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Two way strategy for utilizing agricultural waste 'wheat bran' for production and immobilization of xylanase

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Abstract: This paper reports an economical method for immobilization of xylanase by physical adsorption on residual wheat bran obtained after the extraction of enzyme following solid state fermentation. The optimization of process parameters through response surface methodology gave an immobilization of 83.6 % and protein binding of 82%. The values optimized by RSM were validated with adsorption isotherm (Langmuir and Freundlich isotherm equations). The monolayer capacity (K_L/a_L) for 0.04 g of residual wheat bran was corresponds to 5.25 mg of protein. The pH and temperature stability was enhanced after immobilization. The bound enzyme displayed a higher V_{max} and it could be reused for 15 cycles still retaining 70.0 % of its initial activity. The enzyme was fully stable during storage at 4°C for two months. The immobilized enzyme was found to be effective in removing chromophores from the newspaper pulp. The chromophores released by the enzyme exhibited absorbance at 240 to 360 nm wavelengths with maximum at 300 nm. The immobilization protocol proposed here is apparently cost-effective as it reuses the wheat bran. A considerable stability and reusability of the bound enzyme may be advantageous for its industrial application.

Keywords: Protein recovery; Enzyme activity; Solid-state fermentation; Adsorption; Immobilization

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Xylanase (EC 3.2.1.8) catalyzes the hydrolysis of β-1, 4-linked-D-xylopyranose polymer releasing xylose and xylooligosaccharides (Wong et al. 1988). Xylanases are produced by diverse genera of bacteria [Nagar et al. 2011, 2012; Sanghiet al. 2008; Anuradha et al. 2007), fungi and actinomycetes (Ninawe et al. 2008; Okafor et al. 2007). Over the last decade, xylanases have attracted considerable research interest due to their industrial applications such as bleaching of kraft pulps (Madlala et al. 2001), poultry and animal feed supplement (Buchert et al. 1994), improving dough quality of baked products (Butt et al. 2008), extraction of coffee, plant oils, and starch (Wong and Saddler 1992), clarification of fruit juices (Dhiman et al. 2011) and degumming of plant fibers (Zheng et al. 2000). However, use of the free enzyme for these applications may be restricted by its structural instability and non-recovery of its active form from the reaction mixture for reuse (Krajewska 2004). These limitations may be overcome by using immobilized enzymes which offer the advantages of reusability and enhanced stability leading to a reduced cost of the product. The enzymes whose activity assays are performed at high temperatures (50 or more than 50°C), need rigid support for immobilization. The supports like agarose, gelatin, alginate, chitosan and polyacrylamide gels get damaged during the recycling process of immobilization due to high temperature of the enzyme assay. The use of a support which is stable at a high temperature and pH is generally desirable for enzyme immobilization. Various supports stable under extreme conditions have been reported in the literature, but these are often costly and not easily available. Previously, xylanase immobilization has been reported on various carriers such as chitosan (Chen et al. 2010), chitosan–xanthan hydrogel (Dumitriu and Chornet 1997), Duolite A147 pretreated with glutaraldehyde (Gouda and Abdel-Naby 2002), magnetic latex beads (Tyagi and Gupta 1995), reversibly soluble–insoluble polymer Eudragit S-100 (Gawande and Kamat 1998), Eudragit L-100 (Sardar et al. 2000), cellulose acetate membrane grafted with acrylamide (Sarbu et al. 2006) and multi-walled carbon nanotubes (Shah and Gupta 2008) but not even a

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single report is present on xylanase immobilization on wheat bran. In view of high cost of commonly used immobilization support materials (from Sigma): chitosan (100g) 116.0 US$, sodium alginate (100 g) 10.7 US$, cellulose acetate (100 g) 13.5 US$ and multi-walled carbon nanotubes (10 g) 299.50 US$ approximately, the use of easily available wheat bran (0.02 US$ per 100 g) will be economical for immobilization of enzymes.

The aim of the present work was to develop a cost-effective method for xylanase immobilization by reusing the residual wheat bran (production waste agro-residue) as the support. Further, the potential of the bound enzyme was investigated in removing chromophores from the newspaper pulp.

MATERIAL AND METHODOLOGY

Xylanase production and extraction

Bacillus pumilus SV-85S MTCC 9861, isolated in our laboratory, was used for xylanase production under solid-state fermentation (Nagar et al. 2011). The solid substrate (wheat bran) was moistened with distilled water in the ratio of 1:3 and autoclaved at 15 psi for 20 min. The flasks were inoculated aseptically with 10 % (v/v) of 18 h old inoculum (peptone 0.5%; beef extract 0.3% w/v) and incubated at 37°C for 48 h. Xylanase was extracted from the fermented wheat bran with distilled water added in the ratio of 1:10. The clear cell-free supernatant obtained after centrifugation of the extract at 10,000 x g for 20 min at 4°C was referred to as the crude enzyme.

Xylanase assay and protein estimation

The activity of soluble enzyme was assayed by measuring the amount of reducing sugars (xylose equivalent) liberated from xylan using 3, 5-dinitrosalicylic acid (Miller 1959). The reaction mixture containing 490 µL of 0.5 % birchwood xylan (Sigma) as substrate and 10 µL of appropriately diluted enzyme extract was incubated at 50 °C for 10 min. The reaction was then terminated by adding 1.5 mL of 3, 5-dinitrosalicylic acid reagent. A control was run simultaneously that contained all the reagents but the reaction was terminated prior to the addition of enzyme. The contents were placed in a boiling water bath for 10 min followed by cooling in ice- cold water. The absorbance was measured at 540 nm. Protein was estimated by Lowry's method (Lowry et al. 1951) using bovine serum albumin (BSA) as the standard protein. The reaction mixture containing appropriately diluted sample (1 mL) and 3 mL of reagent (2% Na_2CO_3 in 0.1 N NaOH, 1% $CuSO_4.5H_2O$ and 2% sodium potassium tartarate in a ratio of 9:1:1) was incubated for 10 min. Then, 0.3 mL of 1N Folin-ciocalteau's reagent was added and left for 30 min at room temperature for color development. A blank was run simultaneously that contained all the reagents except protein sample. The intensity of blue color formed was measured against the blank at 660 nm.

Preparation of the immobilization support

The residual wheat bran after the extraction of xylanase from the solid state fermentation medium (as described above) was collected and thoroughly washed 10–12 times with distilled water. The wheat bran was then dried at room temperature, passed through a sieve (mesh No. 12) and autoclaved at 15 psi for 30 min. The resulting bran having uniform particles was used as support for immobilization of xylanase.

Preparation of used spin columns for immobilization

The waste spin columns (used in DNA/RNA extraction) were collected and their DNA/RNA binding membranes were replaced with Whatman filter paper disc (6 mm dia) with the help of forceps. The spin columns were then autoclaved at 15 psi for 30 min and used for immobilization work.

Immobilization of xylanase

The prepared spin columns were filled with 0.05 g of wheat bran, which was moistened with 0.5 mL (1000 IU/mL; 8.75 mg protein/mL) of xylanase. The quantity of wheat bran, volume of the xylanase and incubation time was optimized via RSM. Thereafter, the columns having enzyme adsorbed wheat bran were washed with distilled water till no activity was found in the washing. The washings were pooled and the activity of the enzyme was determined in it. The ready spin columns were stored at 4°C for further studies. The immobilization (%) was calculated as follows:

$$\text{Immobilization (\%)} = \frac{\text{Total activity of immobilized enzyme}}{\text{Total activity of soluble enzyme}} \times 100$$

Total activity of the immobilized enzyme was calculated by subtracting the activity of the unbound enzyme from that of the soluble enzyme. Immobilization was also calculated in terms of protein binding in a similar manner.

$$\text{Immobilization (\%)} = \frac{\text{Protein bound to the support}}{\text{Protein content of soluble enzyme}} \times 100$$

Assay of immobilized xylanase

The activity of the immobilized xylanase was assayed by adding 490 µL of xylan in the spin column and incubated at 50 °C for 10 min. The reaction was stopped by separating the immobilized enzyme from the reaction mixture by centrifuging the spin column at 2000 rpm for 30 sec. Then 1.5 mL of 3, 5–dinitrosalicylic acid reagent was added to the reaction mixture and placed in a boiling water bath for 10 min for color development. The contents were cooled and the absorbance was read at 540 nm.

Statistical optimization of immobilization

The statistical design was applied to evaluate maximum immobilization and protein binding. Three parameters viz. amount of wheat bran, volume of xylanase and incubation time were selected for

optimization. Using statistical software package Design Expert® 8.0.4, Stat-Ease, Inc., a 2^3 full factorial central composite design (CCD) with the above three factors and six replicates at the central points, leading to a set of 20 experiments was used to optimize the immobilization (%) and protein (%) adsorbed on wheat bran. Each variable was used at coded levels (-1, 0, 1) as shown in Table 1. All the variables were taken at a central value represented by '0'. The 20 experiments of CCD included 8 trials for factorial design, 6 for axial point and 6 for replication of central point (Table 2). The response value from each experiment of CCD was the average of triplicates.

Table 1 Experimental range and levels of each variable under central composite design

Name of the variable	Symbol	-1	0	+1
Wheat bran (g)	A	0.02	0.04	0.06
Xylanase (mL)	B	0.10	0.30	0.50
Incubation period (min)	C	30.00	75.00	120.00

The adsorption isotherm

The optimized results were confirmed with adsorption isotherm (Langmuir and Freundlich isotherm equations). The Langmuir isotherm corresponds to the immobilization in a monolayer, and Freundlich isotherm corresponds to multilayer immobilization. The Langmuir constants were calculated using linearization of the Langmuir expression and plotting $1/Q_e$ versus $1/C_e$.

$$Q_e = \frac{K_L C_e}{1 + a_L C_e}$$

Where, Q_e (IU/0.04 g support) representing the enzyme loading (i.e. amount adsorbed) and C_e (IU/mL solution) is the xylanase residual in solution after immobilization. K_L and a_L are the Langmuir constants. K_L/a_L represents the monolayer capacity of adsorbent.

Similarly, the Freundlich constants were determined by the linearization of the Freundlich equation:

$$\ln Qe = \ln K_F + \frac{1}{n} \ln C_e$$

Where, K_F (mL/0.04 g) and 1/n (unitless) are the Freundlich constants. K_F indicates the support capacity, and 1/n is the heterogeneity factor.

Temperature and pH stability of free and immobilized xylanase

Thermal stability was investigated by incubating the free and the immobilized enzyme separately for 1h at various temperatures (30–70 °C) followed by xylanase assay under respective standard conditions. The pH stability of the free and the immobilized xylanase was determined by incubating the free and the bound enzyme separately in 50 mM buffers of different pH values (4.0–11.0) for 1 h at 37°C. The buffers used were citrate (pH 4.0–5.0), phosphate pH (6.0–8.0) and glycine-NaOH (pH 9.0–11.0). The residual enzyme activity was assayed under the standard assay conditions.

Determination of kinetic parameters

The substrate saturation kinetics was determined by varying the concentration of the substrate i.e. birchwood xylan in the range of 0.125 to 8.0 mg/mL at constant concentration of the enzyme. The Michaelis-Menten constant (K_m) and V_{max} for the xylanase were calculated from the Lineweaver–Burk plot.

Storage stability

The storage stability of the free and the immobilized xylanase was evaluated at 4°C for 14 weeks. The spin column containing the bound xylanase was kept at 4°C and the enzyme activity was determined at regular intervals of time.

Re-usability of the immobilized enzyme

The birchwood xylan (0.5%) was added to the spin column containing the bound xylanase, incubated at 50°C for 10 min and centrifuged at 2,000 x g for 30 s. The reaction was then stopped and the enzyme activity determined. The enzyme bound wheat bran was repeatedly used to hydrolyze xylan up to 15 batch reactions.

Scanning electron microscopy (SEM)

The scanning electron microscopy was done for the confirmation of xylanase adsorption on to the wheat bran. The detailed surface morphology was done using a SEM (Zeiss-EVO, MA-10, variable UK).

Application of the immobilized enzyme

Preparation of waste newspaper pulp

Newspaper pulp was prepared by chopping the newspapers to less than 2 cm^2 sizes and then dipping in boiled distilled water for 48 h. The water was removed from the pulp with the help of muslin cloth and the pulp was washed thoroughly with distilled water. The extracted pulp was dried in an oven at 30–40 °C and grinded.

Measurement of chromophores released by the enzyme

The immobilized enzyme was applied to waste news paper pulp and the released chromophores were measured. The pulp at 10% consistency was treated with the immobilized xylanase. After an appropriate time of incubation, the contents were filtered. A portion of the filtrate was centrifuged at 8000 x g for 10 min and the supernatant was collected. The release of chromophores in the supernatant was monitored by recording its absorption spectra (UV/VIS Spectrophotometer) in the wavelength range of 240 to

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360 nm. The experimental conditions for maximum release of chromophores were optimized by treating the pulp with varying amount of the immobilized enzyme, ranging from 1 to 4 g of 10% pulp consistency for variable time intervals up to 300 min, and incubation temperature from 30–60°C. The optimized conditions were further applied to observe the reusability of the process. The immobilized enzyme was sealed in muslin cloth packets for evaluating its reusability in measuring the release of chromophore.

RESULTS AND DISCUSSION

In this study, xylanase was immobilized on residual wheat bran (obtained after the extraction of xylanase from the solid state fermentation medium) by physical adsorption. The graphical representation of the immobilization procedure is shown in figure 1 (Supplementary on website). The label 1, 2 and 3 in the figure 1 reflect the steps, where the waste materials were utilized for immobilization. The waste material (wheat bran) was used twice in our study, for production as well as immobilization. The immobilization conditions were optimized using RSM and potential of the bound enzyme was evaluated in the removal of chromophores from the newspaper pulp.

Optimization of xylanase immobilization through RSM

A central composite design was prepared using statistical software and the values of all the three variables (amount of residual wheat bran, volume of crude xylanase and incubation time) for immobilization of xylanase were optimized. The experimental results of immobilization and protein binding by CCD with six replications of the central point and six axial points are shown in Table 2. The responses of the CCD design were fitted with a polynomial quadratic equation (Eq. 1 and Eq. 2).

The overall polynomial equation for xylanase immobilization and protein binding was:

$$\text{Immobilization (\%)} = 64.39 - 0.49A + 23.46B - 2.05C + 0.94AB - 1.05AC + 3.51BC - 0.15A^2 - 8.16B^2 - 0.39C^2 \quad \text{(Eq. 1)}$$

Where, A is amount of wheat bran; B is volume of xylanase and C is incubation period.

The statistical significance of the model equation was evaluated by the F-test for analysis of variance (ANOVA), which showed that the regression was statistically significant with 99.9 % confidence level. The model F-value for immobilization and protein binding was 151.71 and 12.65 respectively as shown by Fisher's test, which indicated that the model was significant. The value of "P > F" less than 0.05 was desirable for a significant model. The P > F value of the model was 2 x 10^{-4} and 1 x 10^{-4} respectively for immobilization and protein binding; therefore, the model terms were also significant. It implied that the model was statistically significant for xylanase immobilization. There was only 0.01% chance that a ''Model F Value'' was not significant which could arise due to noise. The determination coefficients (R2) of the model were 0.99 and 0.92, which showed the aptness of the model. Moreover, R^2 value was in reasonable agreement with adjusted R^2. The adjusted R^2 corrects the R^2 value for sample size and number of terms in the model (Table 3). The "Adeq Precision" measures the signal to noise ratio. A ratio of greater than 4 was desirable. The ratio of 44.57 and 13.55 indicated an adequate signal. Thus, the model was significant for the process.

The simultaneous effect of all the three variables on xylanase immobilization can be seen in perturbation graph (Fig. 2). The effect of all the three variables on protein binding was in similar pattern with immobilization. The plot reveals that the process of immobilization is most effective to the enzyme load

Table 2 Central composite design for optimization of xylanase immobilization

Std order	A: Wheat bran (g)	B: Xylanase (mL)	C: Incubation period (min)	X: Immobilization (%)		Y: Protein (%)	
				Experimental	Predicted	Experimental	Predicted
1	0.02	0.1	30.0	39.5	38.2	43.6	38.2
2	0.06	0.1	30.0	39.9	37.4	26.1	27.4
3	0.02	0.5	30.0	77.6	76.2	71.3	74.1
4	0.06	0.5	30.0	77.7	79.2	76.5	82.3
5	0.02	0.1	120.0	30.0	29.2	54.7	45.0
6	0.06	0.1	120.0	22.1	24.2	32.6	26.0
7	0.02	0.5	120.0	78.1	81.2	74.9	69.8
8	0.06	0.5	120.0	78.0	80.0	68.1	69.7
9	0.01	0.3	75.0	64.2	64.8	59.4	67.0
10	0.07	0.3	75.0	64.6	63.3	61.9	58.9
11	0.04	0.0	75.0	0.00	10.8	0.00	17.5
12	0.04	0.6	75.0	83.6	81.2	82.0	77.2
13	0.04	0.3	0.0	64.2	66.7	63.4	59.0
14	0.04	0.3	150.7	63.3	59.8	44.1	54.0
15	0.04	0.3	75.0	64.4	64.4	61.8	61.6
16	0.04	0.3	75.0	64.4	64.4	61.8	61.6
17	0.04	0.3	75.0	64.4	64.4	61.8	61.6
18	0.04	0.3	75.0	64.4	64.4	61.8	61.6
19	0.04	0.3	75.0	64.4	64.4	61.8	61.6
20	0.04	0.3	75.0	64.4	64.4	61.8	61.6

while least influenced by the amount of wheat bran. RSM has proven to be an advantageous tool for optimization of conditions.

Table 3 Analysis of variance (ANOVA) of quadratic model for xylanase immobilization

Term	Immobilization (X)	Protein (Y)
Standard deviation (SD)	2.52	7.52
Mean	58.45	56.46
Coefficient of variation %	4.31	13.33
PRESS	494.55	4308.93
R^2	0.99	0.92
Adjusted R^2	0.99	0.85
Predicted R^2	0.94	0.39
Adequate precision	44.57	13.55

PRESS: Predicted residual sum of squares; R^2: Determination of coefficient

The CCD and perturbation plots suggested that the highest immobilization (83.6%) and protein binding (82%) were obtained in the presence of 0.04 g wheat bran, 0.6 mL xylanase and incubation period of 75 min. The result obtained in the experiment was satisfactory as predicted values of immobilization and proteins binding at these conditions were 81.2 and 77.2%, respectively.

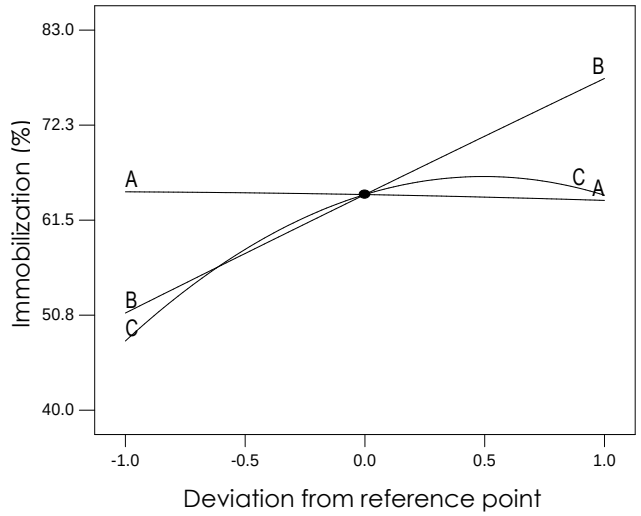

Figure 2 Perturbation graph showing the effect of variables on immobilization (A: wheat bran; B: xylanase; C: incubation period)

The design model was validated by changing the value of parameters on optimized values and found that the conditions mentioned above were appropriate. There was no effect of changing the concentration of each variable against the optimized values. The optimized values were verified by repeating the experiment. There are many reports on immobilization of xylanase (Tan et al. 2008; Hudson et al. 2005) but only a few of these reports used statistical approach.

Adsorption isotherm

In order to evaluate the support adsorption capacity, adsorption isotherms were experimentally determined. Isotherms of Langmuir and Freundlich were applied to describe adsorption isotherms (Gitlesen 1997; Al-Duri and Yong 1997). The monolayer capacity (K_L/a_L) for 0.04 g of residual wheat bran was 500 IU (correspond to 5.25 mg protein). At this value the Langmuir linearity was lost by the immobilization support (Table 4). The Langmuir isotherm reflects irreversible adsorption, where permanent bonds are formed between the enzyme and support surface. The homogeneity of the immobilization support was confirmed by the 1/n value of Freundlich constants. The support surface would be less homogeneous if 1/n is closer to 0. The 1/n value for immobilization support was 0.613 and considered as homogeneous (Table 4). All these experimental data seem to confirm our assumption that the immobilization of xylanase on wheat bran support occurs in a monolayer up to this loading.

Scanning electron microscopy

The SEM of free wheat bran and xylanase adsorbed wheat bran are shown in figure 3. The images were taken at a magnification of 2.0 KX and 5.0 KX at a voltage of 15 kV. The wheat bran basically is the waste product that remained after refining of wheat grain. Bran contains the outer layer of grain and more specifically the pericarp (including exo-, meso- and endocarp) of the wheat grain. The figure 3a and 3b represent the control images for the free wheat bran and figure 3c and 3d showing images for xylanase adsorbed on the surface of wheat bran. As seen from the figures, the enzyme was adsorbed both the external surface of the membrane and within the internal spaces of the pericarp.

Temperature and pH stability of free and immobilized xylanase

Figure 4a shows thermal stability of the free and the immobilized xylanase. The magnitude of activity of both free as well as immobilized enzyme was same up to 40°C, but differed thereafter. It was observed that the free xylanase lost its complete activity after 1 h of incubation at 70°C whereas the bound enzyme preserved 48% of its activity at this temperature. The

Table 4 Values of Langmuir and Freundlich constants for xylanase immobilized on wheat bran

Xylanase loaded (IU)	Protein (mg)	Xylanase bound (IU)	Langmuir constants		Freundlich constants	
400	3.50	305	K_L	0.437	K_F	2.9
600	5.25	465	a_L	8.7×10^{-4}	1/n	0.613
800	7.00	650	K_L/a_L	500		

The calculations were for 0.04 g of immobilization support

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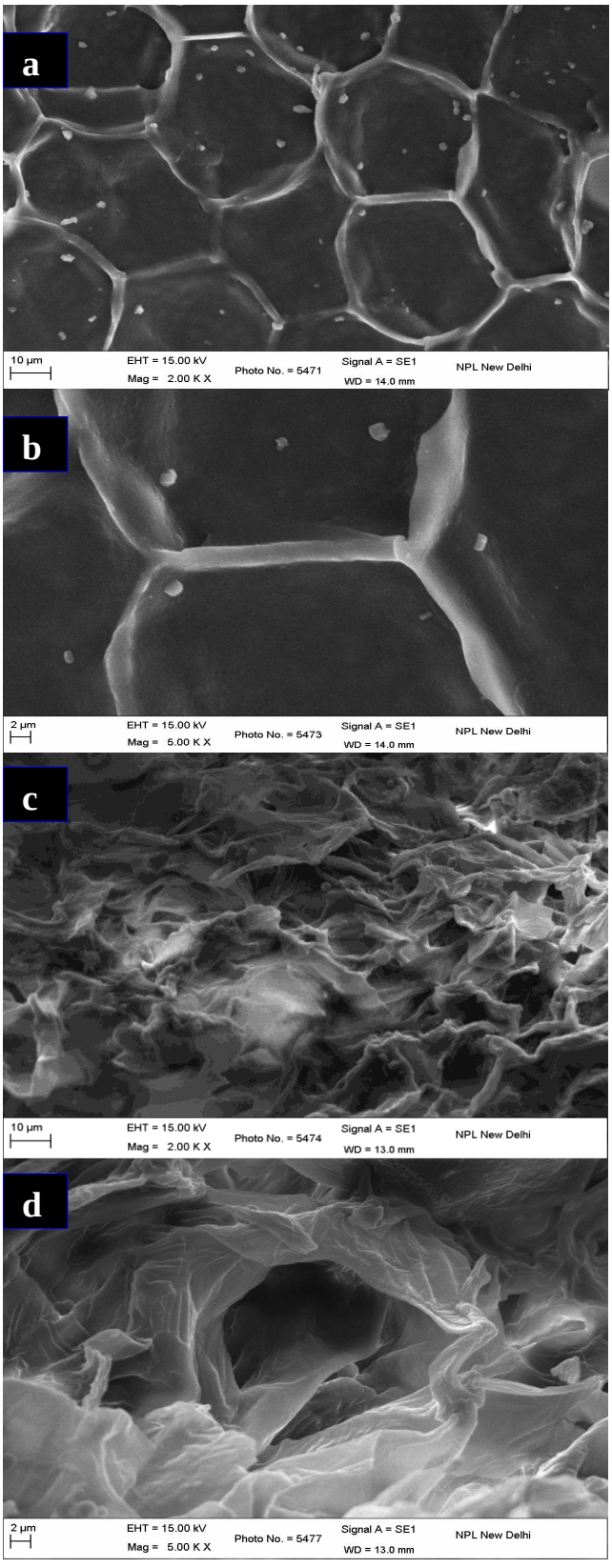

Figure 3 Scanning electron micrographs, control wheat bran at 2 KX (a) and at 5 KX (b); Xylanase adsorbed wheat bran at 2 KX (c) and at 5 KX (d)

increase in temperature stability upon immobilization may be the result of enzyme rigidity (Ortega et al. 2009). Moreover, the substrate may protect the enzyme against inactivation by heat. A similar increase in thermostability on enzyme immobilization has earlier been reported (Tyagi and Gupta 1995; Subramaniyan and Prema 2002).

A xylanase stable at higher temperatures is likely to be more suitable for industrial applications. The pH stability of the immobilized xylanase was greater than that of the free enzyme. The immobilized enzyme was stable at low (pH 4) as well as high (pH 11) pH as compared to the free enzyme (Fig. 4b). The exact reason for the increased pH stability of the enzyme upon immobilization is not clear, however, the immobilization support might have a protective effect on the enzyme at extremes of pH. Ai et al. 2005 demonstrated the xylanase immobilized on Eudragit S-100 exhibited the same pH stability as the free xylanase. The enhanced pH stability of xylanase is desirable for its application in pulp and paper industry (Dalal et al. 2007).

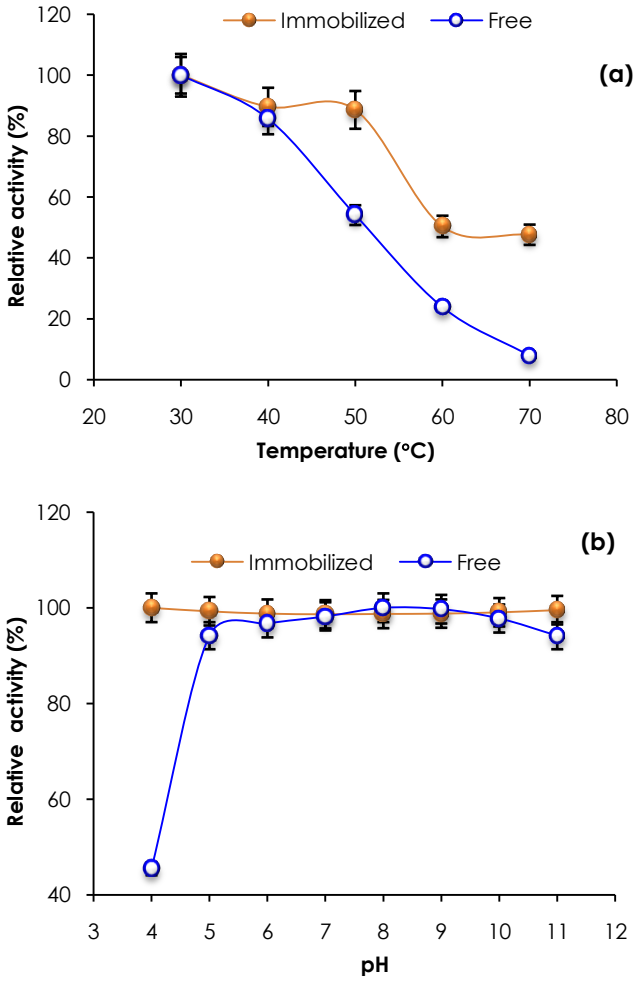

Figure 4 Effect of temperature (a) and pH (b) on the stability of free (hollow circles) and immobilized (solid circles) xylanase

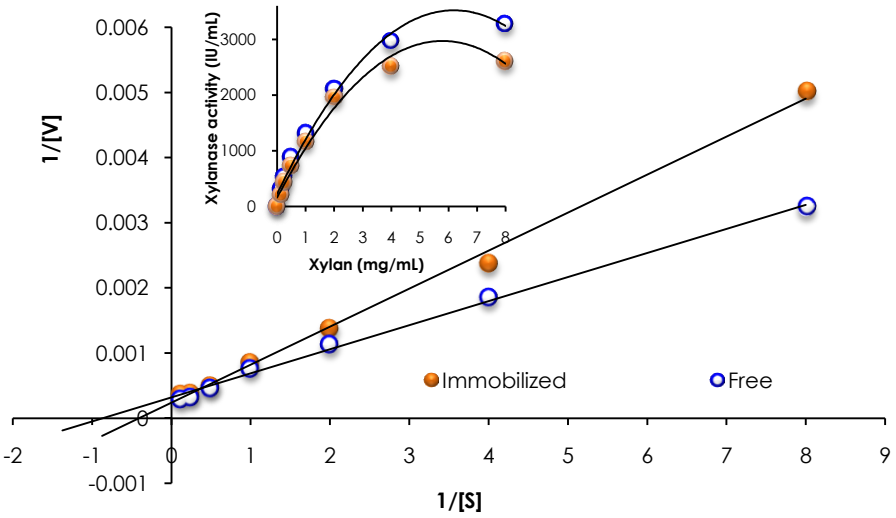

Figure 5 Lineweaver-Burk plot for estimation of kinetic parameters for free (hollow circles) and (filled box) immobilized xylanase. Inset shows the Michaelis - Menten plot

Determination of kinetic parameters

Both free and immobilized xylanase showed a hyperbolic response with increasing substrate concentration (Inset Fig. 5). The Kinetic parameters were calculated using the Lineweaver–Burk plot. The regression equation for L.B. plots of the free and the immobilized enzymes were y = 0.0004x + 0.0003 (R^2 = 0.99) and y = 0.0006x + 0.0002 (R^2 = 0.99), respectively.

The K_m for the free and the immobilized xylanase was 1.33 mg/mL and 3.0 mg/mL, respectively (Fig. 5). The immobilized enzymes have been reported to have higher K_m values especially when assayed with high molecular weight substrates like xylan (Gouda and Abdel-Naby 2002; Bhandari et al. 2009). This might result from the restricted access of the immobilized enzyme to the substrate due to steric hindrance and diffusion effects. The increase in K_m value following immobilization has also been reported earlier by many investigators (Bhandari et al. 2009; Edward et al. 2002). In this study, free and immobilized enzymes exhibited V_{max} of 3333 and 5000 IU/mL, respectively indicating an improvement in the catalytic efficiency of immobilized xylanase (Fig. 5).

Storage Stability

The investigation of storage stability of the free and the immobilized xylanase at 4 °C for 14 weeks showed that the immobilized enzyme was fully stable for 6 weeks but retained 82% of its activity after 14 weeks (Fig. 6). In contrast, the free enzyme retained only 68% of its original activity after 14 weeks. These results showed that xylanase was stabilized by immobilization on residual wheat bran.

Re-usability of the immobilized enzyme

The immobilized xylanase could be reused for 8 consecutive cycles without significant loss of activity and retained 70 % of its initial activity even after 15 cycles. The residual activity of the bound xylanase after 9, 10, 11, 12, 13 and 14 cycles was 84.9, 81.9, 79.5, 74.0, 73.7 and 71.2%, respectively. The reusability of the bound enzyme was superior as compared to earlier reports. Xylanase from *Aspergillus* sp. immobilized on Eudragit S-100 could be reused only for three cycles without any loss in activity in hydrolyzing xylan and wheat bran (Gawande and Kamat 1998). Immobilization of *Thermomyces lanuginosus* SSBP xylanase retained only 62% of its activity after six cycles (Edward et al. 2002). Based on the results of the present study it may be concluded that wheat bran is a novel, cheap and effective support for xylanase immobilization.

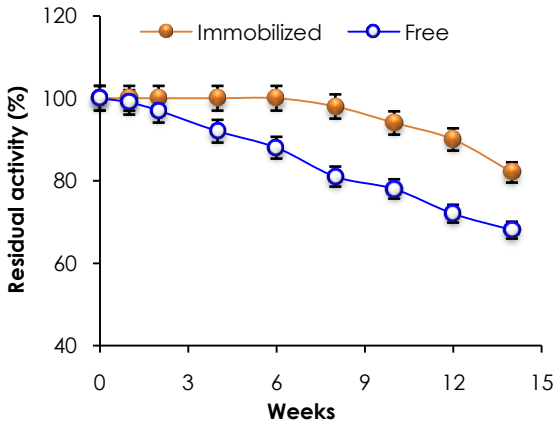

Figure 6 Storage stability of free (hollow circles) and immobilized (solid circles) xylanase

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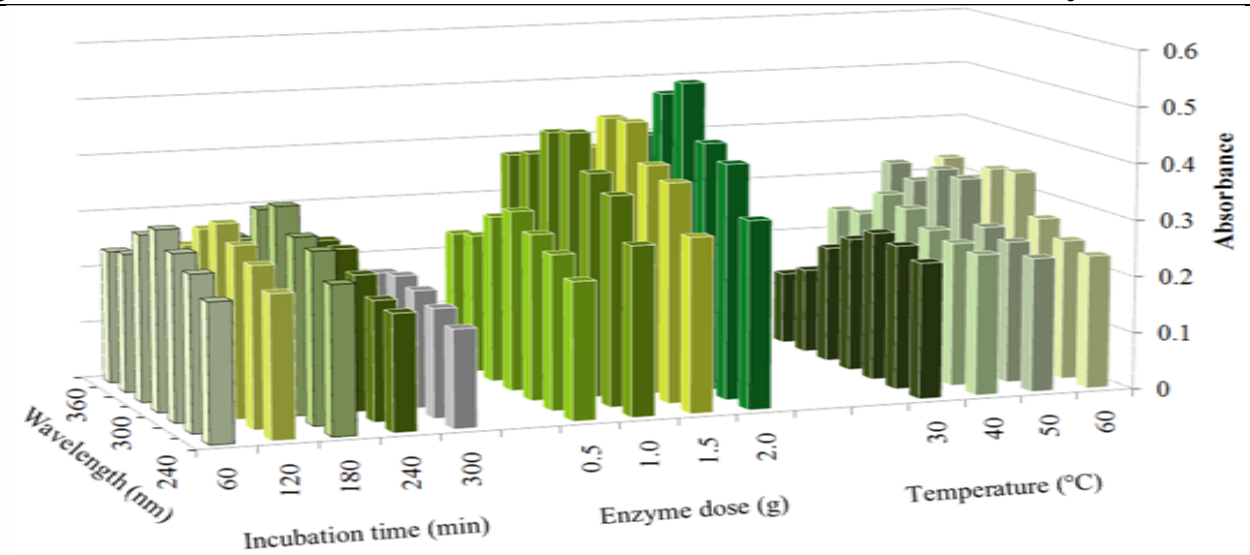

Figure 7 UV spectrum of chromophores released from the newspaper pulp treated with the bound xylanase with respect to incubation time (min), enzyme dose (g) and temperature (°C)

Removal of chromophores from newspaper pulp

The potential of the immobilized enzyme in the removal of chromophores from newspaper pulp was monitored by measuring absorbance in the wavelength range of 240 to 360 nm of the supernatant collected after the treatment of pulp with the immobilized enzyme. The results of this experiment revealed that treatment of newspaper pulp with the enzyme resulted in the removal of chromophores which absorbed at wavelengths ranging from 240 to 360 nm with a peak at 300 nm (Fig. 7). The absorbance values shown in the figure have been obtained after subtracting the absorbance of control from that of the sample. The conditions of chromophore removal have been optimized. On varying incubation time of the bound enzyme with the pulp from 60 to 300 min, it was observed that maximum removal of chromophores occurred after 180 min with a peak at 300 nm. The effect of increasing the enzyme dose from 0.5 to 2.0 g on removal of chromophores showed that the optimum dose was 2.0 g as it gave the highest absorbance of the supernatant. The variation of incubation temperature showed that chromophore removal from the supernatant enhanced with increase in temperature up to 50°C and then it remained constant. The absorbance at 280 nm could be due to the presence of lignins, however, the exact chemical nature of chromophores is not clear. There is no report in the literature on the removal of chromophores from the newspaper pulp. All these experiments were conducted in triplicates and compared with control (untreated) pulp. The optimized conditions (2.0 g immobilized enzyme, 180 min of incubation period and temperature of 50°C) were applied to investigate the reusability of the immobilized enzyme for 10 consecutive cycles in the removal of chromophores from the newspaper pulp at 10% consistency. The data shown in figure 8 revealed presence of chromophores which absorbed at wavelengths ranging from 240 to 360 nm with highest at 300 nm even after 10 cycles. The decline in absorbance at 300 nm after 10 cycles was 40%. These results clearly showed that the bound enzyme was effective in removing chromophores from the pulp even during reuse. Xylanase obtained from *B. pumilus* SV-85S was immobilized on residual wheat bran (obtained after the extraction of xylanase from the solid state fermentation medium) by physical adsorption.

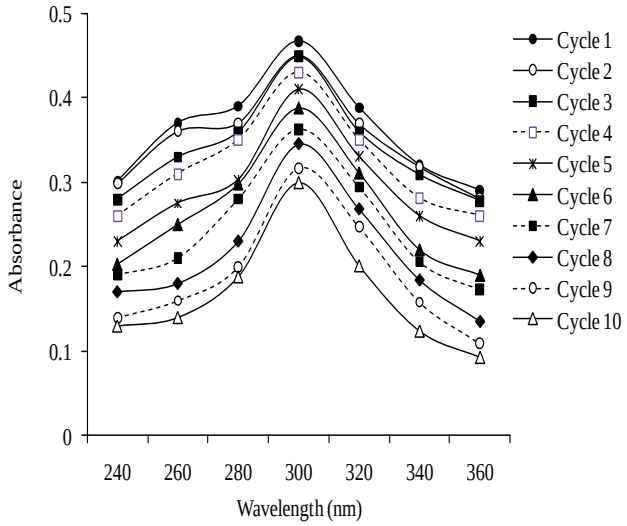

Figure 8 Reusability of the immobilized enzyme for the removal of chromophores from the newspaper pulp

The residual wheat bran was autoclaved and the crude xylanase was allowed to adsorb into it. The amount of residual wheat bran, volume of crude xylanase and incubation time were optimized using RSM and potential of the bound enzyme was evaluated in the removal of chromophores from the newspaper pulp.

Future prospects

Immobilization on wheat bran by physical adsorption is an economically viable and environmental favorable. The immobilization on wheat bran could be a universal support for binding of other enzyme. The support is stable at extremes of pH and temperatures for suitability of various biological or industrial applications. Enzymes immobilized on this material could be reused many times even the support could be reused. The enzyme immobilized on this support can be used in different forms (packets, column, powder) depends upon the requirement of the application.

Conclusion

In this study, xylanase produced from *B. pumilus* SV-85S has been immobilized by physical adsorption on novel support (residual wheat bran). The immobilization conditions were optimized using RSM to improve the immobilization efficiency. The performance of the immobilized xylanase in terms of catalytic activity, thermal and pH stability are more promising than the free enzyme. The bound enzyme exhibited enhanced storage stability and reusability and it was found to be effective in removing chromophores from the newspaper pulp.

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