-lactide has a melting temperature of 175 ^{o}C. The melting point of this polymer can be increased by blending with poly (D-lactide) (PDLA) in solvent. Recently, it was found that the polymer blend of PLLA and PDLA produces stereo-complex with melting temperature around 230 ^{o}C.[2] This finding has attracted more attention to the production of D-lactic acid.

Currently, optically pure lactic acid is produced mainly from corn starch. However, the use of agro-waste materials for lactic acid production appears to be more attractive because they do not impact the food chain for humans. Unfortunately, the process for converting cellulosic material into lactic acid is not yet feasible due to the high cost of cellulase enzymes involved in cellulose hydrolysis (Yanez et al., 2003; Wyman et al., 2005) and also to the use of fastidious organisms (Moldes et al., 2000). In addition, cellulase inhibition by glucose and cellobiose, during the hydrolysis of cellulosic material by cellulases is the main bottleneck, which remarkably slows down the rate of hydrolysis. Thus, it is advantageous to use a lactic acid producing strains that have the ability to utilize both glucose and cellobiose efficiently (Moldes et al., 2000). It

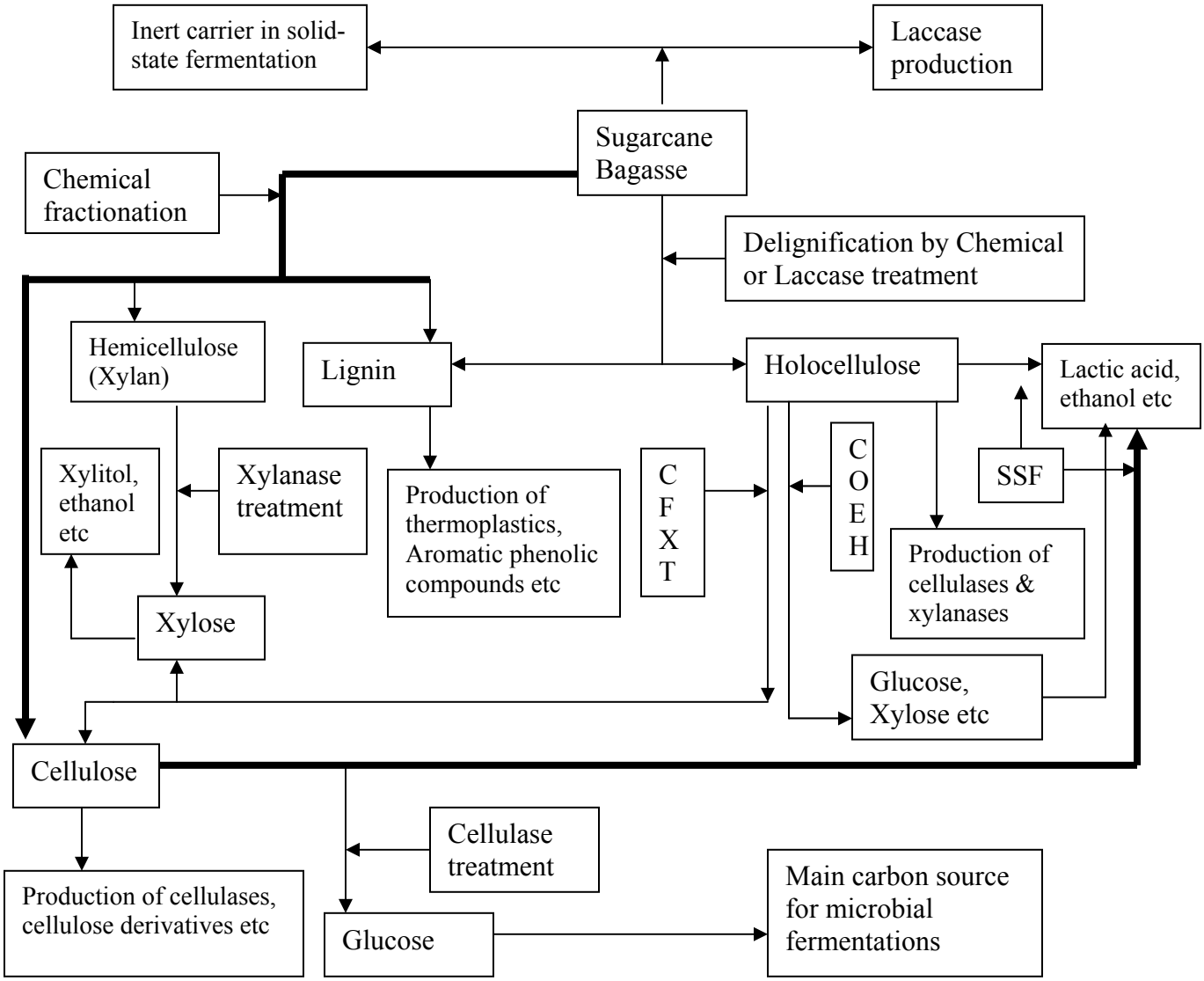
is known that some *Lactobacillus* strains utilize cellobiose as a carbon source (Carr et al., 2002) but very little information is available about lactic acid production from cellobiose. Joshi et al (2010) reported D-lactic acid production from hydrolyzed sucrose, molasses, cellobiose and from bagasse derived cellulose (Singhvi et al., 2010) by *Lactobacillus lactis* mutant RM2-24 with higher productivity.

Lactic acid is produced by oxidizing NADH generated during glycolysis with pyruvate as the electron acceptor. Two separate genes encoding two lactate dehydrogenase enzymes are present in lactic acid bacteria each producing one or other enantiomer (L+ or D-) of lactic acid. The level of purity of these two lactic acid enantiomers depends on the comparative level of expression of these two genes. *Escherichia coli* produces D(-) lactic acid with the help of D-lactate dehydrogenase present in it (Zhou et al., 2003a). A strain of *Escherichia coli* was constructed by transferring L-lactate dehydrogenase gene from *Pediococcus*, which produced L-lactic acid (Zhou et al., 2003b). Lactic acid bacteria are capable of fermenting glucose and other hexoses but lack the ability to ferment pentoses (Patel et al., 2006). For complete conversion of biomass to lactic acid, Lactic acid bacteria should have the capability to ferment pentoses. *E. Coli* strains have been constructed for the production of optically pure lactic acid by deleting the competing pathways (Zhu and Shimizu, 2004; Graber et al., 2006). These *E. coli* strains utilize all the sugars that can be derived from biomass. High sugar and lactic acid concentrations in media and low pH do not support the high growth rates. Higher lactic acid concentration is believed to disrupt membrane potential resulting in growth and fermentation arrest. Directed evolution of the biocatalysts may generate variants that tolerate high acids during fermentation (Graber et al., 2006). These variants can provide clues towards further metabolic engineering of the biocatalysts that can produce much higher concentrations of acidic products. With the advantage of metabolic engineering and directed evolution, strains of *E. coli* were developed which produce 120 g/L of L or D-lactic acid in 48 h with a yield of 98% and chiral purity of 99.9% in simple mineral salt medium (Graber et al., 2006). Genome shuffling approach also improved glucose tolerance (Yu et al., 2008) and acid tolerance in *Lactobacillus* strains (Patnaik et al., 2002; Wong et al., 2007) which produce lactic acid with high productivity at neutral pH. However, lactic acid productivities at acidic conditions are

extremely low. Fermentation of sugars to lactic acid at low pH (below 4.5) is essential to avoid the use of calcium carbonate which generates lot of calcium sulfate during acid hydrolysis to liberate free lactic acid. Such *Lactobacillus* strain capable of producing lactic acid at acidic conditions has not yet been developed. The use of such acid tolerant strains will change the entire scenario of downstream processes for lactic acid purification. Recently, lactic acid (77.0g/l) production from 100 g/l cellulose equivalent of paper sludge was reported using *Bacillus coagulans* strains. The semi-continuous saccharification and fermentation was carried out without pH control since these strains are thermophilic and acid tolerant (Budhavaram and Fan, 2009). Polylactic acid (PLA), a promising biodegradable polymer, is currently synthesized in two steps: fermentative production of lactic acid followed by chemical polymerization. Recently, attempts have been made to produce PLA homopolymer and its copolymer, Poly(3-hydroxybutyrate-*co*-lactate), by direct fermentation by metabolically engineered *E. coli* (Jung et al., 2010). The engineered *E. coli* strain was constructed by introducing heterologous metabolic pathways involving engineered propionate CoA-transferase and polyhydroxyalkanoate (PHA) synthase. This resulted in the efficient generation of lactyl-CoA and incorporation of lactyl CoA into the polymer, respectively. However, the PLA and Poly(3-hydroxybutyrate-*co*-lactate) were synthesized with low frequency in engineered *E. coli*. This strategy of combined metabolic engineering and enzyme engineering could be useful for developing other engineered organisms capable of producing different unnatural polymers by direct fermentation from biomass. Considering large production of sugarcane, India has a great potential for utilizing sugarcane bagasse for both L- and D-lactic acid production which are the key chemicals for production of several chemicals and biodegradable polymers such as PLA. The Fig.3.1 shows the different biotechnological routes of bagasse utilization for the production of value added products.

Very recently, a newly isolated strain of *Enterobacter mundtii* QU25 was reported to metabolize xylose into L-lactic acid in batch fermentation (Abdel-Rahman et al., 2011). The strain produced 87g/L of L-lactic acid from 112g/L of xylose without formation of acetic acid. This is the first report on homo-L-(+)-lactic acid production from xylose by wild type lactic acid bacteria. This could be the ideal bacterial strain for

economical production of lactic acid from renewable biomass substrates which upon hydrolysis release both glucose and xylose.



(CFXT- Cellulase free Xylanase treatment, COEH- Chemical or enzymatic hydrolysis, SSF- Simultaneous saccharification and fermentation, ▬ - Our Work).

Fig.3.1 *Diagrammatic representation of sugarcane Bagasse utilization through different routes for the production of valuable products.*

MATERAILS AND METHODS

Chemicals

Cellulose powder-123 (CP-123) was obtained from Carl Schleicher and Schull Co., Dassel, FRG. *p*-Nitrophenyl-β-Dglucopyranoside (*p*NPG), carboxymethylcellulose (CMC) 3,5- dinitrosalysilic acid were obtained from Sigma–Aldrich Co., St Louis, MO, USA. Sodium azide was obtained from S.D.Fine-Chem (India). Avicel PH-101 was obtained from Fluka Chemie GmbH. The α-cellulose with 0.18% lignin and 98% cellulose was prepared from sugarcane bagasse in our laboratory.

Preparation of sugarcane bagasse cellulose

Sugarcane bagasse was obtained from Tamil Nadu Pulp and Paper Mills, Chennai, India. This bagasse contains about 43% cellulose, 30% xylan, and 20% lignin, in addition to some silica and other constituents. It was cut into small shreds of 1–3 mm size and then pre-treated with steam and alkali by a proprietary process (under patenting) to remove the xylan, lignin, and other impurities. The final product consisted of 93.5% a-cellulose, 5.3% b-cellulose (low molecular weight cellulose and traces of hemicellulose), 1.02% c-cellulose, and 0.18% lignin.

Strain information and cellulase production

Penicillium janthinellum NCIM 1171 was obtained from NCIM Resource Center, Pune, India. Mutants (EMS-UV-8, EU1, EU2D-21) of *P. janthinellum* were generated by exposing conidia of the parent strain to UV-irradiation. The mutant EMS-UV-8 is selected on the basis of hydrolysis of phosphoric acid swollen cellulose. EU1 mutant was selected on the basis of Avicel hydrolysis and EU2D-21 is selected on the basis of phosphoric acid swollen cellulose in presence of 2-deoxyglucose (1.5%). The procedure of generation of these mutants and their crude enzyme mixtures have already been reported in Chapter 1. These cultures were maintained on potato dextrose agar (PDA) and sub-cultured once every three months. PDA contained (g/L) extract from 200 g of potatoes, glucose 20.0; yeast extract 1.0; and agar 20.0. Enzyme production was carried out in a 250 ml Erlenmeyer flask, with 70 ml of production medium containing 1% (w/v) cellulose-123 powder and 2.5% wheat bran (as given in Chapter 2). *Lactobacillus delbrueckii* mutant Uc-3 producing L(+) lactic acid with high productivity was isolated as

described earlier (Kadam et al., 2006). It was maintained in liquid MRS medium supplemented with 0.1% CaCO3.

Enzymatic hydrolysis of Avicel and sugarcane bagasse cellulose

The saccharification experiments were carried out in a 50 ml conical flask with 25 ml citrate buffer (pH 4.5, 50 mM), 1.25 g or 2.5 g of either bagasse cellulose or Avicel, 2.5 mg sodium azide and crude enzyme preparations from *P. janthinellum* NCIM 1171 or its mutants (EMS-UV-8, EU1, EU2D21). This mixture was incubated at 50^0C with shaking at 150 rpm. The samples were analyzed for the reducing sugars after suitable time intervals.

Simultaneous saccharification and fermentation (SSF)

SSF was carried out in a 250 ml screw cap conical flask with the production medium consisting of bagasse sample (10.0 g), CaCO3 (5.0 g), yeast extract (1.0 g) in 125 ml citrate buffer (pH 4.5, 50 mM). The production medium was sterilized at 121^0C for 20 min, the crude enzyme preparation was added, and *Lactobacillus delbrueckii* Uc-3 mutant cells (5%) grown in sucrose based medium (Kadam et al., 2006) were inoculated. The flasks were incubated at 42^0C with shaking at 150 rpm. The other cellulosic substrates used for SSF fermentation were Avicel PH 101, Sigmacell, CP-123 at 50g/L concentration. Bagasse derived cellulose was also used at 50g/L concentration as a control. All the SSF experiments were performed for 72 h in media containing 10 Filter paper units (FPU)/g of substrate. The initial pH of the fermentation medium was 6.0. The samples harvested at various time intervals were centrifuged at 5000 rpm for 20 min to separate the cells. The supernatant was acidified by adding an equal volume of 1 N HCl, where free acid is liberated and analyzed by HPLC for lactic acid.

Analytical methods

The reducing sugar content was estimated as the glucose equivalent by the dinitrosalicylic acid (DNS) method (Fischer and Stein, 1961). Cell growth was monitored by visible spectral peak absorbance (UV–VIS Spectrometer–117, Systronics, Mumbai, India) at a wavelength of 660 nm. The glucose, cellobiose and lactic acid in the samples were determined using a high performance liquid chromatography (HPLC) system

(Dionex India Limited) equipped with UV- or RI-detectors. An ion exclusion column (Aminex HPX-87H, Biorad, Hercules, CA) was used at a temperature of 30^0C, with 0.008 N H_2SO_4 as a mobile phase at a flow rate of 0.6 ml/min. The injection volume of the sample was 50 µl.

RESULTS AND DISCUSSION

Initially, experiments were carried out to evaluate the potential of cellulase enzyme preparations derived from mutants EMS-UV-8, EU1 and EU2D21 towards the hydrolysis of sugarcane bagasse cellulose (lignin content 0.18) and Avicel. Hydrolysis was carried out at two different substrate concentrations (5% and 10%) using 5 FPU/g and 10 FPU/g of the cellulose substrate. It was found that all mutant enzyme preparations gave higher bagasse cellulose/Avicel hydrolysis than that obtained with parent enzyme preparation (Table 3.1). However, hydrolysis of the most crystalline substrate, Avicel, was always lower than that of bagasse sample irrespective of enzyme preparations used. The mutant enzyme preparations derived from EU1 and EU2D-21 yielded maximum hydrolysis at both 5% and 10% bagasse sample. With the same preparations, the Avicel hydrolysis (at 10% concentration) was only 38%, which was better than that obtained with the parental strain (21%). The rate of hydrolysis of both Avicel and bagasse cellulose sample with the mutant enzyme was faster than with the parental enzyme in spite of very low amounts (10 times less) of β-glucosidase in the mutant enzyme preparation compared to the parent enzyme (Fig. 1). The lower hydrolysis (40%) of Avicel could be due to its microcrystalline structure, which prevents an easy access to enzymes. We got approximately 84% hydrolysis (at 5% bagasse cellulose concentration) and 72% hydrolysis (at 10% bagasse cellulose concentration) with EU1 enzyme preparation (10 FPU/g of bagasse sample).

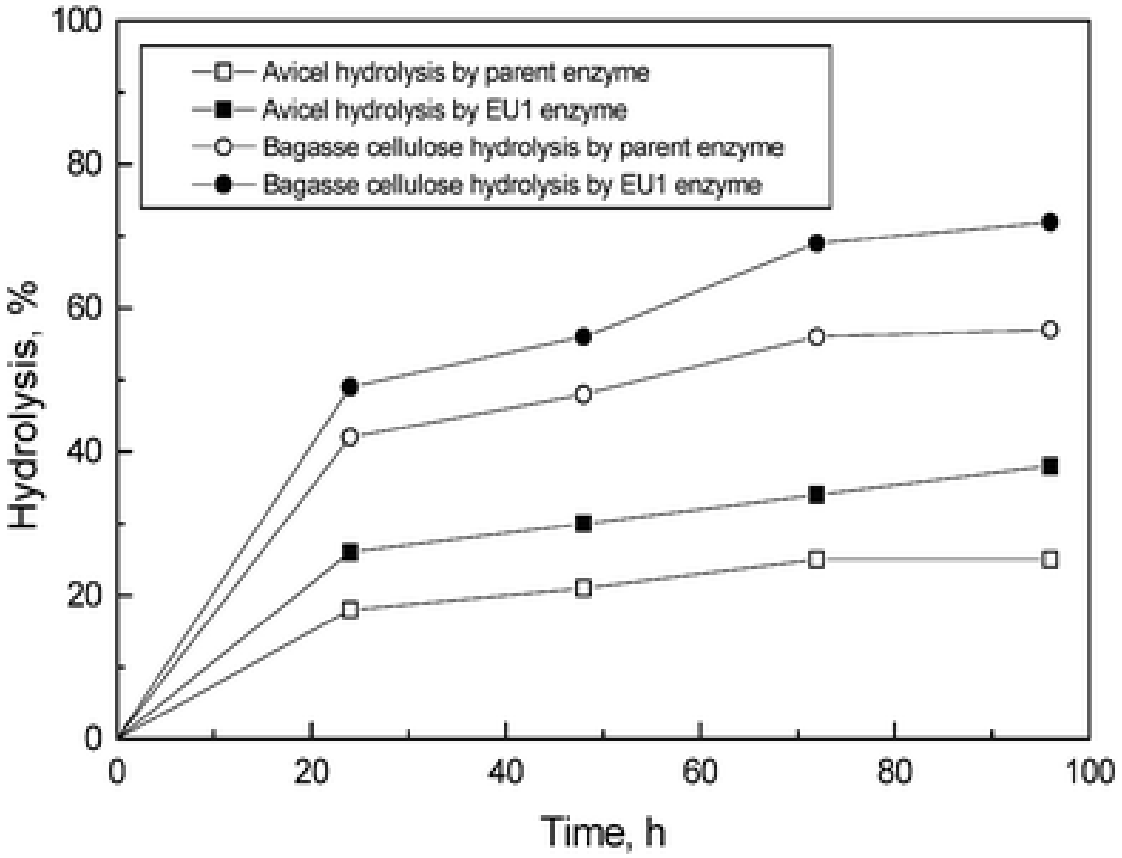


Fig. 3.1 *Profile of hydrolysis of Avicel and bagasse cellulose by parent and mutant (EU1) enzyme preparations. The hydrolysis was carried out using Avicel and sugarcane bagasse cellulose at 10% with parent and mutant enzyme preparations (10 FPU/g of substrate).*

Table 3.1 Saccharification of Avicel and sugarcane bagasse cellulose with enzyme preparations derived from parent and mutant strain

Enzyme activities/g of substrate				% Hydrolysis after 96 h			
Strains	FPU	β-glucosidase	CMCase	Avicel (5%)	Avicel (10%)	BS (5%)	BS (10%)
Parent	5.0	45.0	210	21	21	46	39
EMS-UV-8	5.0	6.5	140	32	33	48	45
EU-1	5.0	9.0	175	41	41	63	62
EU2D-21	5.0	3.8	170	39	38	52	51
Parent	10.0	90.0	420	26	25	64	57
EMS-UV-8	10.0	13.0	280	39	32	70	56
EU-1	10.0	18.0	350	46	36	84	72
EU2D-21	10.0	7.6	340	44	37	80	60

FPU—filter paper cellulase units. CMCase—carboxymethylcellulase activity. BS—sugarcane bagasse cellulose sample.

The hydrolysis of bagasse cellulose sample with parent enzyme preparation resulted in production of glucose as the only end product, probably due to the presence of very high amounts of β-glucosidase. On the other hand, the hydrolysis broth derived from the treatment of bagasse cellulose with mutant enzyme preparations contained both glucose and cellobiose as end products (Table 3.2). The amount of xylose detected was insignificant, indicating a much lower amount of hemicellulose present in sugarcane bagasse cellulose. The mutants produced high levels of glucose because they are selected on the basis of hydrolysis in the presence of 2-deoxyglucose. The presence of cellobiose in the mutant hydrolysate is due to a lower amount of β-glucosidase in the crude enzyme mixture (Table 3.1). The presence of both glucose and cellobiose in the broth may hinder the further hydrolysis to glucose because they are strong inhibitors of cellulases.

However, this drawback can be overcome by SSF methodology to produce lactic acid from bagasse cellulose sample using cellobiose utilizing microbes. Considering the inexpensive nature of bagasse samples, there is no doubt about their high potential as substrates for commercial production of glucose and further fermentation of glucose to other platform chemicals by SSF.

Table 3.2 End product analysis of saccharification of Avicel and sugarcane bagasse cellulose sample using different enzyme preparations

Substrates	Enzyme	Enzyme activity used			
		5 FPU g^{-1} substrate		10 FPU g^{-1} substrate	
		Cellobiose (mg)	Glucose (mg)	Cellobiose (mg)	Glucose (mg)
Avicel (2.5 g)	Parent	3.0	520.0	6.1	610.0
	EMS-UV-8	14.0	805.0	33.0	780.0
	EU1	33.0	1000.0	20.0	870.0
	EU2D-21	31.0	940.0	20.0	900.0
Sugarcane bagasse cellulose (2.5 g)	Parent	ND	970.0	ND	1420.0
	EMS-UV-8	35.0	1090.0	47.6	1345.0
	EU1	84.0	1460.0	48.5	1740.0
	EU2D-21	93.0	1200.0	65.0	1420.0

ND- not detected

SSF experiments were carried out under the selected conditions (42^0C and pH 6.5) because the organism used in this fermentation is a mutant of *L. delbrueckii* (Uc-3) and cannot grow at temperatures more than 42^0C or at pH less than 5.5. We carried out the SSF at pH 6.5, where the cellulases used were active, with retention of 50% activity. The mutant (Uc-3) used in this study produces lactic acid with very high productivity (Kadam et al., 2006). SSF experiments were performed in production media containing cellulases (10 FPU/g of substrate). The pH of the fermentation broth also dropped to 5.3 within 24 h (Fig. 3.2), which is the pH at which the enzymes are most active. There was

no cellobiose accumulation during the fermentation at any time. Cellobiose was either converted to glucose by β-glucosidase present in the cellulase preparations or utilized by the mutant strain to produce lactic acid. We obtained 67 g/L of lactic acid from 80 g/L of bagasse cellulose sample when we used EU1 enzyme preparations for hydrolysis. The yield (g/g) and productivity (g/L/h) of lactic acid were 0.83 and 0.93, respectively. The presence of higher cellobiose concentration could result in significant inhibition, which could be removed by supplementation of the media with additional cellobiase, leading to a remarkable improvement in lactic acid production in fed batch SSF (Moldes et al., 2001). However, in simple batch operations with cellulase from *T. reseei* and *L. delbrueckii*, supplementation of media with fresh cellobiase did not improve the overall process (Parajo et al., 1997).

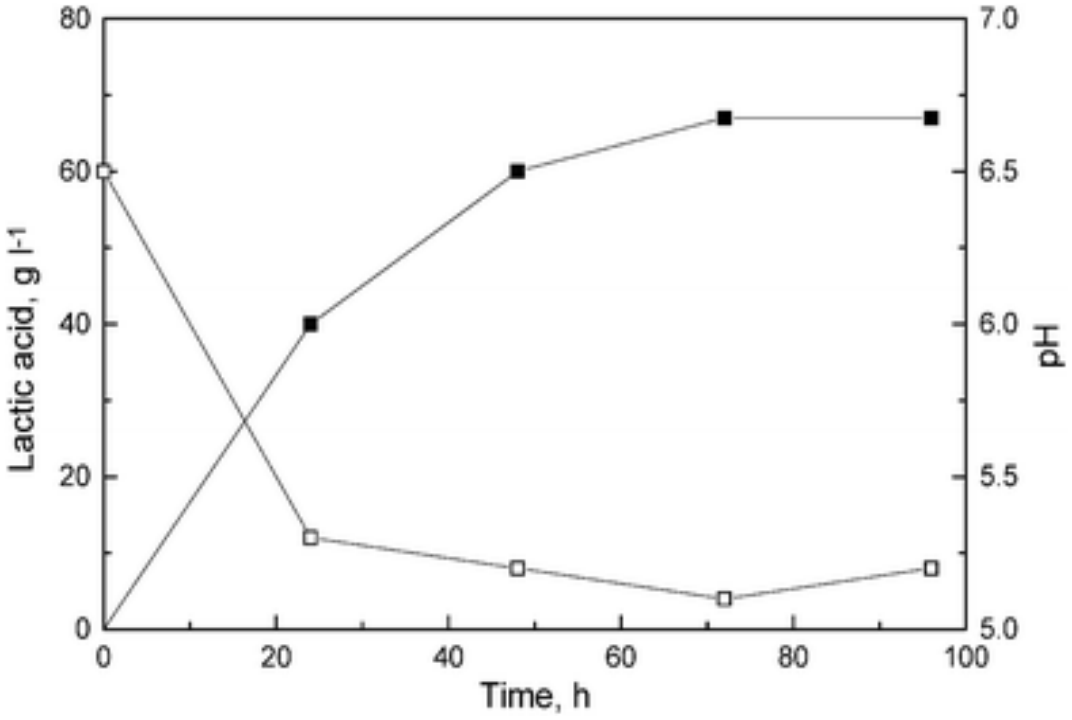


Fig. 3.2 *Course of lactic acid production (■) and pH (□) during SSF of sugarcane bagasse cellulose (80 g/L) using mutant enzyme preparation (EU1, 10FPU/g)*

We also evaluated the capability of *L. delbrueckii* mutant Uc-3 to produce lactic acid from different cellulose substrates (50 g/L) like Avicel PH 101, Sigmacell cellulose, CP-123 (Table 3.3) The amount of lactic acid produced in SSF from the cellulosic materials varied between 18 and 22 g/L after 48 h. The level of lactic acid production from bagasse cellulose was 38 g/L within 60 h of fermentation. In comparison to other reports in the literature (Table 3.4), we obtained the highest yield and productivity of lactic acid reported so far in spite of using less cellulase enzyme (10 FPU/g of the substrate) with low amounts of β-glucosidase in batch SSF. There is one report on maximum lactic acid production (108 g/L), which was achieved by combining multiple substrate addition, supplementation with fresh nutrients and enzymes and removal of lactic acid (Moldes et al., 2001). These results suggest that this *Lactobacillus delbrueckii* mutant Uc-3 has a potential for commercial production of lactic acid from biomass material.

Table 3.3 Lactic acid production from cellulosic substrate in SSF

Substrate (50 g/L)	Lactic acid (g/L)	Fermentation time (hr)
Avicel	18.5	48
Sigmacell	17.9	48
CP-123	22.9	48
Bagasse derived cellulose	38.1	60

Table 3.4 The recent data available on SSF of cellulosic material to lactic acid

Substrate (g/L)	Microorganism	Enzyme (FPU/g)	F. T.	C_{max}	$Y_{p/s}$	Q_p	Ref.
α-cellulose	*L. delbrueckii* NRRL-B445	-	-	75	62	0.34	Iyer and Lee, 1999
Defatted rice bran (100)	*L. delbrueckii* IFO 3202	Cellulase-Y-NC	36	28	28	0.77	Tanaka et al., 2006
Filter paper (33)	*L. coryniformi* ATCC 25600	Celluclast + Novozyme (28)	48	25	75	0.5	Yanez et al., 2003
Solka Floc (60)	*Bacillus sp.* Strain 36D1	Spezyme (10)	192	40	65	0.22	Patel et al., 2005
Solka Floc (20)	*B. coagulans* strain 36D1	Genecore International GC220 (10)	24	13.5	67	0.63	Patel et al., 2006
Preated cardboard (41)	*L. coryniformi* ATCC 25600	Celluclast & Novozyme (22.8)	48	23	56	0.49	Yanez et al., 2005
Paper mill sludge (23.4)	*L. paracasei*	Meicelase MCB8-6 (46)	72	17	72	0.23	Nakasaki et al., 1999
partially-hydrolyzed pulp (100)	R. oryzae UMIP 4.77	-	96	24.1	24	0.5	Vially et al., 2010
paper sludge (100)- semi SSF	Bacillus coagulan strains 36D1	-	100	92	77	0.9	Budhavaram et al., 2009
Sugarcane bagasse cellulose (80)	*L. delbrueckii* Uc-3	*P.janthinellum* EU1 (10)	72	67	83	0.93	This work

F.T.—Fermentation time (h), C_{max}—Maximum lactic acid concentration (g/L), $Y_{p/s}$—% Product yield (g/g), Q_p—Lactic acid productivity (g /L/ h).

CONCLUSION

Batch experiments were conducted for conversion of bagasse sample to lactic acid by simultaneous saccharification and fermentation using a cellulase preparation derived from a mutant of *P. janthinellum* (EU1) and *L. delbrueckii* mutant Uc-3. Saccharification experiments were also carried out using mutant enzymes to characterize the cellulose degradation. Batch SSF yielded 67 g/L of lactic acid from 80 g/L of bagasse sample with yield and productivity of 0.83 g/g of cellulose substrate and 0.93 g/L/ h. Applying SSF to lactic acid production has more advantage than SS since we could operate the SSF at conditions suitable and optimum for both cellulose hydrolysis and growth of *L. delbrueckii* mutant. The further improvements in batch SSF to make it cost effective are necessary, as this work indicates great advantages from the industrial viewpoint. The work on utilization of other biomass materials with proper pretreatment and proper integrated saccharification and fermentation processes may lead to bio-recycling of biomass to produce value added chemicals. We are currently working on improving the techno-economic efficiency of the sugarcane bagasse derived cellulose process, so as to obtain an optimum and inexpensive bagasse constitution (cellulose, xylan, and lignin) more amenable to enzyme/microbial attack and further fermentation to lactic acid and other feedstock chemicals that can take place in a facile manner.

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Chapter 4

Production of Lactic Acid from Cellobiose and Cellotriose by *Lactobacillus delbrueckii* Mutant Uc-3

SUMMARY

Lactobacillus delbrueckii mutant Uc-3 utilizes both cellobiose and cellotriose efficiently, converting it into L(+) lactic acid. The enzyme activities of cellobiose and cellotriose utilization were determined for cell extracts, whole cells, and disrupted cells. Aryl-β-glucosidase activity was detected only for whole cells and disrupted cells, suggesting that these activities are cell bound. The mutant produced 90 g/L of lactic acid from 100 g/L of cellobiose with 2.25 g/L/h productivity.

BACKGROUND

In previous chapter we performed the enzymatic hydrolysis of bagasse derived cellulose followed by simultaneous saccharification and fermentation of hydrolysate to lactic acid. During The end products of cellulose hydrolysis were glucose and cellobiose. However, in SSF we didn't find the presence of cellobiose, we wanted to know the potentiality of mutant *L. delbrueckii* Uc-3 to utilize cellobiose and convert it into lactic acid. The fermentation experiments were carried out in production medium containing cellobiose and cellotriose. We found that the mutant produced 90 g/L of lactic acid from 100 g/L of cellobiose with 2.25 g/L/h productivity. Though it is known that Lactobacillus species utilize cellobiose as carbon source, very little information is available about lactic acid production from cellobiose. We analyzed the enzyme activities present in mutant Uc-3 that are involved in cellobiose and cellotriose utilization and their localization.

INTRODUCTION

Cellulosic biomass represents an abundant natural renewable carbon resource for the production of valuable fuels and biomaterials for both short- and long-term sustainability. The production of value-added products from such renewable feedstock is a present need and perhaps economically and environmentally feasible process. Lactic acid is a commercially viable product, and world consumption of it is estimated to be more than 60,000 metric tons per year. Lactic acid has a wide range of applications in pharmaceutical, cosmetic, textile, and chemical industries (Senthuran et al., 1997; Venkatesh, 1997; Payot el al., 1999; Elezi et al., 2003). It has the potential to become a commodity chemical as feedstock for biodegradable polymers, oxygenated chemicals, plant growth regulators, environmentally friendly solvents, and special chemical intermediates.

The process for converting cellulosic material into lactic acid is yet not feasible due to the high cost of enzymes involved in cellulose hydrolysis (Wyman et al., 2005; Zhang et al., 2006a; 2006b) and also to the use of a fastidious organism (Moldes et al., 2001). The process may involve either a two-step process with complete conversion to sugar, followed by fermentation to lactic acid, or a one-step process in which the saccharification of cellulose by cellulases coupled with fermentation to eliminate the inhibition caused by glucose (Luo et al., 1997; Patel et al., 2006).

During the hydrolysis of cellulosic material by cellulases, the main bottlenecks are cellulase inhibition by glucose and cellobiose (strong inhibitors of cellobiohydrolase), which remarkably slow down the rate of hydrolysis. In simultaneous saccharification and fermentation (SSF), glucose inhibition is totally removed but cellobiose inhibition remains as it is (Caminal et al., 1985; Ramos et al., 1994; Moldes et al., 2000). The addition of β-glucosidase at the beginning of SSF is recommended for the removal of cellobiose inhibition, but sometimes it is not feasible because of rapid deactivation of enzyme (Nakasaki and Adachi, 2003). In some cases, cellobiose inhibition was removed by supplementation of the medium with additional cellobiase, leading to a remarkable improvement in lactic acid production in fed-batch SSF (Moldes et al., 2001). However, in simple-batch operations in SSF with cellulase from *Trichoderma reesei* and *Lactobacillus delbrueckii*, the supplementation of medium with fresh cellobiase did not

improve the overall process (Parajo et al., 1997). To remove these bottlenecks, it is advantageous to use a lactic acid-producing strain that has the ability to utilize both glucose and cellobiose efficiently (Moldes et al., 2000). It is known that some *Lactobacillus* species utilize cellobiose as a carbon source (Carr et al., 2002), but very little information is available about lactic acid production from cellobiose. Specifically, to our knowledge, there is no report on the utilization of cellotriose and cello-oligosaccharides by *Lactobacillus* spp.

we describe the efficient utilization of cellobiose and cellotriose by a mutant strain, *Lactobacillus delbrueckii* Uc-3, for L(+) lactic acid production. The mutant was isolated by UV mutagenesis and selected on the basis of a bigger zone of acid formation on sucrose-based medium (Kadam et al., 2006). We also report the enzyme activities present in *Lactobacillus delbrueckii* mutant Uc-3 that are involved in cellobiose and cellotriose utilization.

MATERIALS AND METHODS

Chemicals

Cellobiose, cellotriose, cellooligosaccharides, sigmacell (cellulose), obtained from Sigma (USA), Avicel PH 101 from Fluka. *p*-nitrophenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-β-D-cellobioside (*p*NPC), and *p*-nitrophenyl-β-D-galactopyranoside (*p*NPgal) are also obtained from sigma. All other chemicals used are analytical grade.

Lactic Acid Production

For the evaluation of lactic acid production from cellobiose, experiments were performed in a 250-ml, screw-cap flask at 42^0C with shaking at 150 rpm. The flask contained 100 ml production medium consisting of 10 g or 50 g cellobiose, 4.5 g CaCO3, and 1 g yeast extract. The flasks were inoculated (5% inoculum) with *Lactobacillus delbrueckii* mutant Uc-3 grown in hydrolyzed, sucrose-based medium (Kadam et al., 2006). After a suitable time interval, the samples for lactic acid and sugar were analyzed by a high-pressure liquid chromatography system equipped with UV or refractive index detectors using an Aminex HPX-87H column. Detail procedure is described in earlier chapter 3.

Separate experiments were carried out to check whether *L. delbrueckii* Uc-3 utilizes cellotriose or cellooligosaccharides as a sole carbon source. Ten milliliters of medium was prepared in 15-ml, screw-cap tubes containing 1% yeast extract, 25 mg of CaCO3, and 20 mg of cellotriose or cellooligosaccharides separately. The tubes were inoculated with a 5% inoculum grown in 100 g/liter of hydrolyzed sucrose and kept at 42^0C under stationary conditions. At 0 h, the samples were analyzed by high-pressure liquid chromatography for the initial concentrations of glucose, fructose, and lactic acid present in the inoculum.

Enzyme activity detection and assay

The cellobiose or cellotriose degrading enzymes by using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-β-D-cellobioside (*p*NPC), and *p*-nitrophenyl-β-D-galactopyranoside (*p*NPgal) as substrates, The total of assay mixture (1 ml) consisting of 0.9 ml of substrate (1 mg/ml) and 0.1 ml of suitably diluted enzyme was incubated at 50^0C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm after developing the colour with 2 ml of sodium carbonate (2%). One unit of enzyme activity is equivalent to one micromole of *p*-nitrophenol generated per minute.

To detect enzyme activities, the cells were grown in different sugars (glucose, lactose, hydrolyzed sucrose and cellobiose) at 42^0C and harvested at late exponential phase by centrifugation. After centrifugation, the supernatant and cells were used for an analysis of aryl-β-glucosidase and aryl-β-galactosidase activities. The cells were washed three times with citrate phosphate buffer (50 mM, pH 6.0) and suspended in the same buffer. This suspension was used for analyzing cell-bound hydrolytic activities.

To check the intracellular localization of these enzymes, cells were subjected to sonication (SONICS Vibra cell; model VC 130) in citrate phosphate buffer (pH 6.0, 50 mM) containing 20 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl2, and phenylmethylsulfonyl fluoride (50 µg/ml). The sonication was performed at a 60% amplitude (125 µm) for 5 min by using a 2-mm probe under cold conditions. Almost 90% of the cells were disrupted by this method. The supernatant and sonicated cell debris were analyzed for all above-mentioned enzyme activities.

RESULTS AND DISCUSSION

Lactic Acid Production

The profile of growth (optical density), pH, lactic acid production, and cellobiose utilization is shown in Fig. 4.1. The maximum amount of lactic acid was produced within 40 h of fermentation, with an increase in optical density from 0.5 to 14 and a decrease in pH from 6.5 to 4.9. The maximum (90 g/L) amount of lactic acid was produced from 100 g/L of cellobiose with a 2.25-g/L/h productivity and a 0.9-g/g yield. These are the highest productivity and efficiency values reported so far. This strain, therefore, proved to be highly efficient for the conversion of cellobiose to lactic acid and could be exploited at a commercial level. In previous chapter we reported the production of lactic acid from bagasse-derived cellulose using the same mutant strain. We could not detect cellobiose in the fermented broth at any point of time in SSF suggesting the possible presence of β-glucosidase activity in the mutant strain.

Susana Romero-Garcia et al (2009) reported that *B. subtilis* ferments cellobiose to produce L-lactate with 82% of the theoretical yield, and with a specific rate of L-lactate production similar to that one obtained fermenting glucose. But this strain also produces low amount of 2,3-butanediol.

Few recombinants and metabolically strain are available which cinvert cellobiose to lactic acid. Miho Sasaki and co-workers (2008) metabolically engineered *C. glutamicum* strain X5C1 to produce chemicals and fuels from sugars derived from lignocellulose. Optically pure D-lactic acid was produced from growth-arrested cells of *C. glutamicum* under oxygen-deprived conditions, production strains derived from strain X5C1 are able to produce ethanol and optically pure D-lactic acid from a sugar mixture containing D-cellobiose, D-glucose, and D-xylose. Similarly, Aspergillus aculeatus β-glucosidase 1 (bgl1) gene was expressed in a lactic-acid-producing *Saccharomyces cerevisiae* strain to enable lactic fermentation with cellobiose.The recombinant β-glucosidase enzyme was expressed on the yeast cell surface by fusing the mature protein to the C-terminal half region of the α-agglutinin. The maximum rate of L-lactate production by this strain on cellobiose is 2.8 g/L/ h (Tokuhiro et al., 2008).

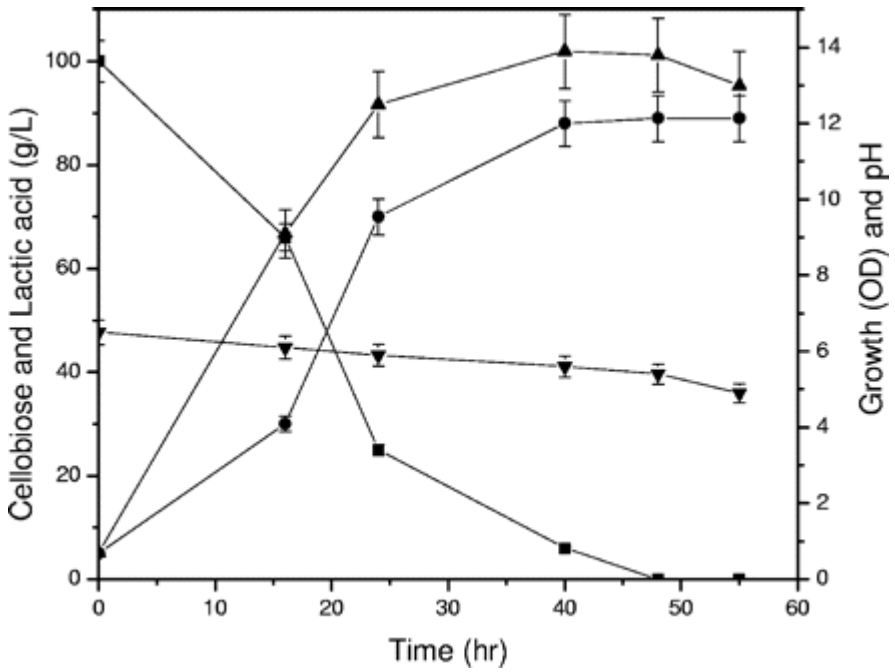

Fig.4.1 *Profile of lactic acid production, growth, pH, and cellobiose utilization during fermentation by Lactobacillus delbrueckii mutant Uc-3 using cellobiose concentration of 100 g/L. ■, cellobiose; •, lactic acid; ▲, growth; ▼, pH. Error bars indicate standard deviations. Symbols with error bars that cannot be seen have standard deviations of 5 to 6%.*

Separate experiments were carried out to check whether *L. delbrueckii* Uc-3 utilizes cellotriose or cellooligosaccharides as a sole carbon source. Initially, totals of 20 mg glucose, 20 mg fructose, and 10 mg lactic acid were present in the 10 ml of medium. Within 18 h of fermentation, the complete utilization of glucose and fructose led to the production of lactic acid. After 18 h of incubation, the mutant started utilizing cellotriose, and within 30 h of incubation, complete utilization of cellotriose was observed with the production of a proportional amount of lactic acid (Table 4.1). The cellooligosaccharides were not found to be utilized by this strain. No lactic acid was produced in medium containing cellooligosaccharides, which shows that the mutant does not utilize cellooligosaccharides as a sole carbon source. The total lactic acid produced from 2 g/L of cellotriose was 1.7 g/L with a 85% yield. This is the first report which shows that *L.*

delbrueckii mutant Uc-3 used cellotriose as a carbon source and produced lactic acid with a significant yield.

Table 4.1 Lactic acid production from cellobiose, cellotriose, and cellooligosaccharides

Substrate	Substrate concentration (g/L)	Time of sampling (h)	Lactic acid (g/L)
Cellobiose	100	40	90 ±4.5
Cellotriose	2	12	1.7 ±0.2
Cellooligosaccharides	2	12	0.1 ±0.12

Values are the average of three independent experiments.

Detection of enzyme activities

This strain was observed to utilize both cellobiose and cellotriose (Table 1). Therefore, we attempted to detect the cellobiose or cellotriose degrading enzymes by using *p*-nitrophenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-β-D-cellobioside (pNPC), and *p*-nitrophenyl-β-D-galactopyranoside (pNPgal) as substrates. We could not detect any enzyme activities in the supernatant.

Table 4.2 presents the data of all the enzyme activities in the mutant strain grown in liquid medium with different sugars. All activities were detected in lactose- and cellobiose-grown cells. However, a higher level of aryl-β-glucosidase was observed in cellobiose grown cells. Other than cellobiose, the lactose grown cells also exhibited all activities. When cells were grown in glucose and hydrolyzed sucrose-based medium, aryl-β-glucosidase and aryl-β-galactosidase activities were detected in small amounts. No activity on pNPC was detected. These results show that the aryl-β-glucosidase or aryl-β-galactosidase gene could be constitutively expressed, and enhancement in activity was observed when the mutant was grown in either cellobiose or lactose, respectively. The activity on pNPC was detected only when culture was grown in cellobiose or lactose. However, the induction in lactose-grown cells is comparatively less than that in cellobiose-grown cells. This result suggested that aryl-β-glucosidase and aryl β-galactosidase enzymes are independent.

Table 4.2 Detection of aryl-β-glucosidase activity from *Lactobacillus delbrueckii* mutant Uc-3 grown in different sugar substrates

Enzyme source	Substrate	Enzyme activity (U/g of cells) in:			
		Glucose	Lactose	Hydrolyzed sucrose	Cellobiose
Cells	pNPG	2.2± 0.20	6.5± 0.55	1.5±0.13	11.8 ±1.0
	pNPgal	4.1± 0.35	6.0± 0.50	4.0 ±0.30	3.0± 0.25
	pNPC	ND	1.3± 0.12	ND	4.5± 0.30
Sonicated cells	pNPG	ND	4.1 ±0.35	ND	5.5± 0.45
	pNPgal	1.0 ±0.10	4.5± 0.30	1.3± 0.10	1.7± 0.15
	pNPC	ND	0.8± 0.07	ND	2.0 ±0.18

The cells were harvested at the onset of stationary phase for the determination of enzyme activity. Values are the averages of two independent experiments.
ND-not detected.

It is noteworthy to say that none of these activities are detected in fermented broth, suggesting the intracellular location of these enzymes. The supernatant and sonicated cell debris was analyzed for all above-mentioned enzyme activities. No activities were detected in supernatant, showing that enzymes are not located in cytoplasm. However, all activities (lactose- and cellobiose-grown cells) were detected with sonicated cells (Table 4.2), suggesting that the enzymes could be cell wall/membrane bound. The activity levels were low compared to those of the intact cells, which could be due to the inactivation of the enzymes during sonication.

CONCLUSION

In conclusion, the *Lactobacillus delbrueckii* mutant Uc-3 is a promising strain for the production of lactic acid from cellulosic materials in SSF. Bottlenecks, like feedback inhibition by glucose and cellobiose, were removed by using such a strain, leading to the complete conversion of cellulosic substrates to the products. This strain utilized both cellobiose and cellotriose effectively and produced lactic acid in a homofermentative way. Such an aryl-β-glucosidase, active on both pNPG and pNPC, has not been reported for any of the *Lactobacillus* species so far. These studies show the potentiality of such a strain, which could produce commodity chemicals from renewable biomass. It could also be possible to generate *Lactobacillus* strains capable of growing at higher temperatures (50^0C), at which the cellulases are most active, which in turn will enhance the efficiency of lactic acid production in SSF.

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Part-B

Yeast Xylanases

Chapter 5

Introduction to xylanases

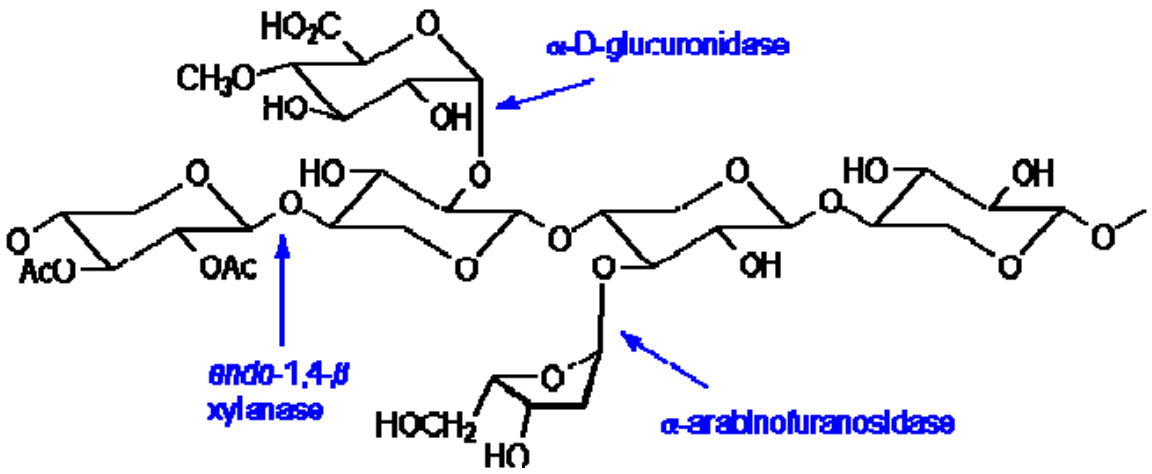
XYLAN AND ITS STRUCTURE

Xylan or hemicellulose is the second most abundant polysaccharide fraction available in nature. Xylan is the one of the major structural polysaccharide in plant cell. The relative distribution of lignocellulosic components in the cell wall is dependent on the plant species and on the stage of growth and development. Xylans are heteropolysaccharides with a homopolymeric backbone chain of β-1, 4-linked β-D-xylopyranose units. The backbone consists of O-acetyl, α -L-arabinofuranosyl, α-1,2-linked glucuronic or 4-O-methylglucuronic acid substituents. Wood xylans exist as O-acetyl-4-O-methylglucuronoxylans in hardwoods or as arabino-4-O methyl glucuronoxylans in softwoods. The cereal xylans are made up of D-glucuronic acid and/or its 4-Omethyl ether and arabinose. Based on side chain the xylans are classified as linear homoxylan, arabinoxylan, glucuronoxylan and glucuronoarabinoxylan. The β 1,4-linked β -D-xylopyranose units are substituted at positions C-2, C-3 and C-5 to varying degrees depending upon the plant and the stage of development of the plant when the polymer is obtained. In monocots, at the C-2 positions 1,3-linked α -D-glucronic acid or 4-Omethyl- α -D-glucuronic acid might occur, while at C-3 of xylopyranose, one frequently finds 1,3 linked α-L arabinofuranose. In some xylans, particularly in hardwoods, xylopyranose residues may be O-acetylated at the C-2 or (more commonly) at the C-3 positions. Again, small amounts of phenolic components, such as ferulic and p-coumaric acids are esterified to xylan *via* their carboxyl groups to C-5 of xylose ring (Bastawde, 1992 and Kulkarni et al., 1999).

Atkins has described the three-dimensional structure of the xylan molecules. The xylan chain shows a threefold left-handed conformation under crystallized conditions; the geometry of the glycosidic linkage is not disturbed by the side chains. Studies show that the Xylan backbone imposes certain minimum constraints and the interactions between the chains determine the final confirmation. The electron diffraction study also shows

that the chains are organized in a trigonal lattice with hexagonal morphology. The single hydrogen substituent at position 5 on the xylose ring has a dramatic effect on the intra- and interchain hydrogen bonding interactions. Energy calculations suggest that the D-xylose ring exists in the common $^4C_1$ chair conformation (Atkins, 1992).

Fig. 5.1 A Representation of Xylan with side chain



XYLANASES

Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which catalyze the hydrolysis of 1,4- β - D-xylosidic linkages in xylan. They are a widespread group of enzymes, and are produced by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods and anthropods (Prade, 1995). First reported in 1955 (Whistler et al., 1955), these enzymes were originally termed pentosanases, and were recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 when they were assigned the enzyme code EC 3.2.1.8. Their official name is endo-1,4- β - xylanase, but commonly used synonymous terms include xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4- β -D-xylanase, β -1,4-xylanase and β -xylanase. Depending upon the mode of action, xylanases are classified as follows.

1. Endo- β - (1, 4)-D-Xylanase (EC 3.2.1.8): These enzymes act randomly on xylan to produce large amounts of xylo-oligosaccharides of various chain lengths.
2. Exo- β -(1, 4)-D-Xylanase: These enzymes remove the single xylose or xylobiose units from the non-reducing end of the xylan chain.

3. β -Xylosidase or Xylobiase. (EC 3.2.1.37*)*: These enzymes hydrolyse disaccharides like xylobiose and the higher xylooligosaccharides with decreasing specific affinity.

Xylanase multiplicity

Most of the microorganisms produce multiple isoforms of xylanases. These multiple forms may have diverse physicochemical properties, structures, specific activities, specificities and yields. The multiplicity increases the efficiency and extent of hydrolysis, and also the diversity and complexity of the enzymes. The *Aspergillus niger* and *Trichoderma viride* sectetes fifteen and thirteen extracellular xylanase respectively (Biely et al, 1985). This type of multiplicity may be due to the genetic redundancy (Wong et al., 1988) or differential post-translational modification (Biely, 1985). The isoenzyme genes may be found as polycistronic or non-polycistronic multiple copies within the genome, and in some cases several xylanases are expressed as a distinct gene product.

Xylanase classification

First time Wong et al (1988) classified xylanases on the basis of their physicochemical properties and proposed two groups: those with a low molecular weight (<30 kDa) and basic pI, and those with a high molecular weight (>30 kDa) and acidic pI. However, several exceptions to this pattern have been found and approximately 30% of presently identified xylanases, in particular fungal xylanases, cannot be classified by this system.

Afterwards more complete classification system was introduced (Henrissat et al., 1989) which allowed the classification of not only xylanases, but glycosidases in general (EC 3.2.1.x). This has now become the standard means for the classification of these enzymes. This system is based on primary structure comparisons of the catalytic domains only and groups of enzymes in families of related sequences (Henrissat et al., 2001). In the beginning, the xylanases are being confined to families 10 and 11. But xylanases are also found in families 5, 7, 8, 16, 26, 43 and 62. Till today, available literature shows that only those sequences classified in families 5, 7, 8, 10, 11 and 43 contain truly distinct catalytic domains with a demonstrated endo-1, 4-β-xylanase activity. Those sequences reported for families 16, 52 and 62 appear, in fact, to be bifunctional enzymes containing

two catalytic domains; a family 10 or 11 xylanase domain as well as a second glycosidase domain.

Xylanases of families 5, 7, 8, 10, 11 and 43 differ in their physico-chemical properties, structure, mode of action and substrate specificities. Furthermore, families 5, 7, 10 and 11 contain enzymes which catalyse hydrolysis with retention of anomeric configuration with two glutamate residues being implicated in the catalytic mechanism in all cases which indicates a double-displacement mechanism., In contrast, enzymes in families 8 and 43 typically operate with inversion of the anomeric centre and a glutamate and aspartate are believed to be the catalytic residues (Nurizzo et al., 2001). Typically the distance between the two residues is around 9.5 A ˚ so as to allow for accommodation of the water molecule between the anomeric carbon and the general base (McCarter et al., 1994; Rye et al., 2000; Zechel et al., 2000). However, this distance is 7.5 A^{o} in the inverting endoglucanase CelA and the distance between the two catalytic residues is less constrained in inverting than in retaining enzymes (Alzari et al., 1996; Guerin et al., 2002). The maximum number of xylanases are found in families 10 and11 i.e. 127 and 173 respectively. Mechanism of xylan hydrolysis is same as cellulases as described in Chapter 1

Source of xylanases

Xylanases are reported to be produced mainly by microorganisms. Large number of xylanolytic system is reported in Fungi and bacteria. Few yeast species are also studied for xylanases. However, there are reports regarding xylanase origin from plants, i.e., endo-xylanase production in Japanese pear fruit and from the flour of European wheat (*Triticum aestivum*). Some members of higher animals, including fresh water mollusc, are able to produce xylanases. There are lots of reports on microbial xylanases starting from 1960 (Subramaniyan et al., 2002).

Among fungi followings are the examples of xylanase producers, *Acrophialophora nainiana*, *Aspergillus niger, Aspergillus aculeatus, Aspergillus awamori, Aspergillus fischeri, Aspergillus fumigatus, Aspergillus kawachii, Aspergillus nidulans, Aspergillus nidulans kK 99, Aspergillus oryzae, Aspergillus sojae, Aspergillus sp., Aspergillus sydowii, Aspergillus terreus, Aspergillus versicolor, Aspergillus*

kawachiiIFO 4308, Aspergillus nidulans, Aspergillus fischeri, Aspergillus sojae, Aspergillus sydowii , Cephalosporium sp., Fusarium oxysporum , Geotrichum candidum, Paecilomyces varioti , Penicillium Purpurogenum, Thermomyces lanuginosus DSM 5826, Thermomyces lanuginosus–SSBP , *Trichoderma harzianum, Trichoderma reesei*, *Penicillium brasilianum , Penicillium capsulatum , Penicillium sp.*

Number of bacteria reported to produce xylanases are, *Acidobacterium capsulatum, Bacillus* sp. W–1,*Bacillus circulans* WL–12, *Bacillus stearothermophilus* T–6, *Bacillus* sp. strain BP-23, *Bacillus* sp. strain BP-7, *Bacillus polymyxa* CECT 153, *Bacillus* sp. strain K-1 ,*Bacillus* sp. NG-27, *Bacillus* sp. SPS-0 *Bacillus* sp. strain AR-009, *Bacillus* sp. NCIM 59, *Cellulomonas fimi Cellulomonas* sp. NCIM. 2353, *Micrococcus*sp. AR-135, *Staphylococcus* sp. SG-13, *Thermoanaerobacterium* sp. JW/SL–YS 485 *Thermotoga maritima* MSB8. The examples of Actinomycetes which produced xylanases are *Streptomyces* sp. EC 10 *Streptomyces* sp. B–12–2, *Streptomyces* T7 *Streptomyces thermoviolaceus* OPC–520 *Streptomyces chattanoogensis* CECT 3336 *Streptomyces viridisporus* T7A *Streptomyces* sp. QG-11-3 2000a *Thermomonospora* 15 *curvata* (Beg et al., 2001).

Production

The large scale production of xylanase is necessary because of its applications in pulp and paper industries, in food and prebiotics. The three major components are necessary for efficient production of xylanolytic enzymes are efficient xylanase secretary organism, suitable substrate and optimum medium composition. Fungi are the most efficient producer of xylanases. The level of extra cellular secretion of xylanases in fungi is more than the bacteria, yeast and actinomycete. In many cases the xylanases are produced with the cellulases by using cellulose as a substrate. The selective production of xylanases is possible by using only xylan as a substrate. The hemicellulose containing substrate such as wheat bran, rice straw, corn cob, , rice bran, ,corn stalk and sugarcane bagasse have also been found to be most suitable for the production of xylanases in the case of certain microorganisms such as *Aspergillus awamori, Penicillium purpurogenum* (Haltrich et al., 1996) *Bacillus sp* (Dey et al., 1992).

The maximum xylanase activities were reported for several fungi, the presence of a considerable amount of cellulase activities and lower pH optima makes the enzymes less suitable for the pulp and paper industries. The *Trichoderma* spp are more studied for higher xylanase production. *Schizophillum commune* is reported as high xylanase producers with a xylanase activity of 1244 U/ml (Steiner et al., 1987). Singh and co-workers (2000) reported the highest xylanase activity i.e. 59,600 nkat/ml (approximately 3576 U/ml) from a *Thermomyces lanuginosus* strain. In case of *Trichoderma reesei* the maximum activity reported is 3350 IU/ml (Haapala et al., 1994). The maximum xylanase activity in solid state fermentation has been obtained from the fungus *Schizophyllum commune* (22 700 IU/g) (Haltrich et al., 1992). *Trichoderma hamatum* is reported to produce 7000 IU/g dry weight using wheat straw as substrate (Grajek, 1987). Although a plethora of xylanase producing strains have been described, their use for commercial production at present is restricted mainly to *Trichoderma sp*. and *Aspergillus sp*. (Haltrich et al., 1996). However, the future scenario may be deferent since several promising strains that produce xylanases in higher yields, with increased stability at extreme conditions of pH and temperature, have been recently identified.

Many factors are reported which influences the yield of xylanases. The standard factors include pH, temperature and agitation. Another major factor is a type of substrate i.e. agriculture residues, chemical or thermal treated agriculture biomass, pure xylan etc. During fermentation, various factors have combined effect includes substrate accessibility, rate and amount of release of the xylooligosaccharides and their chemical nature and quantity of xylose released which acts as the carbon source and as an inhibitor of xylanase synthesis in most of the cases. Generally, the slow release of the inducer molecules and the possibility of the culture filtrate converting the inducer to its non-metabolizable derivative are believed to boost the level of xylanase activity. In many cases the xylanase is tightly bound to the substrate and will not be available in filtrate for further use. The metabolic enzymes of the xylanase producer such as proteases (Penbroke et al., 1992) and transglycosidases also affect the actual yield of the enzyme (Hrmova et al., 1991).

Xylanase is also an inducible enzyme, sometimes it also expressed constitutively with the cellulases (Srivastava et al., 1993). The xylanolytic substrates, substrate

derivatives and the enzymatic end products may often play a key positive role in the induction of xylanases.

Biochemical characteristics of xylanases

The biochemical properties of xylanases are the important part of xylanase characterization. Knowing the optimum conditions (pH and Temperature) of xylanases, which varies from organism to organism and habitat, we may decide their future applications in various fields. The maximum numbers of xylanases have acidic optimum pH and they are active at 50^0C. Optimum temperature and pH of xylanases range from 20^0C to 90^0C and 3 to 10 respectively. The enzyme kinetics gives (km, kcat and Kcat/km) us the details of their efficiency of catalysis and specificity towards the different substrates. The molecular weight is also a major characteristic of xylanases. Molecular weight of xylanases are also varies from species to species (5.5 to 145 kDa). The following table (Table 5.1) shows the examples of xylanases from different species and their biochemical characteristics.

Table 5.1 Characteristics of xylanases from different organisms

Microorganisms	Mr (kDa)	Optimum pH	Optimum Temp. (^{0}C)	pI	Km (mg/ml)	Vmax (μM/min/mg)	References
Bacteria							
Bacillus coagulans	22-26	6	37	10	-	-	Esteban et al., 1983
Bacillus sp 11-1 S	-	4	80	-	1.68	-	Uchino et al., 1981
Acidobacterium capsulatum	41	5	65	7.3	3.5	403	Inagaki et al., 1998
Bacillus sp. W–1	21.5	6	65	8.5	4.5	–	Okazaki et al., 1985
Bacillus circulans WL–12	15	5.5–7	_	9.1	4	_	Esteban et al., 1982
Bacillus stearothermophilus T–6	43	6.5	55	7, 9	1.63	288	Khasin et al., 1993

Bacillus sp. Strain BP-23	32	5.5	50	9.3	–	–	Blanco et al., 1995
Bacillus sp. Strain BP-7	22–120	6	55	7–9	–	–	Lopez et al., 1998
Bacillus polymyxa CECT 153	61	6.5	50	4.7	17.1	112	Morales et al., 1995
Bacillus sp. Strain K-1	23	5.5	60	–	–	–	Ratanakhanokchai et al., 1999
Bacillus sp. NG-27	–	7, 8.4	70	–	–	–	Gupta et al., 1992
Bacillus sp. SPS-0	–	6	75	–	–	–	Bataillon et al., 1998
Bacillus sp. Strain AR-009	23, 48	9–10	60–75	–	–	–	Gessesse, 1998
Bacillus sp. NCIM 59	15.8, 35	6	50–60	4, 8	1.58, 3.50	0.017, 0.74	Dey et al., 1992
Cellulomonas fimi	14–150	5–6.5	40–45	4.5–8.5	1.25–1.72	–	Khanna et al., 1993
Cellulomonas sp. NCIM 2353	22, 33, 53	6.5	55	8	1.7, 1.5	380, 690	Chaudhary et al., 1997
Micrococcus sp. AR-135	56	7.5–9	55	–	–	–	Gessesse et al., 1998
Staphylococcus sp. SG-13	60	7.5, 9.2	50	–	4	90	Gupta et al., 2000
Thermoanaerobacterium sp. JW/SL–YS 485	24–180	6.2	80	4.37	3	–	Shao et al., 1995
Thermotoga aritime MSB8	40, 120	5.4, 6.2	92–105	5.6	1.1, 0.29	374, 4760	Winterhalter et al., 1995
Fungi							
Trichoderma koninjii	17	4.9 to 5.5	50	7.3	4.2	-	Wood et al., 1986
Acrophialophora nainiana	17	6	50	–	0.73, 0.34	–	Ximenes et al., 1999
Aspergillus niger	13.5–14.0	5.5	45	9	–	–	Frederick et al., 1985
Aspergillus kawachii IFO 4308	26–35	2–5.5	50–60	3.5–6.7	–	–	Ito et al., 1992
Aspergillus nidulans	22–34	5.4	55	–	–	–	Fernandez et al., 1992
Aspergillus fischeri Fxn1	31	6	60	–	4.88	5.88	Raj et al., 1996
Aspergillus sojae	32.7, 35.5	5, 5.5	60, 50	3.5, 3.75	–	–	Kimura et al., 1995

Aspergillus sydowii MG 49	30	5.5	60	–	–	–	Ghosh et al., 1994
Cephalosporium sp.	30, 70	8	40	–	0.15	–	Bansod et al., 1993
Fusarium oxysporum	20.8, 23.5	6	60, 55	–	9.5; 8.45, 8.7	0.41, 0.37	Christakopoulos et al., 1996
Geotrichum candidum	60–67	4	50	3.4	–	–	Radionova et al., 2000
Paecilomyces varioti	20	4	50	5.2	49.5	–	Kelly et al., 1989
Penicillium purpurogenum	33, 23	7, 3.5	60, 50	8.6, 5.9	–	–	Belancic et al., 1995
Thermomyces lanuginosus DSM 5826	25.5	7	60–70	4.1	7.3	–	Cesar et al., 1996
Thermomyces lanuginosus–SSBP	23.6	6.5	70–75	3.8	3.26	6300	Lin et al., 1999
Trichoderma harzianum	20	5	50	–	0.58	0.106	Tan et al., 1985
Trichoderma reesei	20, 19	5–5.5, 4–4.5	45, 40	9, 5.5	3–6.8, 14.8–22.3	–	Tenkanen et al., 1992
Actinomycete							
Chainia sp.	5.5 to 6.0	6	60	8.5 – 9.0	5	-	Bastawde, 1987
Streptomyces sp. EC 10	32	7–8	60	6.8	3	–	Lumba et al., 1992
Streptomyces sp. B–12–2	23.8–40.5	6–7	55–60	4.8–8.3	0.8–5.8	162–470	Elegir et al., 1994
Streptomyces T7	20	4.5–5.5	60	7.8	10	7610	Kesker, 1992
Streptomyces thermoviolaceus OPC–520	33, 54	7	60–70	4.2, 8	–	–	Tsujibo et al., 1992
Streptomyces chattanoogensis CECT 3336	48	6	50	9	4, 0.3	78.2, 19.1	Lopez et al., 1998
Streptomyces viridisporus T7A	59	7–8	65–70	10.2–10.5	–	–	Magnuson et al.,1997
Streptomyces sp. QG-11-3	–	8.6	60	–	1.2	158.85	Beg et al., 2000
Thermomonospora curvata	15–36	6.8–7.8	75	4.2–8.4	1.4–2.5	–	Stutzenberge et al., 1992
Streptomyces sp. KT-23	42 to 44	5.5	55	6.9	0.2	-	Nakajima et al., 1984

YEAST XYLANASES

As compared to filamentous fungi, few yeasts were reported to produce complete xylanolytic enzymes. The yeast genera such as *Aureobasidium, Cryptococcus, Pichia, Candida, Trichosporon* produce xylan degrading enzymes. The colour variant strain of *Aureobasidum pullulans* was known to be the best producer of yeast xylanase in early days. Only few strains of *Candida* and *Pichia stipitis* showed the ability to grow on xylan. Within the genus *Candida*, xylan hydrolysis was exhibited by several strains of *Candida shehatae*, the anomorph of *Pichia stipitis*, and by *Candida ergatensis*. These are the only xylanolytic yeasts which are also known to ferment xylose to ethanol (Lee et al., 1986). The yeast xylanolytic system is controlled by induction and catabolite repression. Three enzymes, endoxylanase, xylosidase, and xyloside permease of *Cryptococcus albidus* are produced during the induction with xylobiose or with synthetic, nometabolisable inducer, methyl β-D-xylopyranoside (Biely et al., 1980). In this yeast only endoxylanase is secreted into the extracellular broth. In case of *Cryptococcus flavus* and *A. Pullulans,* the xylanase is induced not only by xylobiose but also by xylose and arabinose (Yasui et al., 1984; Leathers et al., 1986; Pou-Llinas et al., 1987). The endoxylanase of *C. albidus* is the first xylanase gene that was isolated and sequenced (Morosoli et al., 1992).

A highly acidic xylanase form *Cryptococcus* sp. S2 was isolated. This xylanase (xyn-CS2) has optimum pH 2.0 and showed 75% activity even at pH 1.0. The deduced amino acid sequence of xyn-CS2 shows significant similarities with family G-xylanases. Xylanase, xyn-CS2, included two unique cysteine residues in a putative catalytic site, raising the possibility that these residues are at least partially responsible for its acidophilic nature (Iefuli et al., 1996).

Tanaka et al. (2004) isolated gene responsible for acidic xylanase (XynI) from *Aureobasidium pullulans* var. *melanigenum* strain ATCC 20524 and expressed in *Pichia pastoris* under the control of the alcohol oxidase I gene promoter. The 34 amino acid prepro-signal peptide of the *A. pullulans* XynI directed the efficient secretion of 178 mg of active xylanase per liter of the culture medium. The secretion level of the xylanase with its own signal peptide was comparable to that of the mature protein fused to the prepro leader from *Saccharomyces cerevisiae* α-mating factor and twofold higher than

that of the mature protein fused to the pre-type signal peptide from *P. pastoris* acid phosphatase. The N-terminal amino acid sequence and the apparent Mr of 24 kDa of the secreted recombinant protein indicated the native-like processing of the *A. pullulans* XynI signal sequence in *P. pastoris*. The three-dimensional model and mutational analysis of the xynI gene product showed that Asp-73 and Glu-157 residues located at the upper and lower edges of the active site cleft, respectively, play a significant role in its low pH optimum.

Leathers et al. (1986) isolated two colour variants strains of *Aureobasidium pullulans*. These strains produced extracellular xylanases of 20 and 21 kDa. These xylanases represented nearly half the total extracellular protein, with a yield of up to 0.3 g/L of xylanase. The specific activity of partially purified xylanase exceeded 2,000 IU/mg. Xylanase from typically pigmented strains appeared similar to that of color variants with respect to molecular weight, pH and temperature optima, and specific activity of purified (but not crude) enzyme.

Two xylanolytic enzymes, xylanase and xylosidase from the yeast *Pichia stipitis* were purified to homogeneity and characterized. Both enzymes were secreted into the culture medium upon growth on xylan. The xylanase is a glycoprotein with an approximate molecular mass of 43 kDa. The N-linked carbohydrate content was estimated to be 26% by endoglycosidase H digestion. The xylosidase protein has a molecular mass of 37 kDa as determined by SDS-PAGE. Synthesis of xylanase was found to be inducible by xylan and repressible by xylose and glucose. By contrast, xylosidase is synthesized constitutively to a considerable degree. The purified xylosidase was able to hydrolyse aryl-β-D-glucosides with an even higher rate than xylosides. Thus, this enzyme may not be a specific component of the xylan-degrading system of *P. stipitis* (Ozcan et al., 1991).

A xylanase belonging to family 10 is produced by *Cryptococcus adeliae*, an antarctic yeast that exhibits optimal growth at low temperature. The mature glycosylated xylanase secreted by *C. adeliae* is composed of 338 amino acid residues and around 26 osidic residues, and shares 84% identity with its mesophilic counterpart from *C. albidus*. The xylanase from *C. adeliae* is less thermostable than its mesophilic homologue when the residual activities are compared, and this difference was confirmed by differential

scanning calorimetry experiments. In the range 0°–20°C, the cold-adapted xylanase displays lower activation energy and a higher catalytic efficiency. All these observations suggest a less compact, more flexible molecular structure. (Petrescu et al., 2000).

The complete structural characterization of Antarctic yeast *Cryptococcus albidus* TAE85 was achieved both at protein and carbohydrate level by using mass spectrometery analysis. This analysis revealed the presence of N-glycosylation only at Asn254, modified by high mannose structure; moreover the protein resulted to be O-glycosylated with GalGalNAc structures. The N-linked and O-linked data obtained constitute the first description of the glycosylation system in cold-adapted organisms might have similarities as well as differences with respect to mesophilic and thermophilic organisms. The cysteine pairings were eventually identified as Cys173-Cys205 and Cys272-Cys278 showing a free thiol group (Amoresano et al., 2000)

Table 5.2 Characteristics of xylanases from different yeasts

Yeast	Mr (kDa)	Optimum pH	Optimum Temp. (^{0}C)	pI	Km (mg/ml)	Vmax (μM/ min/mg)	Ref.
Cryptococcus sp. S2	22	2.0	40	7.4	-	-	Iefuli et al., 1996
Cryptococcus flavus	31	4.5	55	10	3.1	-	Nakanishi et al., 1984
Aureobasidium pullulans Y-2311–1	25	4.4	54	9.4	7.6	2650	Li et al., 1993
Cryptococcus albidus	48	5	25	–	5.7, 5.3	–	Morosoli et al., 1986
Trichosporon cutaneum SL409	–	6.5	50	–	–	–	Liu et al., 1998
Aureobasidium pullulans var. melanigenum ATCC 20524	24	2.0	50	6.7	-	-	Ohta et al., 2001
Aureobasidium pullulans	20-21	4 to 4.5	35-50	-	-	-	Leathers et al., 1986
Cryptococcus adeliae	43/41.4	5.4	25	-	-	-	Petrescu et al., 2000

APPLICATION OF XYLANASES

Huge amounts of hemicellulose is produced and washed annually which has great potential as raw material if properly exploited. Xylanases are industrially important enzymes (Pandey et al., 1999; Collins et al., 2005) and their judicious use in industries may result in cleaner reactions, higher yields and lower consumption of energy. The xylanases have great potential for increasing the production of several useful products. The major potential applications of xylanases includes bio-bleaching of wood pulp, treating animal feed to increase digestibility, processing food to increase clarification , converting lignocellulosic substances to feedstock and fuels and in pharmaceuticals.

Kraft pulp processing/bleaching

The toxic chemicals produced during pulp processing are resistant to the biodegradation, and create environmental pollutions. Removal of residual lignin from Kraft pulp is physically and chemically restricted by hemicelluloses. Lignin, a major part of the lignocellulose, has been linked with hemicelluloses. The general process for the removal of lignin from wood chips use in a solution of Na2 S/ NaOH at about 170oC for 2 h in industries. The resulting pulp has a characteristic brown color, which is primarily due to the presence of residual lignin and lignin derivatives. The intensity of pulp colour is a function of the amount and chemical state of the remaining lignin. To obtain pulp of very high brightness and brightness stability, all the lignin must be removed from the pulp. For that chemical pulping is more effective than mechanical pulping. However, there is the formation of residual lignin, which has to be removed by bleaching process. The residual lignin in chemical pulp is dark in color because it has been oxidized extensively and modified in the cooking process. This residual lignin is difficult to remove due to its covalent binding to the hemicellulose and perhaps to cellulose fibers. The bleaching of the pulp can be regarded as a purification process involving the destruction, alteration, or solubilization of the lignin, coloured organic matters, and other undesirable residues on the fibres.

Bleaching of chemical pulp to a higher brightness without complete removal of lignin has not been successful in earlier days. Conventionally, chlorine is used for bleaching. Chlorination of pulp does not show any decolorizing effect, and, in fact, the

color of the pulp may increase with chlorination, and it is the oxidative mechanism that aids the pulp bleaching. At low pH the main reaction of chlorine is chlorination rather than oxidation. Thus, chlorine selectively chlorinates and degrades lignin compounds rather than the hemicelluloses / xylan moieties in the unbleached pulp. The dominant role of chlorine in bleaching is to convert the residual lignin in the pulp to water or alkali-soluble products. The effluents that are produced during the bleaching process, especially those following the chlorination, and the first extraction stages are the major contributors to wastewater pollution from the pulp and paper industries.

The use of xylanases treatment step to any conventional bleaching sequence results in a higher final brightness value of the pulp. The chlorine requirement in prebleaching has been shown to be reduced by 20-30%. As a result, the AOX (adsorbable organic halogen) load of the prebleaching effluent has been reduced by 15-20%. The greatest number of mill trials has been performed in Europe, mainly in Scandinavia, where most of the kraft pulp is produced. Xyanase application improves pulp fibrillation and water retention, reduction of beating times in virgin pulps, restoration of bonding and increased freeness in recycled fibres, and selective removal of xylans from dissolving pulps. Xylanases employed in paper technology do not need to be purified, but must be active at high temperatures and alkaline pH, and must not contain cellulolytic enzymes in order to preserve the cellulose fibres.

There are two hypotheses about the role of xylanases in cellulose pulp bleaching. In the first, the xylanases would act on the xylan precipitated on the lignin. This xylan is precipitated due to lowering of the pH at the end of the cooking stage. Its removal by the action of xylanases would leave the lignin more exposed to the compounds employed in the bleaching of cellulose pulp. The second hypothesis is based on the ability of lignin to form complexes with polysaccharides such as, for example, xylan, and the fact that some of the bonds are alkali resistant and might not have been hydrolyzed during the Kraft process .The xylanases act by cleaving the remaining bridges between the lignin and xylan, opening the structure of the cellulose pulp and leading to the fragmentation of xylan and subsequent extractions of the fragments. Treatment with xylanase renders the pulp more permeable to subsequent chemical extraction of the residual brown lignin and

lignincarbohydrate from the fibres (Kulkarni et al., 1999; Subramaniyan et al., 2002 Polizei et al., 2005)

Xylanases in Animal feed

Enzymes for ruminant feed have gained immense importance due to their potential to improve animal performance. Xylanases are used in animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytase, galactosidases and lipases. Pretreatment of agricultural silage and grain feed with xylanases improves their nutritional value. Xylanases break down arabinoxylans in the ingredients of the feed, reducing the viscosity of the raw material (Twomey et al., 2003). The arabinoxylan found in the cell walls of grains has an anti-nutrient effect on poultry. Such components in soluble form may increase the viscosity of the ingested feed, interfering with the mobility and absorption of other components. The addition of xylanase to feed containing maize and sorghum, both of which are low viscosity foods, may improve the digestion of nutrients in the initial part of the digestive tract, resulting in a better use of energy. The joint action of the rest of the enzymes listed produces a more digestible food mixture. Young fowl and swine produce endogenous enzymes in smaller quantities than adults, so that food supplements containing exogenous enzymes should improve their performance as livestock. Moreover, this kind of diet is found to reduce unwanted residues in the excreta such as phosphorus, nitrogen, copper and zinc. Most of the commercial xylanases are produced by *Trichoderma, Bacillus, Aspergillus, Penicillium, Aureobasidium and Talaromyces.* Lignocellulosic wastes from pulp and paper industry, waste from dairy and agricultural industry, are potential substrates to be used as single cell protein for feed and food purposes (Kuhad and Singh, 1993).

Xylanases in Food industries

Xylanases are employed in bread industries together with α-amylase, malting amylase, glucose oxidase and proteases. The xylanases break down the hemicellulose in wheat-flour, helping in the redistribution of water and leaving the dough softer and easier to knead. During the bread-baking process, they delay crumb formation, allowing the dough to grow. With the use of xylanases, there has been an increase in bread volumes, greater absorption of water and improved resistance to fermentation (Maat et al., 1992;

Harbak et al., 2002; Camacho et al., 2003). The arabinoxylooligosaccharides released in bread would be beneficial to health. In biscuit-making, xylanase is recommended for making cream crackers lighter and improving the texture, palatability and uniformity of the wafers.

Recently, the use of xylanases, in conjunction with cellulases, amylases and pectinases, led to an improved yield of juice by means of liquefaction of fruit and vegetables; stabilization of the fruit pulp; increased recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments etc., reduction of viscosity, hydrolysis of substances that hinder the physical or chemical clearing of the juice, or that may cause cloudiness in the concentrate. The desirable properties for xylanases for use in the food industry are high stability and optimum activity at an acid pH. Recombinant yeast containing fungal xylanase was constructed which produces wine with a more pronounced aroma (Ganga et al., 1999). During the manufacture of beer, the cellular wall of the barley is hydrolyzed releasing long chains of arabinoxylans which increase the beer's viscosity rendering it "muddy" in appearance. With the use of xylanases, arabinoxylans are hydrolyzed to lower oligosaccharides diminishing the beer's viscosity and consequently eliminating its muddy aspect (Debyser et al., 1997; Dervilly et al., 2002).

Xylanases in xylooligosaccharides production

Xylooligosaccharides (XOS) acts as the prebiotic and beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (*Bifidobacterium* and *Lactobacilli*). In addition, XOS facilitate the absorption of nutrients and suppress the activity of entero-putrefactive and pathogenic organism. XOS can be used as ingredients of functional food, cosmetics, pharmaceuticals or agricultural products and as a plant growth regulator. In addition to the health effects, XOS present interesting physico-chemical properties, they are moderately sweet, and stable over a wide range of pH and temperatures and have organoleptic characteristics suitable for incorporation into foods (Gibson et al., 1995; Katapodis et al., 2002; Barreteau et al., 2006). They also have importance in decreasing the blood lipids, protecting liver functions, decreasing blood pressure, anticancer and regulating blood

sugar. It was found that the supplementation of the XOS was successful in inhibiting the precancerous lesions, promoting the growth of bifidobacteria and lowering the cecal pH value (Hsu et al., 2004; Moure et al., 2006). Therefore, XOS containing diets are considered to be beneficial in improving gastrointestinal health. Furthermore, the XOS seemed to be more efficient than the fructooligosaccharides in dietary supplementation.

Recently three methods used for XOS production includes the transglycosylation reaction using β-xylosidases, use of glycosyltranferases and synthesis by using glycosynthase (Endo et al., 2000; Perugino et al., 2004; Kim et al., 2006) enzymes. Both the methods are costly and require pure monosaccharides.. Recently the importance of XOs is more as compare to other oligosaccharides in terms of health related products, prebiotics and concentration thresholds, but their comparatively high production costs are hindering a wider and faster market development (Barreteau et al., 2006; Solange et al., 2007). For this purpose, further improvements in processing technology would be necessary. Use of these xylanases for XOS production from hemicellulose will be more feasible environmentally and economically. The endoxylanases play an important role in the selective production of xylooligosaccharides from xylan. The xylotriose to xylohexaose are important for prebiotics applications.

Other applications of xylanases

1. Xylanase treatment of plant cells can induce glycosylation and fatty acylation of phytosterols. Treatment of tobacco suspension cells with a purified endoxylanase from *Trichoderma viride* increased the levels of acylated sterol glycosides and elicited the synthesis of phytoalexins (Moreau et al., 1994).
2. Alkyl glycosides are one of the most promising candidates for new surfactants. Commercially, they are produced from monomeric sugars such as D-glucose and a fatty alcohol. But the direct glycosylation using polysaccharide is more feasible for their industrial production, because hydrolysis of polysaccharide and subsequent steps can be omitted. Thus, use of xylanase in this process provides a challenging opportunity. Recently, xylanase from *Aureobasidium pullulans* has been used for direct transglycosylation of xylan, 1-octanal and 2-ethyl hexanol

3. Some xylanases may be used to improve cell wall maceration for the production of plant protoplasts (Wong et al., 1988).
4. Xylanase in combination with cellulases used in the hydrolysis of cellulose and hemicelluloses for the conversion of sugars and then ethanol. Also used in xylitol production from hemicelluloses.(Dominguez, 1998; Olsson et al., 1996).

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Chapter 6

Xylooligosaccharide producing xylanases from yeast *Pseudozyma hubeiensis*: Biochemical Characterization and analysis of catalytic amino acid residues

SUMMARY

Yeast strain, isolated from decaying scandal wood, is identified as *Pseudozyma hubeiensis* by rDNA D1/D2 sequence analysis (100% matches). This strain produced high levels of cellulase free xylanase. Two distinct xylanases were purified to homogeneity by DEAE cellulose chromatography followed by Sephadex G-50 column chromatography. Molecular masses of two native xylanases were 33.3 kDa (PhX33) and 20.1 kDa (PhX20) confirmed by MALDI-TOF mass spectrometry and also SDS-PAGE. The CD spectra analysis revealed that PhX33 is predominant with α-helix and PhX20 contained predominantly β-sheets. The chemical modification studies revealed the presence of three tryptophan and one carboxyl residues at the active site of PhX33. The active site of PhX20 is comprised of one residue each of tryptophan, carboxyl and histidine. Carboxyl residue is mainly involved in catalysis and tryptophane residues are solely involved in substrate binding. Histidine residue present at the active site of PhX20 appeared to have a role in substrate binding. PhX33 hydrolyzed xylan into xylotriose, xylotetraose and xylopentaose and PhX20 hydrolyzed xylan into xylotriose, xylotetraose, xylopentaose, xylohexaose and xyloheptaose. No xylose and xylobiose were detected in the hydrolyzates. Both the xylanases produced only xylooligosaccharides (XOS) with degree of polymerization (DP) 3 to 7, which could be used in functional foods or as prebiotics. Lc ms-ms ion search of tryptic digestion of these xylanases revealed that there is no significant homology of peptides with known fungal xylanase sequences which indicate that these xylanases appear to be new.

INTRODUCTION

Next to cellulose, xylans are the most important renewable hetero-polysaccharides abundantly found in nature. This abundant hemicellulosic component is composed of a backbone of β-1, 4-linked xylopyranose units of about 100 in soft wood and 200 units in hard woods substituted particularly at 2-OH and 3-OH positions with diverse side groups and also at 1-OH position at the end of xylan chain. Depending upon the plant origin, the xylan backbone was found to be covalently bound to varying degree of acetic acid, arabinose, D-glucoronic acid, 4-*O*-methyl-D-glucuronic acid, ferulic acid and p-cumaric acid. For the hydrolysis of xylan, the synergistic action of several enzymes of different functions is necessary. The main enzyme involved in the fractionation of xylan polysaccharide chain is endo-1, 4-β-xylanase (EC 3.2.1.8). Other enzymes such as β-xylosidase (EC 3.2.1.37), α-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72), α-glucuronidase (EC 3.2.1.139), and feruloyl esterase (EC 3.1.1.73) remove side groups in heteroxylans (Bastawde, 1992; Collins et al., 2005). Most of the fungi and bacteria are known to express functionally diverse multiple forms of xylanases. This multiplicity could be a result of post-translational modification (glycosylation, auto-aggregation or/and proteolytic digestion), genetic redundancy or differential mRNA processing.

The xylanolytic systems are extensively studied in fungi (*Aspergillus* sp, *Trichoderma* sp, *Penicillium* sp etc) and bacteria (Bacillus sp, S*treptomyces* sp) but there are very few examples from the yeast (Biely et al., 1980; Chavez et al., 2006). Cellulase free xylanases are generally studied for its application in paper and pulp, food and textile industries. All endo-xylanases reported so far produced oligosaccharides with xylose as end product of xylan hydrolysis. However, none of the endo-xylanases produce smaller (DP around 3-7) xylooligosaccharides (XOS) without the production of xylose as one of the end products of xylan hydrolysis. The importance of such endo-xylanases, which produce smaller xylooligosaccharides, is yet to be defined due to lack of information on the suitable enzymes.

XOS act as the prebiotic, which beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (*Bifidobacterium* and *Lactobacilli*) by suppressing the activity of enteroputrefactive and

pathogenic organism and also facilitate the absorption of nutrients. XOS can be used as ingredients of functional food, cosmetics, pharmaceuticals or agricultural products and as a plant growth regulator. In addition to the health effects, XOS present interesting physico-chemical properties, they are moderately sweet, and stable over a wide range of pH and temperatures and have organoleptic characteristics suitable for incorporation into foods. (Gibson and Roberfroid, 1995; Katapolis et al., 2002; Barreteau et al., 2006). XOS have importance in decreasing the blood lipids, protecting liver functions, decreasing blood pressure, anticancer and regulating blood sugar. It was found that the supplementation of XOS was successful on inhibiting the precancerous lesions, promoting the growth of bifidobacteria and lowering the colonel pH value (Hsu et al., 2004; Moure et al., 2006). Therefore, XOS containing diets are considered to be beneficial in improving gastrointestinal health. Furthermore, XOS seemed to be more efficient than the fructooligosaccharides in dietary supplementation. The preventive effect of XOS against contact hypersensitivity was also investigated in mice (Yoshino et al., 2006).

We have isolated a new yeast strain, identified as *Pseudozyma hubeiensis*, in our laboratory. It produces very high levels of cellulase free xylanase when grown on xylan containing media (Bastawde et al., 1994).

MATERIALS AND METHODS

Chemicals

Xylan (oat spelts), 3,5-dinitrosalysilic acid, sinapinic acid, DEAE-cellulose, Sephadex-G-50, SDS-PAGE markers, N-ethylmaleimide (NEM), 2,2-dithiobisnitrobenzoic acid (DTNB), Phenylmethylsulfonylfluoride (PMSF), *p*-Chloromercuribenzoate (PCMB), Diethylpyrocarbonate (DEPC), 1-Ethyl-3-(3-dimethylaminoproply) carbodiimide (EDAC), 2,4,6-Trinitrobenzenesulfonic acid (TNBS), N-Bromosuccinimide (NBS), N-Acetylimidazole (NAI), 2,3-Butanedione, Citraconic anhydride, Acetic anhydride, Phenylglyoxal, HEPES, and MES were purchased from Sigma-Aldrich Co. St Louis, mo. USA. TLC plates obtained from Merck. Ampholytes in the range of 3 to 10 from Bio-Rad. All other chemicals used were of analytical grade & of the highest purity available locally. Citraconic anhydride and

Acetic anhydride were prepared in 1,4 dioxane. The stocks of DEP and phenylglyoxal were prepared in acetonitrile and methanol respectively.

Microorganism and production media

The yeast strain (KY-1) was isolated by Bastawde K.B in 1990 from decaying scandal wood (11) and deposited at National collection of industrial microorganism (NCIM) Resource Centre, National chemical Laboratory, Pune, India-411008 with accession no. 3574. This strain was identified at NCYC UK by 26S rDNA D1/D2 sequence analysis and also analyzed for chemotaxonomic characteristic. The strain maintained on MGYP agar slopes or broth. The medium used for production of xylanases contains (g/L), $NaNO_3$ (0.5), KCl (0.5), $MgSO_4$ (0.5), KH_2PO_4 (2.0), yeast extract (1.0), Bactopeptone (5.0), xylan (20.0), pH 5.5.

Enzyme production and purification

The enzyme production was performed in 500ml Erlenmeyer flask containing 200ml above production medium. The flasks were inoculated with 5% inoculum prepared in MGYP and incubated at 28^0C on shaker (170 rpm). The broth was harvested after 48 h, centrifuged (7000Xg, 10 min) and the supernatant was used as a crude enzyme preparation. The broth was concentrated in rotavapour at 40^0C. The 5ml concentrated enzyme sample was loaded on DEAE cellulose column, which was previously equilibrated with phosphate buffer pH 7.0 (50mM). The same buffer was passed through column after loading the concentrated enzyme and 3ml/10min/tube fractions were collected. The active fractions were pooled and concentrated in rotavapour at 40^0C. In second step of purification, the 2ml concentrated sample from DEAE cellulose column was loaded on Sephadex G-50 column, which was equilibrated before loading with 10mM phosphate buffer pH 7. After loading, the 1ml/10min/tube fractions were collected and analyzed for xylanases at pH 4.5. Two distinct xylanase peaks were identified which were designated as PhX20 and PhX33.

Enzyme assay

The xylan substrate was prepared by boiling 1% xylan solution in assay buffer (citrate buffer, 50mM, pH 4.5 or 4.2 or 5.5) in boiling water bath for 20min. The dissolved xylan

(50%) was used for assay. The 1 ml assay mixture consists of 0.5 ml substrate in respective buffer and 0.5 ml suitably diluted enzyme in corresponding buffer. The assay mixtures were incubated at 60^0C for 30 min. The released reducing sugars were analyzed by dinitrosalicylic acid (DNS) method (Fischer and Stein, 1961). One unit (IU) of enzyme activity was defined as amount of enzyme required to liberate 1μmol of reducing sugar equivalents per min.

Enzyme Characterization

The optimum pH of both the enzymes (PhX-20 and PhX-33) was determined by analyzing activities at different pH from 3.0 to 6.5 using citrate buffer 50mM at 50^0C. The optimum temperature of both the enzyme was determined by varying temperature from 40^0C to 75^0C at pH 4.0 for PhX-20 and pH 5.5 for PhX-33 using above 50mM buffer. The pH stability was analyzed by preincubating the enzymes in different buffers ranging from pH 2 to 9 (glycine-HCl pH 2 to3.5, acetate buffer pH 4 to 6, Tris-HCl pH 6.5 to 9) at 30^0C. The samples were analyzed after suitable time interval for the residual enzyme activity. The pH stability was determined by incubating the enzymes at 60^0C and 70^0C at pH 7.0(Tris-HCl, 50mM) and samples analyzed for residual enzyme activity after suitable time interval. The effect of heavy metals was examined by determining the activity in presence of 10 mM of various compounds.

The isoelectric point of both the enzyme determined in polyacrylamide gel (7.5%) using ampholytes in the range from 3 to 10. Protein concentrations were determined according to Lowry using BSA as standard (Lowry et al., 1951). Glycoprotein content of the purified enzyme preparation was determined by phenol sulfuric acid method of Dubois. SDS-PAGE (10 %) was performed in a vertical slab gel apparatus at pH 8.3, according to the method described by Laemmli. Protein bands were detected by Silver staining (0.2 % w/v).

The K_m and K_{cat} values of the native and modified enzymes were determined at standard assay condition (pH 4.2 and 60^0C for PhX-20, pH 5.5 and 60^0C for PhX-33) using 0.5 to 3 mg of soluble xylan. The constant values were calculated by fitting data to linear regression using Lineweaver-Burk plot.

The molecular weight of native enzymes was analyzed by MALDI-ToF mass spectrometry Voyager DE-STR (Applied Biosystems) system equipped with a 337-nm nitrogen laser. The matrix prepared in deionised water containing sinapinic acid (10 mg/ml), 50% acetonitrile and 0.1% TFA. Xylanases were mixed with matrix (1:1) and 2µl spotted on plate, dried at room temperature and analyzed.

CD Spectra's of both the enzymes were recorded on a Jasco-710 spectropolarimeter in the range of 190 to 250 nm using a 250 µl sample holder and 1 cm path length, at 25° C. The concentration of enzyme used was 500 µg/ml for YX-33 and 300 µg/ml for YX-20.

Amino acid analysis of xylanases was done at commercial services (Commonwealth Biotechnologies, Inc., Richmond, Virginia, USA). For analysis, salt free lyophilized enzyme sample 250 µg PhX-33 and 60 µg of PhX-20 was hydrolyzed in gas phase 6N HCl for 20 hours at 110 °C. Following hydrolysis, the sample was taken to dryness, dissolved in 150 µl sample loading buffer (0.4 M sodium borate pH 10.2) and 1 µl of undiluted sample was subjected to analysis on a Hewlett Packard Aminoquant method with Diode array detector. Tryptophan was determined spectrophotometrically. (Spande and Witkot, 1967).

The pH effect on K_m and K_{cat} values of xylanases was determined by varying the substrate concentration in the range of 0.5 to 3.0 mg/ml, using 50 mM citrate buffer (pH range, 3.0 to 6.5). All the assays were performed by standard assay conditions. Kinetic constants were calculated using Lineweaver Burk plots and the pKa values of the ionizable groups in the free enzyme and in enzyme substrate complex were determined according to Dixon.

Analysis of catalytic amino acids

Effect of side chain group specific reagents on xylanase activity

The purified Xylanases PhX-33 (20µg) and PhX-20 (10µg) was incubated with various amino acid functional group specific chemical reagents at specific concentration and reaction conditions (Table 6.2) with 1 ml total reaction system. After 30 minutes of

incubation at room temperature residual activities of enzyme samples were determined by standard assay method.

Modification of Carboxylate

The 1ml of 50 mM MES/HEPS buffer (75:25) pH 6.0 containing 20µg of YX-33 and 10µg of YX-20 was incubated with varying concentration of EDAC (10-100 mM) at 30°C. The control was kept without the addition of EDAC. Samples were withdrawn after suitable interval and reaction was terminated by addition of 100 µl of 1 M acetate buffer (pH 5.5). The residual activity of modified samples was determined by standard assay method. K_m and K_{cat} values of partially modified enzymes also determined using Lineweaver Burk plot.

For the determination of number essential carboxyl group involved in active site, the xylanases(same concentration as above) was incubated with different concentration of EDAC (0, 10, 20, 40, 60, 80mM). The aliquot was removed from each concentration after 15, 30, 45,60, and 75 min for analysis of residual activities. The log of % residual activities plotted against the time and the pseudo-first order rate constant (K_{app}) was determined for each line from the slope at each concentration (EDAC). After that the number of carboxyl groups involved were determined from the double reciprocal i.e. log (K_{app} x 1000) Vs log (mM EDAC)

Modification of Tryptophan

Purified xylanases 1ml (20 and 10 µg) in 100 mM sodium acetate buffer pH 4.5 was incubated with increasing concentration of NBS (1 to 5µM) at room temperature. After 10 min of incubation the aliquots were removed for analysis of residual activity. The residual activity of the enzyme was determined under standard assay conditions. The NBS mediated inactivation was also monitored spectrophotometrically by following the decrease in absorbance at 280 nm. For the determination of essential tryptophan residues the 100 µg of each xylanase was modified with 3µl of NBS (1mM) and incubated for 10 min. This titration was carried out up to the total inactivation of xylanases. The decrease in absorbance and corresponding residual activity was determined. The number of tryptophan residues modified was calculated by assuming Molar absorption coefficient of 5500 M^{-1} cm^{-1}. (Spande and Witkop, 1967).

$$\Delta n = \frac{1.31 \times \Delta A_{280}}{5500 \times \text{molarity of enzyme}}$$

Where ΔA_{280} is the decrease in absorbance at 280 nm, 1.13 is an empirical factor based upon oxidation of model tryptophan peptides by NBS. Δn is the number of tryptophan residues oxidized. The K_m and K_{cat} values of partially NBS modified enzymes were also determined under standard assay conditions.

Modification of Histidine

Purified 10 μg of PhX-20 xylanase in 1ml 50 mM potassium phosphate buffer pH 7.0 was treated with different concentration of DEPC (1 to 5 mM) at 25ºC. After each addition an aliquot 5μl was removed and reaction was arrested by addition of equal volume of 100 mM imidazole buffer, pH 6.8. Subsequently the residual activities were determined under standard assay conditions. Control sample was treated same as above only DEPC in the reaction mixture was replaced by acetonitrile. The DEPC mediated modification was monitored at different concentration (1 to 5 mM) and different time interval for the determination of essential Histidine residues.

The total essential Histidine residues calculated from the slope of line observed in double logarithm [log (K_{app} x 1000) Vs log (mM DEP)]. The procedure is same as above described foe carboxyl group. The K_m and K_{cat} values of partially DEP modified enzymes were also determined under standard assay conditions

Substrate protection studies

The both the xylanases (PhX-33 and PhX-20) were preincubated with the xylan (5mg) in the respective buffer of modifying reagents (EDAC, NBS and DEP). After 5 min incubation at room temperature the suitable concentration of modifying reagents were added and incubated for 30min. The aliquots were removed for the analysis of enzyme activity at standard assay conditions. The controls were kept at the same conditions without the addition of reagents.

Xylan hydrolysis product analysis

The hydrolysis of xylan was performed in 100 ml flask containing 500mg soluble xylan in 50ml citrate buffer (50mM) at pH 4.0 for YX-20 and at pH 5.5 for YX33. The mixtures were kept at 60^0C in water bath using 50 units each xylanase separately. After suitable time interval the aliquots were removed and boiled in water bath for 5 min to stop the reaction. The10 µl of each aliquots were loaded on TLC and analyzed by using butanol: acetic acid: water (3:1:1) solvent system. The TLC plates were developed in duplicates. The one part was visualized by spraying methanol and sulfuric acid (90:10) and then heated on hot plate. Another part of TLC corresponding to the spots were scraped and dissolved in 0.2 ml of distilled water. The dissolved hydrolysis products were centrifuged (5000 x g) and the supernatant was used for the MALDI-TOF mass spectrometry analysis.

The TLC eluted hydrolysis product was analyzed by MALDI-TOF MS using 2, 5 dihydroxybenzoic acid (10mg/ml in methanol) as a matrix. The 1µl of supernatant and 1µl of matrix mixed with each other and spotted on plate and analyzed. The molecular masses observed in spectra were matched with the theoretical masses of xylooligosaccharides.

The hydrolysis product also analyzed by using a high performance liquid chromatography (HPLC) system (Dionex India Limited) equipped with UV- or RI-detectors. An ion exclusion column (Aminex HPX-87H, Biorad, Hercules, CA) was used at 30 ^{0}C temperature, with 0.008 N H_2SO4 as a mobile phase at a flow rate of 0.6 ml/min. Injection volume of the sample was 50 ml.

RESULTS

Yeast Strain

A yeast strain first isolated by Bastawde K.B in 1990 from the decaying scandal wood and again isolated afterwards from wilt leaves by Wang and Bai and identified as *Pseudozyma hubeiensis.* Our isolated yeast strain shows 100% match of 26S rDNA with this strain. The taxonomic and chemotaxonomic details were given by Wang et al for their strain (Wang et al., 2006). We have also characterized our strain for chemotaxonomic characters. The major difference between the two strains (Wang and Bai strain and NCIM 3574) is the inositol and salicin assimilation. The Wang and Bai strain can't utilize the inositol and salicin but our strain assimilates it easily. Our strain grows at 37^0C and on 50% glucose agar but Wang and Bai strain is weakly positive and negative respectively. The detail characteristics of this strain are also available at http://www.ncyc.co.uk/yeast-ncyc-3431.html. The electron micrograph of our strain shows the elongated cells with polar budding (Fig.6.1).

26S rDNA D1/D2 sequence

AAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACGGCGAGTGAAG
AGGGAAGAGCCCAAGATTGAAAGCTGGCGCCTTCGGCGTCCGCATTGTAATC
TCAAGAAGTGTTTTCCGCTTCGGACCATGCCTAAGTCCCTTGGAAAAGGGCAT
CATAGAGGGTGATAATCCCGTACATGGCATGGAGCGCCCGAAGCTTTGTGAT
ACGCTTTCTAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAA
ATGCCATCTAAGGCTAAATATTGGGGAGAGACCGATAGCGAACAAGTACAGT
GATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTTAAACAGTACGTGAAAT
TGCCAAAAGGGAAGGGTAGGAGGTCAGAGATGCGTCGCAGGATTCAGCCTT
GCTTTTGCTTGGTGTTTTTCCTGCGTTGCAGGCCAACGTCGGTTTTGGGCACTG
GAGAAGGGTAGGAGGAACGTGGCACCTCTCGGGGTGTGTTATAGCCTCCTAC
TGGATACAGCGACCGAGACCGAGGAACGCAGCGTACTCGTAAGAGCGGGCC
TTTTGGCACCTTTACGCTTAGGGCGTTGGCATAATGGCCCTCTACCACCCGTC
TT

Species identity: *Pseudozyma hubeiensis* (100% sequence identity)

Strain designation: NCYC 3431, NCIM 3574

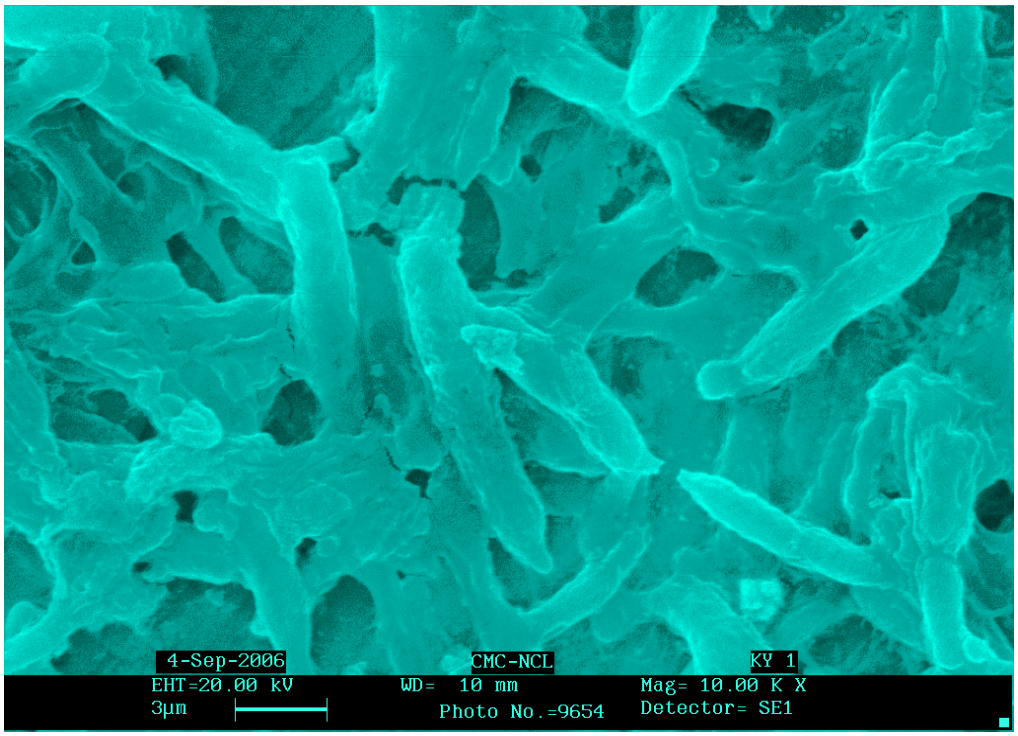


Fig. 6.1 *SEM image of Pseudozyma hubeiensis NCIM 3574*

Table 6.1 Chemotaxonomic characteristics of *Pseudozyma hubeiensis* NCIM 3574 or NCYC 3431

Test	Result
Semi-anaerobic Sugar etc. fermentation	
Glucose fermentation, Sucrose fermentation, Maltose fermentation , Galactose fermentation, Lactose fermentation , Cellobiose fermentation, Trehalose fermentation, Melibiose fermentation, Melezitose fermentation, Raffinose fermentation, α-methyl-D-Glucoside fermentation, Inulin fermentation, Starch fermentation, Xylose fermentation	All Negative
Aerobic utilization and growth (Sole carbon source)	
Glucose assimilation, Galactose assimilation, Sucrose assimilation, Maltose assimilation, Cellobiose assimilation, Trehalose assimilation, Lactose assimilation, Melibiose assimilation, Raffinose assimilation, Melezitose assimilation, Starch assimilation, D-Xylose assimilation, L-Arabinose assimilation, D-Ribose assimilation, Ethanol assimilation, Glycerol assimilation, D-Mannitol assimilation, D-Glucitol assimilation, α-methyl-D-Glucoside assimilation, Salacin assimilation, Inositol assimilation, D-Glucosamine assimilation, Xylitol assimilation	All positive
Inulin assimilation, L-Rhamnose assimilation, Galacitol assimilation, Methanol assimilation	All negative
Sorbose assimilation	+ weak/latent at 3 weeks
D-Arabinose assimilation	+ at 3 weeks
Erythritol assimilation	+ weak/latent at 3 weeks
Ribitol assimilation	+ weak/latent at 3 weeks
DL-Lactic acid assimilation	+ weak/latent at 3 weeks
Succinic acid assimilation	+ weak/latent at 3 weeks
Citric acid assimilation	+ weak/latent at 3 weeks
Glucono-d-lactone assimilation	+ weak/latent at 3 weeks

Sole nitrogen source	
Lysine assimilation, Cadaverine assimilation, Ammonium sulphate assimilation, Ethylamine Hydrogel Chloride assimilation, Potassiun nitrate	All positive
Additional characteristics	
Growth in 100ppm Actidione	-
Growth in 1000ppm Actidione	-
Growth in SPGYE +10% Nacl	+ weak/latent at 3 weeks
Growth in SPGYE +16% Nacl	-
Growth in SPGYE +20% Nacl	-
Growth on 50% Glucose slopes	+
Growth on 60% Glucose slopes	-
Urea hydrolysis	+
Growth on 1% Acetic acid	-
Lipolytic activity	-
Splitting of arbutin	+
Acetic acid production	-
Starch production	-
Growth at 37^0C	+
Growth at 40^0C	-
Pellicle in YM broth	Partial white ring at 2 weeks. Thick non-climbing pellicle at 3 weeks +
Production of Ballistospores	-
Production of Ascospores	-
Production of Pseudomycelia	-

Production of True mycelia

+ Growth on CMA and PDA plates under aerobic and semi-anaerobic conditions: Very extensive production of irregularly branched true mycelium with aerial hyphae consisting of oval to long-oval blastospores in chains.

Cell Morphology

- YM Broth at 2 days: Cells long-oval to elongated. Occurring singly. Polar budding. Cells (2 -3)μ wide x (5 -150)μ long.
- YM Agar at 2 days: Cells long-oval to elongated. Occurring singly and in large clumps. Polar budding. Cells (2 -3)μ wide x (10 -60)μ long.
- Growth on YM agar at 3 weeks +: Dull, rough, tan to pale orange in colour.

+ (positive growth): growth at 1-2 weeks, - (negative): No growth, YM - yeast malt

Enzyme production and purification

Yeast *Pseudozyma hubeiensis* KY-1 produces around 400 IU/ml of xylanase activity within 48 h of incubation at 28^0C with productivity 833 IU/L/h. It also found to produce β-xylosidase and α-arabinofuranosidase (data not shown). On DEAE cellulose column both the enzymes separate out combinely from the other proteins with increase in specific activity from 123 to 988 IU/mg. By using sephadex G-50 column both the enzymes were separated. The combined specific activity, % yield and fold of purification is 1171, 8 and 9.0 respectively. For the purification of these enzymes only two steps were required with high protein yield (10 to 14mg) (Table.6.2). The purity of these enzymes was analyzed by 10% SDS-PAGE.

Table 6.2 Summary of steps involved in purification of xylanases PhX33 and PhX20.

Purification steps		Total activity (U)	Total protein (mg)	Specific activity (U/mg)	% Yield	Fold Purification
Crude broth		165120	1333	123.8	100	1
DEAE-cellulose		87012	88	988	52	7.90
Sepadex-G-50	PhX33	6053	14.85	407	3.66	3.29
	PhX20	7457	10.50	710	4.50	5.73

Characterization of xylanases

Table 6.3 summarizes the characteristics of xylanases. The molecular mass of purified native xylanases were analyzed by MALDI-TOF MS, which shows that the one has 33.5 kDa and another has 20.1 kDa from which we have named them as PhX-33 and PhX-20 respectively. Molecular mass is also confirmed by PAGE. (Figure 6.2)

Table 6.3 Biochemical Characteristics of both the xylanases.

Biochemical Characters	Xylanase PhX33	Xylanase PhX20
Optimum pH	4.7-6.0	4.2
Optimum temp.	60-65^{0}C	55-60^{0}C
Temp. Stability	T/2 70^{0}C,15min	T/2 60^{0}C,10min
pH Stability	3.0-9.0	2.0-9.0
M_r (MALDI-TOF MS)	33.5 kD	20.1 kD
Km (mg/ml)	7.6	11.1
Kcat (per min.)	8.3 x 10^4	2.0 x 10^5
Isoelectric point	Approx. 8.3	Approx. 8.1
Glycoprotein	Present	Present
Secondary structure	Predominant α-helix	Predominant β-sheets

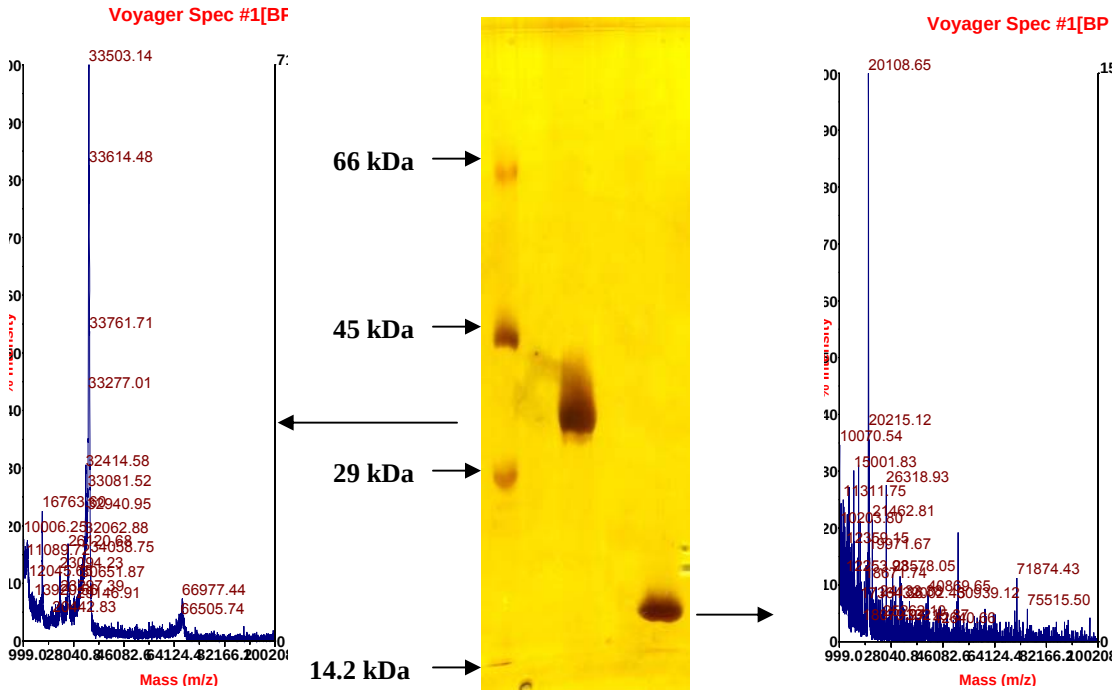


Fig. 6.2 *Determination of Molecular weight of purified xylanases (PhX33 and PhX20) by MALDI-TOF MS and by 10% SDS - PAGE.*

Both the xylanases are active at acidic condition. Xylanase PhX-33 and PhX-20 are highly active at pH 4.7-5.5 and 4.2 respectively. Their optimum temperature ranges from 60-65 and 55-60^{0}C for PhX-33 and PhX-20 respectively. Both are highly stable at wide range of pH. PhY-33 stable at pH 3 to 9 and PhX-20 at 2 to 9 with 90% activity after 48h of incubation at room temperature. It was also found that, they are remains active at pH 7.0 and 4^{0}C up to 2 to3 years. As for as temperature stability is concern the half life of PhX-33 at 70^{0}C is15 min and of PhX-20 at 60^{0}C is 10 min. the heavy metals such as Hg^{2+} and Cu^{2+} found to be inhibitory to both the enzymes. The isoelectric point both the enzymes are at around 8.2 (Table 6.3).

The kinetic parameters like Km and Kcat were analyzed and it was found that the apparent Km forPhX-33 and PhX-20 is 7.6 mg/ml and 11.1 mg/ml using oat-spelt soluble xylan respectively. The Kcat values are 8.3 x 10^4 min^{-1} with apparent second order rate constant 1.09x 10^4 and 2.0 x 10^5 min^{-1} with apparent second order rate constant 1.8 x 10^4 for PhX-33 and PhX-20 respectively.

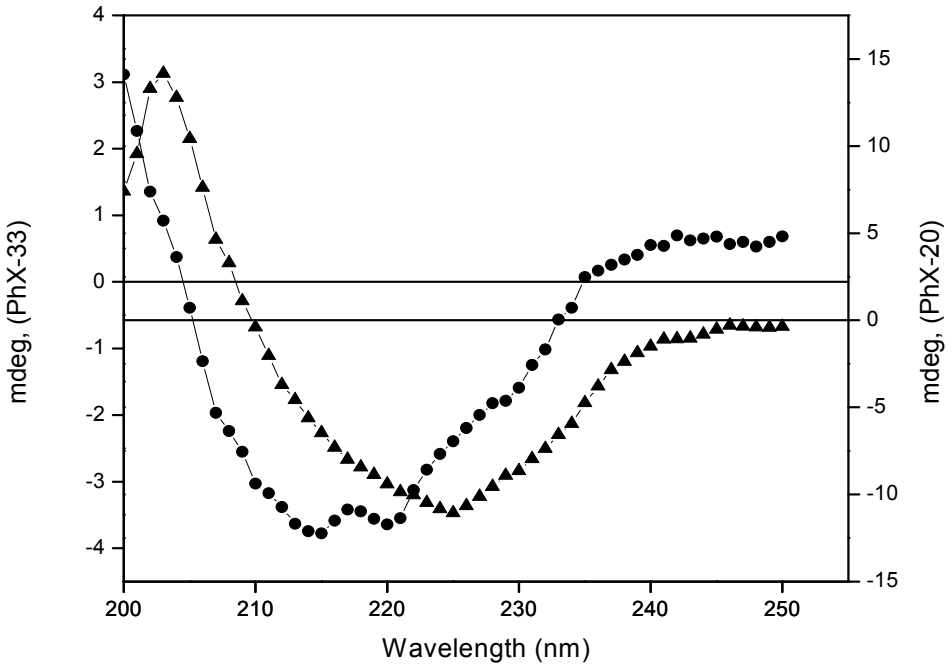


Fig. 6.3 *CD spectra of purified native PhX33 (■) and PhX20 (▲) Xylanases from Pseudozyma hubeiensis at 25^{0}C.*

The secondary structure determined by CD spectra, which shows that PhX-33 shows predominant alpha helix and PhX-20 shows the predominant beta sheet (figure 6.3). Table 6.4 summarizes the total amino acid content for both the xylanases.

Table 6.4 Analysis of total amino acid residues per mole of xylanases

Amino acids	Residues/mole	
	PhX-33	PhX-20
As(x)	45	25
Gl(x)	26	16
Ser	30	17
His	4	2
Gly	26	32
Thr	21	11
Ala	40	23
Arg	8	4
Tyr[a]	7	6
Cys[b]	-	-
Val	19	15
Met	4	2
Trp	5	2
Phe	12	10
Ile	15	6
Leu	21	8
Lys	16	7
Pro	15	10
Total	~314	~196

a-Determined by Spande T. F and Witkop B method, b-not determined.

The pKa values were determined by Dixon plot. It was found that the PhX-33 has pKa value 5.7 and PhX-20 has 4.2. The graph doesn't found to a dome shaped it is a sharp peak. Only one pKa value was found in both the enzymes indicates that the only one amino acid is involved in catalysis (Figure 6.4)

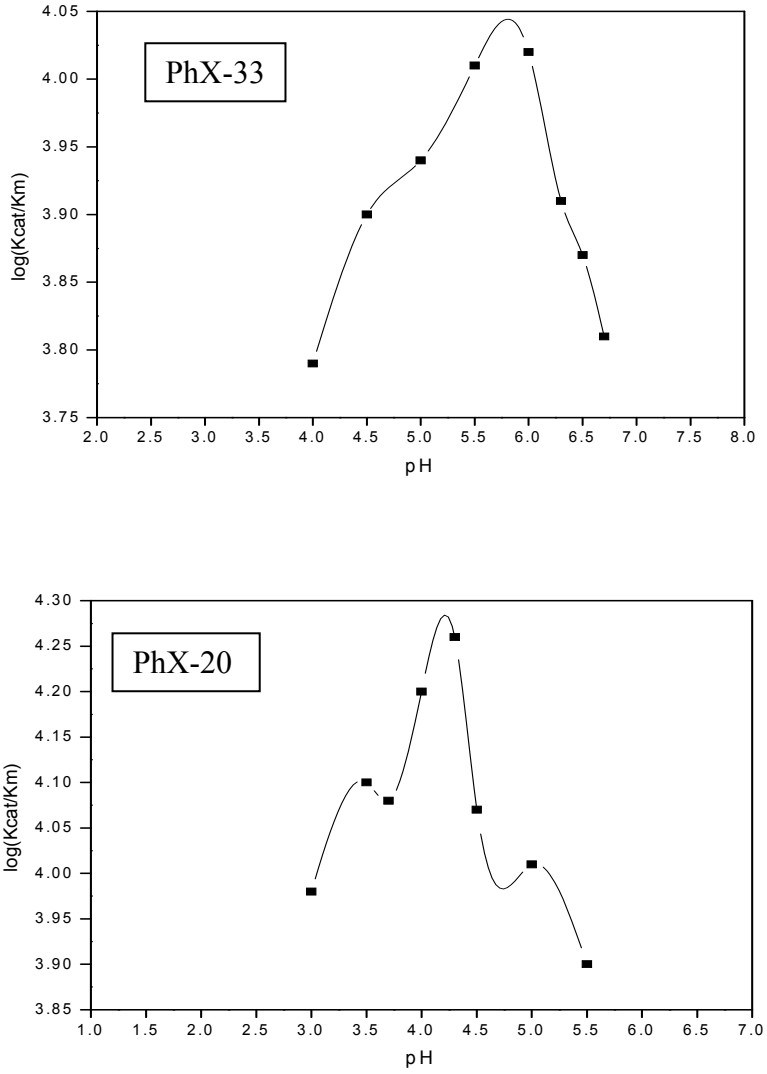


Fig. 6.4 *Determination of pKa: pH dependent Km and Kcat values were determined from Lineweaver-Burk plot by varying substrate concentration at each pH*

Analysis of catalytic amino acids

The table 6.5 shows the modification of the side groups of the amino acids of the two xylanases with side chain specific reagents at specific conditions. Among the reagents tested only NBS and EDAC inactivate the both enzymes and DEP inactivates the PhX-20 only.

Table 6.5 Effect of group specific chemical modifying reagents on activities of yeast xylanases.

Modifying reagents	Concentration	Possible amino acid modification	Buffer system	Residual Activity (%)	
				PhX-33	PhX-20
EDAC	100mM	Asx/Glx	MES/HEPES (75:25), pH 6.0, 50mM	30	20
DEPC	5mM	His	Sodium phosphate, pH 7.0, 50mM	90	5
NBS	1μM	Try	Sodium acetate, pH 4.5, 100mM	0	0
NEM	10mM	Cys	Sodium phosphate, pH 7.5, 50 mM	100	100
Iodoacetate	10mM	Cys	Tris-HCl, pH 8.6, 50 mM	100	93
PCMB	50mM	Cys	Sodium acetate, pH 5.5, 50mM	60	50
NAI	10mM	Tyr	Sodium borate, pH 7.5, 50mM	100	100
PMSF	10mM	Ser	Sodium phosphate, pH 7.5, 50mM	90	85
Phenylglyoxal	10mM	Arg	Sodium bicarbonate buffer, pH 8.5, 100mM	100	75
2,3 Butanedione	50mM	Arg	Sodium borate, pH 7.5, 50mM	100	100
TNBS	1mM	Lys	4% sodium bicarbonate pH 8.4	70	10
Citraconic anhydride	50mM	Lys	Sodium bicarbonate pH 8.4, 100mM	90	100

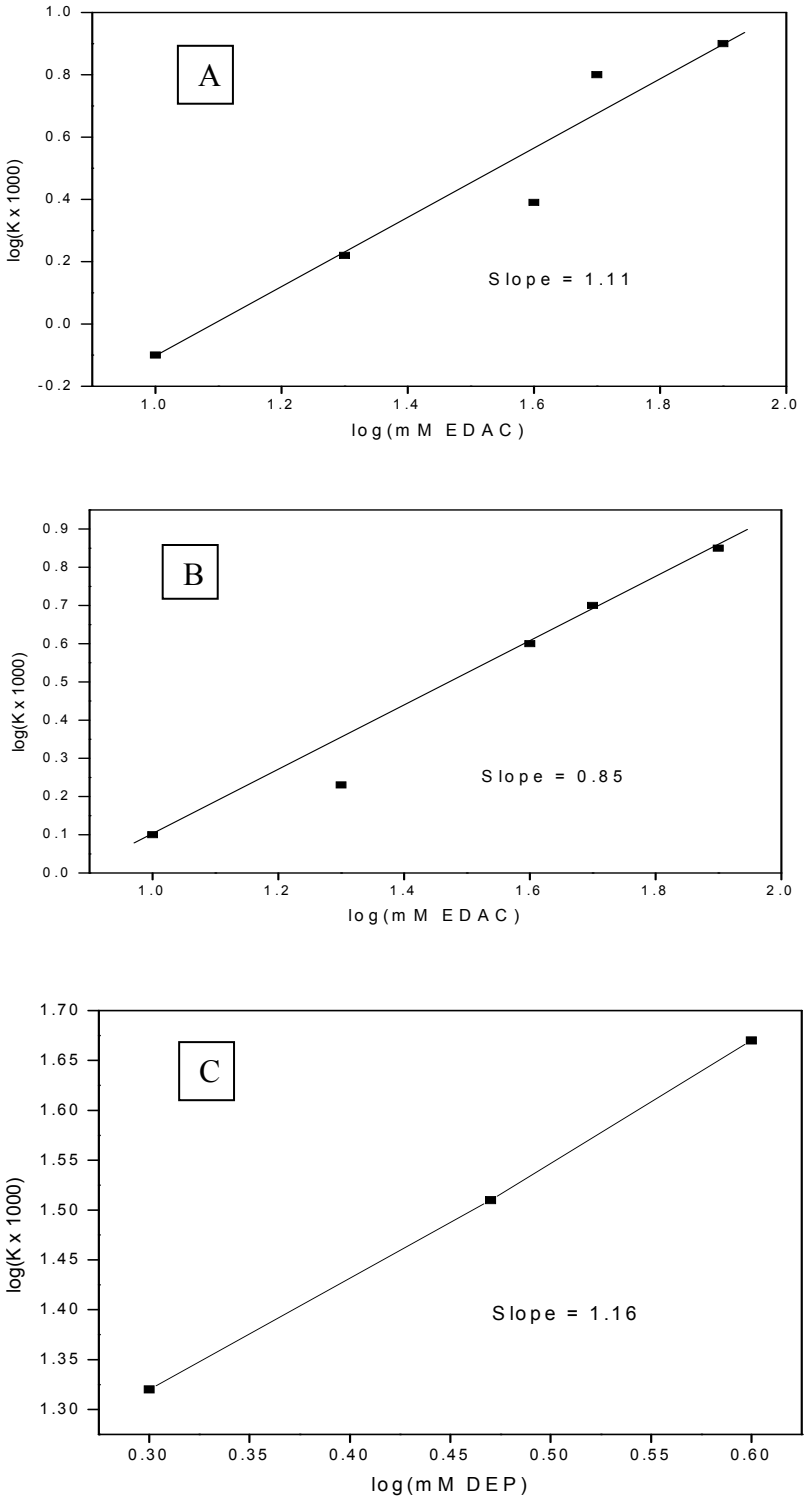
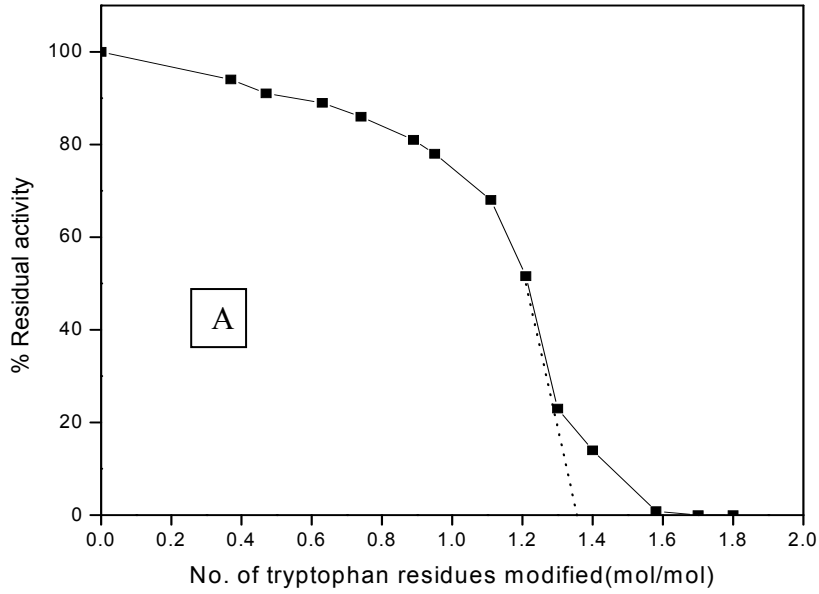


Fig. 6.5 Double logarithmic plot of pseudo first order rate constant (Kapp) and amount of modifying reagents. A) Determination of carboxyl group of PhX-33 by EDAC B) determination of carboxyl group of PhX-20 by EDAC C) determination of histidine residue of PhX-20 by DEP.



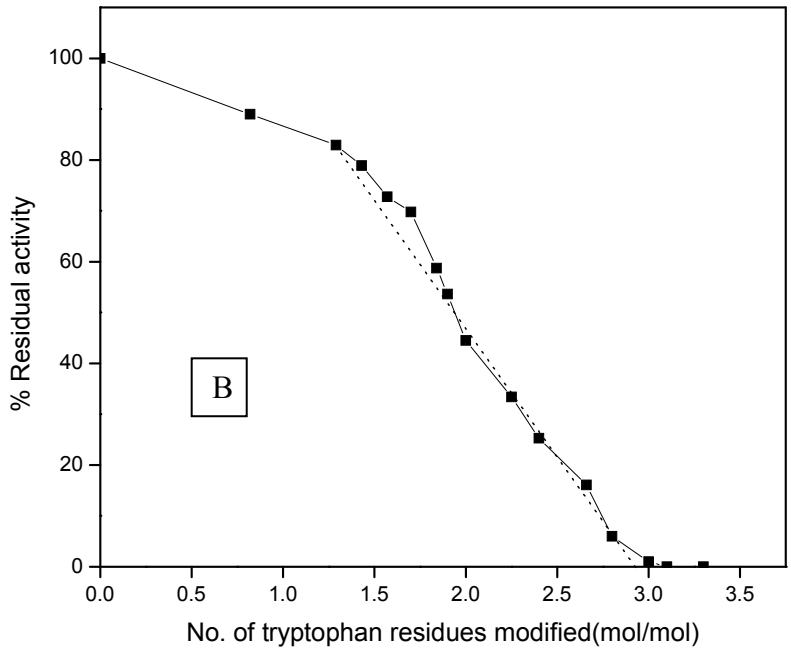


Fig. 6.6 *Titration of Xylanases A) PhX-33, B) PhX-20 with NBS for determination of essential tryptophan residues*

Table 6.6 Substrate protection studies.

Amino acid	Reaction system	Residual activity (%)	
		PhX-33	PhX-20
Tryptophan	Buffer + 10µg enzyme	100	100
	Buffer + 10µg enzyme + 10µM NBS	0.0	0.0
	2.5mg xylan + 10µg enzyme +10µM NBS + Buffer	91	100
Carboxyl group	Buffer + 10µg enzyme	100	100
	Buffer + 10µg enzyme + 80µM EDAC	0.0	0.0
	Buffer + 2.5mg xylan + 10µg enzyme +80µM EDAC	5.3	1.0
Histidine	Buffer + 10µg enzyme	-	100
	Buffer + 10µg enzyme + 10µM DEP	-	0.0
	Buffer + 2.5mg xylan + 10µg enzyme +10µM DEP	-	20

Table 6.7 Km and Kcat values of partially modified xylanase with NBS, EDAC and DEP.

Xylanase	Modifying reagent	Residual activity (%)	Km (mg/ml)	Kcat (min^{-1})
PhX-33	None	100	7.6	8.3×10^4
	NBS	45	20	8.3×10^4
		30	33	8.3×10^4
	EDAC	80	7.6	5.8×10^4
		60	7.6	4.8×10^4
PhX-20	None	100	11.1	2.0×10^5
	NBS	80	15.3	2.0×10^5
		48	25	2.0×10^5
		30	40	2.0×10^5
	EDAC	90	11.1	1.5×10^5
		75	11.1	8.88×10^4
		60	11.1	3.55×10^4
	DEP	60	14	2.0×10^5
		44	20	2.0×10^5
		35	25	2.0×10^5

Table 6.8 Amino acids present at active site of xylanases and their role

Xylanase	Amino acids present at active site	Number of residues	Role of amino acid
PhX-33	Tryptophan	Three	Substrate binding
	Carboxyl group (aspertic acid or glutamic acid)	One	Catalysis
PhX-20	Tryptophan	One	Substrate binding
	Carboxyl group (aspertic acid or glutamic acid)	One	Catalysis
	Histidine	One	Extended substrate binding

Modification of Carboxylate

The modification of both the purified xylanases with 100mM of EDAC resulted in 70 to 80% inactivation within 30 min. (Table 6.5), which shows that the carboxyl group containing amino acids are involved in the active site. The inactivation was dependent on the concentration of the EDAC added. In control there was no loss of enzyme activity detected. For the analysis of the number of carboxyl groups involved in active the xylanases were titrated with different concentration of EDAC with different time interval. There was a gradual decrease in activity with respect to time was found. The second order rates constant were calculated from slope of line of each concentration. The double logarithmic plot of EDAC Vs second order rate constant shows the straight line (figure 6.5). The slope of this line is corresponding to the number of carboxyl group involved in the active site, which is 1.11 for PhX-33 (figure 6.5A) and 0.85 for PhX-20 (figure 6.5B). This shows that only one residue is involved at active site of both the xylanases.

The preincubation of enzyme (substrate protection study) with excess of xylan could not prevent the inactivation (Table 6.6). And also the Km and Kcat values of partially EDAC inactivated xylanases were analyzed (Table 6.7), which shows that the Km remain same in both modified and native xylanases but Kcat decreases(Table 6.7). Both the result suggests that the carboxyl group is involved in the catalysis in case of both the xylanases.

Modification of Tryptophan

The NBS modification of xylanases resulted in the total loss of the enzyme activity with 1μM of reagent (Table 6.5). Both the xylanases inactivates within a few seconds. The inactivation was not dependent on the time. The NBS mediated inactivation was monitored at 280nm and the modified tryptophan residues were calculated as mentioned in materials and methods. The plot of residual activity versus number of tryptophan residues modified(figure 6.6A) shows that the total two residues were modified out of which only one is responsible for the activity in case of the PhX-20. The figure 6.6B shows total three residues required for the PhX-33 xylanase activity. The substrate protection studies (Table 6.6) shows that the preincubation of xylanases with excess amount of xylan prevent total loss of inactivation. More than 90% protection was observed in both the xylanases. The Km and Kcat values of partially modified xylanases indicate that the NBS modification affects only Km but not the Kcat values (Table 6.7). Both substrate protection studies and kinetic analysis suggests that the tryptophan involved in the substrate binding or stabilization of the enzyme substrate complex.

Modification of Histidine

Only in case of the xylanase YX-20 the DEPC inactivate at 5mM concentration within 30 min (Table 6.5). The titration of PhX-20 with DEPC (1 to 5 mM) at different time interval shows the gradual decrease in activity with time. The second order rate constants were calculated as above. The double logarithmic plot shows the straight line and the slope is 1.16. This indicates the only one residue is required for the activity. The little protection to the inactivation was found if enzyme preincubated with xylan (Table 6.6). The kinetic constant of partially modified PhX-20 shows only Km is affected but not the Kcat (Table 6.7). This suggests the histidine may be involved in extended substrate binding or for the stabilization of enzyme substrate complex.

Table 6.8 shows the summary of amino acids, their number and role in active site of both PhX-33 and PhX-20 xylanase.

Hydrolysis product analysis

Both the xylanases hydrolyze the xylan in to the xylooligosaccharides. The masses of hydrolysis product observed in MALDI-TOF MS spectra's are calculated and are corresponding to the theoretical masses of the xylooligosaccharides (figure 6.7). Some times the xylooligosaccharides forms adduct with sodium present in the buffer system. The theoretical and observed masses with calculation are shown in table 6.9. The PhX-33 hydrolyze the xylan in to Xylotriose, xylotetraose and xylopentaose and PhX-20 in to Xylotriose, xylotetraose , xylopentaose, xylohexaose and xyloheptaose. Along with these xylooligosaccharides the acidic xylooligosaccharides are also produced, these are O-methylglucuronic acid, ferulic acid and p-cumaric acid containing xylooligosaccharides (Table 6.9 and Fig.6.7). The acetylating xylooligosaccharides was also detected in the spectra's.

HPLC and TLC analysis shows no xylose and xylobiose was formed. In case of PhX-20 xylanase the arabinose was detected in HPLC analysis, which indicated that, it may be because of arabinopyranosidase activity.

Table 6.9 Identification of masses observed in MALDI-TOF MS spectra

Name of xylooligosaccharide	Theoretical Mw	Observed Mw		Calculations
		PhX-20	PhX-33	
Xylotriose	414.39	413.2	413.32	(150.13x3)-(2x18)
Xylotriose	437.38	437.15	437.25	(150.13x3)-(2x18)+22.99
Xylotetraose	546.5	544.95	545.01	(150.13x4)-(3x18)
Xylotetraose	568.8	569.1	569.20	(150.13x4)-(3x18)+22.99
Xylopentaose	701.11	701.64	-	(150.13x5)-(4x18)+22.99
Xylopentaose	714.6	714.88	714.95	(150.13x5)-(4x18)+22.99+15-1
Xylohexaose	833.77	833.11	-	(150.13x6)-(5x18)+22.99
Xyloheptaose	965.9	965.09	-	(150.13x7)-(6x18)+22.99
4-O-methyl glucuronoxylotriose or ferulic acid + acetyl group	619.53	619.46	619.54	(150.13x3)-(2x18)+(194.14+15-1)+15-18
4-O-methyl glucuronoxylotriose or ferulic acid + three acetyl group	647.53	647.48	647.55	(150.13x3)-(2x18)+(194.14+15-1)+15-18+15-1+15-1

H_2O= 18, xylose=150.13, Na=22.99, CH_3=15, 194.14+15-1= 4-O-methylglucuronic acid

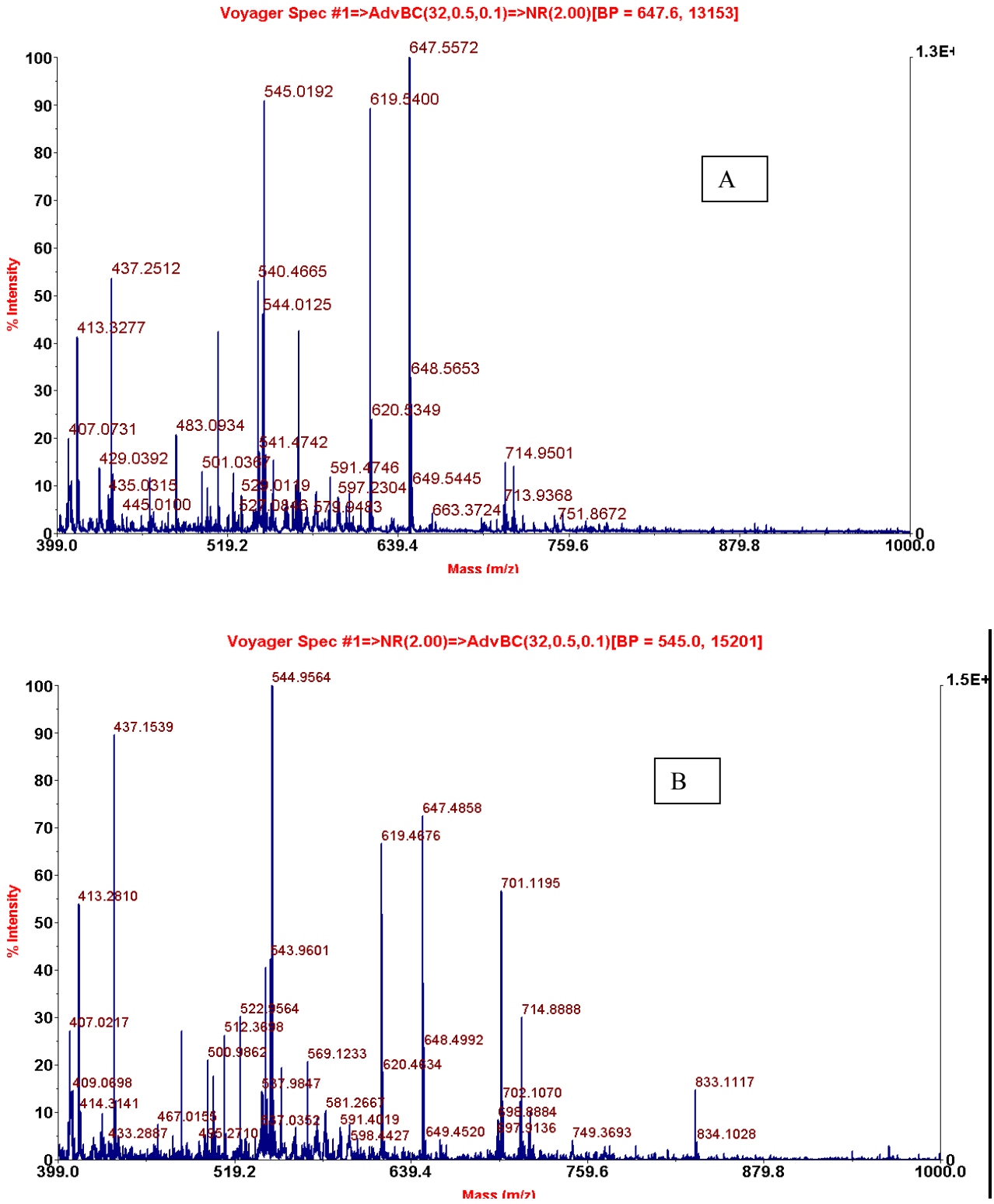


Fig.6.7 *MALDI-TOF mass spectra of Hydrolysis product of soluble oat spelt xylan by A) PhX-33 and B) PhX-20.*

DISCUSSION

The yeast strain KY-1 isolated 1990 and found to produce Xylanases (Bastawde et al., 1994). This is the first report xylanases from *Pseudozyma hubeiensis* with higher extracellular secretion and higher productivity. The information of xylanases from yeast species is very few as compare to the filamentous fungi and bacteria. Earlier only xylanases form the psychophilic yeast *Cryptococcus sp.* was well described. The multiplicity of the enzyme is also found in this case. The purification of xylanase is a two step process. In one step the both the xylanases separate out from the other extracellular proteins. There is nothing wrong of using these enzymes combinely as an application point for xylooligosaccharides (XOS) production. Previously many xylanases are described which produces XOS but they always produces xylose with XOS but not only XOS .The cold adapted xylanases from *Pseudoalteromonas haloplanktis* also produces XOS but they are not stable at higher temperature and they also gives xylose at late hydrolysis (Collins et al., 2002). PhX-33 and PhX-20 are thermozymes i.e. stable and active at higher temperature. Their apparent Km values on soluble olt spelt xylan are higher than the other xylanases, which are generally 1 to 7 mg/ml. The higher Km values are also reported in coldadapted xylanase from *Pseudoalteromonas haloplanktis* (16)**,** xylanases from *Acrophiulophora nainana* (Salles et al., 2000), *Trichoderma reesei* (Tenkanen et al., 1992), and *Streptomyces* T7 (Keskar, 1992). This higher requirement of xylan for saturation may be due to the different mode of hydrolysis giving more XOs production than xylose as a end product which is not the case of other xylanases.

The tryptophan and carboxyl group containing amino acids amino acids present in active site of xylanases are more common but their number of residues varies from enzyme to enzyme. The number of tryptophan found to be more in case of PhX-33, may be because it produces chain of xylose unit and that may require more aromatic residues to bind and stabilize the long chain. In case of PhX-20 only one tryptophan is identified in binding. To stabilize long chain of XOs it may takes help from a single histidine residue. The chemical modification studies are the preliminary studies for the identification of groups involved in the catalysis. The combination of these studies with the determination of three-dimensional structure has helped to ascertain the role of some crucial residues at the active site (Balakrishnan et al., 2006). Generally in the acid base

catalysis two groups are involved in catalysis, one act as a neucleophile and another act as acid base catalyst. But in this case of xylanases we found only one residue. The actual mechanism of hydrolysis using one residue can not be predicted without the confirmation by three-dimensional structure analysis of both the xylanases.

Both the xylanases are totally different from each other as compared with mode of action, pH, temperature optimum, etc. There stabilities are somehow similar at wide range of pH. Both are active at acidic pH, this helps its utilization in the acidic environment of probiotic microflora at the intestine or acidic environment of lactic acid bacterial in vivo. Both the enzymes can be used in combination, in vivo or in vitro, as per the need as a prebiotics. The mode of action of these xylanases is typically different than the xylanases from the other fungi, bacteria and streptomyces. Other xylanases always produces the xylose with XOs (Akpinar et al., 2007) but the xylanases from *Pseudozyma hubeiensis* KY-1 produces XOs with degree of polymerization ranges from three to seven. This is one of the novelty if these xylanases.

Generally the xylanases are more discussed or studied for the application in the paper and pulp industries, but we are diverting their application in prebiotics or as dietary fibers. The hemicellulosic biomass is abundantly available in nature and this biomass not only utilized for biofuels and bioenergy but also a food additives (Moure et al., 2006). The routine method for the production of XOs is acid hydrolysis of hemicellulose, which is not an environmentally feasible method (Sun et al., 2002; Alonso et al., 2003). Recently three methods are incorporated includes the transglycosylation reaction using β-xylosidases, use of glycosyltranferases and synthesis of XOs by using glycosynthase (Endo and Koizumi, 2000; Perugino et al., 2004; Kim et al., 2006)enzymes. Both the methods are costly and require pure monosaccharides.. Recently the importance of XOs is more as compare to other oligosaccharides in terms of health related products,prebiotics and concentration thresholds, but their comparatively high production costs are hindering a wider and faster market development (Barreteau et al., 2006; Mussatto and Mancilha, 2007). For this purpose, further improvements in processing technology would be necessary. Use of these xylanases for XOs production from hemicellulose will be more feasible environmentally and economically. It was also found that xylooligosaccharides are more effective than the froctooligosaccharides in reducing

the colonic lesion development in rats (Hsu et al., 2004). . And also xylooligosaccharides exerted a preferential stimulatory effects on numbers of the health-promoting microorganisms, while xylooligosaccharides inhibited populations of potential pathogens at relatively low level (Rheu et al., 2002). The health-promoting microorganisms such as *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Lactobacillus casei* and *Lactobacillus acidophilus* grew more effectively by xylooligosaccharides than by other carbon source (isomaltooligosaccharides, fructooligosaccharides, sucrose,etc), though xylooligosaccharides inhibited the growth of *Clostridium perfringens*, *Bacteroides fragilis*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhumurium*

The acidic xylooligosaccharides are also has potential importance in health as compare to the plain xylooligosaccharides. The oligosaccharides containing O-methylglucuronic acid, p-cumaric acid or ferulic acid shows strong suppressive activities against various types of tumors. Previously the acidic XOS from wood hemicellulose, *Hericium erinaceum*, *Chamomilla recutita*, *Plantago species* were also reported for anti-inflamatory and antitumor activities (Whistler et al., 1976; Hashi and Takeshita, 1979; Yamada et al., 1985; Mizuno et al., 1992; Samuelsen et al., 1995). The preventive effect of these acidic xylooligosaccharides against contact hypersensitivity was also investigated in mice and suggested that the daily intake of these may prevent contact hypersensitivity in humans (Yoshino et al., 2006).

Japan and US are the leading countries in incorporation and commercialization of oligosaccharides as a functional foods e.g. in combination with soya milk, soft drinks, tea or cocoa drinks, nutritive preparations, dairy products with milk, milk powder and yoghurts, candies, cakes, biscuits, pastries, puddings, jellies, jam and honey products, and special preparations for health food for elder people and children) or as active components of symbiotic preparations (Mussatto and Mancilha, 2007). Apart from prebiotics it is also employed in cosmetics as stabilizers, bulking agents, immunostimulating agents, antioxidant and in pharmaceutical (Moure et al., 2006).

Apart from the XOS production these xylanases (crude preparation) are already used for the removal of hemicellulosic fraction from agro waste material and for the preparation of carbohydrate free (hemicellulose) lignin derived from sugarcane bagasse

(Gokhale et al., 1998; Singh et al., 2005). We are proposing another application includes the purification of the hemicellulose free cellulose derived from natural sources (e.g. sugarcane bagasse, wheat straw etc), which has valuable applications in polymer science as a precursor for synthesis of cellulosic derivatives, by using these cellulase free xylanases.

Further studies, in particular of its three-dimensional structure, the actual number of amino acids residues involved at active site and their function, should give detailed information on the structure-function relationship of these novel xylanases.

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CONCLUSIONS

- A mutant, EMS-UV-8, of *P. janthinellum* capable of showing enhanced zone of hydrolysis on Walseth cellulose was isolated using EMS treatment followed by UV-irradiation of the spores.
- Successive mutation and selection resulted in isolation of two promising mutants, one selected on the basis of Avicel hydrolysis (EU1) and the other on the basis of hydrolysis of Walseth cellulose in presence of 2-deoxy-D-glucose (EU2D-21).
- All these mutants produced two fold filter paper and CMCase activities than the parent strain. These promising strains could be used for large scale production of cellulases by solid state or submerged fermentation.
- Enzyme preparation derived from EU1 mutant hydrolyzed Avicel to greater extent. Such enzyme preparations could be used to hydrolyze highly crystalline cellulosic materials.
- Bagasse derived cellulose, at high concentration (10%), was hydrolyzed by cellulase enzyme preparations (10 FPU/g of cellulose) derived from mutants. We obtained maximum hydrolysis (72%), yielding glucose and cellobiose as the main end products. Such simple sugars have high value for production of value added products such as ethanol, lactic acid.
- Lactic acid was produced from bagasse derived cellulose sample by simultaneous saccharification and fermentation (SSF). A maximum lactic acid concentration of 67 g/L was produced from 80 g/L of bagasse cellulose, the highest productivity and yield being 0.93 g/L/h and 0.83 g/g, respectively.
- Here, we propose to valorise this biomass to produce cellulose and then sugars, which can be fermented to products such as ethanol and lactic acid.
- We also observed that, *Lactobacillus delbrueckii* mutant Uc-3 utilizes cellobiose efficiently indicating possible presence of β-glucosidase.
- *Lactobacillus delbrueckii* mutant Uc-3 is a promising strain for the production of lactic acid from cellulosic materials in SSF.

- Bottlenecks, like feedback inhibition by glucose and cellobiose, were removed by using such a strain, leading to the complete conversion of cellulosic substrates to lactic acid.
- Two Xylanases from *Pseudozyma hubeiensis* were biochemically and biophysically characterized.
- The data of peptide mass fingerprinting and pattern of xylan hydrolysis showed that these xylanases are new.
- We were the first to report that these xylanases only produce xylooligosaccharides (degree of polymerization 3-5 and 3-7) without formation of xylose and xylobiose. These xylooligosaccharides has applications in health as a prebiotics or as a dietary fiber or as a functional food.

PUBLICATIONS

Publications from thesis work

1. **Mukund G. Adsul,** K.B. Bastawde, D.V. Gokhale. (2009) Biochemical Characterization of two xylanases from yeast *Pseudozyma hubeiensis* producing only Xylooligosaccharide. **Bioresource technology**. 100(24), 6488-6495. **(Best paper award From NCL Research foundation-2009)**
2. **Mukund Adsul**, Jayant Khire, Kulbhushan Bastawde and Digamber Gokhale.(2007) Lactic acid production from cellobiose and cellotriose by *lactobacillus delbrueckii* mutant Uc-3. **Applied and Environmental Microbiology.** 73: 5055-5057
3. **Mukund G. Adsul**, Anjani J. Varma, Digamber V. Gokhale. (2007) Lactic acid production from waste sugarcane bagasse derived cellulose. **Green Chemistry**. 9:58-62. **[Top 10 article (4th among top 10)-feb-2007)].**
4. **M.G. Adsul**, K.B. Bastawde, A.J. Varma, D.V. Gokhale. (2007) Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. **Bioresource technology**, 98(7), 1467-1473.

Other related publications:

1. **M. G. Adsul,** M. S. Singhvi, S. A. Gaikaiwari and D.V. Gokhale (2011) Development of biocatalyst for production of commodity chemicals from lignocellulosic biomass. ***Bioresource Technology.*** (Accepted). doi:10.1016/j.biortech.2011.01.002
2. **Mukund G. Adsul**, Diego A. Rey, Digambar V. Gokhale (2011) Combined strategy for the Dispersion/dissolution of single walled carbon nanotubes and cellulose in water. ***Journal of Materials Chemistry***, 21, 2054-2056.

3. M. S. Singhvi, **M. G. Adsul,** D.V. Gokhale (2011) Comparative production of cellulases by mutants of *Penicillium janthinellum* NCIM 1171 and its application in hydrolysis of Avicel and cellulose. ***Bioresource Technology***. (Accepted). doi:10.1016/j.biortech.2011.01.014
4. Mamata Singhvi, Dipti Joshi, **Mukund Adsul**, Anjani varma, Digambar Gokhale. (2010) D(-) Lactic acid production from cellobiose and cellulose by using *Lactobacillus lactis* mutant RM2-24. **Green Chemistry**. 12(6), 1106
5. **Adsul M. G**., Terwadkar, A. P., Varma, A. J., and Gokhale, D. V. (2009). Cellulases from *Penicillium janthinellum* mutants: Solid-state production and their stability in ionic liquids***, BioResources,*** 4(4), 1670-1681.
6. Arti Dumbrepatil, **Mukund Adsul**, Shivani Choudhary, Jayant Khire, and Digamber Gokhale (2008) Utlization of molasses sugar for lactic acid production by *Lactobacillus delbrueckii* mutant UC-3 in batch fermentation. **Applied and Environmental Microbiology.** 74: 333-335.
7. **M.G. Adsul**, J.E. Ghule, R. Singh, H. Shaikh, K.B. Bastawde, D.V. Gokhale, A.J. Varma. (2005) Enzymatic hydrolysis of delignified bagasse polysaccharides. **Carbohydrate Polymers***,* 62:6-10. **Most accessed** (**Among Top 25 it was on 13thposition**) **article within this journal, Apr-Jun 2006].**
8. **M.G. Adsul**, J.E. Ghule, R. Singh, H. Shaikh, K.B. Bastawde, D.V. Gokhale, A.J. Varma. (2004) Polysaccharides from bagasse: applications in cellulase and xylanase production. **Carbohydrate Polymers,** 57:67–72. **[Most accessed (Among Top 25 it was on 5thposition) article within this journal, Oct 2004-Jan 2005].**
9. Anil H. Valekar, Chandrashekhar M. Malba, **Mukund G. Adsul**, Digambar V. Gokhale, and Anjani J. Varma. (2010) Organic solvent facilitates acid hydrolysis of pretreated sugarcane bagasse polysaccharides for production of ethanol. **Trends in Carbohydrate Research**, 2(3), 10-14.
10. Sathish Yadav, **Mukund G. Adsul**, Kulbhushan B. Bastawde, Digambar V. Gokhale. (2011) Differential induction, purification and characterization of cold active lipase from *Yarrowia lipolytica* NCIM 3639. (Communicated).