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Purification of xylanase from *Bacillus pumilus* using Eudragit S-100 and optimization of conditions

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ABSTRACT

Soluble-insoluble polymers have considerable applications in the enzyme purification and these polymers precipitate from the solution by changing the pH. Eudragit S-100, nontoxic polymer of methacrylic acid and methyl methacrylate, has been used in affinity precipitation. The study describes the purification of xylanase from Bacillus pumilus by three phase partitioning (TPP) method and precipitation (PT) method using Eudragit S-100 and to optimize these methods to improve the purification fold. In the TPP method, 14.6 U mL^{-1} of xylanase activity was obtained with a specific activity of 31.74 U mg $protein^{-1}$. The recovery of the enzyme was 52.24 % with 1.79-fold purification. In the PT method, 15 U mL^{-1} of xylanase activity was obtained with the specific activity of 33.4 U mg $protein^{-1}$. The recovery of the enzyme was 52.18% with 1.72-fold purification. Since there is no significant difference in the purification folds of both these methods and to improve the purification fold, the conditions of these methods were optimized. When different concentrations of $(NH_4)_2SO_4$ were used, xylanase with significantly higher specific activity (33.74 U mg $protein^{-1}$) was precipitated with 50% of $(NH_4)_2SO_4$ saturation. Among the different Eudragit concentrations used, 40gL^{-1} Eudragit S-100 yielded significantly higher xylanase activity (18.9 UmL^{-1}) than the other Eudragit concentrations. When the spent medium was treated with 40gL^{-1} Eudragit S-100 and Eudragit bound xylanase was eluted with different concentrations of NaCl, significantly higher activity of xylanase (17.9UmL^{-1}) was eluted with of 0.3 M NaCl. When TPP method of purification was done under optimized conditions (with 50% $(NH_4)_2SO_4$ saturation and usage of 0.3 M NaCl for elution), 17.87U mL^{-1} of xylanase activity was obtained with a specific activity of 38.1 U mg $protein^{-1}$and the xylanase enzyme yield and purification fold were significantly increased to 63.41% and 2.01 than the non-optimized TPP method. When PT method of purification was done under optimized conditions (with 40gL^{-1} Eudragit S-100 and usage of 0.3 M NaCl for elution), 18.96 U mL^{-1} of xylanase activity with the specific activity of 43.09 U mg $protein^{-1}$ was obtained and the xylanase yield and the purification fold were significantly increased to 69.67% and 2.31 respectively than the non-optimized PT method. Since the purification fold of the optimized PT method is significantly higher than that of optimized TPP method, optimized PT method could be recommended for the purification of xylanase from Bacillus type bacteria. When the purified xylanase was subjected to poly acrylamide gel electrophoresis, it gave a single band and the molecular weight of the purified xylanase was determined as 55.4 kDa.

Key words: *Bacillus pumilus, Eudragit S-100, purification fold, three phase partitioning, precipitation, xylanase*

INTRODUCTION

Xylanase enzyme degrades β-1, 4 xylan by cleaving β-1, 4 glycosidic linkage results in xylose and xylo-oligosaccharides like xylobiose (Chidi et al., 2008). Xylanases that are very active under alkaline and thermostable conditions have great potential in the industrial applications to bleach and

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increase the brightness of paper pulp, to improve the digestibility of animal feed and to clarify the fruit juices in the food industry. Bacterial genera have been the principle source of xylanases (Lyon *et al*., 2000; Touzel *et al*., 2000; Roy and Uddin, 2004; Roy, 2004; Lee *et al*., 2006; Ibrahim and El-diwany, 2007; Anuradha *et al*., 2007; Ugwuanyi *et al*., 2008; Yasinok *et al*., 2008; Kapoor *et al*., 2008; Garg *et al*., 2009; Annamalai *et al*., 2009; Kapilan and Arasaratnam, 2010). Some filamentous fungal species (Khan *et al*., 2003; Qinnghe *et al*., 2004; Chidi *et al*., 2008; Kumar *et al*., 2009), Actinomycetes (Ninawe *et al*., 2006; Ball and Mc Carthy, 1989), herbivorus insects (Roy *et al*., 2003) and some crustaceans are the other sources of xylanase enzyme. Secretion of xylanase from diverse microorganisms is often associated with the cellulases. For the industrial pulp treatment, cellulose-free xylanases are highly preferred, since the cellulase may adversely affect the quality of the paper pulp (Archana and Styanarayana, 2003). There have been studies continuing on the purification of crude xylanases from different organisms using different methods. Purification by ammonium sulphate precipitation (Pal *et al*., 2006) followed with DEAE cellulose & column chromatography (Annamalai *et al*., 2009; Kapilan and Arasaratnam 2014), anion - exchange adsorption, ammonium sulphate precipitation & hydrophobic interaction chromatography (Breccia *et al*., 1998), anion - exchange chromatography, gel filtration & affinity chromatography (Bataillan *et al*., 2000) and gel filtration & ion-exchange chromatography (Christalcopoulos *et al*., 1996) are some of the methods in practice.

In addition to the above-mentioned techniques, the aqueous two-phase partitioning (Yang *et al*., 2008), three phase partitioning (Sharma and Gupta, 2002) and precipitation method using Eudragit S 100 (Gupta *et al*., 1994) and some other chromatography techniques (Franco and de Marco, 2020) are some of the latest techniques reported recently. There has been a concern that how these methods are efficient in purifying all types of xylanases isolated from diverse natural sources. Soluble-insoluble polymers have been playing considerable role in the xylanase purification. These polymers are precipitated from the solution by the changing of pH of the media. Eudragit S-100, is an enteric, nontoxic polymer of methacrylic acid and methyl methacrylate and it has been used to precipitate enzymes based on the degree of affinity. Eudragit S-100 which is commercially available will be soluble above pH 6.0 and insoluble below pH 4.0. The study was aimed to purify thermostable alkaline xylanase obtained from *Bacillus pumilus* by three phase partitioning method (Rahman *et al*., 2016; Sharma and Gupta, 2002) and precipitation method (Gupta *et al*., 1994) using Eudragit S-100 and to optimize these methods to recommend the best method.

MATERIAL AND METHODS

Materials

Eudragit S-100 was purchased from Rohm Pharma GmBh, Germany. All the other chemicals used in this study were from the standard sources.

Microorganism

Bacillus pumilus (Kapilan and Arasaratnam, 2010) isolated from open xylan agar plate was used.

Media used and production of crude xylanase

All the plates and slants containing (gL^{-1}) nutrient agar 25.0 and xylan 20.0 were used at pH 8.0 for the storage of the bacterial strain. The activation medium contained

(gL^{-1}) xylan 20.0 and nutrient broth 25.0. Liquid fermentation medium contained (gL^{-1}) xylan, 20.0; peptone, 20.0; yeast extract, 2.5; $CaCl_2.2H_2O$, 0.005; $MgCl_2.6H_2O$, 0.005; $FeCl_3$, 0.005; K_2HPO_4, 2.5; KH_2PO_4,1.0; NaCl, 0.1 and $(NH_4)_2SO_4$, 2.0 at pH 8.0 and 120 rpm. After the completion of fermentation, the whole fermentation media was centrifuged at 15000 rpm at 4°C for 15 minutes and the clear supernatant was recovered. The crude enzyme supernatant was used for the purification studies (Kapilan and Arasaratnam, 2011).

Analytical methods

Xylanase activity was measured in terms of reducing sugar produced by its action on xylan by modified Dinitro salicylic acid (DNSA) method (Miller, 1959; Kapilan and Arasaratnam, 2010). Enzyme was diluted with Tris aminomethane buffer (pH-8.5). Enzyme assay was carried out at pH 8.5 and 60°C by preincubating the enzyme extract with $10gL^{-1}$ xylan in 0.01M Tris aminomethane buffer (pH-8.5) for 5 minutes. Then 0.5mL of the enzyme was mixed with 0.5mL substrate and incubated for 5 minutes at 85°C. Reducing sugar was measured by DNS method (Miller, 1959). Protein concentration of the samples was determined by Lowry's method (Lowry *et al*, 1951). All the experimental set ups were done in triplicates.

Unit of enzyme activity

One unit of xylanase activity is defined as the amount of enzyme that produces one µmole of reducing sugar in one minute at pH 9.0 and 60°C with 20 gL^{-1} xylan.

Purification of xylanase using of Eudragit S 100

Xylanase was purified either by a modified three phase partitioning method (Sharma and Gupta, 2002) or by precipitation method (Gupta *et al*., 1994). All the experimental set ups were done in triplicates.

Purification of xylanase by three-phase partitioning of Eudragit S-100

Eudragit solution (1mL – $20gL^{-1}$) mixed with 2mL of crude enzyme solution, and ammonium sulphate (30%) & butanol (1:1) (v/v), was incubated for 1h. The mixture was centrifuged (2000 rpm MSE bench centrifuge) for 10 minutes. Of the three-phases, top-phase was analyzed for xylanase activity and protein content (Unbound enzyme-Supernatant-1). The middle phase, which contained polymer bound protein, was suspended in 3mL distilled water, and pH was adjusted to 4.5 by the addition of 100µL of 0.1M acetic acid. After centrifugation (at 2000 rpm for 10 minutes), the precipitate was dissolved in 3mL of 0.05M acetate buffer (pH 5.5) and the pH was adjusted to 4.5. After 20 minutes, the suspension was centrifuged (2000rpm for 10 minutes).

The supernatant (Washing) was tested for the xylanase activity and protein content. The precipitated Eudragit bound enzyme was resuspended in 2mL of 0.1 M of phosphate – 1M NaCl buffer (pH 7.0) and the polymer was precipitated by decreasing the pH to 4.5. After 20 minutes, the suspension was centrifuged at 3000rpm for 15minutes. Xylanase activity and the protein content were measured in the supernatant.

Purification of xylanase by precipitating with Eudragit 100

From crude enzyme diluted with 0.01M phosphate buffer (pH 7.0) (1:1 ratio) 1.0mL was mixed with 2mL Eudragit solution ($100gL^{-1}$). After 15 min, the polymer was precipitated at pH 4.5. After 20 min the suspension was centrifuged (2000rpm) for 15 min. The bound enzyme activity and protein content were determined indirectly by

analyzing the supernatant (Supernatant1). The precipitated Eudragit bound enzyme was washed with 2mL of Tris buffer (pH 8.5). The pH was reduced to 4.5 and the suspension was centrifuged at 2000 rpm for 15 minutes. The supernatant (Washing 1) was analyzed for enzyme activity and protein content. Again the precipitate was washed with 2mL of Tris buffer (pH 8.5) (Washing 2). Then to the precipitate, 2mL of 0.1M NaCl was added to elute the bound enzyme. The suspension was centrifuged at 2000 rpm for 15 min. Enzyme activity and protein content were measured in the supernatant.

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.

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 (Gupta *et al*., 1994).

Optimization of conditions for the elution of Eudragit bound xylanase with NaCl

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

Comparison of purification under optimized conditions

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

Statistical analysis

Statistical analyses were performed using R 2.15.3 statistical software at $\alpha = 0.05$ confidence level. The data sets were checked for the parametric assumptions of normality using Shapiro-Wilk and Kolmogorov-Smirnov tests. Box plots were used to identify the outliers they were removed. Analysis of variance was used to analyze the data. Significant differences (at $p \leq 0.05$) were determined by ANOVA and Tukey's multiple comparison test. Each experiment was performed in triplicate and standard deviations for each experimental result were calculated and used for the graphical representation.

SDS Acrylamide gel electrophoretic separation and Molecular weight determination

The pooled sample of purified xylanase was subjected to SDS Acrylamide gel electrophoretic separation, and stained with coomassie brilliant blue (Laemmli, 1970). The molecular weight of the purified xylanase after electrophoresis step was determined (Weber and Osborn, 1969).

RESULTS AND DISCUSSION

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 TPP method, ammonium sulphate and butanol are added to precipitate proteins in an interfacial layer (Lovrein *et al.*, 1987) formed in between the aqueous phase and organic solvent. Butanol adheres proteins and the $(NH_4)_2SO_4$ forms an interfacial precipitate between lower aqueous and upper organic layers (Dennison and Lovrein, 1997). The affinity of xylanase towards Eudragit was used to purify xylanase from the crude mixture (Sharma and Gupta, 2002). In the TPP method, 9.32 and 26.75% of the added xylanase activity and protein were not bound to the polymer (Table 1). To the polymer 22.93% of the added protein and 3.58% of the xylanase activity were loosely bound. With 0.1 M NaCl, 29.3% of the Eudragit bound protein showing 52.3% of xylanase activity was eluted. The crude enzyme had the specific activity of 17.77 U mg protein^{-1} where the eluted enzyme had the specific activity of 31.74 U mg protein^{-1}. Hence 52.24% of the added enzyme was recovered. The purification fold was 1.79. Sharma and Gupta (2002) have reported 60% recovery of activity with 95% fold purification.

Purification of xylanase by precipitating with Eudragit S-100

In the PT method, 20.56% of the added xylanase activity (or 35.57% of protein) was not bound to the polymer (Table 1). The loosely bound enzyme had 3.3% of xylanase activity (or 1.3% protein) with the specific activity of 47.5 U mg protein^{-1}. No detectable amounts of xylanase activity and protein were observed in the washing 2. When the Eudragit bound enzyme was eluted with 0.1M NaCl solution, 15.97 U mL^{-1} of the bound xylanase activity with the specific activity of 33.14 U mg protein^{-1} was obtained. The crude enzyme had the specific activity of 19.26 U mg protein^{-1}. Hence 52.18 % of the added enzyme was recovered and the purification fold by the PT method was 1. 72. Xylanase from *T. viride* has been purified with 4.2 fold by precipitation with Eudragit S100. Despite the random nature of interactions by Eudragit S 100, precipitation method could be used if the elution conditions are optimized and selected carefully (Gupta *et al*., 1994; Nikam, 2011; Rahman *et al*., 2016). For scaling up the isolation of organisms and partial purification of enzymes such as xylanase, precipitation method using Eudragit S 100 is considered as an efficient technique (Franco and de Marco, 2020).

Optimization of conditions

Effect of ammonium sulphate concentration

Many types of salts have been employed for protein separation & purification through salting-out and different proteins precipitate at different salt concentration. Further $(NH_4)_2SO_4$ is also a cheap salt, having high solubility and hence is used commonly (Kapilan and Arasaratnam, 2014). To the crude xylanase (activity 27.9 UmL^{-1}, protein content 1.5 mgmL^{-1}) solid $(NH_4)_2SO_4$ was added. When different concentrations of $(NH_4)_2SO_4$ were used, xylanase with significantly higher specific activity (33.74 U mg protein^{-1}) was precipitated when the concentrations of $(NH_4)_2SO_4$ was kept at 50% saturation (Table 2). This indicated that at 50% $(NH_4)_2SO_4$ saturation non-xylanase proteins got precipitated. The crude enzyme sample precipitated with 50% $(NH_4)_2SO_4$ saturation showed significantly higher specific activity (33.7 U mg protein^{-1}) (Table 2). By this $(NH_4)_2SO_4$ precipitation, the specific activity of xylanase was increased by 1.8 times than that of the crude enzyme.

Xylanase from *Micrococcus* sp.AR135, purified with 70% $(NH_4)_2SO_4$ saturation showed the specific activity of 1.7 U mg

protein^{-1}, which was 34.9-fold higher than that of the crude enzyme with 75% yield (Gessesse and Mamo, 1999). Xylanase from *Bacillus sp* precipitated with 90% ammonium sulphate, showed 1.25Umg protein^{-1} specific activity, with 2.01-fold purification and 80.0% yield (Chauthaiwale and Rao, 1994). When xylanase from *Bacillus amyloliquifaciens* was saturated with 40% $(NH_4)_2SO_4$, 117.0Umg protein^{-1} specific activity was obtained, which was 4.0 fold higher than the crude enzyme in the 61.5% yield (Breccia *et al*., 1997). When xylanase from *Bacillus* sp. Strain SPS-0 was saturated with 20-80% $(NH_4)_2SO_4$, showed 106.3Umg protein^{-1} specific activity with 1.1 fold higher than that crude enzyme with 48.6% yield (Bataillan *et al*., 2000). With 60% ammonium sulphate saturation 55% xylanase was obtained as the yield (Annamalai *et al*., 2009). Gupta *et al*. (1994) have reported on the inhibitory effect of Ammonium sulphate of the binding of endo-xylanase to Eudragit. Ammonium sulphate (50%) precipitation increased the specific activity of xylanase from *Bacillus pumilus* by 1.81 times than that of the crude enzyme.

Effect of Eudragit S 100 concentration

Among the different Eudragit concentrations used, 40gL^{-1} of Eudragit S-100 yielded significantly higher xylanase activity (18.9 UmL^{-1}) than the other Eudragit concentrations (Figure 1). Eudragit-S100 is a copolymer of methylacrylic acid and methylmethacrylate and it shows affinity towards xylanases (Gupta *et al*., 1994). Though xylanase activity was obtained in a broad Eudragit concentration range, very low xylanase activity (12.4 UmL^{-1}) was obtained when higher concentration of Eudragit S-100 (100gL^{-1}) was used. This affinity increased up to certain concentration of Eudragit S-100 and starts to drop beyond that level. Decrease in the xylanase enzyme activity expressed could be due to the intermolecular binding (Cross-linking) between any of the molecular entities of xylanase and Eudragit S100 and also might be due to the enzyme denaturation under the coupling conditions. Some organic solvents slightly increase the enzyme activity and some inactivate the enzyme activity at higher concentrations of Eudragit S100 (Gupta *et al*., 1994; Franco and de Marco, 2020; Niham, 2011). Since Eudragit has liquid-range density, higher solvating capacity, low viscosity, and enhanced diffusivity and mass transfer coefficient, it is considered as a good candidate for replacing the organic solvents in lot of different applications in the chemical, biochemical, and purification industries, polymer sources and particle synthesis techniques (Franco and de Marco, 2020; Niham, 2011). Therefore 40gL^{-1} of Eudragit S-100 concentration was selected for both three phase partitioning and precipitation methods. After optimizing the Eudragit concentration, the effect of NaCl concentration on the elution of Eudragit bound xylanase was studied.

Table 1: Purification of xylanase from thermostable alkaline xylanase producing *Bacillus pumilus* using Eudragit -S 100 -Three phase partitioning method and precipitating with Eudragit S-100.

Sample	**Activity (U mL^{-1})**		**Protein (mg mL^{-1})**		**Specific activity (U mg protein^{-1})**		**Total activity (U)**		**Enzyme yield (%)**		**Purification fold**	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Crude enzyme	27.9	28.7	1.57	1.49	17.77	19.26	111.6	57.3	100.0	100	-	-
Unbound enzyme	2.6	-	0.42	-	6.19	-	7.8	-	6.96	-	-	-
Washing	1.23	-	0.36	-	3.42	-	3.7	-	3.3	-	-	-
Supernatant 1	-	5.9	-	0.53	-	11.13	-	8.8	-	15.36	-	-
Washing 1	-	0.95	-	0.02	-	47.5	-	1.4	-	2.44	-	-
Washing 2	-	No	-	No	-	No	-	No	-	No	-	-
Eluted enzyme	14.6	14.97	0.46	0.45	31.74	33.14	58.3	29.9	52.24	52.18	1.79	1.72

A- Three phase partitioning method (Sharma and Gupta, 2002)

B- Precipitation method (Gupta *et al.*, 1994).

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 different concentration of NaCl significantly higher xylanase activity ($17.9UmL^{-1}$) was eluted with of 0.3 M NaCl. Therefore, it was decided to use 0.8M NaCl to elute xylanase enzyme from the polymer. Xylanase obtained from *Trichoderma viridae* was purified by precipitation method using Eudragit S-100 and the Eudragit bound enzyme was eluted with 0.1MNaCl (Gupta *et al*., 1994). Xylanase of *B. amyloliquefaciens* MIR32 was eluted from Eudragit S-100 with 1M NaCl and 0.2% (v/v) Triton and yielded 83% with a 4.5 fold increased specific activity (Breccia *et al*., 1998). When the xylanase enzyme produced by *Bacillus subtilis* was purified by ammonium sulphate precipitation and with ion exchanger of DEAE –Sepharose and eluted with 0.8M NaCl, the specific activity of was increased from 32.14 to $212.5Umg^{-1}$ protein, which was 6.7 fold higher than that of the crude xylanase and the yield was 85% (Kapilan, 2015).

After optimizing the ammonium sulphate saturation, Eudragit concentration and NaCl concentration, crude xylanase was purified by both three-phase partitioning method and precipitation method under optimized conditions.

Table 2: Effect of ammonium sulphate saturation percentage on the precipitation of xylanase from *Bacillus pumilus.*

$(NH_4)_2SO_4$ (%)	**Precipitate**		
	Xylanase activity (UmL^{-1})	**Protein (mg mL^{-1})**	**Specific activity (U mg $Protein^{-1}$)**
10	10.0	0.54	18.7
20	19.8	0.91	21.7
30	29.4	1.02	28.9
40	33.3	1.10	30.3
50	46.8	1.39	33.7
60	45.0	1.78	25.3
70	43.8	2.64	16.6

Purification of Xylanase under optimized condition

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

In the TPP method of purification, 10% of the added xylanase activity (or 27.52% of protein) was not bound to the polymer (Table 3). The washing contained 9.2% xylanase activity (or 22.15% protein) with the specific activity of 7.97Umg^{-1}. When the enzyme bound to Eudragit was eluted, 63.41% of xylanase activity (or 31.54% protein) was obtained. The crude enzyme had the specific activity of 18.91Umg protein^{-1} where the eluted enzyme had the specific activity of 38.02 U mg protein^{-1}. Hence recovery of 63.41% enzyme was obtained with 2.01 fold purification. The purification fold of the TPP method under optimized conditions was significantly higher (2.02) than the non-optimized TPP method (1.79) when purifying xylanase from *Bacillus pumilus* using Eudragit S-100. Optimized method gave 1.13 times increase in the purification fold, than the non-optimized method. Similar trend was observed in the xylanase enzyme yield also.

Purification of xylanase by precipitation method in the optimized condition

In the PT method, 19.49% of the added xylanase activity (or 39.73% of protein) was not bound to the polymer. Purified enzyme showed 69.7% of xylanase activity with the specific activity of 43.09 Umg^{-1}. When the Eudragit bound enzyme was eluted with 0.1M NaCl solution, 18.96 UmL^{-1} of the bound xylanase activity was eluted (Table 3). Here the recovery of the enzyme was 69.67%. Optimization of the conditions for PT method has significantly increased the purification fold from 1.72 to 2.31. Optimized method gave 1.34 times increase in the purification fold, than the non-optimized method. When the xylanase from *Bacillus subtilis* BS166 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% from 50.1% and the purification fold was increased to 2.33 from 1.73 (Kapilan, 2015).

When PT method of purification was done under optimized conditions (with 40gL^{-1} Eudragit S-100 and usage of 0.3 M NaCl for elution), 18.96 U mL^{-1} of xylanase activity with the specific activity of 43.09 U mg protein^{-1} was obtained and the xylanase yield and the purification fold were significantly increased to 69.67% and 2.31 respectively than the non-optimized PT method (at $p \leq 0.05$). Since the purification fold of the optimized PT method is significantly higher than that of optimized TPP method, optimized PT method could be a better method for the purification of xylanase from *Bacillus* type of bacteria genera.

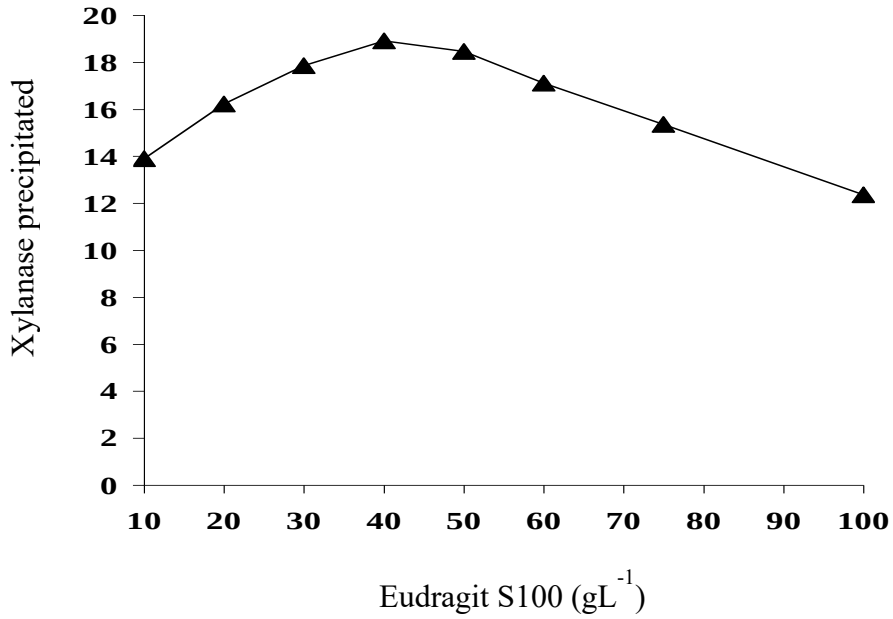

Figure 1: Effect of Eudragit S 100 concentration on xylanase recovery by precipitation method.

After optimizing the conditions for the purification, xylanase activity and purification fold were significantly increased (at $p \leq 0.05$) in the precipitation method than the three-phase partitioning method. Therefore, the precipitation method under optimized conditions could be used for the purification of xylanase obtained from *Bacillus pumilus.*

SDS Acrylamide gel electrophoretic separation and molecular weight determination

When the purified xylanase was subjected to gel electrophoretic separation, and stained with Coomassie brilliant blue, the sample gave single band. This single band indicated that one type of xylanase was produced by this bacterium and further purification steps were not needed. The distance travelled by molecular markers and purified xylanase were measured. The molecular weight of the purified xylanase was 55.4 kDa (Figure 2). The molecular weight of this xylanase (55.3kDa) closely resembled the molecular weight (56000Da) of the xylanase from *Micrococcus* sp AR-135 determined by SDS-PAGE (Gessesse and Mamo, 1998).

Table 3: Purification of xylanase from thermostable alkaline xylanase produced by *Bacillus pumilus* using Eudragit -S 100 -Three phase partitioning method and precipitation method under optimized conditions.

Sample	**Activity (UmL^{-1})**		**Protein ($mgmL^{-1}$)**		**Specific activity (U mg $protein^{-1}$)**		**Total activity (U)**		**Enzyme yield (%)**		**Purification fold**	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Crude enzyme	28.18	27.2	1.49	1.46	18.91	18.63	112.72	54.4	100.00	100.00	1.00	1.00
Unbound enzyme	2.82	-	0.41	-	6.88	-	8.46	-	7.51	-	-	-
Washing	2.63	-	0.33	-	7.97	-	7.90	-	7.01	-	-	-
Supernatant 1	-	5.3	-	0.58	-	9.13	-	7.99	-	14.68	-	-
Washing 1	-	1.2	-	0.13	-	9.23	-	2.4	-	0.04	-	-
Washing 2	-	No	-	No	-	No	-	No	-	No	-	-
Eluted enzyme	17.87	18.96	0.47	0.44	38.02	43.19	71.48	37.9	63.41	69.67	2.02	2.31

A- Three phase partitioning method (Sharma and Gupta, 2002)

B- Precipitation method (Gupta, *et al* 1994).

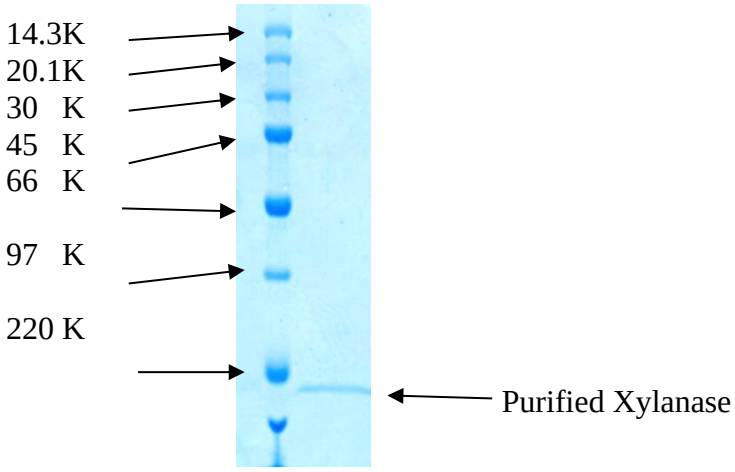

Figure 2: SDS–PAGE pattern of *Bacillus pumilus* xylanase purified by precipitation method. Molecular weight of the purified xylanase was determined as 55.4 kDa

CONCLUSION

Since optimized Precipitation method gave significantly higher xylanase yield and higher purification fold (69.67 and 2.31) than the optimized Three Phase Partitioning method (63.41 and 2.02), optimized Precipitation method could be recommended for the purification of xylanase using Eudragit S-100. The molecular weight of the purified xylanase was 55.4KDa. Further studies need to be done in order to confirm the applicability of this method to all the xylanases obtained from diverse sources.

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