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REVERSE MICELLAR SEPARATION OF PROTEASE FROM FERMENTATION BROTH

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ABSTRACT

Reverse micellar separation of protease fermentation broth has been reported.

Key words: Reverse micellar separation, Protease, Fermentation broth.

INTRODUCTION

Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. Proteases are hydrolytic enzymes showing a wide variety of industrial applications in the detergent, food, pharmaceutical, diagnostics, waste water management, silver recovery and fine chemicals[1]. The protease enzyme constitutes two-thirds of total enzymes used in various industries. Among these, alkaline protease is of particular interest due to its broad applications in detergent, tanning, and dairy industries[2-4].

Since 50–90% of production cost resides in the purification strategy, depending on the purity of the end product many procedures have been developed for alkaline protease downstream processing[5]. Some of these involve two stages of cell separation from fermentation broth using a separating unit such as a flow through centrifuge and product concentration using vacuum evaporation or ultrafiltration[6]. Traditionally protease has been extracted using various buffers[7] and by using ammonium sulphate precipitation. However, these protocols are quite complex, time consuming and scale up is difficult. Therefore, a simple, as well as cost and time – effective, separation methodology with reasonable purification efficiency is needed. Alternative modern technology of using aqueous two phase system has been employed in the extraction of protease[8]. However the use of such system is limited because of the disadvantage of low yield. In recent years liquid- liquid extraction using reverse micelles is an attractive and very versatile tool for separating biomolecules. This process shows a close similarity to the liquid-liquid traditional extraction process. This technique allows the recovery and concentration of a

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specific protein from a dilute aqueous solution containing other bioproducts. Reverse micellar system consists of aggregates of surfactant molecules containing an inner water core, dispersed in an organic solvent medium. In the reverse micellar extraction process, proteins are transferred from an aqueous feed phase to a reversed micellar phase by forward extraction and subsequently to an aqueous stripping phase by back extraction. Reversed micellar systems have great potential for industrial application, since they provide a favorable environment for protein solubilization in the organic phase with preservation of biological activity. Information on reverse micellar extraction of proteins from model system is rich in literature whereas studies on extraction from the fermentation broth, especially on extraction of proteases is very scanty.

There are already several research groups who are engaged in studying the extractions of protein by reverse micelles. The possibility of utilizing AOT reverse micelles for the extraction of protease from fermentation broth was studied. In all work, the extraction efficiency was found to be low[9,10]. Ionic – surfactants are mostly employed to form the extractive reversed micellar system. The attractive electrostatic interaction between the inner micellar charge wall and the biomolecules drives the biomolecule from the aqueous phase to the polar core of reversed micelles. For protein separation, the activity and extraction efficiency is low. Therefore non-ionic surfactant is used to form reverse micelles to weaken the electrostatic interaction between reversed micelles and proteins. The experiments reported in this work show highest yield and activity of protease from fermentation broth by reversed micellar separation process. This work reports recovery of protease from fermentation broth using triton x 100 reversed micelles in toluene. The common approach of studying one variable at a time, while keeping the others constant within a set of selected values, has a drawback of requiring a large number of experiments and missing the interaction among the variables. An alternative is the use of fractional factorial designs, which require fewer experiments. The design of experiments makes it possible to study the effects of the variables individually and their interactions. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors and search for optimum for desirable process. Keeping the above in view, the RSM was employed in the present work to study the effect of various process variables, i.e. pH, triton x 100 concentration and processing time.

EXPERIMENTAL

Materials and methods

Microorganism, cell growth and enzyme production

Bacillus subtilis is the microorganism used for the production of alkaline protease. The solid state fermentation process[7] was carried out in 250 mL Erlenmeyer flask using 30% wheat bran in 25 mL of 0.1M carbonate/bicarbonate buffer (pH 10.0). Cotton-plugged flasks were autoclaved at 121^{o}C, 1.05 kg\cm^2 for 15 min cooled and inoculated (20%). Flask with inoculated wheat bran was shaken at 200 rpm at 37^{o}C C for 120 h. after fermentation, entire fermenting medium was mixed with 20 mL of 0.01M of sodium bicarbonate and filtered. The filtrate was collected. The filtrate containing alkaline protease was termed as fermentation broth in this study. The contents of the filtrate were harvested and assayed.

Protease extraction and back extraction with reversed micelles

The reversed micellar system was prepared by dissolving triton x 100 in toluene to desired concentration. Equal volume of two solutions, fermentation broth (with pH adjusted to the desired level by the addition of 1N NaOH or 1N HCl) and reversed micellar solution was mixed by shaking for desired period of time. The mixture was then centrifuged at 1000 g for 10 minutes to separate the two phases. The protein\enzyme loaded micellar solution from the forward extraction was mixed with an equal volume of aqueous stripping solution (1M KCl solution adjusted to the desired pH) by shaking for desired time period followed by centrifugation at 1000 g for 10 minutes to obtain distinct phase separation.

Protease assay

Alkaline protease activity was assayed given by Takami *et al.*[11] According to this procedure 0.25 mL of glycine : NaCl : NaOH (50 mM, pH 10.5) buffer was incubated with 2.5 mL of 0.6% casein dissolved in the same buffer at 30◦C until equilibrium was achieved. An aliquot of 0.25 mL of the enzyme solution was added to this mixture and incubated for 20 min. The reaction was stopped by adding 2.5 mL TCA solution (0.11M trichloroacetic acid, 0.22M sodium acetate, and 0.33M acetic acid). After 10 min the entire mixture was centrifuged at 5000 × *g* for 15 min. The supernatant in the amount of 0.5 mL was mixed with 2.5 mL of 0.5M Na_2CO_3 and 0.5 mL of Folin-Ciocalteu's phenol solution and kept for 30 min at room temperature. The optical densities of the solutions were determined with respect to the sample blanks at 660 nm using Bio 100 spectrophotometer. One alkaline protease unit was defined as the enzyme amount that could produce 1 g of tyrosine in 1 min under the defined assay conditions.

Total protein content

The total protein contents of the samples were spectrophometrically determined according to the method described by Lowry et al.[12], with bovine serum albumin as the standard.

Experimental design

Statistically designed experiments are a powerful tool for improving the efficiency of experimentation. Response surface methodology (RSM) is a statistical method that uses quantitative data from an appropriate experiment to determine regression model equations and operating conditions. RSM is a collection of mathematical and statistical techniques for modeling and analysis of problems in which a response of interest is influenced by several variables. A standard RSM design called CCD was applied in this work to study the variables affecting the extraction process. This method is suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments, as well as to analyze the interaction between the parameters.

RSM is an empirical statistical technique employed for multiple regression analysis by

using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously. A full factorial design, which includes all possible factor combinations in each of the factors, is a powerful tool for understanding complex processes for describing factor interactions in multifactor systems. In order to describe the effects of pH of fermentation broth, pH of back aqueous stripping phase, concentration of surfactant (mM), forward contact time and back extraction contact time , batch experiments were conducted based on the central composite design. The coded values of the process parameters were determined by the following equation

$$x_i = \frac{X_i - X_o}{\Delta x} \quad \ldots(1)$$

where x_i - coded value of the i^{th} variable, X_i - uncoded value of the i^{th} test variable and X_0 - uncoded value of the i^{th} test variable at center point. The range and levels of individual variables are given in Table 1. The experimental design is given in Table 2, along with experimental response.

Table 1: Levels of different process variables in coded and uncoded form for extraction of protease

Independent variable	Symbol	Level	Real value
pH of fermentation broth	A	-2.328	4
		-1	5
		0	6
		1	7
		2.328	8
PH of stripping phase	B	-2.328	7
		-1	8
		0	9
		1	10
		2.328	11
Concentration of surfactant (mM)	C	-2.328	8
		-1	10
		0	12
		1	14
		2.328	16

Cont…

Independent variable	Symbol	Level	Real value
Forward time of mixing (minutes)	D	-2.328	5
		-1	10
		0	15
		1	20
		2.328	25
Backward time of mixing (minutes)	E	-2.328	5
		-1	10
		0	15
		1	20
		2.328	25

Table 2: Experimental conditions (Coded values) and observed response values of central composite design

A	B	C	D	E	Forward extraction efficiency (%η)	Forward extraction activity recovery (%)
-1	1	1	-1	1	35.13	54.49
-1	-1	-1	1	-1	22.22	29.29
-1	1	-1	-1	-1	25.51	37.91
1	-1	1	-1	-1	65.19	87.73
-1	-1	-1	-1	1	27.28	29.20
-1	-1	-1	-1	-1	27.28	29.20
-1	-1	1	1	-1	35.17	42.85
1	1	-1	-1	1	55.03	73.91
-1	1	1	-1	-1	35.11	54.50
-1	1	-1	1	-1	25.51	31.74
-1	-1	1	-1	-1	35.17	44.88
1	1	1	-1	-1	**71.27**	87.91
0	0	0	0	0	62.19	67.88
1	1	1	1	1	**71.27**	87.91
0	0	0	-2.3784	0	57.54	2.325
0	2.37841	0	0	0	59.55	64.82
0	0	0	0	0	62.19	67.88

Cont…

A	B	C	D	E	Forward extraction efficiency (%η)	Forward extraction activity recovery (%)
0	0	0	0	2.37841	62.88	67.88
1	1	-1	1	1	55.03	73.91
1	-1	-1	1	-1	64.3	77.02
-1	-1	1	1	1	31.05	42.85
0	0	0	0	0	62.19	67.88
0	0	0	0	0	62.19	67.88
0	0	0	0	0	62.19	67.88
2.37841	0	0	0	0	62.64	90.17
0	0	0	0	0	62.19	67.88
-1	-1	-1	1	1	22.22	29.29
1	-1	-1	-1	-1	65.23	77.02
-1	1	1	1	-1	35.11	54.50
0	0	2.37841	0	0	50.27	55.25
0	-2.3784	0	0	0	50.55	64.72
1	1	-1	-1	-1	55.03	73.91
1	1	1	1	-1	**71.27**	87.91
-2.3784	0	0	0	0	2.63	2.325
0	0	0	2.37841	0	60.19	67.88
1	-1	1	1	-1	64.95	87.73
0	0	0	0	0	62.19	67.88
0	0	0	0	-2.3784	60.19	67.88
-1	1	-1	-1	1	30	37.91
-1	1	1	1	1	35.11	54.50
-1	-1	1	-1	1	32.06	44.88
1	-1	-1	1	1	64.3	77.02
1	-1	-1	-1	1	65.23	77.02
1	1	1	-1	1	**71.24**	87.91
1	1	-1	1	-1	55.03	73.91
0	0	0	0	0	62.19	67.88
-1	1	-1	1	1	30	31.74
1	-1	1	1	1	64.95	87.73
0	0	-2.3784	0	0	16.99	24.94
1	-1	1	-1	1	65.19	87.73

RESULTS AND DISCUSSION

The extraction of enzymes from fermentation broth to reverse micellar phase and back extraction from reverse micellar phase to aqueous phase depends on the parameters namely, pH of fermentation broth, pH of back stripping phase, concentration of surfactant, forward mixing time and backward mixing time. The extraction of protease can be optimized by varying these parameters. The changes in the pH and concentration of surfactant affect the protease recovery from reverse micelles to a greater extent. The experiments performed at pH 4 showed low recovery of both protein and activity. At pH 4 the enzyme was observed to get denatured because of acidic conditions. The highest protein extraction was obtained in pH 7 of broth and surfactant concentration at 14 mM. Simultaneous increases in concentration of surfactant causes increase in extraction of 71%. This direct analysis of the results shows different interactions between the variables studied. Thus, the main effects of the factors and their interactions (pH of fermentation broth, pH of back stripping phase, concentration of surfactant, forward mixing time and backward mixing time) can be better evaluated by statistical analysis.

CONCLUSION

Triton X 100 in toluene reverse micellar system has been successfully applied for the extraction of protease from fermentation broth. The experimental data demonstrate the feasibility of forward extraction of proteins by reverse micelles.its extraction efficiency depends on the choice of the extraction parameters.in our study, the highest protein extracted was 71% under the following conditions: broth pH value of 7.2, 8.6 pH value of stripping phase, and concentration of surfactant of 12.24 mM

This work has resulted in devising a new process with higher efficiency (71%) as well as higher activity of (87%) than reported in literature (47%). The proposed reverse micellar extraction would improve the separation economy protease since, both in the yield and activity recovery from fermentation broth.

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