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1 **Reverse micellar extraction of fungal**
2 **glucoamylase produced in solid state**
3 **fermentation culture**

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1 **ABSTRACT**

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

16
17 **Keywords:** *Glucoamylase; Solid-State Fermentation; Purification; Reverse*
18 *Micelle; AOT; Protein.*

19

1. 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(Tanuja, *et al.*, 2000). Since conventional downstream processing methods have their own limitations, i.e. tedious, expensive, and not easily scalable process and resulting in lower product yield due to multiple steps of handling, there has been an increasing interest to develop efficient and economical downstream processing methods for enzyme separation and purification (Hemavathi, *et al.*, 2010, Krishna, *et al.*, 2002, Tanuja, *et al.*, 2000).

Reverse micellar extraction 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(Hayes and Marchio, 1998). RME for separation and purification of proteins is selective, efficient, energy-saving, and easily scalable process, which has a potential for continuous purification of enzymes (Krishna, *et al.*, 2002, Mathew and Juang, 2007, Rodrigues, *et al.*, 1999). 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 (Chen, *et al.*, 2006).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 actual system of fermentation broth using this method (Bera, *et al.*, 2008), and to our knowledge, there are few studies on glucoamylase (GA) purification using RME.

1 In general, large monomeric and oligomeric proteins such as glucoamylase (48-
2 112 kDa) are rather difficult to be extracted into reverse micelles, since they are
3 likely to be excluded by steric interactions with reverse micelles or irreversibly
4 denatured in the reverse micellar system due to electrostatic interactions
5 (Hemavathi, et al., 2010). The activities and conformation of enzymes might be
6 affected by various factors such as ionic strength, pH, and type of the solvents
7 (Mathew and Juang, 2007). Separation of the target enzyme can also be achieved
8 by retaining target enzyme in the aqueous phase, and transferring the impurities
9 into the reverse micellar organic phase (Cortez, *et al.*, 2004, Ferreira, *et al.*, 2005).
10 This strategy may be more advantageous, because of reducing the risk of enzyme
11 denaturation or deactivation. In addition, this procedure eliminates backward
12 extraction, and simplifies the extraction process (Su and Chiang, 2003). One of
13 the most important benefits of this method is possibility of surfactant separation
14 from the contaminant proteins by filtration and its recirculation without
15 deactivation of the target enzyme(Mathew and Juang, 2007). Since solid-state
16 cultures are high complex media, i.e. containing microbial cells, solid substrate
17 particles, and other co-metabolites formed during fermentation, RME could be a
18 convenient process for separation of the produced enzymes. However, there are
19 quite few reports detected in the literature about the enzyme purification by
20 transferring undesired protein from enzyme solution to reverse micellar organic
21 phase (Nandini and Rastogi, 2010, Noh and Imm, 2005, Noh, *et al.*, 2005).

22 Among the industrial enzymes, glucoamylase has a wide application in different
23 industries and corresponds to 25-33% of the world enzyme market and ranks
24 second after proteases (Nguyen, *et al.*, 2002). The most important application of

1 glucoamylase includes production of high glucose syrup and dextrose for
2 confectionary and pharmaceuticals (Bhatti, *et al.*, 2007, Nguyen, *et al.*, 2002).

3 Conventional methods which applied for GA extraction and purification are
4 filtration and HPLC (Mahajan, *et al.*, 1983), electrophoreses and affinity HPLC
5 (Paszczyński, *et al.*, 1982), precipitation and affinity HPLC (Ono, *et al.*, 1988),
6 leaching and HPLC (Yasuda, *et al.*, 1989), pulsed column extractor (Ghildyal, *et*
7 *al.*, 1991), aqueous two phase systems (Ramadas, *et al.*, 1995, Tanuja, *et al.*,
8 1997), ultrafiltration and aqueous two phase systems (Tanuja, *et al.*, 2000),
9 affinity precipitation (Teotia, *et al.*, 2001). However, as previously mentioned
10 most of these conventional methods are tedious, expensive and not easily scalable.

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

2. Materials and methods

2.1. 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).

2.2. Leaching of glucoamylase

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 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.

2.3. Reverse micellar extraction (RME)

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, 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 aqueous phase and 3

1 ml organic phase were mixed for 20 min at ambient temperature. It was then
2 centrifuged for 5 min at 1580 g to separate the phases. Subsequently, the organic
3 phase was removed, and the protein content and enzyme activity of the aqueous
4 phase were measured. The schematic of RME applied here for GA purification is
5 shown in Fig.1

6 **2.4. Enzymes assays**

7 Glucoamylase activity was determined by the amount of glucose released by the
8 action of enzyme on soluble starch. Samples (0.1 ml) were diluted with distilled
9 water (1.0 ml) and added to 1.0 ml starch solution (1% in citrate buffer with pH
10 4.5-4.6) at 60°C for 15 min (Khanahmadi, 2004). The amount of the glucose
11 released after 15 min was then measured using a glucose-oxidase kit (Shim-
12 Enzyme, Iran). One unit of glucoamylase activity (GU) was defined as the
13 enzyme quantity that can release 1.0 μmol glucose per min (Khanahmadi, 2004).

14 The protease assay was performed as described by Ikarashi and Mitchell (Ikarashi
15 and Mitchell, 1996). The proteolytic reaction was performed at 40°C and pH 3
16 using casein (0.006 mg/ml) as substrate. One unit of protease activity (PU) was
17 defined as 1.0 $\mu\text{mol}/\text{min}$ of tyrosine equivalents released at the reaction
18 conditions.

19 The xylanase activity assay was carried out using a colorimetric method (König,
20 *et al.*, 2002). A 0.015 mg/ml azo-xylan solution was prepared in 0.1 M sodium
21 acetate buffer solution at pH 5.0. Xylanase solution and the enzyme control were
22 25-fold diluted using the same buffer. Azo-xylan solution (1.0 ml) was added to
23 each tube containing 0.1 ml xylanase solution, vigorously stirred, and incubated
24 for 20 min at 40°C. To suppress the reaction, 0.5 ml DNS (3,5-Dinitrosalicylic

1 Acid) solution was added to each tube and the tube was shaken for 10 s in boiling
2 water. Thereafter, the tubes were centrifuged at 1580 g for 10 min and the
3 absorbance of their supernatants was measured at 530 nm. One international unit
4 (XU) is the amount of xylanase that can release 1.0 $\mu\text{mol/min}$ xylose equivalents
5 under the assay conditions (König, et al., 2002).

6 **2.5. Protein assay**

7 Protein concentration of the aqueous phase was measured by Lowry method,
8 using bovine serum albumin as standard (Lowry, *et al.*, 1951). The “specific
9 activity” was calculated as the ratio of glucoamylase activity (GU/ml) to protein
10 mass (mg/ml), whereas the “purification factor” was defined as the ratio of
11 specific activities of glucoamylase in aqueous phase before and after the
12 extraction (Hasmann, *et al.*, 2007).

13 **2.6. Experimental design**

14 The experiments were conducted according to CCRD with three factors, each at
15 five levels. The design was generated and analyzed using SAS package (Ver 3,
16 SAS Institute Inc., NC, USA). NaCl concentration and pH of the aqueous phase,
17 and AOT concentration of the organic phase were selected as the design factors,
18 since they are reported to have dominant effects on RME performance (Andrews,
19 *et al.*, 1994, George and Stuckey, 2010). Values of the factors corresponding to
20 their coded levels are given in Table 1. Due to our preliminary experiments, the
21 effect of temperature was not significant; therefore, temperature was not
22 considered as a parameter. Experimental design along with corresponding values
23 of the responses is depicted in Table 2. The response function (Y) of the
24 experimental design was the percentage of protein removed to the organic
25 micellar phase. A second degree polynomial model, based on regression analysis,

1 was fitted to the experimental data of the response variable (Y) as a function of
2 the design factors:

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

4 where, X_i 's stand for design factors according to Table 1. The coefficients B_i , B_{ii} ,
5 and B_{ij} represent the linear and quadratic effects of i-th factor and the cross
6 product effect between i-th and j-th factors, respectively (Dean and Voss, 1999).
7 B_0 is the value of fitted response at the center point of the design, i.e. point (0, 0,
8 0), which is known as an intercept point (Zivorad, 2004). Values of coefficients
9 along with their significance and standard errors were determined by regression
10 analysis using SAS package, which also estimated the optimum extraction
11 conditions.

12 **2.7 Effects of organic to aqueous phase volume ratio and multistage** 13 **extraction on purification**

14 After determination and verification of the optimum conditions obtained in
15 experimental design, the optimum conditions were used for studying the effects of
16 organic to aqueous phase volume ratio (V_{org}/V_{aq}) and multistage extractions. The
17 assessments of V_{org}/V_{aq} were studied at the optimum pH, AOT and NaCl
18 concentrations. After phase separation, glucoamylase activity and protein content
19 of the aqueous phase were measured.

20 For investigation of sequential extraction, predetermined volumes of aqueous
21 phase and organic micellar phase were prepared at the optimum values of pH,
22 NaCl, and AOT concentrations. Proportional volume ratios of these two phases
23 were mixed together. After phase separation at the first stage, the aqueous phase
24 supernatant was exposed to proper amount of fresh organic micellar phase in the

1 second stage. This method was applied for the subsequent stages. The removal of
2 undesired enzymes (i.e. xylanase and protease) from the aqueous phase was also
3 measured and reported.

4 **3. Results**

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

10 **3.1. Effects of NaCl and AOT concentrations and pH on the protein** 11 **extraction**

12 Fig.2 shows the contour plots and response surface curves for protein removal
13 from aqueous phase into organic micellar phase at 200 mM AOT, 100 mM NaCl,
14 and pH 3.0. Fig. 2a and 2b indicate that more than 62% of total protein could be
15 removed into organic phase at pH 2.5. However, increasing pH from 2.5 to 3.8
16 reduced the protein removal to less than 20%. Furthermore, increasing NaCl
17 concentration up to 80 mM showed a positive effect on protein removal, while
18 higher concentration lowered protein removal from more than 60% to less than
19 40% (Fig.2a & 2c). On the other hand, effect of AOT concentration was examined
20 in range of 120 to 280 mM and the results showed 210 mM as an optimum
21 (Fig.2b & 2c).

22 The results of statistical analysis according to second order response model in the
23 form of ANOVA (Analysis of Variance) are given in Table 3. The results
24 indicated that pH and NaCl concentration have significant effects (p-value less

than 0.05) on proteins removal, whereas AOT concentration was not significantly affected the extraction ($p > 0.05$). While significant quadratic effects of the variables observed, there were no significant interactions observed between the parameters.

3.2. Optimum conditions for protein extraction

The mathematical modeling representing protein removal from 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:

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

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

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

Based on the second order regression model, numerical optimization was carried using response optimizer in 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.

3.3. Effect of organic to aqueous phases volume ratio

In order to study the effects of organic to aqueous phases volume ratio (V_{org}/V_{aq}) on glucoamylase purification using RME, a series of experiments performed at optimum conditions obtained from the experimental design, i.e. 100 mM NaCl,

1 200 mM AOT, and pH 2.75. The results are depicted in Fig.3. Enhancement of
2 organic to aqueous phase volume ratio had a minor effect on protein removal,
3 while it shows significant improvements on glucoamylase activity. When the
4 $V_{\text{org}}/V_{\text{aq}}$ was changed from 0.5 to 3, glucoamylase activity in aqueous phase was
5 increased from 86% to 130% of its initial activity value.

6 The purification factor was calculated for different values of $V_{\text{org}}/V_{\text{aq}}$ from 0.5 to 3
7 (Fig.3). In volume ratios 0.5, 0.75, 1, 2, and 3 purification factors were 1.6, 1.8,
8 2.0, 2.3, and 2.6, respectively. The model predicted that purification factors higher
9 than 2.0 was attainable using $V_{\text{org}}/V_{\text{aq}}$ above 2.0, whereas higher ratio of organic
10 to aqueous phase volumes resulted in no significant increase in protein removal.
11 For further experiments the optimum value chosen for $V_{\text{org}}/V_{\text{aq}}$ was 2.

12 **3.4. Effect of sequential extraction**

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

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

1 **3.5. Effect of reverse micellar extraction on xylanase and protease** 2 **activity reduction**

3 In purification processes, it is generally desirable to decrease the amounts of
4 undesired enzymes in the objective enzyme solutions. Thus, the amounts of other
5 enzymes, i.e. xylanases and proteases that should be removed from the aqueous
6 phase were studied. Thereby, the activity of the enzymes were determined at the
7 optimum conditions (100 mM NaCl, 200 mM AOT, pH 2.75, V_{org}/V_{aq} as 2),
8 during 3-stages and the average results are presented in Fig.5.

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

15 **4. Discussion**

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

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

1 The effect of pH was related to the concentration of positive charges on the
2 protein surface (Andrews, et al., 1994, Krishna, et al., 2002). Furthermore,
3 increasing the ionic strength reduces electrostatic repulsion between the charged
4 head groups of the surfactants in a reverse micelle, thereby constricts reverse
5 micelle, which in turn prevents large proteins from extraction (Chen, et al., 2006,
6 Hasmann, et al., 2007). The main driving force of contaminant protein removal
7 from aqueous phase into organic micellar phase is electrostatic interaction
8 between reverse micelles and proteins. However, larger proteins are difficult to be
9 extracted into reverse micelles and for efficient transfer, a pH much lower than its
10 isoelectric point is required for anionic surfactants i.e. AOT. Since glucoamylase
11 isoelectric pH is about 3.6, and it is a large protein (48-112 kDa), high acidic pH
12 is necessary for extraction of this enzyme into AOT-reverse micelles. However,
13 its denaturation at this harsh condition should be considered.

14 Volume ratio of organic to aqueous phase is a critical parameter in extraction of
15 enzymes (Krishna, et al., 2002, Liu, *et al.*, 2006). Increasing V_{org}/V_{aq} is expected
16 to enhance protein extraction due to increasing the number of the reverse micelles
17 that can encapsulate the extractable proteins. On the other hand, when more water
18 is absorbed into the micelles cores, the aqueous phase will be concentrated and
19 result in higher activity of the enzymes. The current study observations indicated
20 that increasing V_{org}/V_{aq} led to more decreasing of aqueous phase volume after
21 phase separation. This could be the reason for increasing the glucoamylase
22 activity by increasing the organic to aqueous phase volume ratio, while no more
23 protein extraction occurred. Water removal into organic phase in subsequent
24 stages might be the reason for improving the glucoamylase activity. Moreover, it
25 is reported that some of the compounds present in wheat bran act as inhibitors for

1 glucoamylase (Kashimura, 2007) and selective extraction of these components
2 may be another reason of glucoamylase activity enhancement.

3 Isoelectric point and molecular weight for xylanases produced by *A. niger* are 9
4 and 13.5-14 kDa, respectively (Beg, *et al.*, 2001). Therefore, in the experimental
5 conditions (pH 2.75), these enzymes charges are highly positive and the great
6 electrostatic attraction transfers the xylanases into reverse micelles. Furthermore,
7 proteases molecular weight is lower than 50 kDa and its isoelectric point close to
8 4.0 (Kalisz, 1988). Therefore, the reason for more than 60% proteases removal
9 from the aqueous phase in this work is due to the electrostatic interactions. The
10 plateau behavior of xylanase and proteases may be attributed to the various kinds
11 of these enzymes in the aqueous phase as well as the electrostatic effects (Krishna,
12 *et al.*, 2002, Pires, *et al.*, 1996). According to the present study observations, it is
13 highly probable that variation of pH and ionic strength of aqueous phase during
14 sequential extraction, led to decreasing protein and enzyme positive charges
15 which resulted in reduction of electrostatic attraction between protein/enzyme and
16 negative head groups of AOT.

17 **5. Conclusion**

18 Glucoamylase produced by solid-state fermentation was successfully purified
19 using RME method. The optimum values for pH, NaCl and AOT concentrations
20 as main effective parameters of purification were 2.75, 100, and 200 mM,
21 respectively. Using organic to aqueous phase volume ratio of two and three stages
22 of extraction at optimum conditions, resulted in extraction of more than 50% of
23 contaminating proteins, while 140% of glucoamylase activity was recovered and
24 the enzyme purified up to 3.0-fold. The proposed extraction procedure has several

- 1 advantages in terms of time and cost and avoiding activity loss of the target
2 enzyme compared to traditional RME.

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5 Science & Natural Resources and supported by Industrial Biotechnology Group at
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7

1 Nomenclature

Abbreviations

ANOVA	Analysis of variance
AOT	Aerosol-OT or Sodium bis-2-ethylhexylsulphosuccinate
<i>A. niger</i>	<i>Aspergillus niger</i>
BSA	Bovine Serum Albumin
CCRD	Central Composite Rotatable Design
DFB	Dried Fermented Bran
DNS	3,5-Dinitrosalicylic Acid
GA	Glucoamylase
RM	Reverse Micelle
RME	Reverse Micellar Extraction
SAS	Statistical Analysis of System

Units

GU	One unit of glucoamylase activity
kDa	kilo Dalton
PU	One unit of protease activity
XU	One unit of xylanase activity

Letters

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	un-coded 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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Table 1

Levels of different process variables in coded (X_i) and un-coded (x_i) forms in experimental design for reverse micellar extraction of glucoamylase by 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

1 **Table 2**

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

5

Trial kind	Trial No.	Coded Variables				
		NaCl (X_1)	AOT	pH	Removal of	Recovery of
			(X_2)	(X_3)	Protein (%)	GA (%)
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	-	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

6

1 **Table 3**
2 Analysis of variance (ANOVA) for quadratic model of protein removal using reverse micellar
3 extraction process.
4

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	2072.2	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	3464.8	384.9	44.7	0.000
(Linear)	3	2266.2	755.4	87.7	0.000
(Quadratic)	3	1193.7	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	3550.9			
R ²	97.6%				
Adjusted-R ²	95.4%				

Figure legends

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

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.

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.

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.

Fig.5. Removal of xylanase and protease during glucoamylase purification using 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, $V_{org}/V_{aq}=2$. 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.

Fig. 1

b

Fig.2

Fig.3

Fig.4

Fig.5