OPTIMIZATION OF PROCESS PARAMETERS FOR FRUIT JUICE CLARIFICATION USING SILICA IMMOBILIZED XYLANASE FROM *PSEUDOMONAS* SP.

Pawan Kumar Sharma,^{a*} Shatavari Kulshrestha,^a Duni Chand,^a

^a Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla, India, 171005.

*Corresponding author: pawan09hpubiotech@gmail.com

Abstract

Xylanase have many applications and fruit juice clarification is one of them. The work was done by taking *Pseudomonas* sp. Xylanase was immobilized on silica with binding efficiency of 46.5% and maximum binding time 6 hours. Maximum amount of juice clarification was in case of Mausambi juice was observed at 40°C and 52 hours in both immobilized and free enzyme treated juice, in case of free enzyme treated juice there is 46.9% increase in clarity and 1.7 fold increase in reducing sugars of the juice and enzyme dose was optimized as 8U with maximum flow rate of 6 ml/min at this dose but in case of immobilized enzyme treated juice there is 47.6% increase in clarity and 1.8 fold increase in reducing sugars, enzyme dose optimized is 7.4U with maximum flow rate of 7.23 ml/min at this dose. In case of orange juice in free enzyme treated juice maximum clarity was observed at 40°C and 52 hours, juice was found to be 42.14 % clear with increase of 1.9 fold of reducing sugars, enzyme dose optimized was 8.06U with maximum flow rate of 0.86 ml/min. In case of immobilized enzyme treated juice maximum clarity was found at 52 hours with 36.46% clarity and 2 fold increase in reducing sugars, enzyme dose optimized was 14.8U with maximum flow rate of 0.9 ml/min at this dose.

Keywords: Fruit juice; *Pseudomonas*; xylanase.

Introduction

Xylan, a heteropolysaccharide containing substituent groups of acetyl, 4-O-methyl-D-glucuronosyl and α -arabinofuranosyl residues linked to a backbone of β -1,4-linked xylopyranose units, is the second-most-abundant renewable resource, with a high potential for degradation to useful end products [1]. These enzymes convert xylan into its constituent sugars, where xylanase is of particular significance because it can catalyze the random endohydrolysis of β -1,4-xylosidic linkages in xylan to produce xylooligosaccharides and xylose [2]. Xylanases are a major group of industrial enzymes due to their biotechnological utility and potential application in a range of industrial processes, such as biobleaching of paper pulp, bioconversion of lignocellulosic material and agro-wastes to fermentative products clarification of juices, and improvement in the consistency of beer and the digestibility of animal feed stock [1]. Current commercial preparations in lignocellulose hydrolysis have primarily been based on dilute-acid pretreatment where hemicellulose is removed before saccharification. With the development of non-acid pretreatment methods where the hemicellulose fraction remains intact, however, xylanases are required [3]. Although the cost of commercial xylanase preparations has been reduced significantly in recent years, enzyme costs are still an obstacle to full-scale process commercialization [4]. Immobilization on an inert carrier offers the prospect of significant cost savings by facilitating enzyme recycling through multiple cycles of batch-wise hydrolysis [5]. Also, enzyme immobilization

frequently results in improved thermal stability or resistance to shear inactivation [6]. The immobilized xylanases are usually packed in a column for convenient application. However, the disadvantage of such an immobilized xylanase column is that the solid property of the substrate xylan restricts passage through the column [7]. In addition, the efficiency of the xylanase is decreased when immobilized on solid supports as the bound enzyme is not accessible to bulky insoluble substrate [8].

Citrus fruit juices have become important in recent years due to overall increase in fruit juice consumption. However raw juice is turbid and viscous hence tends to settle during storage. Therefore it must be clarified before commercialization. Use of enzyme in modifying the nature of food products has exciting potential [9]. The turbidity and viscosity of juice is mainly because of the polysaccharides such as pectin starch and hemicelluloses components [10]. Therefore enzymes have been exploited for degradation of all polymeric carbohydrates such as pectins, hemicellulases and starch, thus increasing the yield of juice by enabling better processing of pulp to improve the yield of substances contained in the fruit such as acids, colouring substances and to liquefy entire pulp for maximum yield [11]. Number of reports is available on the use of pectinases and other enzymes for the clarification of fruit juice but literature citing the use of xylanases for clarification of fruit juice is hardly available.

By keeping above points in view the aims of the present study were; to immobilize the xylanase from *Pseudomonas* sp. on silica and optimize the process

parameter for fruit juice clarification using silica immobilized xylanase.

Materials and Methods

Microorganisms, culture conditions and assay

The culture of *Pseudomonas* sp. procured from the Department of Biotechnology, Himachal Pradesh University was used for this study. Culture was maintained on agar medium (pH-7.5), supplemented with 0.5% (w/v) xylan incubated at 24° C for 12 hours.

All the chemicals used in the experiments were of analytical grade. All the medium components used during the experiments were from Hi-Media (Mumbai, India) and Sigma Aldrich (U.S.A).

A loopfull of bacterial cells were inoculated in seed medium containing peptone 0.5 %, yeast extract 0.2 %, beef extract 0.2 %, dextrose 0.25 % and was incubated at 30° C for 24 hours at 160 rpm. The precultures were added to 50ml production medium containing 0.5% peptone, 0.2% yeast extract, 0.2% beef extract and 0.25% xylan and were incubated at 30°C for 24 hours at 160 rpm. The culture contents were centrifuged at 10,000 g for 20 min, at 0-4°C and supernatant thus collected was further assayed for xylanase activity as per the standard protocol mentioned below.

Xylanase activity was assayed using birchwood xylan 0.5 % as substrate and the amount of reducing sugar released was determined by DNSA (Dinitrosalicylic acid) method given by Miller, [12].

Enzyme activity

One unit of xylanase activity was expressed as the amount of enzyme required to produce 1 µmol of reducing sugar (xylose equivalent) in 1 minute.

Immobilization of xylanase on silica

Immobilization of enzyme was done by dissolving 1gm of dry matrix in minimal buffer (sodium phosphate) and kept overnight. Swelling capacity was calculated as

Wet-Drymatrix/dry matrix

For immobilization, incubated 4ml enzyme with 1gm matrix for 6 hours in shaking incubator. Separated supernatant and enzyme activity from both supernatant and immobilized enzyme was calculated.

Effect of binding time on immobilization

A study was conducted to find out the effect of time on immobilization 2, 4, 6, 8, 10 hours binding time was also provided for binding of enzyme on silica. Binding time was provided under shaking conditions. The enzyme activity and protein content was determined in the supernatant at different time

intervals ranging from 2-10 hours by standard methods.

Optimization of parameter for fruit juice clarification

Juice clarification was carried out on two juice samples

A) Citrus limetta (Mosambi juice) crude fresh juice taken from the juice corner Lakkhar bazaar Shimla.

B) Citrus sinensis (Orange juice) commercial Real orange juice pack.

Juice clarification of 50 ml juice was carried out at 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C by recording transmittance at 650 nm taking distilled water as blank by taking 2ml of sample after every 4 hours till 52 hours. % Clarification was calculated as follows:

$$\% \text{ clarification} = \frac{T_t - T_c}{T_c} \times 100$$

T_t = Transmittance of test.

T_c = Transmittance of control

At the same time amount of reducing sugars were also tested in all samples by using DNSA method taking glucose standard. These experiments were done for both the juices by taking both free and immobilized enzymes.

Effect of enzyme dose for both the juices was studied by taking 3.20U, 8.06U, 16.13U, 32.26U, 64.53U of free enzyme and 1.48U, 3.7U, 7.4U, 14.8U, 29.6U of immobilized enzyme at optimized temperature and time and transmittance was recorded at 650 nm.

The flow rate of juice was tested by filtering 2 ml of juice clarified by different enzyme dose by Whatman filter paper 1 and comparing its value by flow rate of control.

Results and Discussion

Xylan 1%, dextrose, 4% peptone 0.5%, yeast extract 0.2% has shown maximum enzyme activity along with 100 mM sodium phosphate buffer, pH 7.5, at 55 °C for 5 minutes.

Immobilization of xylanase on silica

In the present study xylanase was immobilized by adsorption method on silica. The binding efficiency was found to be 46.5%. A gradual increase in binding efficiency was observed as binding time was increased from 2 to 6 hours. A further increase in binding time led to constant binding efficiency means no further increase in binding efficiency was observed as shown in Fig 1. It may be due to the saturation of binding sites of matrix. Previously reported endoxylanase was immobilized on silica within 3 hours [13], on Eudragit L-100 within 1 hour [14]. Until now xylanase has been immobilized on chitosan beads

[15], silica [13], artificial oil bodies [16], glass fibre disc and glass beads [17].

Fig1. Effect of binding time on binding efficiency

Fig2 Effect of time on % clarification at 40 °C

Fig. 3 Effect of time on reducing sugars at 40 °C

Optimization of parameter for fruit juice clarification

Effect of time and temperature on juice clarification

To determine the effect of time and temperature on fruit juice clarification the range of temperature chosen was 300C to 600C.

Maximum amount of juice clarification in case of Mausambi juice was observed at 40 °C and 52 hours in both immobilized and free enzyme treated juice. Free enzyme treated juice gave 46.9% increase in clarity. In case of orange juice in free enzyme treated juice maximum clarity was observed at 40 °C and 52 hours, juice was found to be 42.14 % clear. The results are shown in figure. The clarification is due to disruption of hemicellulasic material and hydrolysed

all polymeric carbohydrate of juice. There is better %clarification in case of Mausambi juice than in orange juice because may be Mausambi juice was crude having more hemicellulosic material while orange juice was already manufactured one so may be previously treated. Xylanase has been already reported to clarify fruit juice by 27% [18]. Kumar et al., [17] reported clarity in the apple juice by use of xylanase of *Bacillus pumulis* MTCC8964 at 24 hours and at ambient room temperature.

Effect of time on reducing sugars at 40 °C

Reducing sugars were found to increase with time and then became constant. In case of Mausambi juice the value of reducing sugar were maximum at 16 hours in case of immobilized enzyme treatment with value of 58.5 mg/ml while that of untreated juice it was 32 mg/ml and in enzyme treated juice the maximum was at 16 hours with value of 54.5 mg /ml after this time they became constant.

In case of orange juice treated with free enzyme the maximum reducing sugars were formed at 52 hours , with concentration 146 mg/ml while that of untreated juice it was only 84 mg/ml .In immobilized enzyme treated juice value were maximum at 52 hours with value of 132 mg/ml after this value the readings were almost constant. Similarly, *Bacillus stearothermophilus* xylanase has resulted in improvement of two fold in the release of reducing sugar and 35.34% increase in clarity of citrus fruit juice [19].

Effect of enzyme dose on juice clarification and filterability

In present study enzyme dose was optimized to be 7.4U with maximum flow rate of 7.23 ml/min for immobilized enzyme treated Mausambi juice, 8U for free enzyme treated Mausambi juice with maximum flow rate of 6 ml/min, 14.8U with maximum flow rate of 0.86 ml/min for immobilized enzyme treated orange juice and 8.06U with maximum flow rate of 0.9 ml/min for free enzyme treated juice. In another study Citrus sp. Juice clarification showed maximum clarification at 12.5U [19]. Filterability was maximum at optimized enzyme dose as more clarified the juice greater will be its filterability because of disruption of hemicellulosic material and hydrolyses of all polymeric carbohydrates of juice, enhanced extent of liquification of fruit juice, which make it more permeable across filter.

Reference

- 1.Subramaniyan S and Prema P (2002) Biotechnology of microbial l xylanases:

- enzymology, molecular biology, and application
Crit Rev Biotechnol 22: 33–64.
2. Wu S, Liu B and Zhang X (2006) Characterization of a recombinant thermostable xylanase from deep-sea thermophilic *Geobacillus* sp MT-1 in East Pacific Appl Microbiol Biotechnol 72: 1210–1216.
3. Gray KA, Zhao L and Emptage M (2006) Bioethanol .Curr Opin Chem Biol 10: 141– 146.
4. Lynd LR, van Zyl WH, McBride JE and Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16: 577–583.
5. Hudson S, Magner E, Cooney J and Hodnett BK (2005) Methodology for the immobilization of enzymes onto mesoporous materials. J Phys Chem B 109: 19496–19506.
6. Tu M, Zhang X, Kurabi A, Gilkes N, Mabee and Saddler J (2006) Immobilization of β -glucosidase on Eupergit C for lignocellulose hydrolysis Biotechnol Lett 28: 151–156.
7. Liu JH, Selinger LB, Cheng KJ, Beauchemin KA and Moloney MM (1997) Plant seed oil-bodies as an immobilization matrix for a recombinant xylanase from the rumen fungus *Neocallimastix patriciarum*. Mol Breeding 3, 463–470.
8. Tan SS, Li DY, Jiang ZQ, Zhu YP, Shi B and Li LT (2008) Production of xylobiose from the autohydrolysis explosion liquor of corncob using *Thermotoga maritime* xylanase B (XynB) immobilized on nickel-chelated Eupergit C Bioresour Technol 99: 200–204.
9. Yusof S and Ibrahim N (1994) Quality of soursop after pectinase enzyme treatment Food chem 51: 115 – 125.
10. Lee (2006) Optimizing conditions for enzymatic clarification of banana juice using response surface methodology (RSM). J Food Eng 73: 55-63.
11. Gerhartz W (1990) Enzymes in industry .VCH publications , New York.
12. Miller, J. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Biochem 31: 426-428.
13. Kang SC, Kim HJ, Nam SW and Oh DK (2002) Surface immobilization on silica of endoxylanase produced from recombinant *Bacillus subtilis* J Microbiol Biotechnol 12(5):766-772.
14. Roy I, Gupta A, Khare SK, Bisaria VS and Gupta MN (2003) Immobilization of xylan-degrading enzymes from *Melanocarpus albomyces* IIS 68 on the smart polymer Eudragit L-100, Appl Microbiol Biotechnol 61:309–313.
15. Abdel-Naby MA (1992) Immobilization of *Aspergillus niger* NRC 107 Xylanase and β -Xylosidase, and Properties of the Immobilized Enzymes App Biochem Biotech 38:69-81.
16. Hung YJ, Peng CC, Tzen JTC, Chen MJ and Liu JR (2008) Immobilization of *Neocallimastix patriciarum* xylanase on artificial oil bodies and statistical optimization of enzyme activity . Biores Tech 99:8662–8666.
17. Kumar D, Verma R, Savitri , Chand D and Bhalla TC (2009) Immobilization of xylanase produced by new isolate *Bacillus pumilus* MTCC 8964 Int Conf Bioencap Groningen ,Netherland 1-4.
18. Olfa (2007) Induction properties and application of xylanase activity from *Sclerotinia sclerotiorum* S2 fungus. J Food Biochem 31:96-107.
19. Dhiman SS, Garg G, Sharma J, Mahajan R and Methoxy (2010) Characterization of statistically produced xylanase for enrichment of fruit juice clarification process. New Biotechnol 22-44.