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Jacobs Journal of Enzymology and Enzyme Engineering

Research Article

Purification of Extracellular xylanase from *Bacillus subtilis* BS166 by Affinity Precipitation Using Eudragit S-100

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Received: 07-02-2015

Accepted: 07-10-2015

Published: 07-16-2015

Abstract

This study was aimed to compare the purification of xylanase produced by *Bacillus subtilis* BS166 with three phase partitioning and precipitation methods using Eudragit S 100 and to optimize these methods to increase the purification process. In the three phases partitioning method 13.9 UmL^{-1} of xylanase activity was obtained with a specific activity of 30.22 Umg^{-1}. The recovery of the enzyme was 52.3 % with 1.74 fold purification. In the precipitation method, 15.6 UmL^{-1} of xylanase activity was obtained with the specific activity of 36.28 Umg^{-1}. The recovery of the enzyme was 50.1% with 1.73 fold purification. Therefore the three phase partitioning and precipitation methods gave almost same results. To improve the purification, the conditions for purification by these methods were optimized. When the concentrations of $(NH_4)2SO_4$ was increased, at 50% saturation, highest specific activity (31.41 Umg^{-1}) was precipitated. When different concentrations of Eudragit S-100 (10 to 100gL^{-1}) were used in the precipitating method, highest xylanase activity (18.7 UmL^{-1}) was obtained with 40gL^{-1}. Highest Eudragit bound xylanase activity was eluted (23.2 UmL^{-1}) with of 0.8M NaCl. When the three phase partitioning method was repeated with Eudragit 40gL^{-1}, 50% ammonium sulphate and the Eudragit bound xylanase was eluted with 0.8 M NaCl, 17.9 UmL^{-1} of xylanase activity was obtained with a specific activity of 38.10 Umg^{-1}. Xylanase enzyme yield was increased to 57.65 % and the purification fold was increased to 2.07. When the xylanase was purified by precipitation method with Eudragit 40gL^{-1} and the Eudragit bound enzyme was eluted with 0.8MNaCl, 21.6 UmL^{-1} of xylanase activity with the specific activity of 41.54 Umg^{-1} was obtained. Xylanase enzyme yield was increased to 83.72% and the purification fold was increased to 2.33. When the conditions were optimized among the two methods considered, the precipitation method was better than the three phase partitioning method for the purification of xylanase from *Bacillus subtilis* BS166.

Keywords: Eudragit S-100; Purification Fold; Specific Activity; Elution; *Bacillus subtilis*

Introduction

Xylanase which hydrolyses the plant structural materials are very active under alkaline and thermostable conditions. Xylanase has been extracted from many different bacteria, fungi, Actinomycetes, herbivorus insects and from some crustaceans [1]. Xylanases which are very active under alkaline and thermostable conditions have great potential in the modern industrial application. Xylanases are widely used in industries to bleach craft pulp and to increase the brightness in paper industry, to improve the digestibility of animal feed and to clarify the juices in food industry.

Diverse researches are going on the purification of xylanases by ammonium sulphate precipitation [2], ammonium sulphate precipitation followed with DEAE cellulose & column chromatography [1,3]; anion - exchange adsorption, hydrophobic interaction chromatography [4]; anion - exchange and gel filtration & affinity chromatographical systems [5,6]. In addition to these regular purification techniques available, the aqueous two-phase partitioning method, three phase partitioning method [7] and precipitation method using Eudragit S 100 [8] are the other methods reported previously). Soluble-insoluble polymers precipitate from the solution because of the change in the pH. Eudragit S-100, a commercially available enteric, nontoxic polymer of methacrylic acid and methyl methacrylate, is one polymer that falls in this category and it has been used widely in affinity precipitation. It is soluble above pH 6.0 and insoluble below pH 4.0. The objective of this study is to compare and optimize the conditions for the purification of extracellular xylanase from *Bacillus subtilis* BS116 by three phase partitioning method [7] and precipitation method [8], using Eudragit S-100 and to optimize these methods to identify the best method.

Material and Methods

Materials and Culture

Eudragit S 100 was from Rohm Pharma Gmbh, Germany. All the other chemicals used were from standard sources. The culture of *Bacillus subtilis* BS166 strain was provided by Dr.Kanitkar, Department of Biological Science, University of British Columbia, Canada. It was cultured in the optimized fermentation medium [9]. Spent medium was taken at 16 hours and centrifuged at 3000 rpm for 30 minutes. Supernatant was used for the purification studies.

Analytical Methods

Reducing sugar was measured by DNS method [10]. Protein concentration was determined by Lowry's method [11]. One unit of xylanase activity is defined as the amount of enzyme that produces one µmol of reducing sugar in one minute at pH 8.5 and 60°C with $20gL^{-1}$ xylan.

Purification of xylanase using of Eudragit S 100

Xylanase was purified either by three phase partitioning method [7] or by precipitation method[8].

Optimization of conditions for three phase partitioning method

Effect of $(NH_4)_2SO_4$ on the precipitation of xylanase from spent medium

Proteins in the spent medium were precipitated with varying saturations of $(NH_4)_2SO_4$ (10 to 70%). At all the $(NH_4)_2SO_4$ concentrations, the amount of protein and enzyme activity precipitated, were determined after dialyzing the sample against distilled water. Suitable $(NH_4)_2SO_4$ concentration required to precipitate maximum xylanase activity was determined [7].

Optimization of conditions for precipitation method

Effect of Eudragit concentration on the purification of xylanase

Using different concentrations of Eudragit (10 to $100gL^{-1}$) xylanase was purified by precipitation method [8].

Effect of NaCl concentration on the elution of Eudragit bound xylanase

With the optimized amount of Eudragit S-100 xylanase was purified by the precipitation method and to elute the Eudragit bound xylanase, different concentration of NaCl (0.1 to 2M) was used.

Purification of xylanase under optimized condition by three phase partitioning method and precipitation method

Three phase partitioning method was carried out with optimized Eudragit and ammonium sulphate concentration and the bound enzyme was eluted with optimized NaCl concentration and xylanase was purified by precipitation method using optimized Eudragit and to elute the Eudragit bound enzyme, optimized concentration of NaCl was used.

Results

Comparison of the purification of xylanase by Three-phase partitioning method and precipitation method

Purification of xylanase by Three-phase partitioning with Eudragit S-100

In the three phase partitioning method 9.3% of the added xylanase activity (2.6 UmL^{-1}) and 0.42 $mgmL^{-1}$ of protein were not bound to the polymer. The enzyme, which loosely adhered to the polymer showed 1.22UmL^{-1} xylanase activity and 0.35 $mgmL^{-1}$ protein (Table 1). When the enzyme bound to Eudragit was eluted with 0.1 M NaCl, 13.90 UmL^{-1} of xylanase activity and 0.47 $mgmL^{-1}$ of protein were obtained. The crude enzyme had the specific activity of 17.4 Umg^{-1} where the eluted enzyme had the specific activity of 30.22 Umg^{-1}. Hence 52.28 % of the added enzyme was recovered. The purification fold was 1.74 with the specific activity of 30.22 Umg^{-1}.

Purification of xylanase by precipitating with Eudragit S-100

In the precipitation method, 20% of the added xylanase activ-

ity (5.7UmL^{-1}) and 0.52 mgmL^{-1} of protein were not bound to the polymer. The loosely bound enzyme showed 0.89UmL^{-1} of xylanase activity and protein content of 0.16mgmL^{-1} with the specific activity of 5.56 Umg^{-1}. When the Eudragit bound enzyme was eluted with 0.1M NaCl solution, 15.6 UmL^{-1} of the bound xylanase activity with the specific activity of 36.28 Umg^{-1} was obtained (Table 2). The crude enzyme had the specific activity of 21.02 Umg^{-1} where the eluted enzyme had the specific activity of 36.28 Umg^{-1}. Hence 52.21 % of the added enzyme was recovered. The purification fold was 1.73 with the specific activity of 36.28 Umg^{-1}.

Therefore in the three phase partitioning and in the precipitation methods, the specific activity of the enzyme eluted were 30.22 and 36.28 Umg^{-1} respectively with the purification fold of 1.74 and 1.73 respectively.

Table 1. Purification of xylanase from thermostable alkaline xylanase producing Bacillus subtilis using Eudragit -S 100 -Three phase partitioning method [7].

Sample	Activity (UmL^{-1})	Protein (mgmL^{-1})	Specific activity (Umg^{-1})	Total activity (U)	Enzyme yield (%)	Purification fold
Crude enzyme	26.8	1.54	17.40	109.6	100.0	-
Unbound enzyme	2.6	0.42	6.15	7.60	6.93	
Washing	1.22	0.35	3.49	3.60	3.28	
Eluted enzyme	13.9	0.46	30.22	57.30	52.28	1.74

Table 2. Purification of xylanase from the thermostable alkaline xylanase producing Bacillus sp by precipitating with Eudragit S 100 [8].

Step	Activity (UmL^{-1})	Protein (mgmL^{-1})	Specific activity (U/mg)	Total activity (U)	Enzyme yield (%)	Purification fold
Crude	29.9	1.39	21.01	57.3	100	-
Supernatant 1	5.7	0.52	10.96	8.7	15.18	
Washing 1	0.89	0.16	5.56	1.39	2.43	
Washing 2	No	No	No	No	No	-
Eluent	15.6	0.43	36.28	28.7	50.09	1.73

Optimization of conditions

Effect of ammonium sulphate on the precipitation of xylanase

When the saturation percentage of $(NH_4)_2SO_4$ was increased from 10 to 70 %, the precipitation of protein was increased but the enzyme activity increased up to 50% of $(NH_4)_2SO_4$. This is because, above 50% of $(NH_4)_2SO_4$ saturation non-enzyme-protein could be precipitated. The crude enzyme sample precipitated with 50% $(NH_4)_2SO_4$ saturation and (Dialysed against distilled water) showed highest specific activity (33.65Umg^{-1} protein) than the samples precipitated with different concentration of $(NH_4)_2SO_4$ (Table 3). By this $(NH_4)_2SO_4$ precipitation, the specific activity of xylanase has increased by 1.8 times than that of crude enzyme.

Table 3. Effect of ammonium sulphate saturation percentage on the precipitation of xylanase from *Bacillus pumilus.*

$(NH_4)_2SO_4$ (%)	Precipitate		
	Xylanase activity (UmL^{-1})	Protein (mgmL^{-1})	Specific activity (UmL^{-1})
10	10.30	0.59	17.46
20	18.60	0.89	20.90
30	28.40	1.16	24.48
40	33.32	1.21	27.54
50	44.60	1.42	31.41
60	45.30	1.79	25.31
70	42.60	1.86	22.90

Effect of Eudragit S 100 concentration on purification of xylanase by precipitation method

Xylanase enzyme activity recovered was increased with the increase in Eudragit concentration up to 40gL^{-1} (18.7UmL^{-1}, Figure 1). Therefore this concentration was selected for both three phase partitioning and precipitation methods.

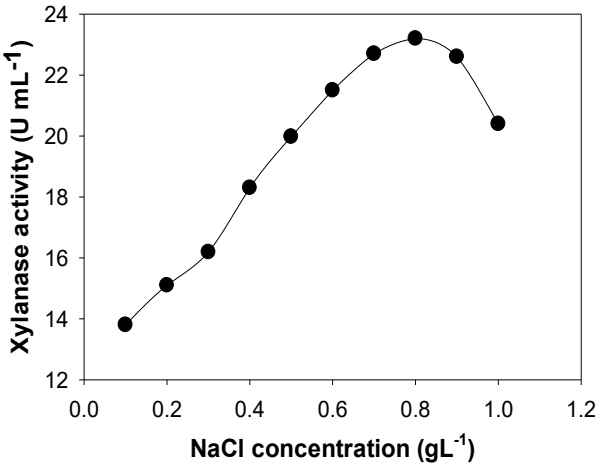

Figure 1. Effect of NaCl concentration in the elution of Eudragit

bound xylanase enzyme by precipitation method of purification using Eudragit S-100.

Effect of NaCl concentration on the Eudragit bound xylanase

When the spent medium was treated with 40gL^{-1} Eudragit S-100 and Eudragit bound xylanase was eluted with increasing concentration of NaCl and highest xylanase activity (23.2 UmL^{-1}) was eluted with of 0.8 M NaCl (Figure 2). Therefore 0.8M NaCl was selected to elute xylanase from the polymer.

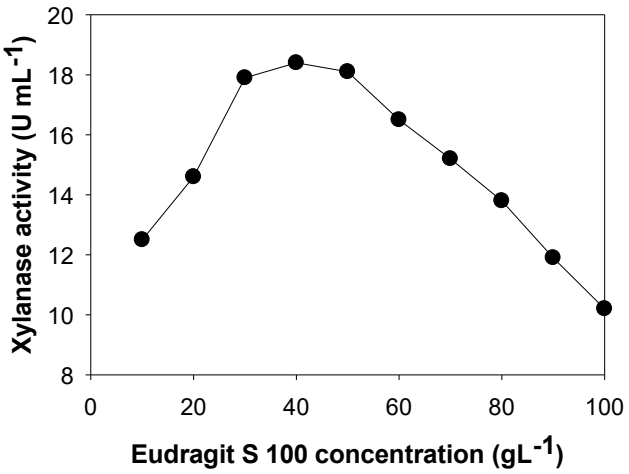

Figure 2. Effect of Eudragit S100 concentration on the xylanase enzyme recovery by precipitation method of purification.

Purification of xylanase under optimized condition

Purification of xylanase by Three-phase partitioning with Eudragit S-100 under the optimized condition

In the three-phase partitioning method, 10% of the added xylanase activity (2.82UmL^{-1}) and 0.41mgmL^{-1} of protein were not bound to the polymer. The washing contained 2.63 UmL^{-1} xylanase activity and 0.33mgmL^{-1} protein with the specific activity of 7.96Umg^{-1}. When the enzyme bound to Eudragit was eluted, 17.9UmL^{-1} of xylanase activity and 0.47 mgmL^{-1} of protein were obtained. The crude enzyme had the specific activity of 18.89Umg^{-1} where the eluted enzyme had the specific activity of 38.10Umg^{-1}. Hence recovery of 57.6% enzyme was obtained with 2.02 fold purification.The crude and purified enzymes had the specific activities of 19.17 and 71.3Umg^{-1} respectively. Here the recovery of the enzyme was 57.6%. Under this optimized conditions in the three phase partitioning method the purification fold heighest specific activity was obtained at 50 % $(NH_4)_2SO_4$. The purification fold was also increased from 1.74 to 2.07 (Table 4). Optimized method gave 1.18 times increase in the purification fold, than the no optimized method.

Table 4. Purification of xylanase in the optimized condition by three phase partitioning method.

Sample	Activity (U mL^{-1})	Protein (mg/mL)	Specific activity (U/mg)	Total activity (U)	Enzyme yield (%)	Purification fold
Crude enzyme	27.8	1.45	19.17	111.2	100.00	1.00
Unbound enzyme	2.82	0.41	6.62	8.7	7.82	
Washing	2.65	0.33	7.36	1.39	1.25	
Eluted enzyme	17.9	0.47	71.28	64.1	57.65	2.07

Purification of xylanase by precipitation method in the optimized condition

In the precipitation method, 8.9 % of the added xylanase activity (2.33 Uml^{-1}) and 0.54 mgml^{-1} of protein were not bound to the polymer. Purified enzyme showed 21.6 UmL^{-1} of xylanase activity with the specific activity of 41.54 Umg^{-1}. When the Eudragit bound enzyme was eluted with 0.8M NaCl solution, 18.96 Uml^{-1} of the bound xylanase activity (83.72%) was recovered. Here the recovery of the enzyme was 83.72%. Optimization of the conditions for precipitation method has improved the purification fold from 1.73 to 2.33 (Table 5). Optimized method gave 1.33 times increase in the purification fold, than the no optimized method. Therefore after optimizing the conditions for the purification, xylanase activity and purification fold were increased in the precipitation method.

Table 5. Purification of Xylanase by Precipitation method under the optimized condition.

Step	Activity (UmL^{-1})	Protein (mg/mL)	Specific activity (U/mg)	Total activity	Enzyme yield (%)	Purification fold
Crude	25.8	1.45	17.79	103.2	100	1
Supernatant1	2.3	0.54	4.26	4.6	8.91	
Washing 1	1.7	0.16	0.11	3.4	6.59	
Washing 2	No	No	No	No	No	
Eluent	21.6	0.52	41.54	86.4	83.72	2.33

Discussion

Eudragit S100 is a water soluble polymer which becomes in-

soluble at below pH 5.5. Around this pH, all the carboxylic acid groups become protonated, the net charge on the polymer becomes nearly zero, electrostatic repulsion ceases, and hydrophobic interactions predominate. Thus the polymer with both its hydrophobic and hydrophilic portions, is similar to the proteins [7]. The polymer aggregates and precipitates out of the solution. Three phase partitioning of proteins uses simultaneous addition of ammonium sulphate and butanol to precipitate proteins in an interfacial layer seperationg the aqueous phase and organic solvent. Butanol adheres to the protein and the $(NH_4)_2SO_4$ forms an interfacial precipitate between lower aqueous and upper organic layers. The affinity of xylanase towards Eudragit, was used in to purify xylanase from the crude mixture. This technique enhances the selectivity of the separation process and this process becomes more predictable and easier to design [7]. Purification of xylanase from *Aspergillus niger* by three phase parttioning method gave 60% recovery of xylanase activity with 95% of purification fold [7].

When the enzyme was added to the polymer, it did bind to the polymer and the binding was electrostatic in nature. The washing of the precipitate removed a small amount of protein. When the pH was raised to 7.0, the enzyme could be dissociated from the polymer. Eudragit bound enzyme was eluted with 0.8MNaCl. Solubility of the Eudragit can be controlled by changing the pH [12]. Xylanase obtained from *Trichoderma viridae* was purified by precipitation method using Eudragit S-100 and the purification fold was 4.2 with 89% yield [8]. Enzyme D-Lactate dehydrogenase was purified by precipitation method using Eudragit S-100 and the purification fold was 9.0 [12].

When xylanase from *Bacillus* sp was purified by DEAE-Sepharose chromatography, specific activity obtained was 213.1Umg^{-1} with the yield of 53.9 and purification fold of 7.3[4]. The molecular weight of the xylanase (55.4kDa) from *Bacillus pumilus* is closely resembled the molecular weight (56000Da) of the xylanase from *Micrococcus* sp AR-135 determined by SDS-PAGE [13].

Conclusion

Among the two different purification methods studied with Eudragit S-100, precipitation method gave better enzyme yield with higher purification fold (2.32) than the three phase portioning method (2.02). Therefore the precipitation method under optimized conditions, could be used for the purification of xylanase.

Acknowledgement

The author thanks Dr.Mohan, T of University of Alberta, for the technical and financial support.

References

1. Annamalai N, Thavasi R, Jeyalakshmi S, Balasubramaniam T. Thermostable and alkaline tolerant xylanase production by Bacillus subtilis isolated from marine environment. Indian Journal of Biotechnology. 2009,. 8: 291-297.

2. Pal A, Roy L, Chattopadhyay P. Purification an immobilization of an A. terreus xylanase: use of continuous fluidized bed column reactor. Indian Journal of Biotechnology. 2006, 5:163-168.

3. Kapilan R, Arasaratnam V. Characterization of xylanase produced by Bacillus pumilus. Journal of National Science Foundation. 2014. 42(4): 363-366.

4. Breccia JD, Siberia F, Baigori MD, Castro GR, Hatti-Kaul R. Purification and characterization of a thermostable xylanase from Bacillus amylolicquefaciens. Enzyme and Microbial Technology. 1997, 22:422-449.

5. Bataillan M, Cardinali A-PN, Castillon N, Duchiron F. Purification and characterization of a moderately thermostable xylanase from Bacillus sp. Strain SPS-O. Enzyme and Microbial Technolog. 2000, 26(2-4): 187-192.

6. Christalcopoulos P, Nerinck W, Kekos D, Macris B, Claeyssens M. Purification and Characterization of two low molecular mass alkaline xylanases from Fusarium oxysporum F3. Journal of Biotechnology. 1996, 51(2): 181-189.

7. Sharma A, Gupta MN. Macroaffinity ligand-facilitated three phase partitioning (MLFTPP) for purification of xylanase. Biotechnology and Bioengineering. 2002, 80(2): 228-232.

8. Gupta MN, Guoqiang D, Kabul R, Mattiasson B. Purificationxylanase from Trichoderma viridae by precipitation with an anionic polymer Eudragit S 100.Biotechnol Tech. 1994, 8(2):117-122.

9. Kapilan R, Vasanthy Arasaratnam. Comparison of the kinetic properties of crude and purified xylanase from Bacillus pumilus with commercial xylanase from Aspergillus niger. Vingnam Journal of Science. 2011, 10(1): 1-6.

10. Miller GL. Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chem. 1959, 31(3): 426-428.

11. Lowry OH, Rosenburg NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 1951, 193: 265-275.

12. Guoqiang D, Ventura MAN, Kabul R, Mattiasson B. Af-

finity thermoprecipitation of yeast alcohol dehydrogenase through metal ion promoted binding with eudragit bound cibracon blue 3GA. Biotechnology Progress. 1995, 11(2): 187-193.

13. Gessesse A, Mamo G. High-level xylanase production by an alkaliphilic Bacillus sp. by using solid-state fermentation. Enzyme and Microbial Technology. 1999,10(11):68-72.