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Response surface methodology and artificial neural network modelling of an aqueous two-phase system for purification of a recombinant alkaline active xylanase

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ABSTRACT

A two-stage polyethylene glycol (PEG)-phosphate aqueous two-phase system was used for purification of a highly thermostable and alkaline active recombinant xylanase. Response surface methodology (RSM) and artificial neural network (ANN) have been used to develop predictive models for simulation and optimization of purification process. Effects of pH, PEG molecular weight, concentrations of phosphate, PEG and NaCl on the partitioning of the target enzyme and the contaminants were studied using a central composite design of experiments. The best first stage purification was achieved using 6% PEG 6000, 20% phosphate and pH 6. The optimum back extraction stage system consist of 10% phosphate, 10% NaCl, pH 10 and the first stage separation top phase. After the two stage phase separations, about 78% of the original enzyme activity was recovered and the specific activity of the purified enzyme was increased by a factor of 6.7. Also, the aqueous two-phase system was scaled-up 100 times. After back-extraction, the specific activity increased 6.56 times with 72% total yield. A similar design was also used to obtain a training set for ANN. A comparison between the model results and experimental data gave high correlation coefficient (R^2) and showed that both models were able to predict the partitioning behavior. The results demonstrated a higher prediction accuracy of ANN compared to RSM. This superiority of ANN over other multi factorial approaches could make this estimation technique a very helpful tool for purification process.

1. Introduction

Xylanases, the enzymes that degrade the backbone of the major hemicellulose xylan have been studied for various applications. One of the main interests in xylanases is their application in pulp and paper production where the use of these enzymes prior to bleaching process substantially reduce the use of bleaching chemicals and improve the pulp quality [1]. Studies revealed that most of xylanases obtained from vast array of organisms are optimally active in acidic to neutral pH range and below 60 °C [2–6]. Only a very small percentage of these xylanases are found to be optimally active at elevated temperature [7–10] or alkaline conditions [11,12]. For the majority of the existing xylanases, application in Kraft pulp processing must be preceded by cooling and pH re-adjustment of the alkaline cooked pulp. Alternatively, the use of alkaline active xylanases is expected to allow direct enzymatic treatment of the alkaline pulp and avoids the cost incurring and time consuming steps of pH re-adjustment. In particular, alkaline xylanases which are operationally stable at higher temperature are more beneficial because of savings in cooling cost and time; however, such xylanases are very scarce [13] and hence the search for novel xylanases for pulp and paper industries has continued. As part of this global effort, we isolated an organism, *Bacillus halodurans* that produces a xylanase which is among the few that are most active and stable at high temperature and pH [1]. This enzyme has great potential for Kraft pulp treatment and other applications that need hydrolysis of xylan at higher pH where solubility of xylan is better. The gene encoding this xylanase has been cloned and expressed in one of the most used bacterial expression host, *Escherichia coli* BL21(DE3) [14–16]. This has also been revealed in the expression profile of the recombinant xylanase from the *B. halodurans* [16,17]. Thus, recovery of the recombinant xylanase

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released to the medium will significantly increase the total recoverable expressed protein. However, the enzyme recovery from the medium is not simple.

The use of the two most frequently used chromatography techniques for recombinant enzymes, affinity and ion exchange chromatography, requires sample pre-treatment step to facilitate the capture of the target protein. The commonly used media such as LB contains yeast extract and salt. Thus, if ion exchange chromatography is an option, the culture medium requires a desalting step. On the other hand, direct use of metal affinity chromatography is impossible as yeast extract is known to contain cysteine rich proteins known as metallothioneins (MT) or other low molecular proteins that are capable of binding to metal ions such as copper [18]. However, as the volume of liquid containing the target protein is large, sample treatment will be expensive. Thus, the use of an alternative method that skips the sample pre-treatment step is beneficial. The use of aqueous two-phase system (ATPS) could be an attractive primary recovery alternative.

ATPS has been used in different process for recovery and purification of biological products [19–22]. It provides a mild and non-denaturing environment for biomolecules, and is easy to scale-up. This would be interesting and suitable from the viewpoint of biotechnology. Often, it involves one or more extraction stages. In the first stage, the target product migrates to one phase, while most of contaminants migrate to the other phase. A further processing of the target phase is necessary to separate the product from the bulk materials. Thus, in the case of a two-stage polymer-salt ATPS process, the first extraction stage eliminates the bottom phase contaminants from the feedstock and result in a top phase enriched in the target product. In the second extraction stage (back extraction), the product of interest is partitioned to a bottom salt-rich phase. Depending on the need, the back extracted product can be further purified, for example using ultrafiltration [23]. As substantial amount of the contaminants are removed during the optimized two-stage ATPS extraction and the product is more concentrated, the subsequent purification can be both simple and cheap.

Several factors such as pH, PEG molecular weight and concentration, phase forming salt concentration and type, sodium chloride concentration and biomolecular properties can strongly affected the partitioning behaviour of biomolecules in aqueous two-phase systems. These factors are inherent to the system (e.g., choice of the system components, polymer molecular weight, concentration of polymers and salts, ionic composition and strength and pH), and to the protein (molecular weight, size, shape, charge, hydrophobicity and conformation) [19]. The mechanism governing the partitioning of biomolecules in ATPS is inadequately understood and this restricts the predictive design of extraction processes. Thus, the success of the purification requires a thorough optimization. Simulation of the extraction processes using conventional mathematical modeling is difficult and time-consuming. Recently, Response surface methodology and artificial neural network have been used as a powerful and reliable modeling tool owing to its simplicity and high prediction power in various chemical engineering fields such as modeling of the Partition Coefficients of Biomolecules in Polymer–Polymer Aqueous Two-Phase Systems [24,25]. Experimental design is a convenient method to study the effect of large number of factors and determine the significant effects that the factors may have on the response of interest. To set up a design, the input of each factor and its level as well as all the responses are required. ANNs are direct inspiration from human brain that the complex information is correlated using billions of processing units that is called neurons. It has attracted increasing attention in recent years, particularly for process modeling [24]. Artificial neural network has a high ability for learning and organizing nonlinear and complicated functions. The basic ANN architecture consists of an input layer (independent variables), a number of hidden layers and an output layer (dependent variables). Each of these layers consists of a number of inter-connected neurons [25]. The input layer receives information from external sources and passes this information to the hidden layer for processing. Before entering the hidden layer, the input values are weighted individually. The hidden layer then does all the data processing and produces output based on the sum of the weighted values from the input layer modified by a sigmoid transfer function. ANN was applied here to provide a nonlinear mapping between input variables and the output variables. Regression-based response surface models require the order of the model to be stated (i.e., second, or third order), while ANN tends to implicitly match the input vectors to the output vectors.

This paper describes the optimization of conditions to achieve a higher yield and purity of the recombinant xylanase from *B. halodurans*, and the extraction carried out was followed by the back extraction of the product into a new phosphate phase and recycling of the top phase. It also demonstrates the effects of studied parameters and their interactions on the xylanase partitioning using Response surface methodology (RSM) and artificial neural network (ANN).

The results of this work may provide valuable information on using ATPS to recover cell free products from *E. coli* culture media and also for optimization of their purification process.

2. Materials and methods

2.1. Materials

Polyethylene glycols (PEG) with average molecular weights of 1000, 3000, and 6000, sodium di-hydrogen phosphate and di-potassium hydrogen phosphate were obtained from Merck (KgaA, Darmstadt, Germany). PEG 400, PEG 8000, Ampicillin, birchwood xylan, bicinchoninic acid (BCA) assay reagents and bovine serum albumin were purchased from Sigma (St., Louis, MO., USA). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Saveen Warner AB (Sweden). All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Production of recombinant B. halodurans xylanase

A single colony of *E. coli* BL21(DE3) harbouring recombinant gene encoding the xylanase from *B. halodurans* S7 cloned in pET-22b(+) plasmid was grown overnight at 37 °C and used to inoculate 10 ml Luria-Bertani (LB) broth containing ampicillin (100 μg/ml). After overnight cultivation at 37 °C, 5 ml of the culture was transferred to a one litter conical flask containing 250 ml of the medium. The culture was incubated at 37 °C with shaking at 200 rpm in an orbital shaker incubator. When the optical density (OD) of the culture at 600 nm was about 0.8, IPTG was added to a final concentration of 1 mM. After 18 h of cultivation, cells and debris were removed from the culture medium by centrifugation at 10 000 × *g* in a Sorvall RC-5B centrifuge for 20 min, and the clear supernatant was used as the xylanase source.

2.2.2. Aqueous two-phase systems

PEG-phosphate ATP systems were prepared by mixing appropriate amounts of PEG and concentrated (40% (w/w)) phosphate solution at required pH, followed by 2 ml of enzyme solution, containing 21.14 mg protein/ml enzyme solution, in 15 ml graduated tubes. The final weight of the system was adjusted to 10 g by addition of Millipore quality water. The pH of the phosphate solution was adjusted by mixing required amounts of di-potassium hydrogen phosphate and sodium di-hydrogen phosphate stock solutions. PEG 400 was used as available in the liquid form while PEG 1000 was used as a 70% (w/w) stock solution and PEG 3000, 6000 and