Immobilization of xylanase purified from *Bacillus pumilus* VLK-1 and its application in enrichment of orange and grape juices

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Abstract This study was conducted to evaluate the efficacy of purified free and immobilized xylanase in enrichment of fruit juices. Extracellular xylanase produced from *Bacillus pumilus* VLK-1 was purified to apparent homogeneity by 15.4-fold with 88.3 % recovery in a single step using CM-Sephadex C-50. Purified xylanase showed a single band on SDS-polyacrylamide gel with a molecular mass of 22.0 kDa. The purified enzyme was immobilized on glutaraldehyde-activated aluminum oxide pellets and the immobilization process parameters were optimized statistically through response surface methodology. The bound enzyme displayed an increase in optimum temperature from 60 to 65°C and pH from 8.0 to 9.0. The pH and temperature stability of the enzyme was also enhanced after immobilization. It could be reused for 10 consecutive cycles with 58 % residual enzyme activity. The potential of purified xylanase (free and immobilized) in juice enrichment from grape (*Vitis amurensis*) and orange (*Citrus sinensis*) pulps has been investigated. The optimization of this process using free xylanase revealed maximum juice yield, clarity and reducing sugar on treatment with 20 IU/g fruit pulp for 30 min at 50°C. Treatment of both the fruit pulps with xylanase under optimized conditions resulted in an increase in juice yield, clarity, reducing sugars, titratable acidity, and filterability but a decline in turbidity and viscosity. Immobilized enzyme was more effective in improving juice quality as compared to its soluble counterpart. The results showed *B. pumilus* VLK-1 xylanase, in both free and

immobilized form, as a potential candidate for use in fruit juice enrichment.

Keywords Xylanase · Aluminum oxide pellets · Response surface methodology · Immobilization · Juice clarification

Introduction

Enzymes are widely used in industrial processes owing to their ease of production, substrate specificity and green chemistry. Xylan, the major renewable hemicellulosic polysaccharide in secondary plant cell walls, consists of a β -1,4-linked D-xylopyranose backbone substituted predominantly with α -L-arabinofuranosyl, acetyl and glucuronosyl residues (Shallom and Shoham 2003). Its complete hydrolysis requires the concerted action of several enzymes including xylanase, β -xylosidase, and enzymes that cleave groups in the side chains. Xylanase (endo- β -1,4-D-xylan xylanohydrolase; EC 3.2.1.8) catalyzes the hydrolytic cleavage of glycosidic bonds in the xylan backbone to produce a mixture of xylo-oligosaccharides of various chain lengths and xylose. It is an industrially important enzyme having applications in biobleaching of pulp, clarification of fruit juices, textile industry, food industry, production of xylo-oligosaccharides, bio-conversion of lignocellulosic feedstock into useful products and improvement of animal feed stock digestibility (Dhiman et al. 2008; Harris and Ramalingam 2010). Xylanase for industrial use is often obtained from bacteria and fungi.

Fruit juices obtained by simple extraction are cloudy, viscous and turbid (Uhlir 1998). The turbidity and viscosity of juice is due the presence of polysaccharides such as starch, pectin, cellulose, hemicelluloses and bound lignin (Lee et al. 2006). The cloudy juices have low yield and less acceptability. These are difficult to pasteurize and concentrate (Rai et al.

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2003). Therefore, juices must be clarified before commercialization. Enzymes are used to obtain optimal juice clarity, yield and a quality product that ensures consumer appeal. Plant cell wall degrading enzymes such as pectinases, hemicellulases and cellulases are used industrially to improve the clarification and yield of fruit juice and to enhance the quality of product (Abdullah et al. 2007; Bajaj and Manhas 2012; Bhat 2000; Pal and Khanum 2011; Rai et al. 2003; Shah 2007; Surajbhan et al. 2012). Enzymes used for fruit juice enrichment should preferably be in purified form. Several reports are available on the use of pectinase for clarification of fruit juice but literature pertaining to the use of xylanase in juice clarification and yield is scanty (Bajaj and Manhas 2012; Dhiman et al. 2011; Pal and Khanum 2011). In these reports, xylanase has been used in free form. The industrial application of a free enzyme may, however, be limited by its high cost and structural instability. The cost of enzyme can be reduced by reusing it through its immobilization on to an insoluble support. Apart from enzyme reusability, immobilization can also improve its chemical and thermal stability by restricting undesirable conformational changes in its structure in unfriendly environments. So, immobilized xylanase is likely to have significant advantage over its soluble counterpart for applications such as clarification of fruit juices.

Different techniques such as physical adsorption, ionic binding, covalent coupling, entrapment and encapsulation may be used for enzyme immobilization. Each of these methods has its own advantages and limitations. To immobilize xylanase it is important that the carrier matrix should be stable at 50–60 °C because the assay of its activity is generally performed in this temperature range. In addition, the efficiency of xylanase is decreased when the bound enzyme doesn't have access to bulky insoluble substrate (Tan et al. 2008). Hence, entrapment of xylanase would not be desirable. Covalent methods involving the formation of a covalent bond between the functional groups of amino acid residues on the surface of the enzyme and the functional groups present on the surface of the carrier are likely to be more appropriate for immobilization of xylanase. The covalently immobilized enzyme preparations are stable and enzyme leaching is minimal. Further, since enzyme molecules are on the surface, contact with large substrates is possible as required in case of xylanase. Immobilization of xylanase has been reported on various supports including polymethyl methacrylate nanofibers membrane (Kumar et al. 2009), Eudragit L-100 (Roy et al. 2003), silica (Kang et al. 2002; Sharma et al. 2012), chitin, HP-20 and gelatin (Kapoor and Kuhad 2007); glass beads (Kumar et al. 2009), chitosan beads (Jingmin et al. 2002), alginate beads (Bhushan et al. 2013) and Eudragit S-100 (Edward et al. 2002; Gawande and Kamat 1998). However, only a few papers have reported the use of immobilized xylanase in enhancing the yield and clarification of fruit juices (Bhushan et al. 2013; Sharma et al. 2012). The potential of

xylanase bound to aluminum oxide pellets has not been studied in juice clarification. Hence, there is a need to investigate the potential of free and immobilized xylanase in clarification of fruit juices. In this study, orange and grape juices were used as these are rich source of vitamin C. Grape juice contains powerful antioxidants, which protect the body from different types of cancers and diseases.

This study reports the purification of extracellular xylanase produced by *Bacillus pumilus* VLK-1 (isolated in our lab from soil) followed by its immobilization on aluminum oxide pellets, which are stable at high pH and temperature. The immobilization process was optimized using statistical approach i.e. response surface methodology (RSM). Xylanase in free and immobilized form was used for clarification of orange and grape fruit juices.

Materials and methods

Chemicals

Aluminum oxide pellets used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) USA. Other chemicals were procured from Sigma-Aldrich Chemical Co. and Hi-Media Laboratories, Mumbai, India.

Microorganism and preparation of inoculum

Bacillus pumilus VLK-1 (Gene bank accession no JQ350583), isolated in our lab from soil, was used for producing extracellular xylanase for the present study. The bacterial strain was grown on xylan-agar medium (pH 7.0) containing 0.5 % peptone, 0.3 % beef extract, 0.1 % xylan, and 2 % agar followed by incubation at 37 °C for 24 h in an orbital shaker at 200 rpm. The culture was maintained at 4 °C by periodic sub-culturing after every fortnight. The inoculum was prepared by inoculating the autoclaved nutrient broth in a conical flask with a loop full of the overnight grown culture of *B. pumilus* VLK-1 followed by incubation at 37 °C in an orbital shaker incubator at 200 rpm.

Xylanase production in submerged fermentation

Xylanase was produced in Erlenmeyer flasks (250 ml) containing 50 ml of autoclaved modified Horikoshi medium (0.29 % peptone, 0.96 % yeast extract, 0.27 % KNO₃, 0.1 % olive oil, wheat bran 4.0, and 0.1 % Tween 80, pH 9.0), which was inoculated with 1 % (v/v) of 8 h old secondary inoculum. Wheat bran, an abundantly available and cost-effective agro-residue, was used as carbon source so as to reduce the enzyme cost. The flasks were incubated in an orbital shaker incubator at 37 °C for 6 h and then at 30 °C for 42 h with an agitation of 200 rpm. The culture filtrate was then centrifuged at 10,000×g

for 30 min at 4°C in a refrigerated centrifuge. The clear supernatant (crude extract) obtained after centrifugation was used for purification of xylanase.

Xylanase assay and protein estimation

Activity of xylanase was assayed according to the method of Bailey et al. (1992) by measuring the amount of xylose released from birch wood xylan using 3,5-dinitrosalicylic acid reagent (Miller 1959). The reaction mixture containing 490 µl of 1 % birch wood xylan (Sigma) as substrate (prepared in 0.05 M potassium phosphate buffer, pH 6.8) and 10 µl of appropriately diluted enzyme was incubated at 60 °C for 10 min followed by addition of 1.5 ml 3,5-dinitrosalicylic acid reagent. The reaction mixture was then heated in a boiling water bath for 10 min followed by cooling in ice-cold water. The absorbance of the resulting color was measured against the control at 540 nm in a spectrophotometer. A control was run under identical conditions except that the reaction was terminated prior to the addition of enzyme. The amount of xylose produced in the reaction was calculated from its standard curve. One international unit (IU) of xylanase activity was defined as the amount of enzyme required to release 1 µmol of xylose per min under the specified assay conditions. Protein content was estimated according to the Lowry's method (Lowry et al. 1951) using bovine serum protein as standard.

Purification of xylanase

The clear supernatant obtained after centrifugation was used for purification of xylanase in a single step by chromatography on CM-Sephadex C-50 column. The enzyme purification was carried out at 4°C. A column (30 cm×2.6 cm) of CM-Sephadex C-50 was packed and equilibrated with 0.02 M potassium phosphate buffer (pH 6.8). The bed volume of column was 159 ml. The crude enzyme extract was filtered using a syringe filter, equilibrated against potassium phosphate buffer (0.02 M, pH 6.8) and loaded on the column. The unbound proteins were eluted through the column by passing about one bed volume of the equilibration buffer and then a continuous gradient of 0–1.0 M KCl was applied to elute the proteins bound to CM-Sephadex. The column was run at a flow rate of 30 ml/h. Fractions (5.0 ml each) were collected and analyzed for protein by measuring their absorbance at 280 nm. The protein containing fractions were tested for xylanase activity using standard assay. The fractions containing xylanase activity were pooled, dialyzed against 0.02 M potassium phosphate buffer (pH 6.8) and concentrated using Millipore Amicon Ultra-15 Centrifugal filter unit (10 kDa cut off). The enzyme activity and protein content were determined in the concentrated extract as well as in the crude extract to calculate specific activity, recovery and purification fold. The

homogeneity of enzyme was checked by SDS-PAGE using 12 % gel (Laemmli 1970). The protein bands were stained with Coomassie brilliant blue R-250. The molecular weight of xylanase was determined through calibration with standard protein molecular weight markers viz. bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.2 kDa), which were run in the marker lane. The purified enzyme was stored in a refrigerator at 4°C.

Immobilization of xylanase on aluminum oxide pellets

Purified xylanase from *B. pumilus* VLK-1 was immobilized on aluminum oxide pellets. The immobilization process was optimized using RSM. The protocol for immobilization of xylanase used for fruit juice enrichment is mentioned here. Two aluminum oxide pellets were activated by dipping in 1 % glutaraldehyde solution at room temperature for 1 h. The excess of glutaraldehyde was removed by washing the pellets with distilled water. The glutaraldehyde-activated pellets were incubated with purified xylanase (500 IU) at room temperature for 30 min followed by washing to remove the unbound enzyme. The activity of xylanase was determined in the supernatant as well as in the enzyme bound pellets. Immobilization yield was calculated in the following manner:

$$\text{Immobilization yield (\%)} = \frac{\text{Total activity recovered on pellets}}{\text{Total activity offered for immobilization}} \times 100$$

Total activity immobilized on pellets refers to the difference in enzyme activity offered for immobilization and activity recovered in the supernatant.

Assay of immobilized xylanase

The activity of immobilized xylanase was assayed by adding 490 µl of 1 % birch wood xylan (Sigma) and 10 µl of the buffer to the immobilized enzyme followed by incubation 60 °C for 10 min. The pellets were separated from the reaction mixture by transferring its contents to another test tube. Then 1.5 ml of 3,5-dinitrosalicylic acid reagent was added to the reaction mixture and the contents were placed in a boiling water bath for 10 min. After cooling, absorbance of the resulting color was read at 540 nm in a spectrophotometer. A control was run simultaneously that contained all of the reagents but 3,5-dinitrosalicylic acid reagent was added prior to the addition of enzyme. The amount of xylose produced was measured from its standard curve.

Statistical optimization of immobilization

Four experimental parameters i.e. number of pellets, glutaraldehyde concentration, enzyme dose, and coupling time were selected on the basis of their influence on immobilization of xylanase on aluminum oxide pellets. The cumulative effect of these four process parameters was optimized through using the statistical software package Design Expert 8.0.1, Stat-Ease, Inc. A 2^4 full factorial central composite design (CCD) with 16 trials for factorial design, 8 trials for axial point and 6 replicate trials at the central point, leading to a set of 30 experiments was designed.

Reusability of immobilized xylanase

Reusability of immobilized xylanase was determined by reusing the enzyme for catalyzing the reaction as described under the section ‘Assay of immobilized enzyme’. After each assay, the enzyme bound pellets were separated, washed with 0.05 M potassium phosphate buffer (pH 6.8) and reused for another reaction cycle. The bound enzyme was repeatedly used to hydrolyze 1 % (w/v) xylan up to 10 batch reactions. The residual activity after each reaction was determined as:

$$\text{Residual activity (\%)} = \frac{\text{enzyme activity in nth cycle} \times 100}{\text{enzyme activity in 1st cycle}}$$

Effect of pH on free and immobilized xylanase

The pH optima of soluble and immobilized xylanase were determined by determining their activities at different pH values (5.0–11.0) using citrate (5.0–6.0), phosphate (6.0–8.0), Tris- HCl (8.0–9.0) and glycine- NaOH (10.0–11.0) buffers, each at a concentration of 0.05 M. The relative activity (%) was calculated with reference to the maximum activity which was taken as 100 %. The pH stability of soluble and immobilized xylanase was investigated by pre-incubating these enzymes separately with the above mentioned buffers for 2 h at room temperature followed by measurement of residual activity under standard assay conditions.

Effect of temperature on free and immobilized xylanase

To determine the temperature optimum, activities of free and immobilized xylanase were measured separately at temperatures ranging from 37 to 75 °C. The relative activity was calculated by considering the highest enzyme activity as 100 %. The temperature at which the enzyme showed maximum activity was taken as the optimum. Thermal stabilities of free and immobilized xylanase were studied by separately pre-incubating these enzymes at different temperatures ranging from 37 to 65 °C for 2 h followed by determination of enzyme

activity using standard assays at the optimum temperature. The residual activity was calculated by taking the enzyme activity at zero time pre-incubation as 100 %.

Preparation of fruit pulp

Ripened fruits of orange (*Citrus sinensis*) and grapes (*Vitis amurensis*) were purchased from the local market, washed thoroughly with water, peeled (in case of orange) and macerated using a blender to get a smooth textured fleshy tissue (pulp), which was filtered through 2–4 layers of muslin cloth to separate juice from the pulp.

Optimization of xylanase treatment conditions for fruit juice enrichment

Orange and grape pulps were treated with soluble xylanase purified from *B. pumilus* VLK-1 for enrichment of fruit juices. The enzyme dose, incubation temperature and time of treatment were optimized to enhance the yield, clarity and other quality characteristics of juice. To optimize the enzyme dose, 20 g of each fruit pulp was treated with 10, 20, 30 and 40 IU of xylanase/g fruit pulp (gfp) at 30 °C for 30 min keeping the untreated pulp sample as control. At the end of incubation period, the enzyme was inactivated by heating the suspension in a boiling water bath for 5 min. After cooling, the fruit pulps were filtered through two layers of muslin cloth and the filtrate was centrifuged at 10,000×g for 15 min to separate the juice. The supernatant (juice) was analyzed for yield, reducing sugars and clarity. The values are represented as percent relative to control. The optimum time of enzyme treatment was determined by incubating 20 g of each fruit pulp with 20 IU xylanase/gfp at 30 °C for a period of 15, 30, 45 and 60 min keeping zero time pulp as control followed by separation and analysis of juice for yield, reducing sugars and clarity as described above. The optimization of incubation temperature was carried out by treating 20 g of each fruit pulp with 20 IU xylanase/gfp at different temperatures (30–60 °C) in a water bath for 30 min. The fruit pulp at 30 °C was kept as control and the juice yield, reducing sugars and clarity obtained from this pulp was taken as 100 %.

Determination of physico-chemical characteristics of fruit juices

Pulp (20 g) each of orange and grape fruits was treated with soluble and immobilized (on aluminum oxide pellets) xylanase separately under optimized conditions and the resulting juice was analyzed for its physico-chemical characteristics (yield, clarity, reducing sugars, viscosity, total acidity and filterability). The yield of juice was determined by measuring the volume of the supernatant obtained after centrifugation of filtered fruit pulp and it was expressed as % (volume

Fig. 1 **a** Elution profile of *Bacillus pumilus* VLK-1 xylanase through CM-Sephadex C-50 column pre-equilibrated with 0.02 M potassium phosphate buffer (pH 6.8). The bound proteins were eluted using a linear gradient of 0–1.0 M KCl, **b** SDS-PAGE of the purified xylanase. The

molecular weight markers run in the marker lane were bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and α -lactalbumin (14.2 kDa)

of juice per 100 g of pulp). The clarity of juice was determined by measuring the percent transmittance (%T) at 650 nm against distilled water using a double beam UV–vis spectrophotometer (Systronics-2203, India). Percent transmittance was considered a measure of juice clarity. Reducing sugars in the juice were estimated by Miller's method (Miller 1959) using 3,5-dinitrosalicylic acid and quantified from a standard curve of xylose. Filterability (per sec) was determined from the reverse of the time taken to filter 50 ml of xylanase-treated juice through Whatman No. 1 filter under vacuum. Total titrable acidity (% citric acid) and viscosity (Pa.s) of the juice were determined as described earlier (Nagar et al. 2012a). All the experiments were performed in triplicates and their mean values are given.

Results and discussion

Purification of xylanase

Xylanase was purified from the culture filtrate of *B. pumilus* VLK-1 in a single step by chromatography on a column of CM-Sephadex C-50 which separates protein on the basis of differences in their charge and size. The enzyme eluted as a single activity peak on applying 0–1.0 M KCl gradient (Fig. 1a). The fractions containing enzyme activity were pooled, dialyzed overnight against 0.02 M potassium phosphate buffer (pH 6.8) to remove the salt and concentrated

using Millipore Amicon Ultra-15 Centrifugal filter unit (10 kDa cut off). The concentrated enzyme showed a single protein band on SDS-PAGE indicating that it was apparently homogeneous (Fig. 1b). The molecular weight of purified xylanase from *B. pumilus* VLK-1 was found to be 22.0 kDa. The purified enzyme exhibited a specific activity of 8406.3 IU/mg protein. The enzyme was purified 15.4-fold with 88.3 % recovery (Table 1). Purification of xylanase from various microorganisms involving different techniques and steps have been reported but with variations in specific activity, recovery % and fold purification (Sa-Pereira et al. 2003). Single step purification of xylanase using CM-Sephadex C-50 is routinely used in our laboratory. Xylanase was purified to apparent homogeneity in one step from *Bacillus subtilis* ASH by 10.5-fold with a recovery of 43 % (Sanghi et al. 2010) and from *Bacillus pumilus* 85S by 25.3-fold with 63.2 % recovery (Nagar et al. 2012a). On the other hand, two forms of alkaliphilic xylanase have been reported from purified from *Bacillus licheniformis* P11(C) (Bajaj and Manhas 2012). Single step purification is likely to reduce the cost of purified enzyme, which is desirable for its use in industry.

Optimization of xylanase immobilization through RSM

Purified xylanase was immobilized on glutaraldehyde-activated aluminum oxide pellets. Four parameters of the immobilization process viz. number of pellets, glutaraldehyde concentration, enzyme dose, and coupling time were

Table 1 Purification of xylanase from culture filtrates of *B. pumilus* VLK-1

Purification step	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg protein)	Recovery (%)	Purification fold
Crude extract	68,535	126.4	544	100.0	1
CM-Sephadex C-50	60,525	7.2	8406.3	88.3	15.4

Table 2 Experimental range of each selected variable for xylanase immobilization

Factor	Name	Unit	Low level (–)	High level (+)	Mean (0)
A	No. of pellets	Numeric	2.0	8.0	5.0
B	Enzyme dose	IU	100	500	300
C	Glutaraldehyde	(% v/v)	1.0	4.0	2.5
D	Coupling period	min	30	120	75

optimized statistically using RSM. Table 2 displays the levels of components ($-\alpha$, -1 , 0 , 1 , $+\alpha$). All the variables were taken at a central value represented by “0”. The experimental design for CCD of RSM and the response values of the experiments are shown in Table 3. The response value from each experiment of CCD is the mean of triplicates. Analysis of the results obtained from RSM showed the highest immobilization yield as 95 %. Responses of the CCD design were fitted in a

polynomial quadratic equation. The overall polynomial equation for the immobilization of xylanase was

$$\begin{aligned} \text{Immobilization Yield (\%)} = & + 87 - 3.875 * A + 18.46 * B + 2.875 * C \\ & + 0.29 * D - 13.69 * A * B + 6.94 * A * C \\ & - 8.06 * A * D - 20.44 * B * C + 7.56 * B * D \\ & + 5.69 * C * D - 12.55 * A^2 - 9.42 * B^2 - 8.18 * \\ & C^2 - 20.80 * D^2 \end{aligned}$$

Table 3 Experimental design (CCD) for xylanase immobilization using RSM

Std	Run	No. of beads	Enzyme dose	Glutaraldehyde (%)	Coupling time (min)	Immobilization (%)
22	1	5	300	5.5	75	86
28	2	5	300	2.5	75	87
5	3	2	100	4	30	2
8	4	8	500	4	30	10
29	5	5	300	2.5	75	87
1	6	2	100	1	30	15
3	7	2	500	1	30	90
14	8	8	100	4	120	49
19	9	5	–100	2.5	75	2
6	10	8	100	4	30	95
25	11	5	300	2.5	75	87
21	12	5	300	–0.5	75	36
15	13	2	500	4	120	91
30	14	5	300	2.5	75	87
24	15	5	300	2.5	165	1
16	16	8	500	4	120	15
2	17	8	100	1	30	1
12	18	8	500	1	120	62
23	19	5	300	2.5	–15	0
13	20	2	100	4	120	2
27	21	5	300	2.5	75	87
18	22	11	300	2.5	75	2
20	23	5	700	2.5	75	90
11	24	2	500	1	120	81
9	25	2	100	1	120	2
26	26	5	300	2.5	75	87
17	27	–1	300	2.5	75	65
7	28	2	500	4	30	2
4	29	8	500	1	30	84
10	30	8	100	1	120	2

Where

- A number of pellets
- B enzyme dose
- C glutaraldehyde concentration
- D coupling time.

The F-value of the model was 4.03 which implied that it was significant. The values of “Prob>F” <0.05 indicate that model terms are significant. On the other hand, values of >0.10 indicate that model terms are not significant. In this case, significant model terms were AB, BC, A², and D² (Table 4). Adequate precision measures the signal to noise ratio. A ratio greater than 4.0 was desirable. In this case, a ratio of 7.65 indicated an adequate signal (Table 5). This model could be used to navigate the design space.

The response plots (3D and contour curves) were analyzed to determine interaction among the variables and the optimum value of each component for maximum immobilization yield (IY). The cumulative effect of number of pellets and enzyme dose on IY at the central level of the remaining two factors is shown in Fig. 2a. It clearly showed an increase in IY with increase in number of pellets from ‘-1’ to central level but thereafter, it remained constant at ‘-1’ level of enzyme dose. Further, IY enhanced with increase in enzyme dose from ‘-1’ to ‘+1’ level at ‘-1’ level of beads. Figure 2 b reveals the variation of IY with coupling time and enzyme dose. Maximum IY was obtained at ‘0’ level of coupling time and at the highest (+1) level of enzyme dose. The effect of glutaraldehyde concentration and enzyme dose on IY (Fig. 2c) showed that IY enhanced as the enzyme dose increased from ‘-1’ to ‘+1’ level and as glutaraldehyde concentration declined from ‘+1’ to ‘-1’ level. The interactive effect of coupling time and number of beads (Fig. 2d) revealed maximum IY close to the central points of both the variables. All the RSM graphs were analyzed in a similar way to determine the optimum value of each factor for maximum IY. The IY was 90 % when two pellets were treated with 1.0 % glutaraldehyde followed by incubation with 500 IU of xylanase for 30 min. These conditions were employed for enzyme immobilization as lower concentration of glutaraldehyde is preferred for activation.

Table 4 Analysis of variance (ANOVA) for response surface quadratic model for xylanase immobilization

Source	F value	p-value	Prob > F
Model	4.03	0.0056	Significant
A- No. of beads	0.55	0.4711	
B- Enzyme dose	12.4	0.0031	
C- Glutaraldehyde conc.	0.3	0.5914	
D- Incubation period	0.003	0.9564	
AB	4.55	0.0499	
AC	1.17	0.2969	
AD	1.58	0.2283	
BC	10.14	0.0062	
BD	1.39	0.2571	
CD	0.79	0.3896	
A ²	6.55	0.0218	
B ²	3.7	0.0737	
C ²	2.78	0.1161	
D ²	18	0.0007	

Table 5 Analysis of variance for response surface quadratic model for xylanase immobilization

Parameters	Values
Std. dev.	24.16
Mean	46.90
C.V. (%)	51.51
PRESS	50424.48
R ²	0.81
Adj R ²	0.63
Adequate precision	7.65

PRESS Predicted residual sum of squares, R² Determination coefficient

1’ level and as glutaraldehyde concentration declined from ‘+1’ to ‘-1’ level. The interactive effect of coupling time and number of beads (Fig. 2d) revealed maximum IY close to the central points of both the variables. All the RSM graphs were analyzed in a similar way to determine the optimum value of each factor for maximum IY. The IY was 90 % when two pellets were treated with 1.0 % glutaraldehyde followed by incubation with 500 IU of xylanase for 30 min. These conditions were employed for enzyme immobilization as lower concentration of glutaraldehyde is preferred for activation.

Reusability of immobilized xylanase

Enzyme cost is important for full-scale process commercialization (Lynd et al. 2005). Immobilized enzymes are preferred as they can be recycled leading to a reduced cost of the product. Reusability of purified xylanase immobilized on aluminum oxide pellets was investigated for ten consecutive cycles, the results of which are shown in Fig. 3. The residual activity of immobilized enzyme was more than 90 %, 85 % and 58 % after four, five and ten reaction cycles, respectively. The decrease in enzyme activity during repeated use with varying extent has been reported by several researchers (Bhushan et al. 2013; Roy et al. 2003). A decline in activity during reuse might occur due to enzyme inactivation as suggested earlier (Nagar et al. 2012b). The considerable stability observed during recycling of the bound enzyme in this study would make the process economical. These results implied that aluminum oxides pellets could be used for xylanase immobilization. The stability of these pellets at high pH and temperature might also be useful during the process.

Effect of pH on immobilized xylanase

Immobilized xylanase showed optimum activity at pH 9.0 whereas its soluble counterpart exhibited pH optimum at 8.0 (Fig. 4a). It is evident from Fig. 4a that the activity of free enzyme was enhanced upon increase in pH up to 8.0 followed by a decline exhibiting 86 %, 79 % and 64 % relative activity at pH 9.0, 10.0 and 11.0, respectively. On the other hand, the activity of immobilized enzyme increased with increase in pH up to 9.0 followed by a decline exhibiting 80 % and 68 %

Fig. 2 Response surface plots showing the effect of interaction between two variables on immobilization yield of xylanase while keeping third variable at '0' level. The interactions are **a** Enzyme dose and number of

pellets, **b** Coupling time and enzyme dose, **c** Enzyme dose and glutaraldehyde concentration **d** Coupling time and number of pellets

Fig. 3 The operational stability of xylanase immobilized on glutaraldehyde-activated aluminum oxide pellets for 10 cycles

activity at pH 10.0 and 11.0, respectively. Thus, pH optimum of xylanase was shifted by one unit (from 8.0 to 9.0) after its covalent immobilization on aluminum oxide pellets which might be due to effect of the microenvironment of the bound enzyme. A shift in pH optimum on immobilization was also recorded earlier (Bhushan et al. 2013; Surajbhan et al. 2012). In contrast, no change in pH optimum was observed after binding of xylanase to aluminum oxide pellets (Nagar et al. 2012) and on Eudragit S-100 (Edward et al. 2002).

The pH stability data revealed significant stability of both free and immobilized xylanase in the pH range 6.0–10.0 after 24 h pre-incubation at these pH values, however, the bound form exhibited better stability (Fig. 4b). At pH 11.0, free enzyme exhibited 48 % residual activity, while immobilized enzyme showed 72 % activity. Hence, it may be inferred from the data that the pH stability of xylanase was significantly enhanced after immobilization. Similarly, soluble form of partially purified xylanase from *B. licheniformis* P11(C) was found to exhibit substantial stability at alkaline pH values (Bajaj and Manhas 2012). The stability of xylanase at alkaline

Fig. 4 Effect of pH on free (●) and immobilized (■) xylanase; **a** pH optimum determined by measuring the enzyme activity using buffers of different pH values viz. citrate (5.0–6.0), phosphate (6.0–8.0), Tris–HCl (8.0–9.0), and glycine–NaOH (10.0–11.0) at 0.05 M each under standard

assay conditions. **b** pH stability investigated by pre-incubating the enzyme in the above buffers for 2 h followed by determination of enzyme activity at the optimum pH under standard assay conditions

pH is desirable for its application in pulp bleaching. Immobilization was found to enhance the pH stability of xylanase by other researchers also. Xylanase entrapped in calcium-alginate beads was found to be stable over a much wider pH range as compared to soluble enzyme (Bhushan et al. 2013). Enhanced stability of *B. pumilus* SV-85S xylanase after immobilization on aluminum oxide pellets has also been documented by (Nagar et al. 2012b). It might be due to multipoint covalent attachment of enzyme to the support preventing enzyme denaturation in acidic or alkaline environments. The increase in stability of immobilized xylanase over a wide pH range is likely to be advantageous during its application.

Effect of temperature on immobilized xylanase

To study the temperature dependence, activity of both free and immobilized xylanase was determined separately at different temperatures (37–75 °C). The activity of free enzyme increased up to 60 °C and decreased thereafter exhibiting

70 %, 45 % and 32 % relative activity at 65, 70 and 75 °C, respectively whereas, the activity of immobilized enzyme increased with rise in temperature up to 65 °C followed by a decline exhibiting 82 % and 74 % activity at 70 and 75 °C, respectively (Fig. 5a). The optimum temperatures of free and the immobilized enzyme were taken as 60 and 65 °C, respectively as maximum enzyme activity was observed at these temperatures. A similar increase in optimum temperature for immobilized xylanase was reported by other researchers (Jingmin et al. 2002; Kumar et al. 2013; Pal and Khanum 2011) and this could be due to enhanced stability or conformational rigidity on immobilization. However, the effect of temperature may vary depending on the support used for immobilization.

Investigation of thermal stability of both free and immobilized xylanase by pre-incubating each enzyme at 37–65 °C for 2 h followed by assay for activity at 60 °C revealed that bound enzyme was slightly more stable at higher temperatures than its free counterpart (Fig. 5b). Free xylanase

Fig. 5 Effect of temperature on free (●) and the immobilized (■) xylanase; **a** Temperature optimum determined by measuring the enzyme activity at different temperatures (37–75 °C), and **b** Thermal stability investigated by pre-incubating the enzyme at different temperatures (37–65 °C) for 2 h followed by determination of enzyme activity at the optimum temperature under standard assay conditions

retained 45 % and 30 % of initial activity after pre-incubation at 55 and 65 °C for 2 h whereas the residual activity of covalently immobilized enzyme at these temperatures was 50 % and 35 % respectively. Increased thermostability of xylanase after immobilization on aluminum oxide pellets was also recorded by Nagar et al. (2012b). Immobilization of an enzyme often protects it against heat inactivation (Gouda and Abdel-Naby 2002). The improved temperature stability of xylanase may boost its suitability for industrial application.

Use of xylanase for enrichment of orange and grape juices

In this investigation, the potential of purified *B. pumilus* VLK-1 xylanase in free and immobilized forms was evaluated in enrichment of fruit juice from orange and grape pulps. The

enrichment process was optimized using free xylanase with respect to its dose, incubation temperature and duration of treatment.

Optimization of enzyme dose

Treatment of grape pulp with purified xylanase (5–30 IU/gfp) at 30 °C for 30 min revealed maximum increase in juice yield (14 %), clarity (22 %) and reducing sugars (12 %) at 20 IU/gfp as compared to enzyme untreated pulp (Fig. 6a). As the enzyme dose was increased or decreased from 20 IU, there was a decline in juice enrichment indicating that 20 IU/gfp was the optimum enzyme dose for treatment of orange fruit pulp. The optimum enzyme dose for treatment of orange pulp also was 20 IU/gfp because maximum yield (14 %), clarity

Fig. 6 Optimization of conditions for treatment of grape and orange pulps with purified free xylanase from *Bacillus pumilus* VLK-1 in terms of juice yield, clarity and reducing sugars: effect of enzyme dose (a & d), incubation period (b & e) and incubation temperature (c & f)

(12 %) and reducing sugars (7 %) were recorded at this concentration (Fig. 6d). The optimum enzyme dose is likely to depend on its characteristics as well as the nature of fruit pulp. Some researchers reported maximum juice yield and clarification by 10–15 IU/gfp of xylanase (Nagar et al. 2012a). Sin et al. (2006) suggested that an initial increase in xylanase dose will cause cloud particles to aggregate and settle down thereby increasing the clarity of juice. However, after a certain enzyme concentration, substrate in the fruit wall will become limited preventing the aggregation of cloud particles. Increase in yield and clarity of juice may be due to the release of water resulting from reduction in water holding capacity of xylan (the major hemicellulosic component of fruit cell wall) after its degradation by xylanase (Grassin and Fauquembergue 1996). The release of xylose and short chain oligosaccharides following treatment of fruit pulp with xylanase might account for enhancement in reducing sugar level of the juice.

Optimization of incubation time

The profile of yield, reducing sugars and clarity on treatment of grape and orange pulps with 20 IU/gfp xylanase for different time duration (15–90 min) revealed maximum efficiency of the enzyme in fruit juice enrichment after 30 min incubation (Fig. 6b, e) which was, therefore, taken as the optimum time. In grape juice, increase in yield, clarity and reducing sugar content was 16, 25 and 12 % respectively whereas in orange juice, the corresponding increase was 15.2, 18 and 8 % respectively. Prolonged incubation time could result in the formation of haze particles consisting of protein-carbohydrate and protein-protein complexes (Sin et al. 2006). Other researchers have documented 30–90 min as the optimum time for xylanase treatment.

Optimization of incubation temperature

The effect of rise in temperature from 30 to 60 °C on the efficacy of xylanase (20 IU/gfp) in juice enrichment of grape and orange pulps showed that the optimum temperature for juice enrichment was 50 °C (Fig. 6c, f). At this temperature, the increase in yield, clarity and reducing sugar content was 20, 27 and 15 % respectively for grape pulp and 16, 19.5 and 12 % respectively for orange pulp. This is also the optimum temperature for *B. pumilus* VLK-1 xylanase. The optimum temperature for juice enrichment is likely to depend on the enzyme used, time of incubation, and nature of pulp among other factors. It was reported to be 37 °C for citrus juice (Dhiman et al. 2011) and 40 °C for apple, pineapple, and tomato juice (Nagar et al. 2012a).

Fruit juice enrichment with free and immobilized xylanase under optimized conditions

The most promising application of xylanase in fruit juice industry is to clarify, liquefy, macerate and increase the yield

of juice. The grape and orange fruit pulps were treated with free and immobilized xylanase separately under optimized conditions (20 IU/gfp xylanase at 50 °C for 30 min) and the resulting juices were analyzed for yield, clarity, reducing sugars, viscosity, acidity and filterability. The results of fruit juice enrichment are shown in Fig. 7. As compared to control, treatment of grapes and orange pulps with soluble purified xylanase increased the juice yield by 25 % and 19 %, whereas immobilized xylanase enhanced the yield by 29 % and 26 % respectively. The clarity of both the juices was also improved after enzyme treatment. The clarity of grape juice (measured in terms of % transmittance at 650 nm) was enhanced by 27 % and 30 % on treatment with soluble and immobilized xylanase respectively over the control. The orange juice also registered a similar increase in clarity (24–29 %) which was slightly greater with immobilized as compared to free enzyme. It could be due to enhanced stability of the enzyme after immobilization.

An increase in juice yield and clarity has been reported earlier after enzymatic treatment of fruit pulp with xylanase alone (Bajaj and Manhas 2012; Nagar et al. 2012a; Olfa et al. 2007; Pal and Khanum 2011) and a combination of pectinase, cellulase, and hemicellulase (Ahmad et al. 2009; Pal and

Fig. 7 Comparison of the efficacy of purified free and immobilized xylanase from *Bacillus pumilus* VLK-1 on juice enrichment (yield, clarity, reducing sugar, viscosity, acidity, and filterability) of grape and orange fruits

Khanum 2011; Shah 2007). However, magnitude of increase or decrease was apparently dependant on several factors including type of fruit pulp and enzyme used (dose, whether used singly or in combination, characteristics, etc.). Treatment with soluble xylanase enhanced the juice clarity of orange by 25–27 % (Olfa et al. 2007), apple by 22.20 % and pineapple by 19.80 % (Nagar et al. 2012a). An increase in juice clarity by xylanase immobilized by adsorption on silica (Sharma et al. 2012) and encapsulation in calcium alginate beads (Bhushan et al. 2013) has been reported. In these reports, bound enzyme was more effective similar to results of the present study. Xylanases may improve the juice yield by different means including liquefaction of fruit, stabilization of fruit pulp, reduction of viscosity and degradation of hemicelluloses (Tajchakvit et al. 2001).

The viscosity of juices obtained after xylanase treatment of pulp was slightly less as compared to control. A comparison of free and immobilized enzyme revealed that the latter was more effective in reducing the viscosity of juices (Fig. 7). A reduction in viscosity of different fruit juices after enzymatic treatment has been documented by several workers (Bhushan et al. 2013; Nagar et al. 2012a; Shah 2007). It could be due to degradation of xylan, which contributed to the turbidity and viscosity of fruit juices (Lee et al. 2006). The decline in viscosity would obviously be accompanied by increase in juice clarity. The reduction in viscosity is advantageous as high viscosity may result in clogging of filtration apparatus due to the cloud particles and can also reduce its filterability (Jacob et al. 2008).

Reducing sugar content of both the juices increased (12–26 %) after xylanase treatment and immobilized enzyme was more effective as compared to its free form. It could be due to the release of sugars from hydrolysis of xylan by the enzyme. Similarly, treatment with soluble xylanase enhanced the reducing sugar content by 22.4, 6.25 and 4.25 % in pineapple, tomato and apple juice respectively (Nagar et al. 2012a) and by 2-fold in citrus juice (Dhiman et al. 2011) and mousambi juice (Bajaj and Manhas 2012). In agreement with the results of present study, increase in reducing sugars of Mousambi and orange juice following treatment with free and silica immobilized xylanase was reported (Sharma et al. 2012).

Titrateable acidity is an important factor in determining the quality of fruit juice. It was found to be higher in grape and orange juices obtained from xylanase-treated fruit pulps as compared to untreated pulps. Kareem and Adebawale (Kareem and Adebawale 2007) also reported an increase in the total titrateable acidity from 0.038 g/100 g in untreated orange juice to 0.047 g/100 g in juice treated with 0.5 % pectinase. An increase in titrateable acidity would increase shelf life of juice (Baker and Bruemmer 1972). The filterability both the juices registered an increase (4–6 %) when fruit pulps were treated with xylanase; immobilized enzyme being more effective than its soluble counterpart. Other researchers

have also reported an increase in filterability of juice after treatment with xylanase (Dhiman et al. 2011).

Thus, the results of this study indicated an improvement of yield and quality of orange and grape juices by the use of purified xylanase in free and immobilized form, the later being more effective.

Conclusion

Xylanase from *Bacillus pumilus* VLK-1 was purified to absolute homogeneity and further immobilized on glutaraldehyde-activated aluminum oxide pellets. The immobilization was optimized using response surface methodology which is known to evaluate the cumulative effects of process variables. The immobilized enzyme exhibited improved stability toward pH, and temperature and it could be reused for ten cycles still retaining 58 % of its activity. Xylanase, in both free and immobilized forms, was effective in enhancing the yield, clarity, reducing sugar content, and filterability but reducing the viscosity of grape and orange juices. The immobilized xylanase showed considerable advantage over its soluble counterpart for application in clarification of fruit juices. This study indicated the potential usefulness of purified free and immobilized xylanase in fruit juice clarification.

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