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

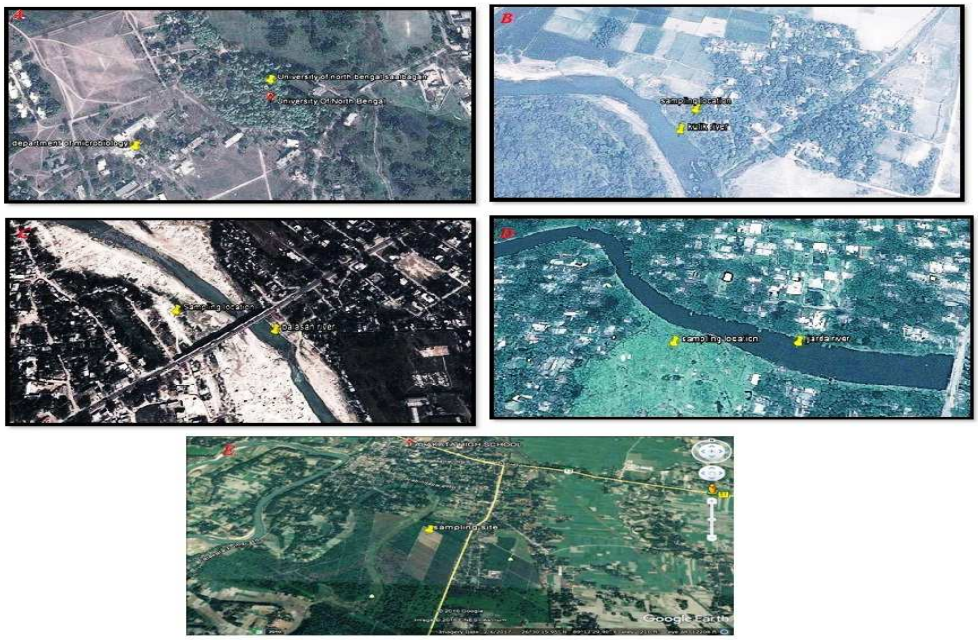


Fig. 2.1. Sampling sites for isolation of xylanase producing microorganisms. (A). Saal bagan, University of North Bengal; (B). Kulik River Basin; (C). Balasan River Basin; (D). Jarda River Basin; (E). Crop field near Falakata. (Image courtesy, Google Earth).

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.

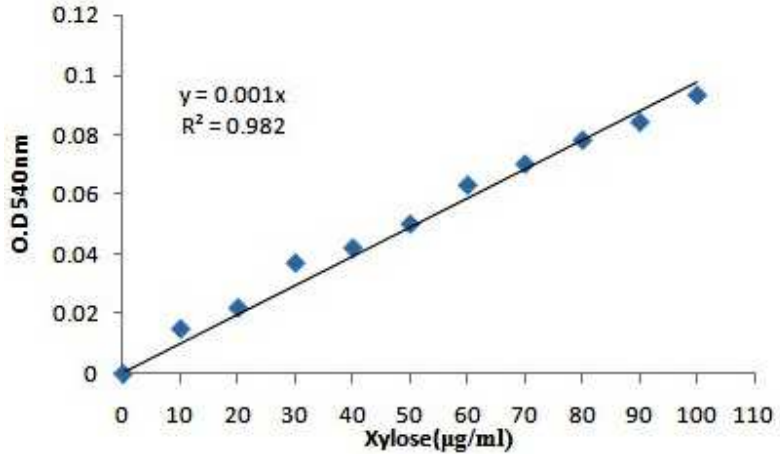


Fig. 2.2. Standard curve for xylose

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.

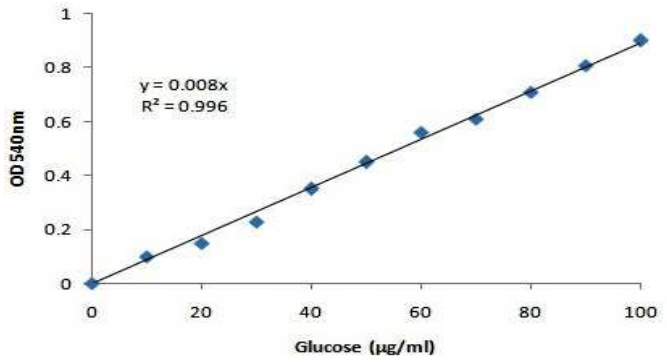


Fig. 2.3. Standard curve for glucose

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.

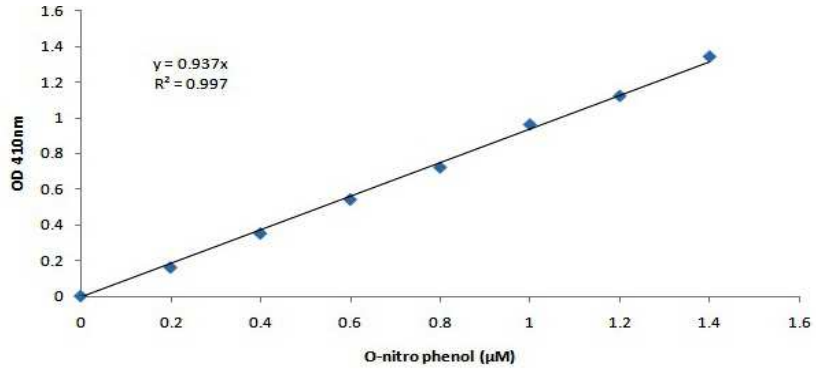


Fig. 2.4. Standard curve for ortho-nitrophenol

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.

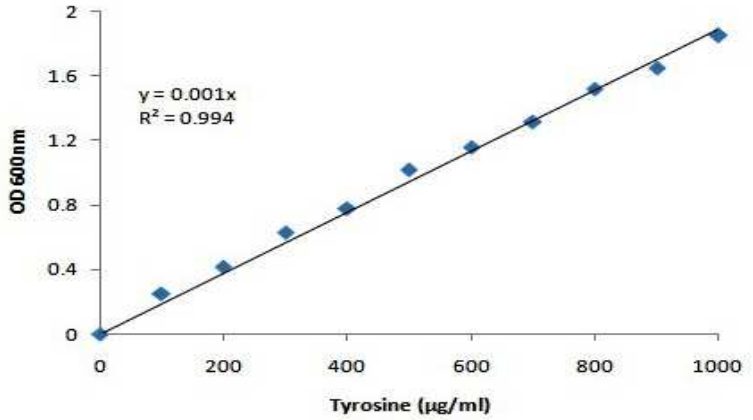


Fig. 2.5. Standard curve for tyrosine

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.

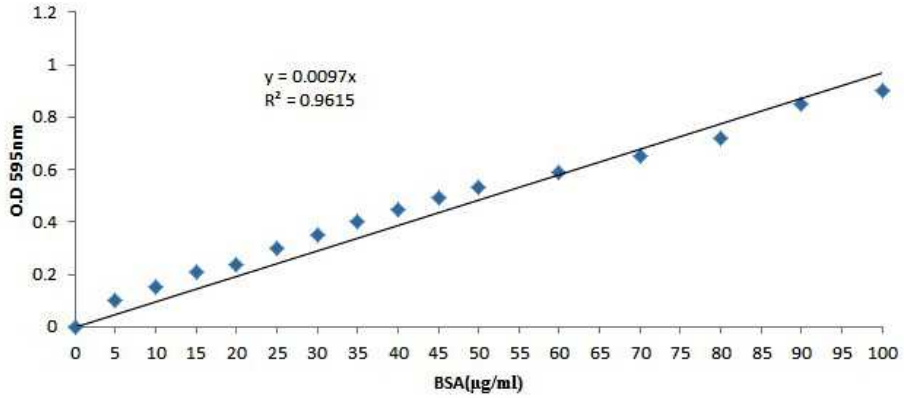


Fig. 2.6. Standard curve for BSA

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.

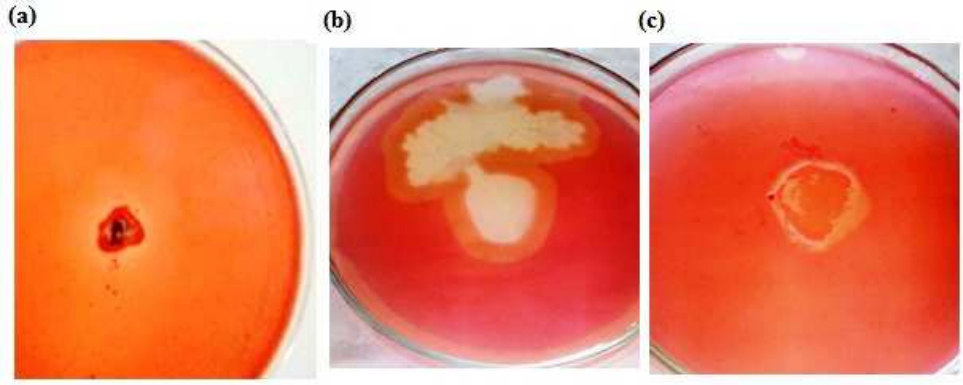


Fig. 2.7. Primary screening for detection of xylanase activity on xylan agar media (XYM) after congo red staining followed by washing with 1M NaCl ,showing yellow zone of clearance against red back ground (a) *Aspergillus flavus* xym4, (b) *Penicillium citrinum* xym2 and (c) *Bacillus subtilis* xym4

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.

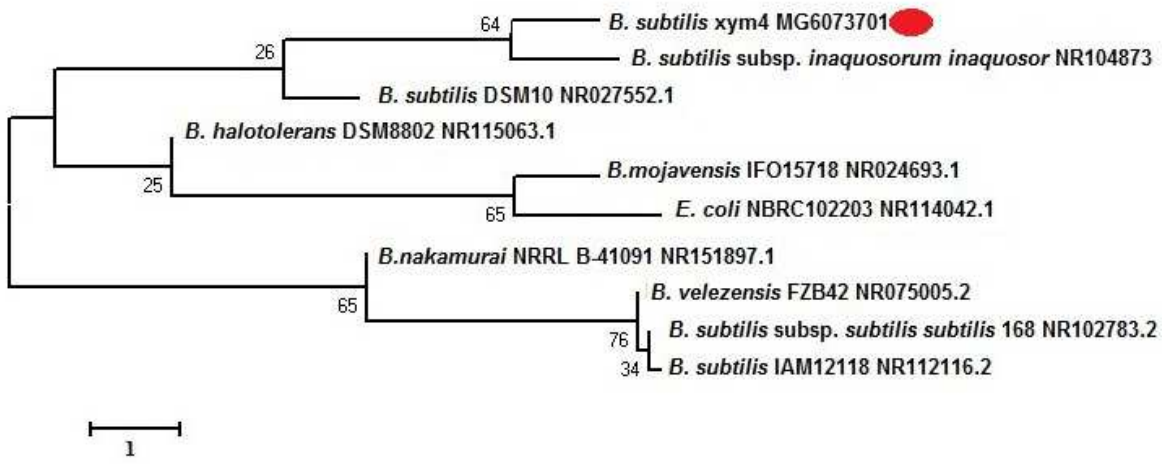


Fig.2.9. Phylogenetic tree constructed using 16S rRNA gene sequences reflecting the position of strain xym4 with the other *Bacillus* species. The sequence of *E. coli* NBRC102203 (accession no. NR114042.1) was incorporated in the tree as on out group. Bar represent 1 nucleotide substitution per base.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

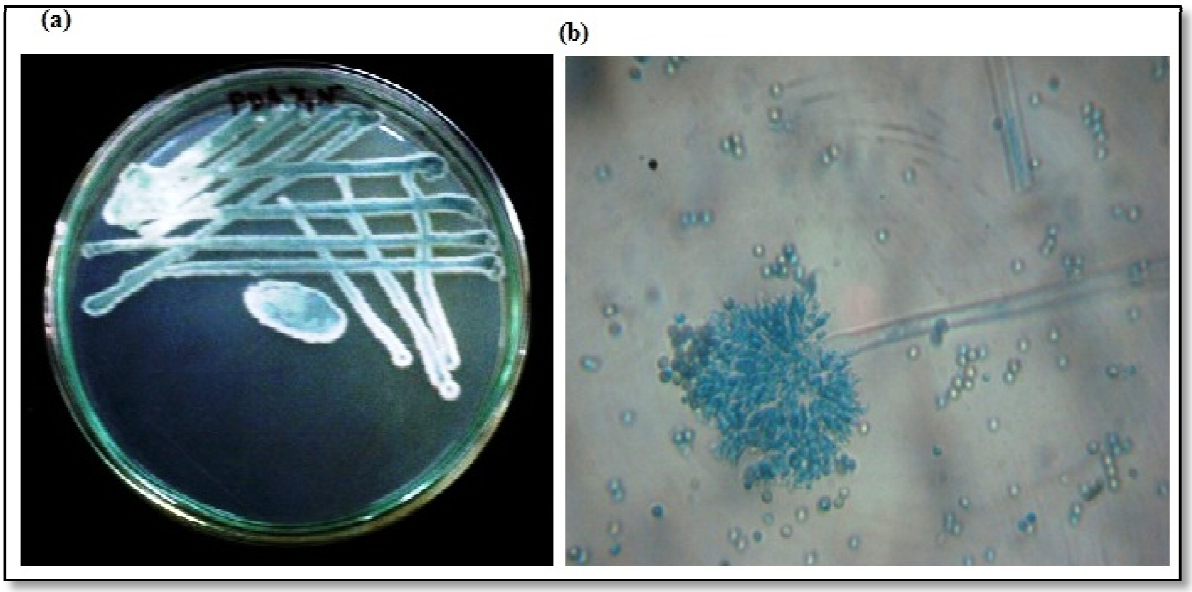


Fig 2.10. (a) Characteristic growth of *Aspergillus flavus* xym4 on PDA plate and (b) microscopic image of its hyphe along with sporangium and spores.

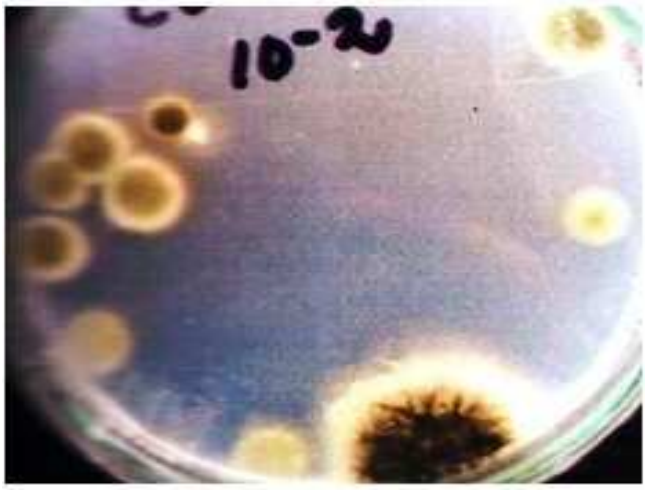


Fig 2.11. Growth of *Penicillium citrinum* xym2 on PDA plate.

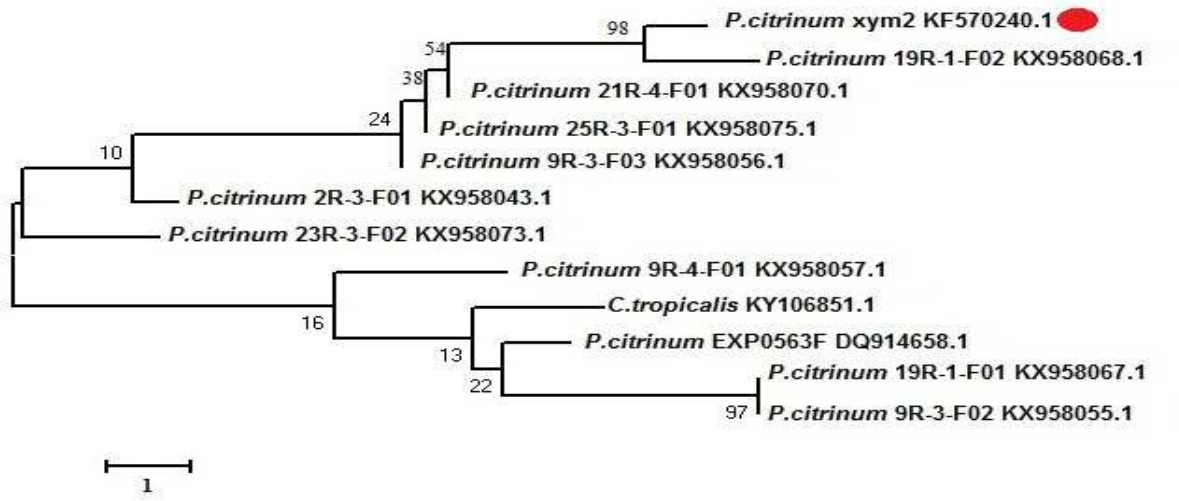


Fig.2.12. Phylogenetic tree constructed using 28S rRNA gene sequences reflecting the position of strain xym2 with the other *Penicillium* species. The sequence of *C. tropicalis* (accession no. KY106851.1) was incorporated in the tree as an out group. Bar represents 1 nucleotide substitution per base. 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.

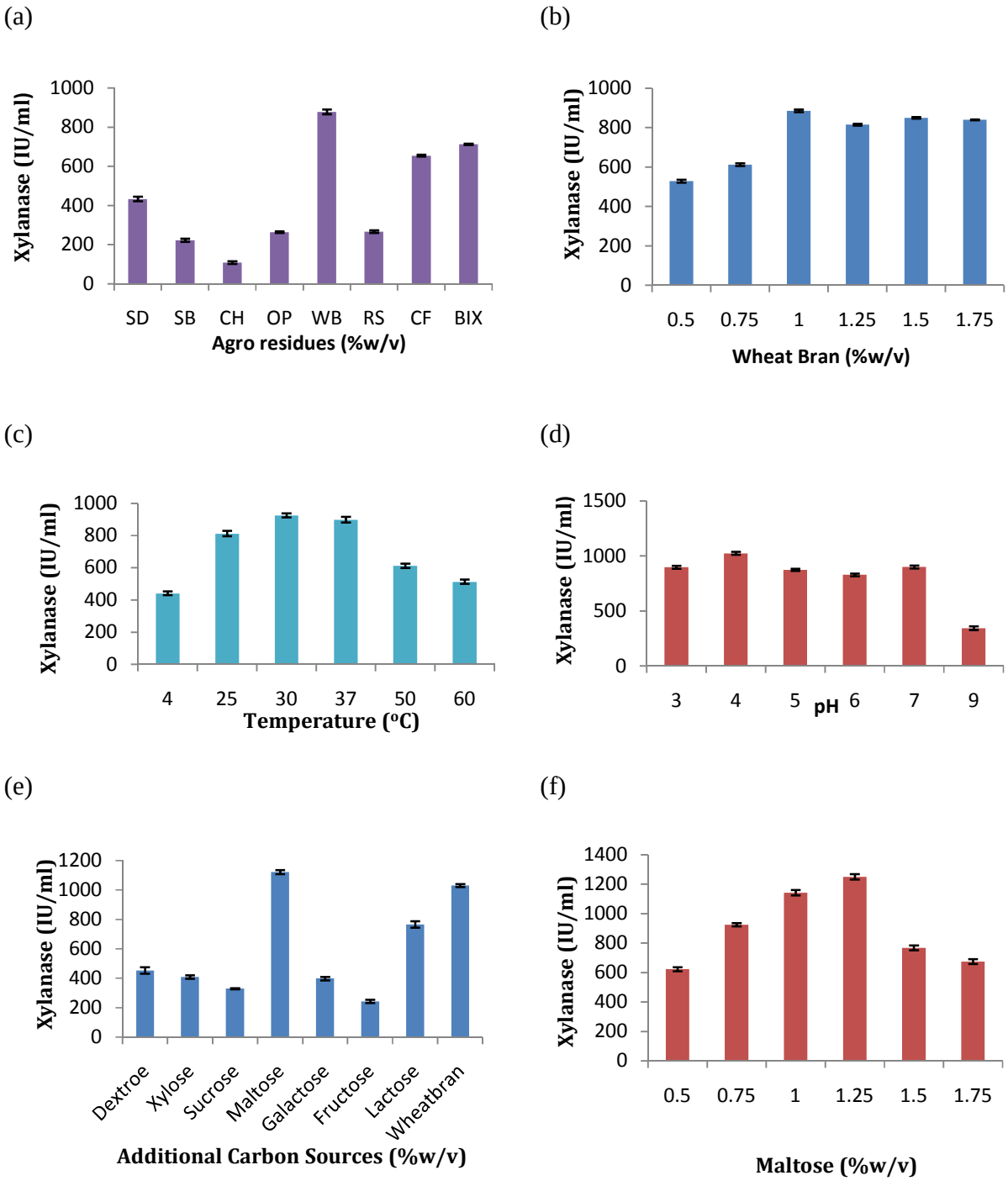
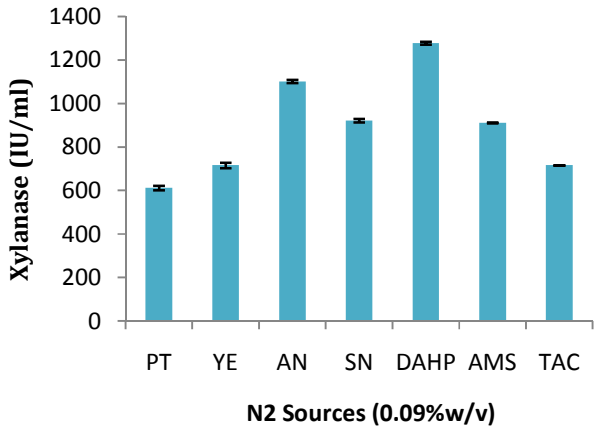
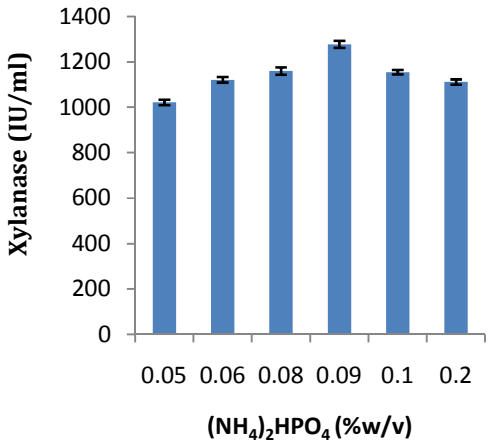


Fig.2.13. Optimization of xylanase production by *P. citrinum* xym2 by OFAT approach. Effect of (a) agro-residues [saw dust (SD), sugarcane baggase (SB), coconut husk (CH), orange peel (OP),wheat bran (WB), rice straw (RS), corn fiber (CF), birchwood xylan (BiX)] (b) wheat bran concentration (c) incubation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

(a)



(b)



(c)

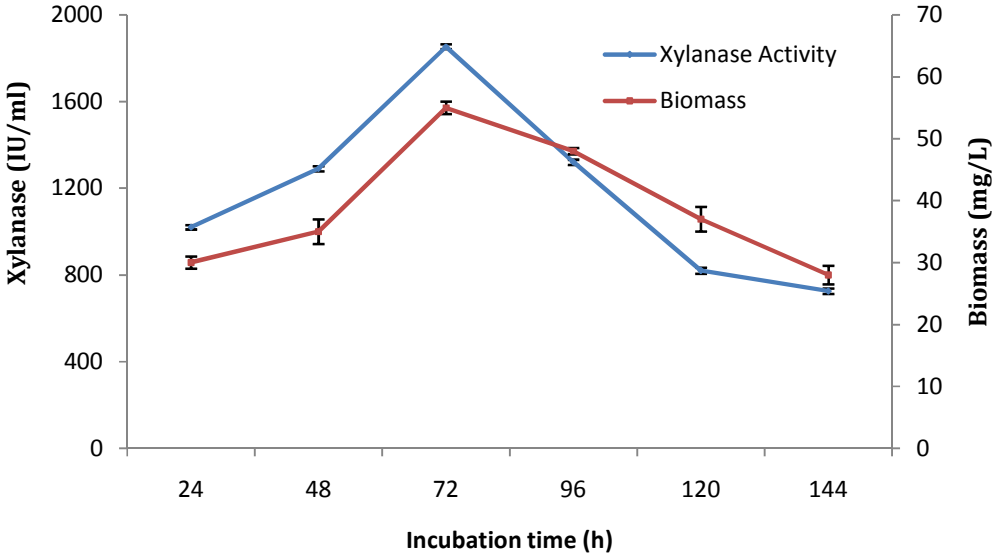


Fig.2.14. Optimization of xylanase production by *P. citrinum* xym2 by OFAT approach. Effect of (a) nitrogen sources [peptone (PT), yeast extract (YE), ammonium nitrate (AN), sodium nitrate (SN), di-ammonium hydrogen phosphate (DAHP),tri ammonium citrate (TAC)] (b) DHAP concentration (c) incubation time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

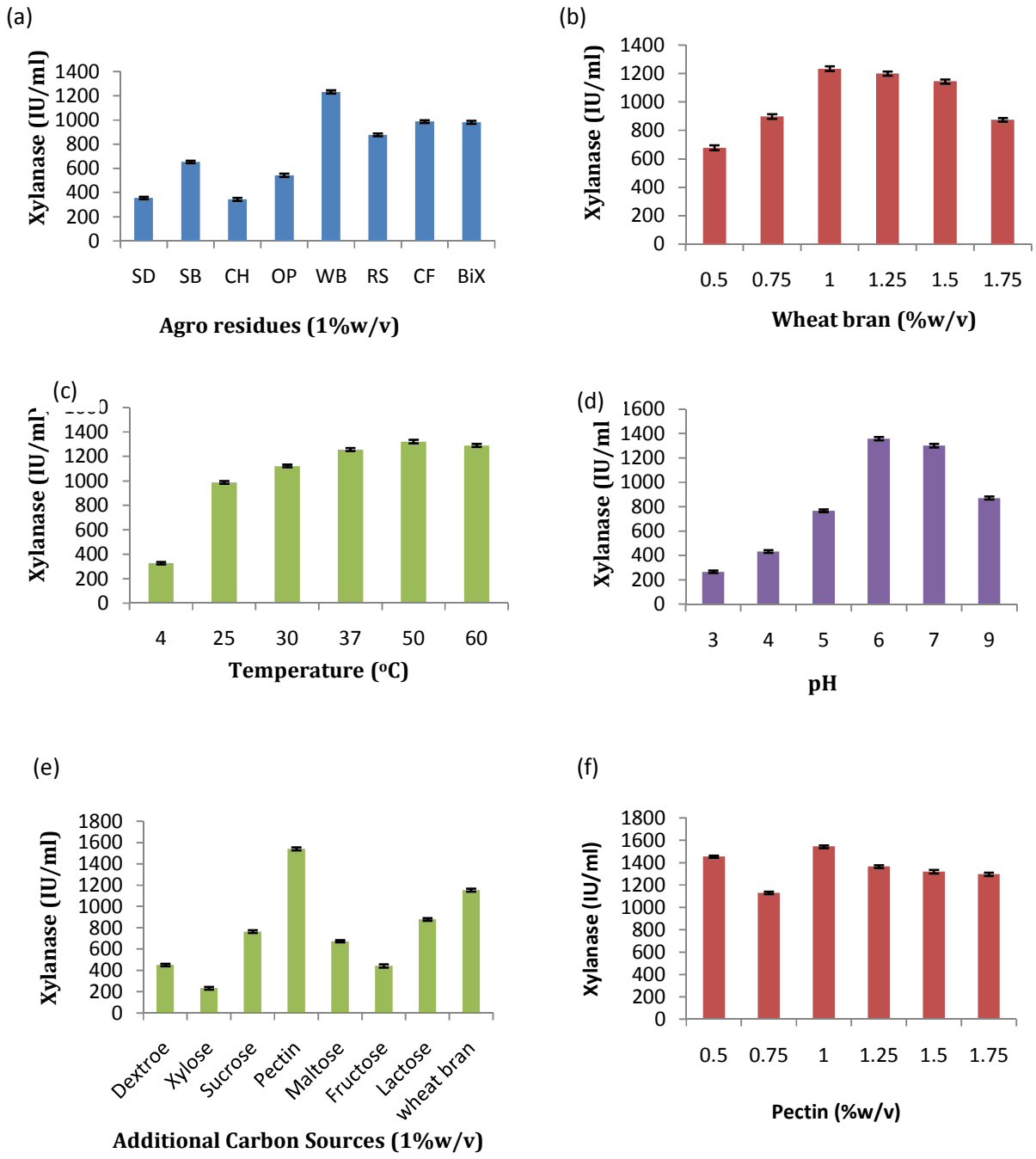
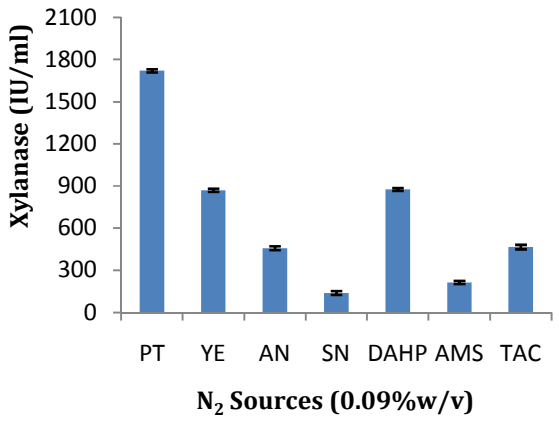
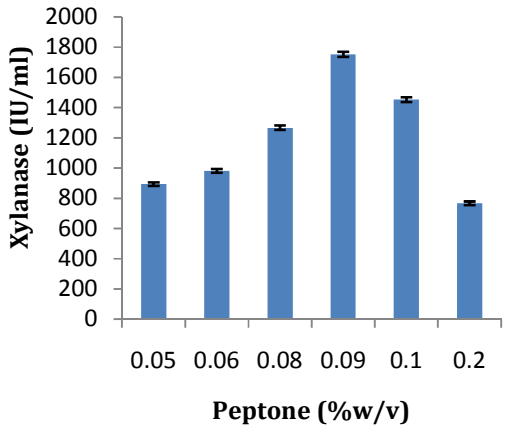


Fig.2.15. Optimization of xylanase production by *B. subtilis* xym4 by OFAT approach. Effect of (a) agro-residues [saw dust (SD), sugarcane baggase (SB), coconut husk (CH), orange peel (OP),wheat bran (WB), rice straw (RS), corn fiber (CF), birchwood xylan (BiX)] (b) wheat bran concentration (c) incubation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

(a)



(b)



(c)

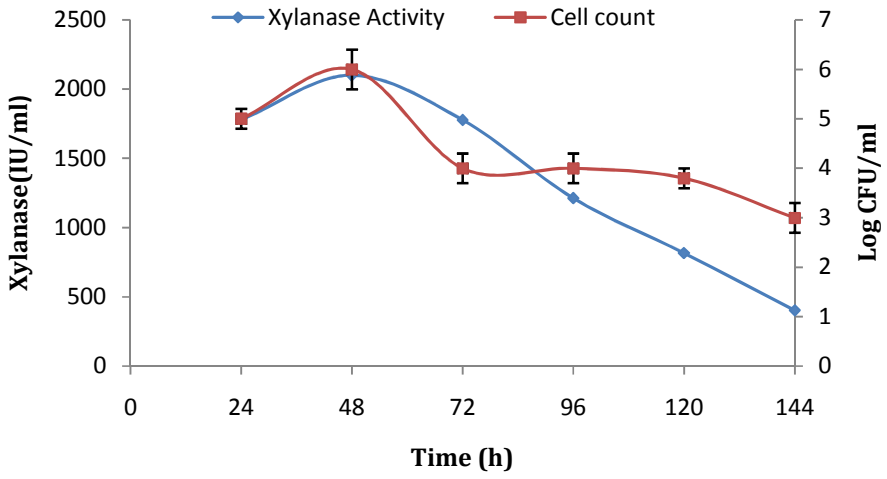


Fig.2.16. Optimization of xylanase production by *B. subtilis* xym4 by OFAT approach. Effect of (a) nitrogen sources [peptone (PT), yeast extract (YE), ammonium nitrate (AN), sodium nitrate (SN), di ammonium hydrogen phosphate (DAHP), tri ammonium citrate (TAC)] (b) peptone concentration (c) incubation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

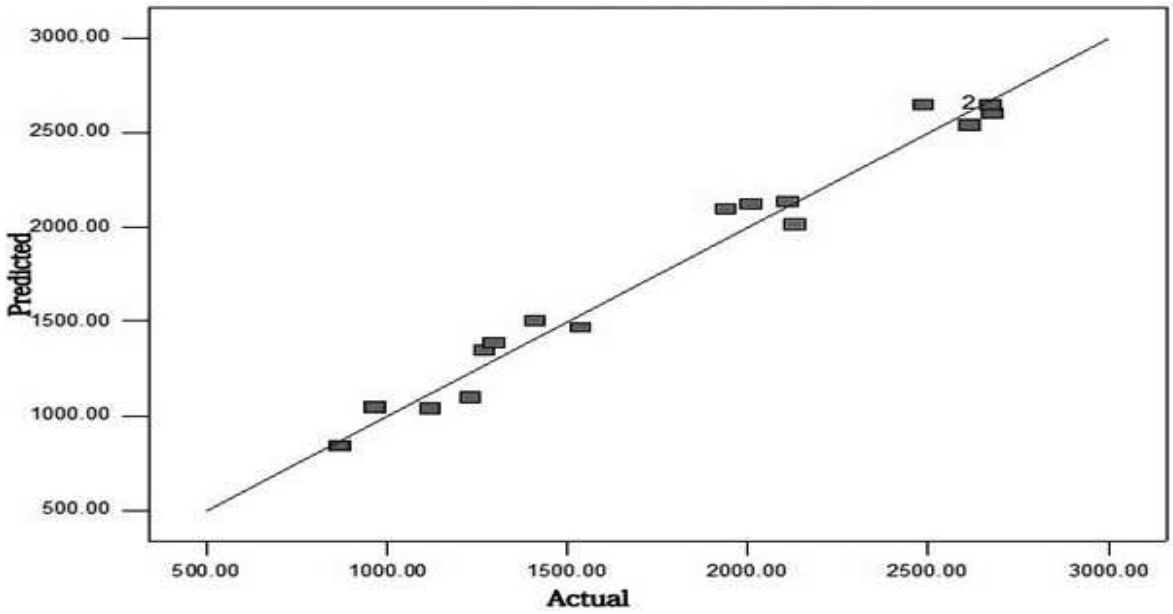


Fig.2.17: Model predicted and actual xylanase activity of *P. citrinum* xym2

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

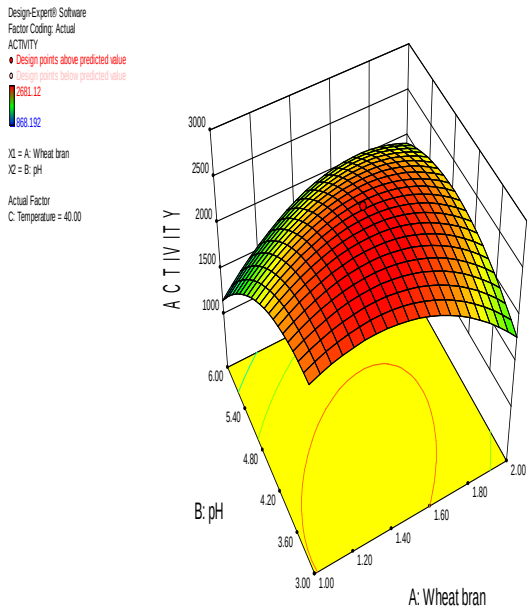
Variables analyzed in OFAT method was further evaluated in CCRD based RSM to further optimize the xylanase production. The effect of the four variables, pectin (A), incubation temperature (B), medium pH (C) and incubation time (D) on xylanase production was investigated. Suitable levels for these parameters 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.

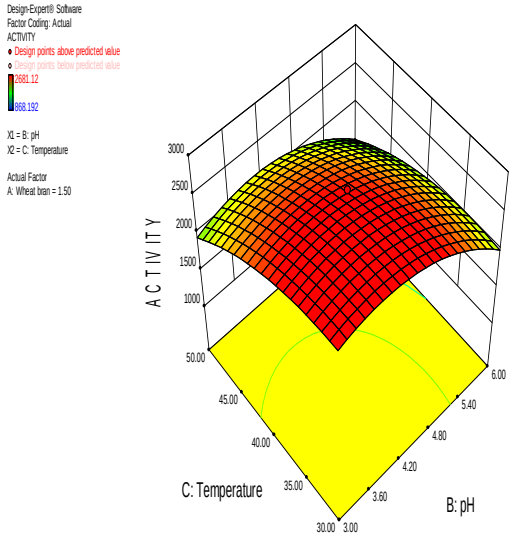
Table 2.8.
Validation of the CCD using different levels of wheat bran concentration, pH and temperature for xylanase production.

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

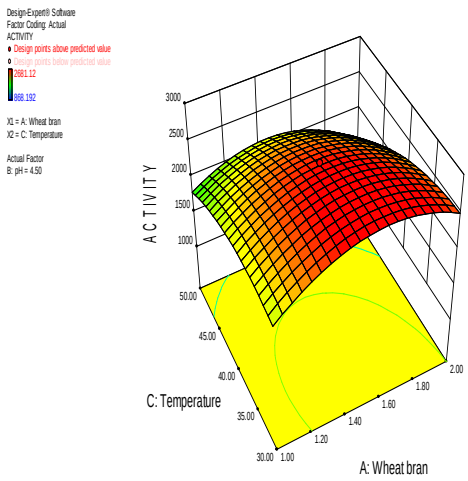
(a)



(b)



(c)



(d)

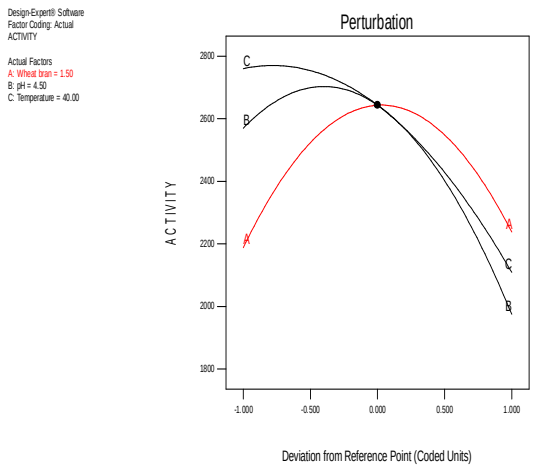


Fig 2.18. Response surface curves of xylanase production from *P. citrinum* xym2, showing interaction between wheat bran and medium pH (a), temperature and medium pH (b) and temperature and wheat bran (c) after 72h of incubation, (d) Perturbation plot of the model identifing the most significant variables.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

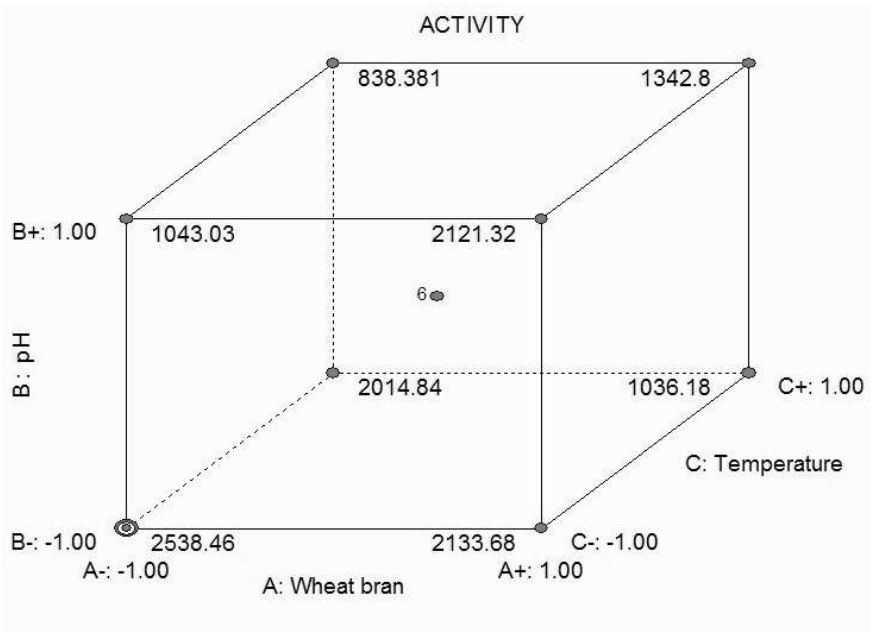


Fig.2.19. Cube plot showing the optimal point of xylanase production.

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.

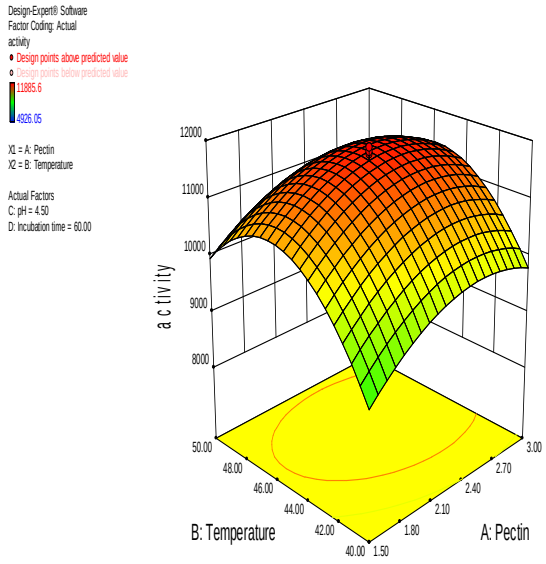
Table 2.9

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

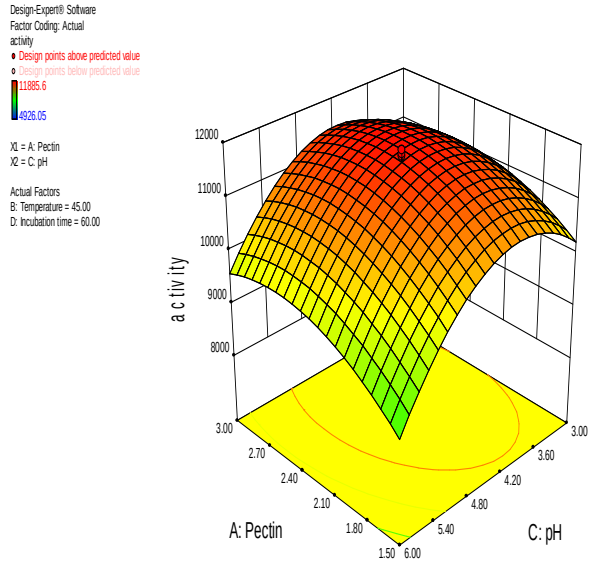
Run	PC[a]	T[b]	pH[c]	IT[d]	Xylanase activity [e](IU/ml)	Xylanase activity [f] (IU/ml)
1	1.5	50	3	72	8282.94	8156.75 ± 10.23
2	1.5	50	6	72	6285.49	6166.45 ± 11
3	2.25	45	4.5	36	5689.43	5772.65 ± 10.26
4	2.25	45	4.5	60	11661.53	11786± 1.23
5	1.5	50	6	48	5529.46	5403.3± 18.23
6	2.25	55	4.5	60	7288.03	7702.25± 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

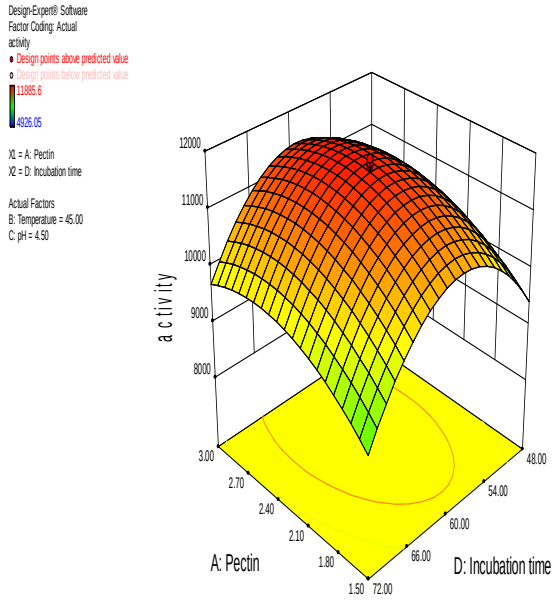
(a)



(b)



(c)



(d)

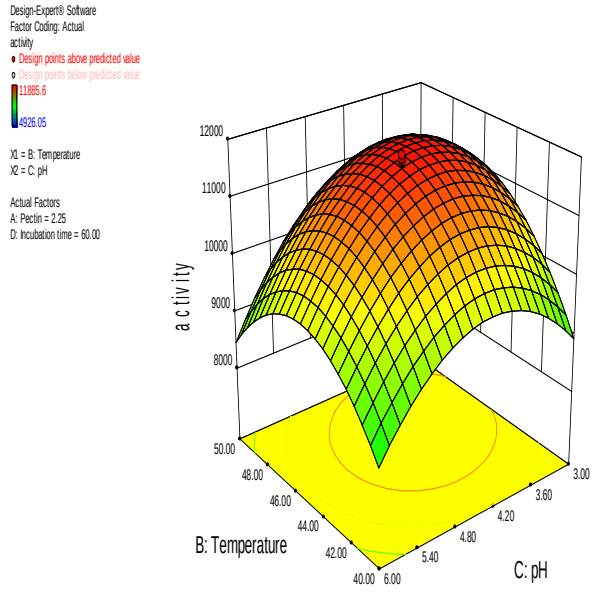
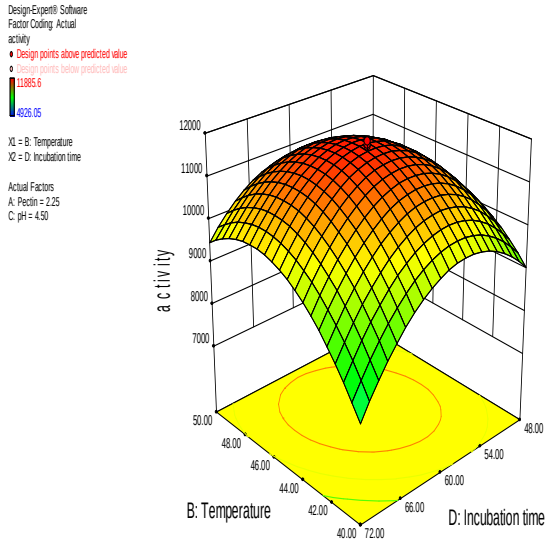
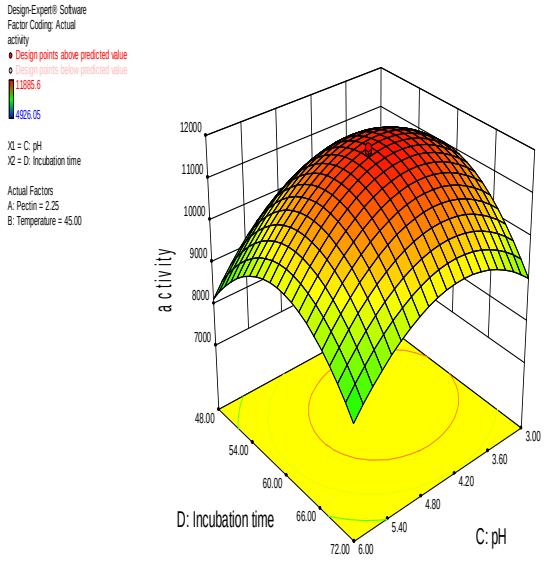


Fig.2.20. Response surface curves of xylanase production from *B. subtilis* xym4, showing interaction between incubation temperature and pectin concentration (a), pectin concentration and medium pH (b), pectin concentration and incubation time (c) and incubation temperature and medium pH (d). [Unit of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

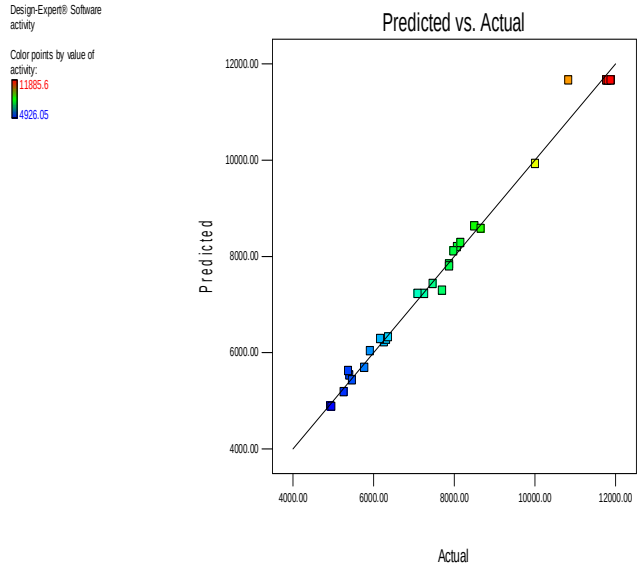
(a)



(b)



(c)



(d)

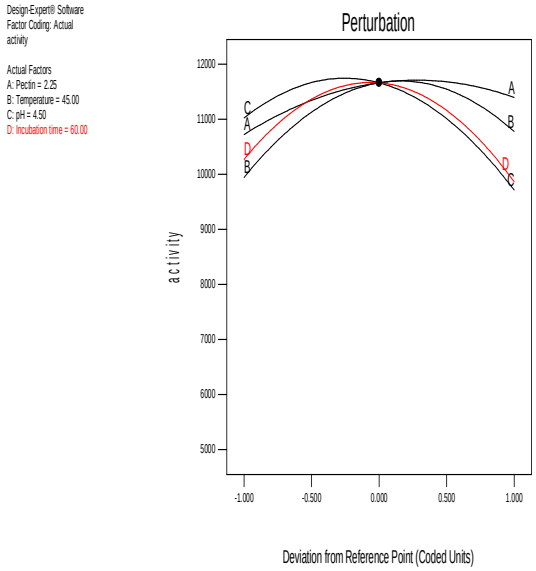


Fig. 2.21.Response surface curves of xylanase production from *B. subtilis* xym4, showing interaction between incubation temperature and incubation time (a), incubation time and medium pH (b), Predicted vs actual xylanase activity (c) and perturbation plot (d) of the model identifying the most significant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

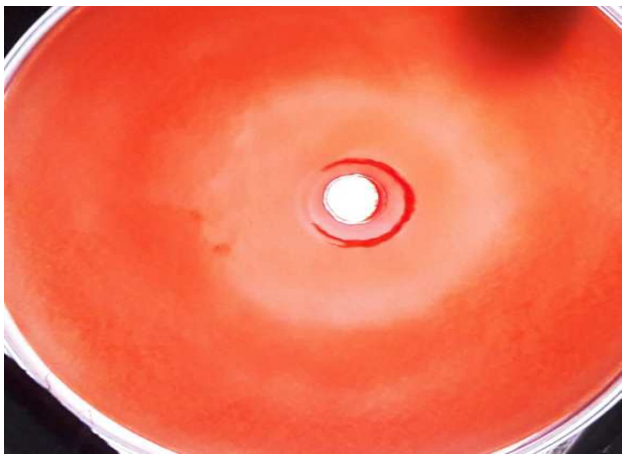


Fig 2.22. Xylan hydrolysis zone on XYM plates using xylanase obtained from *B. subtilis* xym4 cultivated in RSM optimized XPM

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.

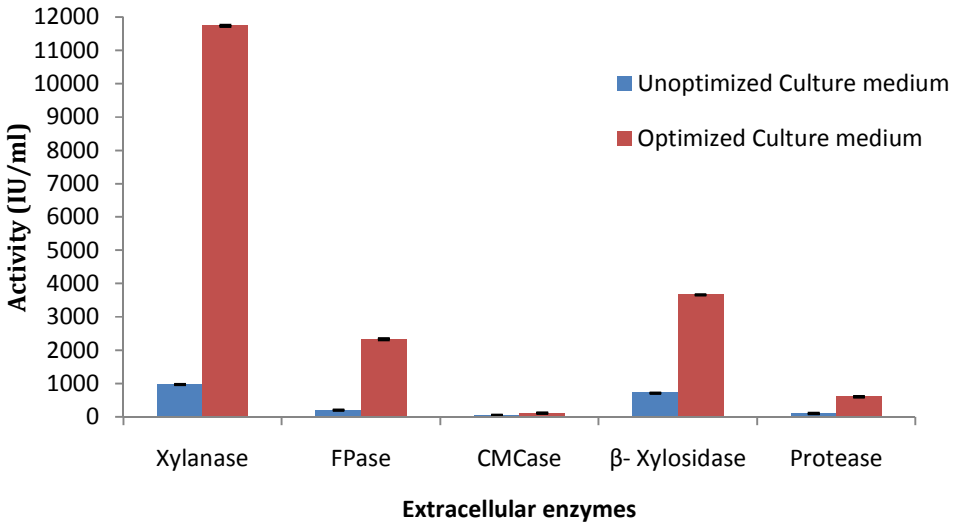


Fig. 2.23. Extracellular enzyme production by *B. subtilis* xym4

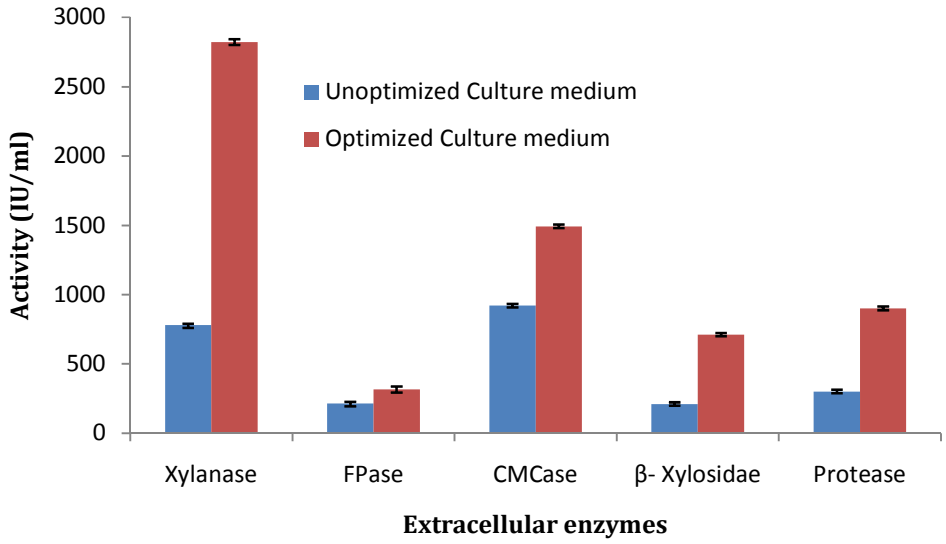


Fig.2.24. Extracellular enzyme production by *P. citrinum* xym2

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

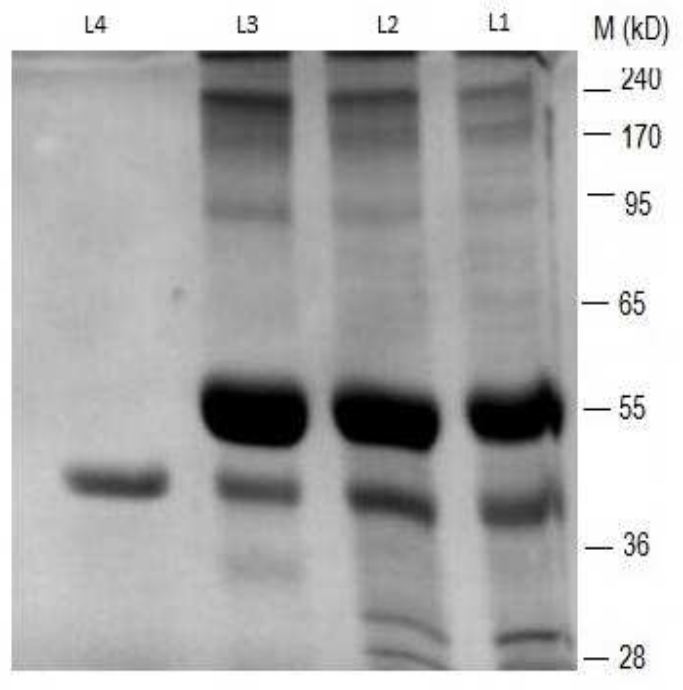


Fig. 2.25. Purification of xylanase from *B. subtilis* xym4. SDS-PAGE analysis of Crude extract (Lane 1). 40-70% ammonium sulphate fraction (Lane2), DEAE eluate (Lane-3), Biogel P100 eluate (Lane 4), Lane M-Protein molecular weight marker.

2.3.6. Characterization of xylanase

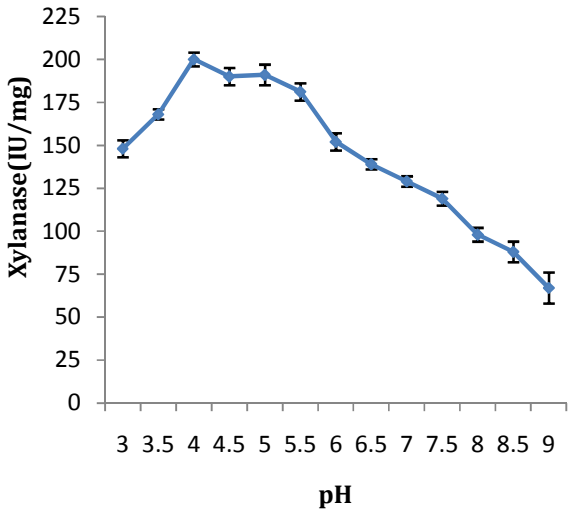
The xylanase enzyme produced by the isolates was characterized for pH optima, temperature optima, thermo stability, cation requirement and Km for birchwood xylan.

2.3.6.1. Determination of pH optimum, temperature optimum and thermostability

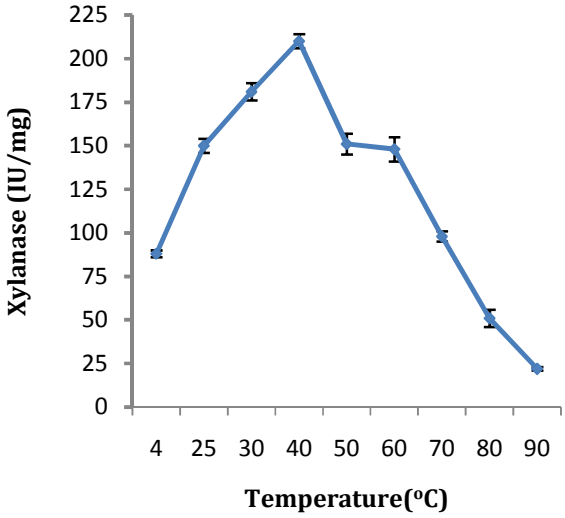
Optimum pH and temperature for the partially purified xylanase from *P. citrinum* xym2 was found to be pH 4 and 40^{o}C with xylanase specific activity of 200 and 210 IU/mg, respectively (Fig 2.26a &b). The enzyme was more active in acidic pH than alkaline pH. The enzyme showed thermostability at higher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



(b)



(c)

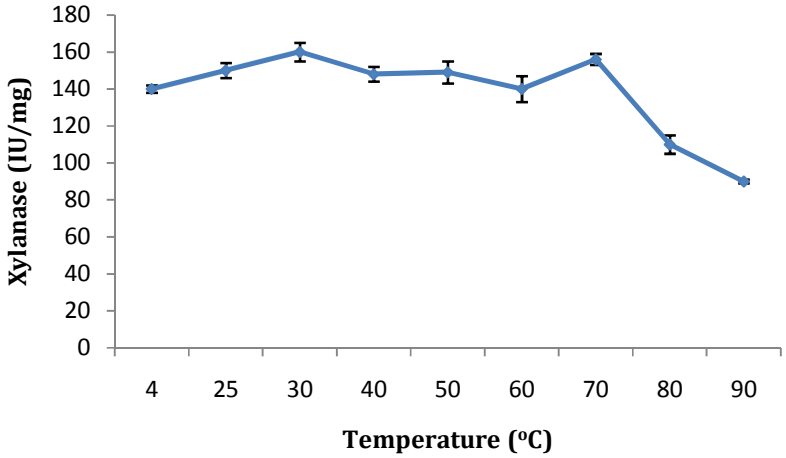
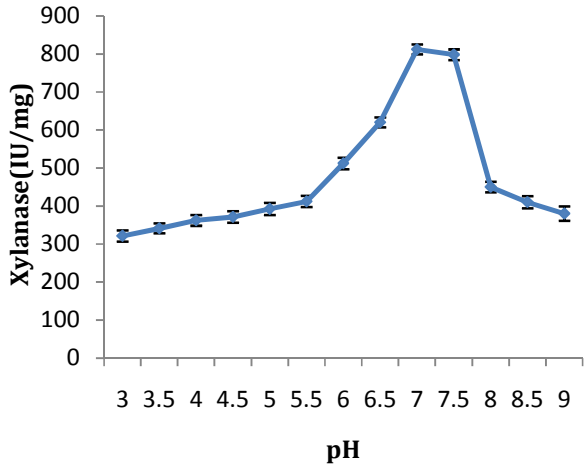
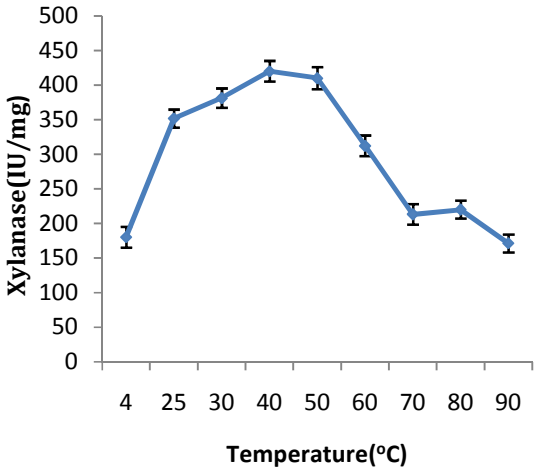


Fig 2.26. Characterization of xylanase obtained from *P. citrinum* xym2. (a) Effect of pH [The effect of pH was determined in 100 mM glycine (pH 3.0 and 3.5), 100 mM sodium acetate (pH 4.0, 4.5 and pH 5.5), 100 mMSodium phosphate (pH 6.0, 6.5 and 7.0) and 100 mMTris- HCl (pH 7.5, 8.0, 8.5 and 9) buffer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

(a)



(b)



(c)

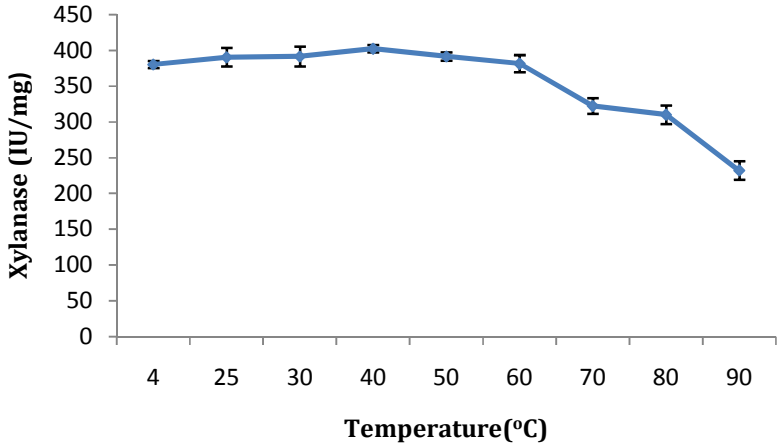


Fig 2.27. Characterization of xylanase obtained from *B. subtilis* xym4. (a) Effect of pH [The effect of pH was determined in 100 mM glycine (pH 3.0 and 3.5), 100 mM sodium acetate (pH 4.0, 4.5 and pH 5.5), 100 mMSodium phosphate (pH 6.0, 6.5 and 7.0) and 100 mMTris- HCl (pH 7.5, 8.0, 8.5 and 9) buffer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

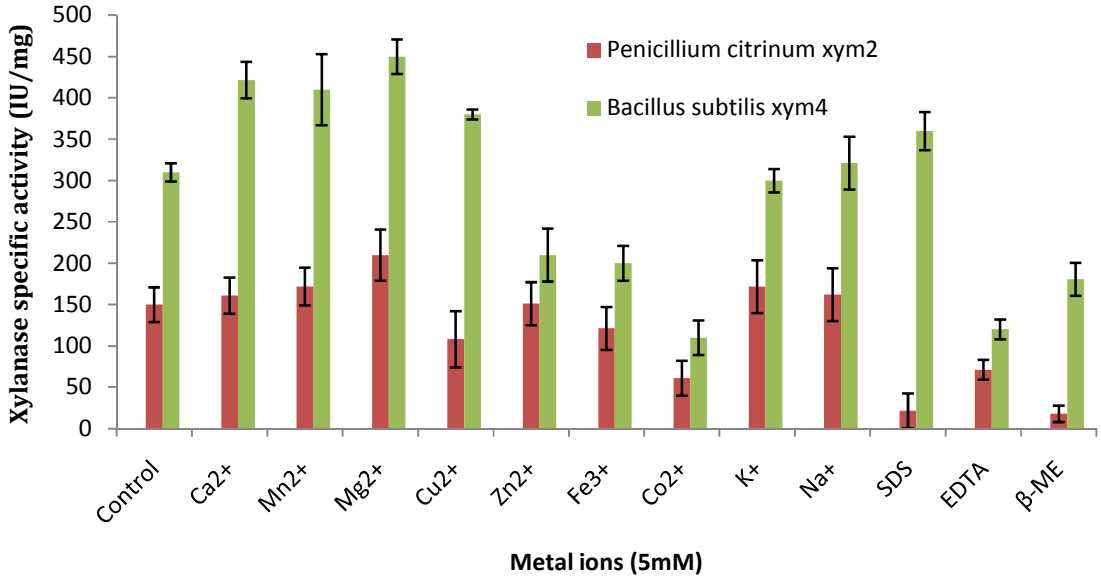


Fig 2.28. Effect of various metal cofactors and chemicals [5mM] on xylanase without any metal cofactors and chemical the enzyme substrate reaction mixture was served as control.

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.

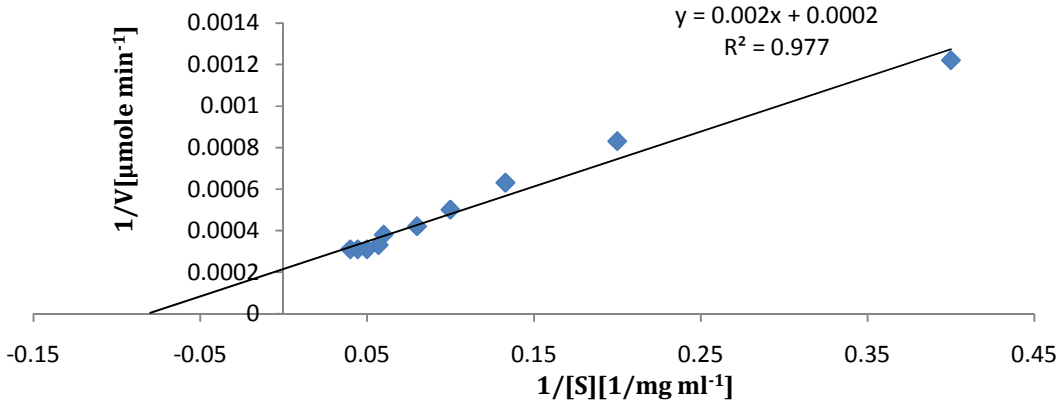


Fig 2.29. Lineweaver-Burk plot for determination of Km & Vmax of xylanase obtained from *B. subtilis* xym4

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.

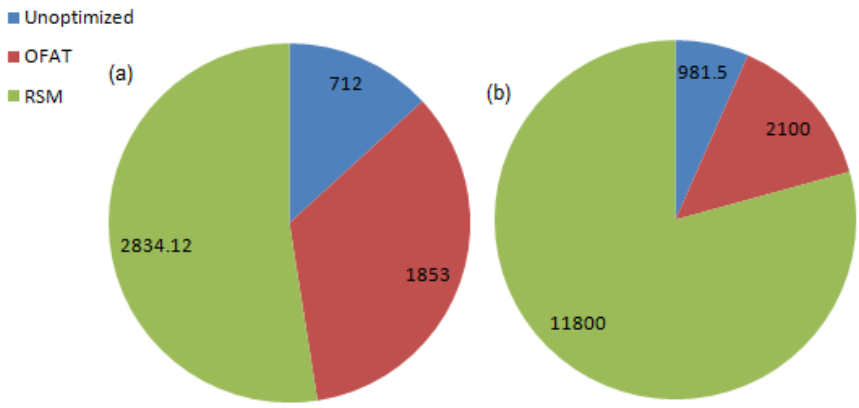


Fig.2.30. Optimization of xylanase production in different methods by (a) *P. citrinum* xym2 and (b) *B. subtilis* xym4