

# Utilization of agrowaste and xylanase production in solid state fermentation

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## Abstract

Aim of the present work was to study the cumulative effect of the environmental and nutritional parameters like pretreatment of substrate, temperature, pH, incubation period, solid to moisture ratio, inoculum size etc. to enhance the yield of extracellular 1,4-β endo xylanase in solid state fermentation using lignocellulosic agrowaste by *Penicillium citrinum* MTCC 9620. To obtain the mutual interaction and optimizing these variables, a 2<sup>4</sup> factorial central composite design was employed using Response surface methodology. The variables investigated were media pH (4.0-6.0); temperature (25-35°C); substrate to moisture ratio (1:4-1:6); and, incubation time (4-6 days). Higher xylanase activity was obtained at 1:5 solid to moisture ratio after 5 days of incubation at pH 5.0 at 30°C using Sugarcane bagasse. Dialysis followed by Q Sepharose column purification yielded 8.51- folds purified xylanase with 63.61% recovery. Presence of Hg<sup>2+</sup>, Cu<sup>2+</sup>, Ca<sup>2+</sup> and EDTA inhibited the activity but Na<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>3+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Tween 80 enhanced the activity.

**Keywords:** Xylanase, *Penicillium citrinum*, Solid State Fermentation, optimization, RSM

## Introduction

According to Sengupta, 2002 about 600 MT of agricultural wastes have been produced in India. The major quantity of agro-industrial wastes generated from sugarcane bagasse, paddy, wheat straw, husk, wastes of vegetables, tea- coffee waste, oil refinery, groundnut shell, coconut husk, etc. Throughout the year rising production of agro-industrial waste is a major concern of

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environmental pollution. The heterogeneous characteristics of the massive quantity of wastes generated annually lead to complexity in recycling and utilization. Solid state fermentation is one of the environmental friendly alternatives to produce different fermented value added products like enzymes, organic acids, bio-color, bio-flavor, bio-pesticides, bio-surfactant etc. using these agro-industrial residues which eventually results greener and cleaner environment (Ghoshal et al., 2012a).

Cheaper hemicellulosic agricultural residues like corn cobs, wheat bran, rice bran, rice straw, corn stalk and bagasse have been found to be the suitable solid substrate for the production of enzyme like xylanase using microorganisms such as *Aspergillus awamori*, *Penicillium purpurogenum* and alkalophilic *Bacillus* species NCIM 59.

Among fungi, the maximum activity in SSF has been obtained from the fungus *Schizophyllum commune* (22700 IU/g). When wheat straw was used as substrate *Trichoderma hamatum* is reported to produce 7000 IU/g of xylanase. Cellulase free xylanase has been produced by *Bacillus sp.* and *Streptomyces sp.* QG 11-3 (Haltrich et al., 1993; Beg et al., 2000).

Commercial applications of xylanase involve conversion of xylan into xylose (Subramanian et al., 2002); clarification of juices and wines, extraction of coffee, plant oils, and starch; improving the nutritional properties of agricultural silage and grain feed, and for the production of fuel and chemical feedstock (Beily, 1991; Wong et al., 1998; Harbak and Thygesen, 2002). Currently use of xylanolytic enzymes in pulp bleaching has been found as one of the major biotechnological applications. Hydrolysis of xylan aids with the release of lignin from paper pulp eventually minimizes the dosage of chlorine as the bleaching agent. The biotransformation of lignocelluloses to fermentable sugars like xylose, xyllobiose, and xylo-oligomers can be attained by the enzymatic hydrolysis of xylan. Xylanases are used in bakery as dough strengthener to provide excellent tolerance to the dough towards altering processing parameters and variation in quality of flour. Xylanases also significantly increase volume of the

baked bread and can be used as anti-staling agent in bread manufacturing (Gaavilighi, 2006; Ghoshal et al., 2013).

Selection of a suitable inducing substrate and the best possible medium composition are the two basic factors for efficient production of xylanolytic enzymes. The substrate serves dual objectives solid support as well as carbon and energy source which provides the necessary inducing factors to the organisms for the production of desirable enzymes. Purified xylan is an excellent carbon source for xylanase production in small scale. Previous reports suggest that higher yield of xylanase with negligible cellulase can be obtained using pure xylan as carbon source by different organisms (Gilbert et al., 1992; Ghoshal et al., 2012b). However, pure xylan is not cost effective for the large scale production process. Therefore xylan rich agricultural residues are being used as substrate at industrial scale. Earlier report (Royer and Nakas, 1989) suggests that better yield of xylanase was achieved from selected lignocellulosic substrate than pure xylan or cellulose when various insoluble lignocellulosic substrates (barley husk, corn cobs, hay, sugarcane bagasse, wheat bran and straw) were compared for xylanase production (Haltrich & Steiner, 1994; Gomes et al., 1992; Haltrich, 1993). Certain problems such as viscous media, agitations, etc. arise while using these insoluble lignocellulosic materials as carbon sources. Therefore, most of the time along with soluble carbon source, insoluble lignocellulosic residues are preferably used for xylanase production (Purkarthofer et al., 1993).

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

To make any process economic, optimization of media composition and process condition is essential. Conventional method is lengthy and often does not reveal the effect of interaction of different parameters correctly. Response surface methodology (RSM) is the frequently used statistical technique for bioprocess optimization. RSM is a combination of numerical and statistical techniques useful for analyzing the effect of selected independent variables. In this process low order polynomial equation containing independent variables in a predetermined region is used. These variables are later examined to establish the optimum values of the independent variables for the best response and also to assess the correlation between a set of controllable experimental factors with the observed outcome. The interaction among the possible influencing parameters can be evaluated with limited number of experiments (Myers & Montgomery, 1995). Although there are reports of optimization of xylanase using RSM with different fungi and bacteria, there are not many expert reports on optimization of xylanase production using *Penicillium citrinum* (Puri et al., 2002; Katapodis et al., 2007).

In the present study, we discussed optimization of incubation parameters and media components of xylanase production by central composite design (CCD) of RSM using Design expert ver 6.0.9 statistical software, purification and characterization of isolated xylanase and effect of different additives on xylanase stability were also investigated.

## Materials and Methods

### *Chemicals and substrates*

All the chemicals used were AR grade. Birch wood xylan, N-Acetyl D- glucosamine and N-N-dimethyl-p-aminobenzaldehyde were purchased from Sigma-Aldrich, Germany. Sugarcane bagasse was purchased from local market of Chandigarh.

### *Microorganisms and growth conditions*

Fungal strain *P. citrinum* MTCC 9620 was isolated from soil of Panjab University, Chandigarh Campus. Czapek Yeast Extract media containing 3.0 g/L NaNO<sub>3</sub>, 0.5 g/L KCl, 0.5 g/L MgSO<sub>4</sub>, 7H<sub>2</sub>O, 0.01 g/L FeSO<sub>4</sub>, 7H<sub>2</sub>O, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 5 g/L yeast extract, 30 g/L sucrose and 1% birchwood xylan was used for isolation of organism. 1 g of paper pulp waste was mixed with 10 mL sterilized distilled water to make slurry and the slurry was used as inoculums. Spread plate method was used to isolate fungal strains. The inoculated plates were incubated at 30°C for 72 h. Identical larger colonies were selected and were further grown in Czapek yeast extract media in 250 mL conical flask containing 50 mL media. The organism was maintained in the Czapek yeast extract agar media and preserved at 4°C.

All the assays were carried out in triplicates along with appropriate buffer and reagent control.

### *Estimation of total sugar*

Total sugar content of the centrifuged supernatant of culture broth was estimated following Somogyi's method using Anthrone reagent (Somogyi, 1945). The absorbance was measured at 625 nm against a reagent blank. The sugar content was expressed in terms of g/L. The absorbance was monitored at 680nm.

### *Estimation of protein content*

Extracellular protein concentration was estimated by Lowry method using bovine serum albumin (BSA) as standard (Lowry, 1951). Standard curve was prepared using BSA (1mg/mL) dissolved in distilled water.

### *Estimation of biomass*

Biomass estimation was carried out during the course of fermentation. Biomass was estimated by indirect method by estimating glucosamine content after acid hydrolysis of the dry fermented substrate. 1g of dry fermented substrate was suspended in 10mL of 6N HCL in screw cap tubes. The tubes were heated in a boiling water bath for 4 h, cooled to room temperature and solids were separated. Supernatants were neutralized with 2N NaOH and used for glucosamine assays. Glucosamine content in the supernatant was determined by Ehrlich's reagent (1.6 g N-N-dimethyl-p-aminobenzaldehyde in a 1:1 mixture of ethanol and concentrated HCl) from standard glucosamine plot. The biomass glucosamine was calculated by measuring glucosamine content in fermented dry substrate minus glucosamine content of the inoculated substrate. The conversion of glucosamine to biomass was adopted according to Said (Said, 2010). The conversion factor for glucosamine content to dry biomass was calculated by estimating glucosamine content of the dry biomass in submerged fermentation. Based on the data of the submerged culture, where the entire nutrient was in soluble form, 120 mg of glucosamine was found equivalent to 1 g dry fungal biomass. Fungal biomass content of each batch of fermentation condition was expressed in g/L.

### *Enzyme assay*

Xylanase activity was measured using 1% birchwood xylan solution as a substrate (Bailey et al., 1992). The release of

reducing sugars in 5 min at 50°C, pH 5.3 (0.05 M citrate buffer) was measured as xylose equivalent using dinitrosalicylic acid method (Miller, 1959). One unit of xylanase activity (U) is defined as the amount of enzyme liberating 1 μmol of xylose per min.

Filter paper cellulase activity (U) was measured according to IUPAC recommendations employing filter paper (Whatman No.1) as substrate (Ghosh, 1994). Release of reducing sugar in 60 min at 50°C at pH 4.8 (0.05 M citrate buffer) was measured as glucose equivalent using dinitrosalicylic acid method. One unit of filter paper cellulase activity is measured as the amount of enzyme liberating 1 μmol of glucose per min.

#### *Optimization of xylanase production in solid state fermentation*

Effect of medium composition and environmental factors of xylanase production by selected isolates was studied in solid state fermentation in shake flask to maximize the xylanase yield.

#### *Effect of different lignocellulosic substrate on xylanase production*

Wheat straw, wheat bran, rice straw, rice bran, sugarcane bagasse, xylan, and corn cobs were used as lignocellulosic substrates for xylanase production in solid state fermentation. Each substrate (5 g) was transferred into 250 mL Erlenmeyer flask and Czapek Yeast Extract media was added such that the final solid to moisture ratio was 1:5. The flasks containing substrate and moistening media were then sterilized for 15 min at 15 psig pressure and 121°C temperature. One loop full of organism was suspended in 10 mL sterilised distilled water. Effect of substrates on xylanase production was evaluated by adding the inoculums maintaining 10<sup>8</sup> spores/g of dry substrate at 5.0 pH. The flasks were incubated at 30°C for 5 days.

#### *Effect of treatment of lignocellulosic substrate on xylanase production*

All the lignocellulosic substrates were dried at 40°C till constant weight in a tray dryer (Basic Technology Private Limited, Kolkata), ground in a mixer (Philips, India), passed through a sieve of 0.5 mm aperture size and used as substrate in SSF.

The selected substrate was cleaned using distilled water, dried, and treated. The treatment included: (i) alkali treatment using 1N NaOH followed by neutralization with 1N HCl and repeated washing with distilled water; (ii) acid treatment with 1N HCl followed by neutralization with 1N NaOH and washing with distilled water; and, (iii) steam treatment at 121°C for 2 h. The substrate was dried at 50°C till constant weight, ground to ≤ 0.5 mm particle size, packed in polyethylene bags and stored until use. Untreated substrate was used as control.

#### *Scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) study of the treated and untreated lignocellulosic residues*

Treated and untreated lignocellulosic residues were tested under scanning electron microscope. SEM was performed on dried powdered samples which were mounted on double side sticky tape on aluminium stubs and sputter coated with gold and palladium alloy. The samples were then visualized and the representative areas were photographed under SEM (JSM-6100, JEOL, Tokyo, Japan) at an accelerated voltage of 10KV and magnification in the range of 100-2000X.

FTIR spectra were recorded at room temperature (28± 2°C) with a Tensor-27 spectrophotometer (Bruker, Germany) in the range of 600-4000 cm<sup>-1</sup> by accumulating 16 scans at 4 cm<sup>-1</sup> resolution. For

spectral measurement of lignocellulosic residue platinum ATR (attenuated total reflectance) from Bruker was used. About 2 mg of dried treated and untreated powdered lignocellulosic residues were placed on ATR for measurement. The peak intensity of each samples were measured and this entire spectra acquisition procedure took 1 min per sample.

#### *Nitrogen source*

The organic nitrogen sources (peptone, meat extract, tryptophane, soya peptone, brain and heart infusion, soya bean meal, beef extract, yeast extract, protease peptone), and inorganic nitrogen source like urea, sodium nitrate, ammonium chloride, ammonium sulphate were used in the basal medium (Czapek yeast extract) without adding any nitrogen source.

#### *Optimization of different environmental factors using response surface methodology*

The substrate giving the maximum xylanase production was subsequently used to investigate the effect of selected process variables (pH, temperature, solid to moisture ratio, and incubation time) on xylanase production. After the addition of inoculums, flasks containing substrate and moistening agent at the ratio of 1:4-1:6, pH 4.0-6.0 were incubated at 25-35°C for 4-6 days.

#### **Experimental design and data analysis**

Optimum operating conditions were determined using RSM with 2<sup>4</sup> factorial designs for the process variables A, B, C, D (pH, temperature, substrate to moisture ratio, and incubation time) with three levels each. Enzyme activity (U/mL) after desired period of incubation was measured as the response (equation 1).

$$Y = b_0 + \sum b_i X_i + b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (1)$$

where, Y is predicted response (xylanase activity); X<sub>i</sub>, X<sub>i</sub><sup>2</sup>, X<sub>i</sub>X<sub>j</sub> are the variables representing linear, quadratic, and interactive effect; b<sub>0</sub> is regression coefficient at the centre of the regression model; and, b<sub>i</sub>, b<sub>ii</sub>, b<sub>ij</sub> are coefficients estimated by the model, representing the linear, quadratic, and interactive effects of the coded variables (A, B, C, D).

Design Expert ver. 6.0.9 statistical software (Stat-Ease Inc, Minneapolis, MN) was used to carry out the statistical analysis. Data was analyzed to estimate whether a given term has a significant effect (p≤0.05) on enzyme activity using the analysis of variance (ANOVA) combined with the Fischer test. Graphical and mathematical analysis was carried out using Design Expert program to determine optimum intensity level of the variables.

The overall second order polynomial relationship between Y and the variables can be represented as:

$$Y = b_0 + b_1A + b_2B + b_3C + b_4D + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{44}D^2 + b_{12}AB + b_{13}AC + b_{14}AD + b_{23}BC + b_{24}BD + b_{34}CD \quad (2)$$

where, A, B, C, D are the coded variables for pH, temperature (°C), substrate to moisture ratio, and incubation time (days); and b<sub>0</sub>, b<sub>1</sub>, b<sub>2</sub>, b<sub>3</sub>, b<sub>4</sub>, b<sub>11</sub>, b<sub>22</sub>, b<sub>33</sub>, b<sub>44</sub>, b<sub>12</sub>, b<sub>13</sub>, b<sub>14</sub>, b<sub>23</sub>, b<sub>24</sub>, b<sub>34</sub> are the coefficients.

### Purification of xylanase

The crude enzyme extract was concentrated by ammonium sulphate precipitation (60%) followed by dialysis for overnight in 0.05M citrate buffer. The concentrated fraction after dialysis was further concentrated using Amicon cones of 10 KDa cut off by centrifuging (Sigma, USA) at 5000 rpm for 5 min. It was then loaded into Q-sepharose anion exchange column of 2 mL capacity equilibrated with 0.1M phosphate buffer containing 1M NaCl (pH 7.0) at a flow rate of 0.1mL/min using Pharmacia® FPLC system. The column was washed with 50 mL loading buffer and eluted with linear gradient 1M NaCl. The active fractions containing xylanase were pooled and concentrated using Amicon cones (UFMC, NMWC, 10 KDa) by centrifuging (Sigma, USA) at 5000 rpm for 5 min. The purification profile of xylanase of different step was estimated by denaturing polyacrylamide gel electrophoresis. Purified fraction was used for characterization study.

### Effect of additives on xylanase activity

Effect of various additives on xylanase activity was determined by incubating xylanase with different additives at room temperature ( $25\pm 2^\circ\text{C}$ ). Selected monovalent and divalent metal ions ( $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Hg}^{2+}$ ), Tween 80, EDTA (10 mM) were used as additives. Xylanase activity with different additives was monitored at every 15 min interval for 1 h.

## Result & Discussion

### Optimization of growth and xylanase production in solid state fermentation (SSF) by *Penicillium citrinum* MTCC 9620 in shake flask

#### Selection of appropriate substrates for xylanase production in SSF

Selection of an appropriate substrate is a crucial step for SSF. Lignocellulosic substrates such as wheat straw (WS), rice straw (RS), wheat bran (WB), corn cobs (CC), rice bran (RB) and sugarcane bagasse (SCB) were selected. Xylanase activity was found to be maximum (156.5 U/gds) with negligible cellulase activity (0.05 U/gds) when sugarcane bagasse ( $\leq 0.5\text{mm}$  size) was used as a substrate. The corresponding xylanase activity pertaining to corn cobs, wheat bran, rice bran, rice straw, and wheat straw were 52.81, 123.54, 93.2, 31.5, 60.09 U/gds respectively. It is apparent from Figures 1a-1c that along with maximum xylanase activity, extracellular protein content and dry cell weight were also maximum when sugarcane bagasse was used as substrate. Bluish green spongy appearance of filamentous fungal growth was observed in all the flasks. Higher xylanase activity using bagasse may be due to its higher hemicellulose content (Palma, 1996). Sugarcane bagasse consists of approximately 50% cellulose, 30% pentosans, and 2.4% ash. Sugarcane bagasse exhibits several advantages over other agro-industrial residues due to its low ash content. Pandey et al. reported that ash content of rice straw and wheat straw is 17.5 and 11.0%, respectively (Pandey, 2000). Milagres et al. (1993) studied xylanase production by a local fungal isolate where enzyme activity was inducible by bagasse.

Sugarcane bagasse is therefore, a better substrate for bioconversion process using microbial cultures. Subsequent experiments were, therefore, carried out using sugarcane bagasse.

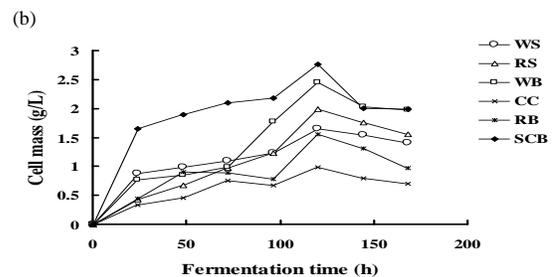
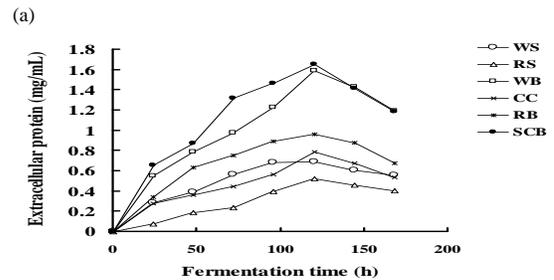
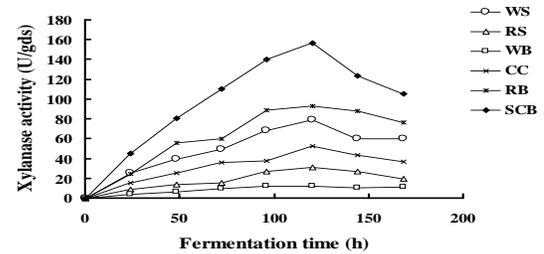


Figure 1. Effect of different lignocellulosic substrates on a) xylanase production b) extracellular protein and c) dry cell weight in Solid State Fermentation. WS, Wheat Straw ; RS, Rice Straw; WB, Wheat Bran; CC, Corn Cobs; RB, Rice Bran; SCB, Sugarcane Bagasse.

### Effect of treatment of sugarcane bagasse on xylanase production

Pretreatment of sugarcane bagasse with alkali, acid and steam was investigated to ascertain whether pre-treatment improves the efficiency of the substrate by altering the topology by removing any functional group or modifying the molecular structure.

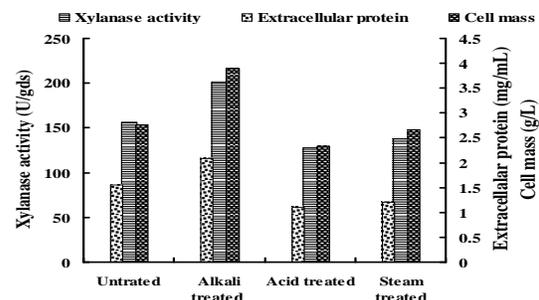


Figure 2. Effect of different treatment on xylanase production in shake flask in solid state fermentation using *P. citrinum* MTCC 9620

Alkali treatment of sugarcane bagasse yielded maximum xylanase activity (201.91 U/mL), extracellular protein content (2.1 mg/mL), and cell mass concentration (3.9 g/L) (Figure 2). Acid treated bagasse produced least xylanase activity (127.86 U/gds). Alkali treatment removes the core and noncore lignin

fractions and improves its digestibility making nutrients easily accessible to microorganisms for their growth (Doran et al., 1994; Alani and Smith, 1998). The alkali pretreatment leads to the fractionation of the three components and opening of cellulose structure. That might cause enlargement of the inner surface area of substrate particles, by partial solubilization and/or degradation of hemicelluloses (Alani and Smith, 1998). A number of physical and chemical pretreatments such as steam explosion, gamma radiation, alkali treatment, hydrogen peroxide treatment, organic solvents treatment, etc have been reported. Among these, chemical pretreatments e.g., treatment with alkali such as 1N NaOH solution on different lignocellulosic substrates have been found effective and economical for the production of different fermented products using several microbial cultures irrespective of bacteria or fungus (Pandey, 2000). Similar observation was found in present study (Figure 2). Figures 3 (a) and 3 (b) show the

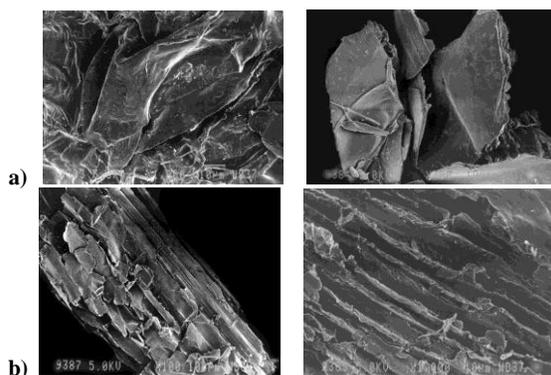


Figure 3. Scanning electron microscopy of (a) untreated (b) alkali treated sugarcane bagasse

scanning electron microgram (SEM) of untreated, and alkali treated sugarcane bagasse at two different (100X and 1000X) magnifications. Higher magnified microgram revealed that untreated bagasse is more compact in nature whereas, treated one is more porous in nature. It is clear that outer surface of alkali treated sugarcane bagasse was rough as compared to untreated bagasse. It can be inferred that alkali treatment might cause removal of some acidic compounds from the surface of the sugarcane bagasse leaving readily utilizable sugars on the surface. The surface of the sugarcane bagasse became rough, and therefore, it is suitable for microorganisms to grow on the surface of alkali treated sugar cane bagasse. It provided larger surface area, for diffusion of nutrients, both at the surface and in the pores of substrates having the same tortuous and sugarcane bagasse fulfilled the above requirements in the present study. As the substrate and microbial interactions are unique, chemical composition and physical properties of the substrate should be taken into consideration. Growth characteristics, physiology and production of metabolites by the organisms are based on the extracellular enzymes in growth-associated metabolism. Previous findings support our result (Bravo et al., 1994; Gomes et al., 1994; Rodriguez-Vazquez et al., 1994; Aiello et al., 1996).

Spectral bands were obtained for alkali treated and untreated sugarcane bagasse. Table 1. lists the vibrational groups and modes of FTIR spectra. The spectra for untreated and treated bagasse were

Table 1. Vibrational groups and modes in the FTIR spectra

Band region (wave number, $\text{cm}^{-1}$ )	Vibrational group and mode
800-1300	C-C, C-O and C-N stretching
1200-1416	Bending of O-C-H, C-C- and C-O-H
1640	Free $\text{COO}^-$
2930	$\text{sp}^3$ C-H stretching
3000-3500	O-H stretching
3000	C-H stretching
1450	C-H <sub>2</sub> bending
1400	C-H <sub>3</sub> bending
1650	Aliphatic C=C stretching
1500	Aromatic C=C stretching
1000	Aromatic C-H bending in phase
675	Aromatic C-H bending out of phase
3300-3500	O-H stretching
1700	C=O stretching of ketone and carboxylic acid
2700-3300	O-H stretching of carboxylic acid

rather similar. However, due to changes in molecular structure in treated sugarcane bagasse (stretching and bending of bonds) remarkable difference in intensities were observed, which suggested that the micro structure of the substrate changed during alkali treatment. Peak intensity decreased in case of alkali treated sugarcane bagasse due to stretching of C-O, C-C and  $\text{sp}^3$  C-H, O-H bonds. Significant decrease in intensity is observed in the region of  $1603\text{-}1631\text{cm}^{-1}$  due to removal of free  $\text{COO}^-$  group. This may be due to removal of organic acids, proteins and free fatty acids in bagasse. The largest vibration in spectra for untreated and alkali treated bagasse was found in the region  $900\text{-}1106\text{cm}^{-1}$ . There may be stretching of C-C and C-H bond. Peak in this region also indicates that bending of aromatic C-H bond in phase and out of phase. Bending vibration in the range of  $900\text{-}1106\text{cm}^{-1}$  indicated that alkali treatment resulted in more solid like compact structure of bagasse. Band intensities decreased in alkali treated sugarcane bagasse and, there may be solubilization of nonstarch polysaccharides (Haltrich et al., 1996). It may therefore, be inferred that the readily utilizable sugars as nutrient for the use of microorganisms increased due to alkali treatment of sugarcane bagasse.

Reduction in xylanase activity after acid and steam treatments may be due to changing of particle size, surface area and porosity in such a way making it unsuitable for microorganism to grow (Shah and Dutta, 2005). Subsequent experiments were, therefore, carried out using alkali treated sugarcane bagasse as the solid substrate.

#### Effect of moistening media on xylanase production in SSF

Effect of different moistening media such as distilled water, minimal media, nutrient broth, and Czapek yeast extract media on xylanase production was studied. Czapek yeast extract media yielded maximum xylanase activity (201.91 U/gds) compared to other moistening media. Protein content and cell mass was also maximum when Czapek yeast extract media was used for solid state fermentation (Figure 4.).

### Optimization of process variables on xylanase activity using Response Surface Methodology (RSM)

After optimization of substrate, pretreatment of substrate, moistening media, inoculum size, other important process variables such as temperature, pH, substrate to moisture ratio, and fermentation time were optimized using RSM. Design of the experiments was carried out using central composite design (CCD) of RSM using Design expert ver 6.0.9 statistical software (Stat-Ease Inc, Minneapolis, MN, USA). Design of

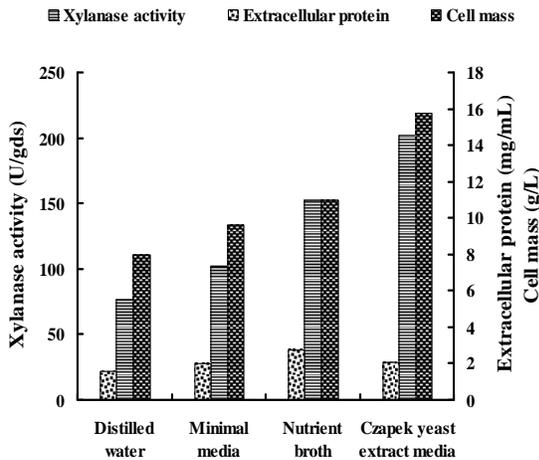


Figure 4. Effect of different media on growth and xylanase production by *P. citrinum* in shake flask in solid state fermentation

experiment and level of the factors chosen on the basis of submerged fermentation, results are reported in Table 2.

Table 2. Levels of factors chosen for the experimental design

Factors	Symbols	Actual levels of coded factors		
		-1	0	+1
pH	A	4.0	5.0	6.0
Temperature (°C)	B	25	30	35
Substrate to moisture ratio	C	1:4	1:5	1:6
Time (days)	D	4	5	6

Activity of xylanase produced under different conditions of initial pH, temperature, substrate to moisture ratio and incubation time is reported in Table 3.

Equation (2) was used to compute the combined effect of the process variables to obtain the optimum conditions for xylanase activity. The resulting equation in terms of coded variables is expressed as

$$\begin{aligned}
 \text{Xylanase activity} = & 1372.4 + 151.6 * A + 11.9 * B + 84.8 * C \\
 & + 133.8 * D + 167.7 * A^2 - 123.5 * B^2 - 100.5 * C^2 - 100.5 * D^2 \\
 & + 10.0 * A * B - 18.7 * A * C + 33.1 * A * D - 41.2 * B * C \\
 & + 70.4 * B * D - 86.3 * C * D
 \end{aligned}
 \tag{3}$$

Fisher's test for analysis of variance (ANOVA) was used for the performance of statistical testing of the model (equation 3) and the results are reported in Table 4.

$R^2 = 0.999$ , Adjusted  $R^2 = 0.9995$ , Predicted  $R^2 = 0.9572$ , C.V. = 0.54, mean = 106.64. Std. dev = 0.58.

Table 3. Experimental design and results of the central composite design for xylanase activity

Run number	Block	Factors				Xylanase activity (U/gds)	
		A	B	C	D	Observed	Predicted
1	1	4.0	35.0	1:4	6	920	922.3
2	1	5.0	30.0	1:5	5	700	700.3
3	1	5.0	30.0	1:5	5	1120	1122.3
4	1	6.0	35.0	1:6	4	920	920.3
5	1	4.0	25.0	1:6	4	1080	1080.3
6	1	5.0	30.0	1:5	5	920	922.3
7	1	6.0	25.0	1:4	6	870	872.3
8	1	6.0	35.0	1:4	4	460	462.3
9	1	6.0	25.0	1:6	6	1370	1367.4
10	1	5.0	30.0	1:5	5	1370	1367.4
11	1	4.0	35.0	1:6	6	1370	1367.4
12	1	4.0	25.0	1:4	4	1370	1367.4
13	2	5.0	30.0	6.68	5	650	648.1
14	2	3.32	30.0	1:5	5	1160	1158.1
15	2	5.0	30.0	1:5	5	1010	1008.1
16	2	5.0	21.59	1:5	5	1050	1048.1
17	2	5.0	30.0	1:5	6.68	950	950.6
18	2	6.68	30.0	1:5	5	1240	1235.7
19	2	7.0	30.0	1:3	5	870	868.1
20	2	5.0	30.0	1:5	3.32	1320	1318.1
21	2	5.0	30.0	1:5	5	1370	1377.5
22	2	5.0	30.0	1:5	5	1370	1377.5

The analysis revealed that the response surface quadratic model was significant because the value of Prob>F was less than 0.05 while model F value was 3227.26 (Table 4.). Non significant value (2.02) for lack of fit indicates that the quadratic model was valid for the present study. Statistical analysis was carried out to determine the experimental factors which generate larger signal to noise ratio. Normally signal to noise ratio greater than 4 indicates adequacy of the model. In our study, signal to noise ratio is 185.393 which indicates the adequacy of the fitting of the model. The  $R^2$  value (0.999) indicated that the data was less variable, more uniform, and homogeneous. Coefficients of variation (CV) specify the degree of precision with which the experimental results are compared. A small value of coefficient of variation (0.54) indicated a high degree of precision and a good deal of reliability of the experimental values (Table 4.). "Prob" value indicates the significance of the coefficients and it is important to consider the pattern of the mutual interaction between the variables. Values of "Prob > F" less than 0.05 for A, B, C, D,  $A^2$ ,  $B^2$ ,  $C^2$ ,  $D^2$ , AC, AD, BC, BD and CD showed that model terms were significant.

The positive linear coefficient of pH (A), temperature (B), substrate to moisture ratio (C) and incubation time (D) demonstrates increased xylanase activity with increased value of these variables. The negative quadratic coefficient for all the variables indicates the existence of highest activity to a point; and, beyond that all these variables had an inhibitory effect on xylanase activity. Substrate to moisture ratio is a vital factor and determines the success of SSF process. Temperature, pH, and fermentation times are important process variables therefore to economize the process, suitable combination of these process variables are prime interest for commercialization of a product. Influence of moisture content on growth of organisms and subsequently biosynthesis of product may be attributed to its effect on the physical characteristics of the solid substrates. Optimum substrate to moisture ratio depends on substrate and its porosity, particle structure, water binding capacity, etc.

Table 4. Analysis of variance (ANOVA) for response surface quadratic model (equation 1)

Source	Sum of squares	DF	Mean of Squares	F value	Prob>F	S
Block	1952.7	1	1952.7			
Model	147338.0	14	10524.1	3227.26	0.0001 <sup>a</sup>	S
A	13005.0	1	13005.0	3864.5	0.0001 <sup>a</sup>	
B	80.0	1	80.0	23.77	0.0028 <sup>a</sup>	
C	9814.2	1	9814.2	2916.31	0.0001 <sup>a</sup>	
D	10125.0	1	10125.0	3008.66	0.0001 <sup>a</sup>	
A <sup>2</sup>	43334.4	1	43334.4	12876.88	0.0001 <sup>a</sup>	
B <sup>2</sup>	23504.7	1	23504.7	6984.46	0.0001 <sup>a</sup>	
C <sup>2</sup>	15571.8	1	15571.8	4627.18	0.0001 <sup>a</sup>	
D <sup>2</sup>	15571.6	1	15571.6	4627.18	0.0001 <sup>a</sup>	
AB	33.4	1	33.4	9.92	0.0198	
AC	281.3	1	281.3	83.57	0.0001 <sup>a</sup>	
AD	364.0	1	364.0	108.16	0.0001 <sup>a</sup>	
BC	1361.2	1	1361.2	404.50	0.0001 <sup>a</sup>	
BD	1641.1	1	1641.1	487.66	0.0001 <sup>a</sup>	
CD	5951.3	1	5951.3	1768.42	0.0001 <sup>a</sup>	
Residual	2.02	6	0.34			
Lack of fit	2.02	1	1.01			
Pure error	0.00	4	0.00			
Cor. Total	14931.09	21				

(S= Significant)

A higher than optimum moisture level causes reduced porosity, change in particle structure, gluey texture, lower gas transfer, and development of the aerial mycelia. It further leads to accumulation of the substrate or sticking of the particles to the wall of the vessel and the substrate becomes vulnerable to contamination, whereas, lower than optimum moisture content may reduce lignin solubility and swelling capability of solid substrate which may cause higher water pressure, consequently reduced growth of microorganisms and eventually lower enzyme production (Shah & Dutta, 2005). Lower or higher than optimum pH cause inadequate growth of microorganism leading to lower enzyme production. Lower temperature results lower capability for the organism to produce enzyme, while higher temperature results lysis of cells.

3D plots were generated to identify the optimal levels of each process variables for maximum xylanase activity. The response surface was selected based on the interactive effect of two independent variables while keeping other variables at fixed level. Six responses were generated (Figures 5a–5f) by taking all the possible combinations. The graphical representations are important to establish the relation between the responses with deviation of each variable, their interactions and finally to obtain the optimum values of the variables for maximum xylanase production. Hump in the 3D plots, or the central point of the contour plots indicates the optimum values.

#### Interactive effect of pH and temperature on the xylanase activity

The Prob > F value less than 0.05 indicates that the interaction is significant; however positive values of coefficients for A, B, AB (equation 3) indicated that pH and temperature have the strong

positive effect and have the most influence on the xylanase activity. Figure 5a indicates that the variables have optimal

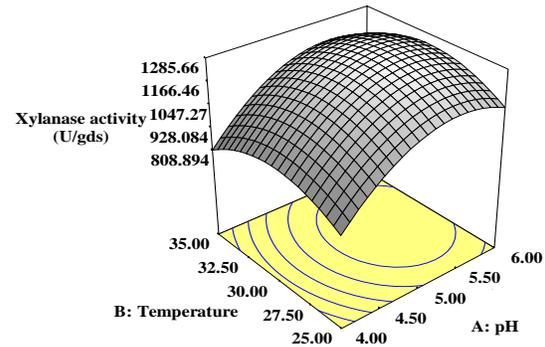


Figure 5a. Interactive effects of pH and temperature on the xylanase activity in shake flask in solid state fermentation

setting as is evident from the peak. The negative value for quadratic coefficient also supported that these variables have negative effect on xylanase activity beyond the optimum limit. “Prob > F” (Table 4) value of interactive effect of pH and temperature was significant (0.0198). Lower and higher temperature had the same response but there is better intermediate temperature axes indicating change in temperature is less important than pH. Inspection of two dimensional contour plot for selected factors indicated the maximum response in the vicinity of a temperature of (30°C) and pH of (5.0) holding mean values of maximum and minimum of other variables.

#### Interactive effect of substrate to moisture ratio and incubation time on xylanase activity

Positive values of linear coefficients indicate that there is positive effect of these variables on xylanase activity which means that xylanase activity increased with increase in substrate to moisture ratio or incubation time. On the contrary, negative quadratic coefficients indicate that the activity increased upto a certain value, and decreasing trend was observed beyond optimum point. Values of “Prob > F” was 0.0001 for CD (Table 4.) indicates that interaction of substrate to moisture ratio and incubation time was significant ( $p < 0.05$ ). Negative value (86.3) of coefficient of interaction (CD) in equation (3) reveals that though the interaction is significant, increased xylanase activity is observed with increase in substrate to moisture ratio and decrease in incubation time and vice versa (Figure 5b). Studies related to such an interaction have not been reported, however, selection of appropriate incubation time for enzyme production has been found to be an important parameter in various studies. Park et al. (2002) reported that fermentation period and media concentration and composition were the most vital parameters for the xylanase production by mutant strain of *Aspergillus niger*. From figure 5b it is clear that optimum substrate to moisture ratio of 1:5 and 5 days incubation period is the optimum time for xylanase production in SSF by *P. citrinum*.

#### Interactive effect of temperature and substrate to moisture ratio on xylanase activity

Positive linear coefficient indicates strong positive effect of these variables on xylanase activity. At the same time, negative quadratic coefficient indicates that with increase of

temperature and substrate to moisture ratio individually keeping other variable

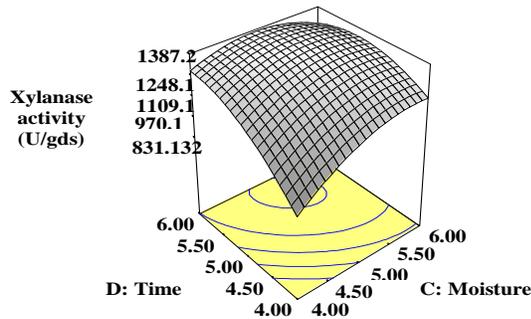


Figure 5b. Interactive effects of incubation time and substrate to moisture ratio on xylanase activity in shake flask in solid state fermentation

constant, xylanase activity increased upto a certain optimum value and after that xylanase activity decreased. Negative interaction coefficient (-41.2) for temperature and substrate to moisture ratio (equation 3) indicates that higher level for both the factors gives a lower response.

Significant values of “Prob>F” (0.0001) for BC (Table 4) indicates that there is interaction within temperature and substrate to moisture ratio but the interaction is lower. It is inferred that there is no change of substrate to moisture ratio with change in temperature or vice versa.

Figure 5c indicates that the maximum response lies in temperature range 29-30°C, beyond that the activity reduced. However, activity increased consistently with substrate to moisture ratio.

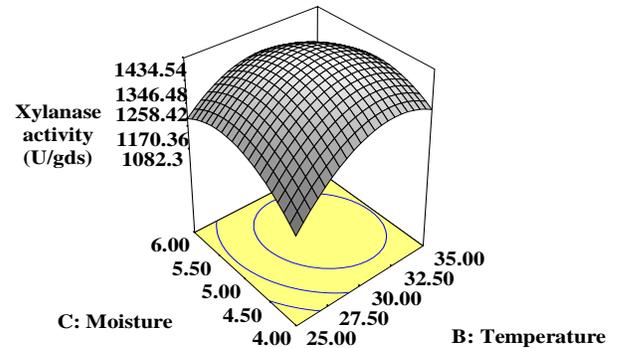


Figure 5c. Interactive effects of substrate to moisture ratio and temperature on xylanase activity in solid state fermentation

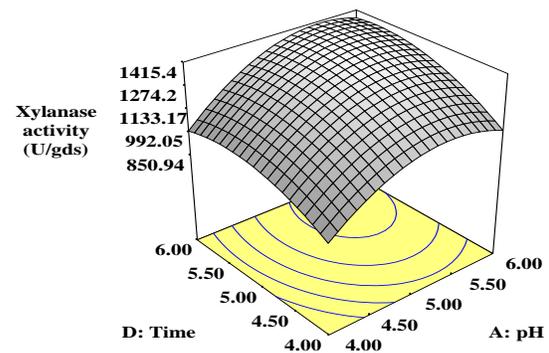


Figure 5d. Interactive effects of pH and incubation time on xylanase activity in solid state fermentation

#### Interactive effect of pH and incubation time on xylanase activity

The positive value (33.1) for interaction coefficient of pH and incubation time in equation (3) infers that there is an interaction effect. Significant values of “Prob > F” (0.0001) for AD (Table 4) indicates that though interaction with pH and incubation time is prominent but the effect is lower. Figure 5d indicates that with increase or decrease of pH above or below pH 5.0, reduction in xylanase activity was observed. At the same time increase of incubation time from 4 days to 5 days increased xylanase activity was observed and decrease in xylanase activity was observed from 5 days to 6 days. Li et al. (2007) reported that at low pH, effect of incubation time on endoxylanase production by *Aspergillus awamori* was negligible; but, when pH in the medium was high, endoxylanase production steadily increased with increasing incubation time, and then decreased slowly. This indicates that choice of correct incubation time is essential to obtain maximum xylanase activity.

#### Interactive effect of temperature and incubation time on xylanase activity in shake flask in SSF

The positive value (70.4) for interaction coefficient in equation (3) infers that there is higher interaction effect. Values (0.0001) of “Prob >F” (Table 4) indicates that there is significant effect within the two variables. Strong interaction is present within temperature and incubation time. Increasing incubation time at low temperature or vice versa enhanced xylanase activity; whereas, increase or decrease of both the parameters at a time increased xylanase activity. Figure 5e indicates that there is an interaction zone of temperature with incubation time in the range of 27.5 to 32.5°C and 4 to 6 days.

#### Interactive effect of pH and substrate to moisture ratio on xylanase activity in shake flask in SSF

The negative value (18.7) for interaction coefficient of pH and substrate to moisture ratio in equation (3) infers that there is mild or nil interaction effect. Significant value (0.0001) of “Prob >F” (Table 4) indicates there is significant effect within the two variables. Figure 5f indicates that with variation of pH above or below 5.0, reduction in xylanase activity. At the same time increase of substrate to moisture ratio causes enhance xylanase activity starting from lower ratio to 1:5 ratio and above that negative effect is observed. The center point in contour plot clearly indicates the optimum point of pH and substrate to moisture ratio for xylanase production in SSF by *P. citrinum*.

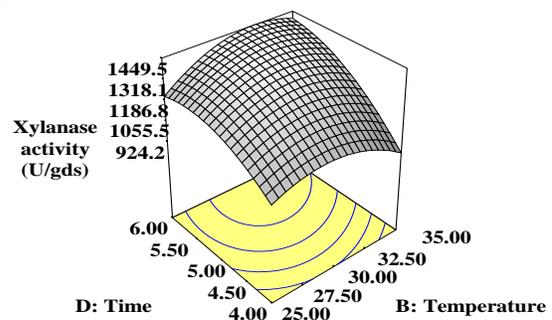


Figure 5e. Interactive effects of incubation time and temperature on xylanase activity in solid state fermentation

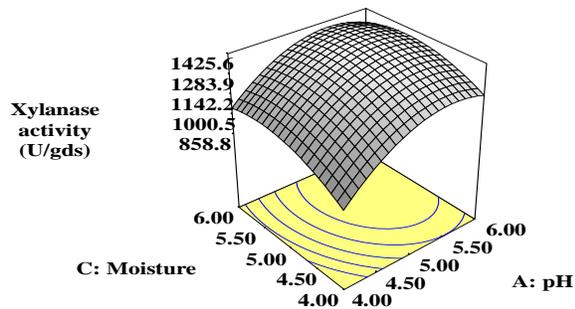


Figure 5f. Interactive effects of pH and substrate to moisture ratio on xylanase activity in solid state fermentation

#### Validation of the experimental model

Validation of the predicted results was carried out by performing triplicate additional experiments with the parameters suggested by the numerical modeling (suggested solution of Design Expert ver. 6.0.9 statistical software). These three sets of reactions yielded an average enzyme production of 1665.8 U/gds against the predicted value of 1370 U/gds (Table 5). Good agreement between the predicted and experimental results confirmed the experimental adequacy of the model and the existence of the optimal point as optimization led to an enhancement of the enzyme activity by 8.25 times higher (from 201.91 to 1665.8 U/gds) as compared to the unoptimized media.

#### Effect of nitrogen source on xylanase production by *P. citrinum* in SSF

Effect of different nitrogen source on xylanase production was studied by *P. citrinum* in SSF. Effect of organic and inorganic nitrogen sources (0.5% concentration) on xylanase production, extracellular protein content and dry cell mass was determined.

As indicated in Figure 6, nitrogen source significantly affected enzyme synthesis. Xylanase production was minimum when no nitrogen source was added in the growth medium i.e. the culture was grown in the presence of only carbon source. Of the various organic nitrogen sources e.g., peptone, meat extract, tryptophane, soya peptone, brain and heart infusion, soya bean meal, beef extract, yeast extract, protease peptone, and inorganic nitrogen sources, like urea, sodium nitrate ( $\text{NaNO}_3$ ), ammonium chloride ( $\text{NH}_4\text{Cl}$ ), ammonium sulphate ( $(\text{NH}_4)_2\text{SO}_4$  etc., maximum xylanase production (2298.7 U/gds) was obtained when meat extract was used followed by protease peptone and yeast extract. In general, organic nitrogen sources being more complex and rich in nitrogen content, favor cell mass production more than inorganic nitrogen sources. Meat extract was therefore used to maximize the xylanase production in SSF by *P. citrinum* in the subsequent experiments. Xylanase production was compared with submerged fermentation by same organism yield and productivity was higher in SSF (Ghoshal et al., 2015), on the other hand the same organism produced little higher xylanase when grown in laboratory scale fermentor (Ghoshal et al., 2014). In our study the organism producing acidic xylanase which is whereas most of the earlier report cover basic xylanase from different strain.

#### Purification of xylanase

Xylanase was produced in modified Czapek yeast extract medium (pH 5.0) using 1% sugarcane bagasse (as carbon source) with 0.5% meat extract (as nitrogen source) at 30°C, 200 rpm. After 5th day culture broth was then filtered using muslin cloth followed by

centrifugation at 10,000 g for 20 min. The supernatant was used for xylanase purification. The purified enzyme was then characterized to determine its molecular properties. Table 6 summarizes the results on purification of extracellular xylanase from *P. citrinum* MTCC 9620. The purified enzyme had specific activity of 1827.62 U/mg and was found to be stable at room temperature for approximately 30 days. In the present study isolated xylanase produced 8.51 fold purified with 63.61% yield of xylanase.

Table 5. Predicted vs experimental values for maximum xylanase production

Variables	Culture conditions	Xylanase activity (U/gds)	
		Predicted	Experimental
pH	5		
Temperature (°C)	30		
Substrate to moisture ratio	1:5	1370	1665.8
Incubation time (Days)	5		

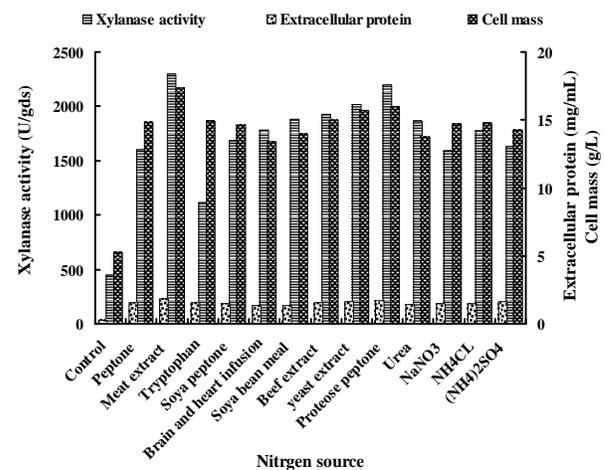


Figure 6. Effect of various nitrogen sources on xylanase activity in solid state fermentation

#### Molecular characterization of purified xylanase

The purified xylanase was characterized using 10% SDS-PAGE with known protein standards under denaturing conditions. Proteins were visualized by staining with coomassie brilliant blue R250. A single band was observed approximately at 29 KDa. It may therefore be inferred that the approximate molecular weight of the isolated xylanase from *P. citrinum* MTCC 9620 is 29 KDa.

#### Biochemical characterization of purified xylanase

##### Determination of pH and temperature optima, pH and thermo stability

Xylanase obtained by *P. citrinum* exhibited a higher range of pH stability and prolong catalytic action of enzyme was observed in a wide range of pH. Optimum pH of the enzyme was found to be 5 and the enzyme was stable in the pH range of 3 to 8. Above pH 8, Kulkarni et al. (1999) reported that most of the xylanases from microbial sources contain single subunit proteins with molecular weight range of 8-145 KDa.

Table 6. Summary of various steps involved in the purification of xylanase for the crude extract

Purification steps→	Crude extract	Ammonium sulphate fraction	Dialysis	Q-sepharose
Volume (mL)	50	5.0	9.5	2.0
Xylanase activity (U/mL)	229.87	115.37	130.82	146.21
Protein (mg/mL)	1.07	0.28	0.20	0.08
Specific activity (U/mg)	24.83	412.04	654.1	1827.62
Yield (%)	100.0	54.54	56.91	63.61
Purification fold	1.00	1.91	3.04	8.51

xylanase activity reduced to 10% of the initial activity after 24 h. Xylanases from various organisms are usually stable over a wide pH range (3-10). It showed optimum pH stability in the range of 4-7. The xylanase from fungi such as *Aspergillus kawachii* and *Penicillium herque* exhibited optimum pH towards acidic side (2-6). The isoelectric points for endoxylanases from various sources ranged from 3-10. Generally bacteria are known to produce two xylanases - high molecular mass acidic xylanase and low molecular mass basic xylanase. However, this type of relationship is not observed in the fungi; but low molecular mass basic xylanases are common. Therefore, our isolated enzyme is not very common and it may be claimed that it is a novel organism which produced unique xylanase.

The enzyme was stable at 30°C for 2 h. The activity reduced to 90% at 40°C and 85% at 50 °C after 2 h. The enzyme was stable up to 80°C. However, the activity reduced to 50% of the initial value after 30 min and 20% of initial value after 2 h at 80°C. Therefore, optimum temperature was found to be 50°C. Presence of xylanase activity above 50°C indicated thermal stability of the xylanase secreted by the organism. Higher temperature caused unfolding of enzyme and thermal inactivation which is to be related to thermal unfolding of the protein structure.

According to Kulkarni et al. (1999), the optimum temperature for endoxylanase activity from bacterial or fungal sources varied between 40 to 60°C. Comparison with bacterial xylanases, fungal xylanases are generally less thermostable.

Table 7 Effect of additives on xylanase activity

Additives	Activity (%)
None	100
Co <sup>2+</sup>	168
Cu <sup>2+</sup>	55
Zn <sup>2+</sup>	104
Fe <sup>3+</sup>	127
Mn <sup>2+</sup>	114
K <sup>+</sup>	106
Mg <sup>2+</sup>	123
Tween 80	122
Ca <sup>2+</sup>	56
Hg <sup>2+</sup>	19
EDTA	09
Cysteine HCL	181
Xylose	147

#### Effect of different metal ions on the xylanase activity

Xylanase activity was repressed by Hg<sup>2+</sup>, Ca<sup>2+</sup>, Cu<sup>2+</sup> and EDTA (Table 7). The repression of xylanase activity by Hg<sup>2+</sup> ions may be due to the interaction with sulphhydryl groups, indicating the presence of cysteine residues very close or within the active site of

the enzyme. Similar results were reported in literature by Khandeparkar and Bhosle, 2006. On the other hand, Nakamura et al., 1993 were unable to detect the presence of cystine residue in the active site of the enzyme, however, strong inhibitory effect of Hg<sup>2+</sup> ion on xylanase activity was observed. Presence of EDTA caused reduction in xylanase activity suggesting the requirement of metal ions for the enzymatic reaction. Xylanase activity increased in presence of K<sup>+</sup>, Mn<sup>2+</sup>, Fe<sup>3+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Tween 80, cystein-HCl and xylose (Table 7). These results further established that metals are required for the enzymatic activity and presence of EDTA as metal chelating agent inhibited the enzymatic reaction. EDTA is a chelating agent used to scavenge heavy metal ions present as impurities in the reagents. However, it may have adverse effect on enzymes. In this study, the presence of EDTA adversely reduced the xylanase activity, which indicates the requirement for some metal ions for its activity. There is no evidence whether these ions/agents could be binding to the enzyme, causing conformational changes that resulted in the enhanced enzyme activity, or metal ions are required by the active site of xylanase. The additives like KCl and MgCl<sub>2</sub> had no significant influence on xylanase activity. Among the monomeric sugars, xylose significantly enhanced xylanase activity. Tween 80 is a non ionic and non denaturing detergent, useful in solubilization and stabilization of proteins. In the present study, Tween 80 enhanced the xylanase activity by 20%. Shah and Madamwar (2005) also reported that the presence of cystein-HCl, CoCl<sub>2</sub>, xylose and Tween 80 remarkably activated the xylanases, while its activity is greatly reduced in the presence of 5 mM CuSO<sub>4</sub> or HgCl<sub>2</sub>. Remarkable activation (175%) by 10 mM cystein-HCl indicated the existence of sulphhydryl group in the catalytic centre of the enzyme. Moreover strong inhibition by heavy metal ions like Cu<sup>+2</sup> and Hg<sup>+2</sup> indicates the sulphhydryl group.

## Conclusion

In present study, agricultural residues were used for xylanase production and sugarcane bagasse was selected as solid support for xylanase production. Alkali treatment on sugarcane bagasse was found beneficial for xylanase production and maximum cell growth was obtained. SEM study confirmed that alkali treatment modified the structure of sugarcane bagasse. To explore suitable nitrogen source, several inorganic and organic nitrogen sources were used at 0.5% level in moistening media (Czapek yeast extract medium). Meat extract was found most suitable organic nitrogen source for maximum xylanase production. The xylanase production by *Penicillium citrinum* MTCC 9620 from sugarcane bagasse was efficient, leading to maximum activity of 2298 U/gds. Optimum conditions for xylanase production were pH 5.0, substrate to moisture ratio of 1:5 at 30°C and 5 days of incubation. In the present study isolated xylanase produced 8.51 fold purified with 63.61% yield of xylanase. Molecular weight was found about 29KDa. Increased activity of xylanase was observed in the presence of K<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>3+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Tween 80, Cystein HCL, xylose whereas Hg<sup>2+</sup>, Cu<sup>2+</sup>, Ca<sup>2+</sup> and EDTA inhibited the xylanase activity. Color of xylanase obtained from SSF process was darker. As our aim was to apply the enzyme in food processing, therefore, before purification bleaching of color is one of the aims for the future work.

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