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Production of Xylanase and β-Xylosidase Enzymes by *Pseudozyma hubeiensis* in Solid State Fermentation

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Abstract

Xylanase is an important enzyme with potential application in the degradation of xylan component in the lignocellulosic biomass. There are very few reports on the production of cellulase free xylanases especially by yeast strains which have great potential in paper and pulp industry in removing the hemicellulose from the treated or untreated pulp. In this study, *P. hubeiensis* NCIM 3574 isolated in our laboratory produced significant levels of extracellular cellulase free xylanase (2480 IU/g DSS) in solid state fermentation (SSF) using wheat bran and xylan. It also produced high levels of β-xylosidase (198 IU/g DSS) when grown in SSF using ground nut oil cake and xylan. These highest activities were obtained when fermented Koji was extracted with 1% NaCl supplemented with 0.5% of Triton X-100. These are the highest activities reported so far from yeast strains in the available literature. The crude xylanase preparation of *P. hubeiensis* produced xylooligosaccharides (XOS) without xylose proving its potential for XOS production with no further requirement of downstream processing. The XOS as prebiotic show beneficial effect on gut microflora such as *Lactobacilli* and *Bifidobacteria* which suppress the activity of pathogenic organisms. This xylanase also has a potential application as a bio-bleaching agent in paper and pulp industry.

Keywords

Pseudozyma hubeiensis, Cellulase-Free Xylanase, β-Xylosidase, Solid State Fermentation, Submerged Fermentation

1. Introduction

Lignocellulosic biomass, being renewable, is the only sustainable source of or-

ganic carbon which is composed of cellulose, hemicelluloses and lignin. Cellulose and hemicellulose can be converted to a number of bulk chemicals. Cellulases and xylanases degrade lignocellulosic biomass in to simple sugars that can be converted to value-added chemicals including biofuels. The high cost of lignocellulose degrading enzymes poses the problems in their application for biofuel production. Hence it is essential to improve the enzyme yields which in turn to decrease the production cost. *Trichoderma* and *Aspergillus* are known to produce large amounts of cellulases which are comprised of endoglucanase, exoglucanase, and β-glucosidase. *Aspergillus* strains are the potent producers of both β-glucosidase [1] and β-xylosidase [2] [3]. Most of the commercial enzyme preparations are deficient in both β-glucosidases and β-xylosidases [4]. It is necessary to supplement the commercial biomass degrading enzymes with such enzyme preparations containing high amounts of β-glucosidases and β-xylosidases to achieve complete biomass hydrolysis.

The conversion of all biomass components, especially hemicellulose and lignin, in particular, would greatly contribute to the economic viability of biomass based processes for second generation biofuels and chemicals. Also, the generation of revenue from each component of biomass is essential for the competitive production of chemicals and fuels from biomass. Due to different structures and chemistries of cellulose, hemicellulose and lignin, effective fractionation of individual components into their native state is very critical. Alonso *et al.* [5] used γ-valerolactone (GVL)/water/acid mixture to dissolve hemicellulose and lignin leaving behind high purity cellulose. Under the conditions, lignin can be separated with high purity and hemicellulose can be converted to furfural. This hemicellulose fraction can be used for the production of xylanases to be used for the production of high-value products such as xylooligosaccharides (XOS). These non-digestible XOS act as prebiotics and they are the most preferred dietary fibers or functional foods. XOS stimulate the growth and activity of bacteria such as *Bifidobacterium and Lactobacillus* present in the colon and also suppress the activity of entero-putretive and pathogenic bacteria. We are the first to isolate the basidiomycetes yeast, *P. hubeiensis,* from decaying sandal wood in our laboratory in 1992. The yeast was found to produce a complete xylanolytic system consisting of cellulase free xylanase [6] and β-xylosidase [7] in submerged fermentation (SmF). The chemical hydrolysis of xylan is faster and applied extensively by industries. However, it results in the formation of toxic compounds that are hazardous to the environment. Hence microbial enzymes mediated xylan hydrolysis provides an alternative to chemical hydrolysis as it is safe and environment friendly. Complete hydrolysis of xylan requires synergistic action of endoxylanase, β-xylosidase and series of other enzymes that degrade side chain groups. The crude xylanase produced by *P. hubeiensis* degraded xylan from different agricultural waste materials to produce xylose that can be the starting material for production of value-added chemicals [8]. Such cellulase-free xylanases have great potential applications in paper and pulp industries to remove xylan in paper and pulp. These biocatalysts (xylanases) would certainly re-

place chlorine which is presently being used in bio-bleaching process. Xylanases of *P. hubeiensis* were purified to homogeneity that only produced XOS with smaller (3 - 7) degree of polymerization [9]. The *β*-xylosidase of *P. hubeiensis* was also purified which was found to be resistant to the heavy metal and ethanol. The mass spectrometric analysis of purified *β*-xylosidase revealed only 26% sequence coverage with *Pseudozyma hubeiensis* SY62 (gi|808364558 glycoside hydrolase). No entries similar to purified *β*-xylosidase were observed after MASCOT searching of an in-house customised SWISS-PROT database indicating its novelty [7].

Solid state fermentation (SSF) involves the growth of microorganisms on moist solids with low water content. The SSF has many advantages over submerged fermentation (SmF) which include the economy of space required for fermentation, simple fermentation media, no need of complex machinery, equipment and control systems, lower capital and recurring expenditure, greater enzyme yields in a shorter time with comparatively less efforts in the downstream processing [10]. In addition, concentrated preparations with high protein content and enzyme activities were obtained in SSF compared to the enzyme preparations obtained in SmF. Although almost all literature on SSF refers to enzyme production by fungi, there are no reports available in the literature on production of biomass-degrading enzymes by yeast under SSF conditions. Earlier we used SSF for cellulase production [11] by mutants of *P. janthinellum* and lipase production [12] by *A. niger* NCIM 1207. The objective of this study was the enhanced production of xylanase and *β*-xylosidase by *P. hubeiensis* NCIM 3574 in SSF using cheap agricultural materials.

2. Materials and Methods

2.1. Microbial Strain, Culture Media and Enzyme Production

P. hubeiensis NCIM3574 was procured from NCIM Resource Center, CSIR-National Chemical Laboratory, Pune 411008, India. The yeast strain was maintained on MGYP agar medium and it was subcultured once in every three weeks. These MGYP grown cultures were used as stock cultures for preparation of inoculum. MGYP agar medium consisted of 0.3% malt extract, 1.0% glucose, 0.3% yeast extract, 0.5% peptone, and 2.0% agar. The fermentation medium consisted of 0.05% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4$, 0.02% K_2HPO_4, 0.1% yeast extract, 0.5% bacto-peptone and 2.0% xylan. The initial pH of the medium was adjusted to 5.5 before sterilization. The SmF was carried out in 250 ml Erlenmeyer flasks with 70 ml of fermentation medium. The flasks were inoculated with 5% inoculum grown in MGYP medium and incubated at 27˚C with shaking at 180 rpm. The cell growth was harvested after specific time period by centrifugation at 7000 rpm for 20 min and the supernatant was used for determination of enzyme activities. The SSF fermentation was carried out in 250 ml Erlenmeyer flasks with 5.0 g of solid substrate, 1.0 g xylan moistened with 10 ml of fermentation medium. The flasks were steam sterilized at 121˚C for 30 min. After cool-

ing, the flasks were inoculated with yeast cells (2 ml) grown on MGYP liquid medium. The content of the flask was thoroughly mixed for uniform distribution of yeast cells. The flasks were incubated at 27˚C in humidity incubator under stationary condition. Each flask was harvested after specific time period and its contents were extracted for enzymes. The extraction was carried out by adding 50 ml of citrate phosphate buffer (50 mM, pH 7.0) to each flask and by shaking the mixture at 180 rpm on rotary shaker for 2 h at room temperature [12]. The mixture was then squeezed with double layer muslin cloth followed by centrifugation at 7000 rpm for 20 min at 4˚C. The supernatant thus obtained was used as a source of extracellular enzymes to determine enzyme activities.

2.2. Xylan Hydrolysis and End Product Analysis

Xylan hydrolysis was carried out in 50 ml conical flask containing 100 mg of soluble xylan in 10 ml of citrate buffer (50 mM, pH 4.5) and 50 IU of crude xylanase with 0.03 IU of β-xylosidase. The reaction mixture was kept at 50˚C with shaking at 150 rpm and the samples were removed after specific time intervals. These samples were kept in boiling water bath for 5 min to stop the reaction and were processed further for analysis of product by TLC. About 5µl of each sample was spotted on TLC plate and subjected to ascending chromatography run using butanol, acetic acid and water (2:1:1, v/v/v) as mobile phase. The TLC plates were developed in duplicates. The bands on one plate were detected by spraying with a staining solution containing 2% (w/v) diphenylamine, 2% (v/v) aniline and 10% (v/v) phosphoric acid in acetone, followed by heating at 120˚C for 10 min. The other plate was used to scrap the bands corresponding to the developed bands and dissolved in 0.2 ml of distilled water. The dissolved hydrolysis products were centrifuged (5000 × g) and the supernatant was used for the MALD ToF MS analysis using 2,5-dihydrobenzoic acid (10 mg/ml in methanol) as a matrix. The one microliter of the supernatant and one microliter of were mixed, spotted on the plate and analyzed. The molecular masses observed in spectra were matched with the theoretical masses of xylooligosaccharides.

2.3. Analytical Methods

The xylanase (EC 3.2.1.8) activity was estimated as reported earlier using beech-wood xylan [8]. The total assay mixture of 1 ml consisted of 0.5 ml xylan solution (1%) in citrate buffer (50 mM, pH 4.5) and 0.5 ml of suitably diluted enzyme. The assay mixture was incubated at 60˚C for 30 min and the reducing sugars released were estimated by dinitrosalicylic acid (DNS) method. One unit (IU) of xylanase activity was defined as the amount of enzyme required to liberate one micromole of reducing sugar as xylose per min. The β-xylosidase (β-D-xylan xylohydrolase, EC 3.2.1.37) activity was estimated as reported earlier [6] using *p*NPX as substrate in citrate buffer (50 mM, pH 4.5). The total 1 ml of reaction mixture consisted of 0.9 ml of *p*NPX (0.5 mg/ml) and 0.1 ml of suitably diluted enzyme. The reaction was initiated by the addition of enzyme followed by incubation at 60˚C for 30 min followed by stopping reaction by the addition

of 2 ml of 2% sodium carbonate. The *p*-nitrophenol released was measured at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate one µmole of *p*-nitro phenol from the substrate per min.

3. Results

3.1. Xylanase and *β*-Xylosidase Production in SmF and SSF

Our earlier work demonstrated that *P. hubeiensis* produced a significant amount of *β*-xylosidase when grown at 27˚C in SmF. Hence we want to evaluate the strain for both xylanase and *β*-xylosidase production at 27˚C in SmF and SSF. The results showed that it produced high activities of xylanase and *β*-xylosidase in both SmF and SSF. Very high xylanase (565 IU/ml) and *β*-xylosidase (5.4 IU/ml) activities were secreted in the medium on 3rd and 5th day respectively in SmF when *P. hubeiensis* was grown in fermentation medium containing 2% xylan (Figure 1). Such high activities are not reported so far for any yeast strain. These results stimulated us to perform further studies on optimization of enzymes production in SSF at 27˚C. The initial SSF studies on time course on enzymes production using wheat bran and xylan were carried out and the results are given in Figure 2. Maximum xylanase activity (1960 IU/g DSS) and *β*-xylosidase activity (52 IU/g DSS) were obtained on 6th and 10th day respectively and hence further studies on optimization of enzyme production were carried out in SSF.

3.2. Effect of Moisture and Inoculum Size on Enzyme Production

The effect of moisture on enzyme production in SSF was tested by adding different amounts of fermentation medium on wheat bran (5.0 g) and xylan (1.0 g) and the results are shown in Table 1. The highest xylanase (1960 IU/g DSS) and *β*-xylosidase (56 IU/g DSS) activities were obtained when the solid substrates were moistened with fermentation medium in 1:2 ratio. Further increase in the

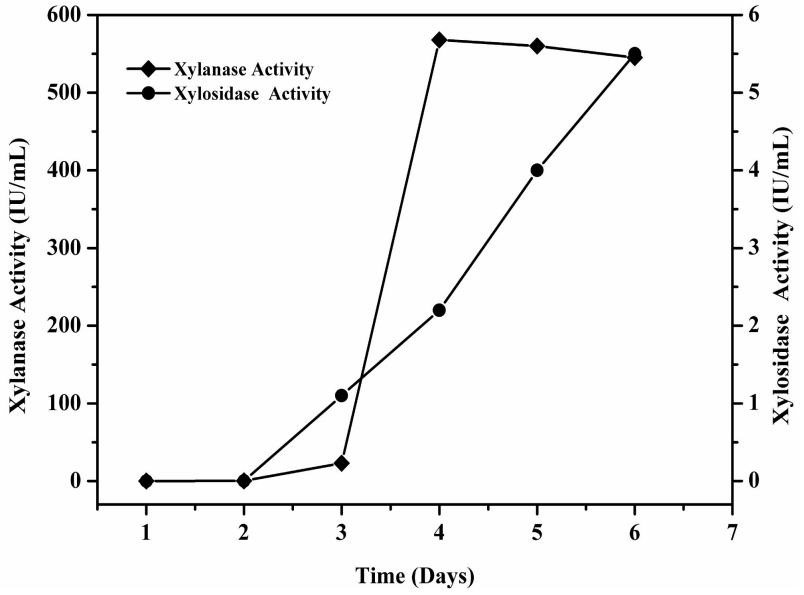

Figure 1. Production of xylanase and *β*-xylosidase in submerged fermentation (SmF).

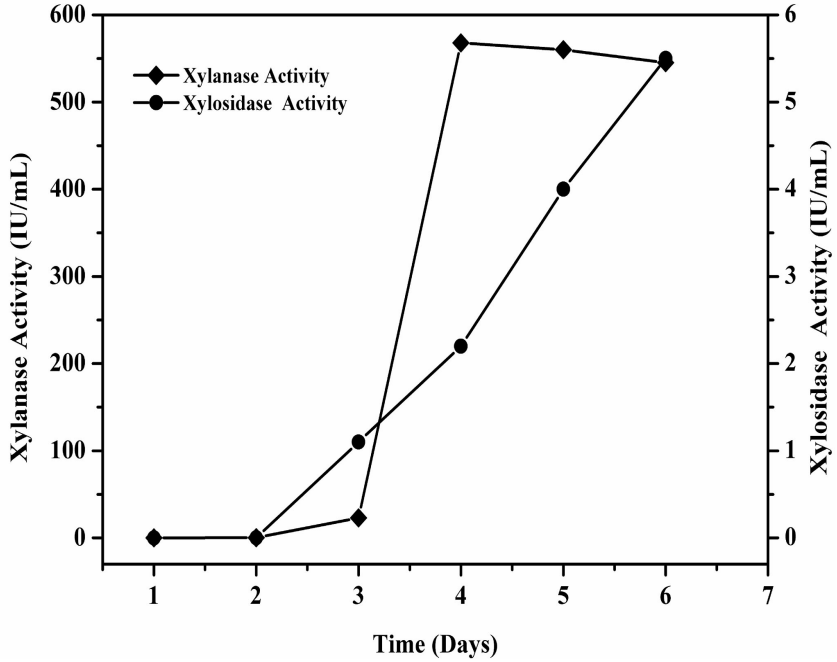

Figure 2. Production of xylanase and xylosidase in solid state fermentation (SSF).

Table 1. Effect of moisture level on enzyme production.

Fermentation medium (ml)	Xylanase activity (IU/g DSS)	β-Xylosidase activity (IU/g DSS)
6	657 ±26	36.2 ± 2.0
8	1143 ± 44	41.5 ± 2.5
10	1960 ± 57	56.0 ± 2.6
12	1782 ± 36	46.9 ± 2.4
14	1694 ± 28	34.0 ± 1.8

ratio resulted in a decline in both the enzyme production probably due to decreased porosity of the medium. To see the effect of different inoculum size on enzyme production, the SSF was carried out using different inoculum size. We found that the cells obtained from 3 ml inoculum yielded high xylanase (2080 IU/g DSS) and xylosidase (67 IU/g DSS) activities (Table 2).

3.3. Effect of Different Agricultural Residues on Enzyme Production

Different agricultural residues were tested as solid substrates for enzyme production by *P. hubeiensis* NCIM 3574. It can be concluded from the results shown in Table 3 that wheat bran was suitable for the production of xylanase (2050 IU/g DSS) and ground nut oil cake was most preferred substrate for high levels (146 IU/g DSS) of β-xylosidase production on 10th day of fermentation. Wheat bran, cotton seed oil cake and rice bran did not support the β-xylosidase production. Thus wheat bran was found to be the most suitable with xylan as inducer and ground nut oil cake with xylan proved to be the appropriate solid substrate for β-xylosidase production.

Table 2. Effect of inoculum size on enzyme production.

Inoculum size (ml)	Xylanase activity (IU/g DSS)	β-Xylosidase activity (IU/g DSS)
1	1940 ± 27	51.5 ± 1.3
2	1970 ± 22	56.0 ± 1.5
3	2080 ± 26	67.0 ± 2.1
4	2050 ± 30	53.0 ± 1.6
5	1660 ± 26	48.5 ± 1.3

Table 3. Effect of various substrates on enzymes production.

Solid substrate	Xylanase activity (IU/g DSS)		β-Xylosidase activity (IU/g DSS)	
	6th day	10th day	6th day	10th day
Wheat bran	2050	1300	43.0	50.5
Rice bran	235	227	3.2	3.5
Cotton seed oil cake	928	730	43.8	48.5
Ground nut oil cake	940	1150	81.5	146.0

3.4. Extraction of Enzymes by Salt Solution with and without Surfactants

Enzymes from fermented solid substrates were extracted with distilled water, citrate phosphate buffer (50 mM, pH 7.0) and 1% NaCl with and without Triton X-100. From the results in Table 4, 1% NaCl supplemented with 0.5% Triton X-100 was found to be very effective for the recovery of both xylanase and β-xylosidase activities. There was no marked difference in the recovery of enzymes when the extraction was carried out with 1% NaCl supplemented with 1% Triton X-100. The use of Tween 80 at 0.5% concentration did not help in the recovery of both the enzymes. The highest yields of xylanase and β-xylosidase were 2480 IU/g DSS and 198 IU/g DSS respectively.

3.5. Xylan Hydrolysis and End Product Analysis

The crude xylanase hydrolyzed xylan into small molecular weight XOS. The hydrolysis product gave arabinose, xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose and xyloheptaose with no xylose detected (**Figure 3**). The earlier report on the purified xylanases of *P. hubeiensis* [9] substantiated the observations in which both the purified xylanases gave XOS with DP of X3-X7.

4. Discussions

Pseudozyma hubeiensis remained unexplored with respect to biomass degrading enzymes. We were the first to report cellulase-free xylanase production [6] at 30˚C by this yeast strain isolated in our laboratory in 1990. It was later identified as *Pseudozyma hubeiensis* in 2007 by National Collection of Yeast Cultures (NCYC) using 26 rDNA D1/D2 sequencing and standard taxonomic tests. It was

Table 4. Effect of different extraction solutions on the recovery of enzymes from the fermented solid substrates.

Salt solution	*β*-Xylosidase activity (IU/g DSS)	Xylanase activity (IU/g DSS)
Distilled water	145	1180
Citrate phosphate buffer (50 mM), pH 7	148	2050
Citrate phosphate buffer (50 mM), pH 7 + Triton X-100 (0.5%)	184	2248
NaCl (1%)	179	2273
NaCl (1%) + Triton X-100 (0.5%)	198	2480
NaCl (1%) + Triton X-100 (1.0%)	198	2470
NaCl (1%) + Tween 80 (0.5%)	185	2108

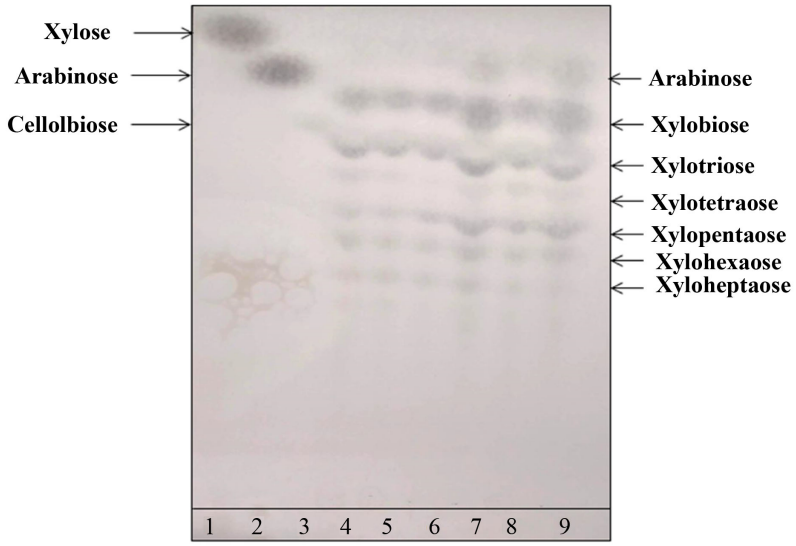

Figure 3. Xylan hydrolysis by crude xylanase and analysis of end products. 1: Standard Xylose; 2: Standard Arabinose; 3: Standard Cellobiose; 4: Hydrolysed sample (15 min); 5: Hydrolysed sample (30 min); 6: Hydrolysed sample (1 hr); 7: Hydrolysed sample (2 hrs); 8: Hydrolysed sample (3 hrs); 9: Hydrolysed sample (4 hrs).

deposited in NCIM Resource Center, CSIR-National Chemical Laboratory, Pune, India with an accession number NCIM 3574. The cellulase-free xylanase produced by *P. hubeiensis* NCIM 3574 degraded different lignocellulosic materials to xylose which can be the starting material for several value added chemicals including xylitol. We also found that the yeast grew significantly at low temperatures (25˚C - 30˚C) and produced high levels of *β*-xylosidase (5.36 IU/ml) at 27˚C with a significant decline in its production at 30˚C [7]. This observation prompted us to continue the study on production of xylanase at 27˚C in both SmF and SSF. *P. hubeiensis* NCIM 3574 produced very high xylanase (565 IU/ml) and *β*-xylosidase (5.4 IU/ml) activities on 3rd and 5th day respectively in SmF when it was grown in fermentation medium containing 2% xylan. The data generated on the temperature effect on xylanase and *β*-xylosidase production

suggests that xylanase produced at 30˚C can be used for XOS production (without purification) since it contains insignificant amounts of β-xylosidase which is evident from **Figure 3**. Presently, XOS (low chain oligosaccharides) are produced by acid hydrolysis followed by chromatographic separations which add to the cost of the process. The production of XOS using crude xylanase does not require chromatographic separation since the xylanase produced low chain xylooligosaccharides. XOS is a prebiotic used to support the growth of probiotic bacteria.

The initial SSF studies were carried out at 27˚C using wheat bran and xylan and the results demonstrated that maximum production of xylanase activity (1915 IU/m DSS) and β-xylosidase activity (52 IU/mL DSS) was achieved on 6th and 10th day respectively. The purified xylanases from *P. hubeiensis* NCIM 3574 produced XOS of less degree of polymerization (DP 3 - 7) which act as bioactive supplements of food and health products. The production of XOS makes these enzymes interesting from industrial point of view. Very recently, *Streptomyces griseorubens* LH-3 was reported to produce thermostable cellulase-free endo-xylanase with bagasse hemicellulose as a carbon source [13]. The enzyme was purified and characterized as a monomeric protein having an apparent molecular weight of 45.5 kDa that produced short chain XOS as hydrolysis products.

The enzymes production by *P. hubeiensis* was attempted in SSF using various solid substrates such as wheat bran, rice bran, cotton seed oil cake and ground nut oil cake. Wheat bran was found to be the most suitable for xylanase production and ground nut oil cake with xylan proved to be the appropriate solid substrate for β-xylosidase production. Wheat bran is known to be a universally suitable substrate for hydrolytic enzymes production due to the presence of sufficient nutrients. It also provides large surface area since it remains loose even under moist conditions. It is noteworthy to mention that ground nut oil cake yielded significantly high β-xylosidase activity and hence the further optimization studies were carried out with wheat bran and ground nut oil cake for xylanase and β-xylosidase production respectively. There are very few reports on the use of ground nut oil cake as substrate for production of xylanase and β-xylosidase. The high activity could be due to the high content of protein (41%) and amino acids present in the ground nut oil cake [14].

The moisture content during the SSF is one of the crucial factors which play an important role in the success of the SSF process [15]. The moisture content in the SSF affects the physiological properties of the solid substrates. High moisture levels decrease the porosity, reduce the oxygen transfer and alter the particle structure of the substrates such as wheat bran. Lower levels of moisture content than the optimum reduce the solubility of the solid substrates and produce the higher water tension. The present study showed that solid substrate to fermentation medium ratio of 1:2 was most suitable for maximum production of xylanase (1960 IU/g DSS) and β-xylosidase (56 IU/g DSS) activities.

The use of surfactants such as Triton X-100, Tween-80 during SmF or SSF was found to increase the enzyme production [16] [17] [18]. In addition, 1% NaCl

supplemented with 0.5% Triton X-100 was also used during extraction of lipase from fermented wheat bran for maximum recovery of lipase [12]. In the present study, we used 1% NaCl supplemented with 0.5% Triton X-100 for extraction of xylanase and β-xylosidase from fermented solid substrates. We could achieve the highest recovery of xylanase (2480 IU/g DSS) and β-xylosidase (198 IU/g DSS). Further increase in Triton X-100 concentration did not help in extraction of both the enzymes.

5. Conclusion

P. hubeiensis NCIM 3574 isolated from decaying sandal wood produced a cellulase free xylanase and β-xylosidase. Elevated amounts of cellulase free xylanase (2480 IU/g DSS) and β-xylosidase (198 IU/g DSS) were obtained when the yeast was grown in SSF using wheat bran and ground nut oil cake respectively. xylanase and β-xylosidase from any yeast strain. The cellulase-free xylanase produced XOS that have great potential as functional foods or prebiotics. The hydrolysis products of xylan by cellulase-free xylanase of *P. hubeiensis* consisted of low chain XOS (X2-X7) and hence can be used directly without further purification. The use of such cellulase-free xylanases also shows great advantages over chlorine bleached processes used to remove xylan from bleached or unbleached pulp. The properties such as high catalytic performance, high metal and ethanol tolerance qualify both xylanase and β-xylosidase of *P. hubeiensis* NCIM 3574 for the use in the hydrolysis of lignocellulosic biomass for biofuel production. Although much information is available about xylanase, to the best of our knowledge, this is the first report on the production of high amounts of cellulase-free xylanase and β-xylosidase by *P. hubeiensis*. Such enzymes would play a major role in valorising xylan component of biomass and making the second generation ethanol production process cost effective.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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