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Reverse Micellar Extraction of Fungal Glucoamylase Produced in Solid-State Fermentation Culture

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Partial purification of glucoamylase from solid-state fermentation culture was, firstly, investigated by reverse micellar extraction (RME). To avoid back extraction problems, the glucoamylase was kept in the original aqueous phase, while the other undesired proteins/enzymes were moved to the reverse micellar organic phase. The individual and interaction effects of main factors (*i.e.*, pH and NaCl concentration in the aqueous phase, and concentration of sodium *bis*-2-ethyl-hexyl-sulfosuccinate (AOT) in the organic phase) were studied using response surface methodology. The optimum conditions for the maximum recovery of the enzyme were pH 2.75, 100 mM NaCl, and 200 mM AOT. Furthermore, the optimum organic to aqueous volume ratio (V_{org}/V_{aq}) and appropriate number of sequential extraction stages were 2 and 3, respectively. Finally, 60% of the undesired enzymes including proteases and xylanases were removed from the aqueous phase, while 140% of glucoamylase activity was recovered in the aqueous phase and the purification factor of glucoamylase was found to be 3.0-fold.

Keywords: Glucoamylase, solid-state fermentation, purification, reverse micelle, AOT, protein

Introduction

Downstream separation and isolation processes are the most difficult and expensive steps in industrial enzyme productions, which stand for up to 70% of the production costs [35]. Since conventional downstream processing methods have their own limitations, such as being tedious, expensive, not easily scalable, and resulting in lower product yield due to multiple steps of handling, there has been increasing interest to develop efficient and economical downstream processing methods for enzyme separation and purification [13, 19, 35].

Reverse micellar extraction (RME) is a promising technique for the recovery and purification of biomolecules from an aqueous culture, which is a liquid-liquid extraction using water-in-oil microemulsion solution [12]. RME for the separation and purification of proteins is a selective, efficient, energy-saving, and easily scalable process, and has the potential for continuous purification of enzymes [19, 23, 32]. However, the reverse micellar systems are very complicated processes, since several factors, including the nature and concentration of the target enzyme, pH and ionic strength of the aqueous phase, type and concentration of the applied surfactant, processing time, and temperature, affect the performance of the separation [5]. This performance has often been studied with model proteins such as cytochrome *c*, ribonuclease, myoglobin, and bovine serum albumin. However, not much work has been dedicated on the actual system of the fermentation broth using this method [3], and to our knowledge, there are few studies on glucoamylase (GA) purification using RME.

In general, large monomeric and oligomeric proteins such as glucoamylase (48–112 kDa) are rather difficult to be extracted into reverse micelles, since they are likely to be excluded by steric interactions with reverse micelles or irreversibly denatured in the reverse micellar system

owing to electrostatic interactions [13]. The activities and conformation of enzymes might be affected by various factors such as ionic strength, pH, and the type of solvents [23]. Separation of the target enzyme can also be achieved by retaining the target enzyme in the aqueous phase, and transferring the impurities into the reverse micellar organic phase [6, 8]. This strategy may be more advantageous, because of reducing the risk of enzyme denaturation or deactivation. In addition, this procedure eliminates backward extraction and simplifies the extraction process [33]. One of the most important benefits of this method is the possibility of surfactant separation from the contaminant proteins by filtration, and its recirculation without deactivation of the target enzyme [23]. Since solid-state cultures are high complex media (*i.e.*, containing microbial cells, solid substrate particles, and other co-metabolites formed during fermentation), RME could be a convenient process for separating the produced enzymes. However, there are few reports detected in the literature about enzyme purification by transferring the undesired protein from the enzyme solution to the reverse micellar organic phase [24, 26, 27].

Among the industrial enzymes, glucoamylase has a wide application in different industries and corresponds to 25–33% of the world enzyme market, ranking second after proteases [25]. The most important application of glucoamylase includes the production of high glucose syrup and dextrose for confectionary and pharmaceuticals [4, 25].

Conventional methods applied for GA extraction and purification are filtration and HPLC [22], electrophoreses and affinity HPLC [29], precipitation and affinity HPLC [28], leaching and HPLC [37], pulsed column extractor [10], aqueous two-phase systems [31, 34], ultrafiltration and aqueous two-phase systems [35], and affinity precipitation [36]. However, as previously mentioned, most of these conventional methods are tedious, expensive, and not easily scalable.

The current study deals with the purification of glucoamylase produced by solid-state fermentation, using the RME method. Here, undesired proteins were removed into the organic micellar phase, and the glucoamylase was kept in the initial aqueous phase. For this purpose, preliminary experiments were carried out to screen the factors that showed dominant effects on the extraction process. Response surface methodology using central composite rotatable design (CCRD) was then applied to study the effects of the main parameters on the enzyme purification. Furthermore, the effects of volume ratio of the two phases and the number of extraction stages were investigated. Removal of xylanase and protease, as the main undesired enzymes, from the glucoamylase solution was investigated at the optimum recovery conditions.

Materials and Methods

Production of Glucoamylase

Glucoamylase was produced by cultivation of *Aspergillus niger* CCUG 33991 (Culture Collection University of Gothenburg, Sweden) on wheat bran in a pilot-scale solid-state fermentation bioreactor (ICRASN, Iran). Sterilized wheat bran with 50% moisture (4 kg) was loaded to the forcedly aerated intermittently mixed packed-bed bioreactor and inoculated with *A. niger* spores, and then cultivated for 40 h at 35°C. Afterward, the moist fermented bran was dried at 40°C to obtain a moisture content of about 5%, and kept in a refrigerator at 4°C. The produced enzyme was purified from the dried fermented bran (DFB).

Leaching of Glucoamylase

A predetermined amount of DFB was mixed with distilled water in order to obtain 50% moisture for DFB. The moist fermented bran was then mixed with 100 ml of distilled water, on a shaker at 120 rpm and ambient temperature for 100 min. The insoluble residues were removed by filtration (Whatman filter paper, No. 2) and centrifugation (at 684 ×*g* for 10 min). The supernatant was used for the enzyme purification by reverse micellar extraction.

Reverse Micellar Extraction

The reverse micellar organic phase was prepared by dissolving a specific amount of anionic surfactant, AOT, in *n*-heptane as a solvent (to obtain 115.9, 150, 200, 250, and 284.1 mM AOT in organic solution). For preparing the aqueous phase, an appropriate amount of NaCl was added to the enzyme solution (to obtain 15.9, 50, 100, 150, and 184.1 mM NaCl in aqueous solution) and the pH was adjusted using citrate buffer with HCl (1N) or NaOH (1N). Afterward, 4 ml of aqueous phase and 3 ml of organic phase were mixed for 20 min at ambient temperature. It was then centrifuged for 5 min at 1,580 ×*g* to separate the phases. Subsequently, the organic phase was removed, and the protein content and enzyme activity of the aqueous phase were measured. The schematic of RME applied here for GA purification is shown in Fig. 1.

Enzyme Assays

Glucoamylase activity was determined by the amount of glucose released by the action of the enzyme on soluble starch. Samples (0.1 ml) were diluted with distilled water (1.0 ml) and added to 1.0 ml of starch solution (1% in citrate buffer, pH 4.5–4.6) at 60°C for 15 min [17]. The amount of the glucose released after 15 min was then measured using a glucose oxidase kit (Shim-Enzyme, Iran). One unit of glucoamylase activity (GU) was defined as the enzyme quantity that can release 1.0 μmol glucose per minute [17].

The protease assay was performed as described by Ikasari and Mitchell [14]. The proteolytic reaction was performed at 40°C and pH 3 using casein (0.006 mg/ml) as the substrate. One unit of

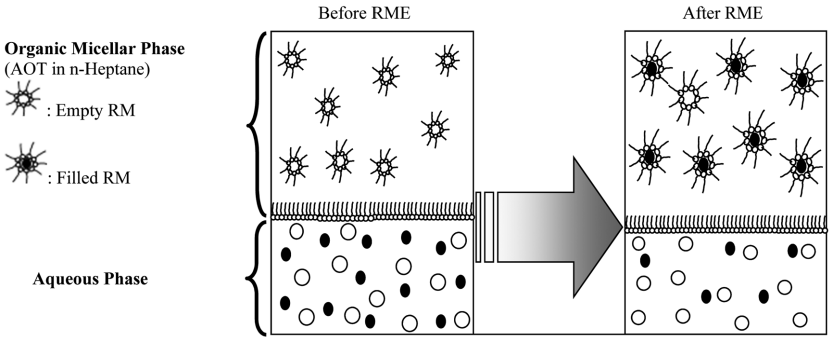

Fig. 1. A schematic of the reverse micellar extraction method applied in the present work for glucoamylase purification.
○: Glucoamylase; ●: Protein.

protease activity (PU) was defined as 1.0 μmol/min of tyrosine equivalents released at the reaction conditions.

The xylanase activity assay was carried out using a colorimetric method [18]. A 0.015 mg/ml azo-xylan solution was prepared in 0.1 M sodium acetate buffer solution at pH 5.0. The xylanase solution and the enzyme control were 25-fold diluted using the same buffer. Azo-xylan solution (1.0 ml) was added to each tube containing 0.1 ml of xylanase solution, vigorously stirred, and incubated for 20 min at 40°C. To suppress the reaction, 0.5 ml of 3,5-dinitrosalicylic acid solution was added to each tube and the tube was shaken for 10 sec in boiling water. Thereafter, the tubes were centrifuged at 1,580 ×*g* for 10 min and the absorbance of their supernatants was measured at 530 nm. One international unit (XU) is the amount of xylanase that can release 1.0 μmol/min xylose equivalents under the assay conditions [18].

Protein Assay

The protein concentration of the aqueous phase was measured by the Lowry method, using bovine serum albumin as the standard [21]. The "specific activity" was calculated as the ratio of glucoamylase activity (GU/ml) to protein mass (mg/ml), whereas the "purification factor" was defined as the ratio of specific activities of glucoamylase in the aqueous phase before and after the extraction [11].

Experimental Design

The experiments were conducted according to CCRD with three factors, each at five levels. The design was generated and analyzed using SAS package (ver. 3, SAS Institute Inc., NC, USA). The NaCl concentration and pH of the aqueous phase, and AOT concentration of the organic phase were selected as the design factors, since they are reported to have dominant effects on RME performance [1, 9]. Values of the factors corresponding to their coded levels are given in Table 1. From our preliminary experiments, the effect of temperature was not significant; therefore, temperature was not considered as a parameter. The experimental design along with corresponding values of the responses is depicted in Table 2. The response function (Y) of the experimental design was the percentage of protein removed to the organic micellar phase. A second-degree polynomial model, based on regression analysis, was fitted to the experimental data of the response variable (Y) as a function of the design factors:

$$Y = B_0 + \sum_i B_i X_i + \sum_i B_{ii} X_i^2 + \sum_i \sum_j B_{ij} X_i X_j \quad (1)$$

where, X_i's stand for design factors according to Table 1. The coefficients B_i, B_{ii}, and B_{ij} represent the linear and quadratic effects of the i-th factor and the cross product effect between the i-th and j-th factors, respectively [7]. B_0 is the value of the fitted response at the center point of the design, point (0, 0, 0), which is known as an intercept point [38]. Values of coefficients along with their significance and standard errors were determined by regression analysis using the SAS package, which also estimated the optimum extraction conditions.

Effects of Organic to Aqueous Phase Volume Ratio and Multistage Extraction on Purification

After the determination and verification of the optimum conditions obtained in the experimental design, the optimum conditions were used for studying the effects of organic to aqueous phase volume ratio (V_{org}/V_{aq}) and multistage extractions. The assessments of V_{org}/V_{aq} were studied at the optimum pH, and

Table 1. Levels of different process variables in coded (X_i) and uncoded (x_i) forms in the experimental design for reverse micellar extraction of glucoamylase by the CCRD method.

Coded levels	Uncoded values of process variables		
	NaCl (mM)	AOT (mM)	pH
X_i	x_1	x_2	x_3
-1.682	15.9	115.9	2.16
-1.000	50	150	2.5
0.000	100	200	3.0
+1.000	150	250	3.5
1.682	184.1	284.1	3.84

Table 2. Experimental designs in coded (X_i) form of process variables and values of experimental data of protein removal, enzyme recovery, and purification factor for the optimization of reverse micellar extraction of glucoamylase using the CCRD method.

Trial kind	Trial No.	Coded variables			Removal of protein (%)	Recovery of GA (%)
		NaCl (X_1)	AOT (X_2)	pH (X_3)		
Core points	1	-1	-1	-1	52	71
	2	-1	-1	1	28	80
	3	-1	1	-1	56	76
	4	-1	1	1	28	90
	5	1	-1	-1	46	81
	6	1	-1	1	20	84
	7	1	1	-1	49	81
	8	1	1	1	21	90
Star-like points	9	-1.682	0	0	50	24
	10	1.682	0	0	38	91
	11	0	-1.682	0	40	101
	12	0	1.682	0	46	101
	13	0	0	-1.682	58	102
	14	0	0	1.682	21	105
Center points	15	0	0	0	53	108
	16	0	0	0	55	110
	17	0	0	0	58	114
	18	0	0	0	58	113
	19	0	0	0	57	116
	20	0	0	0	55	104

AOT: Aerosol OT or Sodium Bis (2-Ethylhexyl) Sulfosuccinate, GA: Glucoamylase.

AOT and NaCl concentrations. After phase separation, the glucoamylase activity and protein content of the aqueous phase were measured.

For investigation of sequential extraction, predetermined volumes of the aqueous phase and organic micellar phase were prepared at the optimum values of pH, and NaCl and AOT concentrations. Proportional volume ratios of these two phases were mixed together. After phase separation at the first stage, the aqueous phase supernatant was exposed to a proper amount of fresh organic micellar phase in the second stage. This method was applied for the subsequent stages. The removal of undesired enzymes (*i.e.*, xylanase and protease) from the aqueous phase was also measured and reported.

Results

Glucoamylase was produced by cultivation of *A. niger* on wheat bran by solid-state fermentation. The fermented wheat bran was mixed with distilled water, and the supernatant was separated from the insoluble residues by filtration and centrifugation. Glucoamylase was purified from the supernatant by reverse micellar extraction.

Effects of NaCl and AOT Concentrations and pH on the Protein Extraction

Fig. 2 shows the contour plots and response surface curves for protein removal from the aqueous phase into the organic micellar phase at 200 mM AOT, 100 mM NaCl, and pH 3.0. Figs. 2A and 2B indicate that more than 62% of total protein could be removed into the organic phase at pH 2.5. However, increasing the pH from 2.5 to 3.8 reduced the protein removal to less than 20%. Furthermore, increasing the NaCl concentration up to 80 mM showed a positive effect on protein removal, while a higher concentration lowered protein removal from more than 60% to less than 40% (Figs. 2A and 2C). On the other hand, the effect of AOT concentration was examined in the range of 120 to 280 mM, and the results showed 210 mM as an optimum (Figs. 2B and 2C).

The results of statistical analysis according to the second-order response model in the form of analysis of variance are given in Table 3. The results indicated that pH and NaCl concentration have significant effects (p-value less than 0.05) on proteins removal, whereas AOT concentration did not significantly affect the extraction ($p > 0.05$). Although significant quadratic effects of the variables were observed, there were no significant interactions observed between the parameters.

Optimum Conditions for Protein Extraction

The mathematical modeling representing protein removal from the aqueous phase during reverse micellar extraction as a function of the most significant variables in coded values of the factors (X_i in Table 1) was given to be

$$\mathbf{Y(\%)} = 56.789 - 3.528\mathbf{X_1} + 1.325\mathbf{X_2} - 12.318\mathbf{X_3} - 5.278\ \mathbf{X_1}^2 - 5.631\ \mathbf{X_2}^2 - 6.869\ \mathbf{X_3}^2 \quad (2)$$

It corresponds to the non-coded factor values (x_i in Table 1) as

$$\mathbf{Y(\%)} = -226.018 + 0.352\ \mathbf{x_1} + 0.927\ \mathbf{x_2} + 140.208\ \mathbf{x_3} - 0.002\mathbf{x_1}^2 - 0.002\mathbf{x_2}^2 - 27.474\ \mathbf{x_3}^2 \quad (3)$$

Based on the second-order regression model, numerical optimization was carried out using the response optimizer

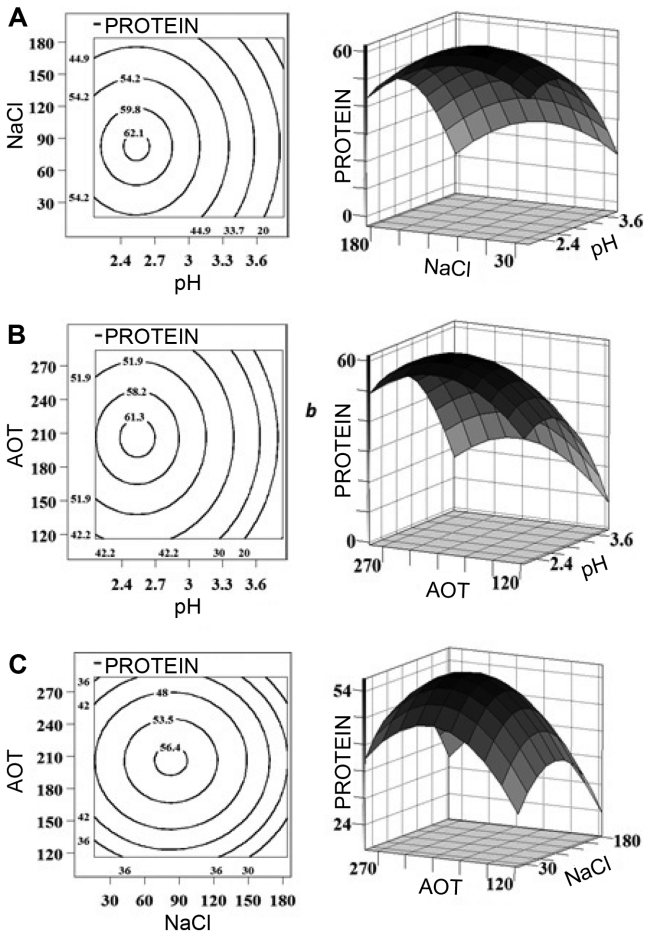

Fig. 2. Contour plots and response surface curves of protein removal *vs*. pH, [NaCl], and [AOT].
(**A**) Protein removal *vs*. pH and [NaCl] at [AOT] = 200 mM. (**B**) Protein removal *vs*. pH and [AOT] at [NaCl] = 100 mM. (**C**) Protein removal *vs*. [AOT] and [NaCl] at pH = 3. Initial GA activity and protein concentration were 10.9 GU/ml and 2.72 mg/ml, respectively. AOT: Aerosol OT or Sodium Bis (2-Ethylhexyl) Sulfosuccinate, GA: Glucoamylase.

in the SAS software. These numerical results showed the optimum pH of 2.5, while experiments show that glucoamylase is significantly deactivated at pH values less than 2.5. Thus, the following point was selected as an appropriate point for enzyme purification: 100 mM NaCl, 200 mM AOT, and pH 2.75. Actual extraction was performed at these conditions and a protein removal of 51% was obtained.

Effect of Organic to Aqueous Phases Volume Ratio

In order to study the effects of the organic to aqueous phases volume ratio (V_{org}/V_{aq}) on glucoamylase purification

Table 3. Analysis of variance for the quadratic model of protein removal using the reverse micellar extraction process.

Source	Degree of freedom	Sum of squares	Mean squares	F-value	*p*-value
NaCl	1	170.0	170.0	19.7	0.001
AOT	1	24.0	24.0	2.8	0.126
pH	1	2,072.2	2072.2	240.6	0.000
NaCl×NaCl	1	367.6	367.6	42.7	0.000
NaCl×AOT	1	0	0	0	1.000
NaCl×pH	1	0.5	0.5	0.06	0.815
AOT×AOT	1	420.8	420.8	48.9	0.000
AOT×pH	1	4.5	4.5	0.5	0.486
pH×pH	1	635.6	635.6	73.8	0.000
Model	9	3,464.8	384.9	44.7	0.000
(Linear)	3	2,266.2	755.4	87.7	0.000
(Quadratic)	3	1,193.7	397.9	46.2	0.000
(Cross product)	3	5.0	1.7	0.2	0.898
Error	10	86.1	8.6		
(Lack of fit)	5	66.1	13.2	3.3	0.108
(Pure error)	5	20	4		
Total	19	3,550.9			
R^2	97.6%				
Adjusted-R^2	95.4%				

using RME, a series of experiments were performed at optimum conditions obtained from the experimental design (*i.e.*, 100 mM NaCl, 200 mM AOT, and pH 2.75). The results are depicted in Fig. 3. Enhancement of the organic to aqueous phase volume ratio had a minor effect on protein removal, while it showed significant improvements on glucoamylase activity. When the V_{org}/V_{aq} was changed from 0.5 to 3, the glucoamylase activity in the aqueous phase increased from 86% to 130% of its initial activity value.

The purification factor was calculated for different values of V_{org}/V_{aq} from 0.5 to 3 (Fig. 3). In volume ratios 0.5, 0.75, 1, 2, and 3, the purification factors were 1.6, 1.8, 2.0, 2.3, and 2.6, respectively. The model predicted that purification factors higher than 2.0 were attainable using V_{org}/V_{aq} above 2.0, whereas a higher ratio of organic to aqueous phase volumes resulted in no significant increase in protein removal. For further experiments, the optimum value chosen for V_{org}/V_{aq} was 2.

Effect of Sequential Extraction

The effects of contact of the aqueous phase supernatant

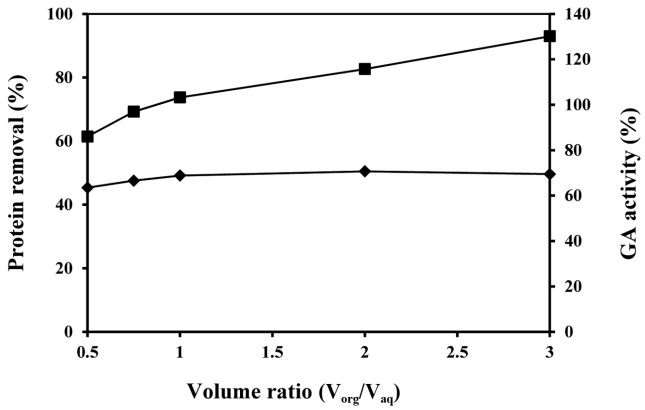

Fig. 3. Effect of organic to aqueous phase volume ratio on the protein removal and enzyme recovery.

The symbols represent glucoamylase (GA) activity (■) and protein removal (◆) at 100 mM NaCl, 200 mM AOT, and pH 2.75. Initial GA activity and protein concentration were 11.6 GU/ml and 2.97 mg/ml, respectively.

containing glucoamylase with the fresh reverse micellar phase from one to four stages were investigated and the results are presented in Fig. 4. In each stage, the volume of the organic phase was twice that of the aqueous phase. The other conditions of these experiments were 100 mM NaCl, 200 mM AOT, and pH 2.75.

The results showed that more extraction stages resulted in higher glucoamylase activity. The activity of the enzyme increased from 107% to 138% (about 30% increment) from stages 1 to 4. However, the protein removal was not significantly affected by increasing the number of extraction stages. Calculation of the glucoamylase purification factor revealed that during the sequential extractions, glucoamylase can be purified more than 3-fold from its initial concentration in the aqueous phase, after 3-stage extraction by the fresh organic micellar phase.

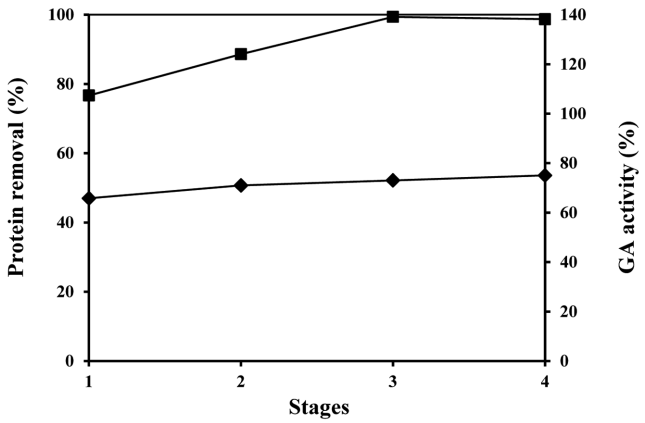

Fig. 4. Effect of sequential extraction on the protein removal and enzyme recovery.

The symbols represent glucoamylase (GA) activity (■) and protein removal (◆) at 100 mM NaCl, 200 mM AOT, pH 2.75, and V_{org}/V_{aq} = 2. Initial GA activity and protein concentration were 11.3 GU/ml and 2.70 mg/ml, respectively.

Effect of Reverse Micellar Extraction on Xylanase and Protease Activity Reduction

In purification processes, it is generally desirable to decrease the amounts of undesired enzymes in the objective enzyme solutions. Thus, the amounts of other enzymes (*i.e.*, xylanases and proteases) that should be removed from the aqueous phase were studied. The activity of the enzymes was determined at the optimum conditions (100 mM NaCl, 200 mM AOT, pH 2.75, V_{org}/V_{aq} as 2), during three stages, and the average results are presented in Fig. 5.

Three-stage extractions increased the glucoamylase activity by a factor of 130%. Furthermore, the activity of xylanase and protease in the aqueous phase before and after each stage demonstrated that more than 60% of these enzymes could be removed by the RME. Moreover, most of these enzymes were removed during the first stage, and further stages had no significant effects on the enzymes removal (Fig. 5).

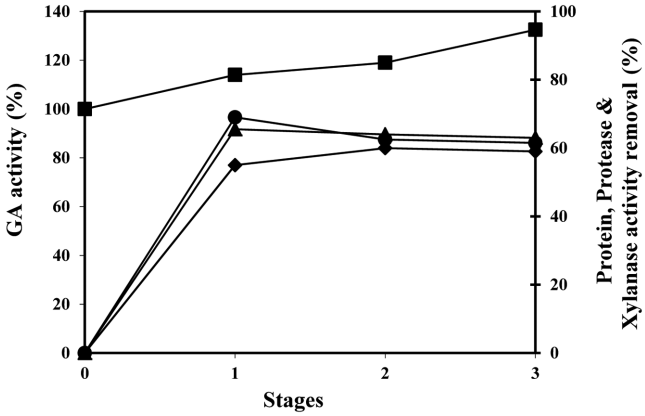

Fig. 5. Removal of xylanase and protease during glucoamylase purification using the reverse micellar process.

The symbols represent glucoamylase (GA) activity (■), protein removal (◆), xylanase removal (●), and protease removal (▲) at 100 mM of NaCl, 200 mM of AOT, pH 2.75, and V_{org}/V_{aq}= 2. Initial GA activity, xylanase activity, protease activity, and protein concentration were 12.9 GU/ml, 120.88 XU/ml, 42.87 PU/ml, and 2.21 mg/ml, respectively.

Discussion

Glucoamylase, one of the most important industrial enzymes, can be successfully purified by reverse micellar extraction from solid-state fermentation by keeping the enzymes in the original aqueous phase and removing the other undesired proteins to the reverse micellar organic phase. the NaCl and AOT concentrations and pH are the effective parameters for removal of contaminant proteins from the aqueous phase in the partial purification of glucoamylase by reverse micellar extraction.

In the current work, pH and NaCl concentration were shown to be the most important factors for the purification of glucoamylase from a solid, state fermentation culture, while the AOT concentration did not significantly affect the purification.

The effect of pH was related to the concentration of positive charges on the protein surface [1, 19]. Furthermore, increasing the ionic strength reduces electrostatic repulsion between the charged head groups of the surfactants in a reverse micelle, and thereby constricts the reverse micelle, which in turn prevents large proteins from extraction [5, 11]. The main driving force of contaminant protein removal from the aqueous phase into the organic micellar phase is electrostatic interaction between the reverse micelles and proteins. However, larger proteins are difficult to be extracted into reverse micelles, and for efficient transfer, a pH much lower than its isoelectric point is required for anionic surfactants such as AOT. Since the glucoamylase isoelectric pH is about 3.6, and it is a large protein (48–112 kDa), high acidic pH is necessary for extraction of this enzyme into AOT-reverse micelles. However, its denaturation at this harsh condition should be considered.

The volume ratio of organic to aqueous phases is a critical parameter in the extraction of enzymes [19, 20]. Increasing the V_{org}/V_{aq} is expected to enhance protein extraction owing to increasing the number of the reverse micelles that can encapsulate the extractable proteins. On the other hand, when more water is absorbed into the micelle core, the aqueous phase will be concentrated and result in higher activity of the enzymes. The current study observations indicated that increasing the V_{org}/V_{aq} led to more decreasing of the aqueous phase volume after phase separation. This could be the reason for increasing the glucoamylase activity by increasing the organic to aqueous phase volume ratio, while no more protein extraction occurred. Water removal into the organic phase in subsequent stages might be the reason for improving the glucoamylase activity. Moreover, it is reported that some of the compounds present in wheat bran act as inhibitors for glucoamylase [16], and selective extraction of these components may be another reason of glucoamylase activity enhancement.

The isoelectric point and molecular mass for xylanases produced by *A. niger* are 9 and 13.5–14 kDa, respectively [2]. Therefore, in the experimental conditions (pH 2.75), these enzyme charges are highly positive and the great electrostatic attraction transfers the xylanases into reverse micelles. Furthermore, the protease molecular mass is lower than 50 kDa and its isoelectric point is close to 4.0 [15]. Therefore, the reason for more than 60% proteases removal from the aqueous phase in this work is due to the electrostatic interactions. The plateau behavior of xylanase and proteases may be attributed to the various kinds of these enzymes in the aqueous phase as well as the electrostatic effects [19, 30]. According to the present study observations, it is highly probable that variation of the pH and ionic strength of the aqueous phase during sequential extraction led to decreasing protein and enzyme positive charges, which resulted in reduction of the electrostatic attraction between protein/enzyme and negative head groups of AOT.

In conclusion, glucoamylase produced by solid-state fermentation was successfully purified using the RME method. The optimum values for pH, and NaCl and AOT concentrations as main effective parameters of purification were 2.75, 100, and 200 mM, respectively. Using the organic to aqueous phase volume ratio of 2 and three stages of extraction at optimum conditions resulted in extraction of more than 50% of contaminating proteins, while 140% of glucoamylase activity was recovered and the enzyme was purified up to 3.0-fold. The proposed extraction procedure has several advantages in terms of time and cost and avoiding activity loss of the target enzyme, compared with traditional RME.

Acknowledgments

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Equation Variables

B_0	Constant value in regression model
B_i	Linear coefficient of X_i in regression model
B_{ii}	Quadratic coefficient of X_i^2 in regression model

B_{ij}	Cross product coefficient of X_i and X_j in regression model
V_{aq}	Volume of aqueous phase
V_{org}	Volume of organic phase
x_i	Uncoded or real value of the main parameter 'i' (i = 1 for NaCl, i = 2 for AOT, and i = 3 for pH)
X_i	Coded value of the main parameter 'i'
Y	Response function of the regression model

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