Production of microbial xylanase under submerged fermentation of agro-residues and its application in xylitol production

Thesis submitted to the University of North Bengal
For the Award of Doctor of Philosophy in Biotechnology

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DECLARATION

I hereby declare that the thesis entitled **"Production of microbial xylanase under submerged fermentation of agro-residues and its application in xylitol production"** has been prepared by me under the guidance of Dr. Shilpi Ghosh, Associate Professor, Department of Biotechnology, University of North Bengal.

No part of thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Xylan, the second most abundant plant polysaccharide comprising a backbone of β-1,4 linked xylopyranosyl residues. It is a hetero polysaccharide that contains repeating group of acetyl, 4-O-methyl-D-glucuronosyl and α-arabinofuranosyl residues linked to the backbone and has binding properties mediated by covalent and non covalent interactions with lignin, cellulose and other polymers. Xylanases are glycoside hydrolases group of enzymes that depolymerize the glycoside linkages in the heteroxylan backbone. The complete depolymerization of xylan requires the interaction of a number of the main-chain and side-chain cleaving enzyme activities, of which endoxylanase (E.C.3.2.1.8), exoxylanase (E.C.3.2.1.37) and β-xylosidase play vital role. These enzymes are of great importance in several industries such as in the paper pulp bleaching industries, animal feed industries, etc. Production cost of xylanase can be reduced by using highly abundant, low cost and easily available xylan rich agro residues as raw material. In India, an agriculture based country, these agro residues are generated throughout the year.

In the present study, two potent xylanase producing microorganisms were isolated from garden soil. They were identified as *Penicillium citrinum* xym2 and *Bacillus subtlis* xym4, on the basis of phylogenetic analysis of 28S rDNA and 16S rDNA sequences, respectively. Both the isolates were employed for production of xylanases under submerged fermentation of various agro residues and both of them showed a significantly higher xylanase yield on wheat bran as compared to that of expensive birchwood xylan. Optimization of cultural conditions and media parameters for both the isolates through one factor at a time (OFAT) and response surface methodology (RSM) approach considerably increased the production of xylanases by them. Consequently, xylanase production by *P. citrinum* xym2 was enhanced from 712 IU/ml (unoptimized) to 1853 IU/ml (OFAT) to 2834 IU/ml (RSM), whereas the enzyme production by *B. subtilis* xym4 was enhanced from 981.5 IU/ml (unoptimized) to 2100 IU/ml (OFAT) to 11800 IU/ml (RSM). *P. citrinum* xym2 also produced 1492 IU/ml of carboxymethyl cellulose, on the other hand, *B. subtilis* xym4 produced significant amount of β-xylosidase and FPase with marginal cellulase activity. Xylanase enzyme cocktail obtained from the *Penicillium* (XEC^P) and *Bacillus* (XEC^B) were further employed in saccharification of wheat bran for liberation of reducing sugar. Reducing sugar released by XEC^B was further optimized through Plackett-Burman (PB) design and RSM. Using these statistical approaches resulted in release of 11.5 mg/l of sugar. The sugar rich broths obtained after XEC^P and XEC^B treatments were fermented to

xylitol by *E. coli* xyl6 with xylitol concentration of 3.2g/l and 4.5 g/l, respectively. *B. subtilis* xym4 and *E. coli* xyl6 were used in a simultaneous saccharification and fermentation (SSF) experiment under various conditions. SSF resulted in markedly higher amount of xylitol production (98.4g/l) under condition4 where *E. coli* xyl6 added to the fermentation medium 24 h after the inoculation of *B. subtilis* xym4.

PREFACE

Xylanses are the important group of carbohydrate active enzymes which have wide range of industrial applications due to its capability to depolymerize xylan, the second most abundant polysaccharide on earth. The enzyme is used for production of fermentable sugars, deinking and recycling of waste paper, biobleaching of paper pulps, treatment of livestock feeds, as an ingredient in detergent for stain removal and many more. In nature xylanases are produced by wide variety of bacteria and filamentous fungi. This industrially important enzyme can be produced in a cost effective manner by using inexpensive carbon sources to support the growth and metabolic activities of xylanase producing microbial strains. Agro residues are low cost xylan rich raw materials that can be used to achieve this goal. Agro residues generated from farming are recycled either by using them as feed for livestock or applying them back in the same field as organic manure. However, due to technological advances the farming has become highly mechanized and depends mainly on commercial fertilizers and thus agriculturally generated crop residues now largely being accumulated as wastes and creates environmental pollution. Lignocellulose present in these agro residues are potential source of fermentable sugar for production of several value added products. Xylitol, a five carbon sugar alcohol, is used as dietary sugar substitute or artificial sweetener. It is widely used in food and pharmaceutical industries due to low calorie content, anti cariogenicity, tooth rehardening, preventive against otitis, ear and upper respiratory infection etc. Due to its health promoting effect the demand of xylitol in the global market is increasing rapidly. Industrial production of xylitol is based on catalytic hydrogenation of pure xylose under high temperature and pressure, which is highly expensive. Therefore, biotechnological intervention for xylitol production is most warranted. Lignocellulosic xylan polymers can be depolymerized by microbial xylanases to xylose that can act as substrate for enzymatic conversion to xylitol. Therefore, the research work presented herein aimed to isolate microorganisms from various environmental samples with the capability to produce xylanase and to convert xylose to xylitol. The production process of microbial xylanase was optimized through conventional one factor at a time as well as by statistical approaches using wheat bran as low cost substrate in submerged fermentation. The xylanases produced were characterized and employed for saccharification of wheat bran for reducing sugar released. The released sugars were further fermented to xylitol in sequential and simultaneous saccharification and fermentation experiments.

ACKNOWLEDGEMENT

"Gratitude can transform common days into thanks giving, turn routine jobs into joy and change ordinary oppurtunities into blessings" – William Arthur Ward

With this, I would like to express my sincere gratitude to all those who have helped me and supported me in overcoming the obstacles and achieving the goal I had set.

I feel myself really privileged to have the oppurtunity to work under the able guidance of my esteemed supervisor Dr. Shilpi Ghosh, Head, Department of Biotechnology, University of North Bengal, West Bengal. Words are actually not enough to describe the constant efforts and inspiration she has given me. Her perfection, vigilant supervision and constructive discussion are the factors which helped me to do hard work and accompolish the work. Iam really grateful to her for having faith on me which is an ignition for me everytime while achieving this scientific goal.

I solicit my obligation and express my heartfelt thanks to Dr. Ranadhir Chakraborty, Professor, Department of Biotechnology, University of North Bengal, West Bengal. He has been always a source of inspiration for me. I acknowledge my sincere thanks to Dr. Dipanwita Saha and Dr. Anoop Kumar, Assistant professors, Department of Biotechnology, University of North Bengal, West Bengal for their valuable suggestions and guidance.

I would like to express my gratitude to Vice Chancellor, Dean science and Registrar, University of North Bengal for permitting me to conduct the research work in the University.

I am also thankful to Dr. Arindam Bhattacharjee, Head, Department of Microbiology, for his constant support and valuable suggestions.

Thanks are also due to all the members of Non-teaching and technical staffs of the department for their help and support.

I express my special thanks to my labmates Ms. Khusboo Lepcha, Mr. Ayan Mahanty, Mrs. Vijeta Rai, Ms. Arijita Basak, Mr. Parmeshwar Vittal Govande and Mr. Vaskar Das for their non-stop contribution and selfless help extended to me.

It would be inappropriate if I do not mention the name of Mr. Sudipta Das for his extensive help and constant support.

No choice of words can add to adequately express my love and gratitude to my father Late. Alokananda Saha, who left me at a very early age but the lessons he imparted to me can never leave me till death.

I would like to express my thanks to my beloved wife cum labmate Mrs. Deepika Mazumdar for helping me in my works and supported me in my difficult times. She has

always given me a constant support and motivation for thinking innovatively while conducting the work.

I bow in gratitude to the Almighty God for my existence as *Homo sapiens* and the strength, knowledge, ability and oppurtunities, He has given to me to fulfill my dreams.

At last, I would like to dedicate this piece of work to my mother Smt. Chhaya Roy Saha who has always seen her dreams in me. God cannot always take care of us and so has send mothers who always do anything without hoping, in return. I would not have been what I am today. My mother is the one who never gave up in her hard times and fought for the existence of both of us. It is her genes in me which has lead me to achieve this aim.

Thanks to everyone,

Shyamaprasad Saha 19/4/18

Shyama Prasad Saha

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Chapter 1

General introduction and Review of Literature

1.1 Introduction

A wide range of biotechnologically important products used in the food, chemicals, pharmaceutical and allied industries are produced by enzymatic biocatalysis of several organic compounds (Uday *et al.* 2016, Bhunia *et al.* 2013, Ratledge and Kristiansen 2008). According to a report, the approximate world market of enzymes was US $30–60 billion, in the year 2010 (Savitha *et al.* 2007). A/S (Denmark), Danisco/Genencor (Denmark and USA), BASF (Germany) and DSM (Netherlands) are the major industries that control about 73% of enzyme market in the world (Uday *et al.* 2016, Jaouadi *et al.* 2008, Ratledge *et al.* 2008). The largest group of industrially important enzymes (75%) belong to the class hydrolases and among these glycoside hydrolase class of enzymes have been marked as the second largest group of industrially important enzymes produced by wide range of bacteria, fungi, animal and plant cells (Bhat 2000). Cellulases and hemicellulases are the two important enzymes of glycoside hydrolase group that can depolymerize cellulose and hemicellulose, respectively. The above two polymers are generally found in lignocellulosic components of plant cells (Bhat 2000). In nature, hemicellulose is the most abundant polysaccharide after cellulose, that mostly contains xylan made up of β-D-xylopyranosyl residues linked via β-1, 4-glycosidic bonds (Saha 2003, Beg *et al.* 2001). The exact amount of xylan present in plant cell wall differs from plant to plant; however, it comprises approximately 30-35% of the total dry weight of plant (Beg *et al.* 2001). In India, an agriculture based country, various agro residues and byproducts such as wheat bran, sugar cane bagasse, corn cobs, rice bran, water hyacinth, corn fiber etc., are generated throughout the year (Cano *et al.* 2007). The improper management of these agro residues creates environmental pollution (Uday *et al.* 2016). Therefore, these inexpensive and easily processed agro residues can be used for production of value added glycoside hydrolase group of enzymes. Due to its immense industrial applications such as in animal feeds, release pentose sugars, bio bleaching of wood pulps, food additives in baking industry, production of xylitol, ethanol, ingredients in laundry detergents or fabric care compositions etc., xylanase production from agro residues has been reported in various work (Khanahmadi *et al.* 2018, Adhyaru *et al.* 2014). Endo-xylanases (EC 3.2.1.8) are important group of industrially applicable enzymes that act in association with β-xylosidase, α-L-arabinofuranosidase and α-glucuronidase for complete breakdown of plant cell

wall polysaccharide to xylo-oligosaccharide and xylose (Collins *et al.* 2005, Chavez *et al.* 2004). Bacteria, fungi, actinomycetes and yeast are reported to produce different family of xylanases (Adhyaru *et al*. 2016, Irfan *et al.* 2016, Thomas *et al.* 2014). Bacteria, due to their faster growth rate and easy to cultivate, are widely used as large scale producers of extracellular xylanases (Aarti *et al.* 2015, Nagar *et al.* 2013). Among the xylanase producing bacteria, *Bacillus* sp has been studied extensively for industrially important xylanase production (Khusro *et al.* 2016, Irfan *et al.* 2016, Thomas *et al.* 2014, Adhyaru *et al.* 2014). On the other hand, filamentous fungi due to their higher rate of production over yeast and bacteria are used in various work as source of xylanase (Ribeiro *et al.* 2014, Sakthiselvan *et al.* 2014, Knob *et al.* 2013, Polizeli 2005).

Xylitol is a naturally occurring sugar alcohol, with sweetness equivalent to sucrose but less calorie content (Edelstein *et al.* 2008, Maguire and Rugg-Gun, 2003). Xylitol has some exclusive health promoting effects such as it can prevent tooth decay and ear infection in infants, used as a substitute of sucrose for diabetic patients due to its non involvement with insulin metabolic pathway, has a low glycemic index, its non fermentable nature retard the formation of bacterial dental plaque in oral cavity and anti cariogenic property and lack of anti nutritional effect on food (Ritter *et al.* 2013, Uittamo *et al.* 2011, Zacharis *et al.* 2012, Chen *et al.* 2010, Khalid *et al.* 2012, Islam 2011). In the recent past, demand for xylitol has steadily increased by 6% per annum owing to its multiple benefits, primarily in the food markets. Two major factors have contributed to the escalating demand; firstly, health concerns (driving the growth of alternative sweetener market) and secondly, increasing awareness of naturally derived low calorie sugar free chemicals. Globally, xylitol has vast market comprising an annual demand of 161.5 million metric tons (MMT) equivalent to a value of US$670 million (in 2013), and forecasted to reach 250 MMT by 2020, nearly 1.5 times its current requirement with an overall market value of US $1 billion (http://www.prnewswire.com). 70% of the global xylitol consumption accounts for manufacturing chewing gums and confectionery products (http://www.marketresearch.com/product/42sample-8164119.pdf). M/s DuPont Danisco, is the leading xylitol manufacturer globally, with plants located in Finland, USA and China (http://www.dupont.com), and followed by M/s Xylitol Canada Inc. in terms of production capacity (Dasgupta *et al.* 2017). Industrially xylitol is produced by catalytic hydrogenation of pure D-xylose solution under high temperature and pressure. With requirement of high purity feed and severe reaction conditions, the process is very expensive and energy intensive. As a counter measure, alternative cost effective production strategies through biotechnology route have been undertaken by the scientific fraternities with attention towards cheap source of xylose and its bio catalytic conversion into xylitol under ambient conditions. Lignocellulosic biomass

from agro-industrial waste was considered to be a potential raw material in this regard (Cheng *et al.* 2014, Franceschin *et al.* 2011).

In the present study, microorganisms isolated from environmental samples were screened for xylanase production followed by characterization of the best producers. Xylanase production by the selected microorganisms were optimized with respect to growth conditions and media composition, using agro residues as sole carbon source under submerged fermentation (SmF). Furthermore, the xylanases producer *B. subtilis* xym4 and *P. citrinum* xym2 were employed in for optimization of sacchrification of agro residues to fermentable sugars. Finally, the reducing sugars obtained after saccharification were used for xylitol production using *E. coli* xyl6. Moreover, wheat bran was subjected to simultaneous saccharification and fermentation (SSF) by *Bacillus* and *E. coli* for xylitol recovery.

1.2 Objectives

1. To isolate the xylanase producing microorganisms from garden soil or other environmental samples.
2. To determine the identity of the isolates with biochemical and molecular techniques.
3. To determine the effect of cheap agro residues on xylanase production under submerged fermentation (SmF) and comparison with production in birchwood xylan, by the isolates.
4. To optimize the culture conditions for enhanced xylanase production with step wise modification of culture media under SmF of agro-residues.
5. To optimize the culture conditions for enhanced xylanase production using statistical model.
6. To purify xylanase from selected isolate by using methods based on solubility and chromatographic techniques and characterization of the enzyme.
7. To optimize the parameters for saccharification of agro residues by xylanase.
8. Isolation and identification of xylitol producing microorganism using biochemical and molecular techniques.
9. Application of xylitol producing microorganism for xylitol production from saccharified broth of agro residues under submerged fermentation.
10. Simultaneous saccharification and fermentation (SSF) of agro residue with xylanase and xylitol producing microorganisms for xylitol production.

1.3 Review of Literature

1.3.1. Xylan structure

Lignocelluloses are mainly composed of cellulose (45-55 %), hemicelluloses (25-35 %) and lignin (20-30%). All these components are major polymers of plant cell wall. Being the second most abundant plant polymer after cellulose, hemicelluloses comprise 25-35 % of weight of terrestrial plants and agro-residues (Mosier *et al.* 2005, Saha 2003, Sun and Cheng 2002). Structurally hemicellulose is a heteropolysaccharide with β-1,4 linked D-xylopyronoside residues as back bone called xylan. In soft wood, xylans make 5-15 % of the total plant dry weight. Depending on the plant varieties and plant tissues the xylose unit of the β-1,4 linked back bone chain often substituted with O-acetyl groups at O-2 and/or O-3, branched (1-3 units/branch) with L-arabinofuranoside (arabinoxylan), or D-glucuronic acid (glucuronoxylan) residues, which can be further linked with O-acetylated or O-methylated at C-4. D-galactose and ferulic acid moieties are also found linked with L-arabinose residues of xylan (Ebringerova 2006, Mosier *et al.* 2005, Saha 2003, Scheller and Ulvskov 2010). Representative chemical structure of xylan is illustrated in the figure 1.1.

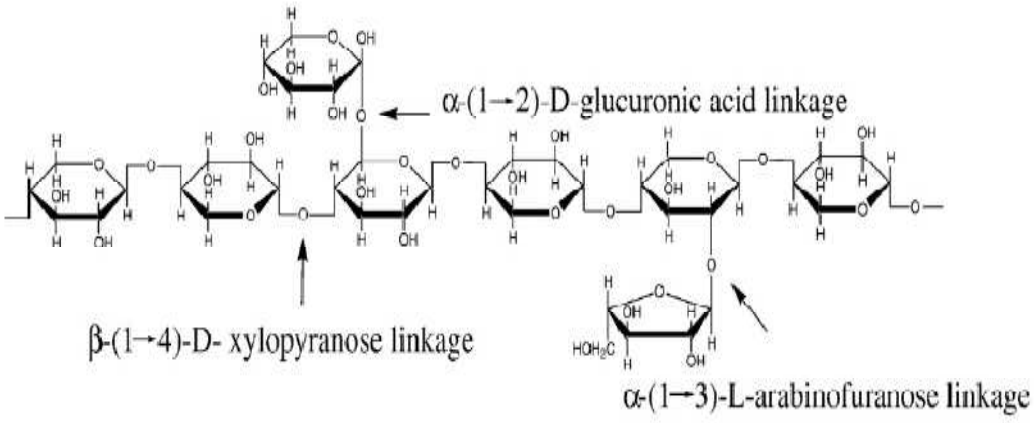


Fig.1.1. Chemical structure of xylan with different side chains attached (Bastawde 1992).

1.3.2. Xylanase and their mode of action

An array of enzymes with vast specificities and mode of actions are required for complete and efficient hydrolysis of xylan heteropolymer. Endo-1,4-β-D-xylanase (E.C. 3.2.1.8) cleaves the xylan back bone randomly and liberates xylooligosaccharides, xylan-1,4-β-xylosidase (EC

3.2.1.37) cleaves xylooligosaccharides from the non reducing end and release xylose monomers while the phenolic and acetyl side groups are removed by the catalytic action of α-L-arabinofuranosidases (EC 3.2.1.55), α–D-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.6) and ferulic acid esterases (EC 3.1.1.73). All these enzymes function synergistically on heteroxylan for its efficient depolymerization (CoIlins *et al.* 2005, Beg *et al.* 2001, de Vries *et al.* 2000, Uffen 1997).

1.3.2.1. Endo-1,4-β D-xylanase (E.C. 3.2.1.8)

The most important xylan degrading enzyme is endo-1,4-β-D-xylanase and it catalyzes the hydrolysis of internal glycosidic bonds in a highly specific manner. The catalytic activity of the enzyme depends on the length and the degree of branching or the presence of specific substituents in the xylan polymer (Coughlan *et al.* 1992).

1.3.2.2. Xylan 1,4-β-xylosidase (E.C. 3.2.1.37)

This is an exo acting enzyme that mainly hydrolyses xylobiose or xylooligosaccharides, and its specific affinity with disaccharides is higher than that of oligosaccharides (Panbangred *et al.* 1984). Hydrolysis of 1,4-β-D-xylooligosaccharides from the non reducing ends by the enzyme liberates xylose monomers. This enzyme has been reported to be present in the microorganisms as monomeric, dimeric or tetrameric forms with molecular weight between 26-360 kDa (lUBMB, 1982).

1.3.2.3. α-L-arabinofuranosidases (E.C. 3.2.1.55)

The terminal, non reducing α-L-arabinofuranosyl groups of arabinans, arabinoxylans, and arabinogalactans are hydrolysed by α-L-arabinofuranosidases (EC3.2.1.55) and liberate arabinose (Gomes *et al.* 2000). Wide range of microorganisms including fungi, actinomycetes and other bacteria has been reported to produce α-arabinosidases (Saha 2000).

1.3.2.4. α–D-glucuronidases (E.C. 3.2.1.139)

The enzyme catalyzes the hydrolysis of α-1,2 linkages between glucuronic acid and xylose residues in glucuronoxylan. Due to the stability of α-1,2 linkages over β-1,4 linkages the enzymatic reaction has been recognized as most crucial for xylan degradation. A number of microorganisms are reported to produce α–D-glucuronidases (Puis 1997).

1.3.2.5. Acetyl xylan esterases (E.C. 3.1.1.6)

Baiely *et al.* (1986) first reported the production of acetyl xylan esterase by cellulolytic and hemicellulolytic fungi, *Trichoderma reesei, Aspergillus niger, Schizophyllum commune* and *Aureobasidum pullulan*. It acts on both xylan and xylooligomers and removes the O-acetyl group from the C-2 and C-3 position of xylose residues (Degrassi *et al.* 2000, Joselau *et al.* 1992).

1.3.2.6. Ferulic acid esterases (E.C. 3.1.1.73)

Removal of ferulic acid moiety from arabinoxylans is catalysed by ferulic acid esterases. Among the fungal species *A. oryaze* was found to be an efficient producer of ferulic acid esterases (Tenkanen *et al.* 1995). Being an acidic monomeric protein with pI 3.6 and mol. wt. 30 kDa, it also liberated p-coumaric and acetic acids from heat treated wheat straw arabinoxylan. The enzyme also hydrolyse of ester bond between hydroxycinnamic acids and sugars in the plant cell walls (Crepin 2004). Figure 1.2 depicts the mode of action of different kinds of xylanases on xylan polymer.

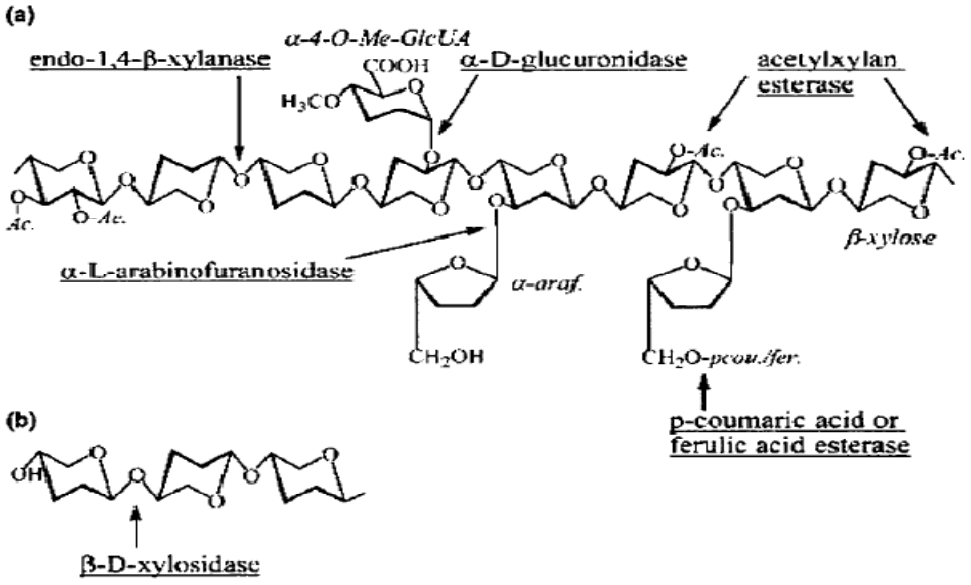


Fig 1.2. (a) Xylan structure of cereals, and mode of action by different xylanolytic enzymes (b) Mode of action of β-xylosidase (Collins *et al.* 2005).

1.3.3. Classification of xylanase

Carbohydrate active enzyme (CAZy) database (http://www.cazy.org) categorize xylanases (EC 3.2.1.8) under glycoside hydrolase (GH) families 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 62 and 98. Families 16, 51 and 62 are composed with bi-functional xylanase enzymes with two catalytic domains, whereas families 5, 7, 8, 10, 11 and 43 are composed of enzymes having a true single catalytic domain with endo-1,4-β-xylanase activity (Collins *et al.* 2005). The residual or secondary xylanase activity was recorded for the enzymes belonging to GH families 9, 12, 26, 30 and 44. However, based on the hydrophobic cluster analysis of the catalytic domains and amino acid sequences similarities, xylanases have been primarily grouped into GH families 10 and 11 (Verma *et al.* 2012). Although the members of these two families have been studied extensively, the information regarding the catalytic activity and chemical properties of the members of other GH families (5, 7, 8 and 43) are very recent and limited (Taibi *et al.* 2012). Collins *et al.* (2005) observed that the members of families 5, 7, 8, 10, 11 and 43 are widely different with respect to their structure, mode of action, substrate requirement and physicochemical properties. The GH 10 family contains the enzymes having high molecular mass, cellulose binding domain (CEBD) and a catalytic domain (CD). The CEBD and CD are connected by a linker peptide which has the isoelectric point (pI) between 8 and 9.5. This family usually has a $(\beta/\alpha)_8$ fold TIM barrel. On the other hand, GH 11 family enzymes have low molecular weight and low pI values. GH family 11 is further subdivided into alkaline and acidic enzyme groups, based on their isoelectric points (Buchert *et al.* 1995, Ahmed *et al.* 2009, Juturu *et al.* 2012).

1.3.3.1. Family 5 glycoside hydrolase

Despite having very limited information the characteristic endo mode of action GH family 5 has been extensively investigated. α-1,2-linked glucuronate moiety directed site specific catalytic hydrolysis of β-1,4 xylan chain is a distinctive ability of this family. The cloning of GH 5 xylanase C (XynC, 90.86 kDa) from *Bacillus subtilis* 168 and its overexpression and crystallization have been done. The crystallographic study revealed its exceptional specificity and catalytic depolyerisation of glucuronoxylan (John *et al.* 2009). Figure 1.3 depicts the biological assembly image of XynC.

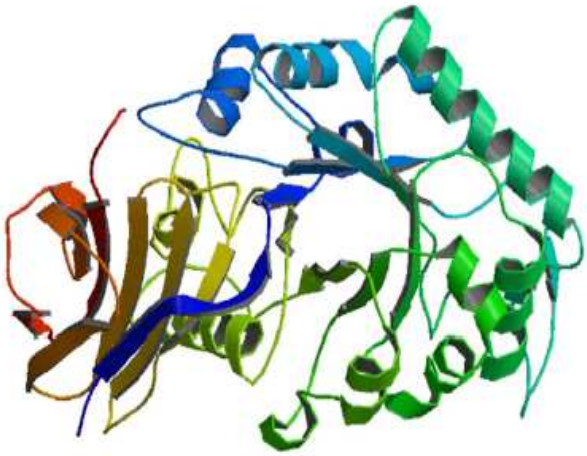


Fig. 1.3 Model assembly image of crystal structure of XynC isolated from *Bacillus subtilis* 168. Protein chains are coloured from the N-terminal to the C-terminal using a rainbow (spectral) colour gradient (http://www.rcsb.org/pdb/home/home.do).

1.3.3.2. Family 8 glycoside hydrolase

The capability to hydrolyse the β-1,4 xylopyranoside chain of xylan is also a characteristic mode of action of this family. The substrate binding to the enzyme active site is highly specific and follows classical induced fit mechanism. The catalytic events are followed by conformational changes of enzyme in number of times due to the proper shiftment of proton donar into a more catalytically competent position. The bacterium *Pseudoalteromonas haloplanktis* TAH3a, isolated from the Antarctic, has been thoroughly studied for its pXyl, the GH family 8 xylanase (Fig. 1.4.) The X-ray chrytallographic study of the enzyme pXyl (kDa 47.34) complex with its substrate xylopentaose and product xylotriose, reveled that subsites from -3 to +3 are mainly responsible for the structure function relationship. Moreover it has been described in the report that the xylan hydrolysis by GH 8 family is preceded by conformational changes with out the ground state conformation of substrate (Vos *et al*. 2006).

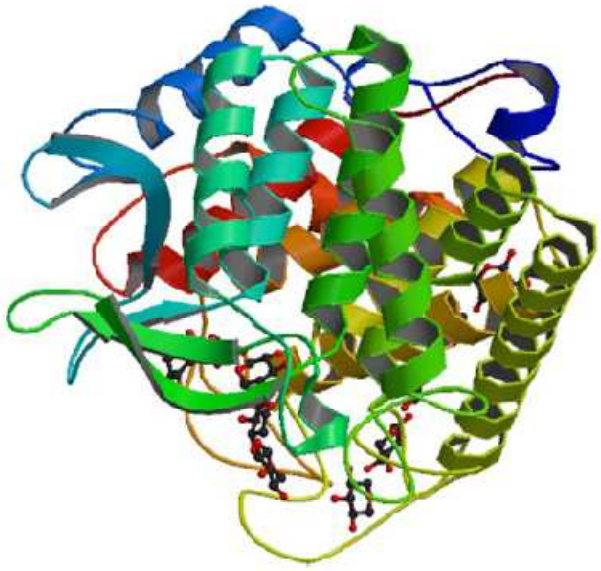


Fig. 1.4. Model assembly image for a cold adapted GH 8 family xylanase in complex with substrate. Protein chains are coloured from the N-terminal to the C-terminal using a rainbow (spectral) colour gradient) (http://www.rcsb.org/pdb/home/home.do).

1.3.3.3. Family 10 glycoside hydrolase

Santos *et al.* (2010) isolated the TpXyl10B (81.62 kDa), a GH 10 family xylanase, from the hyperthermophilic bacterium *Thermotoga petrophila* RKU-1 (Fig. 1.5). Biochemical characterization and crystallographic studies of the enzyme complex with xylobiose in native state at high temperature revealed that there are two catalytic subunits available for bonding with the substrate. Due to the coupling effect of temperature induced structural change the enzyme showed a temperature dependent mode of action. Molecular dynamics simulations method further confirmed the temperature dependent mode of action and also suggested that the release of xylobiose at high temperature is tightly regulated by the significant modification of the catalytic loop Trp297-Lys326 (Santos *et al.* 2010).

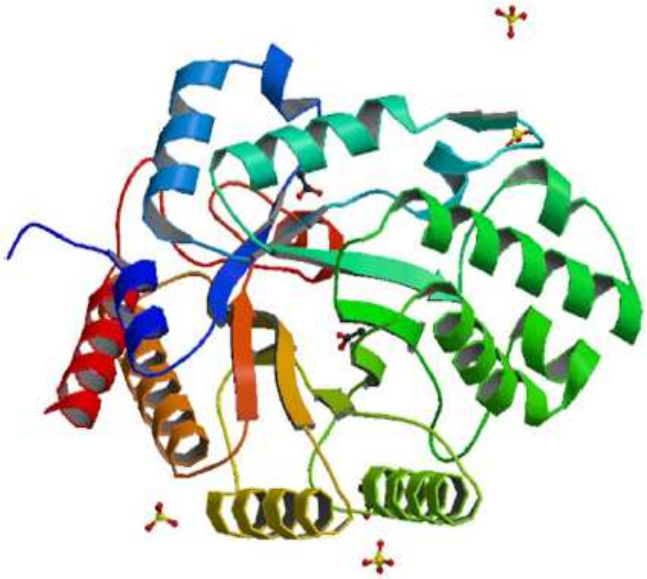


Fig. 1.5. Model assembly image for crystal structure of native xylanase 10B from *Thermotoga petrophila* RKU-1. Protein chains are coloured from the N-terminalto the C-terminal using a rainbow (spectral) colour gradient) (http://www.rcsb.org/pdb/home/home.do).

1.3.3.4. Family 11 glycoside hydrolase

Xylanase enzyme BCX (20.7 kDa) isolated from *Bacillus circulans* by Joshi et al. (2000) was found to be a member of GH family 11 (Fig. 1.6). The enzyme bears an asparagines residue at position 35 and found to influence the pH dependent activity change. This is due to the ionization of the glutamate acid residue at position 78 and 172 with respective pKa values of 4.6 and 6.7. When the asparagines residues at position 35 was substituted with aspartic acid residues, the resulting enzyme N35D BCX showed 20 % increment in its activity as compared to BCX and it was also observed that the optimum pH was shifted from 5.7 to 4.6. The pKa valuesof the enzyme N35D BCX were recorded in the range of 3.5-5.8 and found to follow the double displacement mode of action like that of the native BCX. In this mechanism glutamate at position 78(Glu 78) act as a nucleophile whereas aspartic acid residue at position 35 (Asp 35) and glutamate at position 172 (Glu 172) function together as the general acid/base catalyst. The higher catalytic activity of the mutant N35D BCX was due to the fact that it could catalyzes the reaction through reverse protonation where protonated Asp35 and deprotonated Glu78, stimulate the activity (Joshi *et al.* 2000).

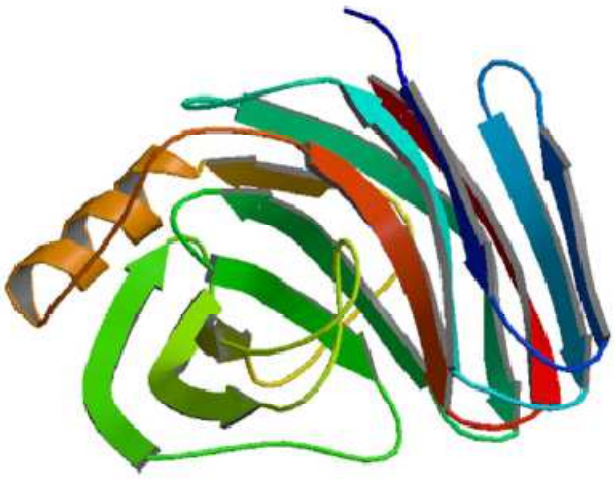


Fig. 1.6. Model assembly image for a GH 11 family xylanase. Protein chains are coloured from the N-terminal to theC-terminal using a rainbow (spectral) colour gradient) (http://www.rcsb.org/pdb/home/home.do).

1.3.3.5. Family 19 glycoside hydrolase

Biochemical analysis and crystal structure study of Xyl-ORF19 (kDa 41.18), an enzyme of GH family 19, was isolated by Han *et al.* (2013) from bacteria isolated from the termite (*Globitermes brachycerastes*) gut. Two domines were reported for the Xyl-ORF19, the catalytic daomin, made up with a C-terminal GH 10 and a non catalytic bacterial Ig-like (Big 2) domain (Fig 1.7). The catalytic domain has a $(\beta/\alpha)_8$ barrel which is similar to the barrel found in the enzymes of GH10 fmily.In addition to that two extra β-strands are also present in that domain. The non catalytic domain is closely similar to that of the immune glubuline like domain of intimins. If the non catalytic domain of the enzymes was removed the catalytic efficiency of the enzymes in terms of kinetic parameters was greatly reduced and alteration its biochemical parameters interms of pH and temperature profiles was altered (Han *et al.* 2013).

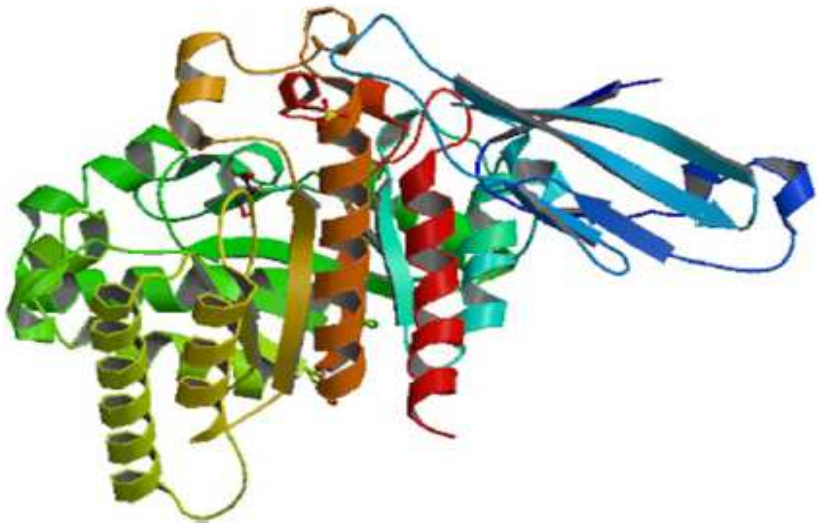


Fig. 1.7. Model assembly image for GH 19 xylanase from bacteria of termite gut. Protein chains are coloured from the N-terminal to the C-terminal using a rainbow (spectral) colour gradient) (http://www.rcsb.org/pdb/home/home.do).

1.3.3.6. Family 30 glycoside hydrolase

The enzyme glucurono xylanase, Xyn30D (kDa 44) belongs to the glycoside hydrolase family 30. It contains a catalytic domain having a carbohydrate binding module (CBM) closely related with CBM35 family (Fig. 1.8). The enzymes contains an $(\beta/\alpha)_8$ barrel as a catalytic domain similar to that of GH family 10 xylanases with an additional β-peptide structure. Two calcium ions are present between the β-sandwich of CBM35 domain. These two domain fold in an independent manner and they are much flexible. The flexibility is due to the presence of linker polypeptide region between them which makes a polar interaction with the catalytic domain. The binding affinity of the enzyme (Xyn30D-CBM35) is highly specific for the substrate with conjugated glucuronic acid moiety. This is occurring due to presence of two successive aromatic amino acid residues in the catalytic pocket. In this pocket a non conserved glutamate residues at position 129 (Glu 129) is also present which binds to the calcium ions (Sainz-Polo *et al.* 2014).

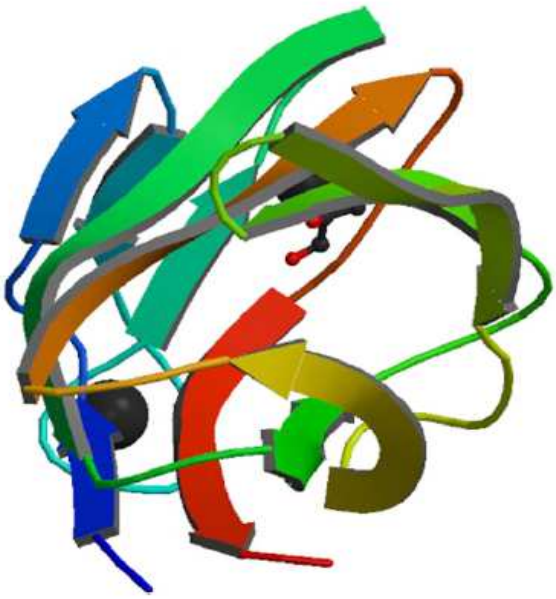


Fig.1.8 Model assembly image for GH family 30 xylanases of CBM35 from *Paenibacillus barcinonensis* (protein chains are coloured from the N-terminal to the C-terminalusing a rainbow (spectral) colour gradient). (http://www.rcsb.org/pdb/home/home.do).

1.3.4. Sources of xylanase

Xylanase, an important group of carbohydrate active enzyme are produced by both prokaryotic and eukaryiotic organisms. It has been reported from marine and terrestrial bacteria, fungi, algae, protozoa, snails, insects and germinating seeds of terrestrial plants (Walia *et al.* 2013a). Apart from soil bacteria, several marine bacteria and cyanobacteria are also reported as xylanase producers (Annamalai *et al.* 2009). Information regarding the presence of xylanase in Japanese pear fruits and in higher animal such as mollusks has also been cited in literature (Yamaura *et al.* 1997). Certain uncommon sources such as immature cucumber seeds and germinating barley were reported as a source of xylanase enzyme (Sizova *et al.* 2011).

1.3.4.1. Bacterial xylanase

A numbers of *Bacillus* species are potential producer of xylanase. *B. circulans, B. stearothermophilus, B. amyloliquefaciens, B. subtilis, B. pumilus, Geobacillus thermoleovorans* and *B. halodurans* were cited in various reports as a significant and considerable producer strain (Subramanian and Prema 2002, Verma and Satyanarayana 2012, Banka *et al.* 2014, Thomas *et al.* 2014, Gupta *et al.* 2015). The bacterial strains *Bacillus* sp, *Stenotrophomonas maltophila, Rhodothermus marinus, Thermotoga* sp, *Clostridium thermocellum* and *Streptomyces* sp, were

found to produce thermostable xylanases that were active in the temperature range 60 - 70 ^{o}C (Kumar and Satyanarayana 2013, Thomas *et al.* 2014). Psychrotrophic or cold adapted xylanase was found to be produced by the bacterial strains such as *Flavobacterium frigidarium* and *Clostridium* sp. PXLY1 (Akila and Chandra 2003, Humphry *et al.* 2001). In terms of industrial production of xylanase, bacterial sources have certain advantages over that of fungi, like optimum pH of the bacterial xylanases is in neutral or alkaline range, whereas for fungal xylanase optimum pH lies in the acidic range thus requiring additional steps in subsequent stages of industrially important process. Moreover, higher growth rate of bacteria increases the volumetric productivity of enzyme in short time (Verma and Satyanarayana 2012). Table 1.1 represents the list of some potential xylanase producing bacteria along with the biochemical properties of the enzyme produced by them.

1.3.4.2. Fungal xylanase

Due to the extracellular secretion of xylanase in the fermentation media in higher titer in comparison to that of bacteria, fungi had been extensively used as a potent source of xylanase enzyme (Polizeli *et al.* 2005). Table 1.2 represents fungi with the ability to produce xylanase and the biochemical characteristics of the enzyme produced by them. Several mesophilic as well as thermophilic fungi are reported to produce xylanase. Among the mesophilic fungi, genera *Aspergillus* and *Trichoderma* and among the thermophilic fungi, genera *Thermomyces* are being the dominant xylanase producers (Kumar *et al.* 2017). Xylanases with high temperature optima were recorded for several fungal strains such as *Aspergillus terreus*, *Penicillium* sp, *Trichoderma* sp, *Thermomyces lanuginosus*, *A.nidulens* etc (Ryan *et al.* 2003, Ghanen *et al.* 2000, Taneja *et al.* 2002). The optimum pH range varied between 4 -7 for xylanase produced by *Gloeophyllum trabeum*, *Humicola insolens* etc. (Kim *et al.* 2014, Du *et al.* 2013) whereas the enzyme from *Malbranchea flava*, *Thermoascus aurantiacus* var.*levisporus* KKU-PN-I2-1 had alkaline pH optima (Ryan *et al.* 2003, Ghanen *et al.* 2000).

Table 1.1
Biochemical characteristics of xylanase produced by bacteria

Bacteria	Substrate	pH	Temp (°C)	Km	Amount	References
Sorangium cellulosum S09733-1	OSX	7	30	38.13	4.11 U/mg	Wang (2015)
Bacillus sp. SN5	Bex	7	40	0.6	104.7 U/mg	Bai *et al.* 2012
Paenibacillus xylanilyticus KJ03	Bix	7.4	40		33U/mg	Park *et al.* 2013
Bacillus sp SV-345	Bix	6.5	50	3.7	2803.1 U/mg	Mittal *et al.* 2012.
Streptomyces sp 76	WB	6	50		79.43U/mg	Bajaj and Singh 2010.
Bacilus sp GRE7	OSX	7	70	2.23	191.1U/mg	Kiddinamoosthy 2008.
Arthrobacter Sp	WB	9	100	0.9	162U/mg	Khandeparkar and Bhosle 2006.
Geobacillus thermoleovorans	Bix	8.5	80	2.6	10.2 U/mg	Verma and Satyanarayan 2012.
Stenrophomonas maltophilia	WB	9	80		82.40U/mg	Raj *et al.* 2013.
Bacillus sp.	OSX	9	55		31.27 U/mg	Annamalai *et al.* 2009.
Actinomadura sp. Strain CPT20	OSX, Bex	10	80		51.06	Taibi *et al.* 2012.
Bacillus brevis	WS	7	55		4380	Goswami *et al.* 2014.
B. pumilus SV-205	WB	10	60		7382.7 IU/ml	Nagar *et al.* 2012.
B. haloduran	CM		80		69 U/ml	Kumar and Satyanarayan 2013.
B. subtilis BS05	SB	5	50		439 IU	Irfan *et al.* 2012.
Paenibacillus macerans		4.5	60		4170 U/mg	Dheeran *et al.* 2012.
Paenibacillus sp.		9	50		24.60 IU/ml	Pathania *et al.* 2012.
Provideacia sp.		9	60		36.3IU/ml	Raj *et al.* 2013.

BiX (birchwood xylan), BeX (Beechwood xylan), OSX (Oat spelt xylan), WB (Whear bran), WS (Wheat straw), CM (Cane molasses), SB (Sugarcane baggase).

Table 1.2
List of fungi producing xylanase with their biochemical characteristics

Fungi	Mw (kDa)	pH	Temp (^{o}C)	Family	References
Aspergillus nidulans KK-99		8	55		Taneja *et al*. 2002
Aspergillus terreus		7	50		Ghanen *et al.* 2000
Acrophialophora nainiana	22	7	55		Salles *et al*. 2000
Myceliophthora sp. IMI 387099	53	6	75		Chadha *et al.* 2004
Penicillium brasilianum	31				Jorgensen *et al.* 2003
Penicillium capsularium	22	3.8	48		Ryan *et al.* 2003
Penicillium sp. 40	25	2	50		Kimura *et al.* 2000
Thermomyces lanuginosus	24.7	6-6.5	70		Singh *et al.* 2000
Gloeophyllum trabeum	50	4-7	70	GH10	Kim *et al.* 2014
Humicola insolens	23	7	60	GH11	Yang *et al.* 2014
Humicola insolens Y1	39-44	6-7	70-80	GH10	Du *et al.* 2013
Malbranchea flava MTCC(MFXI)	25	9	60	GH11	Sharma *et al.* 2010
Malbranchea flava MTCC(MFXII)	30	9	60	GH10	Sharma *et al.* 2010
Thermoascus aurantiacus var. *levisporus* KKU-PN-I2-1	27	9	60		Chanwicha *et al.* 2015
Aspergillus tubingensis FDHN1					Adhyaru *et al.* 2016
Aspergillus tamarii Kita		5.5-6	60		Heinen *et al*. 2017

1.3.4.3. Xylanases from metagenomics libraries

The isolation of novel xylanase enzyme by conventional technique based on the selection of xylanolytic microorganism from environmental samples. But according to the research conducted by Amann *et al.* (1995) it had been obtained that only a small fraction (0.1-1%) of microorganisms were culturable using standard microbiological methods. Whereas with the help of metagenomic approaches a huge genetic diversity was extracted from the environmental samples in short time without laborious microbial culture techniques. Recently, the genes

encoding thermostable xylanases had been extracted from metagenomic library of various echological niches (Table 1.3).

Table 1.3
List of xylanase obtained from metagenomic libraries

Source	Expression host	GH family	Temperature (^{o}C)	pH	References
Volcano crater	*E. coli*	GH10	95	6.8	Mientus *et al.* 2015
Cowdung	*B. megaterium*	GH10	75	7.0	Sun *et al.* 2015
Sugarcane bagasse	*E. coli*	GH11	80	6.7	Kanokratana *et al.* 2015
Hot spring	*E. coli*	GH10	100	5.5-7.0	Sunna and Brqquist 2003.

1.3.5. Xylanase production strategies

Wide range of organisms such as bacteria, fungi, plant and animal cells produce xylanases that are either extracellular or intracellular in nature (Collins *et al.* 2005, Ahmed *et al.* 2009, Knob *et al.* 2010). Due to the several inabilities of plant and animal xylanases, the interest on microbial xylanases has increased to meet the current crisis of energy demand in the world. The improvement of xylanase enzyme production by microorganisms has been based on two major strategies, either through optimization of process parameters for enzyme production by natural wild organisms or by generating recombinant strains.

1.3.5.1. Production using wild strains

Due to the potential industrial applications of xylanase obtained from fungal and bacterial sources, extensive research are being carried out by the scientific communities to improve the xylanase production using wild producers. The process involves preliminary selection of desired microorganism(s) having the potential for novel xylanase production. Also, the enzyme should be free from toxin and undesired products that are produced along with the enzyme. The availability of potent xylanase producer and its applicability as a robust industrially important strain are the main challenges with the wild producer. Although xylanase production by wild producers shows lesser batch to batch variation in xylanase yield, the lower yield using wild producer is a major concern. Moreover, the isolation, screening and production of xylanase with the wild producers through classical microbiological approach is a tedious process. Therefore, the microbial diversity in several ecological niches of hemicellulolytic degradation demands the

proper evaluation for isolation, and screening of new microbial strains with improved industry compatible xylanase production. Several progressive research had been carried out in past for production of xylanase using wild fungal or bacterial cells such as *Burkholderia* sp. (Mohana *et al.* 2008), *Bacillus pumilus* (Battan *et al.* 2008, Poorna *et al.* 2007), *S. actuosus* (Wang *et al.* 2003), *S. cyaneus* (Ninawe *et al.* 2008), *S. matensis* (Yan *et al.* 2009), *Aspergillus niger* (Dobreva *et al.* 2007), *P. thermophila* (Yan *et al.* 2008), *T. longibrachiatum* (Azin et al., 2007), *T. languginosus* (Li *et al.* 2005). Among the microbial strains, filamentous fungal species are reported as potent xylanase producers for commercial applications as they secrete very high amount of xylanase in the fermentation medium. Mesophilic and thermophilic microorganisms were found to secrete significantly high quantity of extracellular xylanase in the medium (Polizeli *et al.* 2005). *Aspergillus*, *Trichoderma* and *Penicillium* species are mesophilic xylanase producers (Taneja *et al.* 2002, Jorgensen *et al.* 2003, Adhyaru *et al.* 2016). Very recently studies have been conducted on extraction of xylanase from thermophilic fungi and as the growth temperature of such fungi is higher, it can be inferred that the enzyme obtained from them also have the high thermostability (Adhyaru *et al.* 2016). Bacterial and fungal xylanases are mainly classified into the glycoside hydrolase family 10 and 11, respectively (Liu *et al.* 2011). Bacterial or fungal endo xylanases are generally produced as monomeric protein with molecular weight ranging between 8.5 to 85 kDa. The enzymes are glycosylated with isoelectric point (pI) ranging between 4.0 and 10.3 (Polizeli *et al.* 2005). Structural comparison between bacterial and fungal xylanases through *in silico* modeling reveals the differences in the secondary structure especially in the loop areas. Xylanase produced by *B. circulans* and *B. subtilis* contained β-pleated sheets in the loop region, whereas the enzyme produced by *Aspergillus niger* mainly contained α-helix in the loop regions. Inspite of having differences in secondary structures both these xylanases were found to contain the glutamic acid residue as nucleophile and proton donor (though the position may change) and thus belong to GH family 11 (Mathur *et al.* 2015). Downstream processing steps are often elabaorative for fungal xylanases as they are produced along with cellulases; on the other hand bacterial xylanases are produced mostly alone and thus shortening the course of downstream processing (Subramanian and Prema 2002).

1.3.5.2. Production using recombinant producer

Recombinant DNA technology (RDT) has been used for modifying the xylanase genes followed by their expression in suitable host. In this strategy the xylanase enzymes with suitable characteristics like theromostability, acid/alkai stability etc. were selected for industrial applications (Li *et al.* 2000). Microorganisms also have the capability to induce xylanase production through synthesis of various kinds of inducers. Therefore, engineering of microbial regulatory pathway for xylanase production happens to be one of the recent and advanced

approach that can improve the xylanase productivity and specificity. The gene encoding xylanase enzyme PoXyn2 (GH 11, 320 aa) was obtained from the cDNA library of *Penicillium occitanis* Pol6, was sub-cloned into the pGAPZαA vector to construct recombinant xylanase (Driss *et al.* 2012). The recombinant PoXyn2 cDNA was ligated to His-tag at the N-terminal end. The resulting construct was further integrated into the genome of *Pichia pastoris* X-33 under the constitutive promoter of glyceraldehyde 3-phosphate dehydrogenase (GAP). The expression of the recombinant xylanase enzyme was confirmed by the activity assay followed by SDS-PAGE analysis. The recombinant enzyme was purified from the cell free supernatant by affinity chromatography on Ni-NTA resin. The engineered *P. pastoris* X-33 was cultivated in the medium containing oat spelt xylan and found to overexpress the PoXyn2 xylanase with specific activity of 8549.85 U/mg (Driss *et al.* 2012).

Overexpression of a xylanase (Xyn 186) from *Alternaria* sp. HB186 in *P. pastoris* GS115 was conducted by Mao *et al.* (2012). Cloning and sequence analysis of the Xyn 186 revealed the presence of an intron of 52 bp within 748 bp nucleotide sequence. The non coding sequence was removed by the restriction digestion using Dpn I and protein encoding cDNA fragment was cloned into the vector pHBM905A. The recombinant vector was inserted into the *P. pastoris* GS115 and the gene copy number was evaluated using real time PCR technique (Mao *et al.* 2012).

1.3.6. Genetic regulation of xylanase

Effect of several carbohydrates along with xylan on genetic regulation and expression of xylanase enzyme has been reported in various scientific reports. It was observed that the microbial strains accomplished xylanase production when they were cultivated in presence of xylan, explaining the induction effect of xylan on xylanase (Biely 1986). An interesting finding was made that xylan having large bulky structure could not directly enter inside the cell to induce the xylanase gene expression. However, some sort of physical contact between the inducer and regulatory apparatus of the cell is required to accomplish xylanase expression and secretion into the fermentation medium. Next, the extracellular enzyme hydrolyses the complex polymers of xylan and liberates the short chain less complex xylooligosaccharides. These products were then transported inside the cell through β-xyloside permeases. The small oligosaccharides now induce the xylanase enzyme expression in a concentration dependent manner (Fig 1.9). In support of the above hypothesis Polizeli *et al.* (2005) observed that not only the xylanase expression but also the permease activity of the cell was increased in presence of xylanolytic inducer. Regulation mechanism for xylanase production has been extensively studied with *Aspergillus* spp. The respective xylanase and β-xylosidase genes xlnA, xlnB, xlnC and xlnD were expressed by the

Aspergillus spp. in presence of xylan and xylose (Colabardini *et al.* 2012). Stricker *et al.* (2008) reported that expression of these genes are under tight transcriptional regulation by transcription factor XlnR and also subjected to catabolite repression mediated by CreA transcription repressor. The repressor protein CreA encodes a 415 amino acid long polypeptide chain having the characteristic features of DNA binding proteins. The presence of zinc fingures motif, alanine rich domain and the occurrence of SPXX and TPXX motifs explaining the strong affinity of this protein to the specific cis acting sites of DNA (Ruijter and Visser 1997). The CreA repressor binds to the CreA binding sites present in the genome of *Aspergillus* spp. (Degraaff *et al.* 1994). The regulation of xylanolytic enzymes by XlnR and CreA ensures a double check mechanism for enzyme expression.

Bacillus subtilis str168 has been studied extensively to understand the regulatory mechanism of xylanase expression. Whole genome sequence analysis of the strain was able to locate the genes for xylanolytic enzyme as well as the transporter protein for xylose uptake. The gene xynA (1241 bp), xynB (1601 bp), xynC (641 bp), and xynD (1241 bp) have been reported as the principal xylanase encoding gene of *B. subtilis* str168 (Fig.1.10a). Endo-1,4-β-xylanase that depolymerises heteroxylan to glucurono xylotetraose and xylooligosaccharides is encoded by xynA gene and is a member of GH 11 family. β-xylosidase is encoded by xynB whereas xynC encodes GH30 family endoxylanase. The product of xynD was found to be active on arabinoxylan. Constitutive expression of xynA has been recorded in contrast to the other extracellular enzymes especially during the logarithmic phase of the bacteria. Expression of β- xylosidase is tightly regulated by the inducer xylose and xylan. The role of xylose as inducer has been previously explained in the study conducted by Gartner et al. (1988) and Hastrup (1988). In a study it was reported that xylA (1337 bp) encodes xylose isomerase while xylulose kinase are encoded by xylB gene. A glycoside pentoside hexuronide transporter protein encoded by xynP (1392 bp) has been found to be present upstream to the xynB gene. The utilization of xylose by xynCB and xylAB has been found to be strongly controlled by transcriptional regulatory protein XylR coded by xylR (1053 bp). The higher amount of XylR negatively regulates the expression of xylAB (Gartner *et al.* 1988, Hastrup *et al.* 1988). Presence of xylanolytic genes both in chromosome and plasmid are reported for *Clostridium acetobutylicum* ATCC 824. Figure 1.10b represents the organization of xylanase encoding genes in the plasmid of *Clostridia*. In addition to the principal xylanolytic genes xynB (957 bp) and xynD (1376 bp), the bacteria contains two homologus copies of xynC (CAP_0118, 1766 bp and CAP_0119, 1758 bp). Involvement of xylulokinase (XylB, CAC2612), a xylose proton symporter (XylT, CAC1345), and a transcriptional regulator (XylR, CAC3673) for xylose utilization by *Clostridium acetobutylicum* has also been noticed (Chakdar *et al.* 2016).

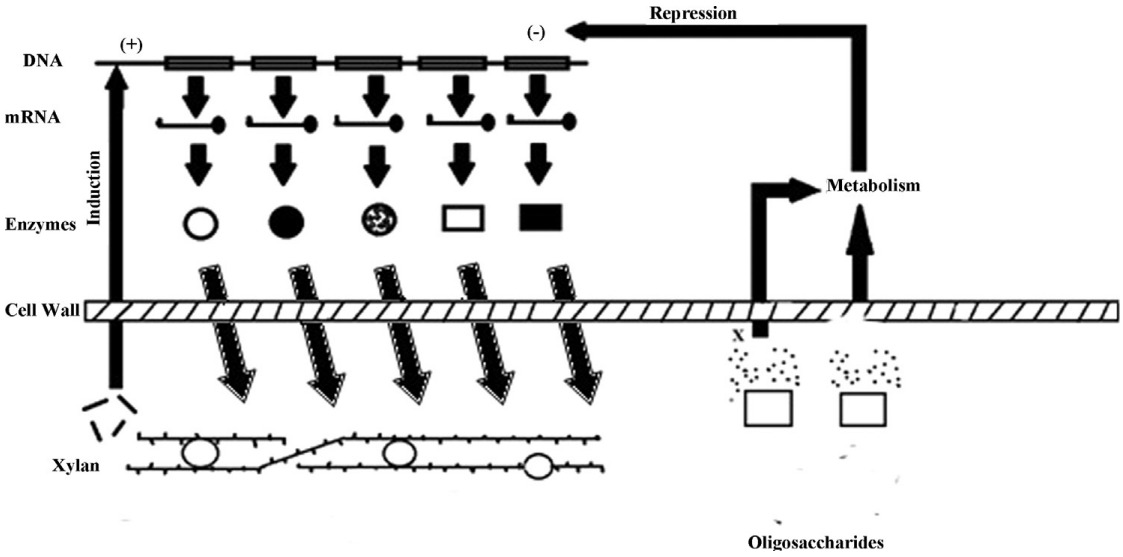


Fig. 1.9. Cellular recognition, genetic regulation and expression of xylanase (Uday *et al.* 2016)

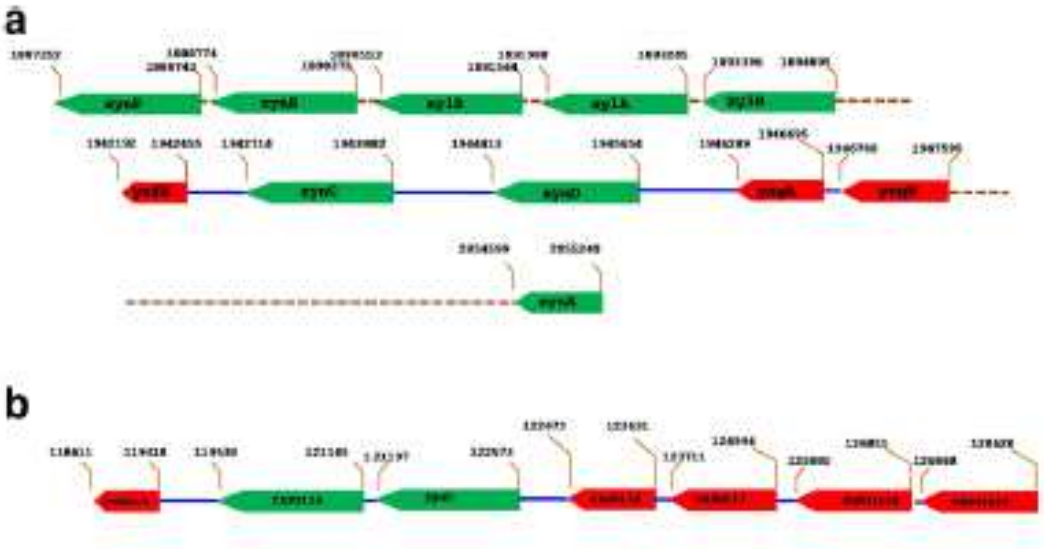


Fig.1.10. (a) Xylanase gene arrangement on chromosome of *B.subtillis* str168 (b) Arrangement of plasmid coded xylanase genes of *C. acetobutylicum* ATCC824 (Chakdhar *et al.* 2016)

1.3.7. Fermentative production of xylanase

Raw materials, cell biomass, media parameters and conditions are crucial factors for efficient fermentation by microorganisms. The sterilized medium inoculated with the starter organism placed/fed into the bioreactor or fermentor, well equipped with agitators, sparger, temperature, pH, and dissolved oxygen sensors, for fermentation. Industrially it has been found that the cost effective production of the final value added product can be achieved by using cheap raw materials (Cheng *et al.* 2014, Franceschin *et al.* 2011).

1.3.7.1. Optimization of media parameters

For maintaining a balance between the medium components and final product yield, medium composition and cultural conditions are required to be optimized. It helps to maximize the effect of each medium component and condition for higher product recovery. Several research have been carried out to evaluate the effect of cost effective agro residues, additional carbon sources, N_2 sources, metal ions, medium pH, incubation temperature, incubation time, agitation speed and inoculum concentration on the yield of xylanase enzyme. Since the nutritional requirements and growth conditions greatly vary between various microorganisms, reports on defined medium and growth conditions for highest xylanase yield are lacking (Bhunia *et al.* 2013).

Using different range of metabolites and transporter proteins microorganisms are capable of using wide variety of carbon and N_2 sources. Two kind of metabolism are generally recorded for microorganism, the primary and secondary metabolism. Primary metabolism helps the organism to go through the exponential phase of growth and the primary metabolites serve as a splitting points of biosynthetic reactions and lead to the production of secondary metabolites in stationary phase. Most of the extracellular enzyme production is reported to be enhanced during the stationary phase and subjected to regulation by various medium components as well as by catabolite repression and feedback inhibition mechanisms (Uday *et al.* 2016).

Although the xylanases can be produced by submerged fermentation (SmF) and solid state fermentation (SsF) processes, environmental parameters during aerobic stage are controlled better in SmF than SsF. Hence, past research works on xylanases production mainly used SmF. Submerged fermentation also produced higher amount of purified enzymes.

A variety of cheap agro residues, such as wheat bran, rice bran, soy meal, sugarcane bagasse, etc., had been found to be effective substrate for xylanase production (Kumar *et al.* 2009, Ras *et al.* 2013). In a study conducted by Sepahy *et al.* (2011) using oat bran as sole carbon source *Bacillus mojavensis* AG137 produced 249.308 IU/ml xylanase under SmF. Saw

dust on fermentation with *Arthrobacter* sp. MTCC6915 under SsF produced 117 U/ml of xylanase (Murugan et al. 2011). Adhyaru *et al.* (2016) reported the xylanase production using sorghum straw by *Aspergillus tubingensis* FDHN1 while Heinen *et al.* (2017) showed the xylanase production by *Aspergillus tamarii* Kita using barley bagasse. Xylanase production from wheat bran, wheat straw, rice husk, saw dust, gram bran, groundnut, and maize bran under SsF by *Bacillus subtilis* ASH has also been observed (Sanghi *et al.* 2008). Production of xylanase using wheat bran as potential substrate has been reported in various research (Kumar *et al.* 2013, Nagar *et al.* 2010, Kapoor *et al.* 2008, Sanghi *et al.* 2008). The higher xylanase productivity from wheat bran could be due to its enriched nutrient content comprising 54% carbohydrates (pentoses and hexoses) and 14% protein, minerals, amino acids, and vitamins (El-Sharnouby *et al.* 2012).

1.3.7.2. Media design by statistical approaches

Fermentation media designing is an important process for the development of microbiotechnological industrial process. The volumetric productivity and the product concentration were greatly affected by fermentation medium. To minimize the overall cost of the process, the medium components must have low cost. Optimization of media components through classical microbiological approach happens to be time consuming, as each of the possible combination of media variables have to be tested in step wise manner. On the other hand statistical approach could identify the significant media variables and study their interaction effect in very short period of time to maximize the yield of response variables. Khusro *et al.* (2016) studied the interaction effect between birchwood xylan concentrations, yeast extract concentration, incubation temperature and incubation time with the help of central composite design (CCD) based response surface methodology (RSM) for optimizing the production of xylanase by *Bacillus tequilensis* ARMATI. The result showed that highest production of xylanase was obtained with the fermentation conditions of birchwood xylan (1.5% w/v), yeast extract (1% w/v), incubation temperature (40°C) and incubation period (24 h). It was also noticed that optimization of medium by RSM caused approximately 3.7 fold enhancement in xylanase activity as compared to that optimized through one factor at a time approach (OFAT). Rosmine *et al.* (2017) conducted a study on xylanase production optimization through Plackett-Burman (PB) design and RSM techniques using *Streptomyces* sp. The significant media variables screened out with the help of PB design were further employed in RSM to maximize the xylanase production. The model predicted the xylanase activity of 10292 U/ml with the optimum concentrations of olive oil, xylan, agitation speed and inoculum age at 33.10 mg/l, 0.37%, 42.87 RPM and 21.05 h, respectively. The observed activity of 10292 U/ml was recorded which was close to the model predicted response (Rosmine *et al.* 2017). Xylanse production by the *A. niger* LPB 326 through SsF using sugarcane bagasse and soybean meal as cheap carbon

and nitrogen source, respectively, was optimized by factorial design. The media containing 6.5 g of sugarcane bagasse, 3.5 g of soybean meal and 85% initial water content showed the maximum xylanase (3099 IU/g of dry matter) production (Maciel *et al.* 2008). Adhyaru *et al.* (2017), using sorghum straw as a principal carbon source in the medium overproduce *Bacillus altitudinis* DHN8 xylanase of 492 IU/ml through RSM technique which was comparable to the xylanase (245 IU/ml) produced in OFAT method as reported in the authors previous study (Adhyaru et al., 2014).

1.3.8. Applications of xylanase

The bacterial xylanases are generally alkali stable and thermostable; moreover, they are devoid of cellulase activity. Due to the above features the bacterial xylanases have played a wide range of role and are industrially important. Along with the bacterial xylanases their production processes were also patented for several industrial applications and have been marketed globally by various commercial companies. Commercial fungal xylanases were also produced in large scale using mainly *Aspergillus niger*, *Trichoderma* sp. and *Humicola insolens* in Japan, Finland, Germany, Republic of Ireland, Denmark, Canada and the USA. Some of the major xylanase producing companies and their purposes are presented in the table 1.4.

1.3.8.1 Paper and pulp industry

The xylanase enzymes active at high temperature and alkaline pH are used for biobleaching of paper pulps without depolymerizing the cellulose polymers (Polizeli *et al.* 2005). Xylanase from various microbial sources has also been employed for bleaching of wood pulp. Xylanse from several bacteria such as *Streptomyces* sp. (Beg et al. 2000; Georis et al. 2000); *Streptomyces galbus* (Kansoh and Nagieb 2004), *Bacillus pumilus* (Bim and Franco 2000; Duarte et al. 2003); *Bacillus circulans* (Dhillon et al. 2000) and from fungus such as *Aspergillus niger* (Zhao et al. 2002); *Aspergillus nidulans* (Taneja et al. 2002); *Aspergillus fumigatus* (Lenartovicz et al. 2002); *Thermomyces lanuginosus* (Haarhoff et al. 1999); *Trichoderma reesei* (Oksanen et al. 2000); *Acrophilophora nainiana*, *Humicola grisea* (Salles et al. 2004) etc. being widely used for biobleaching of paper pulp. There were two proposed mechanisms for biobleaching of paper pulp using xylanase enzyme. According to one mechanism, the industrial scale cooking of primary wood pulp lowers the pH that precipitate plant cell wall xylan over lignin polymers and the depolymerisation of xylan using xylanase expose the lignin and make it more sensitive for successive stages of pulp processing (Viikari *et al.* 1994). On the other hand, second mechanism proposed by Buchert *et al.* (1992) and Paice *et al.* (1992) state that lignin usually form complex with the hemicellulosic material specially with xylan and some of the bonds are alkali resistant

and thus could not been hydrolysed during kraft process. Cleavage of these bonds are catalyzed by microbial xylanases and opening the structure of cellulose fibrils of the paper pulp.

Table 1.4
Commercial products of microbial xylanases

Sl No.	Product	Company	Source	Application
1.	Nutri Xylanase Enzyme	Ultra Biologics Inc., USA	*Bacillus subtilis*	Feed additive
2.	Bleachzyme F	Biocon India, Bangalore	-	Bleaching of pulp
3.	Bacterial Xylanase XBK BX9	Leveking, China	Bacteria	Bakery
4.	Panzea	Novozyme, Denmark	*B. licheniformis*	Bakery
5.	Belfeed B1100	Agrimex, Belgium	Bacteria	Feed additive
6.	Allzym PT	Alltech	*Aspergillus niger*	Animal feed improvement
7.	Bio-Feed Plus	Novo Nordisk	*Humicola insolens*	Animal feed
8.	Solvay pentonase	Solvay Enzymes	*T. reesei*	Starch and bread-making industries
9.	Xylanase GS35	Iogen	T. reesei	Cellulose pulp bleaching, animal feed
10.	Cartazyme	Clariant, UK	*Termomonospora fusca*	-

1.3.8.2. Deinking of waste paper

Deinking is considered as one of the important process for recycling the waste papers. Chemical treatments with chlorine, chlorine based derivatives, sodium hydroxide, sodium carbonate, sodium silicate, hydrogen peroxide, hypochlorites, and chelating agents are being used for deinking purpose which generate large amount of toxic effluents and creating environmental pollution (Maity *et al.* 2012). Removal of ink from the effulent generated from paper and pulp industries had been carried out using xylanase in combination with laccase (Dhiman *et al.* 2014, Chandra and Singh 2012). Synergistic activity of xylanase and laccase had been successfully

employed for recycling the news paper and observed to increase the brightness (11.8 %), whiteness (39 %) and physical properties like breaking length (34.8 %), burst factor (2.77 %), and tear factor (2.4 %) of the recycled paper (Gupta *et al.* 2012).

1.3.8.3. Improvement of feed quality used for livestocks

Digestibility of the feeds for live stock can be improved by xylanase. Certain cereals, such as maize and sorghum have shown very low viscosity due to the presence of arabinixylans which remain undigested in the animal stomach and act as antinutrients. Xylanase treated cereals were digested properly in the upper digestive tract of animal and helped to extract more energy from the feed (Harris and Ramalingam 2010). Diet rich in xylanase treated cereals was found to reduce the level of unwanted residues such as phosphorus, nitrogen, copper and zinc in the excreta and thus playing an important role to minimize the environmental pollution (Polizeli *et al.* 2005).

1.3.8.4. Bakery industry

Wheat, the key ingredient of baking industry contain substantial amount of arabinoxylans. The treatment of wheat flour with xylanase helps to solubilize the water unextractable arabino xylan (WU-AX) and thus improved the do ugh quality (Courtin and Delcour 2002). Butt *et al.* (2008) observed that the viscosity of dough was reduced with treatment of xylanase from *B. subtilis*. Moreover in the same study the negative effect of xylanase on gluten aglomeration was also reported.

1.3.9. Conversion of hemicellulose to fermentable sugar

Heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids together comprises the hemicelluloses. Xylans are mostly present as hardwood hemicelluloses where as glucomannas are the predominant molecule of soft wood hemicelluloses . The hetero polysachharide xylan chains are mostly made up with β-1,4- linked D-xylopyronoside residues along with arabinose, glucuronic acid or its 4-O-methyl ether, and acetic, ferulic, and p-coumaric acids. Different plants varieties such as grasses, cereals, softwood, and hardwood, showed great variation on the content and type of xylan present in the cell wall. Birch wood xylan was found to compose with 89% xylose, 1% arabinose, 1.4% glucose and 8.3% anhydrouronic acid (Kormelink *et al.* 1993), whereas 65.8% xylose, 33.5% arabinose, 0.1% mannose, 0.1% galactose, and 0.3% glucose were found to be the monomeric sugar of wheat xylan (Gruppen *et al.* 1992).Hence, these complex hemicelluloses were treated with xylanolytic enzymes for recovery of several monomeric fermentable sugar.

1.3.9.1. Pretreatment of hemicellulose

Agro residues such as the straws, hulls, stems, stalks etc. of different plant species are the considerable lignocellulosic biomass. Although the composition varied from plant to plant and tissue to tissue, roughly cellulose (35-50%) is the main component which is followed by hemicellulose (20–35%) and lignin (10–25%) (Saha 2003). Disruption of polymeric matrix to monomers is the key chalange for the utilization of lignocellulosic biomass. Thus various research works had been designed aiming to develop an efficient pretreatment method that would assit the enzymatic catalysis of agro residues with higher digestibility (Jorgensen *et al.* 2007). Several numbers of pretreatment methods had been developed such as treatment with dilute acid or alkali, steam explosion, lime treatment, microwave irradiation, wet oxidation, H_2O_2 oxidation, ammonia mediated freeze explosion etc. to make the lignocellulosic residues more sensitive to enzymatic catalysis (Wyman 1994). Removal of hemicelluloses or lignin, reduction of the crystallinity of cellulose and increase the surface area are the main purpose of pretreatment method (Singh *et al.* 2010). Pretreatment with microwave radition found had a positive role on digestion of ligonecllulosic biomass (Maa *et al.* 2009). However, the drawback of the pretreatment procedures is the generation of some microbial growth inhibitory compounds. Degradation of lignin yields phenolic compounds, sugar degradation generates furfural and 5-hydroxymethylfurfural (HMF), and certain aliphatic acids such as acetic acid, formic acid and levulinic acid were found to have microbial growth inhibitory capabilities (Jonsson *et al.* 2016, Ko *et al.* 2015). Microbial growth inhibitory capabilities of furfural and HMF were well documented in the research conducted by Zheng *et al.* (2014). The authors also noticed that these compounds had a negative effect on the production of bacterial nano cellulose. Moreover, the bioconversion of bacterial nono cellulose to oxidized or reduced products was also inhibited by HMF (Zheng *et al.* 2014). Various method of detoxification had been developed, such as filtration of the pretreated broth through ion exchanger resins, charcoal, treatment with laccase, fermentation by filamentous fungus, extraction with organicsolvents etc (Saha 2003, Palmqvist *et al.* 2000). However, another alternative approach was based on the isolation of some microorganism which was resistant to these compounds and could efficiently ferment the released sugar and produced value added products (Jonsson *et al.* 2016). New resistant strains of *K. xylinus* ATCC 23770 had been isolated which showed significant growth on furan aldehydes and phenol and also produced 14.78 g/l bacterial nano cellulose. The conversion of these toxic compounds to less toxic products either by oxidation or reduction was also reported for the *K. xylinus* ATCC 23770.

1.3.9.2 Enzymatic saccharification of hemicellulose

Conversion of polymeric carbohydrates like celluloses and hemicelluloses into monomeric sugars was crried out by the enzymes sucha as cellulase, xylanase, xylosidase etc. obtained from various microbial sources. Among the various ligonocelluosic biomas depolymerizing enzyme producer fungi play crucial role.In addition to the production of cellulolytic and xylanolytic enzymes filamentous fungi also produced hydroxyl radical which oxidized the plant cell wall (Yarbrough *et al.* 2017). Many microorganisms were also reported for production of complete xylanolytic enzyme system such as *Penicillium capsulatum, Talaromyces emersonii* etc. (Filho *et al.* 1991). The thermophilic actinomycete *Thermomonospora fusca* produced an array of endo-xylanase, β-xylosidase, α- arabinofuranosidase and acetyl xylan esterase. These enzyme cocktail was found to act on lignocellulosic biomass synergistically and liberated the fermentable monomeric sugar (Saha 2003).

Bhalla *et al.* (2015) observed that 68.9% birchwood xylan was converted to monomeric sugar when it was acted upon by xylanase from *Geobacillus* sp. WSCUF1. Whereas conversion rate was much lower using commercial enzymes such as Celic HTec2 (49.4 %) and Accelerase XY (28.92 %), at 70 ^{o}C. The enzymatic saccharification of agro residues supplemented with xylanase increase the production of reducing sugar which further fermented for biethanol production. Genetically modified microorganisms were employed for conversion of pentose sugar to ethanol inaddition to the fermentation of hexose sugar generated from lignocellulosicresidues. But as the pentose sugar can simply be converted by microorganism to xylitol, this approach was accssed by many researchers as a promising approach for utlilization of agro residues (Cheng *et al.* 2014, Franceschin *et al*. 2011).

1.3.10. Xylitol, the sweetener

Xyliol ($C_5H_{12}O_5$) is a five carbon sugar alcohol and generally used as sweetener. It was first chemically synthesized by Emil Fisher and Stahel in 1891 (Fischer and Stahel 1891). Its structural formula is shown in Fig. 1.11. Sweetening power of xylitol (sugar alcohol of xylose) is similar to that of sucrose, nearly three times of mannitol (sugar alcohol of mannose) and twice that of sorbitol (sugar alcohol of glucose). As the calorie content of xylitol is less than sucrose, it has the potential to replace sucrose to make low calorie products. The physical and chemical properties of xylitol are listed in Table 1.5. Due to some exclusive properties of xylitol it has been widely used in pharmaceuticals, nutraceuticals, food and beverage industries (Maguire and Rugg-Gun 2003).

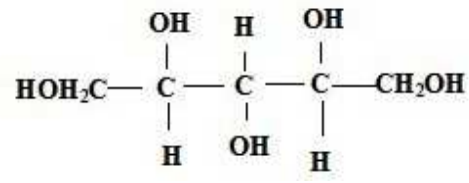


Fig.1.11. Chemical structure of xylitol.

Table 1.5 **Physico-chemical properties of xylitol (Rehman *et al.* 2013)**	
Formula	$C_2H_{12}O$
Molar mass	152.15 g/mol
Appearance	White, crystalline powder
Odour	None
Solubility in water (20^{o}C)	100 g/l
pH in water (1g/10ml)	5-7
Melting point	92 to 96^{o}C
Boiling point	345.39^{o}C
Density	1.52 g/l
Caloric value 4.06 cal/g	16.88 J/g
Density (specific gravity) of aqueous solution (20 °C) 10% 60%	 1.03 1.23
Relative sweetness	Equal to sucrose; greater than sorbitol and mannitol

1.3.10.1. The applications of D-xylitol

D-xylitol is used in chewing gums, mouth rinser and in tooth pestes as an effective safe tooth decaying preventive agent (Ritter *et al.* 2013, Uittamo *et al.* 2011). Most of the human dental decay was found to be associated with *Streptococcus mutans*. This bacterium attached to the enamel pellicle, forms bioflim and interacts directly with salivary components. The intake of xylitol containing products by people suffering from dental plaques caused by *S. mutants*, resulted in decrease of the lactic acid production by the strain. Moreover, xylitol is more alkaline than other sugar thus increasing the salivary pH. High pH facilitates the deposition of calcium and phosphate salts into the exposed enamel parts where it is lacking.

Additionally, the potentiality of saliva in preventing plaque is enhanced due to the acid buffering capacity of xylitol (Chen *et al.* 2010). Xylitol also reduces the bioflim forming ability of *S. mutans* in the enamel (Ritter *et al.* 2013). Uittamo et al. (2011) reported that xylitol at a concentration of 5% to either epithelial cells or pneumococci or both, reduced the adherence of *Pneumococci*. Growth inhibition of *Pneumococci* was found to be xylitol mediated and occurred via fructose phosphotransferase system. In the liver and red blood cell of healthy human beings xylitol is converted to glucose-6-phosphate and this pathway is not regulated by insulin. Glucose-6-phosphate later converted to glucose in a slow catalytic reaction thus raising the insulin level very slowly in the blood. Thus the patients suffering from insulin deficiency are being treated with xylitol. Moreover, due its low calorie content and little thermogenic effect, xylitol appears to be attractive compounds for insulin independent diabetic patient (Chen *et al.* 2010).

1.3.10.2. Chemical method of xylitol production

After extraction and chromatographic separation of D-xylose from woody plant, chemical conversion of xylose to xylitol was first initiated in Finland, during the period of 1970s. Chemically D-xylose was catalyzed in presence of hydrogen with high temperature and pressure for production of D-xylitol. Due to the requirement of pure D-xylose, this process required several purification steps. The process of xylitol production was categorized into four different steps. Firstly, the different biomasses were chemically depolymerised into a mixture of different sugars, mainly rich in pentoses (Kumar *et al.* 2009). Second steps involve the purification of xylose from the chemical hydrolysates with the help of ion exchange resins (Zamani 2015). Activated charcoals were also employed in this step for the removal of salts, metal ions, unpleasant odour and colour (Wei *et al.* 2010). Third, the purified xylose was reacted with hydrogen in presence of Ni as catalyst, under high temperature (140–200 ^{o}C) and high pressure (50–60 bar) until the xylitol production take place (Su *et al.* 2013). In the final step of the process, the xylitol was purified and crystallized (Martinez *et al.* 2015, Sampaioa *et al.* 2006). Although the chemical process of xylitol production showed high yield and high conversion efficiency, but it has some major drawbacks, such as it requires expensive equipments, purification steps, recovery of product etc. which make the overall process energy demanding and expensive.

1.3.10.3. Biotechnological production of xylitol

Due to the major drawbacks and expensive product cost obtained from chemical process creates an urge for intervention of biotechnological process for low cost production of xylitol. As the biotechnological processes operate at relatively mild condition and can be

employed on relatively crude mixture of lignocellulosic hydrolysates, overall cost is greatly reduced. Biotechnological production mainly depends on the live cell of wild microorganisms such as bacteria, fungi or their recombinant strains. Although immobilized cell free catalysts were also employed for bio-conversion of xylose to xylitol, but this process was industrially not economical (Park *et al.* 2005).

1.3.10.4. Microorganisms & metabolic pathway

Microorganisms capable of fermenting xylose to xylitol are cited in various scientific reports and most of them belong to yeast whereas few bacteria are also reported (Rafiqul and Sakinah 2013). Low yielding filamentous fungi were also reported. Table 1.6 represents the list of microorganisms capable of xylitol production. D-xylose is reduced by an intracellular enzyme xylose reductase (XR, EC 1.1.1.21) to xylitol in a single step. The xylitol may either secrete in the fermentation broth or may act upon by xylitol dehydrogenase (XDH, EC 1.1.1.9) and oxidized to xylulose. The cofactor requirement of both the enzyme is just opposite, NADH/NADPH act as a cofactor for XR while XDH requires NAD^+/$NADP^+$ (Granstrom *et al.* 2007). Pentose phosphate pathway recycles the D-xylulose for the production of several biosynthetic precursors and the generation of reducing equivalents. Xylose utilization in bacteria which were devoid of XR and XDH occur through the activity of xylose isomerase enzyme (XI; EC 5.3.1.5) which convert xylose to xylulose and xylulose later enter into the pentose phosphate pathway to support the growth (Dasgupta *et al.* 2017). The pathway for xylose utilization and its conversion to xylitol by microorganisms is presented in figure 1.12. Only few bacteria were reported for xylitol production, such as *Enterobacter liquefaciens* (Ghindae *et al.* 2010, Yoshitake *et al.* 1973), *Corynebacterium* sp. (Rangaswamy and Agblevor 2002). These bacterial strains showed very low productivity and required high incubation period. Due to these features, these strains were not applied for xylitol production in industrial scale (Dasgupta *et al.* 2017). On the other hand due to the stable expression of XR and XDH, and higher rate of pentose conversion to xylitol, yeasts were preferred for xylitol fermentation (Rafiqul and Sakinah 2013). The role of *Candida* sp. as whole cell biocatalysts has been reported by various researchers (Ping *et al.* 2003, Silva *et al.* 2006). Tamburini *et al.* (2015) reported a *Candida* sp. which had approximately 86% conversion efficiency of xylose to xylitol, and showed a considerable amount of volumetric productivity (0.63 g/l/h). Although well known for ethanol production, *Pichia spitis* was also reported as a potential xylitol producer (Rodrigues *et al.* 2008). The diary yeast *Kluyveromyces marxianus* was found ferment pentose sugars under high temperature and reported to produce 0.6 g/g xylitol (Mueller *et al.* 2011). Inspite of its inability to assimilate pentose sugar, *Saccharomyces cerevisae* was

genetically modified for fermentation of xylose to xylitol due to the available vast knowledge about its genomic constitution and scientific data (Kim *et al.* 2002).

1.3.10.5. Genetic modifications of microorganism for high xylitol yield

Major problems with the existing xylitol fermenting yeast are associated with their low xylitol recovery. Genetic stability of microorganisms, their growth patterns, level of expression of proteins, product yield and finally the product recovery are the important factors regarding the production of certain chemical industrially (Ghindae *et al.* 2010). After gradual enrichment of genetic information and knowledge of metabolic pathways in many non *Saccharomyces* yeasts, the product volume and yield of xylitol has increased through specific gene manipulations. Factors influencing the several gene manipulations are briefly discussed in the following sections. Gene manipulations such as gene level optimization of xylose transporter increased the xylose uptake inside the cell, over expression of XR leads to high rate conversion of xylose to xylitol, whereas overexpression of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6-PGD) increase the level of NADPH for xylose reduction by XR and deletion of XDH coding gene stoped the further conversion of xylitol to xylulose (Fig 1.13).

Table 1.6
List of xylitol producing microorganisms.

Microorganism	Temperature (^oC)	pH	Substarte	Xylitol (g/l)	Yield (g/g)	Volumetric productivity (g/l/h)	References
Bacteria *Enterobacter liquefaciens 553*	30	7	Xylose	33.3	0.33	0.35	Yoshitake *et al.* 1973
Corynebacterium sp. B-4247	30	7.2	Xylose	40	0.4	0.8	Rangaswamy and Agblevor 2002
C. guilliermondii FTI 20037	-	-	Rice straw	37.6	0.62	-	Roberto *et al.* 1996
Candida parapsilosis	-	-	Corn cob	36	0.72	-	Kim *et al.*1999
Candida tropicalis KCTC 7221	30	6.5	-	110	0.81	5.4	Kim *et al.* 2004
Pichia sp.	28	-	-	25	0.58	0.5	Rao *et al.* 2006
Kluyveromyces marxianus IMB2	45	4.5	-	7.78	0.42	0.24	Mueller *et al.* 2011
Kluyveromyces marxianus CCA510	30	6	-	12.27	0.50	0.17	De Albuquerque *et al.* 2015
Fungus *Penicillium crustosom CCT 4034*	30	6	-	0.52	-	0.005	Sampaio *et al.* 2003

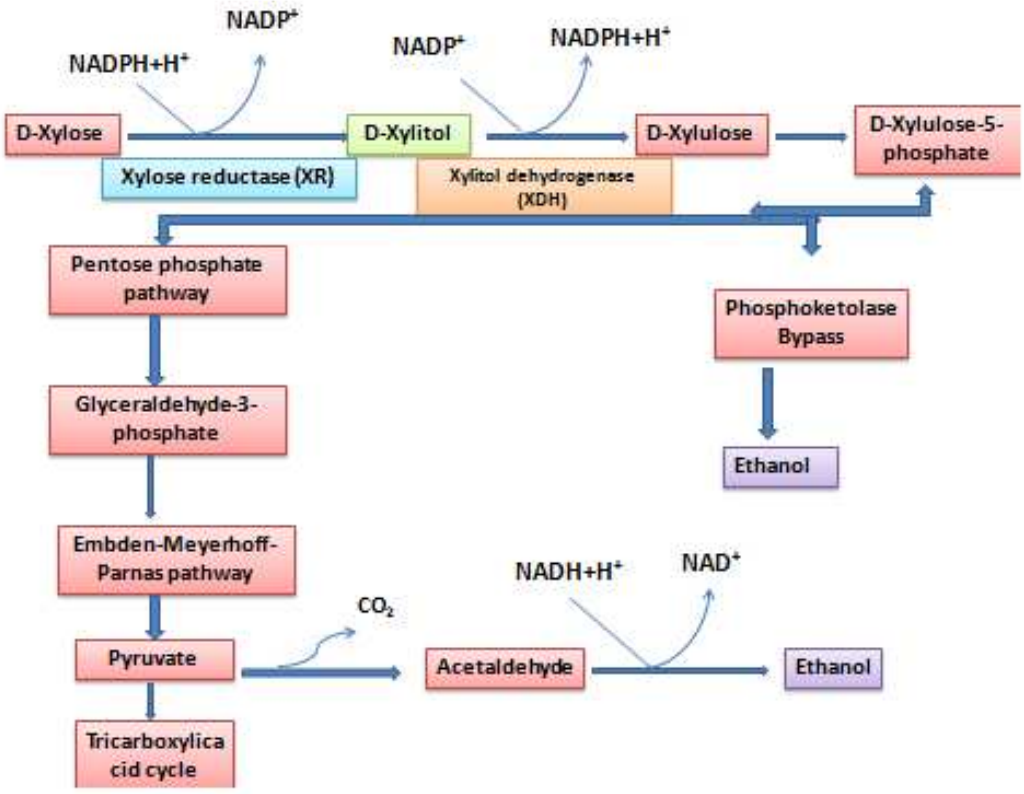


Fig. 1.12. Metabolic pathway of xylose utilization in microorganism (Granstrom *et al.* 2007).

1.3.10.5.1. Transport optimization

Pentose sugars are mainly transported into the cell through the hexose transporter proteins such as Hxt4, Hxt5, Hxt7 and Gal2 in yeast (Jeffries *et al.* 2007). These transporter proteins are neither selective for xylose nor for other sugars (Farwick *et al.* 2014). In hemi ascomycetes yeasts transporters Hxt 7 and Hxt5 are mainly responsible for xylose uptake when it is present as sole carbon source in the media. Fernandes and Murray (2010) successfully cloned and expressed the native transporter proteins of yeast involving in xylose uptake. *Candida* sp xylose symporter GFX1 and GXS1 and xylose symporter SUT from *Pichia* sp has been expressed in *Saccharomyces* and *Kluyveromyces* and found to be functional (Zhang *et al.* 2015). However, the modified transporters suffer from low xylose uptake hence low xylitol yield (Apel *et al.* 2016). In order to regulate the xylose uptake and flux control, a group of transporter proteins which act cooperatively might be essential

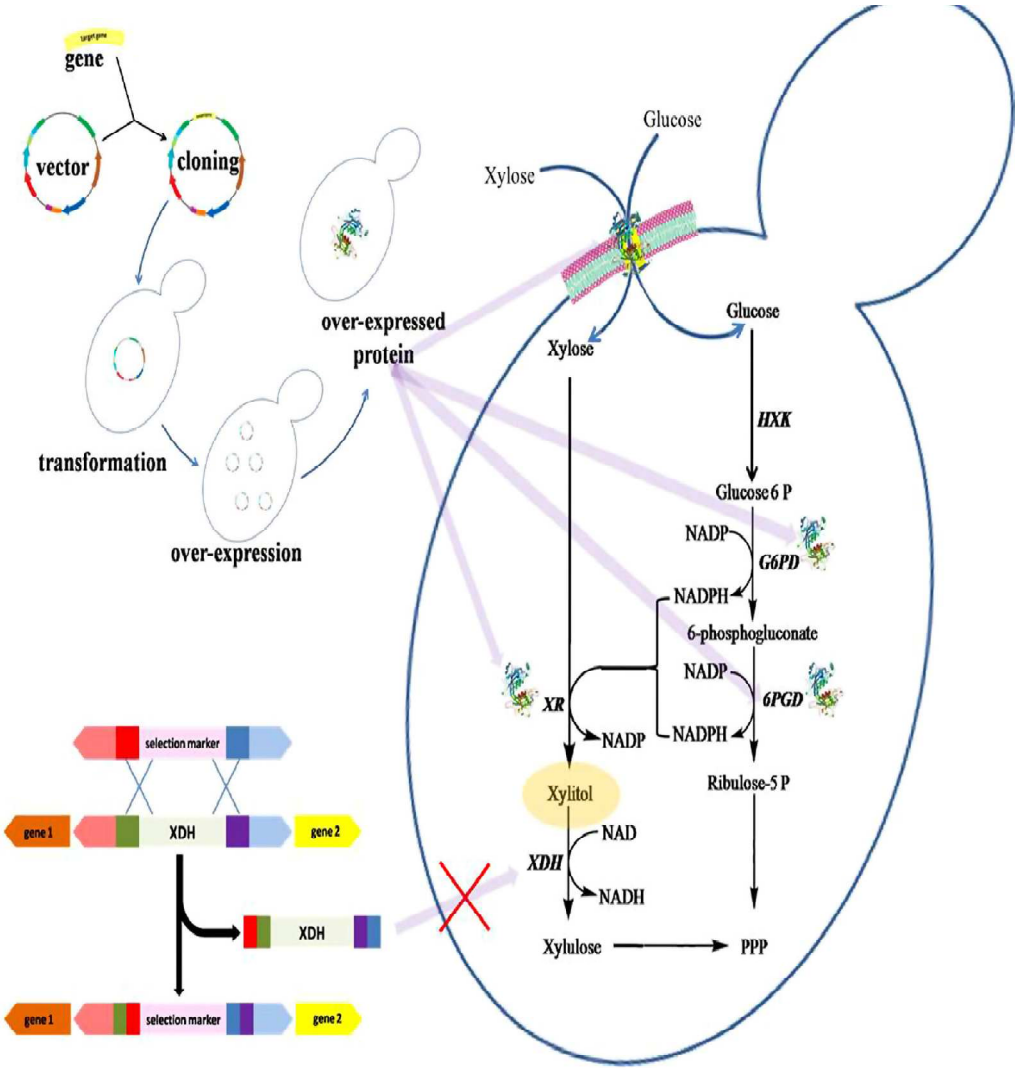


Fig 1.13. Several types of gene manipulation conducted in yeast for high xylitol yield (Dasgupta *et al.* 2017).

1.3.10.5.2. XR overexpression

The principal enzyme for xylitol production pathway is xylose reductase. Most of the xylitol producing strains often suffer from low expression level of XR and hence low xylitol yield. Native XR enzyme coding genes were modified through recombinant DNA technology for their stable expression or exogenous XR genes might be overexpressed in the endogenous

host under a strong constitutive promoter. In *S. cerevisae* XR genes from different sources were expressed after modification of the codon usage according to the *S. cerevisae*, and had shown to improve the xylose reduction rate to xylitol (Kogje and Ghoshalkar 2016). Baker's yeasts harbouring the exogenous XR genes of *Candida* and *Pichia*, under constitutive promoter, showed the production of 0.86 g/g and 0.9-1.0 g/g of xylitol, respectively (Bae *et al.* 2004, Kim *et al.* 2002).

1.3.10.5.3. Cofactor supply and engineering

Xylose to xylitol conversion by XR enzyme required reduced form of NAD in phosphorylated or unphophorylated state. Product yield become greatly reduced due to the unavailability of reducing equivalent. To enhance the product yield, reduced cofactors might be externally added to the media. But due to the high cost of the reductant overall cost of the process becomes expensive. Xylitol production pathway in *C. utilis* CBS-621 has been studied thoroughly and found that accumulation of NADPH/NADH principally depends on hexoses monophosphate pathway (HMP) and the redox imbalance between XR and XDH (Bruinenber *et al.* 1983). Berg et al. (2002) observed that regeneration of NADPH occurred in oxidative pentose phosphate pathway, during the generation of cell biomass. Two key enzymes were involved in this mechanism G6PD (glucose-6-phosphate dehydrogenase) and 6-PGD (6-phospho-deleta gluconolactonase). Over expression of these proteins in the presence of fermentable sugars helped to regenerate the cofactors (Uppada *et al.* 2014). Recombinant *Saccharomyces* BJ3505, harbouring XR gene from *P. stipitis,* was modified by genetical tools to overexpress the endogenous G6PD gene that found to improve the NADPH accumulation. Moreover, the strains showed 6 fold increase in XR activity and xylitol volumetric productivity was increased from 1.6 to 2 g/l/h (Kwon *et al.* 2006).

1.3.10.5.4. Gene deletion

Downstream to the xylose metabolism pathway, XDH is a key enzyme. In absence of the XDH enzyme their might be accumulation of xylitol provided cofactors were regenerated through some other route. Knocking out the XDH gene pairs, using the disruption cassettes (URA3 and His3) resulted the formation of mutant version of *C. tropicalis* ATCC 20913 which showed approximately 98% xylose conversion efficiency to xylitol (Ko *et al.* 2006). In a separate experiment *Candida* sp. was subjected to UV mutagenesis and a mutant strain was found to have point mutation in the XDH gene. The mutant accumulated 1-2 fold more xylitol as compared to wild type strain (Kumar *et al.* 2010, Rao *et al.* 2006).

1.3.10.6. Bioprocess

Xylitol production through biological process depends on several factors such as, low cost raw materials or carbon source, nitrogen source, incubation temperature, media pH, aeration, reaction mode, inoculum stage and volume and finally the product recovery with downstream processing. Lignocellulosic agro residues were widely used as low cost raw materials for xylitol production. These complex polymers were initially depolymerised with physical, chemical and enzymatic treatment for liberation of monomeric sugars (Binder and Raines 2010). Xylose from lignocellulosic residues was converted to xylitol, thus the process of xylose production from lignocellulosic wastes through hydrolysis using xylanolytic enzyme system proved to be a promising approach. In addition to being a law cost feed stock, lignocellulosic agro residues require mild pretreatment and moderate detoxification was required for removal of toxic compounds from saccharified hydrolysates. Microbial xylitol fermentation route via xylose reduction also has the process advantage. There is no requirement of extra pure D-xylose, as required in chemical process. Thus mild detoxification with over liming or by activated charcoal removed the toxic compound from sachharified hydrolysates and makes the broth suitable for microbial fermentation (Tanifuji *et al.* 2013). Figure 1.14 represents the overall scheme of xylitol production from agro residues.

1.3.11. Future prospects and conclusions

Production of xylitol from lignocellulosic materials involved three principal steps, which are pretreatment, saccharifion and fermentation. The main focus of the process happens to be the degradation of complex carbohydrate polymers into fermentable sugars. Among the several methods of pretreatment, dilute acid or alkali pretreatments were used as method of choice. The pretreated agro residues were subjected to enzymatic hydrolysis using xylanolytic enzyme system. In addition to fermentable sugar, pretreatments led to the generation of undesirable toxic compounds in the hydrolystaes, such as phenolics, furfuryl, HMF etc. The toxic compounds can be removed by over liming or activated charcoal treatment. Moreover, some microorganisms are also reported to ferment the pentoses in presence of these toxic compounds. Industrially xylanases are mainly produced by some filamentous fungi and bacteria. Large scale industry compatible xylanase enzyme can be produced by the genetic modification and site specific mutagenesis of the endogenous xylanase coding gene as well as by optimization of media components and process parameters through OFAT and statistical approaches. Xylanases produced in these processes had wide range of applications; especially in remarkable sachharification of agro residues.

Although the xylanase mediated fermentable sugars were employed for xylitol production, the global need of xylitol has been met mostly through chemical hydrogenation process. However, in terms of energy expense and environmental compatibility the biological process of xylitol production is very much warranted. This biological process not only helps to produce value added products but also recycle the renewable agro wastes. Although majority of works in this field was carried out in laboratory scale, to meet up the global need the process should be conducted in industrial scale through the intervention of chemistry, chemical engineering and biotechnological aspects. Hence validation of bioprocess has been required for the development of a economic industrial process. Lignocellulosic agro residues will be proved as economic feedstock for generation of xylitol in cost effective manner and to meet the world need of xylitol demand. Although many fungus are reported that can be used in bioconversion of xylose to xylitol, the number of reported bacterial isolates are very few. Due to their rapid growth rate, bacteria can be used as effective producer with high volumetric productivity of xylitol in shorter period of time and hence, lowering down the production cost. Moreover, bacteria resistant to the phenolic inhibitory compound can also be used without much genetic alternation for xylitol production.

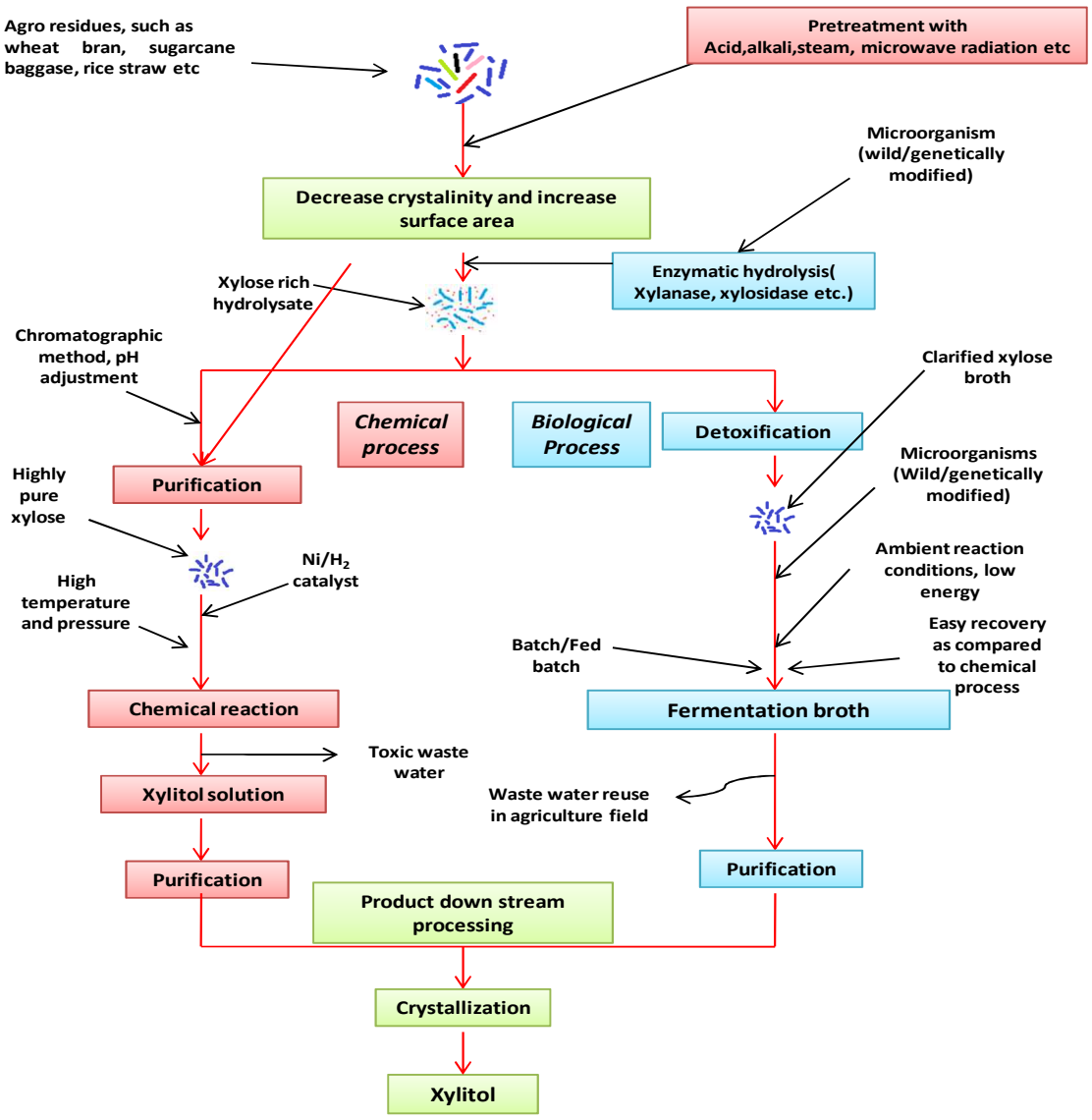


Fig.1.14. Overall scheme of xylitol production from agro residues by chemical and biological process

Chapter 2

Characterization and optimization of xylanase production by microorganisms isolated from soil

2.1. Introduction

Plant lignocellulosic wastes such as agricultural and forestry residues can be used for the biological production of fuels and several value added chemicals that ultimately offer economic, environmental and strategic advantages to the society (Wang *et al.* 2016, Gladden *et al.* 2012). The lignocellulosic materials generally contain up to 25% lignin, 35% hemicellulose and 40% cellulose. In nature, cellulolytic microorganisms can degrade cellulosic materials by producing multiple enzyme systems of different substrate specificities (cellulases, xylanases and other carbohydrate active enzymes) and catalytic mechanisms, which can be either free or cell associated (Mazzoli *et al.* 2012).

Xylan is the most abundant hemicellulose present in both hardwoods and annual plants (Ghosh and Saha 2014, Fang *et al.* 2010). It accounts for 20-25 % of the dry weight of hardwood and 7-15 % of soft woods. In all terrestrial plants, xylans are characterized by β-(1,4) linked D-xylopyranosyl main chain carrying a variable numbers of neutral or uronic monosaccharide subunits or short oligosaccharide side chains. They greatly vary in amount and complexity in different plant species and cell types (Huisman *et al.* 2000). The β-(1, 4) xylan chain has been reported to be more flexible than the two helix of β-(1, 4) cellulose because of the presence of only one hydrogen bond between adjacent xylosyl residues in contrast to the two hydrogen bonds between adjacent glycosyl residues of cellulose (Subramaniyan and Prema 2002). The complete cleavage of glycoside linkages in the heteroxylan backbone requires the interaction of a number of the main-chain and side-chain cleaving enzyme activities, of which endoxylanase (E.C.3.2.1.8), exoxylanase (E.C.3.2.1.37) and β-xylosidase play vital role (Carvalho *et al.* 2013, Collins *et al.* 2005, Beg *et al.* 2001).

Microbial xylanases have fascinated researchers because of their potential applications in industrial processes such as, bio-bleaching of pulp in paper industry,

conversion of biomass waste to fermentable sugar for production of biofuel and other chemicals, animal feed quality improvement, clarification of fruit juices and wines (Ghosh and Saha 2014, Fang *et al.* 2010, Kumar and Wyman 2009, Jordan *et al.* 2001, Beg *et al.* 2001). Microbial xylanases are advantageous over the enzymes derived from plant or animal origin because of their easier availability, structural stability and ease of genetic manipulations (White *et al.* 2008, Van-ooyen *et al.* 2006, Chand and Mishra 2003, Subramaniyan and Prema 2002). Among microbial xylanase producers, fungi are of special interest because they secrete substantially greater amounts of xylanases into extracellular culture medium than do bacteria. Furthermore, xylanases produced by many filamentous fungi have special properties like pH stability, high temperature optima and thermostability (Uday *et al.* 2016, Ghosh and Saha 2014, Polizeli *et al.* 2005). Such thermotolerant and alkalitolerant activities are important in industries especially in paper and pulp bio-bleaching processes that require both high temperature and basic pH (Kumar *et al.* 2017). Filamentous fungi such as *Aspergillus* and *Trichoderma* species produce significantly higher levels of xylanases and therefore have been widely used to produce industrially important xylanases (Heinen *et al.* 2018, Uday *et al.* 2016, Polizeli *et al.* 2005). Xylanases obtained from bacteria such as *Bacillus altitudinis* DHN8 and *Bacillus subtilis* Lucky9 are thermotolerent as well as alkali and acid tolerant, which make them beneficial for paper industries (Adhyaru *et al.* 2017, Chang *et al.* 2017).

The successful industrial applications of xylanase require its cost effective production in bulk quantity. The production cost can be reduced by optimization of cultural conditions, using cheap agro residues as carbon source and better microbial strains. The optimization of cultural parameters or variables for enzyme production is generally carried out using one factor at a time (OFAT) approach. However, this approach is an underestimation of the enzyme production as it does not consider interactions among variables (Gupta *et al.* 2012). The optimal design of the cultural medium for enzyme production by statistical approaches such as Placket-Burman (PB) design and Response Surface Methodology (RSM) are alternative strategies that offer minimum number of experiments for a large number of process variables and modeling of interaction among them. Recently, RSM has been utilized successfully to improve product yield and to reduce development time and cost of biotechnological processes (Rosmine *et al.* 2017, Kumar *et al.* 2017, Kaur *et al.* 2016, Khusro *et al.* 2016,).

In the present study, microorganisms isolated from environmental samples were screened for xylanase production followed by characterization of the best producers. Further, xylanase production by the selected microorganisms was optimized by one factor

at a time (OFAT) and response surface methodology (RSM) with respect to growth conditions and media composition, using agro residues as sole carbon source under submerged fermentation (SmF). Finally, the enzyme was partially purified and characterized.

2.2. Materials and methods

2.2.1. Strains, Chemical and reagents

Birch wood xylan was purchased from HiMedia Laboratory, India. Oat spelt xylan and DEAE-sepharose were purchased from Sigma, USA. Biogel P-100 were from BioRad, USA. Dialysis membrane sack with MWCO-12KDa was purchased from Sigma-Aldrich, India. The fungal strains *Aspergillus flavus* xym4, *Penicillium citrinum* xym2 (Accession no.KF570240) and the bacterial strain *Bacillus subtilis* xym4 (Accession no. MG607370) were isolated from soil of the different regions of North Bengal. Agro residues were purchased from the local market near University of North Bengal. All other biochemicals and microbiological media were from Sigma-Aldrich, USA; E. Merck, Germany and HiMedia Laboratory, India.

2.2.2. Sample collection

For screening and selection of xylanase-producing microorganisms, a wide range of environmental samples such as garden soil, rhizospheric soil, dung and rotten wood log were collected from different regions of North Bengal (Table 2.1 and Fig 2.1). About 100 g of soil or environmental sample were taken in sterilized zipper bags and they were immediately transported to the laboratory for gradient dilution using autoclaved saline (0.85 % w/v) water, and aliquots were transferred to appropriate growth media.

2.2.3. Isolation and screening of xylanase producing microorganism

10 g soil or environmental sample was diluted in 90 ml of sterile saline (0.85 % w/v) water and serially diluted (10^{-1} to 10^{-8}). The consecutive propagation of microorganisms having the property of xylanase production was conducted by spreading 0.1 ml of the serially diluted samples on the xylan agar media (XYM, pH 7) containing (g/l): peptone, 0.90; $(NH_4)_2HPO_4$, 0.40; KCl, 0.10; $MgSO_4,7H_2O$, 0.10; birch-wood xylan, 5 and 2% w/v agar and incubating at 37 ^oC for 48 h. The representative individual colonies obtained on XYM plates were selected based on their morphology. After obtaining pure cultures, all the isolated strains were qualitatively screened for xylanase production. For this, selected microorganisms were spot inoculated in XYM plates and incubated at 37 ^oC for 48 h The

plates showing the growth of microbial colonies were flooded with congo red solution (0.5% congo red dye in 5% ethanol) and were kept at room temperature for 30 min for reaction between congo red and xylan polymer. The plates were then thoroughly washed with 1M sodium chloride solution to remove the unbound congo red. Microorganisms forming yellow zone of clearance around their colony against red background were primarily selected as xylanase producers.

Table 2.1
Sampling sites for isolation of xylanase producing microorganisms

Sl. No	Sampling sites	GPS
1.	**Saal bagan (North Bengal University)**	Latitude 26”42’45.03 °N Longitude 88”21’15.28°E
2.	**Kulik River Basin**	Latitude 25°38’13.03”N Longitude 88°07’16.28”E
3.	**Balasan River Basin**	Latitude 26°42’45.03”N Longitude 88°21’15.28”E
4.	**Jarda River Basin**	Latitude $26^0$34’4.01 °N Longitude $88^0$49’13.37°E
5.	**Crop Field, Falakata**	Latitude $26^0$30’05.37’’ N Longitude $88^0$12’19.77’’ E

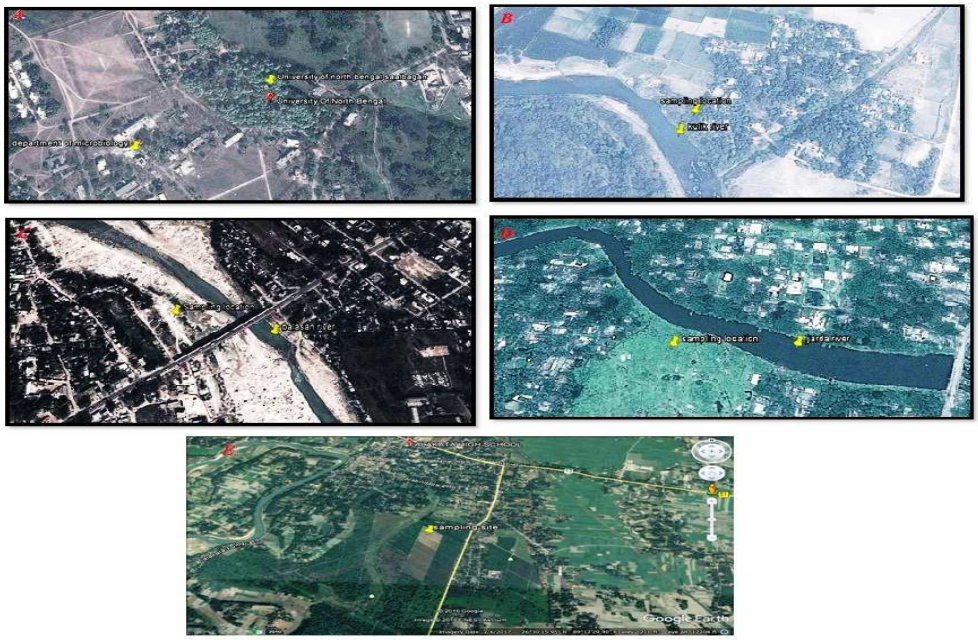


Fig. 2.1. Sampling sites for isolation of xylanase producing microorganisms. (A). Saal bagan, University of North Bengal; (B). Kulik River Basin; (C). Balasan River Basin; (D). Jarda River Basin; (E). Crop field near Falakata. (Image courtesy, Google Earth).

Xylan hydrolysis by the bacterial and fungal isolates has been represented as zone diameter and zone clearance ratio, respectively. Zone clearance ratio of the fungal isolates was calculated using the equation 1.

Zone clearance ratio = Diameter of hydrolysis zone (mm)/Diameter of colony in mm(Eq 1)

The bacterial isolates were grown in nutrient broth at 37 ^{o}C for 24 h and fungal isolates were grown in potato dextrose broth (PDB) at 30 ^{o}C for 48 h. Then equal volumes of individual culture and glycerol (80 %) were mixed and stored at -20 °C, until further use. The cultures from the glycerol stocks were propagated for two generations before the performance of experiments.

2.2.4. Quantitative screening of xylanase producing microorganisms

Microorganism(s) showing significant zone diameter or zone clearance ratio were further screened for quantitative xylanase activity.

2.2.4.1. Preparation of cell free extracts (CFE)

The isolated microbial strains were inoculated in xylanase production media (XPM = XYM-Agar). Fungal cultures were incubated at 30 ^{o}C for 48 h, whereas bacterial cultures were incubated at 37 ^{o}C for 24 h. An aliquot of culture broth was withdrawn at various time intervals and centrifuged at 8,000 RPM at 4 ^{o}C for 10 min. The supernatant after filtration (through 0.2 µm membrane filter) was designated as cell free extract (CFE) was used for various enzymatic assays.

2.2.4.2. Determination of total reducing sugars

Total reducing sugar was determined by the method of Miller (1959). Reducing sugars/ oligosaccharides released during enzymatic reaction or saccharification were reacted with dinitrosalicylic acid (DNS) reagent (solution A containing 24 g sodium potassium tartarate in 12 ml 2M NaOH was mixed with solution B containing 0.874 g DNS in 40 ml water). The reaction mixture was then heated in a boiling water bath for 10 min, cooled at room temperature followed by measurement of absorbance at 540 nm. Glucose and xylose served as the calibration standard for estimation of enzyme activities of cellulases and endoxylanases, respectively.

2.2.4.3. Xylanase assay

Xylanase was assayed according to the method of Bailey *et al.* (1992) based on the reaction of liberated reducing sugar (xylose equivalent) with 3, 5-dinitrosalicylic acid (DNS) reagent. The reaction mixture containing 0.25 ml CFE and 0.25 ml xylan solution (1% w/v in 100 mM citrate buffer, pH 5.4 for fungal xylanase or in 100 mM phosphate buffer, pH 7 for bacterial xylanase) was incubated at 37 ^{o}C for 20 min. The reaction was then stopped by adding 0.50 ml DNS reagent followed by incubation in boiling water bath for 10 min. After cooling at room temperature, absorbance was recorded at 540 nm and the amount of reducing sugar released was estimated from the reference curve prepared for 10 to 100 µg/ml of xylose (Fig 2.2). One international unit (IU) of xylanase activity is defined as the amount of the enzyme required to release 1 µmol xylose/min under standard assay condition. Reaction mixture without incubation served as blank.

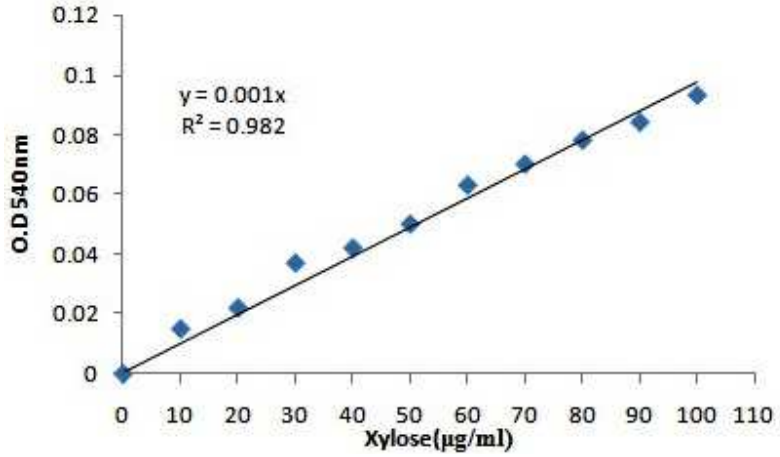


Fig. 2.2. Standard curve for xylose

2.2.5. Identification of the bacterial and fungal isolates

2.2.5.1. Morphological and biochemical characterization

Morphological characterization of bacterial isolates was done by growth pattern, motility test, spore formation test and gram staining. Growth pattern of the bacteria was monitored in XPM media for identification of their aerobic/anaerobic nature; motility test was performed in the sulphide indole motility agar medium; spore formation capability was checked by malachite green staining; and gram staining was performed to classify the microorganisms in specific genera. The biochemical characterisation studies included catalase test, Voges-Proskaur test, methyl red test, urease test, nitrate reduction test, oxidase

test, citrate utilization, indole test, starch hydrolysis test, casein hydrolysis, gelatin liquefaction test, lipid hydrolysis, growth at 45 ^{o}C, growth in 7% NaCl, fermentation of sugars (glucose, fructose, mannitol, lactose, sucrose, maltose, starch, xylose, sorbitol, mannose) (Smibert and Kreig 1994). Fungal isolates were identified by colony morphology, growth pattern on potato dextrose agar (PDA), lactophenol cotton blue staining.

2.2.5.2. Molecular analysis of isolates

2.2.5.2.1. Isolation of genomic DNA from bacterial isolates

Genomic DNA was isolated by Murmur's method (Murmur 1961). Bacterial culture was inoculated in Luria Bertani (LB) broth and incubated for overnight at 37 ^{o}C and then centrifuged at 8,000 RPM for 10 min at 4 ^{o}C. The cell pellet was washed with 0.1M EDTA: 0.15M NaCl solution (1:1) followed by centrifugation at 10,000 RPM for 5 min. Cell pellet was resuspended in 2-3 ml of 0.1 M EDTA: 0.15 M NaCl solution and was stored at -20 °C for 4 h. Frozen cells were thawed in a 55 ^{o}C water bath till dissolution and incubated with 50 µg/ml solution of lysozyme (prepared in 0.1 M Tris-HCl pH 8) at 37 °C for 30 min. To the cell lysate SDS was added and incubated at 55 °C for 15 min. The resulting mixture was then treated with proteinase K (4 µg/ml) at 55 °C for 30 min. Genomic DNA was purified from the lysate by sequential extraction with equal volume of Tris-saturated phenol (pH 8), Tris-saturated phenol:chloroform (1:1) and chloroform (Sambrook *et al.* 1989). DNA was precipitated from the aqueous phase by adding two volume of absolute ethanol followed by centrifugation at 10,000 RPM for 10 min at 4°C. The DNA pellet was washed with 75 % ethanol, air dried and dissolved in TE buffer [10 mM Tris HCl and 1 mM EDTA (pH 8.0)].

2.2.5.2.2. Isolation of genomic DNA from fungal isolate

Fungal genomic DNA was isolated according to the method describe by Sambrook *et al.* (1989). The fungal culture was inoculated in PDB and incubated for 48 h at 30 ^{o}C. The fungal mass was obtained by filtering the culture broth through a whatmann No.1 filter paper that allowed the medium to pass through and retained the fungal mass. The fungal mass was homogenized with a pestle and mortar using lysis buffer [100 mM Tris HCl (pH 8.0), 50 mM EDTA and 3% SDS]. The tissue homogenate was centrifuged at 13,000 RPM for 10 min and supernatant was transferred to a fresh centrifuge tube. To the supernatant, 2 µl of RNase A (10 mg/ml) was added and incubated at 37 °C for 15 min. To it equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well and

subjected to centrifugation at 12,000 RPM for 10 min (Note: this step was repeated once more to completely get rid of proteins/cell debris). The upper aqueous layer was taken in a fresh micro centrifuge tube; DNA was precipitated by adding twice volume ethanol followed by incubation at -20 ^{o}C for 30 min and centrifugation at 10,000 RPM for 10 min at 4 ^{o}C. The DNA pellet was washed with 75 % chilled ethanol, air dried and dissolved in TE buffer.

2.2.5.2.3. Agarose gel electrophoresis of DNA

1% agarose in 1X Tris-acetate EDTA (TAE) buffer [1 litre of 50X solution contains 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA] was melted and then cooled to 50-60 °C. It was then supplemented with 5µg/ml ethidium bromide (EtBr). The melted agarose was then poured in a casting tray fitted with a teflon comb forming wells. DNA sample was mixed with 1X DNA loading dye [0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in distilled water] and loaded onto the wells. Electrophoresis was performed in a horizontal electrophoresis tank using 1X TAE buffer at 100 V. DNA band was visualized on a UV-transilluminator.

2.2.5.2.4. PCR amplification of 16S rRNA and 28S rRNA

Bacterial genomic DNA was used as template for PCR amplification of 16S rRNA gene. The reaction mixture in total volume of 25 µl contained; 9.5µl ultrapure water, 5µl 5X PCR buffer (100 mM Tris-HCl, 500 mM KCl pH 8.3), 2µl $MgCl_2$(2mM), 1µl dNTP's (10 mM), 1µl forward primer(10 µM) 27F (5'AGAGTTTGATCCTGGCTCAG3'), 1µl reverse primer(10 µM) 1492R (5'TACGGTTACCTTGTTACGACTT3'), 5µl genomic DNA (20ng) and 0.50 µl DNA polymerase enzyme (5 U/µl). PCR was performed with initial denaturation at 94 ^{o}C for 5 min followed by 30 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 58 ^{o}C for 30 sec and extension at 72 ^{o}C for 1 min and then a final extension at 72 ^{o}C for 7 min. The PCR product was separated on 1% agarose TAE gel, cut from the gel, and then extracted and purified using gel extraction kit (QIAGEN, India). The purified PCR product was sequenced.

The D1/D2 (forward primer 5'GCATATCAATAAGCGGAGGAAAAG3' and reverse primer 5'GGTCCGTGTTTCAAGACGG3') region of 28S rRNA gene was amplified by PCR using fungal genomic DNA as template. PCR mix was prepared as mentioned above and PCR was performed with initial denaturation at 94 ^{o}C for 5 min followed by 30 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 58 ^{o}C for 30 sec and extension at 72 ^{o}C for 1 min and then a final extension at 72 ^{o}C for 7 min. The PCR product was separated on 1%

agarose TAE gel, cut from the gel, and then extracted and purified using gel extraction kit (QIAGEN, India). The purified PCR product was sequenced.

2.2.5.2.5. Phylogenetic analysis

The phylogenetic relationship of the bacterial and fungal isolates was determined by comparing their 16S and 28SrDNA sequences, respectively, with closely related neighbour sequences retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI), via BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.* 1990). Phylogenetic analysis was performed by using the software package MEGA 4 (Tamura *et al.* 2007) after obtaining multiple alignments of the data available from public database by Clustal W (Thompson *et al.* 1994). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 replicates (Nei and Kumar 2000).

2.2.6. Production of xylanase, carboxy methyl cellulose (CMCase), cellobiohydralase / filterpaperase (FPase), β-xylosidase and protease enzymes by the isolated strains

Fungal and bacterial inoculums were prepared by growing them, respectively in nutrient broth (NB) at 37 ^{o}C for 24 h and PDB at 30 ^{o}C for 48 h. Xylanase, CMCase, FPase, β-xylosidase and protease production ability of the isolated strains was monitored by cultivating the isolates on XPM. XPM prepared in 100 mM sodium citrate buffer (pH4) inoculated with fungal culture [2%(v/v) or 1g/l biomass] and grown for 48h at 30 ^{o}C, whereas the XPM prepared in 100 mM phosphate buffer (pH7) inoculated with bacterial culture [2% (v/v) or 10^{4}CFU/ml] and grown for 24 h at 37 ^{o}C. The CFEs prepared from the bacterial and fungal cultures by method described in section 2.2.4.1, were assayed for the various extracellular enzyme activities. All the assays for fungal and bacterial enzymes were carried out using 100 mM sodium citrate buffer (pH 4) and 100 mM phosphate buffer (pH 7), respectively.

2.2.6.1. Carboxymethyl cellulase assay

Carboxymethyl cellulase (E.C.3.2.1.4) was assayed by the method of Dahlberg and Kristijansson (1993). 1 % (w/v) sodium salt of carboxymethyl cellulose (CMC) in 100 mM buffer was used as substrate. 0.25 ml of CFE was mixed with 0.25 ml of substrate solution and the resulting reaction mixture was incubated at 37 ^{o}C for 20 min. The amount of reducing sugar released was determined by DNS method using the reference curve prepared for 10 to 100 µg/ml of glucose (Fig 2.3). Reaction stopped before incubation served as blank. One international unit (IU) of carboxymethyl cellulase activity is defined as the

amount of the enzyme required to release 1 µmol glucose/min under standard assay condition.

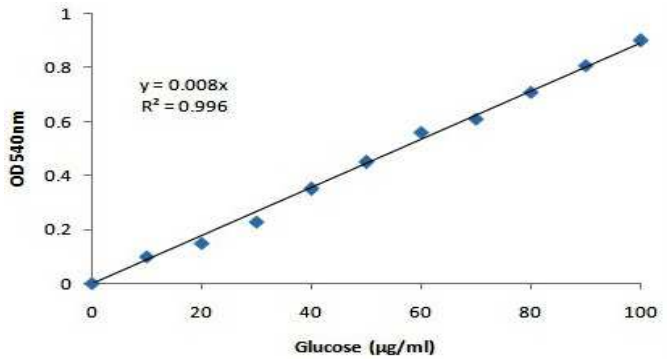


Fig. 2.3. Standard curve for glucose

2.2.6.2. Cellobiohydrolase assay

Cellobiohydrolase or FPase (E.C.3.2.1.91) assay was carried out by method of Mandel and Stenberg (1976), with some modification. 0.5 g of whatman no.1 filter paper was cut into 0.5 cm pieces and suspended in 0.5 ml of 100 mM buffer. To 0.5 ml of substrate solution, 0.5 ml of CFE was added and the mixture was incubated at 37 ^{o}C for 20 min and then the reaction was stopped by adding 1 ml DNS reagent. The mixture was kept in boiling water bath for 10 min,cooled then the amount of reducing sugar released was estimated by measuring the absorbance at 540 nm using the reference curve prepared for 10 to 100 µg/ml of glucose (Fig 2.3). Reaction stopped prior to incubation served as blank. One international unit (IU) of cellobiohydrolase is defined as the amount of the enzyme required to release 1 µmol glucose/min under standard assay condition.

2.2.6.3. β-xylosidase assay

β-xylosidase (E.C.3.2.1.37) assay was carried out by the method of Flanigan and Sellars, (1977). Reaction mixture containing 0.1ml of 10 mM ortho-nitrophenyl-β-D-xylopyranoside in 0.1 ml of 100 mM buffer and 0.1 ml of CFE was incubated at 37 ^{o}C for 20 min followed by termination of reaction by adding 2 ml of 1M Na_2CO_3. The liberated ortho-nitrophenol was measured at 410 nm using the reference curve prepared for 0.2 to 1.4 µM of ortho-nitrophenol (Fig 2.4). Reaction stopped prior to incubation served as blank. One international unit (IU) of β-xylosidase is defined as the amount of the enzyme required to release 1 µmol ortho-nitrophenol/min under standard assay conditions.

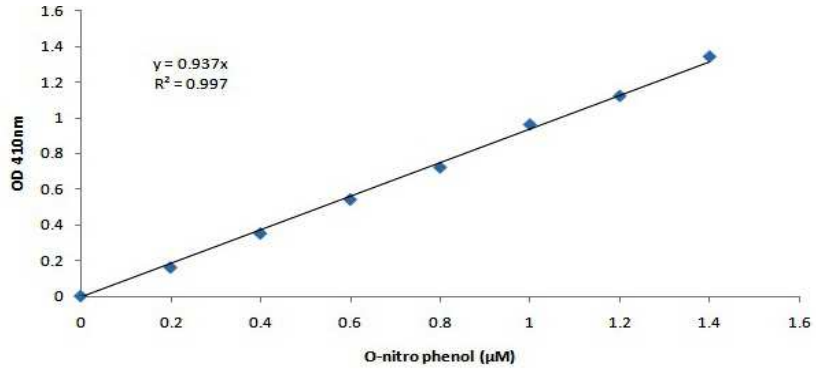


Fig. 2.4. Standard curve for ortho-nitrophenol

2.2.6.4. Protease assay

Protease assay was carried out by TCA method (Subramaniyan *et al.* 2001). The reaction mixture contained 0.5 ml of casein [2% w/v casein was dissolved in 100 mM phosphate buffer pH 7] and 0.5 ml of CFE. The resulting mixture was incubated for 40 min at 37 ^{o}C and the reaction was terminated by adding 10 % tricholoro acetic acid (TCA). The protein precipitate was removed by centrifugation at 10,000 RPM for 10 min at 4 ^{o}C. To 0.5 ml of supernatant, 5 ml of 0.5 M Na_2CO_3 was added and kept for 10 min at room temperature; to this 1ml of 1N Folin-ciocalteu's reagent was added followed by incubation for 30 min in dark at room temperature for development of colour. The liberated free amino acids were measured at 600 nm using a reference curve prepared for 100 to 1000 µg/ml of tyrosine (Fig. 2.5). One international unit of protease activity is defined as the µmol of tyrosine liberated/min under standard assay condition.

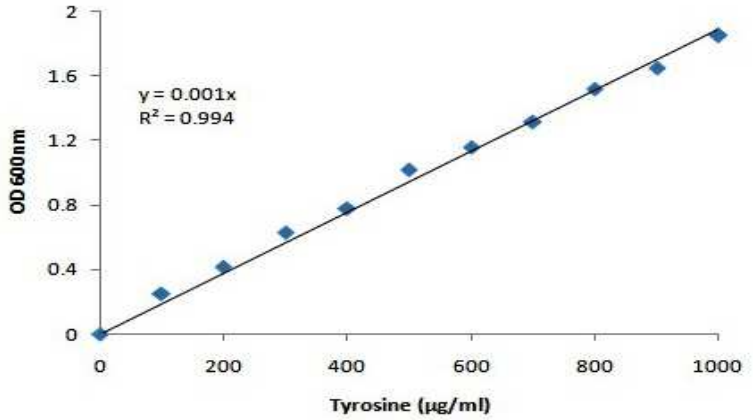


Fig. 2.5. Standard curve for tyrosine

2.2.7. Protein estimation

Protein was estimated by the method described by Bradford *et al.* (1976), using BSA as standard (Fig.2.6). 100 µl of protein sample was mixed with 3.0 ml of Bradford reagent [100 mg Coomassie Brilliant Blue G-250 (CBG-250) was dissolved in 50 ml 95 % ethanol; to the solution 100 ml 85 % (w/v) phosphoric acid was added and total volume was made 1 litre with distilled water]. The reaction mixture was incubated at room temperature for 10 min and the absorbance was recorded at 595 nm.

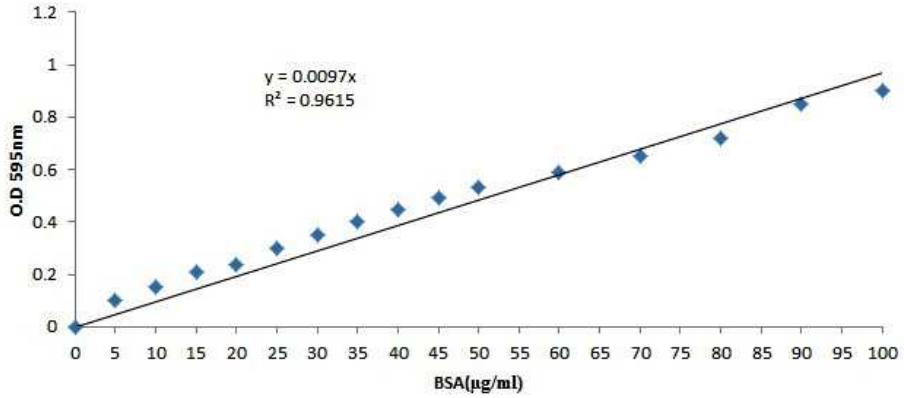


Fig. 2.6. Standard curve for BSA

2.2.8. Optimization of xylanase production

2.2.8.1. Optimization of xylanase production by OFAT (one factor at a time) approach

Xylanase production by *Penicillium citrinum* xym2 and *Bacillus subtilis* xym4 was optimized by OFAT using various agro residues. The cultural parameter optimized was incorporated in the subsequent steps of optimization.

Inoculum preparation

Inoculum for bacterial and fungal isolates were prepared by the method given in section 2.2.3. The inoculum at 2% (v/v) fungal mass or 10^4 CFU/ml of bacterial count was added in xylanase production medium (XPM).

Effect of agro-residues on xylanase production

The agro-residues such as, saw dust, sugarcane bagasse, coconut husk, orange peel, wheat bran, rice straw and corn fiber, were washed thoroughly with water, air dried and cut into small pieces for use as carbon source. The birch wood xylan in the XPM was replaced with either of the carbon source at 1.0 % (w/v) concentration. The media were inoculated with

either bacterial or fungal culture and the fermentation was allowed to proceed at 37 ^{o}C for 24 h (bacterial) or 48 h (fungal). Thereafter, xylanase activity in the CFE was monitored by the method given in section 2.2.4.2. Agro residues showing the maximum xylanase activity was further tested for its optimum concentration.

Effect of temperature and pH on xylanase production

The cultivation temperature was optimized by growing the organism at 4, 25, 30, 37, 50 and 60 ^{o}C for 24 h (Bacterial) or 48 h (fungal), followed by determination of enzymatic activity in culture CFE.

Xylanase production was studied at pH values ranging from 3 to 9. The media of different pH (100 mM citrate buffer pH 3, 4 and 5, 100 mM phosphate buffer pH 6, 7 and 100 mM Tris–HCl buffer 9) were inoculated with bacterial or fungal culture and incubated at 50 ^{o}C for 24 h (bacterial culture) or 30 ^{o}C for 48 h (fungal culture). Thereafter, CFE was assayed for xylanase activity.

Effect of additional carbohydrates on xylanase production

The production media having optimized agro residue as carbon source and pH, was supplemented with either of the monosaccharide/disaccharide such as dextrose, xylose, sucrose, maltose, galactose, fructose, pectine and lactose at 1% (w/v) each and subsequently inoculated with bacterial or fungal culture. The medium without additional sugar served as control. Fermentation was allowed to proceed at respective optimized incubation temperatures for 24 h (bacterial) or 48 h (fungal).Thereafter, CFE was assayed for xylanase activity. The sugar molecule showing maximum xylanase production was varied further to determine its optimum level.

Effect of nitrogen sources on xylanase production

In order to determine the effect of different nitrogen sources on xylanase production, the bacterial or fungal culture was inoculated in the production media with previously optimized parameters and 0.09% (w/v) of either of the nitrogen source, such as, peptone, yeast extract, ammonium nitrate, sodium nitrate, di-ammonium hydrogen phosphate, Tri-ammonium citrate and ammonium sulphate. Fermentation was allowed to proceed under submerged condition at optimized temperature for 24 h (bacterial) or 48 h (fungal) and xylanase activity in the CFE was determined.

Time course of xylanase production

To determine the time course of xylanase production, the fungal and bacterial strains were grown in their respective optimized XPM. Samples were withdrawn at 24h intervals for a period of 144h and xylanase activity in the CFE was monitored.

2.2.8.1.2. Biomass or cell count

Fungal and Bacterial cultures were inoculated in the 250ml of their respective OFAT optimized XPM and incubated for 144 h in six Erlenmeyer flask (500ml). One flask was withdrawn at an interval of 24 h for the quantification of fungal biomass or bacterial cell count. To determine the fungal biomass, 100 ml of culture broth was centrifuged at 10,000 RPM for 20 min. The cell pellet was washed three times with distilled water (10 ml).The washed pellet was dried at 80^{o}C in a hot air oven until till constant dry weight. XPM without inoculation served as control. Fungal biomass (g/l) was calculated according to the equation 2.

$$\text{Fungal Biomass (g/l)} = \frac{W2-W1}{V} \times 1000 \quad \text{.....(Eq.2)}$$

Where, W_2 is the weight of centrifuge tube with dried fungal biomass, W_1 is the weight of centrifuge tube with dried media residues only and V is the volume of culture broth used for centrifugation.

For determination of bacterial viable count 1ml of culture was serially diluted in saline water and spread plated on nutrient agar (NA) plates. The plates were then incubated at 37 ^{o}C for 24 h to get viable bacterial colonies. Colony forming unit (CFU) of the bacteria were enumerated according to the standard protocol and expressed in CFU/ml.

2.2.8.2. Optimization of xylanase production by *P. citrinum* xym2 and *B. subtilis* xym4 using Response surface methodology (RSM)

Optimization of xylanase production by RSM using central composite rotatable design (CCRD) was used to the study of interaction among three effective parameters selected from the OFAT method. Wheat bran, medium pH and cultivation temperature were selected to study their effect on xylanase production by *P. citrinum* xym2, whereas pectin, cultivation temperature, pH and incubation time were chosen for *B. subtilis* xym4 xylanase. All other media variables were kept constant at their OFAT optimized level. CCRD contains a factorial matrix with a centre point and “axial points” around the centre point that allow the

curvature of the model to be established. The distance from the centre point to the factorial point is ± 1 unit for each factor, and the distance space from the centre of the design space to the axial point is ± α, where $\alpha=(2^k)^{1/4}$ [k = number of independent factors]. The variables optimized for xylanase obtained from *Penicillium* were wheat bran (A), medium pH (B) and incubation temperature (C) with five different coded levels [-α, -1, 0, +1, +α]. The independent variables such as pectin (A), incubation temperature (B), medium pH(C) and incubation time (D) were also selected with the above said coded levels. Table 2.2 and 2.3 represent the coded value and the actual value of each of the these independent variables selected for generation of model for xylanase production by *P. citrinum* xym2 and *B. subtilis* xym4, respectively.

Table 2.2.
Relation between the independent variables selected for *P. citrinum xym2* xylanse production with their coded and actual level

Factor Code	Factor	Levels				
		-α	-1	0	+1	+α
A	Wheat bran (% w/v)	0.66	1	1.50	2	2.34
B	Medium pH	1.98	3	4.50	6	7.02
C	Incubation temperature (^oC)	23.18	30	40	50	56.82

Table 2.3.
List of variables used for xylanase production by *B. subtilis* xym4 using RSM

Factor Code	Factor	Levels				
		-α	-1	0	+1	+α
A	Pectin (% w/v)	0.75	1.5	2.25	3	3.75
B	Temperature (^oC)	35	40	45	50	55
C	pH	1.5	3	4.5	6	7.5
D	Incubation time (h)	36	48	60	72	84

The relation between the coded forms of the input variable and the actual value of the wheat bran, medium pH and incubation temperature are described in Eq (3).

$X_a = (Z_a - Z_0)/\Delta Z$……(Eq.3)

Where Xa is a coded value, Z_a is the actual value of the factor; Z_0 is the actual value of the same variable at the centre point and ΔZ the step change of the variable. According to the CCRD model total number of the experimental run is determined by the following equation Eq (4)

$R= 2^k + 2k+ n_0$ ……(Eq.4)

Where k is the number of independent variable and n_0 is the number of repetitions of the experiments at the centre point. Total number of experimental runs was found to be 20 (8 factorial, 6 axial and 6 centre point runs) for xylanase produced by *Penicillium* whereas 30 experimental runs (16 factorial, 8 axial and 6 center point runs) were conducted for xylanase production by *Bacillus*. Xylanase produced (Activity IU/ml) from all the experimental runs was analyzed by a second order polynomial regression equation [Eq. (5)] to better estimate the experimental error.

$Y= =a_0+a_1x_1+a_2x_2+a_3x_3+a_{11}x_{12}+a_{22}x_2^2+a_{33}x_3^2+a_{12}x_1x_2+a_{13}x_1x_3+a_{23}x_2x_3$……..(Eq. 5)

where Y is the predicted xylanase activity, a_0 is the intercept terms, x_i (x_1,x_2,x_3,x_{12},x_{22},x_{32}) is the independent factors and a_i (a_1,a_2,a_3,a_{11},a_{22},a_{33},a_{12},a_{13},a_{23}) is the model coefficient parameters. With the help of Eq. (3), all the independent variables are optimized to get a better response.

2.2.9. Purification of xylanase

2.2.9.1. Preparation of DEAE-Sephadex column

The pre-swollen DEAE-Sephadex (Sigma-Aldrich, USA) was suspended in 0.1M NaOH containing 0.5 M NaCl for 10 min with intermittent stirring. The ion-exchanger was then allowed to settle and excess of solution was decanted. The process was repeated with only 0.5 M NaCl and then with 0.1 M HCl containing 0.5 M NaCl. The slurry was then repeatedly washed with distilled water till the pH became about neutral. Finally, the charged DEAE-Sephadex was suspended overnight in 100 mM sodium phosphate buffer, pH 7 and then packed into glass column.

2.2.9.2. Purifications of xylanase from *B. subtilis* xym4 and *P. citrinum* xym2

B. subtilis xym4 and *P. citrinum* xym2 was grown in 250 ml of media containing (g/l): peptone, 0.90; $(NH_4)_2HPO_4$, 0.40; KCl, 0.10; $MgSO_4,7H_2O$, 0.10; birch-wood xylan, 5 and 2% w/v agar and incubating at 37 ^oC for 24 h (Bacterial) or 30 ^oC for 48 h (Fungal). The bacterial/fungal culture was centrifuged at 10,000 RPM for 10 min at 4 °C to collect the supernatant. The supernatant was further passed through a 0.2 μm of membrane filter and the filtrate was used as bacterial crude enzyme extract (BCE) or fungal crude enzyme extract (FCE). To CEs ammonium sulphate salt was added gradually at 0 ^oC with continuous stirring to achieve 30% saturation and kept at 4 ^oC for 30 min. The precipitated proteins were separated from the supernatant by centrifugation at 10,000 RPM for 20 min and the pellet was stored at 4 ^oC. The supernatant obtained was subjected to ammonium sulphate precipitation to achieve 70% saturation. After allowing to stand for 30 min, it was centrifuged at 10,000 RPM for 20 min. The pellet fractions obtained after 30 and 70% saturation of ammonium sulphate were dissolved in 2ml of 100 mM Tris-HCl pH 7.10 (BCE) or 100mM sodium citrate buffer pH 4 (FCE) and desalted by using dialysis sacs (Sigma-Aldrich, MWCO-12KDa). The desalted bacterial enzyme was loaded onto anion-exchange (DEAE-Sephadex) column (2x 15 cm) pre-equilibrated with 100 mM Tris-HCl pH 7 (Buffer A). The column was washed with Buffer A to remove the unbound proteins and then eluted as 1 ml fractions using linear gradient of 0-0.50 M NaCl in Buffer A followed by determination of xylanase activity in each fraction. The active fractions were pooled, concentrated and subjected to gel filtration chromatography on a P-100 column (Bio Rad, 1x25 cm) equilibrated Buffer A. Fractions of 1 ml each were collected and analyzed for protein and xylanase activity. The active fractions were pooled and stored at 4 ^oC for further studies.

2.2.9.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the method described by Laemmli (1970). The enzyme fractions from various steps of purification were resolved by 12 % SDS-PAGE. Resolving gel solution was composed of 30 % acrylamide, 4 ml; 1.5 M Tris-HCl (pH 8.8), 2.50 ml; 10 % SDS, 0.10 ml; 10 % APS, 0.10 ml; TEMED 0.004 ml and 3.30 ml water and was poured between two glass plates (5 cm height) which were clamped together but held apart by plastic spacers and was allowed to set. The stacking gel mixture made up with 1M Tris-HCl (pH 6.8), 0.38 ml; 10 % SDS, 0.03 ml; 10 % APS, 0.03 ml; TEMED, 0.003 ml; 2.10 ml water, was poured on the top of resolving gel (1 cm in length) and a plastic comb was placed on the stacking gel. After polymerization the comb was carefully removed to provide

loading wells. Glass plate assembly with the gel was placed in vertical electrophoresis system with running buffer tank containing the running buffer [25mM Tris-HCl (pH 8.0), 250 mM glycine, 0.10% (w/v) SDS]. The protein sample was mixed with SDS gel loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2 % (w/v) SDS, 0.10 % bromophenol blue, 10% glycerol] and heated in a boiling water bath for 10 min. Protein samples and prestained protein molecular weight markers (Fermentas) were loaded in the wells and electric field was applied. When the dye reached at the bottom of the tank, power was turned off. Gel was removed carefully from the glass plates and stained with CBB R-250 reagent [0.1 % Coomassie brilliant blue R-250 in 10 % acetic acid and 40 % methanol] for overnight, distained to remove unbound stains and visualized under transilluminator.

2.2.10. Characterization of xylanase

The purified xylanase of *B. subtilis* xym4 and partially purified xylanase of *P.citrinum* xym2 were characterized for pH optima, temperature optima, thermostability, Km for birchwood xylan, and metal ions requirement.

2.2.10.1. pH optimum, temperature optimum and thermostability

The pH optimum for both the bacterial and fungal xylanases were determined by measuring the enzyme activity at pH 3.0-9.0 in the following buffers: 100 mM glycine (pH 3.0 and 3.5), 100 mM sodium acetate (pH 4.0, 4.5 and pH 5.5), 100 mM phosphate (pH 6.0, 6.5 and 7.0) and 100 mM Tris-HCl (pH 7.5, 8.0, 8.5 and 9). Xylanse activity was determined as described in materials and method section 2.2.4.2. except for varying the buffer solution. The optimum temperature for both the xylanase were determined at optimum pH and at temperature ranging from 4° to 90 °C. The incubation temperature was varied for the standard assay procedure. For determination of thermal stability, the desalted enzyme preparation was pre-incubated at various temperatures ranging 20-100 ^{o}C for 30 min, followed by determination of activity under standard conditions**.**

2.2.10.2 Effect of metal ions

The effect of metal ions and chemical reagents on both the xylanase were determined by measuring enzymatic activity in presence of 5 mM Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, K^{+}, Na^{+}, SDS, EDTA, β-ME. Activity without addition of ions or reagents was used as control.

2.2.10.3. Determination of K_m

Purified bacterial xylanase preparation was incubated with birchwood xylan concentrations ranging 2.5-25 mg/ml in 100mM phosphate buffer pH 7 at 37 ^{o}C. K_m for xylan was determined form Lineweaver-Burk double reciprocal plot.

2.3. Results

2.3.1. Isolation, Screening and Identification of xylanase producing microorganism

Xylanase producing microorganisms were isolated from the environmental samples by serial dilution method on XYM media. Fifteen microorganisms showing growth on XYM plates were examined for microscopic and colony characteristics. Among these five isolates showed morphological features of fungi, whereas ten were bacteria. All the strains were qualitatively screened for xylanase production based on the formation of zone of clearance on xylan-agar plates and zone clearance ratio for the fungal isolates was determined. Nine isolates (seven bacteria and two fungi) showing relatively greater zone clearance ratio was further screened for quantitative extracellular xylanase activity after growing them in XPM media. Two fungal strains xym2, xym4 and one bacterial strain xym4.1 showed significantly higher xylanase activity of 712 ± 0.66, 1420 ± 0.27 and 981 ± 0.58 IU/ml, respectively (Table 2.4) and hence selected for further studies. A representive figure for xylan hydrolysis by these isolates and by their CFEs on XYM plate, are depicted in figure 2.7 and 2.8 respectively.

Table 2.4.
Xylanase producing microorganisms isolated from soil of different region of north bengal

Sl.No	Isolates	Activity (IU/ml)	Zone diameter(mm)	Zone clearance ratio	Location	Isolate Type
1	Xym1	185.93±0.72	0.6	-	Kulik River Basin	Bacteria
2	**Xym2**	**712±0.66**	3	1.5	**Saalbagan (NBU)**	**Fungus**
3	Xym3	185.703±0.60	0.6	-	Balasan River Basin	Bacteria
4	**Xym 4**	**1420±0.27**	**3**	**2.1**	**Saalbagan (NBU)**	**Fungus**
5	**Xym 4.1**	**981.52±0.58**	**0.7**	**-**	**Saalbagan (NBU)**	**Bacteria**
6	Xym 5	140.901±0.79	0.6	-	Jarda River Basin	Bacteria
7	Xym 6	138.76±0.66	0.6	-	Balasan River Basin	Bacteria
8	Xym 7	188.86±0.86	0.7	-	Crop Field Falakata	Bacteria
9	Xym 8	158.76±0.76	0.7	-	Saalbagan (NBU)	Bacteria

Activities are presented as triplicates of mean±standard deviation

Isolated bacteria were identified based on their colony morphology in nutrient agar plates, gram nature and biochemical reaction up to genus level. The strain xym4.1, showing the maximum xylanase activity, was morphologically characterized as gram positive, rod shaped, motile bacteria. The colonies of this isolates were creamy white, irregular in margin and convex in appearance in nutrient agar plates. The bacterium was characterized biochemically as negative in citrate utilization, Voges-Proskauer tests, urease test, oxidase test (table 2.5). Strain xym4.1 was further identified using phylogenetic analysis based on 16S rRNA gene sequence comparisons which showed that the isolate xym4.1 (GenBank accession number MG607370) belong to the branch encompassing members of genus *Bacillus* and was most closely related to *Bacillus subtilis* with 99% 16S rDNA sequence similarity (Fig. 2.9) and hence identified as *Bacillus subtilis* xym4. Xylanase producing fungal strain xym4 was identified on the basis of microscopic observation and colonoy morphology. Microscopic image and the colony morphology of the isolate are represented in the Fig 2.10. As can be observed colonies are yellowish green in colour with velvety appearance, white peripheral apron and distinct margin. Conidiophores are long (200-400 μ) and have club shaped vesicles that are 20-40 micron in diameter. Conidia arise in chains and

tend to sweep toward the central axis. On the basis of these observations the strain xym4 was identified as *Aspergillus flavus* and hence named as *Aspergillus flavus* xym4. The fungal strain xym2 was identified by microscopic observation, colony characteristics (Fig 2.11) and by 28S rDNA sequencing followed by phylogenetic tree construction using MEGA 4 software (Fig.2.12). Analysis of phylogeny revealed that xym2 is closely related to *Penicillium citrinum* and hence named as *Penicillium citrinum* xym2 (Gene bank accession no. KF570240).

2.3.2. Production of other carbohydrate active enzymes by the isolates

The three selected srtains *Bacillus subtilis* xym4, *Aspergillus flavus* xym4 and *Penicillium citrinum* xym2 were also analysed for production of other carbohydrate active enzymes such as FPase, CMCase and β-xylosidase, as well as protease. Although *A. flavus* xym4 produced higher amount of xylanase (1400 ± 7.1 IU/ml**)** and cellulase (817 ± 6.1 IU/ml) as compared to other two strains, it was completely unable to produce FPase and β-xylosidase. On the other hand, *B. subtilis* xym4 and *P. citrinum* xym2 showed FPase activity of 211 and 216 IU/ml, respectively, and β-xylosidase activity of 720 and 211 IU/ml, respectively (Table 2.6).

Production of xylanase along with significant amount of FPase and β-xylosidase by the microorganisms happens to be beneficial for efficient saccharification of lignocellulosic agro residues to xylose, the substrate for synthesis of xylitol. Therefore, *B. subtilis* xym4 and *P. citrinum* xym2 were selected for optimization of xylanase production using one factor at a time and statistical approach.

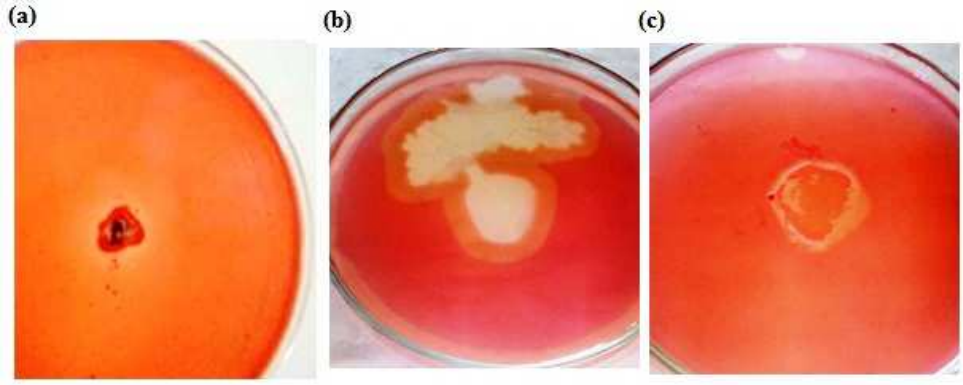


Fig. 2.7. Primary screening for detection of xylanase activity on xylan agar media (XYM) after congo red staining followed by washing with 1M NaCl ,showing yellow zone of clearance against red back ground (a) *Aspergillus flavus* xym4, (b) *Penicillium citrinum* xym2 and (c) *Bacillus subtilis* xym4

Table 2.5.
Morphological and biochemical characteristics of the xylanase producing microrganism

Sl.No		Xym1		Xym3		Xym 4.1		Xym 5		Xym 6		Xym 7		Xym 8	
1	Gram staining	+		+		+		+		+		+		+	
2	Shape of vegetative cell	Rod		Coccus		Rod		rod		rod		coccus		rod	
3	Diameter of colony(mm)	5		9		3.4		1.2		2.4		10		3.1	
4	Colony Morphology	Larger, round, smooth, convex, colorless colonies		Round, circular, raised, margin, smooth, opaque colonies		creamy white, irregular margin and convex		Circular, entire edge, flat surface, non-mucoid, translucent colonies		Round, irregular margin, raised, smooth, opaque colonies		white, irregular margin and convex		white, regular margin and convex	
5	Spore formation	-		-		+		-		+		+		-	
6	Motility	-		-		+		-		+		+		-	
7	Growth on usual media (Aerobic)	+		+		+		+		+		+		+	
8	Growth on NaCl	-		+		+		-		+		+		-	
9	Starch hydrolysis	+		+		+		+		-		+		+	
10	Casein hydrolysis	+		+		+		+		+		+		+	
11	Gelatin hydrolysis	-		-		+		+		+		+		+	
12	Lysine decarboxylase	+		-		+		+		+		+		+	
13	Urease test	-		+		-		-		+		+		+	
14	Catalase test	+		+		+		+		+		+		+	
15	Oxidase test	+		+		-		-		-		+		+	
16	Lipase test	-		+		+		+		+		+		+	
17	Citrate utilization	-		-		-		-		+		+		+	
18	Voges-Proskauer test	-		-		-		-		-		-		-	
19	Methyl Red test	+		+		+		+		+		+		+	
20	Nitrate reduction test	+		+		+		+		+		+		+	
21	Fermentaion/Oxidation	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
														+	+
21.1	Starch	+	+	+	+	+	+	+	+	+	+	+	+	-	+
21.2	Lactose	-	+	+	+	-	+	-	+	-	+	-	+	-	+
21.3	Glycerol	+	-	+	+	-	+	-	-	-	+	+	+	+	+
21.4	Mannose	+	-	+	+	-	+	-	+	-	+	+	+	+	+
21.5	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21.6	Fructose	+	+	+	+	-	-	+	+	-	+	+	+	-	+
21.7	Manitol	-	+	+	+	-	-	+	-	+	-	-	+	+	+
21.8	Xylose	+	-	+	+	-	-	+	+	+	+	+	+	+	+
21.9	Arabinose	-	+	+	+	+	-	-	-	+	+	+	+	-	+
21.10	Cellobiose	+	+	+	+	+	+	+	+	-	+	+	+	-	+
21.11	Sucrose	+	+	-	+	-	-	+	-	-	+	+	-	-	+
21.12	Maltose	-	-	+	+	-	-	+	-	-	+	+	+	-	+
21.13	Sorbitol	-	+	-	+	-	-	+	+	-	+	+	+	-	+
	Identity of Bacteria	***Geobacillus sp***		***Panebacillus sp***		***Bacillus sp***		***Geobacillus sp***		***Salinicoccussp***		***Bacillus sp***		***Bacillus sp***	

Fig. 2.8: Zone of xylan hydrolysis on XYM plates after congo red staining, using extracellular CFE of (1) *B. subtilis* xym4 (2) *P. citrinum* xym2 (3) *A. flavus* xym4.

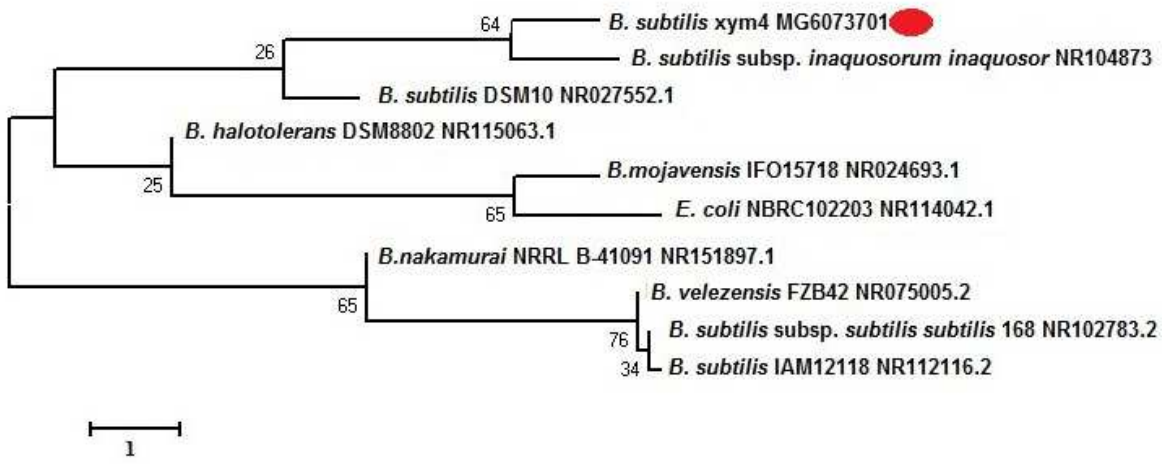


Fig.2.9. Phylogenetic tree constructed using 16S rRNA gene sequences reflecting the position of strain xym4 with the other *Bacillus* species. The sequence of *E. coli* NBRC102203 (accession no. NR114042.1) was incorporated in the tree as on out group. Bar represent 1 nucleotide substitution per base. Numbers at nodes represent bootstrap values. Accession numbers are given at extreme left of each strain.

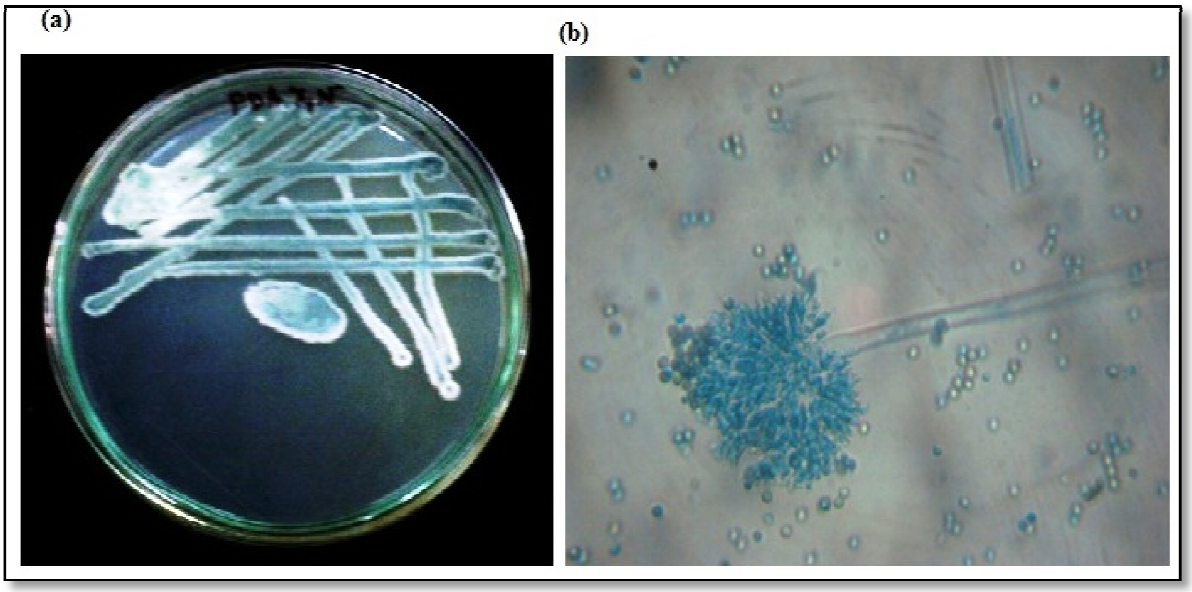


Fig 2.10. (a) Characteristic growth of *Aspergillus flavus* xym4 on PDA plate and (b) microscopic image of its hyphe along with sporangium and spores.

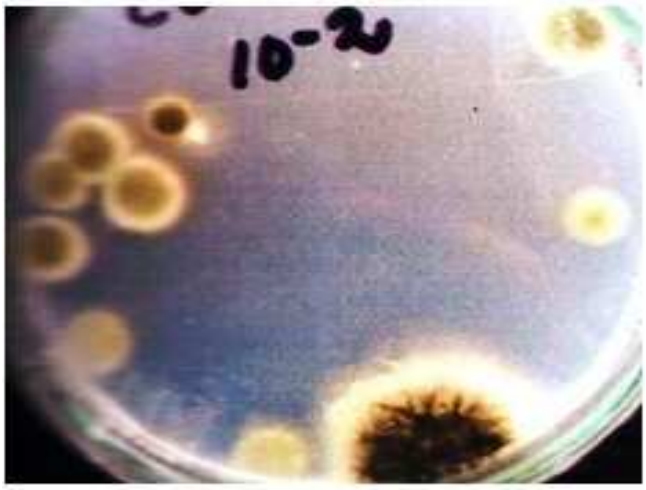


Fig 2.11. Growth of *Penicillium citrinum* xym2 on PDA plate.

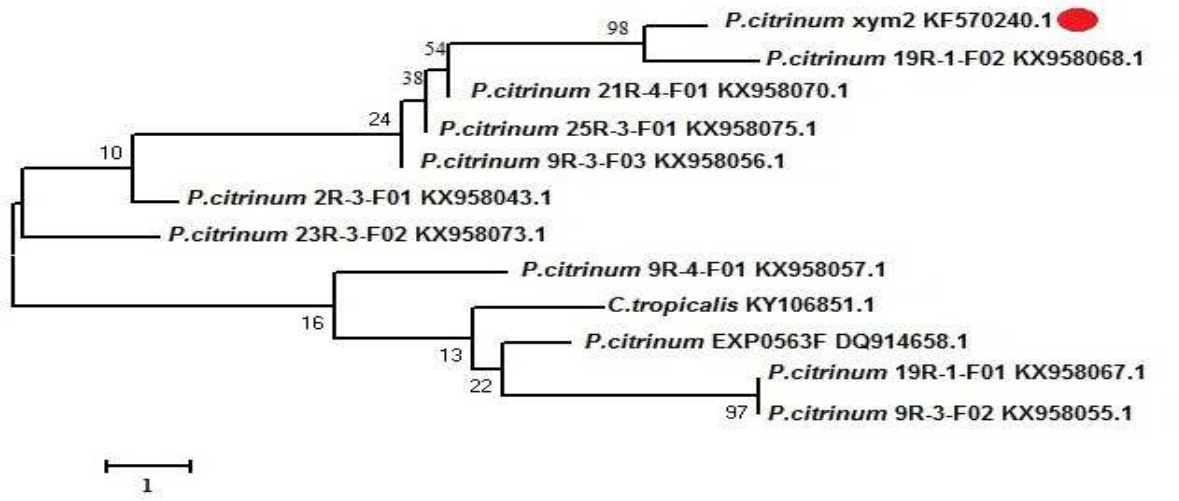


Fig.2.12. Phylogenetic tree constructed using 28S rRNA gene sequences reflecting the position of strain xym2 with the other *Penicillium* species. The sequence of *C. tropicalis* (accession no. KY106851.1) was incorporated in the tree as an out group. Bar represents 1 nucleotide substitution per base. Numbers at nodes represent bootstrap values. Accession numbers are given at extreme left of each strain.

Table 2.6
Production of xylanase, FPase, CMCase, β-xylosidase and protease by *B. subtilis* xym4, *A. flavus* xym4 and *P. citrinum* xym2

Enzymes Activity(IU/ml)	*B. subtilis* xym4	*A. flavus* xym4	*P. citrinum* xym2
Xylanase	980 ± 4.2	1400 ± 7.1	780± 10.2
FPase	211± 11	0	216± 11.3
CMCase	56± 8.1	817±6.1	921± 1.2
β- Xylosidase	720± 6.0	0	211± 1.2
Protease	106± 15.7	110	301±7

The CFE was prepared for determination of enzyme activity from *B. subtilis* xym4 grown at 37 ^{o}C for 24 h , *A. flavus* xym4 and *P. citrinum* xym2 were grown at 30 ^{o}C for 48 h.

2.3.3. Optimization of xylanase production

2.3.3.1. OFAT approach

The production of xylanase by *P. citrinum* xym2 was carried out in presence of either of the agro residue such as saw dust, sugarcane bagasse, coconut husk, orange peel, wheat bran, rice straw and corn fiber (1.0 % w/v each) as sole carbon source in xylanase production medium (XPM) at 37 ^{o}C for 48 h. As shown in the Fig.2.13a, wheat bran showed the significantly higher xylanase yield of 878 ± 12.33 IU/ml as compared to that of

Birchwood xylan (712 ± 3.23 IU/ml). Further, wheat bran at 1% (w/v) supported maximum xylanase activity of 885 ± 6.7 IU/ml and production of the enzyme gradually declined above and below the optimum concentration (Fig. 2.13b). For determination of optimum incubation temperature for enzyme production *P.citrinum* was grown in XPM containing 1% wheat bran, at the temperature range was 4-60 ^{o}C keeping the other growth variables constant. Although *P. citrinum* xym2 produced notable amount of xylanase at 4 and 60 ^{o}C, the maximum level of xylanase activity (925 ± 2.8 IU/ml) was obtained at 30 ^{o}C (Fig.2.13c). The optimum pH for production of xylanase was determined by growing the fungal strain in the above optimized media of pH range 3 to 9 at 30 ^{o}C followed by determination of extracellular enzymatic activity. Xylanase production was maximum (1025 ± 4.3 IU/ml) in the medium with pH 4 and a progressive and significant ($p<0.001$) loss of activity was noted below and above 4 (Fig.2.13d). Xylanase production medium prepared in 0.1 M citrate buffer at pH 4, containing 1% (w/v) wheat bran, supplemented with either of the sugars (1% w/v) such as, dextrose, xylose, sucrose, maltose, galactose, fructose and lactose was inoculated with *P. citrinum* xym2 and the medium was incubated at 30 ^{o}C for 48h. Xylanase production increased significantly to 1122 ± 14.22 IU/ml on supplementation of maltose (Fig.2.13e). However, other carbohydrates reduced the enzyme production with maximum decline in presence of fructose 243 ± 11.23 IU/ml. Effect of varying concentration of maltose (0.50 to 1.75% w/v) was also studied and the results revealed 1.25 % (w/v) concentration as the optimal level with maximum xylanase yield of 1250 ± 8.2 IU/ml. Further increase in maltose concentration resulted in decline of activity (Fig.2.13f). Nitrogen source is an important media parameter that influences the cellular growth and thus enzyme production. To check the effect of nitrogen on xylanase yield, XPM was supplemented with various organic and inorganic nitrogen compounds such as peptone, yeast extract, ammonium nitrate, sodium nitrate, di ammonium hydrogen phosphate, ammonium sulphate and tri ammonium citrate at a concentration of 0.09 %(w/v) individually. As shown in results of Fig. 2.14a, di-ammonium hydrogen phosphate showed significantly ($p<0.001$) higher xylanase production of 1278 ± 6.43 IU/ml. In addition to $(NH_4)_2HPO_4$, substantially higher activity was also noted with ammonium nitrate (1102 ± 6.8 IU/ml), sodium nitrate (922 ± 7.77 IU/ml) and ammonium sulphate (912 ± 2.11 IU/ml). The effect of varying concentration of di-ammonium hydrogen phosphate on enzyme production was showed 0.09 % (w/v) as the optimum for xylanase activity (1278 ± 6.43 IU/ml) (Fig.2.14b). Finally, xylanase yield by *P. citrinum* xym2 was evaluated at different incubation time keeping all other variables constant at their optimal levels. A maximum xylanase activity of 1853 ± 7.77 IU/ml was obtained after 72 h of incubation, with a fungal biomass yield of 55 ± 1.43 mg/l (Fig.2.14c). Biomass and enzyme activity was found to be

positively correlated with a pearson correlation coefficient of 0.862. Thus, optimization of xylanase production by OFAT approach revealed that *P. citrinum* xym2 produced maximum xylanase of 1853 ± 7.77 IU/ml, when grown on media prepared in 0.1 M citrate buffer at pH 4, containing 1 %(w/v) wheat bran, 0.09 %(w/v) of di ammonium hydrogen phosphate, 0.1g/l KCl, 0.1g/l $MgSO_4$, $7H_2O$, supplemented with 1.25 %(w/v) maltose, at 30 ^oC of incubation temperature after 72 h of submerged fermentation.

Bacillus subtilis xym4 was also cultivated in XPM media for optimization of xylanase production using OFAT approach. The results in Fig.2.15 a and b reveal that among the various agro residues tested, wheat bran at a concentration of 1% (w/v) showed the highest xylanase activity of 1232 ± 7.89 IU/ml, which was significantly higher than that obtained in presence of commercial birchwood xylan (980 IU/ml). The XPM containing 1% (w/v) wheat bran as sole carbon source was further inoculated with the bacteria and incubated at different temperatures ranging from 4 to 60 ^oC. Although, notable amount of xylanase activity was seen at 37 ^oC, maximum yield of 1321 ± 5.6 IU/ml was achieved at 50 ^oC and further increment of incubation temperature lowered the enzymatic activity (Fig.2.15c). To check the effect of medium pH, the XPM was prepared in different buffer solutions (as mention in material and method section) of pH range 3 to 9. The highest xylanase yield (1358 ± 11.1 IU/ml) was obtained at medium pH 6 (Fig. 2.15d). Additional carbon sources such as dextrose, xylose, sucrose, pectin, maltose, fructose and lactose were added individually to the above optimized XPM (pH 6) at a concentration of 1% (w/v) and the resulting media was inoculated with the bacterial strain and incubated at 50 ^oC for 24 h. Among the carbon sources pectin showed significantly higher amount of xylanase activity (1542 ± 12.71 IU/ml) as compared to that in medium where no additional carbon source was added (1154 ± 10.71 IU/ml) (Fig 2.15e). All other additional sugars had detrimental effect on enzyme production. The effect of different amount of pectin was also tested and the maximum xylanase (1545 ± 4.5 IU/ml) production was achieved at a concentration of 1 % (w/v), further increase in pectin concentration decreased the enzyme production (Fig. 2.15f). In next step of this approach the effect of nitrogen sources was tested and peptone at a concentration of 0.09 % w/v showed the maximum xylanase activity of 1720 ± 11.1 IU/ml (Fig.2.16a & b). Finally, xylanase activity and bacterial colony count were evaluated at various incubation times in the range of 24 -144 h keeping all other variables of the XPM medium constant in their optimal levels. It was observed that maximum xylanase activity of 2100 ± 5.17 IU/ml was obtained after 48 h of incubation with a bacterial count of 6 Log CFU/ ml (Fig.2.16c). Bacterial colony count and enzyme activity was found to be positively correlated with a pearson correlation coefficient of 0.791.

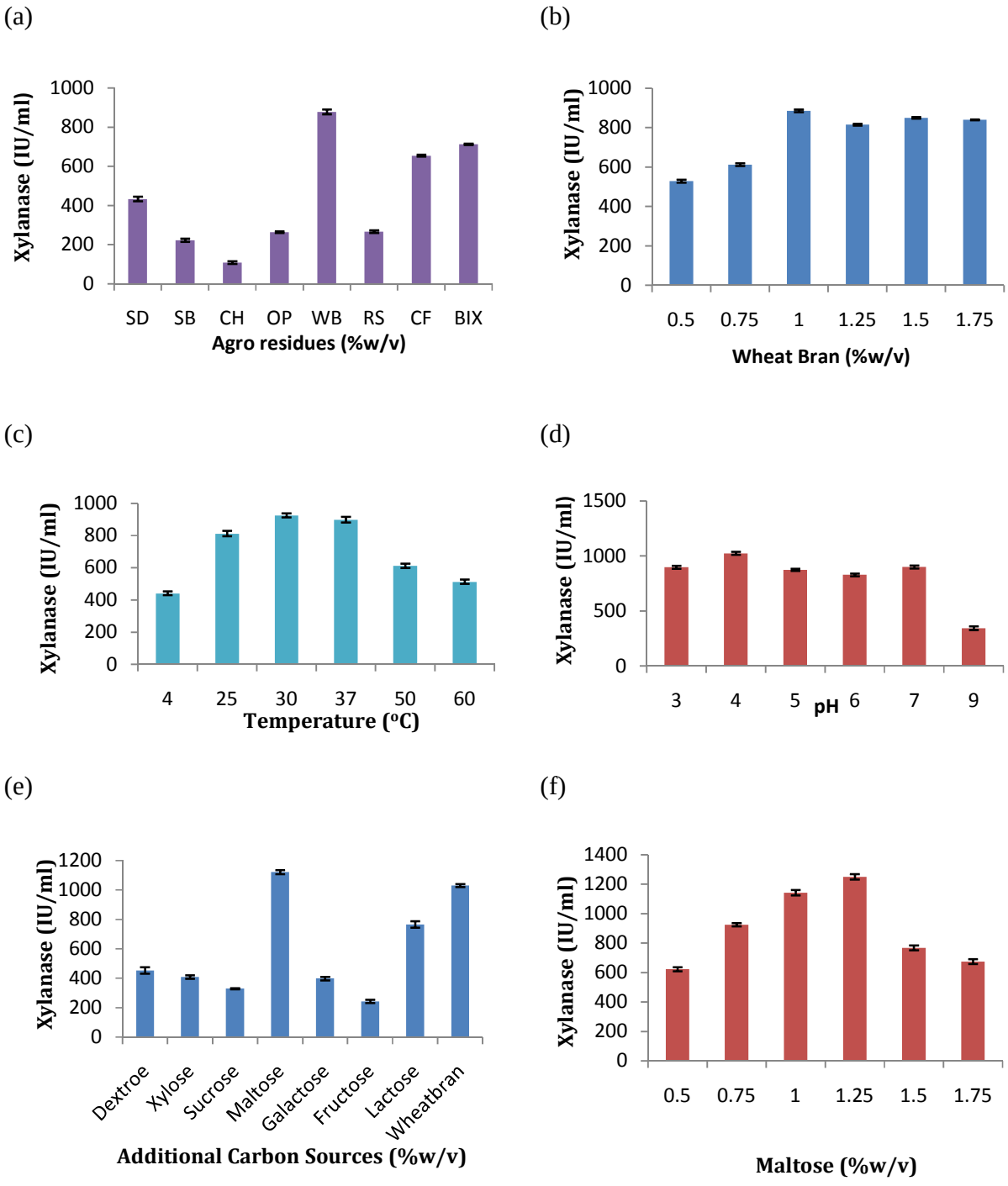
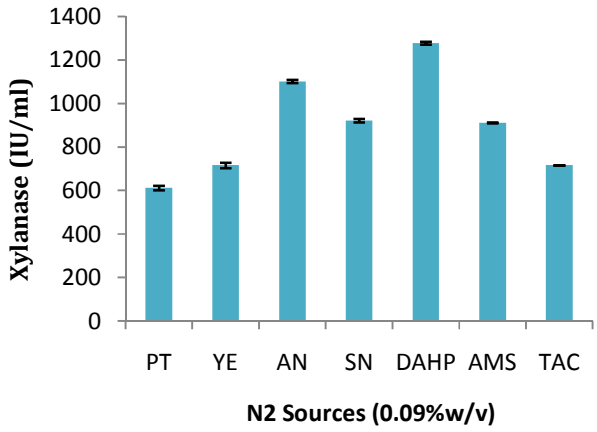


Fig.2.13. Optimization of xylanase production by *P. citrinum* xym2 by OFAT approach. Effect of (a) agro-residues [saw dust (SD), sugarcane baggase (SB), coconut husk (CH), orange peel (OP),wheat bran (WB), rice straw (RS), corn fiber (CF), birchwood xylan (BiX)] (b) wheat bran concentration (c) incubation temperature (d) pH (e) additional carbon source (f) maltose concentration on xylanase production. The parameter optimized was incorporated in subsequent experiment.

(a)



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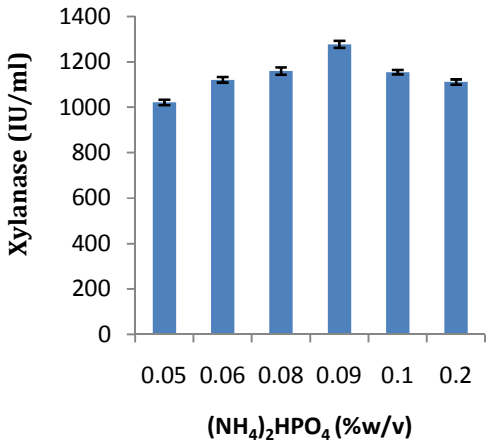


(c)

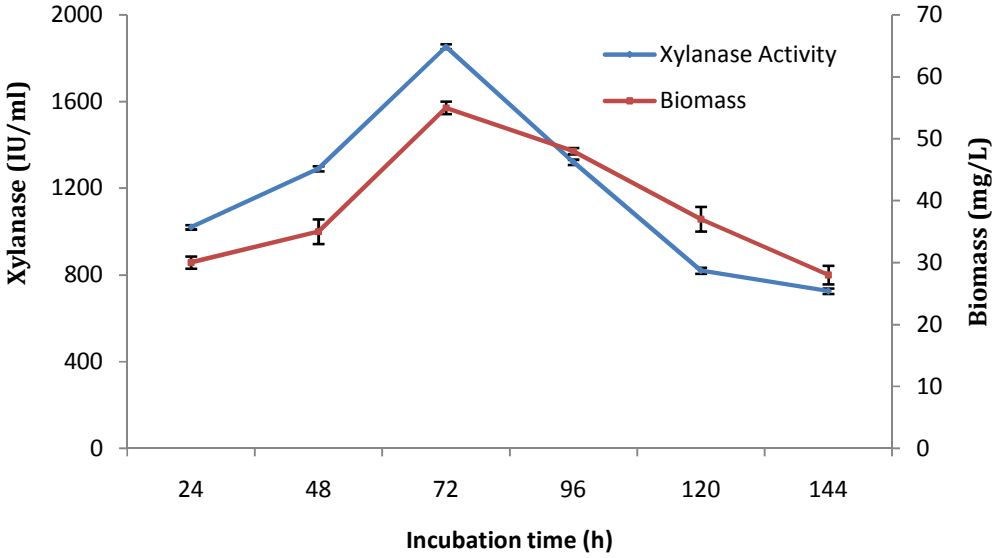


Fig.2.14. Optimization of xylanase production by *P. citrinum* xym2 by OFAT approach. Effect of (a) nitrogen sources [peptone (PT), yeast extract (YE), ammonium nitrate (AN), sodium nitrate (SN), di-ammonium hydrogen phosphate (DAHP),tri ammonium citrate (TAC)] (b) DHAP concentration (c) incubation time on xylanase production. The parameter optimized was incorporated in subsequent experiments.

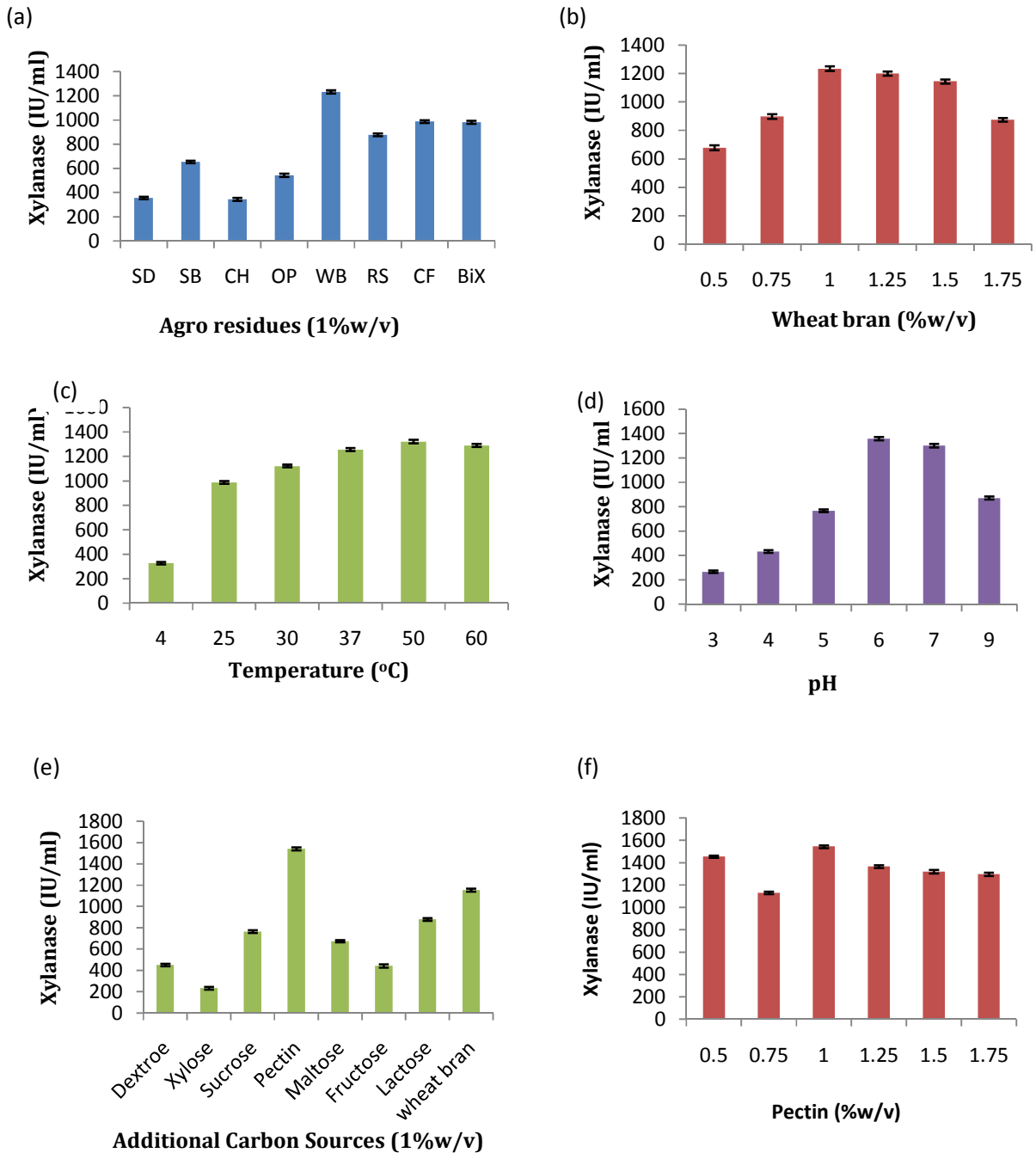
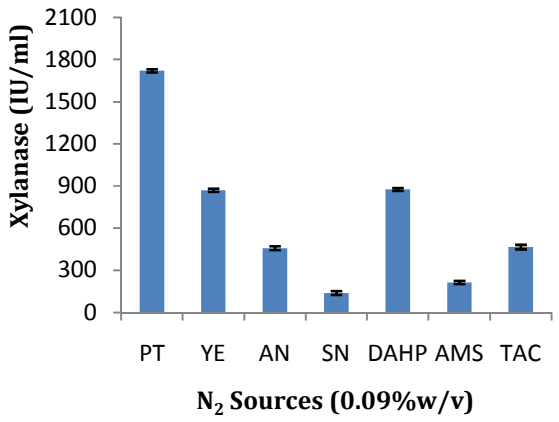
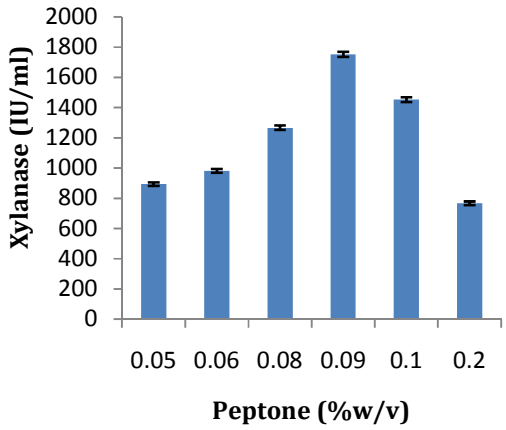


Fig.2.15. Optimization of xylanase production by *B. subtilis* xym4 by OFAT approach. Effect of (a) agro-residues [saw dust (SD), sugarcane baggase (SB), coconut husk (CH), orange peel (OP),wheat bran (WB), rice straw (RS), corn fiber (CF), birchwood xylan (BiX)] (b) wheat bran concentration (c) incubation temperature (d) pH (e) additional carbon source (f) pectin concentration on xylanase production.

(a)



(b)



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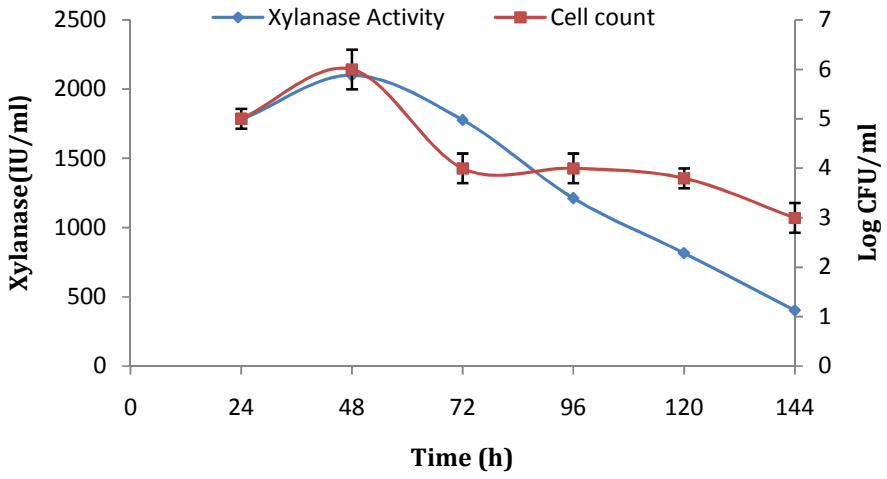


Fig.2.16. Optimization of xylanase production by *B. subtilis* xym4 by OFAT approach. Effect of (a) nitrogen sources [peptone (PT), yeast extract (YE), ammonium nitrate (AN), sodium nitrate (SN), di ammonium hydrogen phosphate (DAHP), tri ammonium citrate (TAC)] (b) peptone concentration (c) incubation time on xylanase production. The parameter optimized was incorporated in subsequent experiments.

2.3.3.2. Optimization of xylanase production by *P. citrinum* xym2 using response surface methodology (RSM)

The effect of three variables, wheat bran (A), medium pH (B) and incubation temperature (C), on xylanase production was investigated. Suitable levels for these parameters were determined by CCRD of RSM. For xylanase production optimization twenty experimental runs were performed using different combinations of the variables as per CCRD. The medium components used for CCRD are given in the methods section 2.2.8.2 and the experimental design and results of CCRD are shown in table 2.7. Analysis of variance (ANOVA) was performed to the experimental design used. The result of ANOVA shows that model as highly significant ($p<0.001$) with better prediction of data. Within the model, B (medium pH), C (incubation temperature), AB (wheat bran X medium pH), AC (wheat bran X incubation temperature), A^2 (wheat bran^2), B^2 (medium pH^2), and C^2 (incubation temperature^2) are the significant ($p<0.001$) model terms. Using the results of these experiments, second-order polynomial regression equation for xylanase yield was obtained as represented in Eq. (6) and (7) in coded and actual form, respectively.

$$\text{Activity} = +2643.49 +24.91*A -297.20*B -325.54*C +370.77*A*B -143.47*A*C +79.75*B*C -430.25*A^2 -370.94*B^2 -208.71*C^2 \quad \text{(Eq. 6)}$$

$$\begin{aligned}\text{Activity} = &-3215.30566+4135.96558*\text{Wheatbran}+331.4237*\text{pH}+153.53134*\text{Temperature}\\ &+494.3596*\text{Wheatbran}*\text{pH}-28.6935*\text{Wheatbran}*\text{Temperature}\\ &+5.31635*\text{pH}*\text{Temperature}-1721.00820*\text{Wheatbran}^2-164.86141*\text{pH}^2-\\ &2.08710*\text{Temperature}^2 \qquad \text{(Eq.7)}\end{aligned}$$

The fit of the model can be evaluated by using various criteria. In the present study, coefficient of determination (R^2), adjusted R^2, predicted R^2 and Lack of Fit were taken into consideration .The R^2value of 0.9825 indicated that the model could explain 98.25 % of the variability in the response. The pedicted R^2 of 0.8869 is in resonable agreement with adjusted R^2 of 0.9667. The 'Lack of Fit F-value' of 4.27 implied to be insignificant. 'Adequate percision' measures the signal to noise ratio and a ratio greater than 4 is desirable. Our model ratio of 20.750 indicates an adequate signal. Hence, the model can be used to navigate the design space.

Table 2.7

Central Composite rotatable design (CCRD) of factors in coded levels with enzyme activity as response for *P. citrinum* xym2.

Run No	Type	Factor1 A:Wheat bran (%w/v)	Factor 2 B: pH	Factor 3 C: Temp (^{0}C)	Xylanase activity (IU/ml) Experimental	Xylanase activity (IU/ml) Predicted
1.	Axial	1.50	4.5	23.18	2681.12	2600.65
2.	Factorial	1	3	50	2133.23	2014.84
3.	Factorial	1	3	30	2618.12	2548.36
4.	Axial	1.50	7.02	40	1234.23	1094.48
5.	Centre	1.50	4.5	40	2675.23	2643.49
6.	Centre	1.50	4.5	40	2674.12	2643.49
7.	Factorial	2	-1	30	2113.12	2133.68
8.	Centre	1.50	4.5	40	2675.12	2643.49
9.	Centre	1.50	4.5	40	2489.12	2643.49
10.	Axial	1.50	4.5	56.82	1412.12	1505.68
11.	Factorial	1	6	50	868.192	838.38
12.	Factorial	2	3	50	1123.23	1036.18
13.	Centre	1.50	4.5	40	2474.45	2643.49
14.	Centre	1.50	4.5	40	2675.12	2643.49
15.	Axial	1.50	1.98	40	1941.31	2094.15
16.	Axial	2.34	4.5	40	1540.12	1468.44
17.	Axial	0.66	4.5	40	1299.89	1384.66
18.	Factorial	1	6	30	965.23	1043.03
19.	Factorial	2	6	50	1272.4	1342.80
20.	Factorial	2	6	30	2012.18	2121.32

Comparision of observed and predicted xylanase activity

Using the second-order regression equation,the model predicted the response (xylanase activity) corresponding to particular values of the regressor variables. The plot for the observed xylanase activity (the response) versus model predicted xylanase activity has been shown in Fig.2.17. As can be seen that the observed xylanase activity (response) and model predicted xylanase activity data points are split by 45^{o} line indicating a reasonable agreement of the predicted response with the observed ones.

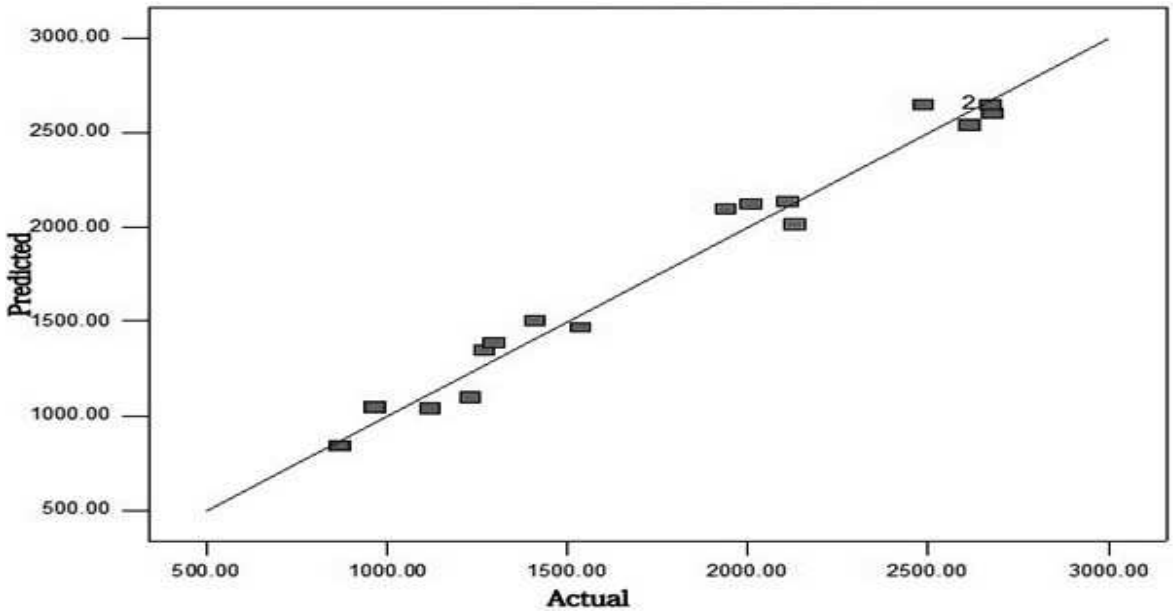


Fig.2.17: Model predicted and actual xylanase activity of *P. citrinum* xym2

Interpretation of interaction effects between the independent factors and localization of optimum condition for xylanase production

The response surface plots and their contour plots described by second-order polynomial equation were generated in order to investigate the interactions among variables and optimal level of variables for xylanase production (Fig. 2.18). From the result in Fig.2.18a, it is evident that xylanase production varied significantly by medium pH with higher activity in the pH range 3.0 – 4.5. Xylanase production was not much affected by wheat bran and its activity remained almost unaltered in the concentration range 1.0 - 1.7 %. However, a high level of interaction was observed between these two variables, like xylanase production was significantly reduced on increment in wheat bran concentration beyond 1.7% , keeping the medium pH fixed at 4.5. Similarly, increment of medium pH beyond 4.5 at fixed

concentration of wheat bran resulted in reduction of xylanase activity. Interaction between these two variables was also reflected in ANOVA with AB as significant model term.

The interaction between medium pH (B) and cultivation temperature (C) for xylanase production is shown in Fig. 2.18b. Maximum xylanase production was noted in the pH and temparature range 3 - 4.8 and 30-40 ^{o}C, respectively.

The response surface plot and contour plot for interaction between wheat bran (A) and cultivation temperature reveal that xylanase production was significantly enhanced in wide ranges of wheat bran concentration (1.20 – 1.98 % w/v) and cultivation temperature (30-40 ^{o}C).Further increase in temperature decreased xylanase activity markedly (Fig. 2.18c). From the model generated perturabation plot (Fig. 2.18d) it was clearly observed that all thesse three varibales had significant effect on xylanase production.

The optimal points for xylanase production was analysed by the cube plot. The result in Fig. 2.19 indicates that optimal predicted xylanase activity of 2538.46 U/ml was obtained at points –A,-B,-C corresponding to decreasing level of wheat bran, pH and cultivation temperature.

Validation of the model

The validity of the model was confirmed by testing xylanase production by random set of ten experiments. The results in table 2.8 clearly show that actual values were very close to the predicted values and thus the model was succesfuly validated. These validation experiments suggested wheat bran 1.5 % (w/v), medium pH 3.5 and cultivation temperature 30^{o}C as optimal condition for xylanse production. The above condition led to xylanase production of 2834.12 ±0.75 U/ml, which was comparable to the experimental data (2845.66 IU/ml). Hence, the CCRD based RSM models were considered to be accurate and reliable for predicting the production of xylanase by *P. citrinum* xym2.

2.3.3.3. Optimization of xylanase production by *B. subtilis* xym4 by RSM

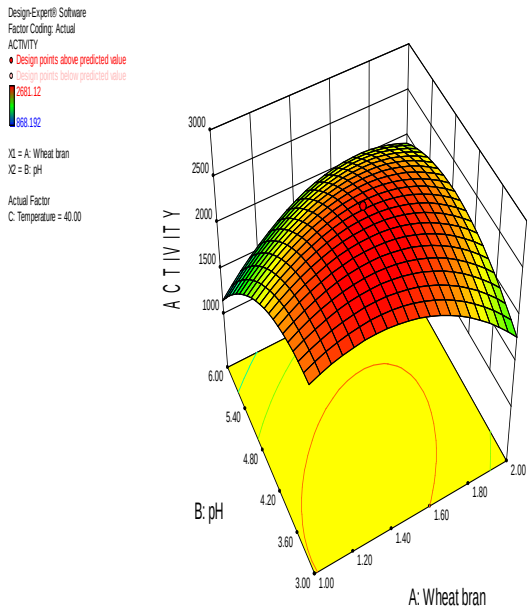
Variables analyzed in OFAT method was further evaluated in CCRD based RSM to further optimize the xylanase production. The effect of the four variables, pectin (A), incubation temperature (B), medium pH (C) and incubation time (D) on xylanase production was investigated. Suitable levels for these parameters were determined by CCRD of RSM. Total thirty experimental runs were performed using different combinations of the variables according to CCRD model. The levels of medium components used for CCRD are present in table 2.3 and experimental design with actual and model predicted xylanase activity is shown in table 2.9. Analysis of variance (ANOVA) was performed to the experimental design used.

The result of ANOVA shows that model is highly significant ($p<0.001$) and can better predict the data. Within the model, A (Pectin), B (Incubation temperature), C (Medium pH), D (Incubation time), BC (Incubation temperature X Medium pH), BD (Incubation temperature X (Incubation time), CD (Medium pH X Incubation time), A^2 ($Pectin^2$), B^2 (Incubation $temperature^2$), C^2 (Medium pH^2), D^2 (Incubation $time^2$) are the significant ($p<0.001$) model terms. Using the results of these experiments, second-order polynomial regression equation for xylanase yield was obtained which is represented in Eq. (8) and (09) in coded and actual form, respectively.

Table 2.8.
Validation of the CCD using different levels of wheat bran concentration, pH and temperature for xylanase production.

Run No	Wheat bran(%w/v)	pH	Temperature (^{0}C)	Xylanase (IU/ml) Predicted	Xylanase (IU/ml) Actual
1.	1.5	4.5	40	2643.49	2643.21 ± 0.23
2.	1.5	4.5	30	2760.31	2698.45 ± 0.5
3.	1.5	3.5	40	2674.95	2656.18 ± 0.28
4.	1.5	3.5	30	2845.66	2834.12 ± 0.75
5.	2	3.5	30	2333.26	2254.76 ± 0.22
6.	1	3.5	30	2497.55	2478.45 ± 0.28
7.	1	3	30	2538.03	2435.21 ± 1.45
8.	1.5	4.5	50	2129.17	2089.56 ± 1.68
9.	1.5	6	40	1975.34	1878.45 ± 0.33
10.	2	4.5	40	2238.14	2217.12 ± 1.09

(a)



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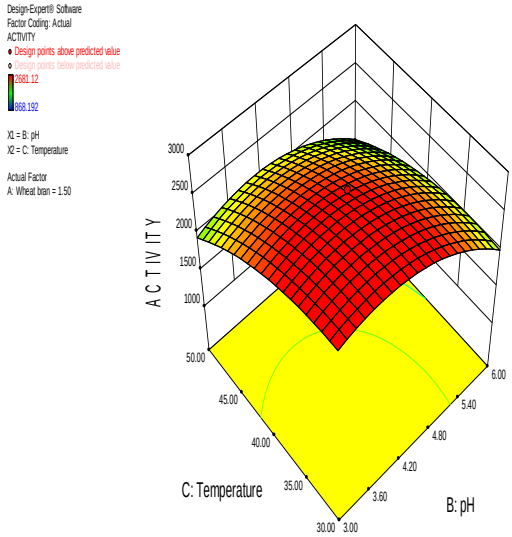

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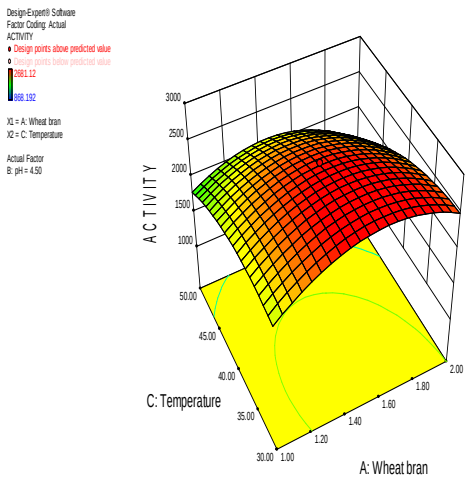


(d)

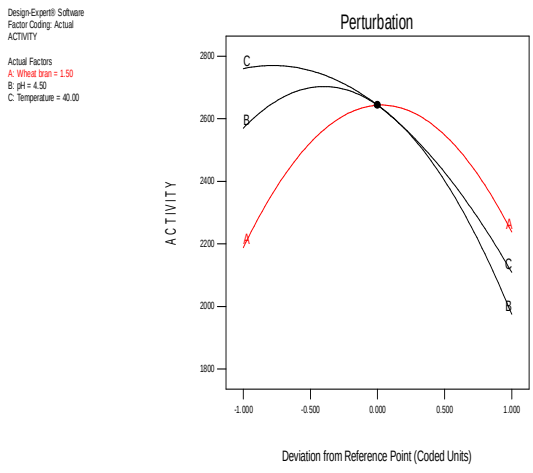


Fig 2.18. Response surface curves of xylanase production from *P. citrinum* xym2, showing interaction between wheat bran and medium pH (a), temperature and medium pH (b) and temperature and wheat bran (c) after 72h of incubation, (d) Perturbation plot of the model identifing the most significant variables. [Unit of xylanase activity was recorded in IU/ml]

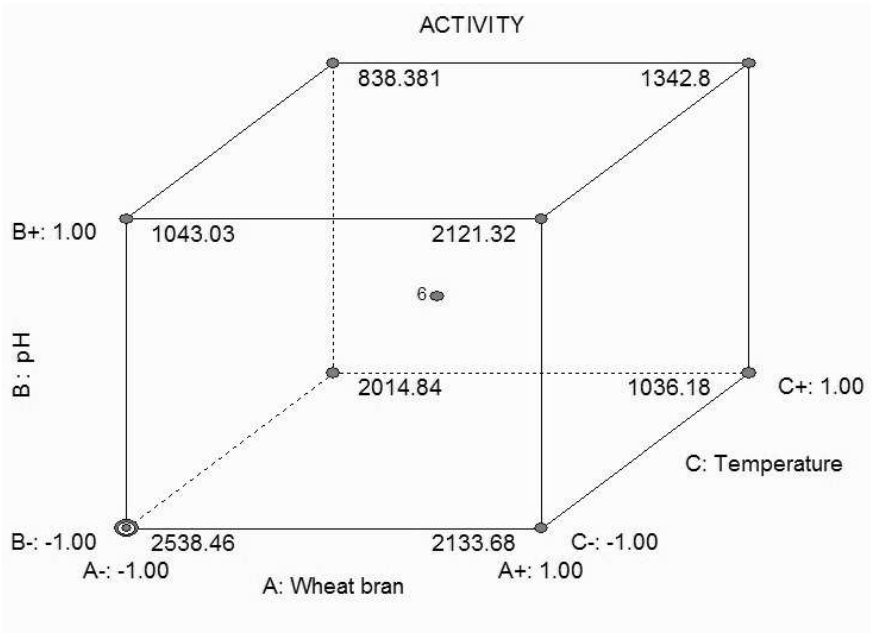


Fig.2.19. Cube plot showing the optimal point of xylanase production.

Activity (IU/ml) = +11661.53 + 336.60XA +416.95 XB – 653.55XC – 202.77 XD - 124.86XAXB + 148.26 XAXC+108.58 XAXD -364.06 XBXC- 522.22 XBXD+167.14 X CXD-603.08XA^2-1301.85XB^2-1294.05XC^2-1594.41XD^2 (Eq 8)

Activity (IU/ml) = -1.36741E +005 +5454.79 X Pectin + 4541.18 X Temperature +6071.18 X pH +851.18 X Incubation time -33.29 X Pectin X Temperature + 131.78 X Pectin X pH +12.06 X Pectin X Incubation time – 48.54 X Temperature X pH +8.70 X Temperature X Incubation time + 9.28 X pH X Incubation time -1072.13 X $Pectin^2$ -52.07 X $Temperature^2$ -575.13XpH^2-11.07XIncubationtime^2 (Eq 9)

The fit of the model can be evaluated by using various criteria. In the present study, coefficient of determination (R^2), adjusted R^2, predicted R^2 and Lack of Fit were taken into consideration .The R^2value of was 0.9920 indicated that the model could explain 99.20 %of the variability in the response. The predicted- $R^2$of 0.9769 is in reasonable agreement with adjusted -R^2 of 0.9844. The 'Lack of Fit p-value' of 0.9710 implied to be insignificant. 'Adequate precision' measures the signal to noise ratio and a ratio greater than 4 is

desirable. Our model ratio of 33.34 indicates an adequate signal. Hence, the model can be used to navigate the design space.

Comparison of observed and predicted xylanase activity

Using the second-order regression equation, the model predicted the response (xylanase activity) corresponding to particular values of the regression variables. The plot for the observed xylanase activity (the response) versus model predicted xylanase activity is shown in Fig. 2.21c. As can be seen that the observed xylanase activity (response) and model predicted xylanase activity data points are split by 45^{o} line indicating a reasonable agreement of the predicted response with the observed ones

Interpretation of interaction effects between the independent factors and localization of optimum condition for xylanase production

The response surface plots and their contour plots described by second-order polynomial equation were generated in order to investigate the interactions among variables and optimal level of variables for xylanase production. From the result in Fig. 2.20 a, it is clear that xylanase production varied significantly by pectin concentration with higher activity in the concentration (% w/v) range 2.10 – 3. Xylanase production was affected by incubation temperature and its activity was found to be higher in the temperature range of 45-48 ^{o}C, whereas below and above the temperature range enzyme activity was significantly reduced. The interaction effect between pectin concentration (A) and incubation temperature (B) was found to be non significant as also suggested by the ANOVA result with a p-value of 0.10.

The interaction between pectin concentration (A) and medium pH (C) for xylanase production has been shown in Fig. 2.20b. Maximum xylanase production was noted in the pectin concentration and pH range 1.90 - 3.00 and 3.80 - 4.70, respectively. Although the interaction effects between these two variables were found to be non significant but they individually affected the enzyme production significantly.

The response surface plot and contour plot for interaction between pectin concentration (A) and incubation time (D) reveal that xylanase production was significantly enhanced in wide ranges of pectin concentration (1.9 – 3.00 % w/v) and incubation time (55-62 h). Further increase or decrease in incubation time decreased xylanase activity markedly (Fig. 2.20 c).

The interaction between the independent variables medium pH (C) and Incubation temperature (D) are presented in the fig.2.20 d. High yield of xylanase was obtained at a pH

and Temperature range of 4.20 - 4.50 and 45 – 47 ^{o}C. Below or above the temperature range enzyme activity significantly declined.

However, a high level of interaction was observed between two variables, incubation temperature (B) and incubation time (D). If the temperature kept constant at 46^{o}C, xylanase production varied in the incubation time range 48 h to 60 h, with yield of 8149 IU/ml and 11120 IU/ml, activity respectively (Fig 2.21a). ANOVA ($p<0.0001$) result also supported the significant interaction effect between these two variables.

The interaction between pH (C) and incubation time (D) for xylanase production has been shown in Fig. 2.21b. Maximum xylanase production was noted in the medium pH and incubation time (D) range 3 - 4.8 and 54 - 65h respectively. Interaction effect these two variables was also found to be significant ($p<0.05$). The significant level of individual medium variables were depicted in the perturbation plot (Fig 2.21d), and from the plot it was obtained that all the variables had significant effect on xylanase production.

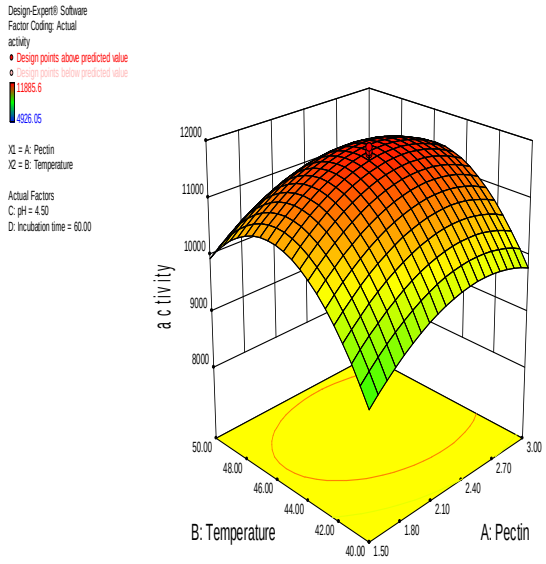
Table 2.9

Central composite experimental design data for optimization of xylanase production with predicted and experimental values by *B. subtilis* xym4.

Run	PC[a]	T[b]	pH[c]	IT[d]	Xylanase activity [e](IU/ml)	Xylanase activity [f] (IU/ml)
1	1.5	50	3	72	8282.94	8156.75 ± 10.23
2	1.5	50	6	72	6285.49	6166.45 ± 11
3	2.25	45	4.5	36	5689.43	5772.65 ± 10.26
4	2.25	45	4.5	60	11661.53	11786± 1.23
5	1.5	50	6	48	5529.46	5403.3± 18.23
6	2.25	55	4.5	60	7288.03	7702.25± 12.21
7	0.75	45	4.5	60	8576.01	8656.9± 14.23
8	3	40	6	48	7220.66	7262.5± 15.23
9	3	40	6	72	6322.14	6366.1± 11.24
10	1.5	40	6	72	4885.54	4926.05± 8.27
11	2.25	45	1.5	60	7792.43	7876.7± 8.25
12	3	40	3	72	6270.34	6312.15± 11.23
13	2.25	45	4.5	60	11661.53	11885.6± 15.23
14	1.5	40	3	48	7428.19	7470± 15.23
15	3.75	45	4.5	60	9922.43	10005.7± 11.2
16	1.50	50	3	48	8195.48	8071.75± 10.2
17	3	50	6	72	7222.66	7096.5± 19.23
18	3	50	3	72	8627.08	8503.35± 8.23
19	1.5	40	3	72	5426.76	5469.7± 1.23
20	2.25	45	4.5	60	11661.53	11881.5± 7
21	2.25	45	7.5	60	5178.21	5258.05± 6
22	1.5	40	6	48	6218.39	6262.35± 7.6
23	3	40	3	48	7837.43	7878.7± 8.5
24	2.25	35	4.5	60	5620.21	5370.1± 4.4
25	2.25	45	4.5	84	4878.36	4959.25± 4.3
26	2.25	45	4.5	60	11661.53	10835.8± 3.4
27	2.25	45	4.5	60	11661.53	11810.9± 5.5
28	3	50	3	48	8105.30	7980.45± 4.9
29	3	50	6	48	6032.31	5909.6± 7.8
30	2.25	45	4.5	60	11661.53	11769.4± 8.6

[a]Pectin (%w/v), [b] Incubation Temperature ([o]C),[c]pH of medium, [d] Incubation time(h), [e] Predicted xylanase activity (IU/ml), [f] Actual Xylanse activity (IU/ml), Data were presented as triplicate of mean ±SD

(a)



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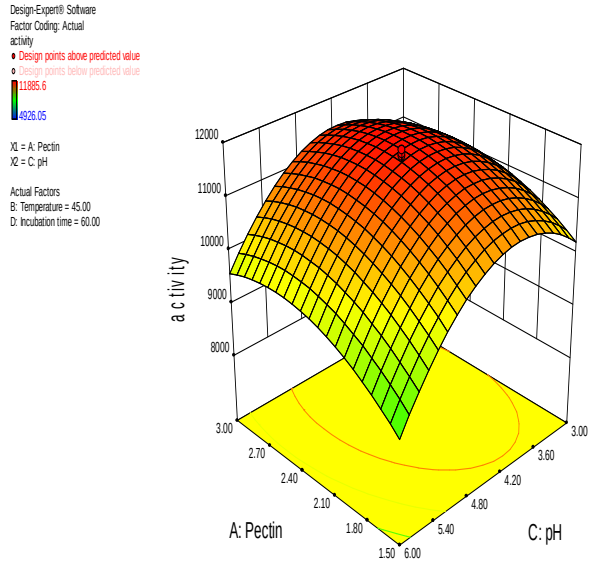

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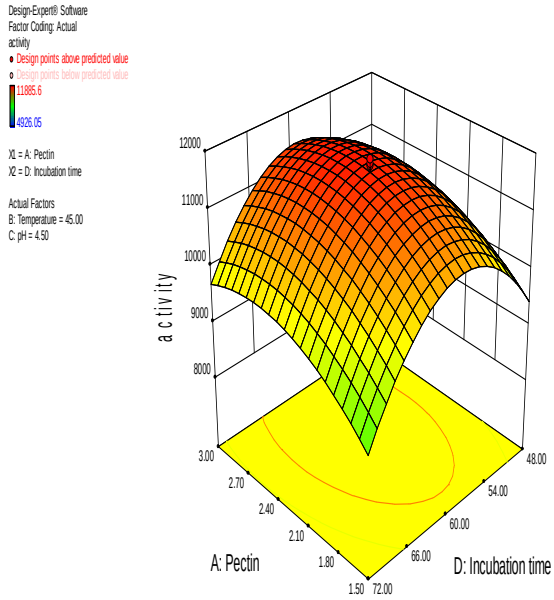

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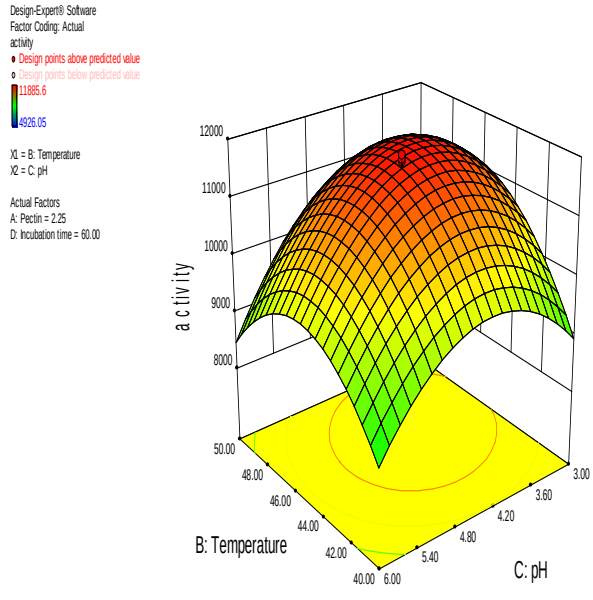
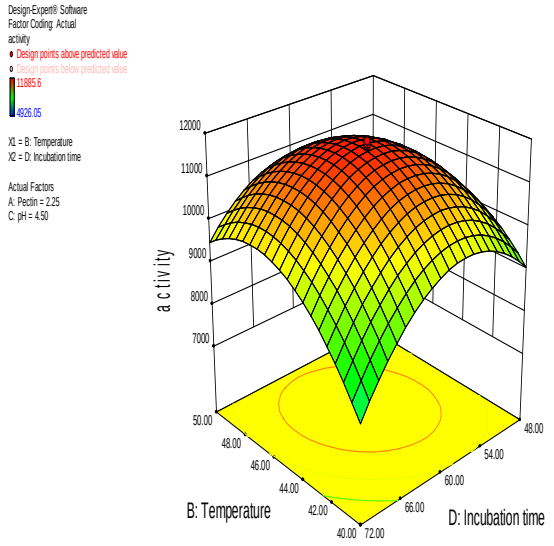


Fig.2.20. Response surface curves of xylanase production from *B. subtilis* xym4, showing interaction between incubation temperature and pectin concentration (a), pectin concentration and medium pH (b), pectin concentration and incubation time (c) and incubation temperature and medium pH (d). [Unit of xylanase activity was recorded in IU/ml]

(a)



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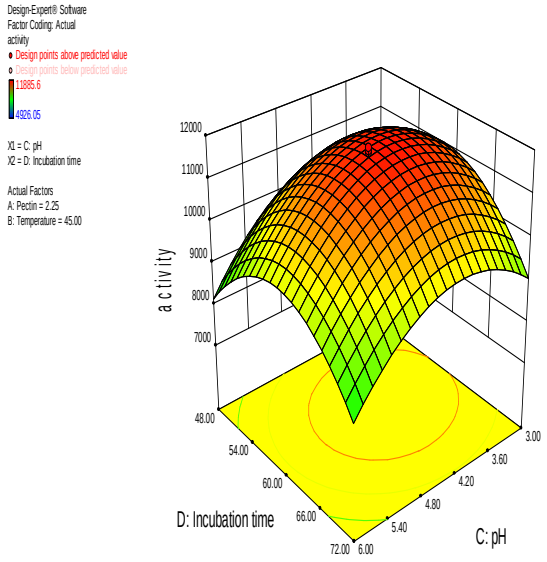

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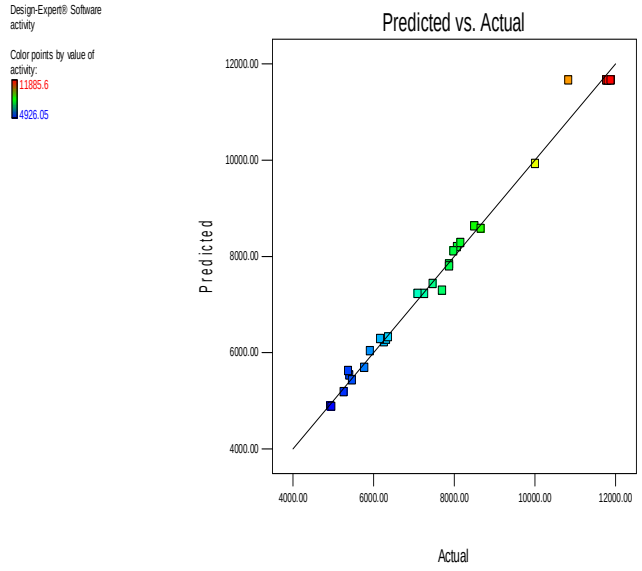


(d)

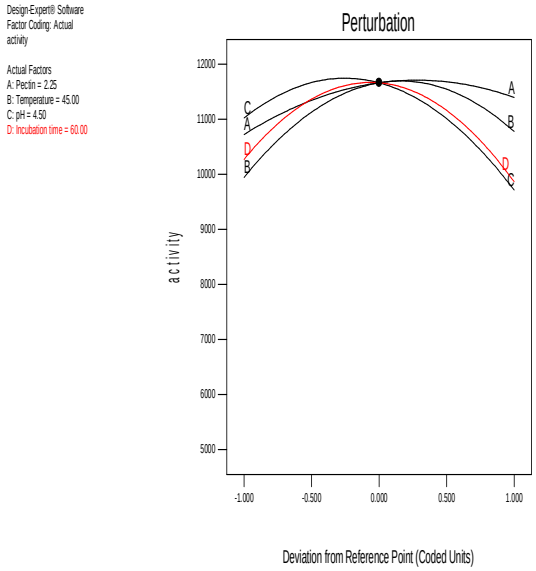


Fig. 2.21.Response surface curves of xylanase production from *B. subtilis* xym4, showing interaction between incubation temperature and incubation time (a), incubation time and medium pH (b), Predicted vs actual xylanase activity (c) and perturbation plot (d) of the model identifying the most significant variables. [Unit of xylanase activity was recorded as IU/ml]

Validation of the model

The model was finally employed to find out the optimum parameter for highest xylanase yield. Model predicted optimum parameters that showed the maximum xylanase activity are presented in the table 2.10. In the model optimized conditions, it was observed that model predicted xylanase yield of 11700.53 IU/ml was obtained at pectin concentration 2.25 %(w/v), temperature 45 ^{o}C, medium pH 4.5 and 60 h of incubation which was in reasonable agreement with the actual xylanase activity of 11800 ± 11.3 IU/ml. Xylanase produced by the *B. subtilis* xym4 on the RSM optimized medium was also used in agar diffusion plate assay in XYM plates followed by congo red staining and NaCl wash. The Fig. 2.22 represents formation of xylan hydrolysis zone of 2.1 mm.

Table 2.10
Validation of the CCD using different levels of wheat bran concentration, pH and temperature for xylanase production.

Run No	Pectin (%w/v)	Temperature (^{0}C)	pH	Incubation time(h)	Xylanase Activity (IU/ml) Predicted	Xylanase Activity (IU/ml) Actual
1.	3	45	4.2	59	11470	11476± 10.23
2.	3	41	5	65	9888.04	9865± 7.5
3.	3	44	3.4	60	10946.4	10986± 10.28
4.	**2.25**	**45**	**4.5**	**60**	**11700.53**	**11800± 11.3**
5.	3	46	4.1	62	11409	11520± 10.22

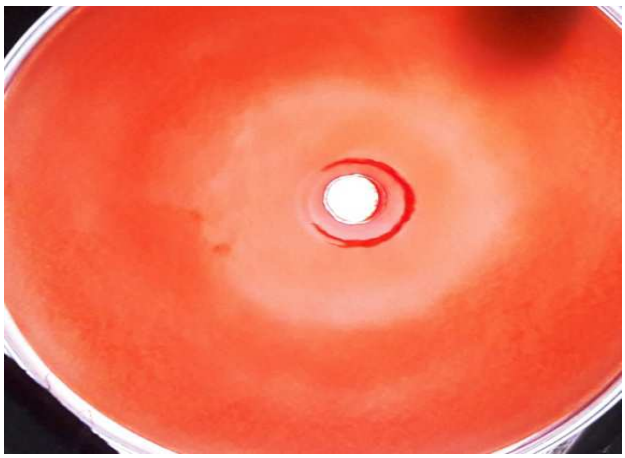


Fig 2.22. Xylan hydrolysis zone on XYM plates using xylanase obtained from *B. subtilis* xym4 cultivated in RSM optimized XPM

2.3.4. Carbohydrate active enzymes production by the isolates in optimized fermentation medium:

Production of carbohydrate active enzymes such as FPase, CMCase, β- Xylosidase as well as the protease enzyme by *B. subtilis* xym4 and *P. citrinum* xym2 were compared with their unoptimized and optimized levels of xylanase activities (Fig 2.23 and 2.24). In unoptimized culture medium *B. subtilis* xym4 produced significant amount of xylanase, FPase and β-xylosidase, whereas little activities of CMCase and protease were obtained. After optimization of culture medium, xylanase production by *B. subtilis* xym4 was increased by approximately 12 fold, whereas Fpase and β-xylosidase activities were enhanced by 11 and 5 folds, respectively. Due to its low CMCase and protease activities the xylanase obtained from *B. subtilis* xym4 can be used for higher xylose yield from agro residue for xylitol production. *P.citrinum* xym2 produced xylanase with 2821 IU/ml activity. The fungus also produce CMCase (1492 IU/ml), β-xylosidase (711 IU/ml) and FPase (316 IU/ml), and proteases (901 IU/ml). Due to the presence of high amount of xylanase, and cellulase activities, the extracellular enzyme cocktail of the fungal species can be used for sachharification of agro residues to yield fermentable sugars for synthesis of value added products. However, the enzyme cocktail with higher activity of cellulase is quite unsuitable for efficient production of xylitol.

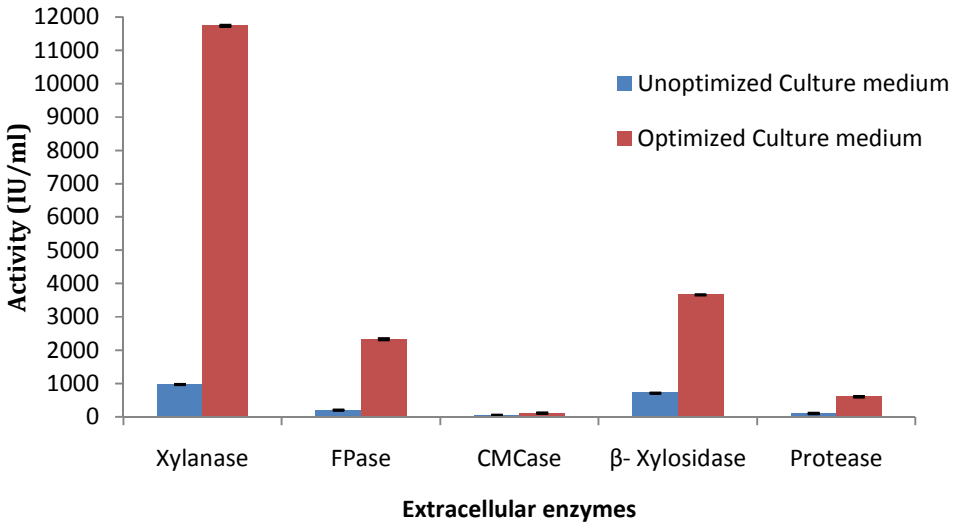


Fig. 2.23. Extracellular enzyme production by *B. subtilis* xym4

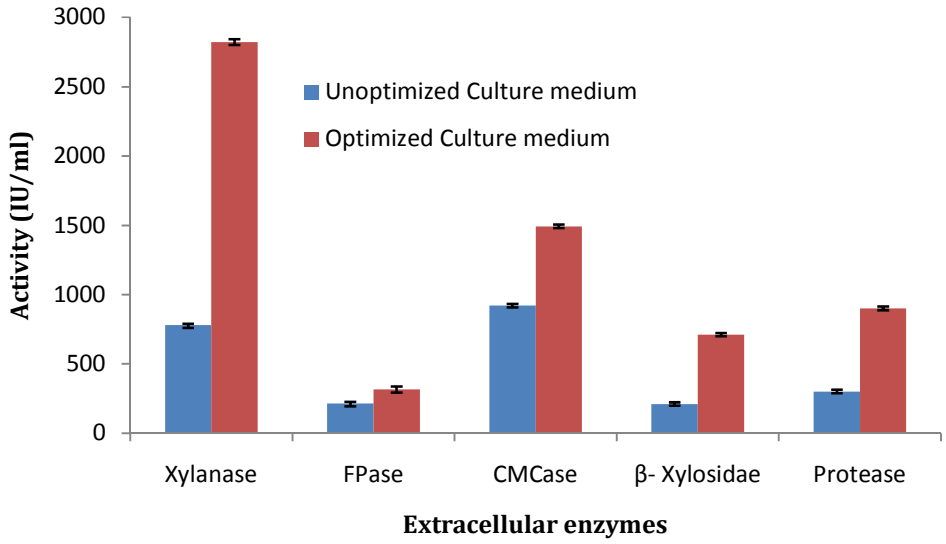


Fig.2.24. Extracellular enzyme production by *P. citrinum* xym2

2.3.5. Purification of xylanase from *Bacillus subtilis* xym4

Xylanase from *Bacillus subtilis* xym4 was purified from 24 h grown culture on XPM by $(NH_4)_2SO_4$ precipitation, anion-exchange chromatography and gel filtration and the results of enzyme purification are shown in table 2.10. The bacterial CFE were subjected to $(NH_4)_2SO_4$ precipitation and about 78.74% of the enzyme was recovered in 40-70% saturated $(NH_4)_2SO_4$ fraction. The desalted enzyme preparation was subjected to DEAE-sephadex chromatography, which resulted in removal of substantial amount of proteins and enhanced the specific activity to about 4140.64 IU/mg. The active fractions were pooled and subjected to gel filtration chromatography using Biogel P-100. The enzyme was finally purified with a purification fold and specific activity of 26.34 and 13432.1 IU/mg protein, respectively. Finally 32.71 % enzymes were recovered. The purified protein appeared as a single band on the SDS-PAGE with an apparent molecular mass of about 42 kDa (Fig.2.25).

Table 2.11
Purification of xylanase obtained from *Bacillus subtilis* xym4.

Purification Step	Volume (ml)	Total xylanase activity (IU*)	Total protein (mg)	Sp. Activity (IU/mg)	Fold Purification	% Yield
Crude	100	78000	153	509.80	1.00	100.00
Ammonium sulphate	2	61417.32	56	1096.73	2.15	78.74
DEAE-Sephadex	10	45547.1	11	4140.64	8.12	58.39
Biogel P-100	6	25521	1.90	13432.1	26.34	32.71

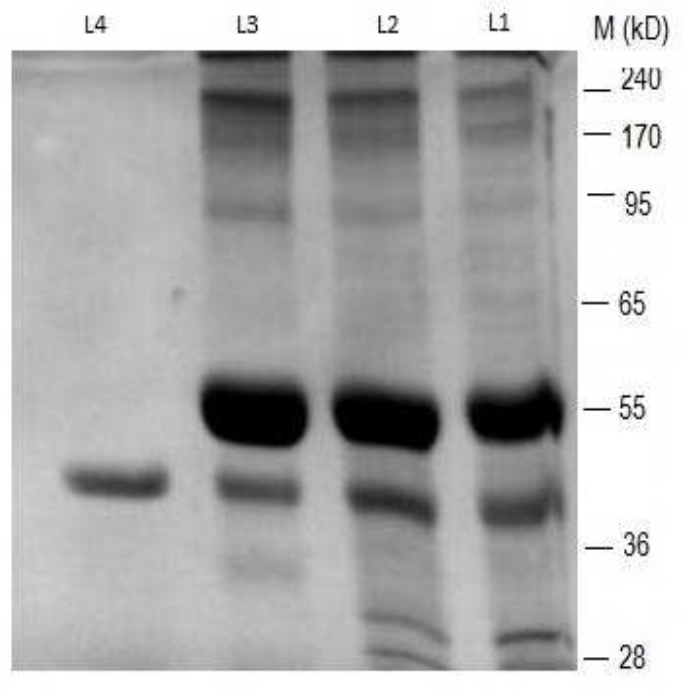


Fig. 2.25. Purification of xylanase from *B. subtilis* xym4. SDS-PAGE analysis of Crude extract (Lane 1). 40-70% ammonium sulphate fraction (Lane2), DEAE eluate (Lane-3), Biogel P100 eluate (Lane 4), Lane M-Protein molecular weight marker.

2.3.6. Characterization of xylanase

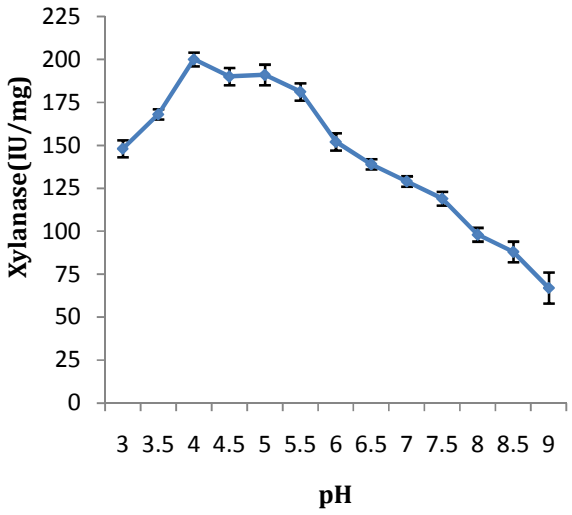
The xylanase enzyme produced by the isolates was characterized for pH optima, temperature optima, thermo stability, cation requirement and Km for birchwood xylan.

2.3.6.1. Determination of pH optimum, temperature optimum and thermostability

Optimum pH and temperature for the partially purified xylanase from *P. citrinum* xym2 was found to be pH 4 and 40^{o}C with xylanase specific activity of 200 and 210 IU/mg, respectively (Fig 2.26a &b). The enzyme was more active in acidic pH than alkaline pH. The enzyme showed thermostability at higher temperature as it retained 80% and 50%of activity on preincubation for 30 min at 80^{o}C and 90^{o}C, respectively (Fig 2.26c).

Purified xylanase from *B. subtilis* xym4 was found to be showed maximum xylanase specific activity of 812 and 420 IU/mg , at pH 7 and 40^{o}C respectively (Fig 2.27a &b). The enzyme was stable in alkaline as well as in acidic pH. The enzyme retains its 39% and 46% activity in pH 9 respectively as compared to its activity in optimum pH 7. The enzyme showed thermostability at higher temperature as it retained 77%and 57%of activity on preincubation for 30 min at 80^{o}C and 90^{o}C, respectively (Fig 2.27c)

(a)



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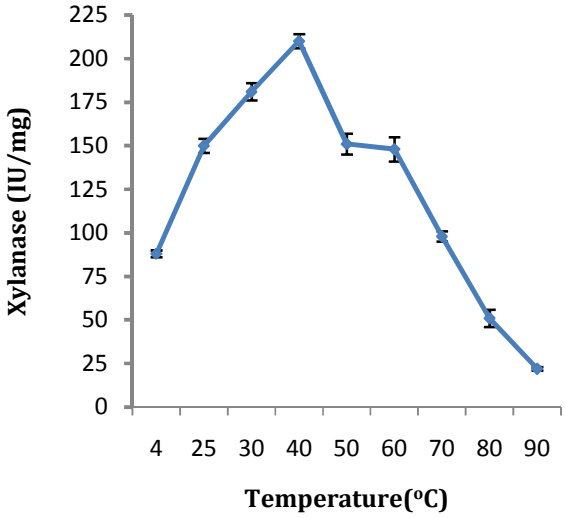

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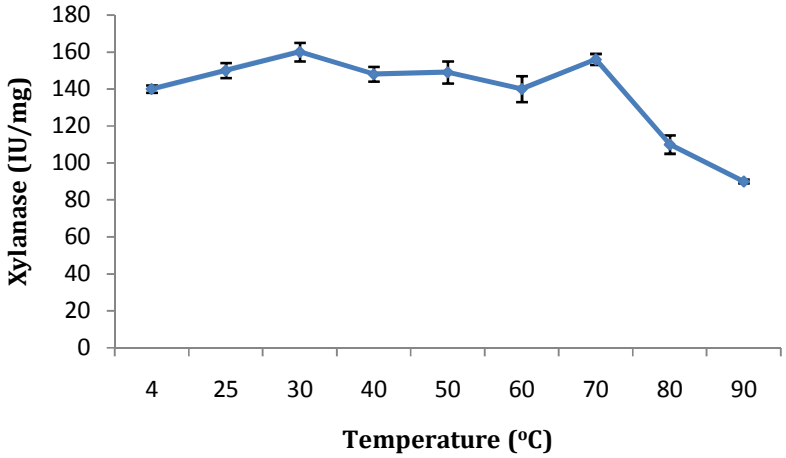
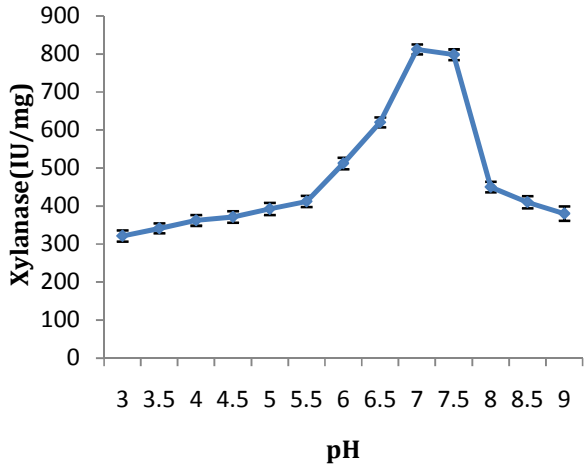
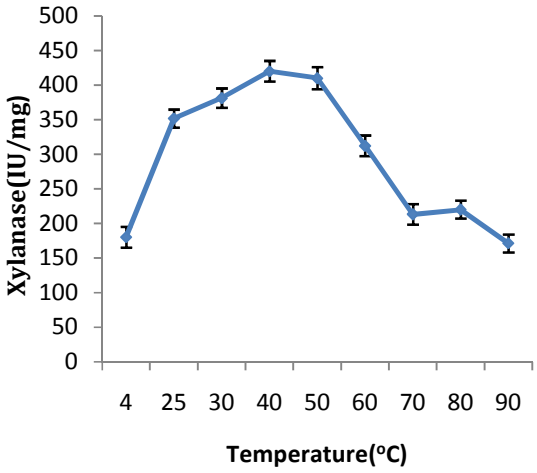


Fig 2.26. Characterization of xylanase obtained from *P. citrinum* xym2. (a) Effect of pH [The effect of pH was determined in 100 mM glycine (pH 3.0 and 3.5), 100 mM sodium acetate (pH 4.0, 4.5 and pH 5.5), 100 mMSodium phosphate (pH 6.0, 6.5 and 7.0) and 100 mMTris- HCl (pH 7.5, 8.0, 8.5 and 9) buffer at 37°C] (b) Effect of temperature and(c) Thermo stability of xylanase.

(a)



(b)



(c)

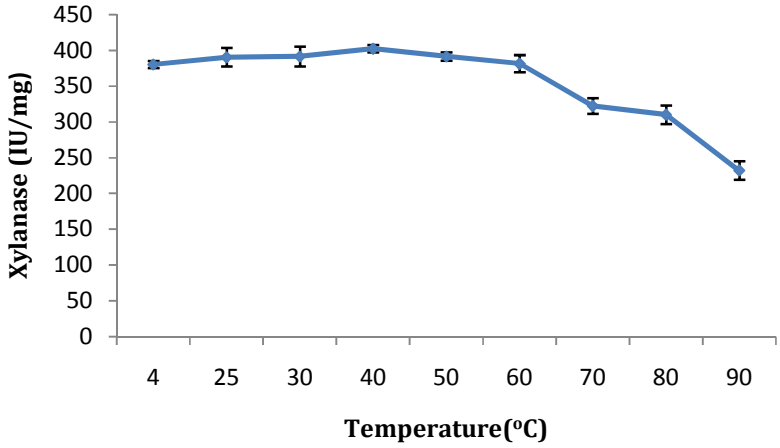


Fig 2.27. Characterization of xylanase obtained from *B. subtilis* xym4. (a) Effect of pH [The effect of pH was determined in 100 mM glycine (pH 3.0 and 3.5), 100 mM sodium acetate (pH 4.0, 4.5 and pH 5.5), 100 mMSodium phosphate (pH 6.0, 6.5 and 7.0) and 100 mMTris- HCl (pH 7.5, 8.0, 8.5 and 9) buffer at 37°C] (b) Effect of temperature and(c) Thermo stability of xylanase.

2.3.6.2. Effect of cations and chemicals on xylanase activity

The effect of metal ions and chemical reagents on xylanase produced by the isolates were determined by measuring enzymatic activity in presence of 5 mM Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+},Zn^{2+}, Fe^{3+} , Co^{2+},K^{+},Na^{+}, SDS, EDTA and β-ME. Activity without addition of ions or reagents used as control.

Penicillium xylanase activity was inhibited by the presence of Co^{2+},$Cu^{2+}$and Fe3+, whereas, the addition of Mg^{2+}, Mn^{2+},K^{+},Na^{+},Ca^{2+}, led to increase in activity as 140%, 114%, 114.66%, 108%, 107.33%, respectively. Moreover, SDS, EDTA and β-ME have also showed the inhibitory effect on enzyme activity (Fig 2.31).

Xylanase activity of *Bacillus* was significantly inhibited in the presence of Co^{2+}, Fe^{3+} and Zn^{2+}, whereas, the addition of Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+} led to increase in activity as 145%, 136%,132% and 122.58% respectively. Moreover, EDTA and β-ME have also showed the inhibitory effect on enzyme activity. SDS had shown stimulatory effect on enzyme activity. Other metal ions or chemicals had little or no effect (Fig 2.28).

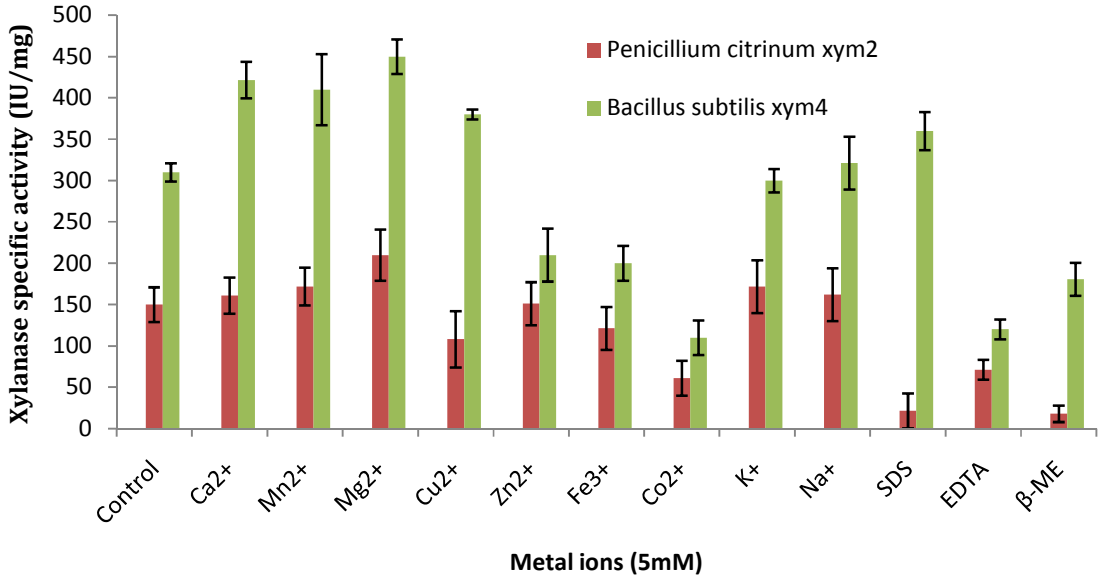


Fig 2.28. Effect of various metal cofactors and chemicals [5mM] on xylanase without any metal cofactors and chemical the enzyme substrate reaction mixture was served as control.

2.3.6.3. Km and Vmax for xylanase:

In order to determine the Km for xylan, xylanase activity of *B.subtilis* xym4 was measured in presence of increasing concentration of birchwood xylan. The enzyme showed hyperbolic response to increasing concentrations of xylan. Apparent Km and Vmax value for xylanase

was determined by Lineweaver-Burk plot (Fig 2.29). Enzyme was found to have a Km, Vmax, Kcat and Kcat/Km of 10 mg/ml, 2631.57 µmole/min/mg, 110.52/min and 11.05, respectively. Low Km, high Vmax and Kcat/Km ratio of the xylanase signify its high affinity and catalytic efficiency towards xylan substrates.

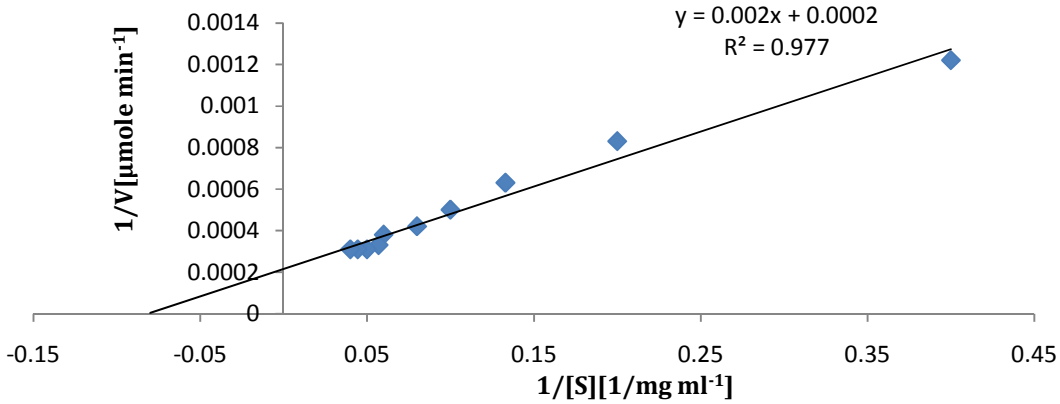


Fig 2.29. Lineweaver-Burk plot for determination of Km & Vmax of xylanase obtained from *B. subtilis* xym4

2.4. Discussion

Xylan, the complex polysaccharide of xylose is the main component of hemicellulose found plentifully in plant biomass. It consist of β-1,4-linked D-xylose backbone and can be substituted with different side groups such as L-arabinose, D-galactose, acetyl, feruloyl, p-coumaroyl and glucoronic acid residues **(**Wilkie 1983**).** Endoxylanase (E.C.3.2.1.8) is the principal enzyme that randomly hydrolyzes the β-1,4- xylosidic bond of xylan to form xylooligosaccharides, which are then degraded by accessory xylanolytic enzymes such as β-xylosidase **(**Chavez *et al.* 2006**).** The possible biotechnological applications of xylanases in various industries include the food, feed, fuel, textile, detergents, paper and pulp industries and in waste treatment **(**Dhiman *et al.* 2008**).** Xylanase improves the elasticity and strength of dough in baking industry (Shah *et al.* 2006). It helps to reduce the intestinal viscosity and therefore improve both the weight gain of chicks and their feed conversion efficiency (Bedford and Classen 1992). In the recent years, xylanases have been used for bio energy production from hemicelluloses (Dhiman *et al.* 2008). Moreover, xylanase can also be employed for production of several value added products such as xylitol from agro residues **(**Cheng *et al.* 2014, Franceschin *et al.* 2011). The high demand and cost of available

commercial enzymes create need to find a renewable source of xylanase which will be cost effective and industrially comparable (Uday *et al.* 2016). The successful industrial application of xylanase requires its economic production in bulk quantity (Gupta *et al.* 2012). Moreover, xylanase with wide range of thermal stability and acid/alkali stability have been proved to be the best for application in various biotechnological processes (Bouacem *et al.* 2014, Zhou *et al.* 2007).

Present study describes the isolation of xylanase producing microorganisms from various environmental samples and their identification, optimization of cultural conditions for xylanase production by the isolates by cultivating them with various agro residues under submerged fermentation condition employing OFAT and other RSM based statistical tools and finally characterization of xylanases. Initial experiments were carried out to isolate novel xylanase producing microorganism from soil of various environmental locations. Previous studies have reported the isolation of xylanase producing microorganism from wide range of geographical location such as, hot spring (Daupan and Rivera 2015), pulp and paper industry soil (Gaur *et al.* 2015), stored seeds, and decomposing organic matter (Ahirwar *et al.* 2017), ruminant dung (Thite and Nerurkar 2015), humic soil, the inner part of rock, rock surface and grass land (Amore *et al.* 2014), termite gut (Dheeran *et al.* 2012) etc. Conventional biochemical characterization and microscopic observation revealed that among the isolated xylanase positive bacterial strains, three belong to the genera *Bacillus,* two belong to the genus *Geobacillus*, one belongs to *Paenibacillus* and the other one in *Salinicoccus*. All the bacteria were gram positive in nature and are belongs to the phylum firmicutes. There are several reports on production of xylanase by firmicutes such as *Bacillus subtilis* (Chang *et al.* 2017), *Bacillus amyloliquefaciens* (Liu *et al*. 2017), *Bacillus brevis* (Mathur *et al.* 2017), *Bacillus licheniformis* (Kumar *et al.* 2017), *Paenibacillus* sp (Zheng *et al.* 2014, Shi *et al.* 2010), *Paenibacillus macerans (*Dheeran *et al.* 2012), *Geobacillus* sp (Bhalla *et al.* 2015), *Geobacillus stearothermophilus* (Bibi *et al.* 2014).

In this study, two fungal strains with the abilility to degrade xylan were also isolated and they were identified as *Aspergillus flavus* xym4 and *Penicillium citrinum* xym2. *Penicillium* fungi are primarily saprophytic in nature, and numerous species have been used in commercial food and antibiotic penicillin production. Several previous reports suggest that they can also be used for the production of xylanolytic enzymes (Vardakou *et al.* 2008). Driss *et al.* (2013) reported the production of GH11 family xylanase by *Penicillium occitanis* Pol6 and *Penicillium funiculosum*. Increased production of cellulases and xylanases by *Penicillium echinulatum* S1M29 in batch and fed-batch culture was obtained in research conducted by Reis *et al.* (2013). Other studies also showed the xylanase

production ability of *Aspergillus* sp. such as in *Aspergillus tamarii* (Heinen *et al.* 2017), *Aspergillus Niger* (Fakhari *et al.* 2017), *Aspergillus tubingensis* (Adhyaru *et al.* 2016).

Xylan hydrolysis requires the activities of different xylanolyticenzymes such as such as endoxylanase, β-xylosidase, α-arabinofuranosidase, acetyl esterase, and α-glucoronidase. Although among the isolated microorganisms *Aspergillus flavus* xym4 produced markedly highest level of xylanase, it showed marginal activities of FPase and β-xylosidase and thus failed to meet our objective of xylitol production; and hence this strain was not incorporated for future studies. *Bacillus subtilis* xym4 and *Penicillium citrinum* xym2 were selected for further studies due to their high xylanase as well as appropriate amount of carbohydrate active enzyme production capability.

In the present study production of xylanase by *Bacillus subtilis* xym4 and *Penicillium citrinum* xym2 was optimized sequentially using one factor at a time approach (OFAT) and Response Surface Methodology (RSM). The application of agro-industrial residues as sole carbon source in xylanase production provides not only an alternative low cost substrate, but also a promising approach to reduce the pollution problems related to inappropriate management of lignocellulosic waste (Botella et al., 2007). Therefore in OFAT method, xylanase production by both *B. subtilis* xym4 and *P. citrinum* xym2 were optimized with respect to agro residues as sole carbon source under submerged fermentation. Both the organisms showed the maximum amount of enzyme production in wheat bran as compared to other agro residues as well as birchwood xylan. High xylanase activity in presence of wheat bran could be due to its high nutrient supporting initiation of growth and replication of microorganisms and also wheat bran remains loose even under moist conditions providing a large surface area for microbial nutrient uptake during SmF (Gupta *et al*. 2012; Kuhad and Singh 1993). Incubation temperature was found to be one of the significant parameter of OFAT approach. Although *B. subtilis* xym4, mesophilic bacteria, showed detectable xylanase activity at 37 ^{o}C, it produced highest amount of xylanase at 50 ^{o}C. Similarly, Simphiwe *et al.* (2011) reported higher amount of xylanase production by *Bacillus* sp. at 55 ^{o}C. However, the results of Sepahy *et al.* (2011) and Monisha *et al.* (2009) showed maximum production of xylanase at 37 ^{o}C by *B. mojavensis* AG137 and *B. pumilus*, respectively. The optimization of cultivation temperature of *P. citrinum* xym2 for xylanase production by OFAT approach indicated mesophilic nature of the fungus with maximum enzyme production at 30 ^{o}C incubation temperature. The result is supported by the previous studies showing the maximum xylanase activity near about 30 ^{o}C by various species of *Penicillium* (Cui and Liming 2012, Lacis *et al.* 1993, Haas *et al.* 1992).

Numerous enzymatic catalysis and transport of nutrients and several other components across the cell membrane are strongly affected by the pH of medium. In the present study medium pH 6 was found to be optimum for maximum xylanase yield by *B. subtilis* xym4. The results also indicated that the bacterial strains can tolerate in acid and alkaline conditions. Similar finding was also noted by Dheeran *et al.* (2012) in their work with *Paenibacillus* sp. A59 showing pH 6.5 as optimum medium pH for xylanase stability. On the other hand, *B. mojavensis* AG137 and *B. pumilus* showed highest xylanase activity yield at pH 8 and 7, respectively, in submerged fermentation (Sepahy *et al.* 2011, Monisha *et al.* 2009). The optimum pH for enzyme production by *P. citrinum* xym2 was 4.0. As reported previously, other filamentous fungi also produced maximal level of xylanase in the acidic pH range. *Penicillium purpurogenum* and *Penicillium janthinellum* showed maximum xylanase yield at pH 5.5 (Oliveira *et al.* 2007, Eyzaguirre *et al.* 1994, Lacis *et al.* 1993) whereas xylanase production by *Penicillium* sp. ZH-30 was highest at pH6 (Cui *et al.* 2007).

The supplementation of pectin as additional carbon source in the production media significantly induced the xylanase production by *B. subtilis* xym4 with approximately 1.33 fold higher production in comparison to birchwood xylan alone. However, presence of other sugars in the medium declined the production of enzyme with most severe decline in presence of xylose. Contrary to the finding of present study, Azeri *et al.* (2010) reported different strains of *Bacillus* sp. exhibiting maximum xylanase production in birchwood xylan as a carbon source. Another study showed that sucrose supplementation to the fermentation medium significantly enhanced the production of xylanase by *B. subtilis* (Saleem *et al.* 2002).

The optimization of enzyme production by *P. citrinum* xym2 using OFAT approach indicated that the supplementation of maltose and di-ammonium hydrogen phosphate as additional sugar and nitrogen source, respectively, positively influenced the xylanase production and proved to be valuable parameters. The sugar mediated regulation of xylanase gene expression has been studied with various strains of *Penicillium*. The production of β-xylosidase and xylanase by *Penicillium pinophilum*, *Penicillium persicinum* and *Penicillium brasilianum* were induced by xylose and repressed by glucose. Glucose repression has also been demonstrated in *P. purpurogenum* and *Penicillium chrysogenum* by northern blot analysis and β-glucosidase reporter gene assay, respectively (Bull *et al.* 2003, Chavez *et al.* 2004, 2002, Jorgensen *et al.* 2004). As observed in this study, *Penicillium oxalicum* produced maximal level of xylanase using inorganic nitrogen source NH_4Cl instead of organic nitrogen sources (Abt *et al.* 2000). Among all the tested

inorganic and organic nitrogen sources, peptone at a concentration of 0.09 %(w/v) was proved to be the best nitrogen source for *B. subtilis* xym4. In earlier studies with *Bacillus* species reported stimulating effects of organic nitrogen sources on xylanase production (Battan *et al.* 2007). In a similar study by Sepahy *et al.* (2011) xylanase production ability of *B. mojavensis* AG137 was noted to enhance in presence of yeast extract with tryptone and yeast extract with NH_4NO_3 in the medium as nitrogen sources. Similarly, Sharma and Bajaj (2005) isolated different species of *Streptomyces* and found best xylanase production with soybean meal and yeast extract as nitrogen sources in the medium.

In this investigation, a correlation was found between kinetics of xylanase production, and CFU and biomass yield of *B. subtilis* xym4 and *P. citrinum* xym2, respectively. Xylanase yield and CFU count of *B. subtilis* were maximum after 48 h of incubation. Similar finding was also observed in a research conducted by Irfan *et al.* (2016) who showed fermentation period of 72 h as optimum for xylanase production by *B. megaterium* BM07, while *B. subtilis* BS04 exhibited maximum production after 48 h of fermentation period. As observed, the report also suggests that further increase in fermentation period beyond the optimum incubation period resulted in decline of xylanase production by *B. subtilis* BS04 and *B. megaterium* BM07, which might be due to the production of toxic metabolites or proteases during microbial growth that either inhibit or degrade the enzyme (Irfan *et al.* 2016). Gupta and Kar (2009) studied xylanase production by *Bacillus* sp. and found that highest xylanase yield was obtained after 48 h and 72 h of fermentation using wheat bran and corn cob as carbon source, respectively. In another study some strains of *Bacillus* showed maximum xylanase yield after 24 h of fermentation using digested bran and after 48 h of fermentation using saw dust as substrate, respectively (Simphiwe *et al.* 2011). *B. subtilis* and *B. licheniformis* MTCC 9415 were observed to exhibit highest xylanase activity after 72 h of solid state fermentation (SsF) (Gupta & Kar 2009, Heck *et al.* 2002) while Sepahy *et al.* (2011) reported fermentation period of 48 h as optimum for *Bacillus mojavensis* AG137 in submerge fermentation using oat bran as substrate. In the present study of xylanase production by *P. citrinum* xym2, biomass yield and xylanase activity were maximum at 72h of cultivation and then they declined gradually. The reduction of xylanase yield beyond 72h could be either due to the depletion of nutrients in the media or due to the reduction in fungal biomass.

The high demand and cost of available commercial enzymes require bioprospecting and modulation of microbes for higher productivity, which could enhance the yield of existing commercial enzymes (Khusroo *et al.* 2016, Kumar *et al.* 2014). Enzyme productivity can be increased by optimizing the production processes (Kumar *et al.* 2014). The optimization

of xylanase production conditions by OFAT approach enhanced the xylanase yield of *B. subtilis* xym4 from 980 IU/ml to 2100 IU/ml and that of *P. citrinum* xym2 from 712 IU/ml to 1853 IU/ml. Attempts were made to further enhance the enzyme production by employing statistical experimental design. The use of Response Surface Methodology (RSM) as a efficient tool to get better enzyme yield by designing minimum number of experiments for a large number of independent variables was cited in various report (Walia *et al.* 2015, Bocchini 2002). RSM justifies the combined effects of all the independent variables in a production process and explores estimated interactions between a response variable and the set of independent variables (Khusro *et al.* 2016). The significant factors for xylanase production identified through OFAT approach were further employed in CCRD based RSM for xylanase production by *B. subtilis* xym4 keeping the other variables constant in their level as suggested by OFAT approach. RSM identified the optimal level of variables as wheat bran 1 % (w/v), peptone 0.09 % (w/v), pectin 2.25 % (w/v), incubation temperature 45 ^{o}C, pH 4.5, incubation time 60 h, KCl 0.1 g/l and $MgSO_4$, $7H_2O$ 0.1 g/l and using these values enhanced the xylanase production up to 11800 IU/ml, which was about 12 fold higher than that of unoptimized level. The optimization of xylanase production of *P. citrinum* xym2 by OFAT approach identified wheat bran, incubation temperature and medium pH as the most influential variables. These three variables were hence further optimized by RSM, keeping the levels of other variables same as appeared in OFAT approach. In RSM the optimal level of variables were, wheat bran 1.5% w/v, incubation temperature 30^{o}C, medium pH 3.5, maltose 1.25% w/v, di-ammonium hydrogen phosphate 0.09% w/v, incubation time 72h, and using these levels increased the xylanase production to 2834.12 IU/ml, which was about 4 fold higher than that of unoptimized level. The substantial increase in xylanase production by RSM optimization could be due to significant interaction between the independent variables, wheat bran, incubation temperature and medium pH. In a study conducted by Adhyaru *et al.* (2016) obtained 1.3 fold enhanced production of xylanase by *Aspergillus tubingensis* FDHN1 using the statistical approach. It has also been reported that statistical optimization of fermentation process conditions resulted in 7.2 fold increase in xylanase activity from unoptimised 198 IU/ml to 1430 IU/ml in *Bacillus pumilus* 3GAH (Kaur *et al.* 2016). The xylanase production by *P. citrinum* xym2 in the present study was substantially higher than the previously reported activity values of 40.50 U/ml, 6.47 U/ml, 3.46 U/ml, and 7.82 U/ml for *Penicillium* wx-z1, *P. chrysogenum* (PCL 501), *P. oxalium* and *P. sclerotionum*, respectively (Banerjee *et al.* 2012, Knob and Canocarmona 2008, Ashok *et al.* 2007, Emezue *et al.* 2007).

The xylanase produced by *B. subtilis* xym4 and *P. citrinum* xym2 were characterized with respect to pH and temperature optima, thermal stability and metal ion requirement. Xylanase obtained from *B. subtilis* xym4 and *P. citrinum* xym2 was found to have a pH optima of 7 and 4, respectively and both the enzymes showed maximum activity at 40-50 ^{o}C. Moreover, xylanases from both the sources were stable in the pH range of 3-9 and retained more than 80% activity in the temperature range of 25-60^{o}C. Similarly, xylanase produced by *Bacillus altitudinis* DHN8 exhibited residual xylanase activity in the temperature range of 35-55 ^{o}C, whereas the optimum activity of the enzyme was at 45-55 ^{o}C (Adhyaru *et al.* 2017). In a research work, Bai *et al.* (2010) observed optimum pH 7.0 for recombinant xylanase XynA4 from *Alicyclibacillus* sp. A4 and more than 40% of its activity was retained by their reported xylanase across the pH range 3.8–9.4. It was also showed that *Bacillus altitudinis* DHN8 xylanase exhibited maximum activity in 50 mM sodium phosphate buffer (pH 6.0–8.0) with an optimum pH 8.0. Xylanase from *B. subtilis* xym4 and *P. citrinum* xym2 had remarkable thermal stability and thus retained 100% activity in the preincubation temperature range of 4-60 and 4-70 ^{o}C, respectively. Previous studies on xylanase from *B. pumilus* SS1, the enzyme showed good stability at 50°C for 60 min, however, its stability was reduced at 60°C (Bajaj *et al.* 2012). *T. lanuginosus* VAPS-24 xylanase was quite stable from the temperature ranges 50–80 °C and had a pH optima of 7 but was found less stable below pH 3.0 and above pH 9.0 (Kumar *et al.* 2017). Overall, *B. subtilis* xym4 and *P. citrinum* xym2 xylanases showed good temperature-stability as compared to the above reports. High temperature and pH tolerance of both the xylanases might be due to the translational modifications occurred during enzyme excretion, may be occurred during glycosylation, which results in the improved stability of an enzyme (Savitha *et al.* 2007).

Metal dependent stimulation in xylanase activity was observed and Mg^{2+}, Mn^{2+}, Ca^{2+}, Na^{+}, K^{+}, Zn^{2+} were found to stimulate xylanase activity of *P. citrinum* xym2, whereas Co^{2+}, Fe^{3+}, Cu^{2+} have reduced the activity of the enzyme. Xylanase from *B. subtilis* xym4 was also stimulated in presence of Mg^{2+},Ca^{2+}, Mn^{2+},Cu^{2+}, whereas Co^{2+}, Fe^{3+} and Zn^{2+} had significant inhibitory effect. The stimulatory effects of Ca^{2+}, Mn^{2+}, Fe^{2+}, K^{+} and Na^{+} on xylanase activity had been described by various researchers (Bajaj and Manhas 2012, Bajaj *et al.* 2012, Lv *et al.* 2008). The strong inhibitory action of heavy metal like, Hg^{2+} on xylanase was also reported by Bajaj and Manhas (2012), and Jiang *et al.* (2010). In our study, loss of activity was observed in presence of EDTA and β-ME for both xylanases. It was also found that SDS could slightly stimulated xylanase of *B. subtilis* xym4 but completely inhibited the *P. citrinum* xym2 xylanase.

In conclusion, two potent xylanase producing microorganisms *Bacillus subtilis* xym4 and *Penicillium citrinum* xym2 were isolated from the environmental samples. Production of xylanase by the two organisms was optimized sequentially by OFAT and RSM approach. Using these two approaches enhanced the xylanase production capability of *B. subtilis* xym4 from 2100 to 11800 IU/ml and that of *P. citrinum* xym2 from 1853 to 2834.12 IU/ml (Fig. 2.30). Both the xylanases were showed activity in the pH range of 3-9, and were thermostable. The high catalytic efficiency of xylanase from *Bacillus subtilis* xym4 (K_{cat}/Km=11.05) markedly greater in comparison to the other reports. The cellulase free xylanase from *Bacillus subtilis* xym4 could be a good candidate for sachharification of agro residues to fermentable sugar especially pentoses such as xylose, for xylitol production, whereas the enzyme obtained from *P. citrinum* xym2 can be a potent candidate for saccharification of lignocellulosic wastes.

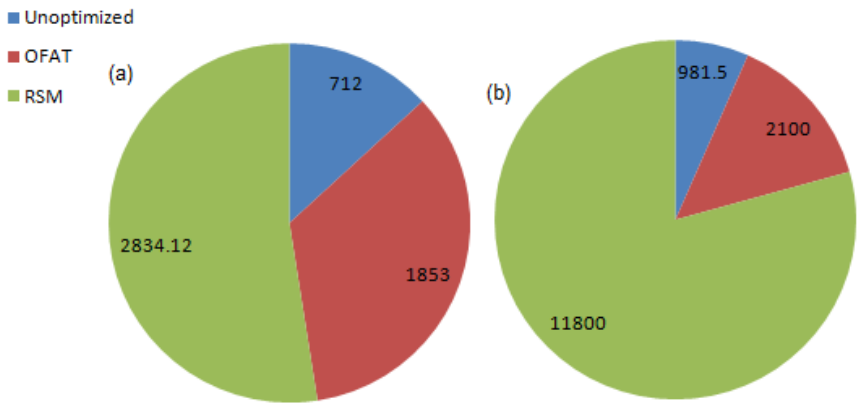


Fig.2.30. Optimization of xylanase production in different methods by (a) *P. citrinum* xym2 and (b) *B. subtilis* xym4

Chapter 3

Sequential and simultaneous strategies for xylitol production from agro residues

3.1. Introduction

Cellulosic and hemicellulosic materials present in agro residues are biodegraded to monomeric sugars by the concerted activities of enzymes, such as cellulase, ligninase and xylanase (Liu *et al.* 2006). Several research works have been conducted to isolate or to develop microbial strains that could produce these enzymes in higher titre for saccharification. Alternatively, research on optimization of process parameters for sachharification of agro residues can achieve the same goal (Singh *et al.* 2011). The bioconversion of various polymers present in the agro residues to fermentable sugars has been identified as a promising approach for production of different value added products. Utilization of these lingocellulosic wastes for generation of fermentable sugars by microbial enzymes also helps to reduce the pollution problems that arise from their frequent and inappropriate dumping in environment (Botella *et al.* 2007). Concerted activities of several enzymes such as xylanases, cellulases, ligninases, β-glucosidase, β-xylosidase as well as various other hemicellulases are required for complete depolymerization of agro residues to generate fermentable sugars (Gupta *et al.* 2012, Koseki *et al.* 2006). Heteroxylan is a highly branched hemicellulosic component of plant biomass and is made up of β-1, 4 linked xylopyranosyl backbone with different side chains of arabinosyl, glucuronyl and acetyl residues (Khanahmadi *et.al.* 2018). Xylanase in association with β-D-xylosidase (E.C.3.2.1.37), α-L-arabinofuranosidase (E.C.3.2.1.55), α-D-glucroronidase (E.C.3.2.1.139) and acetyl-xylan esterase (E.C. 3.1.1.72) is needed for complete breakdown of hemicellulose rich in heteroxylan (Fang *et al.* 2008).

Xylitol, a five carbon sugar alcohol, is used as dietary sugar substitute or artificial sweetener. It is widely used in food and pharmaceutical industries due to low calorie content, anti cariogenicity, tooth rehardening, preventive against otitis, ear and upper respiratory infection etc (Islam 2011, Uittamo *et al.* 2011). Due to its health promoting effect the demand of xylitol in the global market is increasing rapidly. Worldwide

consumption of xylitol was approximately 160 thousand metric tons in 2013, which corresponds to a market value of US$ 670 million and predicted to reach 242 thousand metric tons by 2020 with a market value of US$ 1 billion (http://www.prnewswire.com). As most of the xylitol is produced through expensive catalytic hydrogenation of pure xylose under high temperature and pressure, an intervention of biotechnological processes for xylitol production with low cost raw materials is required (Albuquerque *et al.* 2014).

Wheat bran, wheat straw, corn stover, corn cobs etc. are the few examples of low cost lignocellulosic agro residues that can be used as source of xylose for xylitol production (Rao *et al.* 2006). Pretreatment of these residues with acid, alkali, steam exploitation, microwave radiation etc. has been found to be essential for separation of lignin and reduction of crystallinity of the substrate used for xylitol production. Acid pretreated residues are widely used for sachharification by xylanase with a focus on maximum xylose recovery. Optimization of process parameters through several statistical approaches has been used for higher xylose yield from agro residues which can be converted to xylitol by microbial fermentation (Rao *et al.* 2006).

In the present investigation, microorganisms isolated from soil were screened for xylitol production followed by characterization of the best producers. Furthermore, the xylanases produced by *B. subtilis* xym4 and *P. citrinum* xym2 (Chapter-2) were employed in optimization of sacchrification of agro residues to fermentable sugars. Finally, the reducing sugars obtained after saccharification were used for xylitol production by sequential strategies. Moreover, simultaneous saccharification and fermentation (SSF) experiment was also conducted for production of xylitol using wheat bran as substrate.

3.2. Materials and Method

3.2.1 Strains, Chemical and reagents:

Xylitol, Nicotinamide Adenine Dineucleotide (NAD), Reduced Nicotinamide Adenine Dineucleotide (NADH), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), phenazine methosulfate (PMS), and Sorbitol dehydrogenase (SDH) were purchased from Sigma, USA. The bacterial strain *Escherichia coli* xyl6 (Accession no. MG607371) was isolated from the soil of Salbagan, University of North Bengal (Latitude 26”42’45.03 °N, Longitude 88”21’15.28°E). The fungal strains *Penicillium citrinum* xym2 (Accession no.KF570240) and the bacterial strain *Bacillus subtilis* xym4 (Accession no. MG607370) were isolated from garden soil of University of North Bengal, as discussed in chapter 2, section 2.2.2. *Candida tropicalis* ATCC 96745 was procured from American Type Culture Collection. Agro residues were collected from the local market near University of North

Bengal. All other biochemicals and microbiological media were purchased from Sigma-Aldrich, USA; E. Merck, Germany and HiMedia Laboratory, India.

3.2.2. Sample collection

For screening and selection of xylitol producing microorganisms, the garden soil of Salbagan, University of North Bengal, was used. About 100 g of soil collected in sterilized zipper bags. The samples were immediately transported to the laboratory for gradient dilution using autoclaved saline (0.85 % w/v) water and aliquots were transferred to appropriate growth medium.

3.2.3. Xylitol dehydrogenase (XDH) assay

3.2.3.1. Xylitol dehydrogenase enzyme preparation

Candida tropicalis ATCC 96745 was grown in a growth medium containing (g/ l): yeast extract, 3; K_2HPO_4, 3; $MgSO_4.7H_2O$, 1 and D-xylose, 20 (pH 5); at 30 °C for 48 h with 120 RPM shaking. Thereafter, the culture was harvested by centrifugation at 10,000 RPM for 10 min at 4 ^oC. The cell pellet was washed with 0.5 M potassium phosphate buffer pH 7, suspended in 2 ml phosphate buffer (pH 7) and then vortexed with sterilized glass beads (Sigma). The crude extract was centrifuged at 10,000 RPM for 15 min and the supernatant was filtered by passing through 0.2 µm syringe membrane filter. To the filtrate ammonium sulphate was added till 70% saturation and the resulting mixture was centrifuged at 10,000 RPM for 15 min at 4 ^oC to collect the precipitated proteins. The pellet was suspended in 2 ml of 0.5 M potassium phosphate buffer pH 7, dialyzed against the same buffer and used for xylitol dehydrogenase assay.

3.2.3.2. Xylitol dehydrogenase assay

Xylitol dehydrogenase was assayed by the method of Yokoyama *et al.* (1995), with certain modifications. The assay is based on oxidation of xylitol in presence of NAD as cofactor with concomitant production of xylulose and $NADH+H^+$. The reaction mixture in a final volume of 1ml contained: 50 mM sodium phosphate buffer pH 7, 400 µl; 2M xylitol, 100 µl; 100 mM β-mercaptoethanol, 100 µl; 40 mM NAD, 100 µl; yeast cell free extract, 100 µl and de-ionized water, 200 µl. In the control assay, xylitol in the reaction mixture was replaced by de-ionized water. The progress of reaction was monitored by recording increase in absorbance at 340 nm due to conversion of NAD to NADH. One unit (U) of xylitol dehydrogenase activity is defined as the amount of enzyme required to oxidize 1.0 µmole of xylitol in one minute. The xylitol dehydrogenase activity was calculated using the following equations 1 and 2:

$dA/dt\ (min^{-1}) = [Rate]_{Test} - [Rate]_{control}$ ……(Eq.1)

$$\text{Activity (U/ml)} = \frac{TV \times D \times dA/dt}{\varepsilon \times V} \quad \text{……..(Eq.2)}$$

TV: Total volume in cuvette

D: Dilution factor of the cell free extract.

V: Volume of cell free extract/enzyme used

ε: Molar extinction coefficient for NADH (6.22 L/mmol for a path length of 1.0 cm)

3.2.4. Isolation and screening of xylitol producing microorganism

10 g soil sample was diluted in 90 ml of sterile saline (0.85 % w/v) water and serially diluted (10^{-1}-10^{-8}). The consecutive propagation of microorganism having the property of xylitol production were conducted by spreading 0.1 ml of the serially diluted samples on the xylose agar media (XLM, pH 7) containing (g/l): peptone, 0.90; $(NH_4)_2HPO_4$, 0.40; KCl, 0.10; $MgSO_4$,$7H_2O$, 0.10; xylose, 5 and 2% w/v agar and incubated at 37 ^oC for 48 h. The representative individual colonies obtained from XLM plates were selected based on their morphology. After obtaining pure cultures, all the isolated strains were qualitatively and quantitatively screened for xylitol production in solid and liquid medium, respectively.

3.2.5. Preparation of standard curve for xylitol

Standard curve of xylitol (100 to 1000 µg/ml) was prepared according to the chromomgenic reaction method describe by Beutler (1984). 500 µl of xylitol (Sigma, USA) was added to 750 µl of chromomgenic reaction mixture containing 100 µl of 1 mM INT, 100 µl of 1 mM PMS, 50 µl of 1mM NAD and 500 µl of SDH/XDH enzyme. The reaction mixture was incubated at room temperature for 1 h and then optical density was measured at 650 nm. Blank was prepared with the reaction mixture that contain 500 µl of distilled water instead of xylitol. The mechanism of the assay is explained by the equation 3 and 4.

$$\text{Xylitol} + \text{NAD} \xrightarrow{\text{Xylitol dehydrogenase}} \text{Xylulose} + \text{NADH} + H^+ \quad \text{……(Eq. 3)}$$

$$\text{NADH} + H^+ + \text{INT} + \text{PMS} \longrightarrow \text{NAD}^+ + \text{INT-formazon (Coloured)} \text{……(Eq. 4)}$$

Absorbance at 650 nm (A_{650}) obtained using the two enzymes were tested using two sample t-test assuming equal variance. The two regression curves were not significantly different as

T_c (0.93) < T_α (2.08) at 0.05 level of significance. Moreover, these two curves showed apparently same and high coefficient of determinant value (R^2_{SDH} = 0.996 and R^2_{XDH} = 0.993). Hence, it can be inferred that xylitol concentrations can equally be estimated using both the regression line.

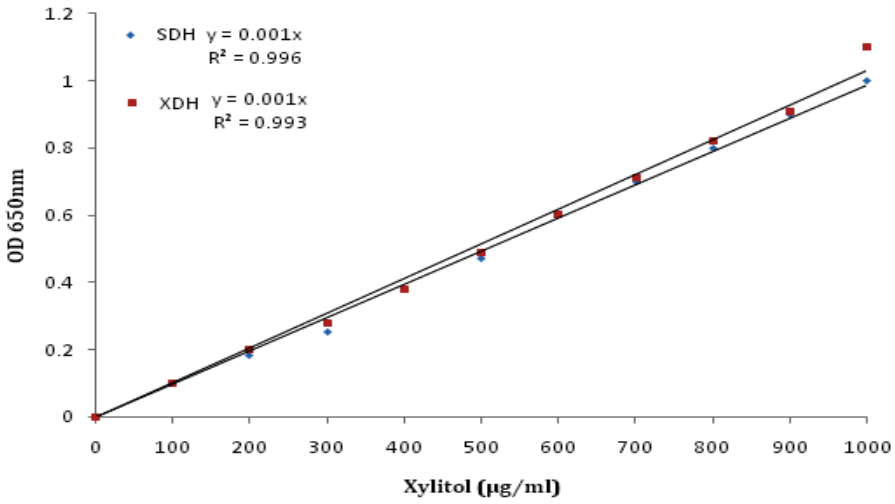


Fig. 3.1. Standard curve for xylitol

3.2.6. Screening of xylitol producing bacteria

3.2.6.1. Primary screening in solid medium

Bacteria isolated from the XLM plates were purified and then individually spread plated in XLM and incubated for 48 h at 37 ^oC to produce bacterial mat. Xyitol production by each of the bacterial mat was tested according to the method of Beutler (1984). Sterilized short strips of whatmann no.1 filter paper soaked in chromomgenic reaction mixture containing 100 µl of 1 mM INT, 100 µl of 1 mM PMS, 50 µl of 1 mM NAD and 500 µl XDH or SDH (10 IU/ml) were placed over the bacterial lawn for 12 h to monitor change in strip colour. The background colour was verified by placing filter-paper bits soaked with the chromogenic mixture on the XLM plates without culture. In the control assay, no enzyme was added. The background color was verified by placing filter-paper bits soaked with the assay mixture on the agar petri dishes without any culture. Filter paper strips soaked in reaction mixture without the enzyme preparation placed over the bacterial mat was used as control assay.

3.2.6.2. Secondary screening and xylitol estimation in broth medium

Bacterial culture isolated after primary screening in XLM plate was further inoculated in xylose broth medium (XBM = XLM-Agar) and incubated for 24 h at 37 ^{o}C. Thereafter culture broth was centrifuged at 8000 RPM for 10 min at 4^{o}C and the supernatant obtained was passed through a 0.2 µm membrane filter and the resulting filtrate was used as cell free supernatant (CFS). 500 µl of CFS was mixed with 750 µl of chromogenic reaction mixture as described above and incubated for 1 h at RT. The absorbance of the colour developed was recorded at 650 nm using UV-Vis spectrophotometer (Shimadzu 1800). Quantification of xylitol was inferred from the standard curve prepared using 100 to 1000 µg/ml of xylitol (Fig.3.1). For the blank, sterile water was added to the assay mixture instead of culture supernatant (substrate). In the control assay, sterile water instead of the enzyme solution was used in the assay.

3.2.7. Xylose reductase (XR) assay

3.2.7.1. Xylose reductase enzyme preparation

10^4 CFU/ml of *Escherichia coli* xyl6 was inoculated in the XLB medium and then incubated at 37 ^{o}C for 24 h; thereafter the cells were pelleted by centrifugation at 8000 RPM for 15 min at 4 ^{o}C. The cell pellet was washed twice with 100 mM citrate buffer pH 5 and resuspended in the same buffer. The cell suspension was vortexed with sterilized glass beads (Sigma) in a microfuge tube at about 4 °C to obtain the crude extract, which was centrifuged at 10,000 RPM for 15 min. The supernatant after passing through 0.2 µm syringe membrane filter was used as cell free extract (CFE) for determination of xylose reductase activity.

3.2.7.2. Xylose reductase assay

The xylose reductase (XR) activity in *E.coli* xy16 cell free extract was determined spectrophotometrically based on oxidation of NADPH by the method outlined by Yokoyama *et al.* (1995). The reaction mixture contained 100 mM citrate buffer (pH 5), 200 µl; 100 mM β-mercaptoethanol, 200 µl; cell free extract, 100 µl; 3.4 mM NADPH, 100 µl; and 1.2 ml of deionized water. After mixing properly, the reaction mixture was allowed to stand for 1 min to eliminate the endogenous NADPH oxidation to $NADP^+$. The reaction was started by the addition 200 µl of 500 mM D-xylose. The reaction mixture with heat inactivated cell free extract served as control. The rate of NADPH oxidation was recorded at 340 nm using UV-Vis Spectrophotometer (Shimadzu 1800) at 1 min intervals for 5 min. One unit (U) of xylose reductase is defined as the amount of enzyme required to catalyze the

oxidation of 1 μ mol of NADPH/min under standard assay conditions. The XR activity was calculated using the extinction coefficient of NADPH through Eqs. (5) and (6) (Nidetzky *et al.* 1996), and expressed as U/ ml and U/mg protein.

OD_{340}/min= [OD_{340} at 1min – OD_{340} at 5 min]/4min……(Eq. 5)

Activity (U/ml) = [OD_{340}/min (Test)- OD_{340}/min(Control)]X TV XD/ε XV……(Eq. 6)

Where,
OD_{340}/min = Rate of decrease in absorbance at 340 nm per min;
TV=Total volume of assay (ml);
D= Dilution factor of CFS
V: Volume of CFS/enzyme used
ε: Molar extinction coefficient for NADPH(6.22 mM^{-1} cm^{-1})

3.2.8. Characterization of xylose reductase

E.coli xy16 cell free extract was prepared as described in section 3.2.7.1. The extract was subjected to 30-70% ammonium sulphate precipitation. The resulting mixture was centrifuged at 10,000 RPM for 15 min at 4 ^{o}C to collect the precipitate. The pellet was dissolved in 2 ml of 100 mM citrate buffer pH 5 and dialyzed against the same buffer. The dialyzed extract was used for characterization of XR with respect to pH optima, temperature optima, thermal stability and metal ions requirement.

3.2.8.1. Determination of pH optima, temperature optima and thermostability of XR

The optimum pH of XR was determined by measuring the XR activity at pH 3.0-9.0 in the following buffers: 100 mM glycine (pH 3.0 and 3.5), 100 mM sodium acetate (pH 4.0, 4.5 and pH 5.5), 100 mM phosphate (pH 6.0, 6.5 and 7.0) and 100 mM Tris-HCl (pH 7.5, 8.0, 8.5 and 9). The normal assay procedure was followed except for varying the buffers of specified pH. The optimum temperature of the enzyme was determined by measuring the enzymatic activity at optimum pH and at temperatures ranging from 4 to 90 °C. For determination of thermal stability, the desalted enzyme preparation was pre-incubated at various temperatures ranging 4-100 ^{o}C for 30 min, followed by determination of enzymatic activity under standard conditions**.**

3.2.8.2. Effect of metal ions

The effect of metal ions and chemical reagents on XR was determined by measuring enzymatic activity in presence of 5 mM Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, K^{+}, Na^{+},

SDS, EDTA, β-mercaptoethanol. Activity without addition of ions or reagents was used as control.

3.2.9. Saccharification of agro residues

3.2.9.1. Preparation of agro residues

Agro residues such as sawdust, grass, water hyacinth, petals, sugarcane bagasse, coconut husk, orange peel, mango leaf, wheat bran, rice straw and corn fiber were washed, dried and sieved to 0.4-0.8 mm particle size. They were pre-treated with either 0.1N NaOH or 0.1N HCl (20 % w/v) for 16 h. The pre-treatment was followed by washing with distilled water till neutrality and drying in oven at 80 ^{o}C till constant dry weight.

3.2.9.2. Xylanase cocktail preparation

Penicillium citrinum xym2 and *Bacillus subtilis* xym2 were cultivated in their respective optimized media for xylanase production as described in chapter 2. After fermentation the culture broth was centrifuged at 10,000 RPM for 15 min at 4 ^{o}C. The filtered supernatant was used as xylanase enzyme cocktail (XEC) in sachharification studies. XEC obtained from *Penicillium citrinum* xym2 and *Bacillus subtilis* xym4 are henceforth mentioned as XEC^{P} and XEC^{B}, respectively. XEC^{P} and XEC^{B} were appropriately diluted with 100 mM sodium citrate buffer (pH 4.2) and 100 mM sodium phosphate buffer (pH 7), respectively, and applied for saccharification of agro residues.

3. 2.9.3. Saccharification of agro residues

Each agro residue was separately suspended in 100 mM sodium citrate buffer (pH 4.2) and 100 mM sodium phosphate buffer (pH 7) at 3% (w/v) and supplemented with XEC^{P} and XEC^{B} (500 IU/gds), respectively. XEC^{P} contained (IU): xylanase 500; CMCase, 266; β-xylosidase, 126.96; FPase, 56.42 and protease, 160.88, whereas XEC^{B} contained (IU): xylanase, 500; CMCase, 4.90; β-xylosidase, 113; FPase, 99.18 and protease 25.8. The reaction mixtures were incubated at 40 ^{o}C for 1h and then the amount of reducing sugar released was estimated by DNS method. Agro residues treated only with buffer solution served as blank, whereas XECs mixed with respective buffers only, were used as negative control.

3.2.9.4. Estimation of reducing sugar released (RSR)

The quantification of reducing sugars released in the saccharification reaction was done by 3,5-dinitro salicylic acid (DNS) method using xylose as standard (Miller 1959). After the saccharification, the whole reaction content was centrifuged at high speed. Then equal

volumes of saccharified supernatant and DNS reagents were mixed thoroughly and heated in a boiling water bath for 10 min. After cooling at RT, absorbance was recorded at 540 nm and the concentration of reducing sugar was inferred from the calibration curve of xylose ranging 10-100 μg/ml.

3.2.10. Screening of parameters affecting sachharification of wheat bran using XEC^B by Plackett Burman (PB) design

Plackett–Burman design has been proved to be a powerful and efficient mathematical approach for screening of the most influential parameter(s) of a study. Nine different independent variables such as soaking time (h) of wheat bran in 0.1N HCl (20 % w/v), enzyme loading (IU/gds), wheat bran concentration (g/l), pH, incubation temperature (^oC), reaction time (h), particle size (mm), $MgCl_2$ concentration (g/l) and agitation speed (RPM), were screened using the PB design for the response variable, reducing sugar release (RSR in mg/ml). These variables were investigated for RSR in total 12 experiments (Table 3.1). Each of the independent variables was incorporated in the design at its high and low levels and denoted by +1 and -1 signs, respectively.

The effect of each variable was calculated from the difference between the averages of measurement made at high and low levels, using Eq. 7:

$$E_{(Xi)} = 2(\Sigma^{Pi+} - \Sigma^{Pi-}) / N \quad \text{(Eq. 7)}$$

Where, $E_{(Xi)}$ is the concentration effect of tested variable, and Pi+ and Pi- represent the RSR from the trials, where the variables (Xi) being measured were added to production medium at high and low concentrations, respectively, and N is the number of experiments carried out.

Table 3.1
List of variables with their coded values used for Placket Burman experimental design for reducing sugar released (RSR)

Coded values	Soaking time (h)	Enzyme dose (IU/gds)	Wheat bran (g/l)	pH	Incubation temp. (^oC)	Reaction time (h)	Particle size (mm)	$MgCl_2$ (g/l)	Agitation (RPM)
+1	8	500	5	4	30	3	0.2	0.10	50
-1	16	1000	50	8	50	6	0.8	0.50	100

3.2.11. Response surface optimization of significant parameters for sachharification of wheat bran using XEC^{B}

Central composite rotatable design (CCRD) based response surface methodology was used to further optimize the conditions for sachharification of wheat bran by XEC^{B}. Four independent variables wheat bran concentration (g/l), reaction time (h), agitation speed (RPM) and soaking time (h) of wheat bran in 0.1N HCl were selected in five different coded levels to optimize the response variable reducing sugar release (RSR). The investigated range and levels of the variables are given in Table 3.2. In total 30 experimental runs were performed to optimize the saccharification parameters. RSR obtained (mg/ml) from all the experimental runs was analyzed by a second order polynomial regression equation (Eq. 8) to better estimate the experimental error.

$$Y= =a_0+a_1x_1+a_2x_2+a_3x_3+a_{11}x_{12}+a_{22}x_2^2+a_{33}x_3^2+a_{12}x_1x_2+a_{13}x_1x_3+a_{23}x_2x_3 \quad \text{(Eq. 8)}$$

where Y is the predicted xylanase activity, a_0 is the intercept term, x_i (x_1,x_2,x_3,x_{12},x_{22},x_{32}) is the independent factor and a_i (a_1,a_2,a_3,a_{11},a_{22},a_{33},a_{12},a_{13},a_{23}) is the model coefficient parameter.

Table 3.2
Actual and coded levels of variables used in CCRD based RSM design

Factor Code	Factor	Levels				
		-α	**-1**	**0**	**+1**	**+α**
A	Soaking Time(h)	4	8	12	16	20
B	Incubation time (h)	1.5	3	4.5	6	7.5
C	Agitation speed (RPM)	25	50	75	100	125
D	Wheat bran (g/l)	12.5	25	37.5	50	62.5

3.2.12. Xylitol production from saccharified wheat bran

Saccharified wheat bran extract was filtered through a sterilized muslin cloth to remove the undigested agro residues and the clear filtrate was collected. 50 ml of filtrate was supplemented with 0.9 g/l peptone and inoculated with 10^6 CFU/ml of 24 h old culture of *E.coli* xyl6, and the resulting mixture was incubated for 24 h at 37^{o}C. After fermentation the resulting extract was centrifuged at 8000 RPM at 4 ^{o}C for 10 min and the supernatant was

used for quantification of xylitol by the method described in section 3.2.6.2. Also, the 24 h fermented culture broth was serially diluted in 0.85 % (w/v) sterilize saline water and 0.1ml of the diluted sample was spread plated over Eiosine Methelene Blue (EMB) agar plate and viable count in (CFU/ml) was recorded according to standard plate count method.

3.2.13. Production of xylitol through simultaneous saccharification and fermentation (SSF) of wheat bran

3.2.13.1. Development of inoculum

The pure culture of xylanase producing *B. subtilis* xym4 was cultivated in xylanase production medium (XPM, pH 7) [containing (g/l): peptone, 0.90; $(NH_4)_2HPO_4$, 0.40; KCl, 0.10; $MgSO_4$.$7H_2O$, 0.10; birchwood xylan], whereas xylitol producing *E. coli* xyl6 was cultivated in XLB (pH 7). Both the cultures were incubated at 37 ^oC for 24h. *B. subtilis* xym4 and *E.coli* xyl6 culture with viable cell counts of $5.2X10^6$ CFU/ml and $6.2 X 10^6$ CFU/ml, respectively, were used as inoculum source.

3.2.13.2. Preliminary optimization of culture media for xylitol production

In preliminary experiment, the optimum inoculum size of xylanase and xylitol producing bacteria for the simultaneous saccharification and fermentation (SSF) was determined by varying the inoculum quantity (1%, 2%, 3%, 4% and 5% v/v) in their respective culture broths. *B. subtilis* xym4 was inoculated in the 100 ml of primary SSF medium (SSF^P) (pH 7), containing (g/l); wheat bran, 15; peptone, 0.90; KCl, 0.10; $MgSO_4$,$7H_2O$, 0.10; whereas *E.coli* xyl6 was inoculated in XBM (pH 7), and they were incubated at 37 ^oC for 24 h. Thereafter, colony counts (CFU/ml) of the serially diluted culture broth was measured by standard plate count (SPC) method using nutrient agar plates. Estimation of xylanase and xylitol from the CFS of *B. subtilis* xym4 and *E. coli* xyl6 culture broth were also carried out.

The effect of incubation temperature (25, 30, 37, 40, 50 and 60 °C) on the yield of viable bacterial colony counts of both the bacteria was studied by inoculation of optimum level of bacterial inoculum to their respective culture broths and incubation for 24 h at above mentioned incubation temperatures while the other conditions were kept constant. After incubation colony count (CFU/ml) of the serially diluted culture broths was measured by SPC method.

The effect of medium pH on the yield of viable bacterial colony counts of both the bacteria was studied by inoculation of optimized level of inoculum to their respective culture media of pH ranging between 3-9 (100 mM citrate buffer pH 3, 4 and 5; 100 mM phosphate buffer pH 6, 7 and 100 mM Tris–HCl buffer 9) and incubation at 40 ^oC for 24 h followed by

measurement of colony count (CFU/ml) of the serially diluted culture broths by SPC method.

The effect of incubation time on the yield of viable bacterial colony count was also studied. For this the inoculated culture broths (*Bacillus* and *Escherichia)* were incubated for various time intervals ranging from 24 to 114 h keeping all the other optimized conditions constant. After incubation colony count (CFU/ml) of the serially diluted culture broths was measured by SPC method.

3.2.13.3. Simultaneous saccharification and fermentation (SSF) of wheat bran

B. subtilis xym4 and xylitol producing *E. coli* xyl6 were cultivated in a secondary SSF medium (SSF^S) with 10 fold higher concentration of medium components as compare to that of $SSF^{P.}$ Six different fermentation conditions were used to evaluate xylitol production from wheat bran, which are as follows:

(i) *B. subtilis* xym4 inoculated (1% v/v) in 500 ml of SSF^S (pH 7) [containing (g/l); wheat bran, 150; peptone, 9; KCl, 1; $MgSO_4$, $7H_2O$, 1] in an Erlenmeyer flask and incubated at 40 ^oC for 72 h.

(ii) *E. coli* xyl6 inoculated (1% v/v) in 500 ml of above mentioned culture medium and incubated at 40 ^oC for 72 h.

(iii) Both the bacteria together, at inoculum concentration of 1% v/v each, were inoculated in the 500 ml of above mentioned culture medium and incubated for 72h.

(iv) *B. subtilis* xym4 was inoculated in the culture medium first and incubated for 24 h at 40 ^oC. The culture was then inoculated with *E. coli* xyl6 and further incubated at the same temperature for 48h.

(v)*B. subtilis* xym4 was inoculated in the culture medium, grown for at 40 ^oC for 48 h and then *E. coli* xyl6 was inoculated and grown at same temperature for another 24 h.

(vi) *E.coli* xyl6 was inoculated into the culture broth first, incubated for 24 h at 40 ^oC, and then *B. subtilis* xym4 was inoculated into the same culture broth and incubated for 72 h.

Aliquots from each of the six flasks were aseptically withdrawn at 24 h of interval and analyzed for viable colony count of both the bacteria (CFU/ml), medium pH, dissolved oxygen (DO) percentage, xylanase activity (IU/ml), xylose reductase activity (U/ml), total reducing sugar (mg/ml), total protein (mg/ml) and xylitol content (g/l).

3.2.14. Analytical techniques

3.2.14.1. Xylitol quantification through gas chromatography mass spectrometry (GC-MS)

Each fermentation broth was centrifuged at 10,000 RPM at 4 ^{o}C for 8 min to collect the clear supernatant. The supernatant was filtered through 0.2 µm membrane filter to remove any insoluble material. All the samples were freeze dried in a freeze dryer (condenser temperature -50 °C and chamber pressure P < 0.08 mbar) for 48 h. The dried samples were further derivatized for GC-MS analysis. The derivatization of possible sugar and sugar alcohols in the sample was performed according to the method of Namgung *et al.* 2010 and Sweeley *et al.* 1963. 1mg of samples was mixed with 500 µl solution of methoxyamine hydrochloride in pyridine (20 mg/ml). The mixture was vortexed for 1 min and incubated at 37 ^{o}C for 90 min. After incubation 700µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added to the mixture and incubated at the same temperature for 30 min. The reaction mixture was kept at room temperature at least for 2 h before analyzed through GC-MS. Derivatized samples were further mixed with isobutanol as the IS (Internal standard) in 2ml screwcap septum vials and then loaded onto the auto sampler.

Agilent 7890A gas chromatograph coupled with a 5975C mass spectrometer and a DB-5MS column (30 m length × 0.25 mm i.d. × 0.25 µm film thickness) was employed for GC–MS analysis of sugars and sugar alcohol. Helium was used as carrier gas that was maintained at a flow rate of 1 ml/min. 1µl injection volume of sample was used with split ratio of 10:1. The oven temperature was held at 70 °C for 4 min, and then increased to 310 ^{o}C at 5 °C/min and held at the temperature for 10 min. All samples were analyzed in both full scan (mass range of 40–510 amu) as well as in selective ion scan mode. The injector inlet and transfer line temperature were 290 and 280 °C, respectively. Xylitol along with other sugar alcohol and sugar residues in the extract were identified by comparing retention time or mass fragment characteristic of targets with that of standard compounds, and NIST data. Calibration curve approach was used to quantify the xylitol. Chromatographic data were recorded and integrated using Agilent Chemstation software. Xylitol yield was calculated according to the following equation 9.

$$\text{Xylitol yield (g/g)} = \frac{\text{Xylitol (g/l)}}{\text{Reducing sugar (g/l)}} \quad \text{OR} \quad \frac{\text{Xylitol (g/l)}}{\text{Wheat bran (g/l)}} \qquad \text{……. (Eq. 9)}$$

3.2.14.2. Viable colony count

Culture broth obtained after completion of fermentation was serially diluted in 0.85% (w/v) saline water (10^{-1} - 10^{-9}). Total bacterial count (CFU/ml) was obtained by spreading 100 µl of serially diluted culture in nutrient agar plate. *E. coli* xyl6 colonies were enumerated by spreading 100 µl of serially diluted culture in Eiosine Methelene Blue (EMB) agar plate. The count of *B. subtilis* xym4 was conducted by plating the serially diluted culture in phenyl ethyl alcohol (Himedia) agar. The culture plates were incubated at 37 ^{o}C for 24 h. After incubation viable colony counts were taken and expressed as CFU/ml.

3.2.14.3. Total Protein

Protein was estimated by the method described by Bradford *et al.* (1976) as explained in chapter 2, section 2.2.7.

3.2.14.4. Dissolve oxygen (DO)

DO in the fermented broth was recorded by using Eutech Instrument Cyber Scan series 600 probe and measured in percentage (100%).

3.2.14.5. Xylanase activity

Xylanase activity in the fermentation broth was measured according to the method describe in chapter 2, section 2.2.4.2.

3.2.15. Statistical analysis

All the experiments were performed in duplicate or in triplicate. The data were represented as mean with standard error and their significance level were further evaluated by analysis of variance (ANOVA), t-tests using SPSS version 16. Homogeneity of mean was further checked by Duncan's multiple range test (DMRT) at a level of significance $P < 0.05$. Pearson correlation tests were also performed to check the interaction effects among fermentation variables using SPSS version 16. Principal component analysis (PCA) for reduction of data obtained from SSF experiments was carried out using the SPSS.

3.3. Results

3.3.1. Isolation, screening and identification of xylitol producing bacteria

Xylitol producing microorganisms were isolated from the soil samples by serial dilution method on XLM media plates. Seven microorganisms showing growth on XLM plate were isolated and selected for primary screening. Isolated microorganisms were individually spread-plated at high concentration on XLM plates and incubated at 37 ^{o}C for 24 h to form mat like growth. Thereafter, a short strip of whatman no.1 filter paper soaked in chromogenic reaction mixture placed in the middle of bacterial lawn. One isolate named xyl6 turned the colourless filter paper to pink suggesting the production of xylitol (Fig 3.2). The bacterial strain was further grown in XBM medium at 37 ^{o}C for 24 h and xylitol level in the culture supernatant was quantified by chromogenic assay method with SDH or XDH

enzyme. The bacterial strain xy16 produced 5.2 g/l of xylitol. The strain xyl6 was maintained in nutrient agar slant for further studies. The xylitol producing bacteria, xy16 was identified based on its colony morphology in nutrient agar plates, gram nature and biochemical reaction up to genus level. The strain was morphologically characterized as gram negative, rod shaped, motile bacteria. The colony of the isolate was circular in shape with entire margin, raised elevation,

nonpigmented, translucent, small size with smooth texture. The bacterium was characterized biochemically as negative in citrate utilization, Voges-Proskauer tests and positive in indole fermentation test (Table 3.3).

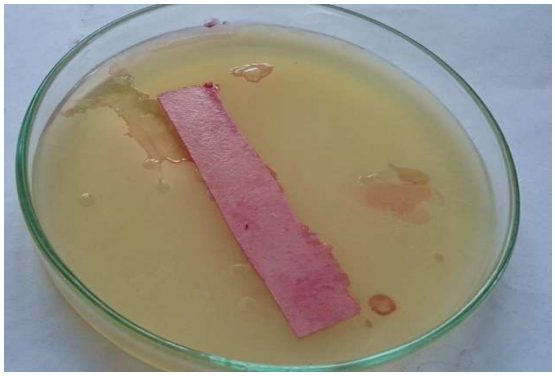


Fig.3.2. Xylitol production by *E.coli* xyl6.

Figure 3.3. Growth of *E. coli* xyl6 in EMB agar.

Table 3.3 **Morphological and biochemical characteristics of the xylitol producing bacterial strain xyl6**			
1	Gram staining	-	
2	Shape of vegetative cell	Rod	
3	Diameter of colony(mm)	2	
4	Colony Morphology	Circular shape, entire margin, raised elevation, non pigmented, translucent, small size with smooth texture.	
5	Spore formation	-	
6	Motility	+	
7	Growth on usual media (Aerobic)	+	
9	Starch hydrolysis	+	
10	Casein hydrolysis	+	
11	Gelatin hydrolysis	-	
12	Lysine decarboxylase	+	
13	Urease test	-	
14	Catalase test	+	
15	Oxidase test	-	
16	Lipase test	-	
17	Citrate utilization	-	
18	Voges-Proskauer test	-	
19	Methyl Red test	+	
	Indole	+	
20	Nitrate reduction test	+	
21	Fermentaion/Oxidation	Acid	Gas
21.1	Starch	+	+
21.2	Lactose	+	+
21.3	Glycerol	+	-
21.4	Mannose	+	-
21.5	Glucose	+	+
21.6	Fructose	-	-
21.7	Mannitol	+	+
21.8	Xylose	+	-
21.9	Arabinose	+	+
21.10	Cellobiose	+	+
21.11	Sucrose	+	+
21.12	Maltose	-	-
21.13	Sorbitol	+	+
	Identity of Bacteria	***Escherichia coli***	

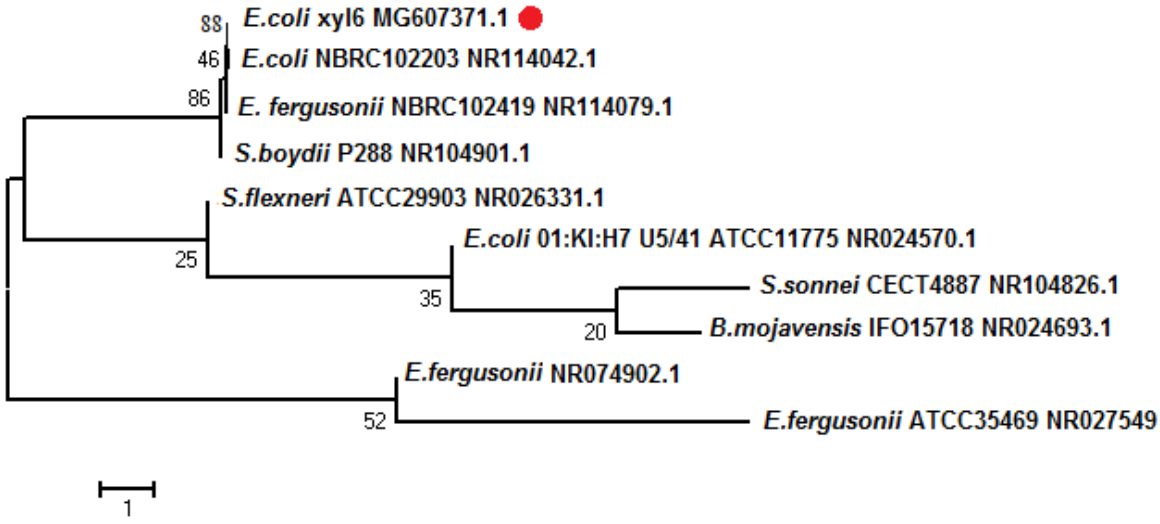


Fig.3.4. Phylogenetic tree constructed using 16s rRNA gene sequences reflecting the position of strain xyl6 with the other *Escherichia* species. The sequence of *B. mojavensis* (accession no. NR024693) was incorporated in the tree as on out group. Bar represent 1 nucleotide substitution per base. Numbers at nodes represent bootstrap values. Accession numbers are given at extreme left of each strain.

3.3.2. Characterization of xylose reductase (XR)

To determine the effect of pH on XR activity, XR was reacted with D-xylose in the pH range 3-9. From the result in Fig. 3.5a shows that XR was active in a wide range pH 3 to 7 with optimum at pH 5. Although the enzyme retained its activity in acidic pH, it gradually lost its function at alkaline pH. XR showed highest activity at 30 ^{o}C and retained 86% and 49 % at incubation temperature of 40 and 50 ^{o}C, respectively, and the enzymatic activity declined sharply beyond 50 ^{o}C incubation (Fig 3.5b). For determining the thermal stability of XR, the enzyme preparation was pre-incubated for 30 min at 4, 25, 30, 40, 50, 60, 70, 80 and 90 ^{o}C followed by determination of enzymatic activity. From the result in Fig. 3.5c it is apparent that XR showed stability in the temperature range from 4-50 ^{o}C, and the activity declined sharply at higher pre-incubation temperature. The activity of XR was increased in presence of Ca^{2+}, Mg^{2+} and Mn^{2+}, whereas the other metal ions had inhibitory effect on the enzyme (Fig. 3.5d). XR activity was reduced in large extent in the presence of SDS, EDTA and β-ME.

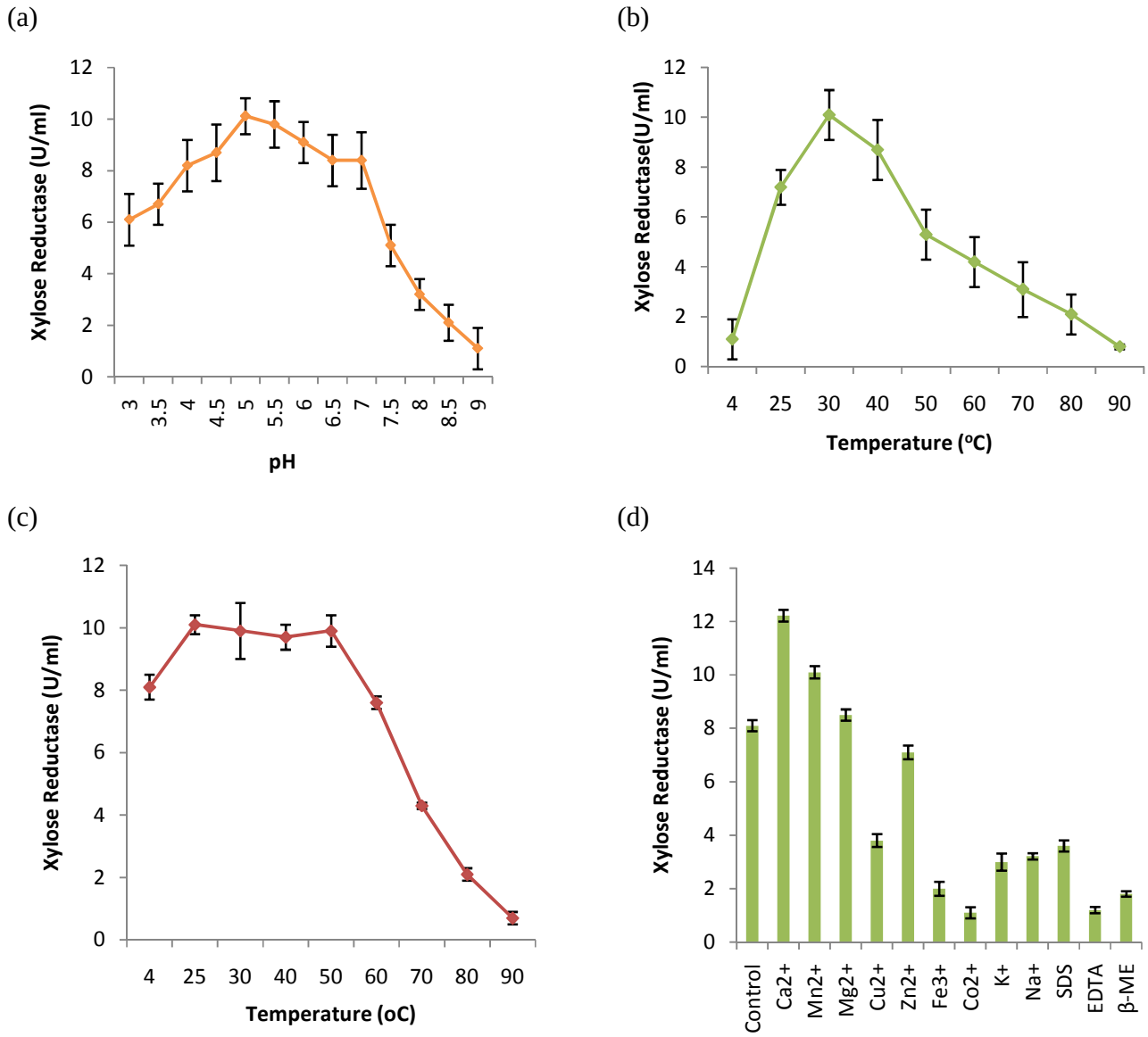


Figure 3.5. Characterization of xylose reductase from *E.coli* xy16 (a) optimum pH , (b) optimum temperature, (c) thermal stability and (d) metal ion requirement

3.3.3. XEC^{P} and XEC^{B} mediated saccharification of agro residues for xylitol production

Xylanase enzyme cocktail (XEC) obtained from *P. citrinum* xym2 (XEC^{P}) and *B. subtilis* xym4 (XEC^{B}) were used for saccharification of various acid/alkali pretreated agro residues. The results in Fig.3.6 represent the amount of reducing sugar released from various agro residues by XEC^{P} of *P. citrinum* xym2. Among the various lignocellulosic wastes, acid

pretreated wheat bran was the most effective substrate for saccharification. XEC^P dependent enzymatic hydrolysis of acid pretreated wheat bran resulted in 7.02 ± 1.2 mg/ml of reducing sugar released, which was higher than that from both untreated (0.75 ± 0.06 mg/ml) and alkali pretreated (4.6 ± 1.1 mg/ml) conditions. In case of most of the other agro residues, reducing sugar released was much higher under acid pretreated condition and was found to be in the following order; wheat bran (7.02 mg/ml) > grass (6.84 mg/ml) > saw dust (6 mg/ml) > orange peel (5.5 mg/ml) > water hyacinth (4.3 mg/ml) > corn fiber (3.8 mg/ml) > mango leaf (3.2 mg/ml) > sugarcane bagasse (2.6 mg/ml) > rice straw (2.5 mg/ml) > coconut husk (2.4 mg/ml) > petals (1.9 mg/ml).

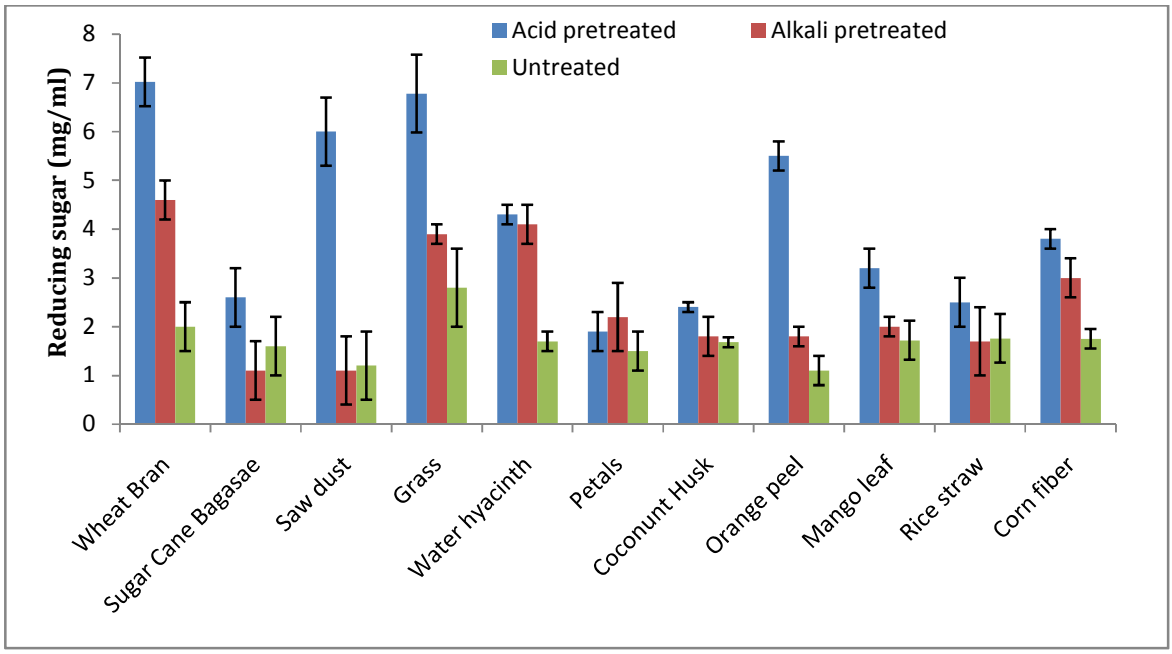


Figure 3.6. Saccharification of untreated, acid/ alkali pretreated lignocellulosic waste using XEC^P

As because the acid pretreated wheat bran showed highest level of sugar production in XEC^P mediated saccharification, the resulting saccharified extract was subjected to GC-MS analysis. The study explored the presence of various sugar, sugar derivatives and phenolic compound in the sample. The total ion chromatogram (TIC) in Figure 3.7, shows the appearance of a large peak of 2-deoxy D- glucose in 17.1 min of retention time (RT) which was followed by D-xylose (RT 17.55 min), D-glucose (RT 19.53 min), D-mannose (RT 19.77 min) and phenol 2,6 bis [1,1 dimethylethyl 4 ethyl]. Moreover the fraction bearing retention time of 10.7, 12.2 and 13.58 min indicated the presence of 3,4-di isopropoxy-3 cyclo butene dione,1-amino 2 hydroxymethyl anthraquinone and glycyl-D-aspartic acid, respectively.

Attempts were made to use the XEC^P treated saccharified product of wheat bran for production of xylitol by *E.coli* xyl6. The fermented extract yielded 3.2 g/l of xylitol and had *E.coli* viable count of 4.2 X10^4 CFU/ml. The TIC image of the fermentation extract (Figure 3.8) also revealed the presence of xylitol corresponding to the retention time of 18.83 min.

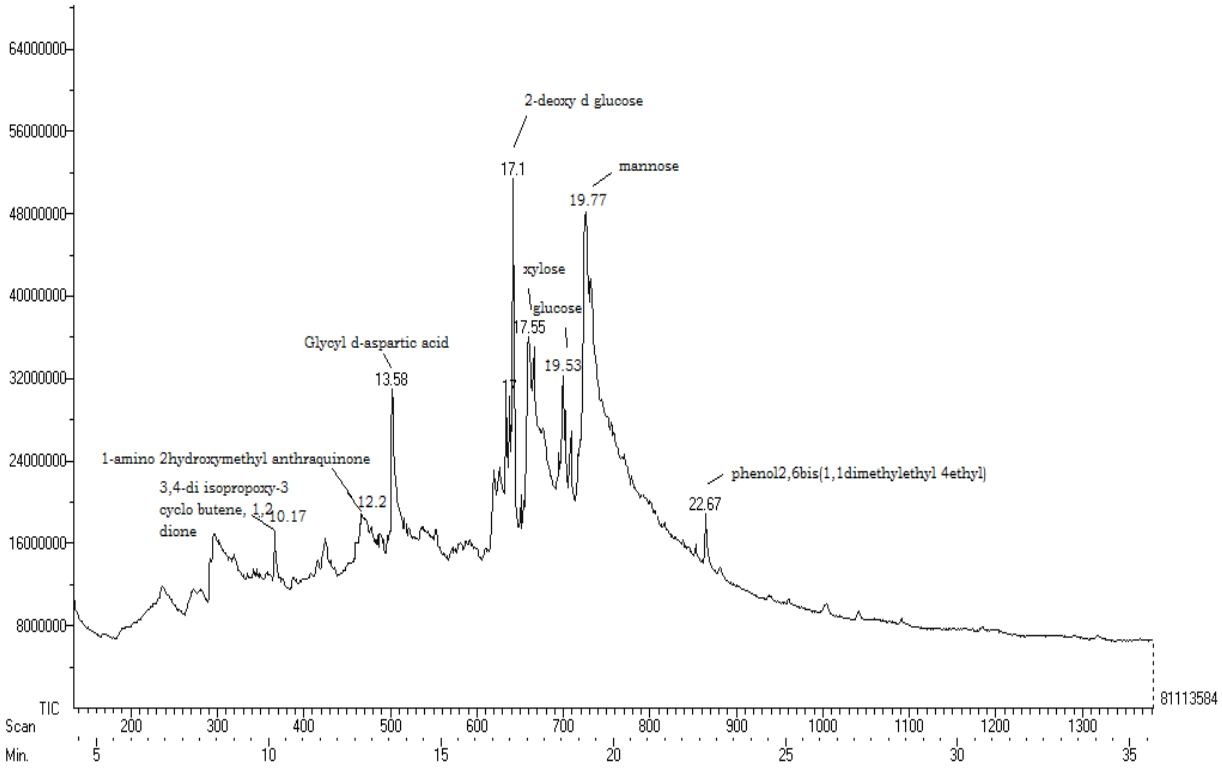


Fig 3.7. GC-MS total ion chromatogram (TIC) of supernatant obtained after saccharification of acid pretreated wheat bran using XEC^P

Comparing the TIC image of XEC^P saccharified supernatant (Figure 3.7) with that of the fermented extract (Figure 3.8) revealed that the most of the peak visible in the Figure 3.7, such as the peaks of 2-deoxy-D-Glucose, D-xylose, D-Mannose, Phenol-2,6-bis-[1,1 dimethylethyl-4-ethyl], 3,4-diisopropoxy-3-cyclobutenedione-1-amino-2-hydroxymethyl anthraquinone and glycyl D-aspartic acid were replaced by the peaks of xylitol (RT 18.83 min), mannitol (RT 17.12 min) and phenol-2,6-dimethoxy -4(2 propenyl) (RT 20.77 min) in Figure 3.8. Although the peak for glucose at retention time 19.53 was present in both the extract, its relative abundance was decreased in fermented extract. Further mass spectrometry analysis of *E.coli* fermented extract fraction with RT 18.83 min confirmed the

presence of xylitol (Fig 3.9). The production of xylitol was also confirmed from the MS spectra of standard xylitol and NIST data base (Fig. 3.10).

Acid or alkali pretreated agro residues were also subjected to saccharification by the XEC^B, obtained from *B. subtilis* xym4. As depicted in the Figure 3.11, acid pretreated wheat bran was found to liberate highest amount of reducing sugar 8.2 mg/ml as compared to the other

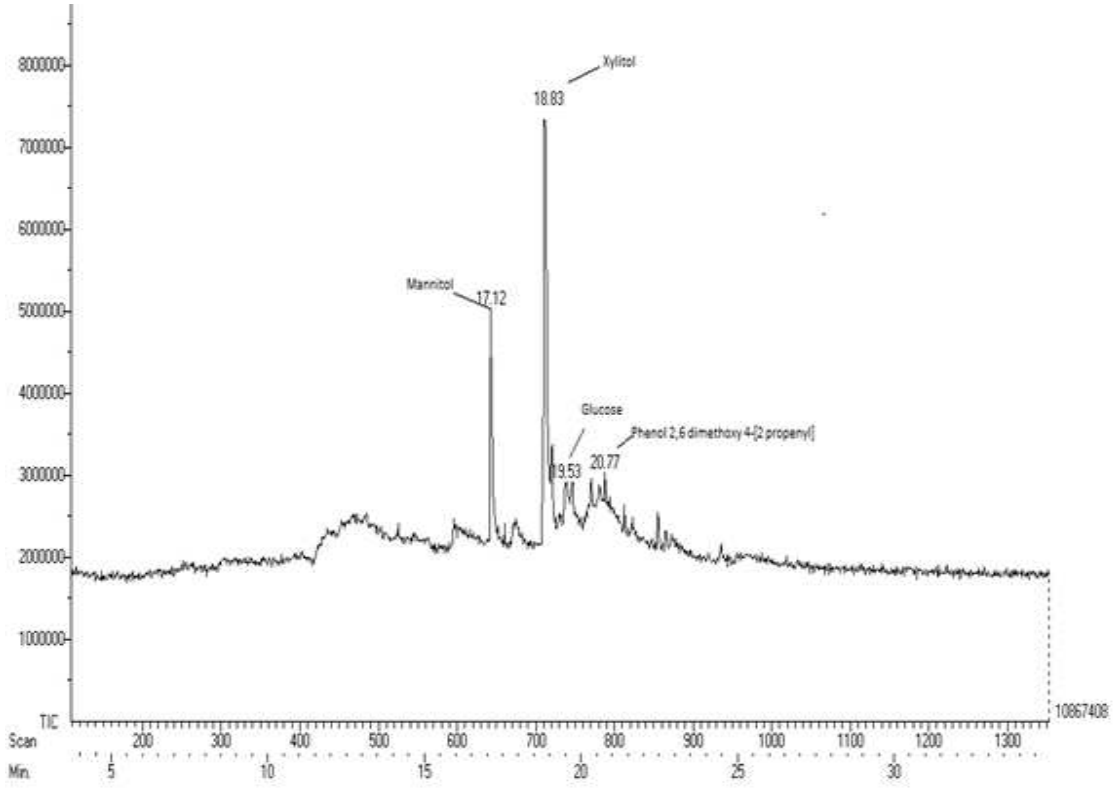


Fig 3.8. GC-MS total ion chromatogram (TIC) of XEC^P treated wheat bran supernatant fermented with *E. coli* xyl6

agro residues used in the study. Therefore, the saccharified wheat bran extract was subjected to GC-MS analysis. The result of its total ion chromatogram (TIC) (Fig. 3.12) revealed the presence of peaks of various sugar, sugar derivatives and phenolic compound, such as 1,3-pentanedione-4-methyl-1-phenyl (RT 13.55 min), 2-deoxy-D-glucose (RT 17.1 min), xylose (RT 17.75 min), glucose (RT 19.53 min), mannose (RT 19.77 min) and phenol 2,6-bis [1,1 dimethyl ethyl 4 –ethyl]. When the saccharified extract was inoculated with *E. coli* xyl6 and the fermentation was allowed to proceed at 37 ^oC for 24 h, xylitol was produced at an amount of 4.5 g/l with *E.coli* xy16 viable count of 5.1 X 10^5 CFU/ml.

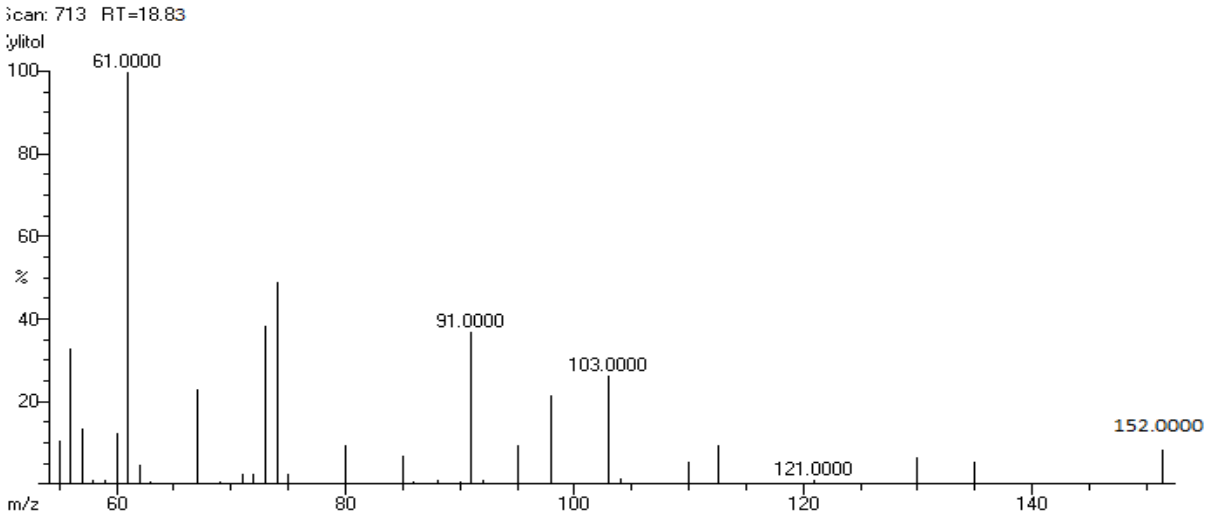


Fig. 3.9. Mass spectra of xylitol (RT 18.83 min) obtained from XEC^{P} treated wheat bran supernatant fermented with *E. coli* xyl6

A comparison of total ion chromatogram (TIC) of wheat bran supernatant before (Fig.3.12) and after (Fig.3.13) fermentation indicated the appearance of a peak of xylitol with RT of 18.88 min and a new peak of α-D-gluco pyranoside methyl with RT of 20.78 in the later. Peaks of 2-deoxy-d-glucose, xylose and mannose were greatly reduced in the fermented extract as compared to that of XEC^{B} saccharified broth, whereas the peak representing glucose in XEC^{B} sachharified extract was altogether absent in fermented extract. Mass spectra of *E. coli* fermented extract fraction with retention time 18.83 min confirmed the presence of xylitol (Fig 3.14).

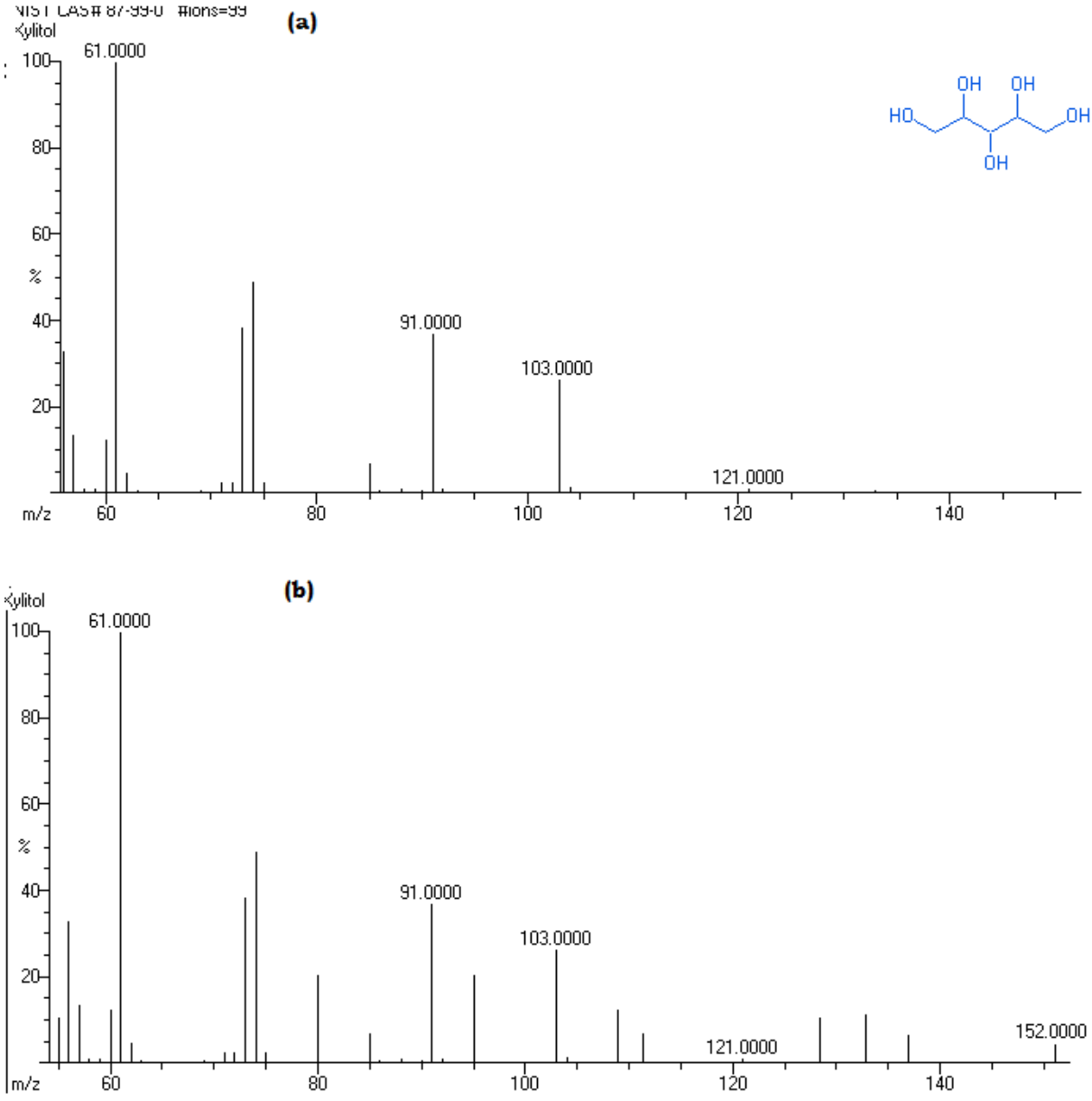


Fig.3.10. Mass spectra of xylitol obtained from NIST database (a) and standard xylitol (b)

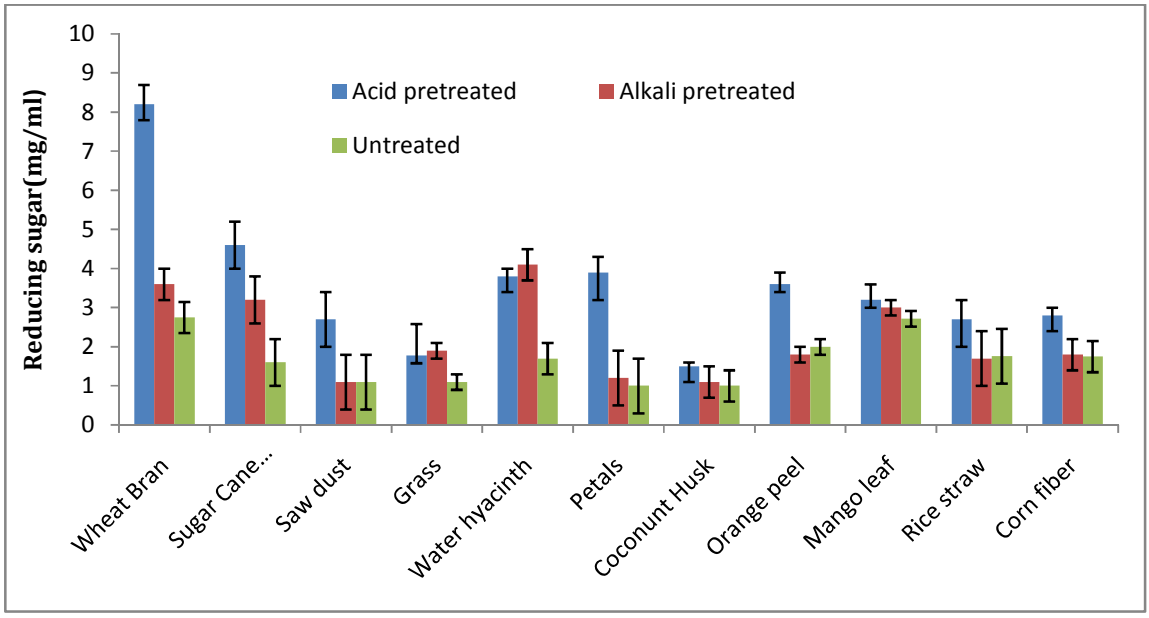


Fig 3.11. Saccharification of untreated, acid/ alkali pre-treated ligno-cellulosic waste using XEC^{B} of *B. subtilis* xym4

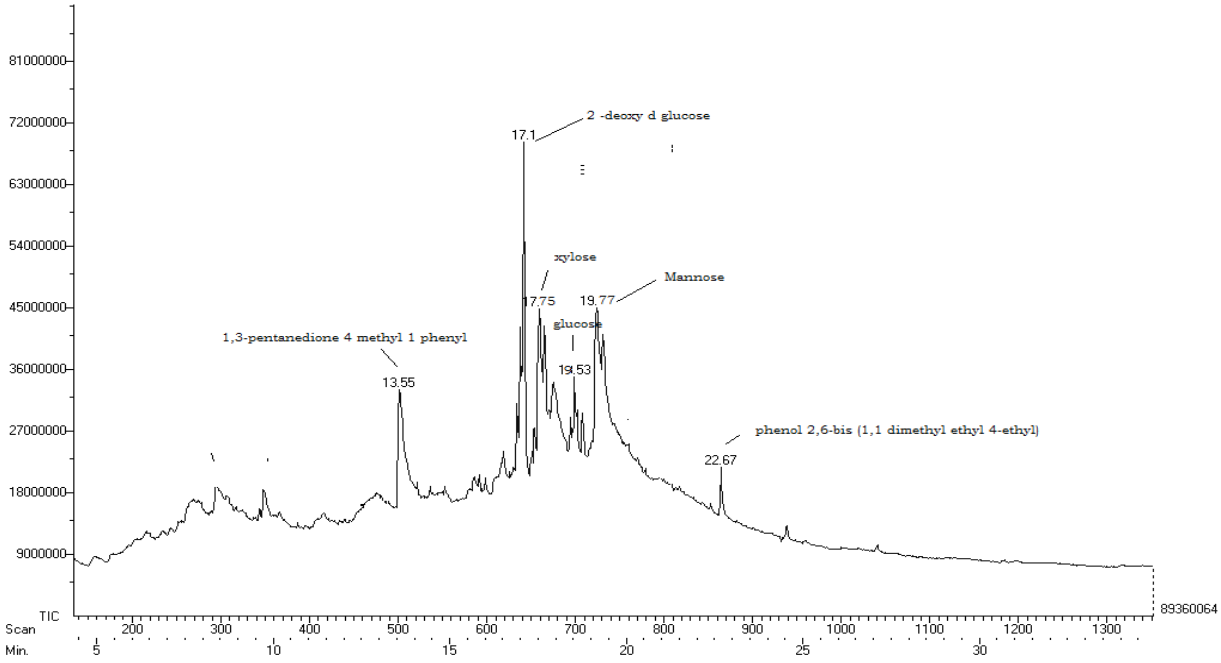


Fig 3.12. GC-MS total ion chromatogram (TIC) of supernatant obtained after saccharification of acid pretreated wheat bran using XEC^{B} of *B. subtilis* xym4.

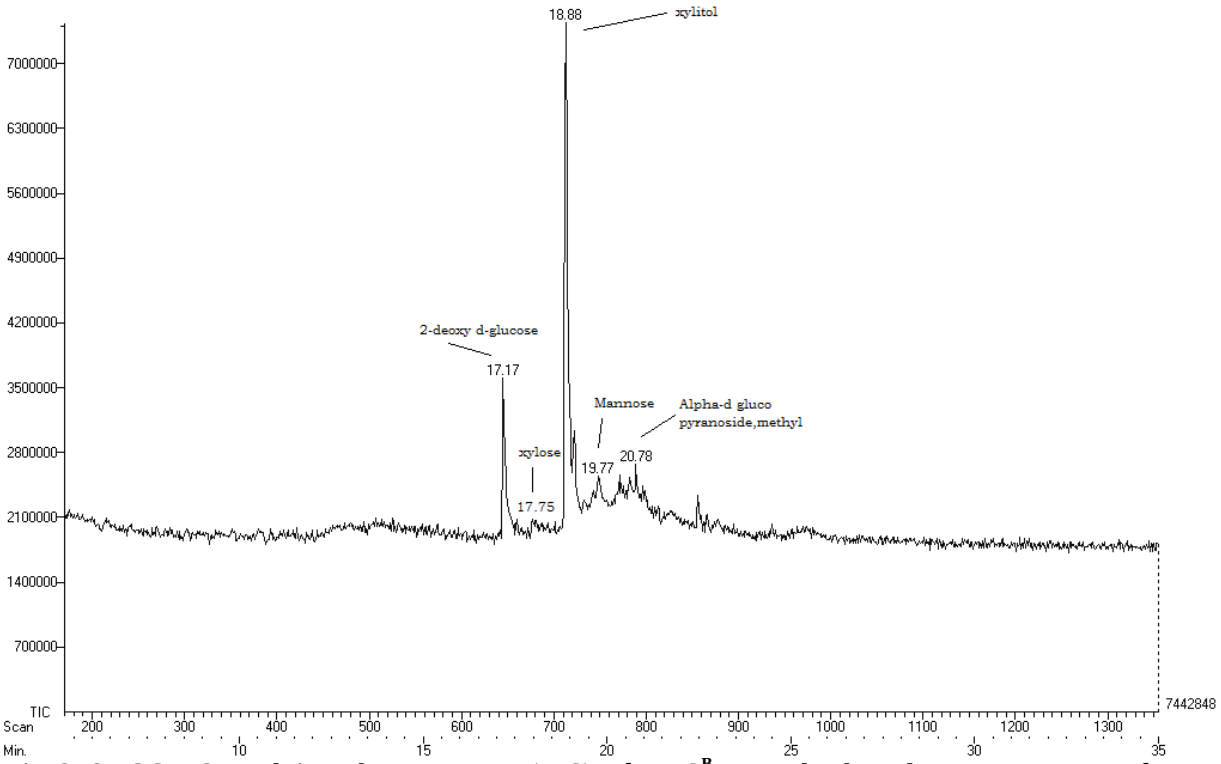


Fig 3.13. GC-MS total ion chromatogram (TIC) of XEC^{B} treated wheat bran supernatant after fermentation with *E. coli* xyl6.

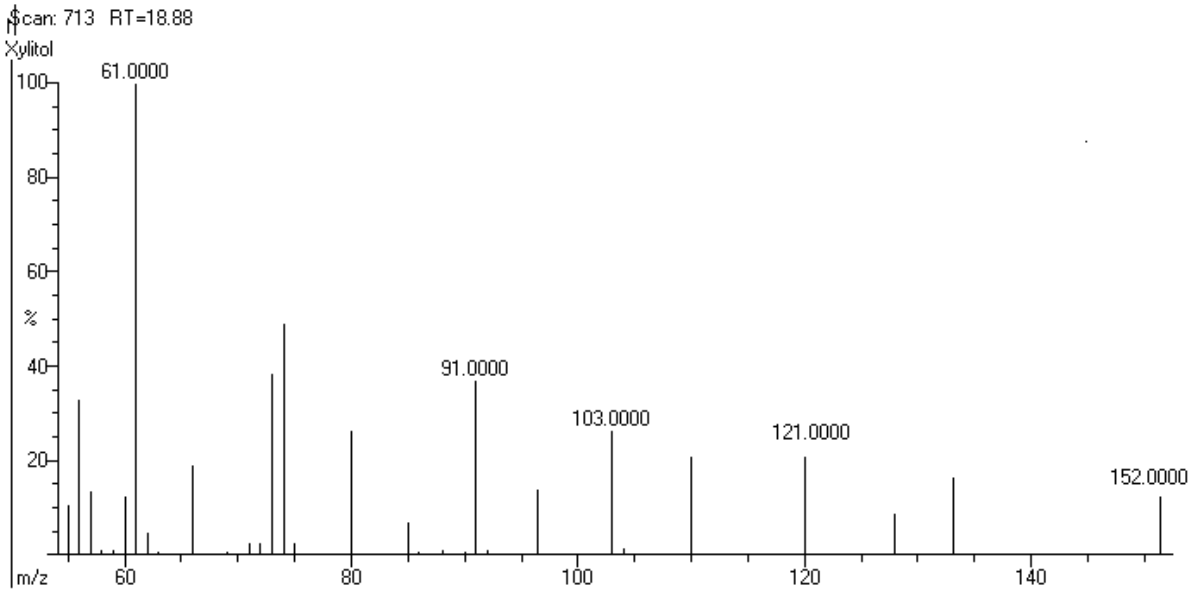


Fig.3.14. Mass spectrometry analysis of xylitol obtained from XEC^{B} treated wheat bran extract at retention time 18.83 min.

3.3.4. Sachharification of wheat bran using Placket Burman (PB) design

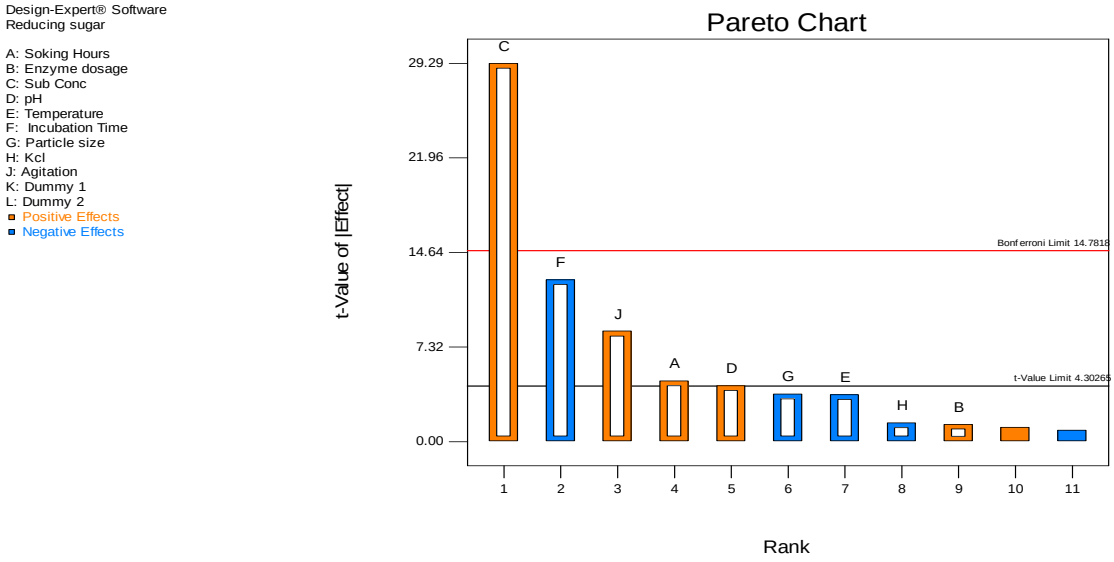
Enzymatic hydrolysis of the wheat bran for liberation of reducing sugar was conducted using PB design to determine the principal factors and their appropriate ranges in which the optima persist. The effect of different factors on the saccharification of wheat bran in terms of reducing sugar released has been represented in Table 3.4. Analysis of results through the generation of Pareto chart and variance analysis (Fig 3.15 and Table 3.4) suggest that wheat bran concentration, Incubation time, Agitation speed and Soaking time of wheat bran on 0.1 (N) HCl, were identified as the most significant and contributory factors with a range of 5-50 g/l, 3-6 h, 50-100 RPM and 8-16 h, respectively. Among these four variables only incubation time showed the negative effect which implies the high degree of sachharification at low incubation time.

Table 3.4
Plackett-Burman Design for screening important variables for xylanase production

Run	ST[a]	ED[b]	WB[c]	pH[d]	T[e]	IT[f]	PS[g]	$MgCl_2$[h]	AS[i]	XA[l]	XA[m]
1	8	1000	50	4	50	6	0.8	0.1	50	3.2±0.07	3.4
2	16	500	50	8	50	3	0.2	0.1	100	9.7±0.8	9.9
3	8	1000	5	8	50	3	0.8	0.5	100	2.3±0.06	2.39
4	16	1000	5	4	30	6	0.2	0.5	100	1.2±0.06	1.4
5	8	500	5	8	30	6	0.8	0.1	100	0.66±0.05	0.64
6	16	500	5	4	50	3	0.8	0.5	50	0.51±0.04	0.49
7	8	1000	50	8	30	3	0.2	0.5	50	8±0.06	7.98
8	16	1000	50	4	30	3	0.8	0.1	100	9.5±0.05	9.3
9	8	500	50	4	50	6	0.2	0.5	100	5.5±0.04	5.3
10	8	500	5	4	30	3	0.2	0.1	50	1.2±0.07	1.3
11	16	1000	5	8	50	6	0.2	0.1	50	0.34±0.03	0.14
12	16	500	50	8	30	6	0.8	0.5	50	5.4±0.03	5.4

[a]Soaking time (h),[b]Enzyme dosage (U/gds), [c]Wheat bran/substrate concentration (g/l),[d]pH, [e]Temperature (^oC) [f]Incubation time(h), [g]Particle size (mm),[h]$MgCl_2$ (g/l), [i]agitation speed (RPM), [l]Actual Reducing sugar(mg/ml),[m]Predicted Reducing sugar(mg/ml) ,Actual responses are presented as triplicate of mean±SD

(a)



(b)

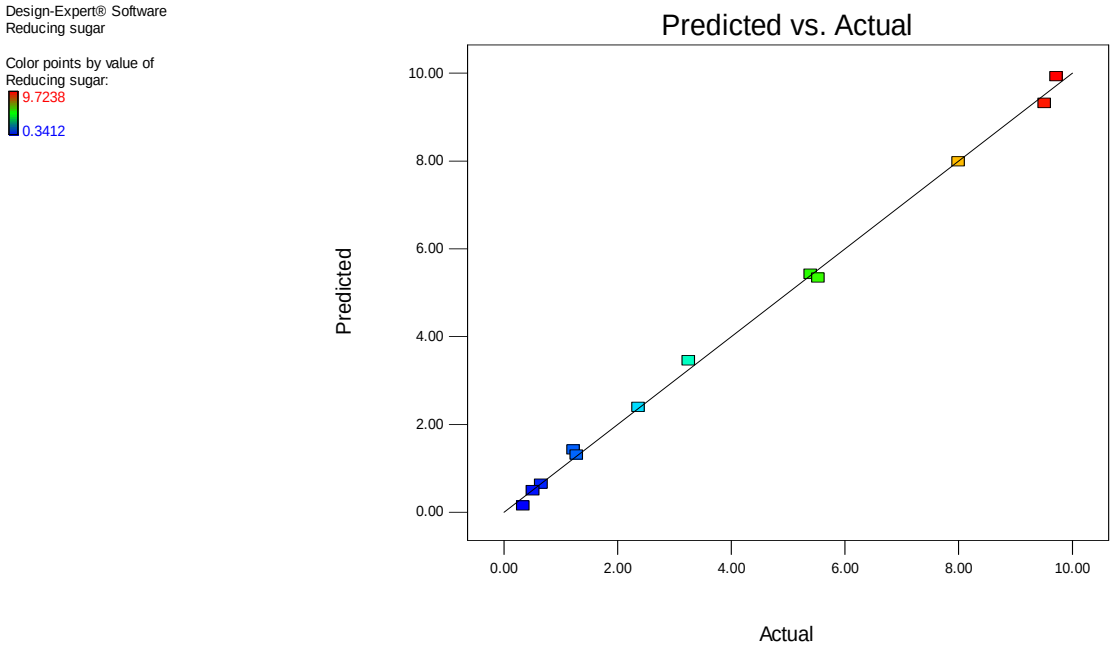


Fig 3.15. Model generated plots, (a) Pareto chart showing the positive and negative variables affecting saccharification of wheat bran by XEC^{B} of *B. subtilis* xym4 and (b) model predicted vs actual xylanase activity.

Table 3.5
Analysis of variance of the variables used in the PB design for sachharification of wheat bran using XEC[B]

Source	Sum of squares	df	F-value	p-value
Model	138.27	9	128.79	0.0077
A- Soaking time	2.63	1	22.02	*0.0425*
B-Enzyme dosage	0.21	1	1.75	0.31
C-Wheat bran	102.31	1	857.62	.0012
D-pH	2.24	1	18.75	.0494
E-Temperature	1.58	1	13.22	.0680
F-Incubation Time	18.72	1	156.95	.0063
G-Particle Size	1.62	1	13.54	.0666
H-$MgCl_2$	0.25	1	2.12	0.28
J-Agitation speed	8.72	1	73.13	0.01
Residual	0.24	2		
Cor Total	138.51	11		

R^2= 0.9983, adjusted R^2=0.9905, Predicted R^2=0.9380, Adequate Precision 31.01
p-value <0.05 implies the significance of model terms.

3.3.5. Optimization of saccharification parameters using CCRD based RSM

Four independent variables such as wheat bran (g/l), soaking time (h), agitation speed (RPM) and incubation time (h) were selected according to the result obtained in PB design. These variables were used in different level as mention in section3.2.1, whereas the composition of other variables were kept constant (enzyme dosage, 500 IU/gds; $MgCl_2$, 0.1(g/l); pH 7; particle size of wheat bran, 0.8 mm and incubation temperature 40 ^oC). The result of RSM with four variables (optimized through PB design) was presented in Table 3.6. The response variables reducing sugar released as a function of wheat bran (g/l), soaking time (h), agitation speed (RPM) and incubation time (h) was estimated using the following Eq. 6.

Reducing sugar released = +30.40 -2.21X soaking time - 2.11 X incubation time - 0.21 X agitation - 0.26X wheat bran + 0.066X soaking time X incubation time + 3.37E - 003 soaking time X agitation + 7.45 E-003 soaking time X wheat bran + 3.26 E-003 incubation time X agitation - 0.025 X incubation time X wheat bran - 4.00E - 005 agitation X wheat

bran + 0.063 X soaking time^2 + 1.04 E - 003 agitation^2 + 2.97 E - 003 wheat bran^2 (Eq 6)

Table 3.6
Central composite experimental design data for optimization of reducing sugar released from wheat bran with predicted and experimental values.

Run	Soaking time(h)	Incubation time (h)	Agitation (RPM)	Wheat bran (g/l)	Reducing sugar (mg/ml) Actual	Reducing sugar (mg/ml) predicted
1	4	4.5	75	37.5	6.1 ±1.1	5.33
2	12	4.5	75	37.5	2.43±1.2	2.42
3	8	6	50	25	8.92±1.3	9.15
4	12	4.5	75	62.5	2.57±1	2.54
5	12	1.5	75	37.5	1.12±0.09	1.08
6	16	6	100	25	10.48±1	10.35
7	8	6	50	50	5.52±0.8	5.71
8	16	3	100	25	3.44±0.9	3.26
9	16	6	50	50	7.92±0.7	7.77
10	8	3	50	50	2.24±0.6	2.62
11	16	6	100	50	8.52±0.8	8.35
12	12	4.5	75	12.5	6.28±1.07	6.05
13	8	6	100	25	8.24±1.08	8.43
14	20	4.5	75	37.5	7.2±1.03	7.71
15	16	6	50	25	9.56±0.09	9.72
16	16	3	50	50	3.28±0.3	3.09
17	12	4.5	125	37.5	4.8±0.8	4.74
18	16	3	50	25	3.24±0.7	3.11
19	12	4.5	75	37.5	2.4±1	2.43
20	12	4.5	75	37.5	2.43±0.65	2.43
21	8	3	100	25	2.52±0.64	2.93
22	16	3	100	50	3.16±0.7	3.19
23	12	4.5	75	37.5	2.43±1.6	2.43
24	12	7.5	75	37.5	11.48±0.08	11.26
25	12	4.5	75	37.5	2.44±0.09	2.43
26	12	4.5	75	37.5	2.43±0.9	2.43
27	12	4.5	25	37.5	5.57±0.07	5.36
28	8	3	100	50	1.52±0.4	1.37
29	8	6	100	50	4.56±0.6	4.94
30	8	3	50	25	3.96±0.7	4.31

Data were presented as duplicate of mean ±SD.

Analysis of varience (ANOVA) of the model data (Table 3.7), showed to have a p value <0.0001, which suggests that model could efficiently predict the response variable. Model

R^2 and predicted R^2 of 0.99 and 0.95, respectively, are in a reasonable agreement with adjusted R^2 of 0.98. Signal to noise ratio of the model was estimated by adequate precision measurement and the value greater than 4 is desirable. Our model shows an adequate precision of 41.194, indicating a good signal and less noise. Moreover, the model lack of fit was found to be non significant ($p > 0.05$). So overall the model was found to be good and could better estimate the optimum level of each of the independent factors for reducing sugar yield from wheat bran.

Table 3.7
Analysis of variance table of RSM model used to optimize reducing sugar released from wheat bran

Source	Sum of squares	df	F-value	p-value
Model	245.29	14	143.5	<0.0001
A-Soaking time	8.54	1	69.95	<0.0001
B-Incubation time	155.45	1	1272.69	<0.0001
C-Agitation	0.58	1	4.74	0.0458
D-Wheat bran	18.49	1	151.40	<0.0001
AB	2.53	1	20.70	0.0004
AC	1.82	1	14.92	0.0015
AD	2.22	1	18.18	0.0007
BC	0.24	1	1.97	0.1813
BD	3.72	1	30.50	<0.0001
CD	2.500E-003	1	0.020	0.88
A^2	28.62	1	234.33	<0.0001
B^2	23.93	1	195.91	<0.0001
C^2	11.77	1	96.37	<0.0001
D^2	5.93	1	48.52	<0.0001
Lack of fit	0.83	10	1.9	0.19

R^2 0.9926, Adj R^2 0.9857, Pred R^2 0.9573, Adeq Percision 41.194
p-value <0.05 implies the significance of model terms.

Model uses the second order regression equation to calculate the predicted response which corresponds to the values of the regressor varibles. When model predicted response (reducing sugar released) was plotted (Fig 3.16) aginst the corresponding actual response, a stright line with a slope of 45^o was obtained. This suggest the strong association between predicted and actual reducing sugar released.

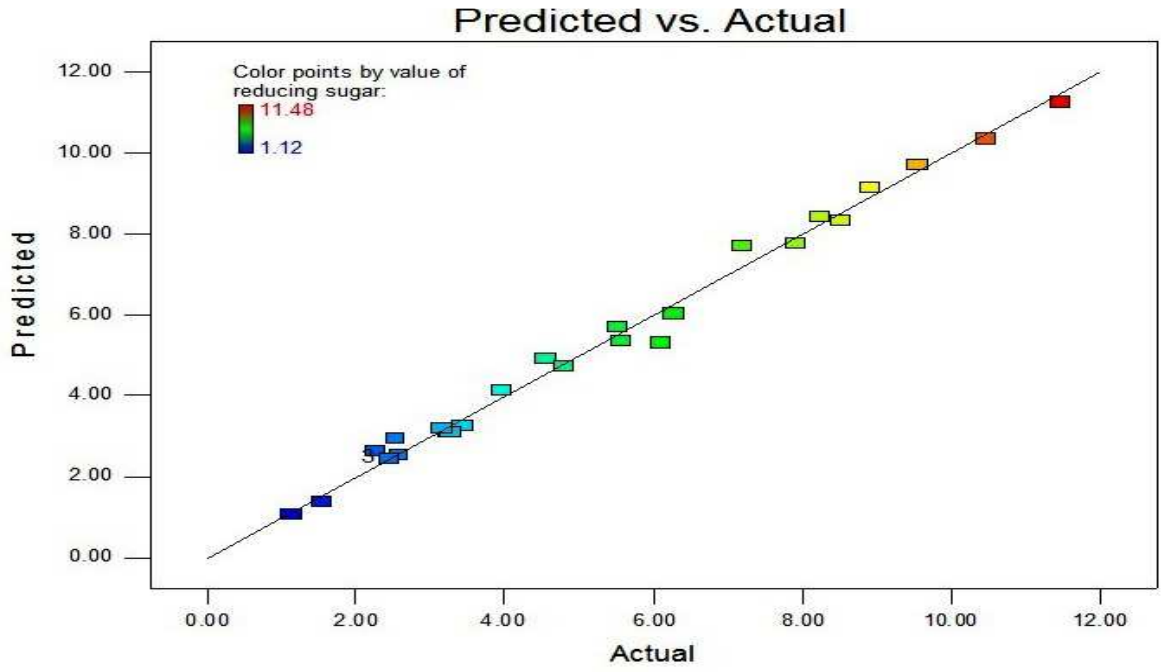


Fig.3.16. Predicted vs Actual response curve of reducing sugar relaesed by XEC^{B} from wheat bran using CCRD based RSM

The response surface plots were generated using the second order polynomial regression equation in order to find out the interaction effects between the independent variables. Intearction effect was analysed for two variables at a time using the plot keeping the other variables constant at their center point. Figure 3.17a depicts the interaction effect between soaking time and incubation time interms of contour and response surface plot. From the figure it is evident that increasing the soaking time along with the incubation time enhanced the reducing sugar released. Moreover, if the incubation time was varied keeping soaking time constant, a greater fluctuation of reducing sugar released was observed. For example if soaking time of 16 h was monitored with 3 and 6 h of incubation time, the corresponding reducing sugar released varied from 3.16 mg/ml to 9.56 mg/ml. This suggest a strong interaction effect between these two variable and was also supported by the ANOVA result in Table 3.7 with $p < 0.0004$.

Another significant interaction that existed between soaking time (A) and agitation (C), ($p < 0.0015$) has been represented by Figure 3.17b. A careful analysis of both the response surface showed that the maximum amount of sugar released was achieved when both the variables were present in their highest level. Interaction between wheat bran concentration or

substrate concentration (D) and soaking time (A) has been depicted in the Figure 3.17c. It can be seen that wheat bran at a concentration of 45 g/l soaked for 10 h resulted in lower amount of sugar released by the XEC^B, however, when the same amount of wheat bran was soaked for 16 h the higher amount of reducing sugar was obtained. Figure3.17d depicts the interaction between incubation time (B) and agitation (C). Agitation individually had moderately significant ($p=0.04$) effect on sugar released, whereas incubation time was found to be a highly significant ($p<0.0001$) independent variable greatly affecting the sachharification process. The plot suggests that the interaction effect between these two was not significant ($p=0.18$). Very strong interaction effect ($p<0.0001$) was found between wheat bran (D) and incubation time (B). As shown in the Figure 3.18a, when wheat bran concentration was kept constant at 30g/l, varying the incubation time from 3-6 h greatly influenced the response variable (reducing sugar). Maximum amount of sugar released was achived at higher incubation time. Figure 3.18b shows the response surface interaction between agitation (C) and wheat bran (D). The reducing sugar released remained almost constant in various combination of agitation speed and wheat bran concentration indicating non significant interaction and hence had a littile effect in sachharification process. Fig.3.18 c and d represent the predicted vs actual plot and model perturbation plot which showed the significant model terms.

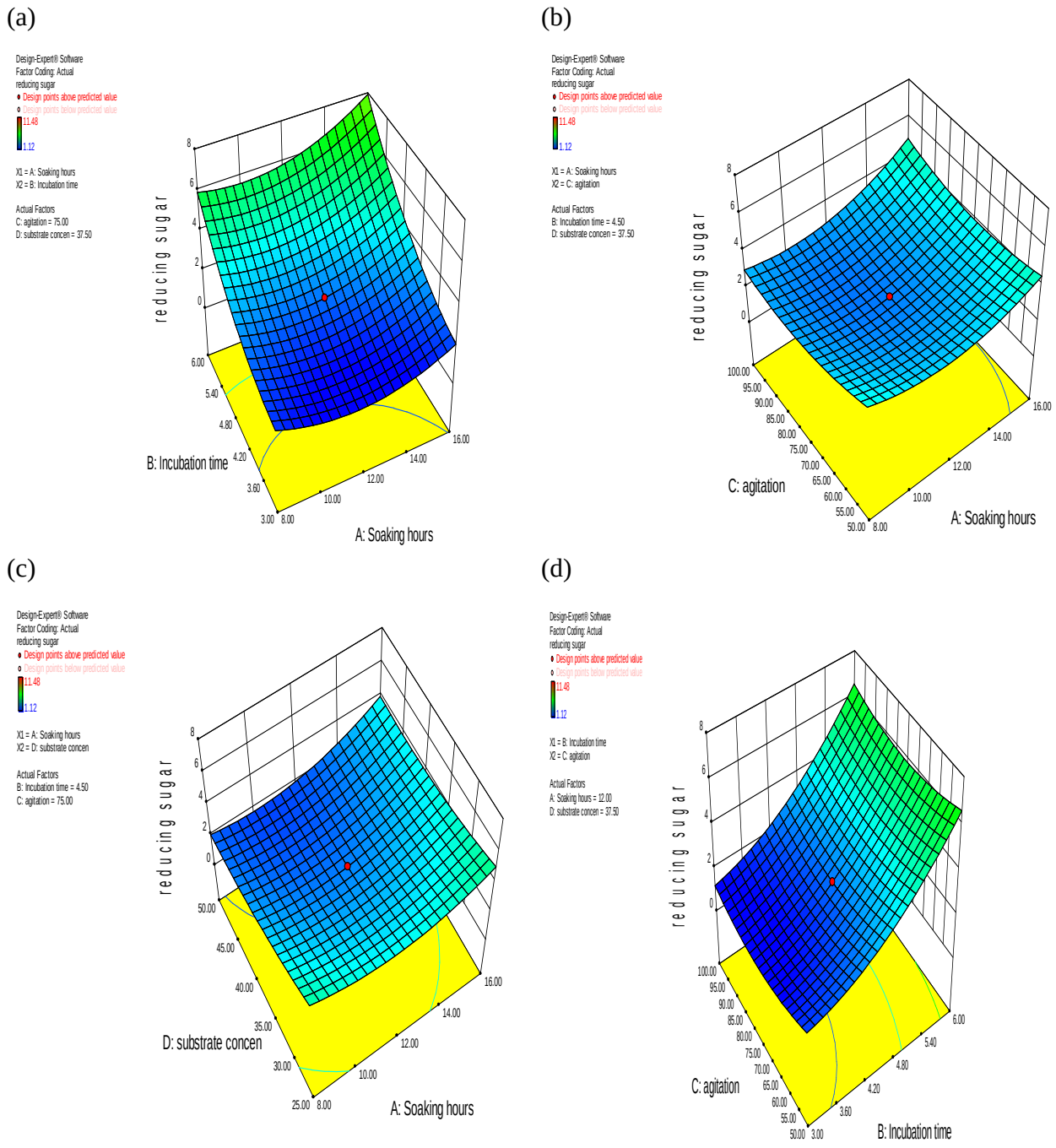


Fig 3.17. Response surface curves of reducing sugar released from wheat bran using XEC^{B},showing interaction between (a)incubation time and soaking time, (b)Agitation and soaking time, (c) substrate concentration and soaking time (d) incubation time and agitation speed. [Unit of reducing sugar was recorded in mg/ml]

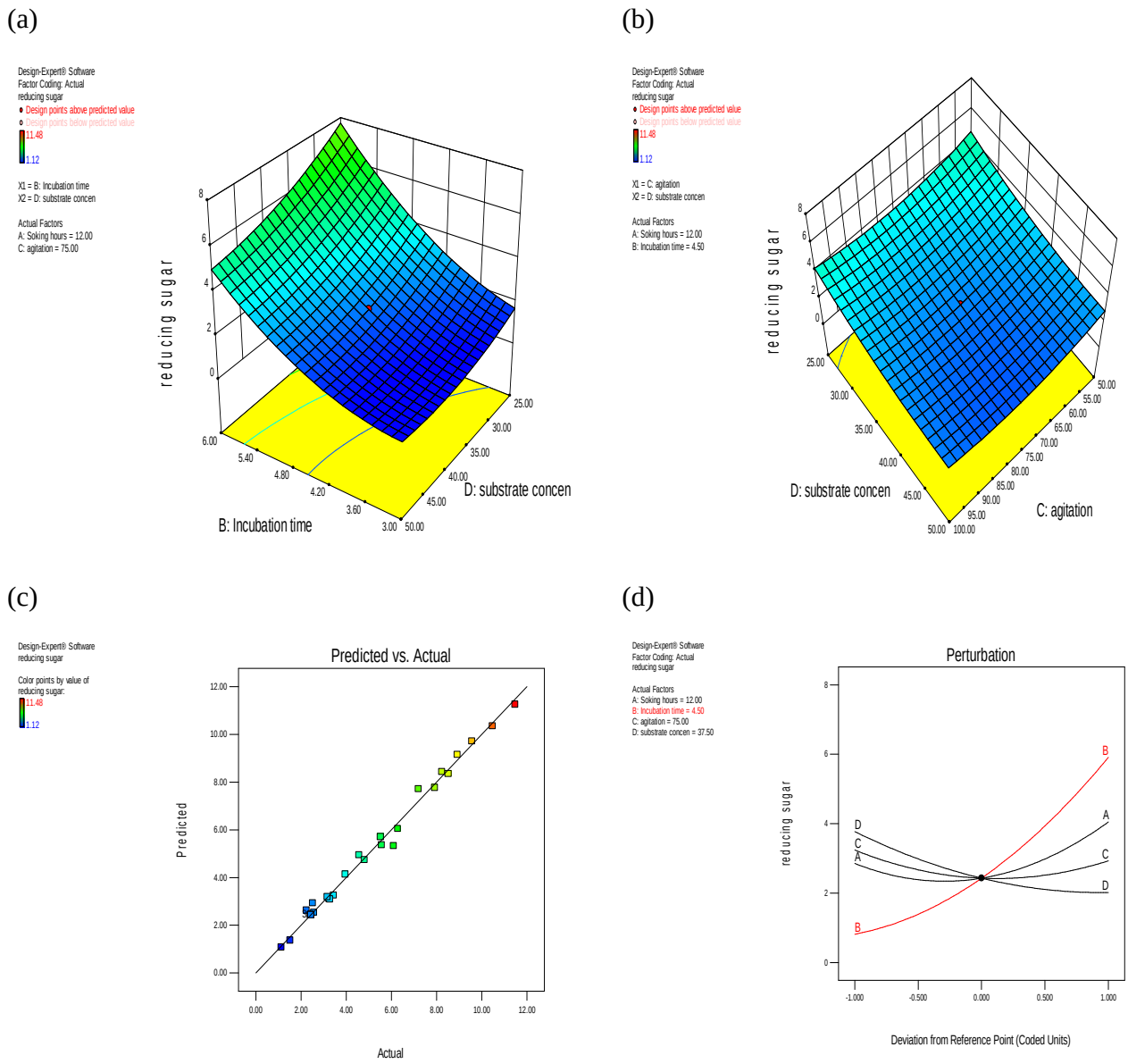


Fig 3.18. Response surface curves of reducing sugar released from wheat bran using XEC^{B},showing interaction between (a) incubation time and substrate concentration, (b) Agitation and substrate concentration. (c) model predicted vs actual sugar released and (d) Perturbation plot to identify significant model terms. (Unit of reducing sugar was recorded in mg/ml)

The model was finally employed to find out the optimum parameters for highest reducing sugar yield. Optimal levels were identified through numerical method and the result has been depicted in the Figure 3.19. Model predicted optimum parameters that led to the maximum saccharification of wheat bran were soaking time 12 h, incubation time 7.5 h, agitation 75 RPM and wheat bran 37.5 g/l. Using this RSM optimized conditions the saccharification of wheat bran resulted in the release of 11.5 mg/ml of reducing sugar which was same as the predicted response.

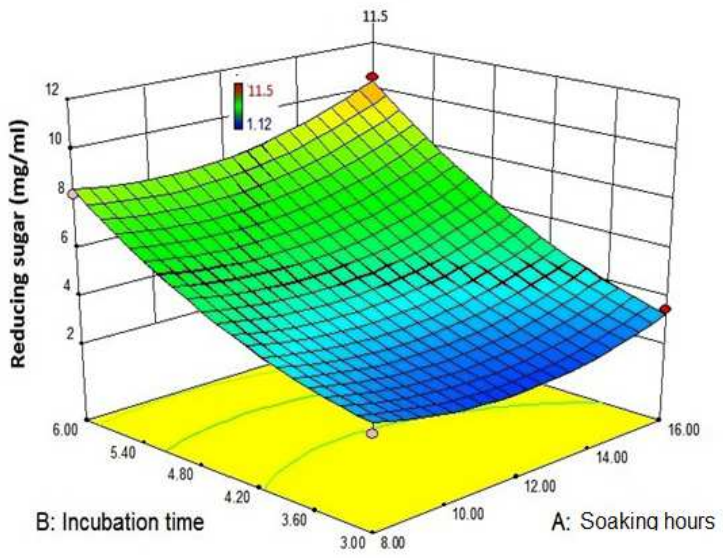


Fig 3.19. Graphical optimization of the independent variables for the sachharification of wheat bran and reducing sugar released

3.3.6. Xylitol production in sachharfied (RSM optimized) wheat bran extract

Sachharified supernatant from wheat bran under RSM optimized conditions was inoculaed with *E.coli* xyl6 and incubated for 24 h at 37 ^{o}C. Thereafter the viable bacterial cell count count and the production of xylitol were monitored, which were 8.11 g/l and 3.4X10^{5} CFU/ml, respectively. The results in Figure 3.20 depicts the total ion chromatogram (TIC) of the fermented broth obtained through GC-MS analysis. A major peak of xylitol was observed with retention time of 18.83 min. The result also shows the presence of D-glucose and 1-deoxy-d-mannitol having retention time of 19.43 and 12.93, respectively. Mass spectrometry analysis of the fraction with RT of 18.83 min, confirmed the presence of xylitol (Fig 3.21).

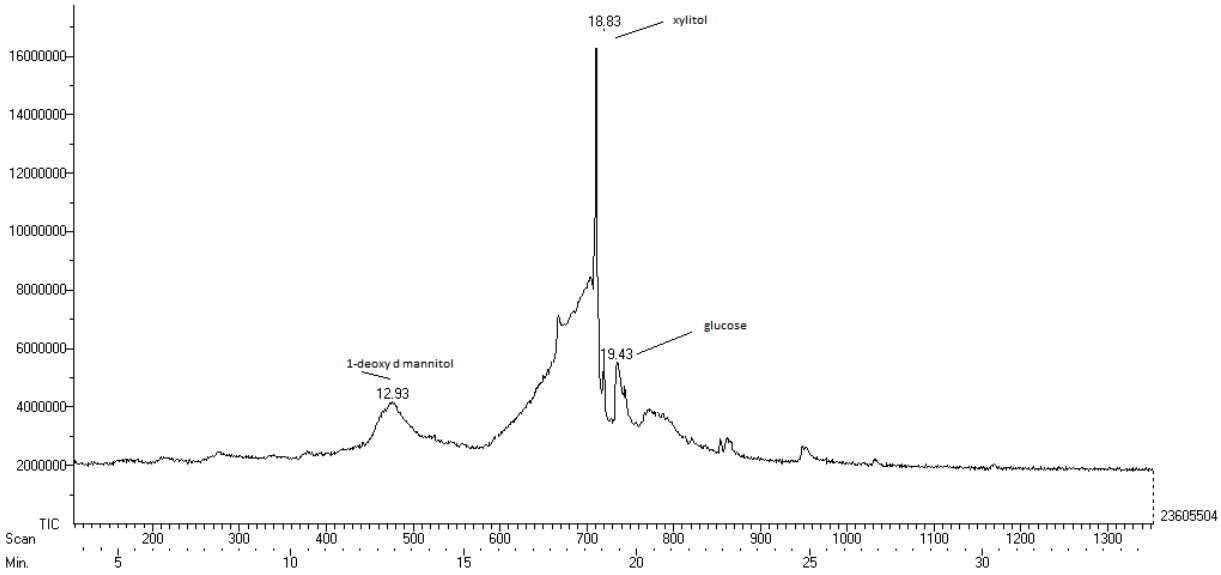


Fig 3.20.Total ion chromatogram (TIC) of GC-MS analysis of supernatant obtained after *E. coli* xyl6 mediated fermentation of XEC^{B} catalyzed and RSM optimized saccharification products.

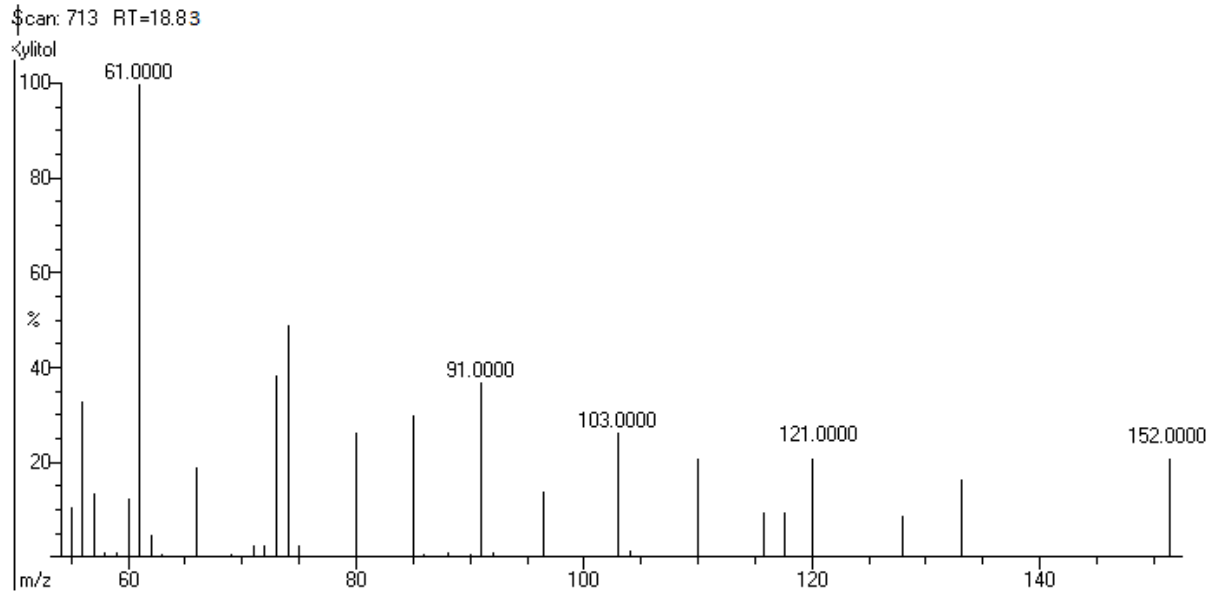


Fig 3.21. Mass spectrometric analysis of the GC-fraction with RT of 18.83 obtained after fermentation of RSM optimized sachharified suparnatent by *E.coli* xyl6.

3.3.7. Preliminary optimization of culture media for SSF

Simultaneous saccharification and fermentation (SSF^{S}) media contained 150 g/l of wheat bran so that *B. subtilis* xym4 could produce markedly high amount of xylanase for efficient liberation of reducing sugars that in turn could be used by *E. coli* xyl6 for xylitol production. *E.coli* xy16 showed very little growth in wheat bran containing SSF^{P} and hence, the growth

parameters of the bacteria was analyzed in XBM medium having the composition same as that of SSF^P except for replacing wheat bran by xylose.

The effect of inoculum concentration on viable plate count of *B. subtilis* xym4 and *E .coli* xyl6 and their respective production of xylanase and xylitol are depicted in the Figures 3.22 and 3.23. The result shows that *B. subtilis* xym4 at 1 %(*v/v*) inoculum volume yielded highest bacterial count and xylanase activity. Also, xylitol production and *E. coli* xyl6 viable count were found to be optimum at 1 % (*v/v*) of the inoculum. Xylanase production and viable count of *B. subtilis* xym4 were found to be positively correlated with a pearson correlation coefficient of 0.87, whereas xylitol production and *E. coli* growth had a coefficient of 0.76.

The results in Figure 3.24 show the effect of incubation temperature on viable plate count of both the bacteria. As can be seen optimum temperature for growth of both *B. subtilis* xym4 and *E.coli* xyl6 was 40 °C.

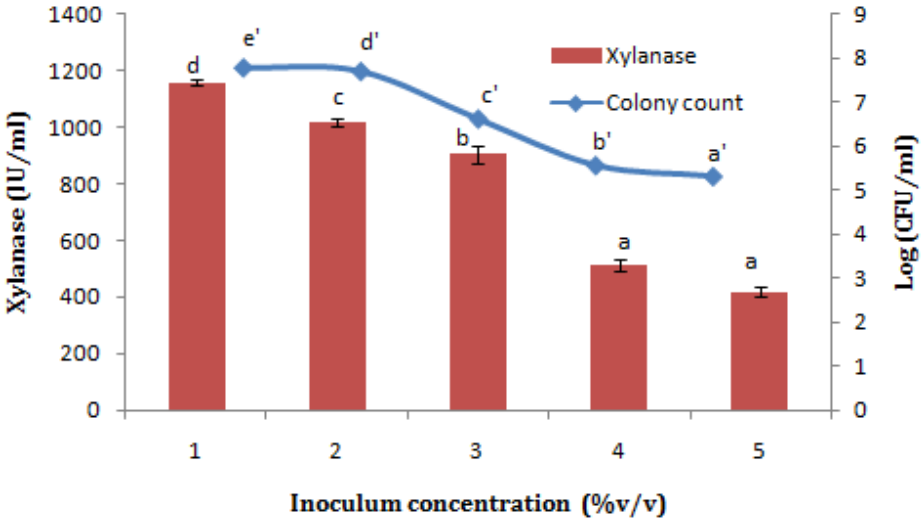


Figure 3.22. Effect of inoculum size on xylanase production and growth of *B. subtilis* xym4. Bar with different letters show significant difference at $p<0.05$ as suggested by Duncan multiple range test.

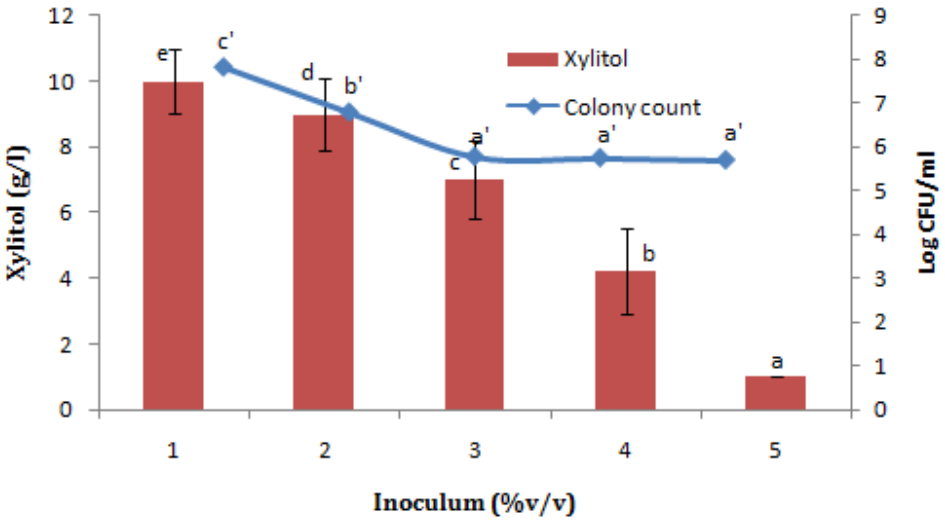


Fig.3.23. Effect of inoculum size on xylitol yield and growth of *E. coli* xyl6. Bar with different letters show significant difference at p<0.05 as suggested by Duncan multiple range test

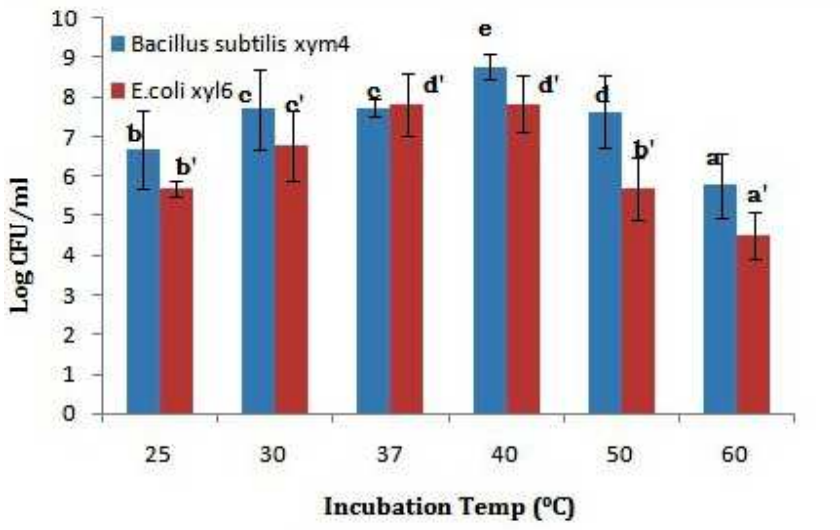


Fig. 3.24. Effect of incubation temperature on the growth of *B. subtilis* xym4 and *E. coli* xyl6. Bar with different letters show significant difference at p<0.05 as suggested by Duncan multiple range test

The effect of medium pH on viable count of *B. subtilis* xym4 and *E. coli* xyl6 was studied. The optimum amount of growth of both the bacterial isolates was achieved in medium of pH 7 (Fig.3.25).From the result obtained, it is also apparent that *B. subtilis* xym4 showed the ability to tolerate wide range of pH with significant viability count at acidic pH, whereas alkaline pH was found to support the moderate growth of *E.coli* xyl6.

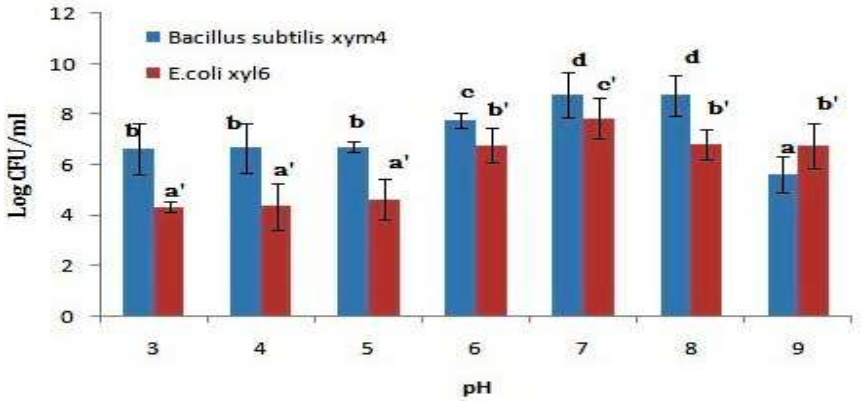


Fig 3.25. Effect of medium pH on growth of *B. subtilis* xym4 and *E. coli* xyl6. Bar with different letters show significant difference at p<0.05 as suggested by Duncan multiple range test

For determining the effect of incubation time *B. subtilis* xym4 and *E. coli* xyl6 were grown on SSF^P and XBM, respectively, and cell viability count was monitored at an interval of 24 h for 144h. Both the bacteria showed maximum growth when they were incubated for 48 h. A notable amount of growth was observed up to 72 h incubation, beyond that the growth rate started declining (Figure 3.26).

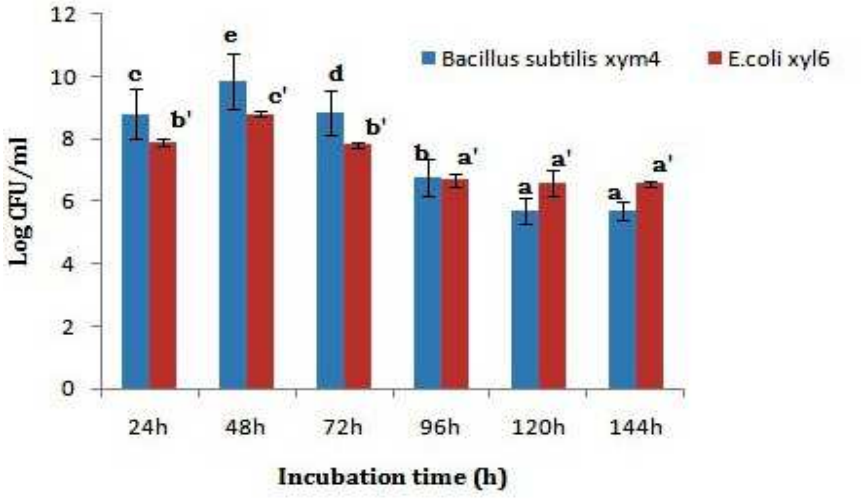


Fig. 3.26. Effect of incubation time on growth of *B. subtilis* xym4 and *E.coli* xyl6. Bar with different letters show significant difference at p<0.05 as suggested by Duncan multiple range test

The results of above preliminary optimization study used to formulate the SSF medium variables and process parameters. SSF was carried out using culture media with 10 fold higher concentration of wheat bran as compared to preliminary SSF^P medium. The media were inoculated with 1% (v/v) of both the bacteria which were incubated for up to 72 h at 40 ^oC.

3.3.8. SSF experiments:

Six different fermentation conditions in terms of inoculum addition schedule were evaluated for SSF of wheat bran with xylanase producing *B. subtils* xym4 and *E. coli* xyl6. The results are represented in the Table 3.8. Highest amount of xylitol (98.4±1.08 g/l) was obtained with fermentation condition 4, where *E.coli* xyl6 was added into the fermentation broth 24 h after the addition of *B. subtilis* xym4. After 24 h of incubation significantly higher growth of *Bacillus* (6.7 x 10^8 CFU/ml) led to production of high amount of xylanase (4531 IU/ml) with release of 2.8 g/l of reducing sugar in the medium that in turn supported increased growth of *E.coli* xyl6 (5.8 x 10^6 CFU/ml). As the medium pH and DO were decreased from 8 to 4.5 and 63 % to 42 %, respectively, the activity of xylose reductase increased to 25.34 IU/ml, with concomitant accumulation of 98.4 g/l of xylitol. The viable cell count of *E. coli* xy16 was reduced on further incubation with subsequent reduction in xylitol production. GC-MS (TIC) analysis (Figure 3.27) of the fermented broth under condition 4 revealed the presence of xylitol (RT = 18.83 min), which was also confirmed by its mass spectra (Figure 3.28). The chromatogram also showed the presence of other peaks of 2 deoxy d- glucose (RT 17.12 min), D-xylose (RT 17.75 min) and D-mannose (RT = 19.48 min).

Table 3.8
SSF of wheat bran using *B.subtilis* xym4 and *E.coli* xyl6 for xylitol production

Fermentation conditions		Viable count (CFU/ml)		pH	DO (%)	Xylanase (IU/ml)	Total reducing sugar (g/l)	Xylose reductase (U/ml)	Total protein (mg/ml)	Xylitol (g/l)
	Total incubation time (h)	*B. subtilis* xym4	*E. coli* xyl6							
Condition1	24 h	$6.8X10^{8}$± 1.2	0	8± 0.04	63± 10.2	4250± 21.8	2.7± 0.05	0	1.5± 0.08	0
	48 h	$7.2X10^{9}$± 1.4	0	7.5± 0.06	52± 4.2	6851± 12.9	3.2± 1.03	0	2.1± 0.05	0
	72 h	$6.6X10^{8}$± 1.3	0	6.5± 0.08	57± 4.1	3122± 13.7	3.9± 0.09	0	1.9± 0.04	0
Condition 2	24 h	0	$1.7X10^{5}$± 0.2	6.5± 0.12	55± 4.7	0	0.4	2.3± 0.05	1.1± 0.08	1.2 ± 0.1
	48 h	0	$1.2X10^{6}$± 0.1	7± 0.43	48± 3.2	0	0.3	4.2± 0.15	1.2± 0.19	1.6 ± 0.8
	72 h	0	$2.5X10^{4}$± 0.7	7.2± 0.76	32± 2.3	0	0.2	3.3± 0.23	0.8± 0.04	1.3 ± 0.08
Condition3	24 h	$2.8X10^{6}$± 0.2	$4.2X10^{5}$± 0.7	6± 0.56	51± 8.9	3218± 26.7	0.9± 0.05	9± 0.44	3.2± 0.03	22 ± 0.07
	48 h	$15.2X10^{5}$± 0.6	$1.2X10^{6}$± 0.8	5.5± 0.32	42± 4.6	2176± 32.1	1.2± 0.08	11.12± 0.34	3.7± 0.07	35± 1.09
	72 h	$18X10^{4}$± 1	$3.8X10^{5}$± 0.8	4.5± 0.16	30± 6.9	1143± 34.5	1.5± 0.07	13.43± 0.78	2.8± 0.04	47± 1.06
Condition 4	24 h	$6.7X10^{8}$± 1.2	0	8± 0.32	63± 4.1	4531± 43.1	2.8± 0.04	0	1.6± 0.08	0
	48 h	$4.8X10^{5}$± 0.8	$5.8X10^{6}$± 0.4	4.5± 0.17	42± 4.4	3532± 12.8	0.8± 0.03	25.34± 0.23	3.4± 0.04	98.4± 1.08
	72 h	$5.9X10^{4}$± 0.5	$4.7X10^{5}$± 0.6	6± 0.32	21± 1.5	1800± 23.9	0.6± 0.02	22.22± 0.56	2.8± 0.07	88.4± 0.04
Condition 5	24 h	$6.9X10^{8}$± 1.5	0	8± 0.12	66± 3.7	4122± 11.5	2.8± 0.06	0	1.7± 0.03	0
	48 h	$7.8X10^{9}$± 1.7	0	7.5± 0.23	52± 6.5	6120± 17.6	3.5± 0.04	0	2.1± 0.34	0
	72 h	$4.7X10^{6}$± 1.8	$3.1X10^{5}$± 0.6	5± 0.12	40± 3.7	3246± 16.4	2.3± 0.16	9.8± 0.21	1.1± 0.67	26.4± 2.1
Condition 6	24 h	0	$1.6X10^{5}$± 0.9	6± 0.32	56± 2.7	0	0	2.5± 0.50	1.2± 0.68	1.3 ± 0.1
	48 h	$2.4X10^{5}$± 1.1	$6.6X10^{6}$± 1.1	5.5± 0.21	42± 2.4	2176± 15.3	1.1± 0.45	10.21± 1.09	1.8± 0.45	26.6± 2.1
	72 h	$1.2X10^{4}$± 2.6	$3.2X10^{4}$± 0.8	6± 0.32	28± 1.9	1543± 16.7	1± 0.08	7.4± 0.07	0.6± 0.21	10.4± 1.6

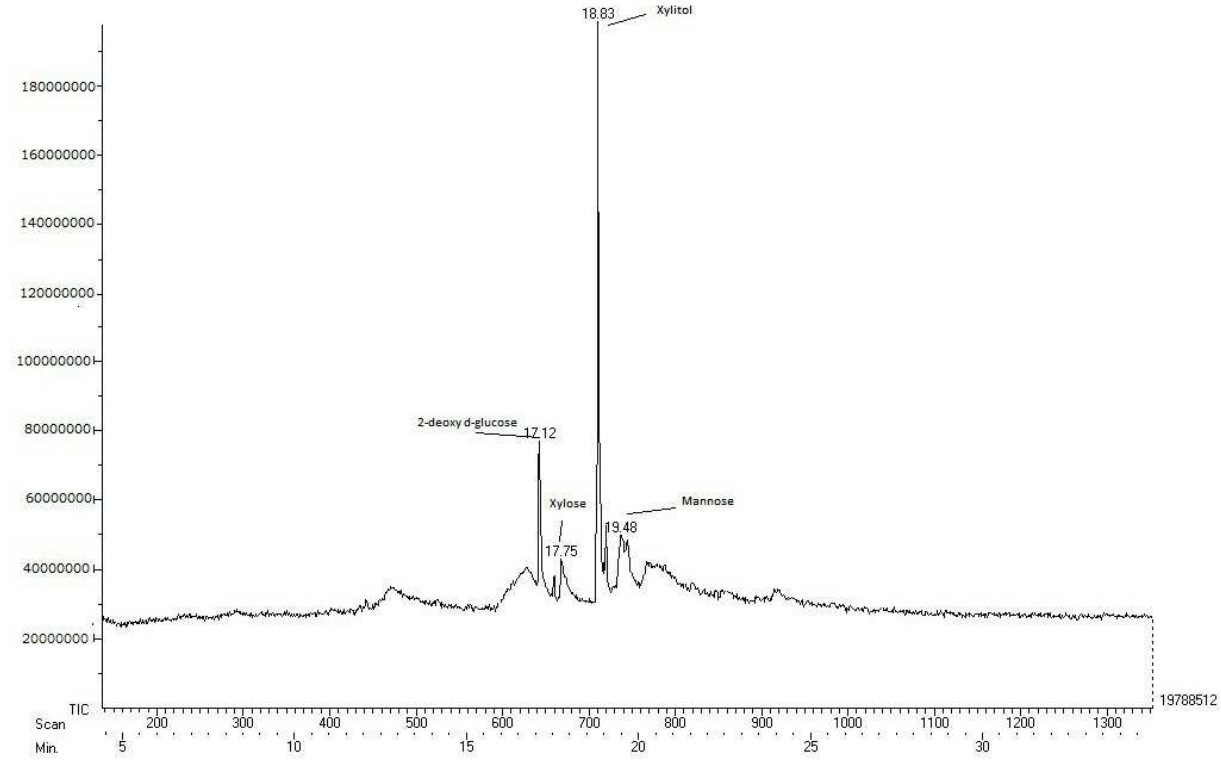


Fig.3.27. GC-MS (TIC) analysis of fermented broth obtained from SSF under condition 4.

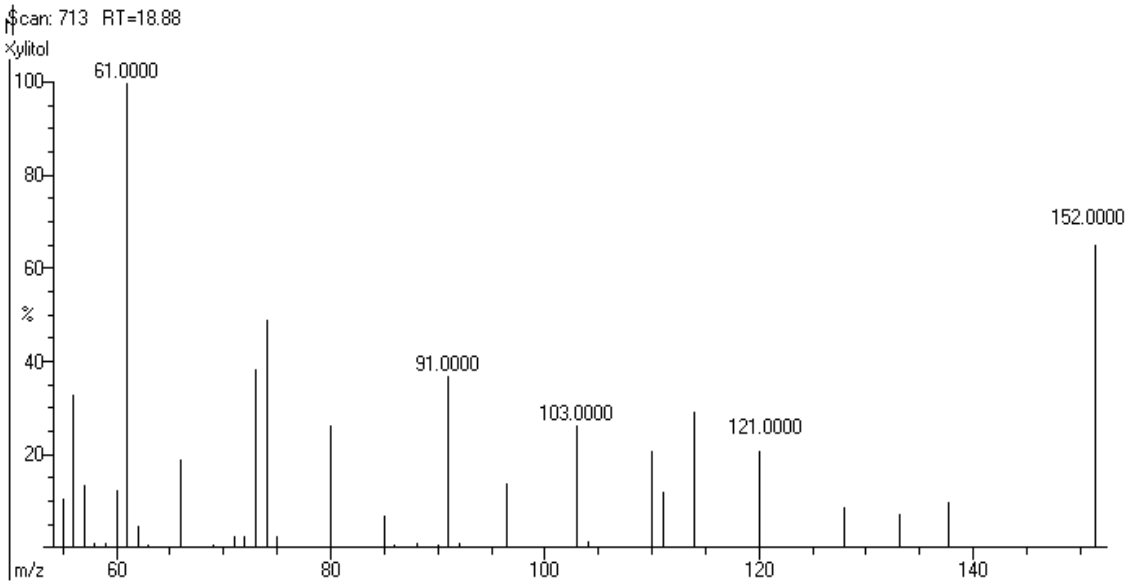


Fig. 3.28. Mass spectra of xylitol present in GC-fraction with RT=18.83 min.

3.3.9. Principal component analysis (PCA) and correlation analysis of SSF data

Factors affecting the xylitol production in SSF experiments were identified according to their relative importance by Principal component analysis (PCA) using statistical tool, SPSS. Combined approach such as eigen values, scree plot and variance explained criterion of 55%, were considered for identification of influential fermentation factors. Three principal components (PCs) PC1, PC2 and PC3 which explain 44.4%, 20.8% and 13.16% variability, respectively, were extracted. The major contributory factors to PC1 (eigen value 4.8) were xylose reductase (0.946), pH (-0.87), DO (-.0.776), reducing sugar (-0.60), xylanase (-0.58), *E. coli* count (0.62) and *B. subtilis* count (-0.51). Xylitol produced after co-fermentation contributed to PC1 with a correlation coefficient of 0.887. The second component PC2 (eigen value 2.28) represented xylanase (0.77) and protein (0.76), whereas the third component PC3 (eigen value 1.4) represent fermentation time (0.84) and DO (-0.54). The results of PCA loading plots (Figure 3.29, 3.30 & 3.31) suggest that the xylitol production had significant positive correlation with the attributes of PC1 as reflected by the formation of tight cluster with xylose reductase (XR), *E. coli* count and protein in the loading plane of p1 vs p2 plot. Whereas xylitol formed cluster with xylose reductase (XR), *E. coli* count, protein and DO in the loading plane of p2 vs p3 plot. In p1 vs p3 loading plot, xylitol formed aggregate with xylose reductase (XR), *E.coli* count, protein concentration and fermentation conditions. Thus PCA analysis was successfully exploited for the extraction of most influential factors affecting xylitol production in different conditions of SSF according to their ascending order of correlation coefficients, which are as follows (Figure 3.32): Fermentation conditions (0.98) > XR(0.96) > xylanase (0.95) > Fermentation time (0.94) > DO(0.92) > Reducing sugar (0.83) > protein (0.82) > pH(0.76) > *E.coli* (0.75) > *B. subtilis* (0.70). Interaction effects among the variables were also evaluated in terms of correlation coefficient (Table 3.9) and it was found that XR interaction with xylitol production was much higher.

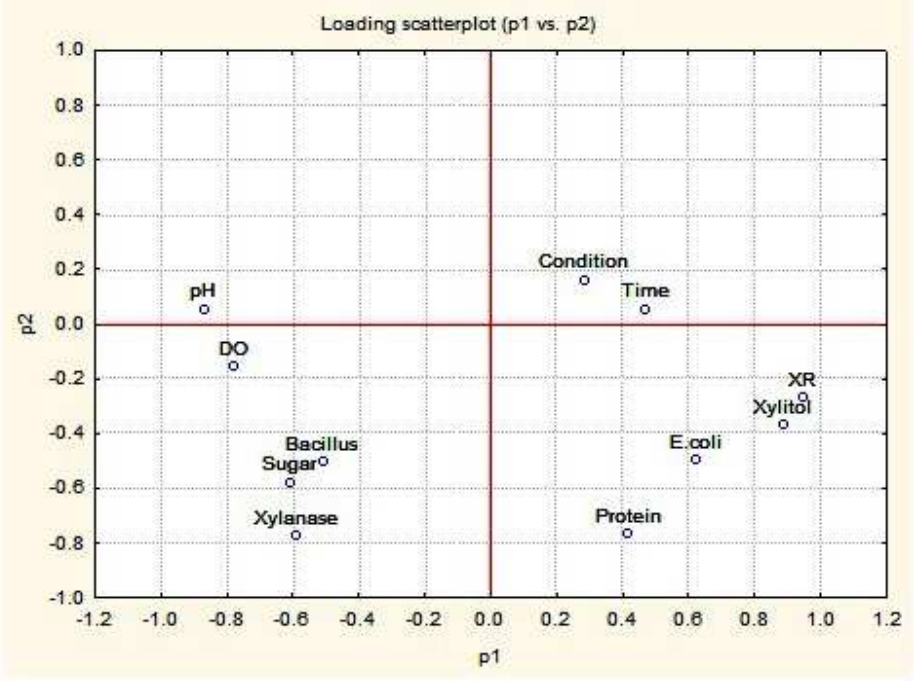


Fig. 3.29. Principal component analysis (PCA) loading plot of p1 vs p2 for the factors affecting xylitol production in SSF.

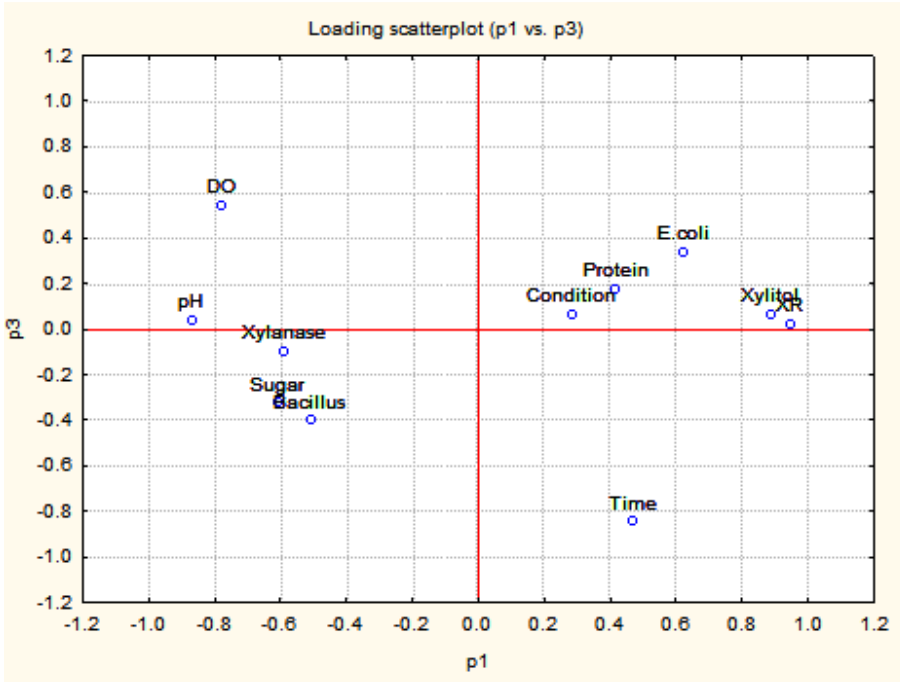


Fig 3.30. Principal component analysis (PCA) loading plot of p1 vs p3 for the factors affecting xylitol production in SSF.

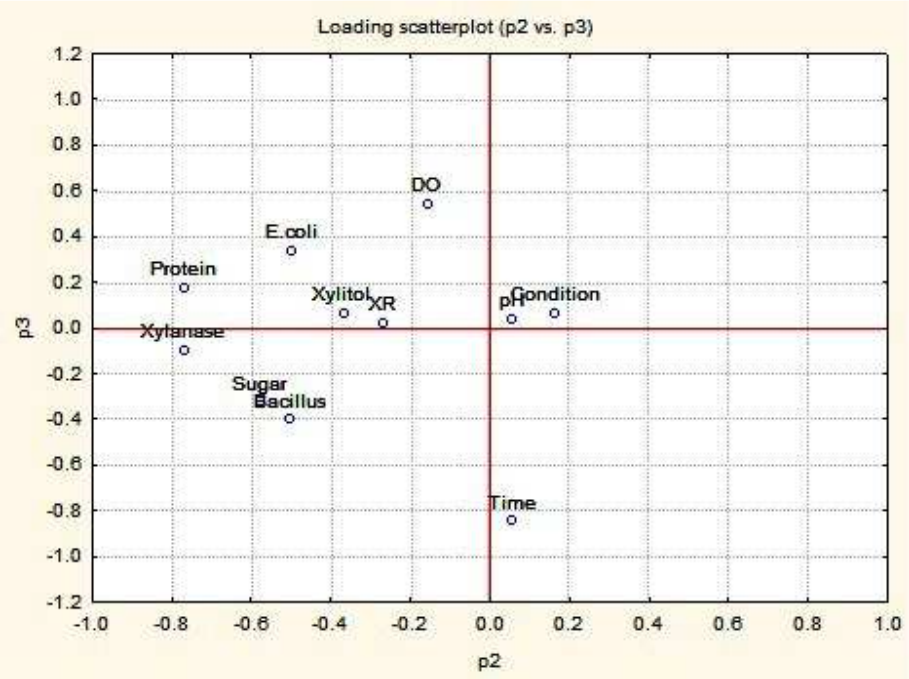


Fig.3.31. Principal component analysis (PCA) loading plot of p2 vs p3 for the factors affecting xylitol production in SSF.

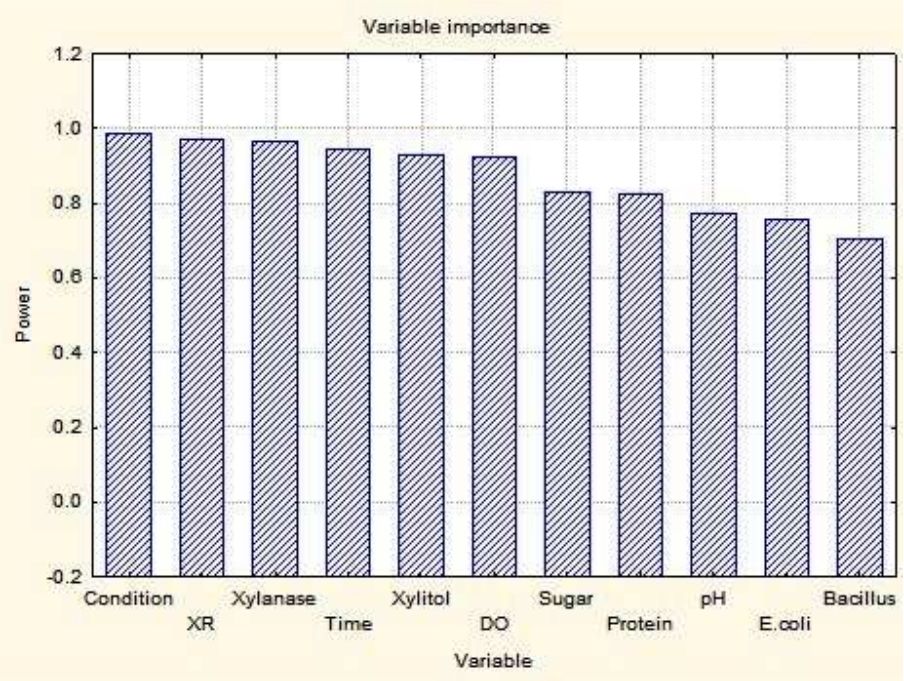


Fig 3.32. PCA extracted fermentation factors represented according to their influence for xylitol production.

Table 3.9
Pearson correlation analysis of SSF variables.

	Time	***B. subtilis***	***E. coli***	**pH**	**DO**	**Xylanase**	**Sugar**	**XR**	**Protein**	**Xylitol**
Time	1.00	-0.02	0.03	-0.4	**-0.78**	-0.25	0.005	0.38	-0.02	0.31
B. subtilis	-0.02	1.00	-0.16	0.4	0.21	**0.68**	**0.56**	-0.36	-0.05	-0.29
E. coli	0.03	-0.16	1.00	**-0.53**	-0.18	-0.03	-0.19	**0.71**	**0.54**	**0.74**
pH	-0.44	-0.40	**0.53**	1.00	**0.61**	**0.48**	0.40	**-0.78**	-0.41	**-0.70**
DO	**-0.78**	0.21	-0.181	**0.61**	1.0	**0.50**	0.46	**-0.69**	-0.14	**-0.60**
Xylanase	0.253	**0.689**	-0.03	**0.48**	**0.50**	1.00	**0.874**	-0.33	0.30	-0.24
Sugar	0.01	**0.56**	-0.19	0.40	0.46	**0.87**	1.00	-0.43	0.06	-0.34
XR	0.38	-0.36	**0.71**	**-0.78**	**-0.69**	-0.33	-0.43	1.00	**0.58**	**0.97**
Protein	-0.02	0.05	**0.54**	-0.4	-0.14	0.30	0.06	**0.58**	1.00	**0.63**
Xylitol	0.31	-0.29	**0.74**	**-0.7**	**-0.6**	-0.24	-0.34	**0.97**	**0.63**	1.00

Bold numerics suggest that the correlation coefficients are significant at the 0.05 level.

3.4. Discussion:

Xylitol ($C_5H_{12}O_5$), the sugar alcohol, is a derivative of xylose and possesses a high sweetening power. Despite sweetening activity it generates 40% less calories than sucrose (Albuquerque *et al.* 2015). Due to organoleptic characteristic and health promoting effects such as low glycemic index (Islam 2011), high degree of solubility in water (Khalid *et al.* 2012), non mutagenic/carcinogenic traits (Uittamo *et al.* 2011) and cariostatic activities (Ritter *et al*. 2013), it has been widely cited in numerous scientific journals. Industrial production of xylitol is mainly achieved by chemical methods where purified D-xylose is treated with high temperature (80-140 ^{o}C) and pressure (>50 atm) until and unless it gets converted to polyol (Chen *et al.* 2010). The operating conditions and complexity in purification process make xylitol an expensive product (Albuquerque *et al.* 2015). In order to reduce the production cost agro residues such as bamboo (Miura *et al.* 2013), vegetable wastes (Zhang *et al.* 2012), sunflower stalks (Martínez *et al.* 2012) etc. are being utilized as a renewable source of biomass for xylitol production. The agro residues on chemical, physical and enzymatic treatments produce xylose, which can be further reduced to xylitol by microbial activity (Prakash *et al.* 2011). In the present investigation, a xylitol producing microorganism with xylose reductase activity was isolated from soil. Morphological and biochemical characterization studies and phylogenetic analysis of 16S rRNA gene sequence

identified the organism as *E. coli* and hence, named as *E. coli* xyl6. As in our study, another member of family enterobacteriaceae, *Enterobacter liquefaciens*, has been reported to produce xylitol (Ghindae *et al.* 2010). In a previous research, Rangaswamy and Agblevor (2002) isolated 17 numbers of xylitol producing bacterial strains which belonged to the genera of *Serratia, Cellulomonas* and *Corynebacterium*. Among these strains *Corynebacterium* sp. B-4247 produced 10.05 g/l of xylitol. Several other genetically modified organisms have also been used for xylitol production studies. Genetically modified microorganisms like *Bacillus subtilis* and *Corynebacterium glutamicum* were found as good candidate for production of xylitol directly from agro residues (Sasaki *et al.* 2010, Cirino *et al.* 2006).

The bioconversion of various polymers present in the agro residues to fermentable sugars has been identified as a promising approach for production of different value added products. Xylan polymer present in hemicellulose fraction of agro residues is an abundant and sustainable source of xylose for production of xylitol. However, production of xylitol in higher quantity requires efficient saccharification of xylan. In the present study, xylanase enzyme cocktails (XEC, 500 U/gds) produced by *Penicillium citrinum* xym2 (XEC^P) and *B. subtilis* xym4 (XEC^B) were used for saccharification of cheaper agro residues such as wheat bran, sugarcane bagasse, saw dust, grass, water hyacinth, petals, coconut husk, orange peel, mango leaf, rice straw and corn fiber, after pretreatment with acid and alkali. Acid pretreated agro residues were more efficiently depolymerized by both the XECs with release of greater quantity of reducing sugars. Saccharification of acid pretreated wheat bran yielded 6.82 and 7.2 mg/ml of reducing sugar by XEC^P and XEC^B treatment, respectively, which was markedly higher in comparison to that of other tested agro residues. Similarly, Adhyaru *et al.* (2016) reported the release of 97.28, 68.71, 64.34, 72.15 and 83.75 mg reducing sugar/g of 1 M HCl pretreated sorghum straw, wheat straw, rice straw, barley straw and corncob, respectively, using xylanase from *Aspergillus tubingensis* FDHN1. Using the crude xylanase of *Scytalidium thermophilum*, Kocabas *et al.* (2015), achieved reducing sugar yield of 0.66 mg/ml form corn cob. In this same study, alkaline hydrogen peroxide pretreatment was found to be the effective method of saccharification. Sorghum straw pretreated with 3% alkaline hydrogen peroxide yielded maximum reducing sugar (34.94 mg/g) after 36 h enzymatic hydrolysis using xylanase obtained from *Bacillus altitudinis* DHN8. Whereas, alkali, acid and untreated biomass of sorghum straw liberated 29.56, 23.81 and 2.58 mg/g reducing sugar, respectively, after 48 h of incubation (Adhyaru *et al.* 2014). Alkali pretreated corncob when hydrolyzed by xylanase from *Bacillus subtilis* Lucky9 at pH 6.5 and 50 ^oC, liberated l8.7 mg/ml of reducing sugars after 8 h of incubation (Chang *et al.* 2017). Reis *et al.* (2013) reported that a cocktail of enzymes having 43.73

U/mg of xylanase, 1.93 FPU/mg of cellulase and 0.93 U/mg of β-glucosidase from *Penicillium echinulatum* S1M29, released 15% more of the sugar from sugar cane bagasse pretreated by steam explosion followed by partial delignification. Comparison between the TICs of sachharified extract of acid pretreated wheat bran with XEC^P and XEC^B, in this study, revealed the presence of peak of xylose, glucose, and mannose, 2 deoxy d-glucose at retention time of 17.75, 19.53, 19.77 and 17.1 min, respectively. Presence of phenol 2,6 bis [1,1 dimethyl ethyl 4 –ethyl] was found to be the common phenolic derivatives present in the chromatogram. Whereas in XEC^B treated wheat bran, 1,3 pentanedione 4 methyl 1 phenyl (RT 13.55 min) was found to be a phenol derivatives. Moreover, in XEC^P treated wheat bran extract, glycal d-aspartic acid (RT 13.58 min), 2 hydroxy anthraquinone (RT 12.2 min) and 3,4-di isopropoxy -3 cyclo butane,1,2 dione (RT 10.17 min) were also observed. Minghua *et al.* (2012), reported the presence of monomeric sugars, such as xylose, glucose, arabinose and so on from the dilute sulfuric acid pretreated corn cob. Besides sugars several inhibitors for microbial growth including furfural, HMF, acetic acid and phenolic compounds were also found to be generated in the hydrolysate (Minghua *et al.* 2012). Formation of monomeric sugars such as glucose, mannose and xylose from sugarcane bagassae and rice straw was also reported using xylanase and cellulase of *Aspergillus tubingensis* NKBP-55. In our present research XEC^B, obtained from *B. subtilis* xym4, were further employed for sachharification of wheat bran using PB design and RSM technique. XEC^P from *P. citrinum* xym2 was not incorporated for statistical optimization of sachharification study of wheat bran, because this enzyme cocktail contain higher level of protease (160.88 IU/ml), which may inactivate the xylanase in the mixture and decreased the sugar yield from wheat bran. Moreover, due to the presence of some phenolic, acetic and quinine derivatives which may exert their inhibitory effect on the growth of *E .coli* xyl6 hence reduced the xylitol production. When the XEC^P treated wheat bran extract was used for xylitol production, 3.2 g/l xylitol was obtained which was much lower than the xylitol yield (4.5g/l) obtained after fermentation of XEC^B treated wheat bran by the same bacteria. The observation made in this context of low sugar yield and low xylitol yield may also be due to the fact that polysaccharides related to xylan were present in amorphous form. This occurs because of the inefficient accessibility of the enzyme active sites by the substrate (Mohapatra *et al.* 2018). Response surface optimized XEC^B treated wheat bran extract when fermented with *E. coli* xyl6, 8.11g/l of xylitol was achieved, more importantly the inhibitory phenolic derivatives were not observed in TIC of the fermented broth.

Simultaneous saccharification and fermentation (SSF) of wheat bran with *B. subtilis* xym4 and *E. coli* xyl6 was successfully operated in different conditions for xylitol production in one pot. Among the various parameter affecting xylitol production in the

fermentation broth, cell count of both the bacteria were important one. To quantify the metabolically active cell in the mixed culture, agar plating using selective and differential agar media plates, for specific microorganism was used. Eiosine methylene blue (EMB) agar plates are commonly used to enumerate the number of *Escherichia coli* cells in a mixed culture (Horvath and Ropp 1974). Methylene blue and Eosin-Y present in the medium inhibit the growth gram-positive bacteria such as *B. subtilis*. (Howard 1994). For selective estimation of *B. subtilis* from mixed culture, phenyl ethyl alcohol agar plates are used (Nandy and Venkatesh 2014, Duteau *et al.* 1998). Presence of phenyl ethanol in the medium, gram positive organisms such as *B. subtilis* can grow well while growth inhibition of the gram negative organisms such as *E. coli* was occured (Lilley and Brewer 1953). Phenyl ethyl alcohol specifically inhibits the DNA synthesis of gram negative bacteria (Dowell and Altemeier 1964). Results obtained after fermentation were presented in the table 3.8 and the data were further processed through principal component analysis (PCA) and pearson correlation coefficient determination. Cell counts of *B. subtilis* xym4 were found to form a cluster in all the three PCA loading plots (Fig.3.29,3.30, 3.31) with xylanase and reducing sugar in the medium. A strong significant correlation coefficient of 0.68 and 0.56 were existing between *Bacillus* cell count with xylanase production and with reducing sugar, respectively, in the medium. Adhyaru *et al.* (2014) reported that during early to late exponential phase of *Bacillus altitudinis* DHN8 on sorghum straw, the bactrium reaches to their maximum number which was also associated with the maximum enzyme titer. Kumar *et al.* (2012) and Nagar *et al.* (2010) found that 18 h old inoculums of *Bacillus* in fermentation medium lead to high cell count and xylanase activity.

A negative coefficient of -0.34 between xylitol yield and the available reducing sugar in the medium implies that xylitol yield was maximum when the reducing sugar in the medium was low. This may be due to the fact that the reducing sugar liberated by the xylanase from wheat bran was consumed by the bacteria to support their high growth rate as well as were used for xylitol production by *E. coli* xyl6. Mussatto *and* Roberto (2005) and Walther *et al.* (2001) reported that high substrate could improve the yield of biomass and xylitol.

More over in our study, it was observed that the growth of *Bacillus* was slightly suppressed by the growth of *E. coli*, which was statistically confirmed by a negative correlation coefficient of -0.21 between these two variables. Nandy and Venkatesh (2014) examined the growth of live cell through methelene blue reduction test (MBRT) and viable plate count with the mixed culture of *E. coli* and *B. subtilis* and found that although the fraction of metabolically active cell of *E. coli* in a mixed culture was initially lower than that of *Bacillus* but as long as the fermentation proceed, *E. coli* was found to be dominant over *Bacillus.* In the present study a strong association had been reveled between xylose

reductase (XR) and xylitol production by the *E. coli* xyl6. XR (EC 1.1.1.21) is the principal enzyme in the microbial xylose utilization pathway which can leads to the single step reduction of D-xylose to xylitol. Few bacteria like *Enterobacter liquefaciens* (Ghindae *et al.* 2010, Yoshitake *et al.* 1973), *Corynebacterium* sp. (Rangaswamy and Agblevor 2002) were reported so far were found to ferment xylose to xylitol and hence expressed XR enzyme. However, due to their low productivity rate of xylitol over fermentation time restrict their utilization as an industrial strain to produce xylitol. Yeasts such as *Candida* sp. (Petschacher *et al.* 2005), *Pichia spitis* (Rodrigues *et al.* 2008), *Kluyveromyces marxianus* (De Albuquerque *et al.* 2015; Mueller *et al.* 2011) were preferred for xylitol production in industrial scale due to their high rate of pentose conversion pathway to xylitol. The higher amount of xylitol production by this strain was due to their capability of stable expression of XR and xylitol dehydrogenase (XDH) enzyme (Rafiqul and Sakinah 2013). XDH (EC 1.1.1.9) is the enzyme which converts the xylitol into xylulose in a NAD^+ requiring reaction. Xylose metabolism of bacteria which are devoid of XR and XDH enzymes uses xylose isomerase enzyme to convert xylose directly into xylulose bypassing the xylitol production pathway. High amount of xylitol secretion in extracellular media thus require an intricate balance between XR and XDH activity (Granstrom *et al.* 2007). The present research probably is the first report for efficient utilization of *E. coli* xyl6 in SSF of wheat bran with *B. subtilis* xym4 for significant amount of xylitol production. In our study, condition 4 revealed that the first 24 h of aerobic stage [DO (63 %)] promote the growth of *B. subtilis* xym4, with large amount of xylanase secretion in the medium followed by high reducing sugar released in the media. In the second stage decreased DO (42%) and acidic pH 4.5, in the medium enhance the xylitol accumulation. Under this stage the recorded maximum xylitol concentration was 98.4g/l. Vendeska *et al.* (1995) reported that the lower amount of dissolved O_2 and acidic pH in the fermentation broth stimulates the accumulation NADH which will in turn improved the xylose reductase activity and inhibited the NAD dependent xylitol dehydrogenase activity, resulting the high rate of xylose to xylitol conversion in *Candida* sp. As presented in the table 3.9, DO and xylitol yield had a significant negative correlation coefficient of -0.60, which implies the high DO could lower down the xylitol production. This could be due to the fact that aerated condition oxidized NADH to NAD^+ and the NAD^+/NADH ratio was much higher to activate XDH enzyme, which will in turn reduce the xylitol activity by converting xylitol to xylulose. In the present work, although maximum xylitol production was archived after 48 h of fermentation in condition 4, but further increment of fermentation time reduced the xylitol production as well as the *E .coli* viable cell count. This result supports the fact of impairment of xylose uptake and its consumption by *Candida* sp., which led to reduce amount of xylitol (Granstrom *et al.* 2001).

In conclusion, xylitol production from agro residues was carried out by sequential sachharification and simultaneous sachharification and fermentation (SSF) methods using *E. coli* xyl6. The quantity of reducing sugar released and xylitol produced, and viable count of bacteria were greatly varied under different experimental conditions (Table 3.10). Highest amount of xylitol production was achieved in SSF experiment with condition 4, where *E. coli* xyl6 was added to the SSF^S broth 24 h later the addition of *B. subtilis* xym4. The experimental condition resulted in conversion of xylose to xylitol at 98.4 g/l concentration that was found to be highly depended on viable count of *E. coli* xyl6, availability of reducing sugar in the media, activity of XR and most importantly with the dissolved O_2 level of the fermentation broth and medium pH. Although the xylitol yield was found to be higher when fermentation was performed in PB and RSM optimized saccharified wheat bran extract, its concentration was higher in SSF study. The production of xylitol at higher concentration could be achieved by concentrating the reducing sugars of the statistically optimized saccharified broth. However, a previous report by Minghua *et al.* (2011) suggests that during the process of concentration the saccharified broth some non-volatile toxic compounds, especially phenolic compounds, are also get concentrated that in turn reduce or inhibit the microbial growth. Hence, the SSF (condition 4) can be employed for efficient production of xylitol directly from wheat bran.

Table 3.10
Compression of different method of xylitol production

Method	**Reducing sugar (g/l)**	***B. subtilis* xym4 (CFU/ml)**	***E. coli* xyl6 (CFU/ml)**	**Xylitol (g/l)**	**Xylitol yield (g/g)**
XEC^P treated wheat bran	7.02	-	$4.2x10^4$	3.2	0.45
XEC^B treated wheat bran	8.2	-	$5.1X10^5$	4.5	0.54
PB+RSM optimized XEC^B treated wheat bran	11.50	-	$3.4X10^5$	8.11	0.70
SSF (Condition 4)	150 g/l (Wheat bran)	$4.8X10^5$	$5.8X10^6$	98.4	0.65

Summary and Conclusion

Xylanases are hydrolases group of enzymes and due to their depolymerisation activity they have great importance in several industries such as in the paper pulp bleaching industries. Several microorganisms including fungi and bacteria have been reported to readily hydrolyze xylans by expressing 1,4-β-D endoxylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37). Bacteria, just like in the case of many industrial enzymes, fascinated the researchers for alkaline thermostable xylanase producing trait. Noteworthy members producing high levels of xylanase activity at alkaline pH and high temperature are *Bacillus* spp. The importance of xylanases has not been limited to the paper and pulp industry. Potential applications of xylanases also include bioconversion of lignocellulosic material and agro-wastes to fermentative products, clarification of juices, improvement in consistency of beer and the digestibility of animal feed stock. The enzyme can also be applied for the biosynthesis of xylitol, a polyol alcohol that is used as a sweetener with low calorific value. Since the biotechnological applications require large amounts of low cost enzymes, one of the appropriate approaches for this purpose is to utilize the potential of lignocellulosic wastes.

In this current study two potent xylanase producing microorganism (*P. citrinum* xym2 and *B. subtilis* xym4) have been isolated from the environmental samples. Both the fungal and bacterial isolates were found to produce substantial amount of enzyme using wheat bran as low cost substrates. Moreover, in this research a xylitol producing bacteria, *E. coli* xyl6 was successfully isolated. The research concluded with the following out comes.

1. Xylanase production by the *P. citrinum* xym2 and *B. subtilis* xym4 was optimized to 4 fold and 12 fold, respectively, using wheat bran as raw materials through OFAT and RSM approach.
2. Xylanase from both the sources showed activity in the pH range 3-9 and were thermostable, retaining 80 % activity in the temperature range of 4-60 ^{o}C.
3. *B. subtilis* xym4 was found to be superior than *P. citrinum* xym2 in terms of saccharification and release of xylose from agro residues.
4. Xylanase from *B. subtilis* xym4 was found to have molecular weight of 42 kDa and showed higher catalytic efficiency (Kcat/Km= 11.05).
5. Acid pretreated wheat bran was proved to be the best agro residual substrate that liberated higher quantity of reducing sugars upon XEC^{P} and XEC^{B} treatments.

6. Saccharified broths were used for fermentation by *E. coli* xyl6. XEC^{B} treated wheat bran produced higher level of xylitol in comparison to that treated with $XEC^{P.}$
7. PB and RSM based statistical designs together optimized the process of reducing sugar released by XEC^{B} treatment and fermentation of the extract with *E. coli* xyl6 achieved highest xylitol yield.
8. Among the six different fermentation conditions used in SSF experiments, condition4 showed highest production of xylitol, which was confirmed by GC-MS spectral data.
9. Xylitol production was found to be strongly associated with xylose reductase activity.
10. Low DO and pH facilitated the xylitol production.
11. Higher xylitol concentration (98.4 g/l) and high volumetric productivity of 2.05 g/l/h, under SSF process indicated greater efficiency of the process over sequential statistical optimization method.

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Appendix A

LIST OF ABBREVIATIONS

^{o}C	Degree Celcius
µg	Microgram
µl	Microliter
µmol	Micromole
µm	Micrometer
$	Dollar currency of United States
α	Alpha
β	Beta
B-ME	Beta Mercaptoethanol
AN	Ammonium Nitrate
ANOVA	Analysis of Variance
APS	Ammonium per sulphate
AS	Agitation Speed
ATCC	American Type Culture Collection
BCE	Bacterial Crude Enzyme Extract
BeX	BeechwoodXylan
BiX	Birchwood Xylan
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
cal	Calorie
CAZy	Carbohydrate Active Enzyme
CBB	Coomassie Brilliant Blue
CBG-250	Coomassie Brilliant Blue G-250
CBM	Carbohydrate Binding Module
CCRD	Central Composite Rotatable Design
CD	Catalytic Domain
CE	Crude Extract

CEBD	Cellulose Binding Domain
CF	Corn Fiber
CFE	Cell Free Extracts
CFU	Colony Forming Unit
CH	Coconut Husk
CM	Cane Molasses
CMC	Carboxymethyl Cellulose
CMCase	Carboxy Methyl Cellulose
DEAE	Diethylaminoethyl
df	Degree of Freedom
DHAP	Diammonium Hydrogen Phosphate
DMRT	Duncan's Multiple Range Test
DNA	Deoxyribonucleic Acid
DNS	Dinitro Salicylic Acid
dNTP	Deoxynucleotide Triphosphate
DO	Dissolved Oxygen
EC	Enzyme Commission
ED	Enzyme Dose
EDTA	EtheleneDiamine Tetra Acetic Acid
EMB	EiosineMethelene Blue
EtBr	Ethidium Bromide
FCE	Fungal Crude Enzyme Extract
FPase	Filterpaperase
g	Gram
G6PD	Glucose 6 Phosphate Dehydrogenase
GC	Gas Chromatography
gds	Gram per dry solid
GH	Glycoside Hydrolase
GPS	Global Positioning System
h	Hour
HMF	Hydroxymethylfurfural

HMP	Hexoses Monophosphate Pathway
IT	Incubation Time
IU	International Unit
J	Joule
kDa	Kilo Dalton
l	Liter
LB	Luria-Berteni
lUBMB	International Union of Biochemistry and Molecular Biology
min	Minute
ml	Mililiter
MM	Milimole
mm	Millimetre
MS	Mass Spectroscopy
MTCC	Microbial Type Culture Collection
Mw	Molecular Weight
MWCO	Molecular Weight Cut Off
NA	Nutrient Agar
NAD	NicotinamideAdinineDineucleotide
NADH	NicotinamideAdinine Dinucleotide (Reduced)
NADP	NicotinamideAdinine Dinucleotide Phosphate
NADPH	NicotinamideAdinine Dinucleotide Phosphate (Reduced)
NCBI	National Center for Biotechnology Information
NIST	National Institute of Standard and Technology
OD	Optical Density
OFAT	One Factor At A Time Approach

OP	Orange Peel
OSX	Oat spelt xylan
PAGE	Polyacrylamide Gel Electrophoresis
PB	Plackett-Burman
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PGD	Phospho Glucono Delta Lactonase
PMS	PhenazineMethosulfate
PS	Particle Size
PT	Peptone
RNase	Ribonuclease
RPM	Rotation Per Minute
RS	Rice Straw
RSM	Response Surface Methodology
RSR	Reducing Sugar Released
RT	Retention Time
SB	Sugarcane Baggase
SD	Saw Dust
SD	Standard Deviation
SDH	Sorbitol Dehydrogenase
SDS	Sodium Dodecyl Sulphate
SmF	Submerged Fermentation
SN	Sodium Nitrate
SPC	Standard Plate Count
SSF	Simultaneous Saccharification and Fermentation
SsF	Solid State Fermentation
SSF^P	SSF Medium Primary
SSF^S	SSF Medium Secondary

ST	Soaking Time
TAC	Tri Ammonium Citrate
TAE	Tris Acetate EDTA
TCA	Tricholoro Acetic Acid
TE	Tris EDTA
TEMED	Tetramethylethylenediamide
TIC	Total Ion Chromatogram
TIM	TriosephsphateIsomerase Metabolic conseved
U	Unit
UV	Ultraviolet
V	Volt
v/v	Volume by Volume
Vis	Visible
w/v	Weight by Volume
WB	Wheat Bran
WS	Wheat Straw
WU-AX	Water UnextractableArabinoXylan
XBM	Xylose Broth Medium
XDH	Xylitol Dehydrogenase
XEC	Xylanase Enzyme Cocktail
XEC^{B}	XEC of *Bacillus*
XEC^{P}	XEC of *Penicillium*
XI	Xylose Isomerase
XLM	Xylose Agar Medium
XPM	Xylanase Production Media
XR	Xylose Reductase
XYM	Xylan Agar Media
YE	Yeastextract

Appendix B

THESIS RELATED PUBLICATIONS AND ABSTRACTS

(A) Publications

Shyama Prasad Saha and Shilpi Ghosh (2014). Optimization of xylanase production by *Penicillium citrinum* xym2 and application in saccharification of agro-residues.Biocatalysis and Agricultural Biotechnology 3:188-196.

Shyama Prasad Saha, Deblina Mukherjee and Shilpi Ghosh (2012).Submerged cultivation of *Aspergillus flavus* xym4 with water hyacinth as substrate for production of a highly active, thermostable xylanase. Annals of Biological Research 3(10):4884-4892.

(B) Abstracts

Shyama Prasad Sahaand ShilpiGhosh (2015). Production of microbial xylanase under submerged fermentation using wheat bran as agro residues and its role in xylitol Production: A statistical experimental design based study. International symposium on Biodiversity, Agriculture, Environment and Forestry, held on 11-12 December, 2015, organized by Association for the Advancement of Biodiversity Science (Oral).

Shyama Prasad Saha and Shilpi Ghosh (2015).Application of wheat bran as potential agroresidue for enhanced production of xylanase and xylitol by statistical experimental design. 22nd West Bengal State Science and Technology Congress held on 28th February-1st March, 2015 organized by University of North Bengal (Oral). .

Shyama Prasad Saha and Shilpi Ghosh (2012). Saccharifying enndoxylanase production from the submerged fermentation of water hyacinth by *Aspergillus* sp. xym4. National seminar on Emerging trends in Microbiology, Microtrends, held on 16th March, 2012, organized by Department of Microbiology, University of North Bengal (Oral). .

Shyama Prasad Saha and Shilpi Ghosh (2017). Production of xylitol by bacterial co-culture from wheat bran and sugarcane bagasse through simultaneous saccharification and co-fermentation. National seminar on Applied Microbiology: Microbial world 2017, held on 4th September, 2017, organized by Department of Microbiology, University of North Bengal (Oral).

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Biocatalysis and Agricultural Biotechnology 3 (2014) 188–196

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Original Research Paper

Optimization of xylanase production by *Penicillium citrinum* xym2 and application in saccharification of agro-residues

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ARTICLE INFO

Article history:
Received 3 November 2013
Received in revised form
8 March 2014
Accepted 11 March 2014
Available online 25 March 2014

Keywords:
Xylanase
Penicillium citrinum xym2
CCRD
RSM
Saccharification

ABSTRACT

The production of thermostable xylanase by a newly isolated *Penicillium citrinum* xym2 was optimized by one factor at a time (OFAT) and response surface methodology (RSM) approaches. Optimization of xylanase production by OFAT approach indicated medium pH 4.0, wheat bran 1% (w/v), maltose 1.25% (w/v), di-ammonium hydrogen phosphate 0.09% (w/v), cultivation temperature 30 °C, and incubation time 72 h as optimal conditions for xylanase production of 1853 IU/ml. Three effective variables, wheat bran concentration, medium pH and cultivation temperature were optimized by central composite rotatable design (CCRD) based RSM. The interactions between these variables contributed to significant increased xylanase production. The RSM optimized wheat bran level 1.5% (w/v), pH (3.5) and temperature (30 °C) enhanced xylanase production to 2834 IU/ml. The production value or enzyme production was closer to the model diagnosed value of 2845 IU/ml and was about 4 fold higher than in unoptimized basal medium. The enzyme showed potential in saccharification of second generation feed stocks into sugars.

1. Introduction

Plant lignocellulosic biomass accumulates in large quantity in environment through forestry and agro-industrial processes. These biodegradable wastes are recognized as potential sustainable source for production of various value added products like, biofuel, animal feed, chemicals and enzymes.

Xylanases (1,4-β-xylanohydrolase; EC 3.2.1.8) are important enzymes that degrade xylan, the most abundant hemicellulose present in both hardwoods and annual plants (Fang et al., 2010; Ghosh et al., 2012). Microbial xylanases have fascinated researcher because of their potential applications in industrial processes such as, biobleaching of pulp in paper industry, conversion of biomass waste to fermentable sugar for production of biofuel and other chemicals, animal feed quality improvement, clarification of fruit juices and wines (Beg et al., 2001; Chandra and Uma Maheswari, 2000; Fang et al., 2010; Ghosh et al., 2012).

Among microbial xylanase producers fungi are of special interest because they secrete substantially greater amounts of xylanase into extracellular culture medium than do bacteria. Xylanase produced by many filamentous fungi have special property like pH stability, high temperature optima and thermostability (Haltrich et al., 1996; Maheshwari et al., 2000).

The successful industrial application of xylanase requires its cost effective production in bulk quantity. The production cost can be reduced by using cheap agro-residue as carbon source and better microbial strains. The optimization of variables for enzyme production is generally carried out using OFAT approach, but it does not consider interaction among variables (Gupta et al., 2012). The optimal design of the cultural medium for enzyme production by statistical approaches such as response surface methodology (RSM) is an alternative strategy that offers minimum number of experiments for a large number of process variables and modelling of interaction among them. Recently, RSM has been utilized successfully to improve product yield and to reduce development time and cost of biotechnological processes (Bocchini, 2002; Ghanem, 2000; Senthil Kumar, 2005). In the present study, the production of thermostable xylanase by a newly isolated *Penicillium citrinum* xym2 in submerged fermentation was optimized by one factor at a time (OFAT) and CCRD based RSM approaches. The enzyme showed potential in saccharification of second generation feed stocks into sugars.

2. Materials and methods

2.1. Raw material and chemicals

Birchwood xylan was purchased from Sigma-Aldrich, USA. All other biochemicals and microbiological media were from Hi-Media, India or E. Merck, Germany. Agro-residues were

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http://dx.doi.org/10.1016/j.bcab.2014.03.003
1878-8181/

collected from either local agro-industries or university garden. They were washed with distilled water; oven dried (60 °C for 72 h), ground in an electric grinder and packed in air-tight containers for use as the substrate for submerged fermentation.

2.2. Fungal strain

Xylanase producing fungal strains were isolated from soil sample collected from University of North Bengal, India. One gram of soil was suspended in sterile distilled water, serially diluted and spread on xylan agar medium (XYM, pH 6.5) containing (g/l): peptone 0.90; $(NH_4)_2HPO_4$ 0.40; KCl 0.10; $MgSO_4 \cdot 7H_2O$ 0.10; birch-wood xylan 5 and 2% agar and incubated at 37 °C for 48 h. The colonies were then transferred on fresh XYM and screened for xylanase production using congo red plate assay and selected on the basis of hydrolysis zone. Zone clearance ratio was determined by calculating ratio between inhibition zone diameter and colony diameter.

The identification of potent xylanase producing fungal isolate was done by PCR amplification of a 612 bp region of 28S rRNA gene by using universal primers followed by sequencing (Fritsch et al., 1989). The nucleotide sequence has been deposited in the GenBank database (Accession no. DQ914658.1).The identity of the isolate was confirmed by phylogenetic analysis of 28S rRNA sequence using package Mega-4 (Dudley et al., 2007).

2.3. Microorganism maintenance and preparation of inoculums

P. citrinum xym2 was maintained in XYM at 4 °C and subcultured at two weeks intervals. The inoculum was prepared by cultivating the organism in potato dextrose broth for 24 h at 30 °C under shaking. The inoculum at 2% (v/v) was used for inoculation of culture media.

2.4. Xylanase production in submerged fermentation (SmF)

Xylanase production by *P. citrinum* xym2 was carried out in xylanase production medium (XPM) having same composition as XYM but without agar (XPM=XYM—Agar). The autoclaved medium was inoculated at 2% (v/v) fungal culture and incubated for 48 h at 30 °C under shaking. The culture was then centrifuged at 10,000 rpm for 10 min at 4 °C and the clear cell-free supernatant was assayed for xylanase activity.

2.5. Xylanase assay

Xylanase was assayed according to the method of Bailey et al. (1992) by measuring the amount of reducing sugar (xylose equivalent) liberated from xylan using 3,5-dinitrosalicylic acid. The reaction mixture containing 0.25 ml diluted enzyme extract and 0.25 ml xylan solution (1% w/v in 100 mM citrate buffer, pH 5.4) was incubated at 37 °C. After 20 min the reaction was stopped by adding 0.50 ml 3,5-dinitrosalicylic acid reagent and the amount of reducing sugar released in the reaction was estimated by measuring the absorbance at 540 nm. One international unit (IU) of xylanase activity is defined as the amount of the enzyme required to release 1 μmol xylose from xylan per minute under standard assay condition.

2.6. Optimization of xylanase production by OFAT method

For optimization of xylanase production by *P. citrinum* xym2, the effect of various process variables such as carbon source, initial medium pH, cultivation temperature, additional sugar, nitrogen source and cultivation time, were studied under SmF using OFAT method. Each parameter optimized was incorporated further in the subsequent experiments.

2.6.1. Effect of agro-residues on production

Birch wood xylan in the XPM was replaced with agro-residues such as coconut husk, sugarcane bagasse, rice straw, corn fibre, orange peel, saw dust and wheat bran, each at 1% (w/v). The fermentation was allowed to proceed at 37 °C for 48 h under shaking. The culture was then centrifuged at 10,000 rpm for 10 min and the clear cell-free supernatant was assayed for xylanase activity. The agro-residue showing maximum xylanase production was varied further to determine its optimum level.

2.6.2. Effect of pH and temperature on production

Xylanase production was studied at pH values ranging from 3 to 9. The medium of different pH (100 mM citrate buffer pH 3, 4 and 5, 100 mM phosphate buffer pH 6, 7 and 100 mM Tris–HCl buffer 9) was inoculated with fungal culture and incubated at 37 °C for 48 h. The clear supernatant obtained after centrifugation of culture was assayed for xylanase activity. The cultivation temperature was optimized by growing the organism at 4, 25, 30, 37, 50 and 60 °C for 48 h, followed by determination of enzymatic activity in culture supernatant.

2.6.3. Effect of monosaccharide and disaccharides on production

The production medium was supplemented with monosaccharide or disaccharide such as dextrose, xylose, sucrose, maltose, galactose, fructose and lactose at 1% (w/v) each. The medium without sugar served as control. Xylanase was assayed in the clear culture supernatant. The sugar molecule showing maximum enzyme production was varied further to determine its optimum level.

2.6.4. Effect of nitrogen sources on production

To determine the effect of nitrogen sources, different organic and inorganic nitrogen sources such as peptone, yeast extract, ammonium nitrate, sodium nitrate, di-ammonium hydrogen phosphate, ammonium sulphate and tri-ammonium citrate at a concentration of 0.09% (w/v) each, were used in the production medium. Further, effect of different concentration of the selected nitrogen source on enzyme production was investigated.

2.6.5. Effect of cultivation time on production

For determining the cultivation time for highest xylanase production, the fungal culture samples were withdrawn at 24 h intervals for a period of 144 h and enzymatic activity was monitored in the culture supernatant.

2.7. Optimization of xylanase production using CCRD based RSM

Further optimization of xylanase production by RSM using CCRD was based on the study of interaction among three effective parameters selected from the OFAT method i.e. wheat bran, medium pH and cultivation temperature. CCRD contains a factorial matrix with a centre point and “axial points” around the centre point that allow the curvature of the model to be established. The distance from the centre point to the factorial point is ± 1 unit for each factor, and the distance space from the centre of the design space to the axial point is $\pm\alpha$, where $\alpha=(2^k)^{1/4}$ [k=number of independent factors]. The variables optimized were wheat bran (A), medium pH (B) and incubation temperature (C) with five different coded levels [$-\alpha$, -1, 0, $+1$, $+\alpha$]. For wheat bran (A) the coded levels $-\alpha$, -1, 0, $+1$, $+\alpha$ are 0.66, 1, 1.50, 2 and 2.34% w/v, respectively. For pH (B) the coded levels $-\alpha$, -1, 0, $+1$, $+\alpha$ are 1.98, 3, 4.50, 6 and 7.02, respectively, whereas for incubation

temperature (C) the coded levels $-\alpha$, -1, 0, $+1$, $+\alpha$ are 23.18, 30, 40, 50 and 56.82 °C, respectively.

The relation between the coded forms of the input variable and the actual value of the wheat bran, medium pH and incubation temperature are described in Eq. (1).

$$X_a = (Z_a - Z_o)/\Delta Z \quad (1)$$

where X_a is a coded value, Z_a is the actual value of the factor, Z_0 is the actual value of the same variable at the centre point and ΔZ the step change of the variable. According to the CCRD model total number of the experimental run is determined by the following equation (Eq. (2)).

$$R = 2^k + 2k + n_0 \quad (2)$$

where k is the number of independent variable and n_0 is the number of repetitions of the experiments at the centre point. Total number of experimental runs was 20 with 8 factorial, 6 axial and 6 centre point runs. Xylanase produced (activity IU/ml) from all the experimental run was analyzed by a second order polynomial regression equation (Eq. (3)) to better estimate the experimental error.

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_{12} + a_{22}{x_2}^2 + a_{33}{x_3}^2 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 \quad (3)$$

where Y is the predicted xylanase activity, a_0 is the intercept terms, x_i is the independent factors and a_i is the model coefficient parameters. With the help of Eq. (3), all the independent variables are optimized to get a better response.

2.8. Characterization of xylanase from P. citrinum xym2

Xylanase from *P. citrinum xym2* was partially purified using 30–70% ammonium sulphate precipitation and characterized with respect to pH optima, temperature optima and thermostability. The pH optimum was determined by measuring the enzyme activity at pH 3–9 in the following buffer: 100 mM citrate buffer (pH 3, 4 and 5), 100 mM phosphate buffer (pH 6 and 7) and 100 mM Tris–HCl buffer (pH8 and 9).The optimum temperature for xylanase activity was determined at optimum pH and at temperatures 4, 25, 30, 40, 50, 60, 70, 80 and 90 °C. For determining thermostability enzyme preparation was pre-incubated at temperatures 4, 25, 30, 40, 50, 60, 70, 80 and 90 °C for 30 min followed by determination of activity under standard conditions.

2.9. Saccharification of agro-residues

Agro-residues such as saw dust, grass, water hyacinth, petels, sugarcane bagasse, coconut husk, orange peel, mango leaf, wheat bran, rice straw and corn fibre were washed, dried and sieved to 4–8 mm particle size. They were pre-treated with either 0.1 N NaOH or 0.1 N HCl (20% w/v) for 16 h or autoclaved at 121 °C for 1 h. The pre-treatment was followed by washing in distilled water till neutrality and drying in oven at 80 °C. Each agro-residue was suspended in 50 mM sodium citrate buffer (pH 4.2) at 3% (w/v) and supplemented with xylanase enzyme preparation (250 IU/g substrate), and the reaction mixture was incubated at 40 °C. After 1 h the reaction mixture was centrifuged at 5000 rpm at 4 °C for 10 min and the supernatants were used for quantification of reducing sugar by 3,5-dinitrosalicylic acid method using xylose as standard. Saccharification percentage was calculated by the following equation (Baig et al., 2004) (Eq. (4)).

$$\text{Saccharification}(\%) = \frac{\text{Xylose (mg/ml)}}{\text{Substrate (mg/ml)}} \times 100 \quad (4)$$

Time profile for saccharification of wheat bran and grass was also determined by incubating the saccharification reaction mixture for 1–7 h and the amount of reducing sugar released was assayed using the above method.

2.10. Interpretation and data analysis

The Design Expert Software (Version 8.0.7.1, State-Ease, Minneapolis, MN, USA) was used for the statistical design of the experiments, regression analysis of experimental data, analysis of variance and lack of fit test etc. Prediction of optimal submerged fermentation parameters and creation of response surface plot were also conducted by the same software.

Experimental results of agro-residues selection and saccharification of agro-residues were analyzed in the form of one way and two-way ANOVA, respectively. The homogenous subsets for mean were determined by Duncan's multiple range test (DMRT) at a level of significance of $p < 0.05$.

3. Results

3.1. Selection and identification of potent xylanase producing fungal strain

Fungal strains isolated from garden soil were screened for xylanolytic activity. Among these one isolate XYM-2 was produced the largest zone of hydrolysis on xylan agar plate flooded with congo-red having the zone clearance ratio of 2.13. The fungal isolate was identified by phylogenetic analysis of 28S rRNA sequence as *P. citrinum* and hence named as *P. citrinum* xym2 (Accession no KF570240).

3.2. Optimization of xylanase production by OFAT method

The effective parameters for xylanase production were determined by studying the process variables like, carbon source, pH, temperature, additional sugars, nitrogen source and incubation period, by OFAT approach.

3.3. Effect of agro-residues xylanase production

Plant lignocellulosic biomass has been recognised as a potential substrate for production of cellulases and xylanases. For determining the influence of agro-residues on xylanase production, xylan in the production medium was replaced with either of the agro-residues, such as coconut husk, sugarcane bagasse, rice straw, corn fibre, orange peel, saw dust and wheat bran at 1% (w/v) concentration. The results in Table 1 indicate that xylanase showed maximum activity (878 IU/ml) in presence of wheat bran, and was comparable to that of expensive birch wood xylan (712 ± 3.23 IU/ml).

3.4. Effect of pH and temperature on xylanase production

The cultivation temperature and medium pH are regulatory parameters affecting production of enzyme during fermentation. The temperature of the production medium was varied between 4 to 60 °C. Substantial xylanase activity was noted between 25 and 50 °C with highest at 30 °C (925 IU/ml). The enzyme production declined by about 36% at medium temperature of 50 °C (Table 2). Effect pH on xylanase production was determined by growing the fungal isolate in medium of varying pH (3–9). Xylanase production was significantly higher in the pH range 3–7 with highest (1025 IU/ml) in medium of pH 4.0 (Table 2).

Table 1
Effect of agro-residues, carbon sources and nitrogen sources on xylanase production by *Penicillium citrinum* xym2.

Agro-residues* (1% w/v)	Xylanase activity (IU/ml)[a]	Carbon** sources (1%w/v)	Xylanase activity (IU/ml)[a]	Nitrogen** sources (0.09%w/v)	Xylanase activity (IU/ml)[a]
Saw dust	433 ± 11.34	Dextrose	453 ± 22.1	Peptone	612 ± 10.44
Sugarcane bagasse	222 ± 7.88	Xylose	409 ± 11.2	Yeast extract	716 ± 12.2
Coconut husk	108 ± 6.65	Sucrose	329 ± 3.86	Ammonium nitrate	1102 ± 6.8
Orange peel	264 ± 4.11	Maltose	1122 ± 14.22	Sodium nitrate	922 ± 7.77
Wheat bran	878 ± 12.33	Galactose	398 ± 12.21	Di ammonium hydrogen phosphate	1278 ± 6.43
Rice straw	266 ± 6.5	Fructose	243 ± 11.23	Ammonium sulphate	912 ± 2.11
Corn fibre	654 ± 4.56	Lactose	766 ± 22.12	Tri ammonium citrate	716 ± 1.22
Birchwood xylan	712 ± 3.23	Birchwood xylan	712 ± 3.23		

* Culture medium was incubated at 37 °C for 48 h.
** Culture medium was incubated at 30 °C for 48 h.
[a] 1 IU = μmol of xylose released/min. Results are presented as mean (n=3) +/− SD.

Table 2
Parameters optimization for xylanase production by *P. citrinum* xym2, using OFAT method.

Variables	**Wheat bran (% w/v)**					
	0.5	0.75	1	1.25	1.5	1.75
Activity (IU/ml)[a]	528 ± 7.4	612 ± 6.9	885 ± 6.7	815 ± 4.3	850 ± 4.5	840 ± 2.5
Variables	**Temperature (°C)**					
	4	25	30	37	50	60
Activity (IU/ml)[a]	441 ± 11.5	812 ± 6.1	925 ± 2.8	898 ± 17.3	612 ± 3.7	513 ± 3.5
Variables	**pH**					
	3	4	5	6	7	9
Activity (IU/ml)[a]	898 ± 3.2	1025 ± 4.3	875 ± 10.1	828 ± 2.5	885 ± 12.6	344 ± 6.76
Variables	**Maltose (% w/v)**					
	0.5	0.75	1	1.25	1.5	1.75
Activity (IU/ml)[a]	622 ± 3.8	925 ± 11.2	1142 ± 7.8	1250 ± 8.2	768 ± 6.5	740 ± 6.6
Variables (% w/v)	**$(NH_4)_2HPO_4$**					
	0.05	0.06	0.08	0.09	0.1	0.2
Activity (IU/ml)[a]	1022 ± 2.62	1122 ± 7.12	1160.28 ± 3.66	1278 ± 6.43	1155 ± 6.22	1112 ± 1.67

[a] 1 IU = μmol of xylose released/min. Results are presented as mean (n=3) +/− SD.

3.5. Effect of sugar supplementation on xylanase production

Xylanase production medium containing 1% (w/v) wheat bran was supplemented with either of the sugars (1% w/v) such as, dextrose, xylose, sucrose, maltose, galactose, fructose and lactose. The supplementation of maltose significantly increased the xylanase production to 1122 IU/ml (Table 1). However, other carbohydrates inhibited the enzyme with maximum decline in activity in presence of fructose. Effect of varying concentration of maltose ranging from 0.50 to 1.75 (w/v) was studied on xylanase production. The results revealed 1.25% (w/v) maltose as optimal level for maximum xylanase yield of 1250 IU/ml. Further increase in maltose concentration resulted in decline of activity (Table 2).

3.6. Effect of nitrogen sources on xylanase production

To optimize the nitrogen source for xylanase production, XPM was prepared with different organic and inorganic nitrogen sources. Di-ammonium hydrogen phosphate was the most favourable nitrogen source for xylanase production (Table 1) with maximum activity of 1278 IU/ml at 0.09% (w/v) concentration (Table 2). Substantial activity was also noted in presence of NH_4NO_3, $NaNO_3$ and $(NH_4)_2SO_4$.

3.7. Time course of xylanase production

All the parameters for highest xylanase activity were taken into consideration for determining the time course of xylanase production by *P. citrinum* xym2. Xylanase yield was found to be correlated with the fungal biomass. Xylanase production was maximum (1853 IU/ml) at 72 h of incubation followed by a gradual decline till 144 h.

3.8. Central composite rotatable design based response surface methodology for optimization of xylanase production

The effect of three variables, wheat bran (A), medium pH (B) and incubation temperature (C), on xylanase production was investigated. Suitable levels for these parameters were determined by CCRD of RSM. For xylanase production optimization twenty experimental runs were performed using different combinations of the variables as per CCRD. The medium components used for

Table 3
Central composite rotatable design (CCRD) of factors in coded levels with enzyme activity as response.

Run no.	Type	Factor1 A: wheat bran (% w/v)	Factor 2 B: pH	Factor 3C: temp (°C)	Xylanase activity (IU/ml)[a] experimental	Xylanase activity (IU/ml)[a] predicted
1.	Axial	0	0	$-\alpha$	2681.12	2600.65
2.	Factorial	−1	−1	1	2133.23	2014.84
3.	Factorial	−1	−1	−1	2618.12	2548.36
4.	Axial	0	$+\alpha$	0	1234.23	1094.48
5.	Centre	0	0	0	2675.23	2643.49
6.	Centre	0	0	0	2674.12	2643.49
7.	Factorial	1	−1	−1	2113.12	2133.68
8.	Centre	0	0	0	2675.12	2643.49
9.	Centre	0	0	0	2489.12	2643.49
10.	Axial	0	0	$+\alpha$	1412.12	1505.68
11.	Factorial	−1	1	1	868.192	838.38
12.	Factorial	1	−1	1	1123.23	1036.18
13.	Centre	0	0	0	2474.45	2643.49
14.	Centre	0	0	0	2675.12	2643.49
15.	Axial	0	$-\alpha$	0	1941.31	2094.15
16.	Axial	$+\alpha$	0	0	1540.12	1468.44
17.	Axial	$-\alpha$	0	0	1299.89	1384.66
18.	Factorial	−1	1	−1	965.23	1043.03
19.	Factorial	1	1	1	1272.4	1342.80
20.	Factorial	1	1	−1	2012.18	2121.32

[a] 1 IU = μmol of xylose released/min.

CCRD are present in the methods section and the experimental design and results of CCRD are shown in Table 3. Analysis of variance (ANOVA) was performed to the experimental design used. The result of ANOVA shows that model is highly significant ($p < 0.001$) and can better predict the data. Within the model, B (medium pH), C (incubation temperature), AB (wheat bran × medium pH), AC (wheat bran × incubation temperature), A^2 (wheat bran^2), B^2 (medium pH^2), and C^2(incubation temperature^2) are the significant ($p < 0.001$) model terms. Using the results of these experiments, second-order polynomial regression equation for xylanase yield was obtained which is represented in Eqs. (5) and (6) in coded and actual form, respectively.

$$\begin{aligned}\text{Activity} = {} & +2643.49 + 24.91 \times A - 297.20 \times B - 325.54 \times C \\ & + 370.77 \times A \times B\ 143.47 \times A \times C \\ & + 79.75 \times B \times C - 430.25 \times A^2 - 370.94 \\ & \times B^2 - 208.71 \times C^2 \end{aligned} \quad (5)$$

$$\begin{aligned}\text{Activity} = {} & -3215.30566 + 4135.96558 \times \text{wheat bran} + 331.4237 \\ & \times \text{pH} + 153.53134 \times \text{temperature} + 494.3596 \\ & \times \text{wheat bran} \times \text{pH} - 28.69355 \times \text{wheat bran} \\ & \times \text{temperature} + 5.31635 \times \text{pH} \times \text{temperature} \\ & - 1721.00820 \times \text{wheat bran}^2 - 164.86141 \times \text{pH}^2 \\ & - 2.08710 \times \text{temperature}^2 \end{aligned} \quad (6)$$

The fit of the model can be evaluated by using various criteria. In the present study, coefficient of determination (R^2), adjusted R^2, predicted R^2, adequate precision and 'Lack of Fit' were taken into consideration. The R^2 value of 0.9825 indicates that the model could explain 98.25% of the variability in the response. The pedicted $-R^2$ of 0.8869 is in resonable agreement with adjusted $-R^2$ of 0.9667. The 'Lack of Fit F-value' of 4.27 implies to be insignificant. 'Adequate percision' measures the signal to noise ratio and a ratio greater than 4 is desirable. Our model ratio of 20.75 indicates an adequate signal. Hence, the model can be used to navigate the design space.

3.9. Comparision of observed and predicted xylanase activity

Using the second-order regression equation, the model predicted the response (xylanase activity) corresponding to particular values of the regressor variables. The plot for the observed xylanase activity (the response) versus model predicted xylanase activity shows that these are very close with each other. As can be found that the observed xylanase activity (response) and model predicted xylanase activity, data points are split by 45° line indicating a reasonable agreement of the predicted response with the observed ones.

3.10. Interpretation of interaction effects between the independent factors and localization of optimum condition for xylanase production

The response surface plots and their contour plots described by second-order polynomial equation were generated in order to investigate the interactions among variables and optimal level of variables for xylanase production (Fig. 1). From the result in Fig. 1A, it is evident that xylanase production varied significantly by medium pH with higher activity in the pH range 3.0–4.5. Xylanase production was not much affected by wheat bran and its activity remained almost unaltered in the concentration range 1.0–1.7%. However, a high level of interaction was observed between these two variables. Xylanase production was significantly reduced on increment of wheat bran concentration beyond 1.7%, keeping the medium pH fixed at 4.5. Similarly, increment of medium pH beyond 4.5 at fixed concentration of wheat bran resulted in reduction of xylanase activity. Interaction between these two variables was also reflected in ANOVA with AB as significant model term.

The interaction between medium pH (B) and cultivation temperature (C) for xylanase production is shown in Fig. 1B. Maximum xylanase production was noted in the pH and temparature range 3–4.8 and 30–40 °C, respectively.

The response surface plot and contour plot for interaction between wheat bran (A) and cultivation temperature reveal that xylanase production was significantly enhanced in wide ranges of wheat bran concentration (1.20–1.98% w/v) and cultivation temperature (30–40 °C). Further increase in temperature decreased xylanase activity markedly (Fig. 1C).

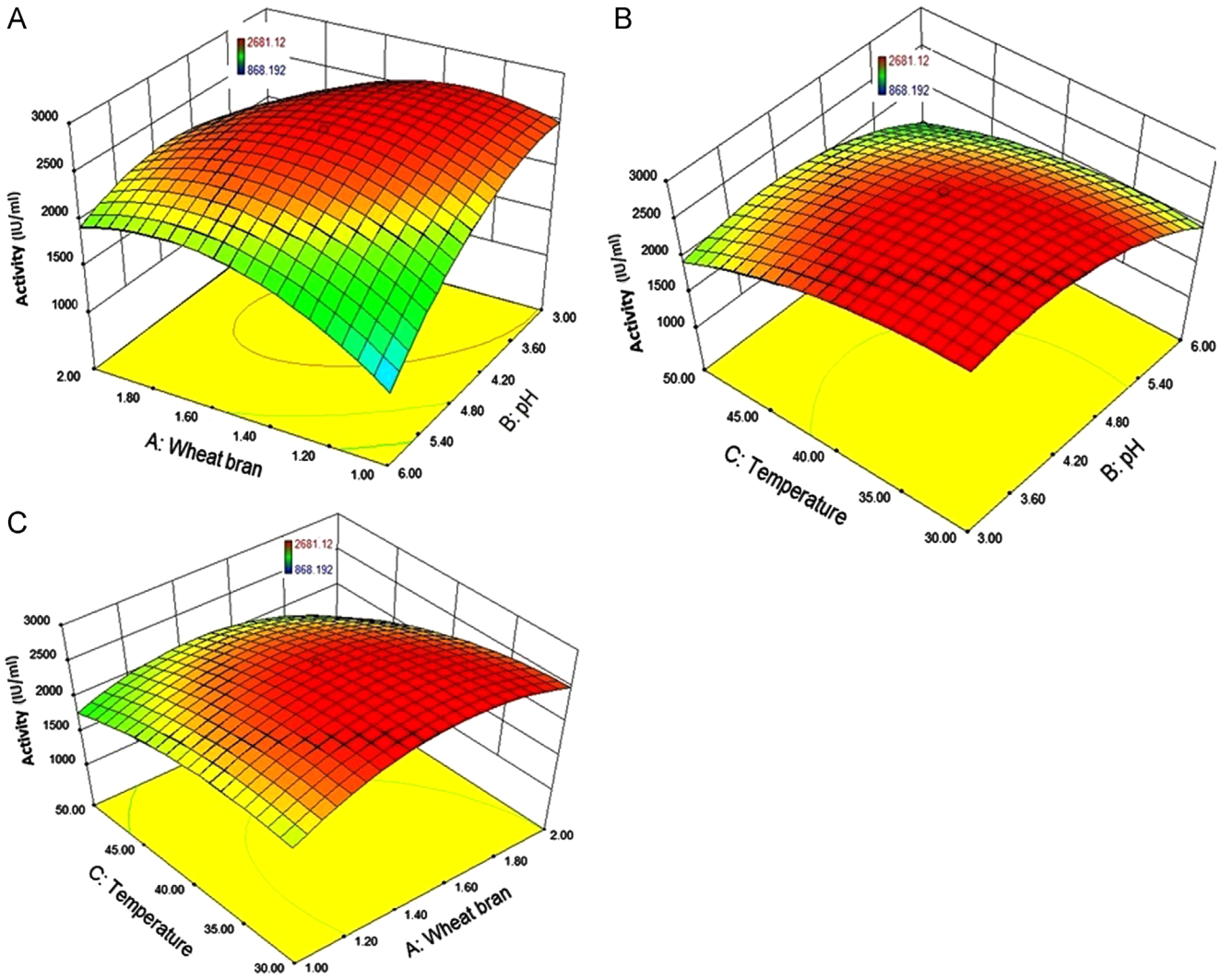

Fig. 1. Response surface curves of xylanase production from *Penicillium citrinum* xym2, showing interaction between wheat bran and medium pH (A), temperature and medium pH (B) and temperature and wheat bran (C) after 72 h of incubation.

The optimal points for xylanase production was analysed by the cube plot.The result indicates that optimal predicted xylanase activity of 2538.46 IU/ml was obtained at points −A, −B, −C corresponding to decreasing level of wheat bran, pH and cultivation temperature.

3.11. Validation of the model

The validity of the model was confirmed by testing xylanase production by random set of ten experiments. The results clearly show that actual values were very close to the predicted values and hence the model was succesfuly validated. These validation experiments suggested wheat bran 1.5% w/v, medium pH 3.5 and cultivation temperature 30 °C as optimal condition for xylanse production. The condition led to xylanase production of 2834.12 ± 0.75 IU/ml, which was comparable to the model predicted data (2845.66 IU/ml). Hence, the CCRD based RSM models were considered to be accurate and reliable for predicting the production of xylanase by *P. citrinum*.

3.12. Characterization of xylanase from P. citrinum xym2

Optiomum pH and temperature for the partially purified xylanase from *P. citrinum* xym2 was found to be pH 4 and 40 °C respectively. The enzyme was more active in acidic pH than alkaline pH. The enzyme showed thermostability at higher temperature as it retained 80% and 50% of activity on preincubation for 30 min at 80 °C and 90 °C, respectively (Fig. 2A–C).

3.13. Enzymatic hydrolysis of agro-residues and saccharification

In the present study the saccharification of various agro-residues by xylanase produced by *P. citrinum* xym2 under submerged fermentation was studied. Agro-residues were pre-treated individually with either 0.1 N NaOH or 0.1 N HCl and heat followed by washing till neutrality and then treated with xylanase preparation. The sacchafication value of untreated agro-residue was about 40%, which was enhanced by various pre-treatments. Acid pre-treatment was the most effective pre-treatment assisting mean saccharification of 67% followed by alkali (58%) and heat (51%) pre-treatments (Fig. 3). The percentage saccharification of various agro-residues were in the following increasing order, coconut husk (45%), rice straw (48%), sugarcane bagasse (48%), corn fiber (53%), water hyacinth (57%), orange peel (58%), saw dust (59.02%), wheat bran (65%), grass (66%). Two agro-residues showing high saccharification i.e. wheat bran and grass were selected for evaluating the time course of enzymatic saccharification and the results reveal that the sugar yield increased till 4 h and thereafter it increased marginally.

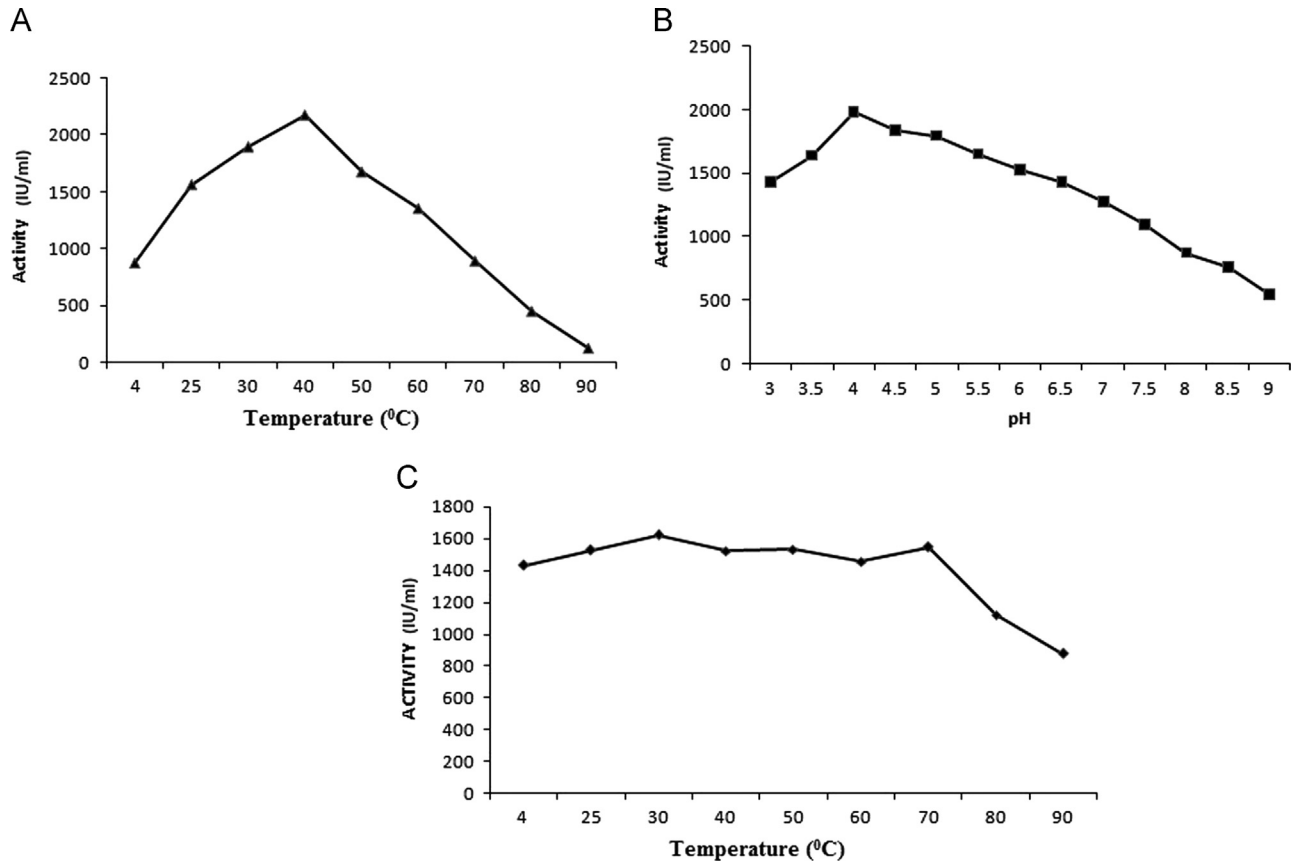

Fig. 2. Influence of different parameters on xylanase activity produced by *P. citrinum* xym2. pH optimization of xylanase enzyme. Xylanase activity was assayed at 40 °C for 10 min using different buffers [100 mM citrate buffer (pH 3–5), 100 mM phosphate buffer (pH 6–7) and 100 mM Tris–HCl buffer (pH 8–9)] (A), Temperature optimization xylanase enzyme. Xylanase activity was assayed at pH 4 for 10 minutes at different temperature (B) and thermostability of the xylanase enzyme after pre- incubation at different temperature (C).

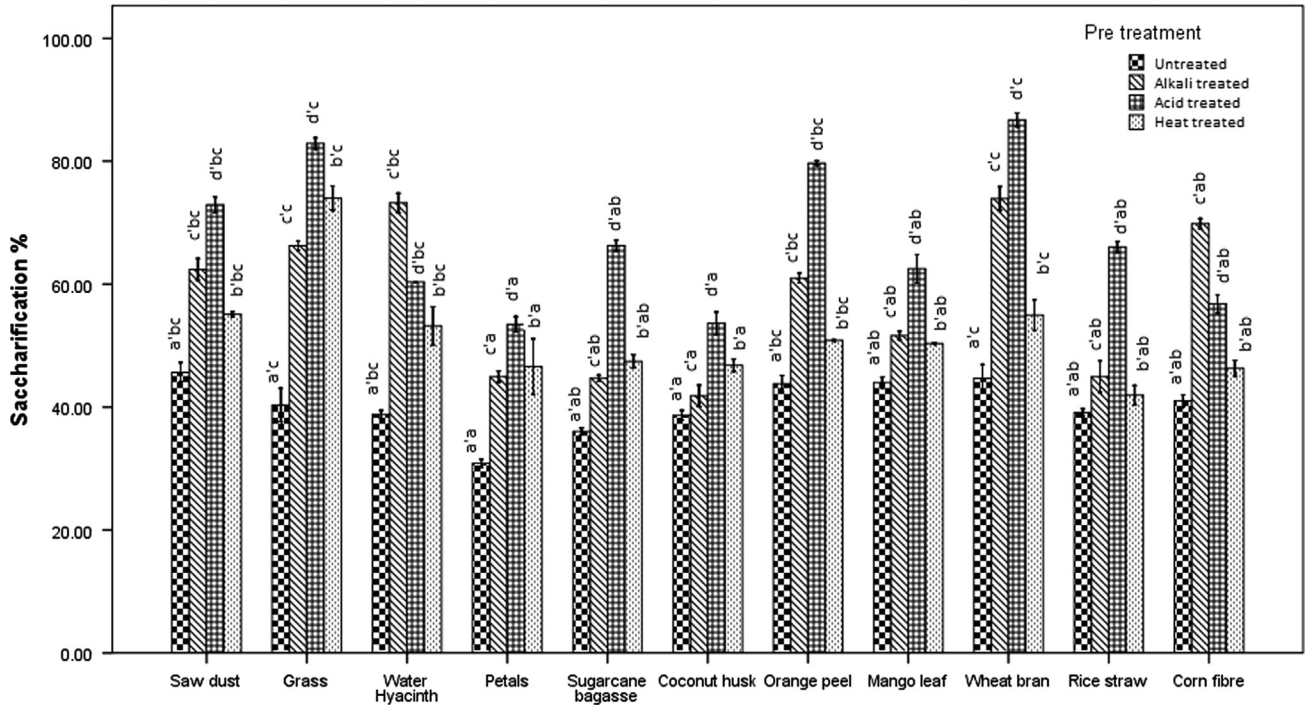

Fig. 3. Effect of different pretreatment process on sachharification of agro-residues by xylanase enzyme obtained from *P. citrinum* xym2. Each bar having different latter are significantly different in their mean saccharification value at a level of significance of $p < 0.05$, obtained by Duncan's multiple range test. 250 IU/g substrate, enzyme dose was used for saccharification.

4. Discussion

Present study indicated wheat bran is the most effective agro residue for xylanase production by *P. citrinum* xym2 under SmF. In previous studies wheat bran has been used for production of xylanase by several strains of *Penicillium* and other microorganism (Araujo et al., 2006; Cui et al., 2007; Emezue et al., 2007). High xylanase activity in presence of wheat bran could be due to its high nutrient contents supporting initiation of growth and replication of microorganisms and it remains loose even under moist conditions providing a large surface area for microbial nutrient uptake during SmF (Gupta et al., 2012; Kuhad and Singh, 1993).

The optimization of cultivation temperature of xylanase production by OFAT approach indicates mesophilic nature of the fungus. The result is supported by the previous studies showing the maximum xylanase activity near about 30 °C by various species of *Penicillium* (Cui and Liming, 2012; Haas et al., 1992; Lacis et al., 1993).

The optimization of xylanase production by OFAT approach enhanced the xylanase activity from 712 IU/ml (unoptimized level) to 1853 IU/ml by OFAT. Among the various parameters optimized by OFAT approach pH was one of the most important parameter for xylanase production. The optimum pH for enzyme production was 4.0. As reported earlier for other filamentous fungi also produce maximal level of xylanase at acidic pH range. *Penicillium purpurogenum* and *Penicillium janthinellum* showed maximum xylanase yield at pH 5.5 (Lacis et al., 1993; Oliveira et al., 2006; Eyzaguirre et al., 1994) whereas xylanase production by *Penicillium* sp.ZH-30 was highest at pH 6 (Cui et al., 2007).

The supplementation of maltose and di-ammonium hydrogen phosphate as additional sugar and nitrogen source, respectively, positively influenced the xylanase production and proved to be valuable parameters of OFAT approach. The sugar mediated regulation of xylanase gene expression has been studied with various strain of *Penicillium*. The production of β-xylosidase and xylanase by *Penicillium pinophilum, Penicillium persicinum* and *Penicillium Brasilianum* were induced by xylose and repressed by glucose. Glucose repression has also been demonstrated in *P. purpurogenum* and *Penicillium chrysogenum* by northern blot analysis and β-glucosidase reporter gene assay, respectively (Bull et al., 2003; Chavez et al., 2004, 2002a; Jorgensen et al., 2004). As observed in the present study, *Penicillium oxalicum* produced maximal level of xylanase using inorganic nitrogen source NH_4Cl instead of organic nitrogen source (Abt et al., 2000).

A correlation was found between kinetics of xylanase production and biomass yield of *P. citrinum* xym2. Both biomass yield and xylanase activity were maximum at 72 h of cultivation and then they declined gradually. The reduction of xylanase yield beyond 72 h could be either due to the depletion of nutrients in the media or due to the reduction in fungal biomass.

The xylanase production optimization by OFAT approach showed wheat bran, incubation temperature and medium pH as the most influential variables. These three variables were hence further optimized by RSM, keeping the level of other variable same as appeared in OFAT approach. In RSM the optimal level of variables were, wheat bran 1.5% w/v, incubation temperature 30 °C, medium pH 3.5, maltose 1.25% w/v, di-ammonium hydrogen phosphate 0.09% w/v, incubation time 72 h, and using these levels increased the xylanase production to 2834.12 IU/ml, which was about 4 fold higher than the unoptimized level. The substantial increase in xylanase production by RSM optimization could be due to significant interaction between the independent variables, wheat bran, incubation temperature and medium pH.

The enzyme also showed high saccharification activity with acid treated wheat bran and grass as substrate. Several *Penicillium* species have been reported to produce xylanase. The xylanase production by *P.citrinum* xym2 in the present study was substantially higher than the previously reported activity values of 40.50 U/ml, 6.47 U/ml, 3.46 U/ml, and 7.82 U/ml for *Penicillium* wx-z1, *P. chrysogenum* (PCL501), *P. oxalium* and *P. sclerotionum*, respectively (Ashok et al., 2007; Banerjee et al., 2012; Emezue et al., 2007; Knob and Canocarmona, 2008).

The production of thermostable xylanase by *P. citrinum* was optimized by using OFAT and CCRD. They appeared to be valuable tools for optimization as xylanase production increased by about four folds. The enzyme showed potential in saccharification of second generation feedstocks into sugar for various applications.

Acknowledgements

Financial support for SG from University Grant Commission, Govt. of India F. No. 41-547/2012 (SR) is gratefully acknowledged.

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CODEN (USA): ABRNBW

Submerged cultivation of *Aspergillus flavus* xym4 with water hyacinth as substrate for production of a highly active, thermostable xylanase

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ABSTRACT

Xylanase (E.C.3.2.1.8), converting xylan to xylose is an enzyme of immense industrial importance. In the present study, a highly active extracellular xylanase producing strain of Aspergillus flavus was isolated from garden soil. The highest xylanase activity was obtained with water-hyacinth as sole carbon source and was comparable to that in presence of birchwood xylan. Di-ammonium hydrogen phosphate was the most appropriate inorganic nitrogen source. The best xylanase production was achieved at 30°C and 72 h incubation time in a medium of pH 6.5. Optimization of cultural conditions resulted in about two fold increase in xylanase activity (3292 U ml^{-1}). A significant xylanase activity was also supported by the production medium containing xylose as sole carbon source. The optimum temperature and pH for xylanase activity were 40°C and 6.5, respectively. The enzyme was acid-alkaline stable. It retained almost 100% activity on pre-incubation at 70^{o}C for 30 min showing thermal stability. The enzyme could saccharify various agro-residues, of which orange peel and water hyacinth were proved to be the best substrates for sugar production.

Key words: Optimization, production, Xylanase, *Aspergillus flavus* xym4, thermostable

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INTRODUCTION

Lignocellulosic wastes are generated in large quantity through forestry, agricultural practices and industrial processes. These wastes are biodegradable and can be converted to valuable products, such as, biofuel, chemicals or cheap energy source for fermentation [1]. Lignocellulose consists of cellulose, hemicellulose and lignin. Xylan, a component of hemicelluloses is the second most abundant polysaccharide. The heteropolysaccharide consists of a homopolymeric backbone of β-1, 4-linked xylopyranose units, with short chains of the substituent groups of acetyl, 4-Omethyl-D-glucuronosyl and a-arabinofuranosyl residues [2, 3, 4]. Xylan interacts covalently and non-covalently with lignin, cellulose and other polymers. The degradation of xylan is catalysed by cooperative action of xylanase (1,4-β-D-xylanohydrolase, E.C. 3.2.1.8), β-xylosidase (1,4-β-D-xylanohydrolase, E.C. 3.2.1.37), α-L-arabinofuranosidase and acetyl xylanesterase [2,4]. Xylanase, which acts on the internal xylosidic linkages on the xylan backbone to produce xylooligosaccharides and xylose, is the most important of these enzymes. Xylanases have potential applications in the food, feed, beverage and textile industries and in waste treatment. In the food and beverage industry xylanases are used for clarifying juices and wines, for extracting coffee and plant oils. In paper and pulp industry xylanase have played essential role in preparation and bleaching of the pulp. This enzyme is also used for the degradation of agricultural wastes and hydrolyzing materials are used as a source of biofuel. Due to its immense application xylanase is regarded as an industrially important enzyme and research are conducted for the production of this enzyme using different strategies [2, 3].

The successful biotechnological applications of enzymes require their cost effective production. The use of purified xylan as inducer for enhanced xylanase production is uneconomical. One of the appropriate approaches for this

purpose is to use lingo-cellulose rich plant residue, which not only serve as economic substrate but also offer environmental advantages. Xylanases are reported to be produced mainly by bacteria and fungi. Filamentous fungi are more promising xylanase producers because of their capability to produce the extracellular enzyme. In the present study, we describe the optimization of production of a highly active, thermally stable xylanase from *Aspergillus flavus xym4* under submerged fermentation and its application in saccharification of agro-residues.

MATERIALS AND METHODS

Chemicals

Birchwood xylan was purchased from Sigma Chemical Co., USA. All other biochemicals and microbiological media used were from Hi Media Laboratories, India or E. Merck, Germany.

Isolation and screening of xylanase producing fungus

Xylanase producing fungal strains were isolated from soil sample collected from the university garden during the month of March. A known amount of soil suspended in sterilized distilled water was poured and spread onto xylan agar medium (XAM) containing (% w/v): birchwood xylan, 0.5; Peptone, 0.09; Potassium chloride, 0.01; Diammonium hydrogen phosphate, 0.04; Magnesium sulphate, 0.01%; agar, 1; pH 7.0 and incubated at 37^{o}C for 48 h. The colonies on the plates were transferred onto fresh XAM and incubated at 37^{o}C for 48 h. The plates were then counter stained with Congo red (1mg/ml) followed by NaCl washing [5]. Colonies producing yellow zone in the red background were selected.

Fungal strains hydrolyzing xylan were analyzed for quantitative production of xylanase in the xylanase production medium (XPM= XAM-Agar). For this fungal strains were grown in XPM at 37^{o}C for 48 h under shake flask culture in a shaker incubator (Labtech, Korea). The culture was centrifuged at 10,000 rpm for 10 min and the xylanase activity in the supernatant was determined. Finally one fungal isolate XYM4 with highest xylanase activity was selected for further studies. The fungus was characterized by morphological and microscopic studies.

Xylanase assay

Xylanase activity was measured by the reducing sugar method [6] with modifications. The reaction mixture contained 0.5 ml of 0.1% (w/v) birchwood xylan in 0.1 M sodium phosphate buffer (pH 6.5) and 0.1 ml of cell-free culture supernatant. The mixture was incubated at 37^{o}C for 30 min. The reducing sugar released was measured using 3,5-dinitrosalicylic acid (DNSA) at 575nm using xylose as a standard. One unit (U) of activity was defined as amount of enzyme required to liberate 1µmol of xylose per minute under the assay conditions.

Effect of agro-residues on xylanase production

The agro-residues such as, coconut husk, sugarcane bagasse, rice straw, mango leaf, cornfibre, orange peel, water hyacinth, wheat bran and saw dust were washed thoroughly with water, air dried and cut into small pieces and used as carbon source. The birchwood xylan in the XPM was replaced with either of the carbon source at 1.0 % (w/v) concentration. The fermentation was allowed to proceed under shaking (150 rpm) at 37 ^{o} C for 48h and xylanase activity in the culture supernatant was monitored.

Effect of nitrogen sources on xylanase production

In order to determine the effect of different nitrogen sources on xylanase production, the production medium contained water hyacinth as sole carbon source and 0.09%w/v of either of the nitrogen source, such as, peptone, yeast extract, ammonium nitrate, sodium nitrate, di-ammonium hydrogen phosphate and ammonium sulphate. The fermentation was allowed to proceed under submerged condition at 37 ^{o} C for 48 h and xylanase activity was determined for each medium.

Effect of temperature and pH on xylanase production

The fungal strain was grown in production medium containing water hyacinth and di-ammonium hydrogen phosphate as carbon and nitrogen source, respectively, for 48 h. The pH of the medium (3.0 to 9.0) and incubation temperature (4 to 50 ^{o} C) were varied and their effect on xylanase production was investigated by determining xylanase activity in the culture supernatant.

Time course of xylanase production

To determine the time course of xylanase production, the fungal strain was grown in production medium (pH 6.5) containing water hyacinth and di-ammonium hydrogen phosphate as carbon and nitrogen source, respectively, at 30^{o}C under shaking. Samples were withdrawn at 24h intervals for a period of 144h and xylanase activity in the culture supernatant was monitored.

Effect of alternative carbon source on xylanase production

To investigate the effect of alternative carbon sources on xylanase production by the fungus, water hyacinth in the production medium was replaced by xylose, maltose, fructose, carboxy methyl cellulose (CMC), galactose, dextrose, maltose and xylan at a concentration of 0.05% w/v. The fermentation was allowed to proceed under submerged condition at 30 ^{o} C for 72 h and xylanase activity was determined for each medium.

Enzyme extraction

The fungal culture was grown in 100 ml of XPM for 72 h at 30^{o}C, followed by harvesting the cells by centrifugation at 10,000 rpm for 10 min at 4^{o}C. The supernatant obtained after centrifugation was fractionated by step-wise precipitation with $(NH_4)_2SO_4$ at 0-30% and 30-70% saturation. The protein pellet from 30-70% $(NH_4)_2SO_4$ saturation was resuspended in 0.1 M phosphate buffer (pH 6.5) and desalted in a P-2 column (Bio Rad, 2 × 10 cm). The desalted enzyme preparation was used for characterization of the enzyme.

Effect of pH and temperature on xylanase activity

The pH optimum was determined by measuring the enzyme activity at pH 3.0-9.0 in the following buffers: 100 mM glycine (pH 3.0 and 3.5), 100 mM sodium acetate (pH 4.0, 4.5 and pH 5.5), 100 mM phosphate (pH 6.0, 6.5 and 7.0) and 100 mM Tris-HCl (pH 7.5, 8.0, 8.5 and 9). The optimum temperature for activity was determined at optimum pH and at temperatures ranging from 4^{o} to 90^{o}C.

Effect of temperature on xylanase stability

The desalted enzyme preparation was pre-incubated at various temperatures ranging 20-100 ^{o} C for 30 min, followed by determination of activity under standard conditions**.**

Saccharification of agro-residues by the enzyme

The agro-residues were washed thoroughly with water, air dried and cut into small pieces, before using as substrate for saccharification. They were treated with 0.1N HCl for 1 hour and then neutralized by washing with distilled water for 1 hour. These residues were then again dried in a hot air oven at 55^{0}C for 4h.

Different agro-residues at a concentration of 5 mg/ml in 0.1M phosphate buffer (pH 7) were incubated with constant volume (1ml) of enzyme preparation for 5h at 30^{0}C. After incubation the mixture was centrifuged at 3000 rpm for 10 minutes and the resultant supernatant was analyzed for reducing sugar by DNS method [6] .using xylose as a standard. The saccharification percentage was calculated using the following formula [7]:

$$\text{Saccharification (\%)} = \frac{\text{Xylose (mg/ml)}}{\text{Substrate (mg/ml)}} \times 100$$

RESULTS AND DISCUSSION

Isolation and screening of fungal strain for xylanase production

The bioconversion of lignocellulosic biomass to fermentable sugar has captured worldwide attention of industrial and scientific research, since it represents an alternative feed stock for the production of biofuels, chemicals, cheap energy source for fermentation and improved animal feed. In a preliminary experiment, fungal strains from garden soil were screened for xynolytic activity in xylan agar medium and examined further by counter staining. A xylanase producing isolate XYM 4 produced the largest zone of clearance and also showed the highest extracellular xylanolytic activity under submerged fermentation. The fungal isolate was characterized by colony morphology and microscopic examination. The colonies were green in colour with 19 mm diameter. The organism formed septate hyphae with conidial head that radiate to loosely columnar with age, conidiospores were circular with 30-50µm diameter. The fungus was identified using standard reference manual [8] as *Aspergillus flavus* and hence, tentatively named as *Aspergillus flavus* xym4. Xylanases are previously reported to be produced by many bacterial and fungal genera such as *Bacillus*, *Penicillium*, *Streptomyces*, *Schizophyllum*, *Thermomyces*, *Trichoderna*, *Humicola* and by *Aspergillus* [9, 10, 11, 12, 4]

Effect of lignocellulose rich agro-residues on xylanase production

The carbon source is a key factor affecting the production cost and yields of lignocellulosic enzymes [2]. Hence, lignocellulosic residues and agro-industrial wastes are increasingly being investigated as substrate for production of cellulases and hemicellulases [11, 13, 14, 4]. In the present study, agro-residues such as, wheat bran, rice straw, coconut husk, sugarcane bagasse, cornfibre, mango leaf, orange peel, water hyacinth, and sawdust (1.0 % w/v) were used as sole carbon source in the xylanase production medium. From the results in Figure1, it is evident that xylanase production by *Aspergillus flavus* xym4 was maximum in the presence of water-hyacinth as carbon source.

The xylanase activity (1600 Uml^{-1}) in water-hyacinth containing production medium was comparable to that in presence of birch-wood xylan. Other substrates like coconut husk, sugarcane bagasses, orange pill and mango leaf also showed significant enzyme production. However, rice straw, wheat bran and sawdust were inadequate substrate for xylanase production. Although, production of xylanase by several mesophilic and thermophilic fungi in wheat bran, barley husk, sugarcane bagasse has been reported [15, 16, 4], no information is available regarding the use of water hyacinth as xylan source. The xylanase activity of *Aspergillus flavus* xym4 with water hyacinth as substrate was remarkably greater than that from other *Aspergillus* species [16, 17].

Effect of nitrogen sources on xylanase production

The nitrogen source is one of the major factors affecting enzyme production. The selection of nitrogen source for the production medium was based on previous studies. The production medium contained water hyacinth as carbon source and supplemented with different inorganic nitrogen sources (0.09 %). Among the various nitrogen sources tested, $(NH_4)_2HPO_4$ was the most favourable nitrogen source for xylanase production (1573.3 U ml^{-1}), however, substantial activity was also noted with NH_4NO_3 (1453 Uml^{-1}), $(NH_4)_2SO_4$ (1186 Uml^{-1}), and $NaNO_3$ (1120 Uml^{-1}) (Fig. 2a). The effect of varying concentration of $(NH_4)_2HPO_4$ in the production medium was also examined. $(NH_4)_2HPO_4$ at a concentration of 0.27 % raised the xylanase activity to about 2006 U ml^{-1} (Fig. 2b). As in the present study xylanase production by *Thermomyces langinosus* was suitable with $(NH_4)_2HPO_4$ [18]. The production of xylanase by *Tricoderma harziznum* was maximum with $(NH_4)_2SO_4$ as nitrogen source [11], while in another study with the same organism $NaNO_3$ and peptone gave better results [19].

Effect of pH, temperature and incubation time on Xylanase production:

The optimum temperature and pH for production of xylanase were determined by growing the fungal isolate in the optimal medium of varying pH (3-9) and at varying temperature (4-50^oC), followed by determination of extracellular enzymatic activity. Although, the enzyme was active throughout the experimental pH range, the maximum activity was noted in production medium of pH 6.5 and a progressive loss in activity was observed below and above 6.5 (Fig. 3a). The cultivation temperature does not only affect the growth of organism but it also has a marked effect on the enzyme production. Xylanase production was maximum at 30^oC, which was also the optimum temperature for growth of the organism. The reduction in xylanase production at temperatures below and above the optimum might be due to inhibition of fungal growth (Fig. 3b).

For determining the time for maximum xylanase production all the parameters for highest activity were taken into consideration. The fungal isolate was grown in production medium (pH6.5) containing water hyacinth and $(NH_4)_2HPO_4$ as carbon and nitrogen sources, respectively, at 30^oC. This resulted in a significant increase in xylanase activity to about 2500 U ml^{-1} at 24h of growth with a maxima (3292 U ml^{-1}) at 72h of incubation. The activity then declined slowly to 58 % at 144h (Fig. 3c).

Effect of alternative carbon sources on xylanase production

Xylan in the production medium was replaced with different carbon sources (0.05% w/v). A significant xylanase activity was also detectable when xylan in the production medium was substituted with other carbon sources, such as, xylose, maltose, CM-cellulose, lactose. Xylanase activity was maximum with xylose as carbon source. The results imply that xylanase production was not subject to xylose repression. Moreover, the enzyme was not specific to xylan and can be produced constitutively to a higher level. Xylose has been described as effective inducer of xylanase in several organisms [20, 21]. However,the activity was reduced considerably with glucose, fructose and galactose, which may be due to inhibition of enzyme synthesis in presence of easily metabolizable carbohydrates (Fig. 4). Similar findings were reported by Seyis and Aksoz (2005), who studied the xylanase produced by *Trichoderma harzinum*.

Effect of pH and temperature on xylanase activity:

Xylanase activity at different pH showed that the enzyme had maximum activity(1106 Uml^{-1}) at pH 6.5 (Fig. 5a). Significantly high activity was also present at pH 4.5 (706 Uml^{-1}), 5.0 (866 U ml^{-1}), 5.5 (1040 Uml^{-1}), 6.0 (1066 Uml^{-1}), 7.0 (773Uml^{-1}) and 7.5 (659 Uml^{-1}). However, the activity decreased reasonably in the pH ranges 3-4 and 8-9. The pH optimum of xylanase from other *Aspergillus* species were quite similar except from *A.niger*, which was more active in the alkaline pH range [22, 23, 24, 25]

The temperature profile xylanase was determined from 4^oC to 90^oC using the standard xylanase assay at the given temperature. The enzyme activity increased with increasing temperature up to 40^oC and declined above 50^oC (Fig. 5b). Thermal stability analysis of *Aspergillus flavus* xym4 xylanase showed that the enzyme was absolutely maintained at 70^oC for 30 min. No xylanase activity was detectable at pre-incubation at 90-100^oC (Fig. 5c). The optimum temperature of most xylanases from *Aspergillus* strains ranged from 45^oC to 60^oC [22, 16,26]. As in the present study, xylanase from other *Aspergillus* species has shown thermostability property [22, 23, 24, 25].

Saccharification activity of xylanase on plant-residues

The plant lignocellulosic mass can be enzymatically hydrolyzed to provide sugar for the production of second generation biofuel or other industrial products through fermentation. Since, it is naturally recalcitrant to enzymatic hydrolysis pretreatment is essential for improving its enzyme digestibility and also for obtaining solubilized sugars. Saccharification of plant residues by xylanase of *Aspergillus* sp.xym4 was investigated.

The pre-treated plant-residues were incubated with crude enzyme preparation at pH 7 for 5h, followed by analysis of released reducing sugar. As depicted in Figure 6, saccharification activity was highest with orange peel (79%), followed by water hyacinth (65%), and grass (55%). All other agro-waste showed saccharification activity below 50%. Due to high sachhrification activity, xylanase produced by *Aspergillus* sp.xym4 has potential as a commercial xylanase production system for various biotechnological applications. Previous studies have indicated an increase in saccharification by xylanase supplementation. Among different strategies of xylanase supplementation, the 24-h xylanase treatment before cellulase addition yielded an increase of 40 and 10 % in glucose and xylose production, respectively [27].

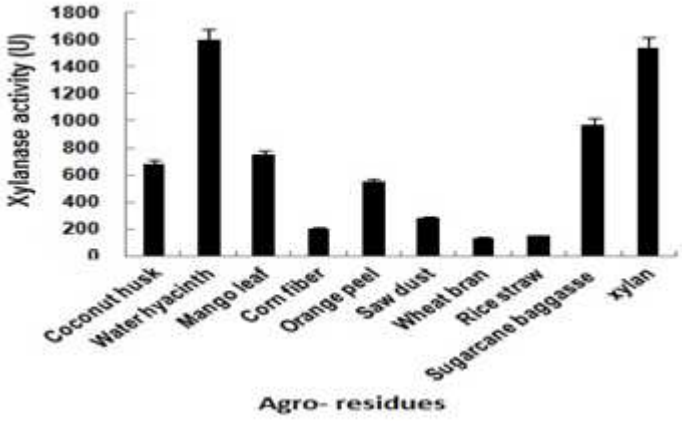

Fig 1: Effect of different carbon sources on the xylanase production by *Aspergillus flavus*.xym4 (values are mean of three replicates ± SD). *1U xylanase activity= 1µmol xylose min^{-1}

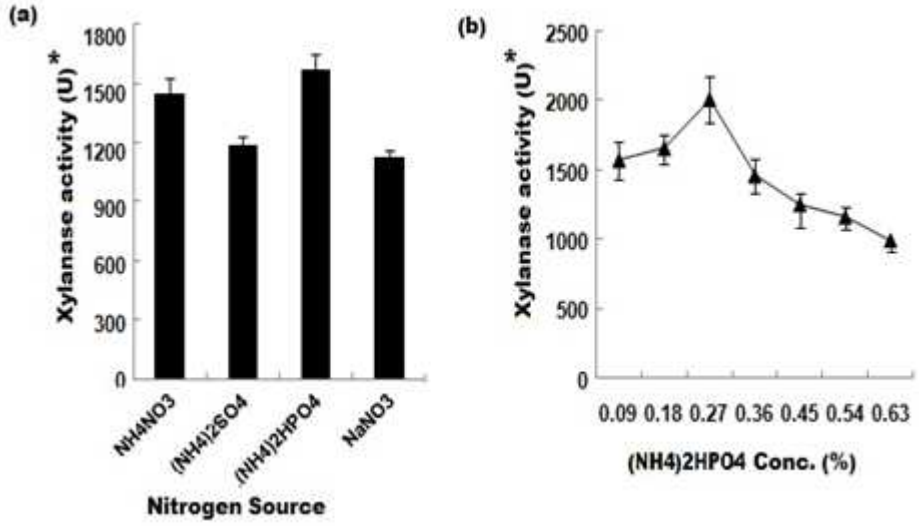

Fig 2: Effect of different nitrogen sources (a) and different concentration of $(NH_4)_2HPO_4$ (b) on the production of xylanase by *Aspergillus flavus* xym4 (values are mean of three replicates ± SD) *1U xylanase activity= 1µmol xylose min^{-1}

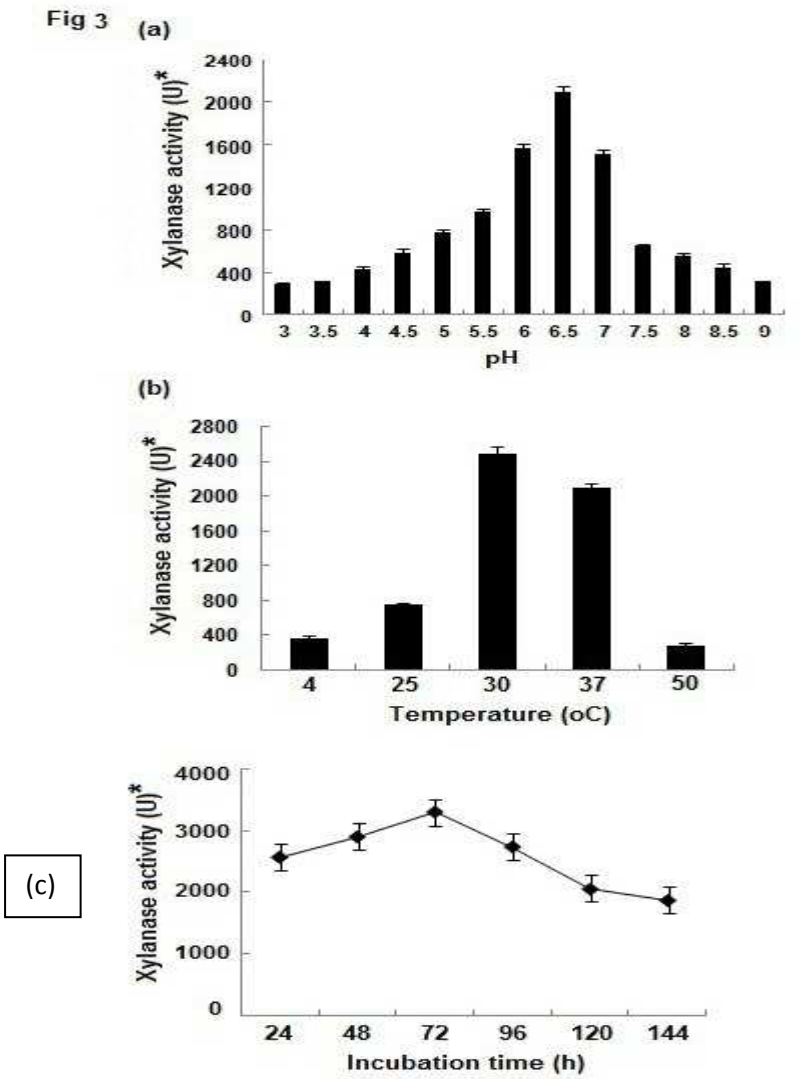

Fig 3: Effect of pH (a), temperature (b) and incubation time (c) on xylanase production by *Aspergillus flavus* .xym4 (values are mean of three replicates ± SD). *1U xylanase activity= 1µmol xylose min^{-1}

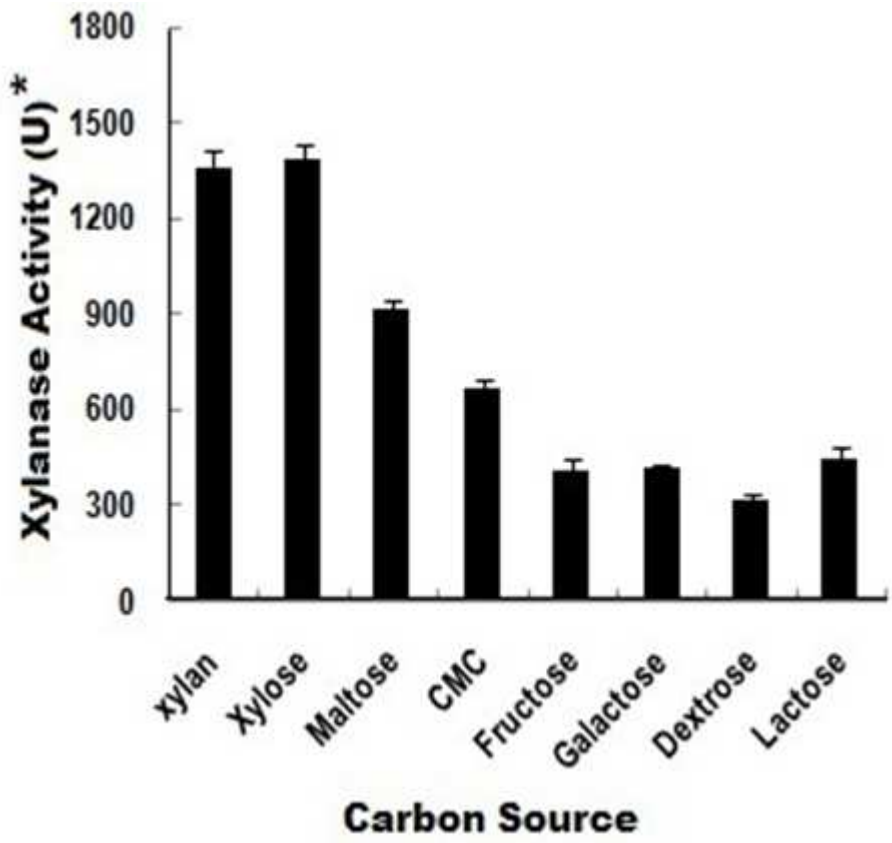

Fig 4: Effect of alternative carbon sources on the xylanase production by *Aspergillus flavus* xym4 (values are mean of three replicates ± SD). *1U xylanase activity= 1µmol xylose min^{-1}

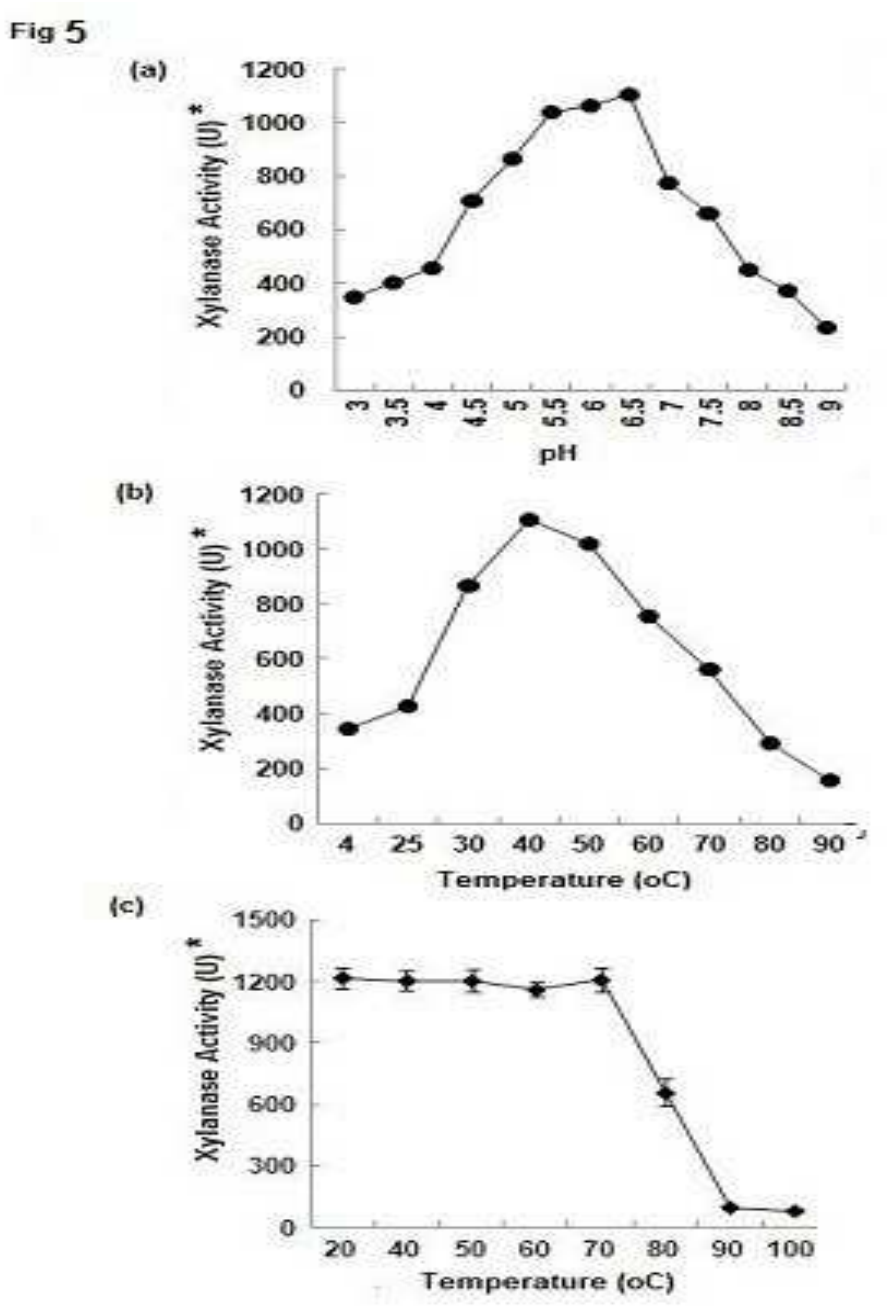

Fig 5: Effect of pH (a) and temperature (b) on xylanase from *Aspergillus flavus* xym4. [(a) The effect of pH was determined in 100 mM glycine (pH 3.0 and 3.5), 100 mM sodium acetate (pH 4.0, 4.5 and pH 5.5), 100 mM phosphate (pH 6.0, 6.5 and 7.0) and 100 mM Tris-HCl (pH 7.5, 8.0, 8.5 and 9) buffer at 37ºC (b) Profile of xylanase activity at optimum pH and at temperature ranging from 4º to 90ºC; and (c) Effect of temperature on stability xylanase. Purified enzyme preparation was incubated at indicated temperature for 30 min and enzyme activity was determined under standard conditions as described in 'Materials and Methods' section]. *1U xylanase activity= 1µmol xylose min^{-1}

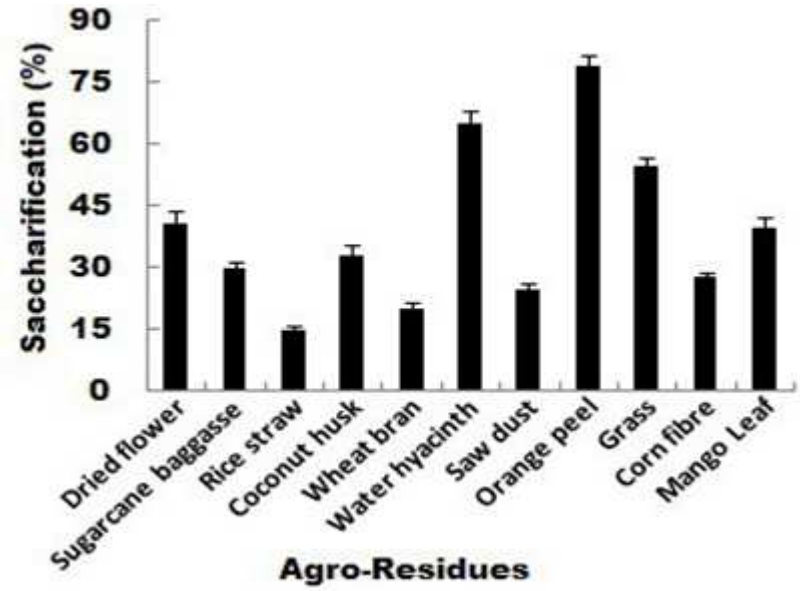

Fig 6: Saccharification activity of *Aspergillus flavus* xym4 xylanase on agro-residues. Agro-residues at a concentration of 5 mg/ml in 0.1M phosphate buffer (pH 7) were incubated with enzyme preparation for 5h at 30^{0}C.

Acknowledgement
Financial support for SG from Department of Biotechnology, Govt of India is gratefully acknowledged.

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