EXTRACTION OF ENDO-PECTINASE ACTIVITY FROM THE CULTURE FILTRATE OF Polyporus squamosus BY AQUEOUS TWO-PHASE SYSTEMS COMPOSED OF LOW MOLECULAR MASS POLYETHYLENE GLYCOL AND PHOSPHATE SALT

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Separation of endo-pectinase activity from the culture filtrate of Polyporus squamosus - strain MMOL76, by aqueous two-phase partitioning technique in polyethylene glycol/sodium dihydrogen phosphate system was investigated as the first operation in the downstream processing of enzyme. The best results concerning the partitioning coefficient and the top phase yield were achieved in the polyethylene glycol 400/sodium dihydrogen phosphate system, at the tie-line length 78.9 % at pH 3.8 (K = 8), with a 90% yield.

KEYWORDS: Endo-pectinase activity; Polyporus squamosus; aqueous two-phase system (ATP-system)

INTRODUCTION

Discovery and application of novel biotechnological processes for the production of mycelia biomass of edible mushroom, means not only the step forward in increasing the yield or in obtaining the biomass product with standard and improved characteristics, but contributes also to the expansion in the food and pharmaceutical industry. Some time ago, a novel fermentation process has been developed in order to obtain simultaneously the mycelia biomass of Polyporus squamosus - strain MMOL76 with an antioxidant potential and a dietary fiber and pectinases (1). During this process, P. squamosus produced endo- and exo–pectinases (endo-p and exo-p) in a homogeneous medium (1) and in aqueous two-phase systems (ATP-systems) in a submerged culture, using sugar beet pulp as a carbon source (2). This fungus produces an abundant endo-p activity, which is the major active component of complex pectinases (3,4). This enzymatic activity activity has a crucial role in obtaining the intact cells. The basis of most manufacturing processes for functional food production are intact cells of plant tissues.
The preparation of baby food and functional food requires maceration, i.e. separation, of whole cells. This treatment keeps the cells intact, vitamins, color and aroma are preserved, and cell contents are protected from oxygen. For these applications, those enzyme preparations are preferred that contain mainly endo-pectinase activity and are free from other pectinases (5). This is achieved either by purifying the commercial pectinases, which are almost exclusively derived from Aspergillus sp., or by using strains that synthesize only endo-p (but not other pectinases), such as P. squamosus. Since the applications of pectinases in various fields are widening, it is important to achieve an economical and cheap separation from the fermentation broth. For the past few years, vigorous research has been carried out on isolation and characterization of different enzymes, but reports on the use of aqueous two-phase systems (ATP-systems) for partitioning of endo-p are not available.

Effective separation of extracellular endo-p from the fermentation broth is an important technique in the industrial production of enzymes. The first operations for the downstream processing of endo-p from a complex culture medium are filtration and microfiltration. ATP-systems could be a good alternative to the first step of downstream processing of endo-p.

Extraction in ATP-systems is now attractive as an industrial unit operation for the downstream processing of enzymes and other biologically active proteins. Only polyethylene glycol (PEG)/salt systems are reported to be in industrial use today (6). In such a system, PEG and certain salts (phosphate, sulphate) are needed in moderate concentrations to accomplish phase formation (7).

The distribution of an enzyme in such systems can be described with the partition (K) and distribution (G) coefficient (8). During the process optimization, one has to compromise between activities and yield of the target enzyme, the compromise being dependent on K. The partition coefficient, K, is governed by several factors, some of which can be related to the system parameters and others to the target protein enzyme (9). Enzyme extraction by ATP-systems offers several advantages, such as high product yield and high potential for industrial application, when compared with conventional processes. Economically, ATP-systems have certain advantages especially in respect to cost effectiveness, and their performance can be controlled and optimized by varying the solution conditions. In addition, being a liquid-liquid extraction type operation, these systems can also be readily scaled up. However, few attempts of separating the pectinases by using ATP-systems have been reported in the literature (10-12). Therefore, it is of great interest to develop a separation process for pectinases as an industrial unit operation based on the ATP-system technique.

The basic aim of this study was to optimize the ATP-systems as a potential industrial unit operation, and as the first purification step for the downstream processing of endo-p, since such systems allow removal of several contaminants by a simple and economic process. Culture filtrate contains hyphen cells and their fragments, different degradable fragments produced from carbon source and other enzymatic activities, such as cellulase, xylanase, etc. The aim of the present study was to select those ATP-systems that may replace conventional microfiltration step in the manufacturing of extracellular endo-p from culture filtrate. The partitioning of endo-p from the culture filtrate of P. squamosus into the PEG/ sodium dihydrogen phosphate ATP-system was investigated, and the influence of polymer molecular mass (Mr) and the pH of solution on partitioning and distribution coefficients
was examined, with the objective of finding a suitable system for the separation of endo-p into the top phase.

EXPERIMENTAL

Microorganism

Polyporus squamosus - strain MMOL76, obtained from the Research Institute NPO “Biotechnology” Moscow, Russia, was stored on Saburaud maltose agar slant at 4°C. The microorganism, washed from the slant surface with 10 ml sterile water, was inoculated into a 300 ml shake flask containing 100 ml of liquid sugar beet pulp medium. The culture was cultivated at 28°C for 48 h in a shaker, at 200 rpm. The first vegetative generation obtained in this way was used as inoculum.

Media and fermentation procedure

The medium for \textit{P. squamosus} growth was prepared by using sugar beet pulp as a carbon source. The liquid sugar beet pulp medium contained: 0.2\% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 1.5\% sugar beet pulp, and 0.15 mol/l KH\textsubscript{2}PO\textsubscript{4}. Sterilization was accomplished for 30 min at 120°C. Initial pH, before sterilization, was 4.5. Shake flasks experiments (100 ml in 300 ml flasks, inoculated with 2.5\% of inoculum) were carried out at 28°C.

Determination of protein concentration

Concentration of protein was determined according to Lowry \textit{et al}. (13).

Preparation of ATP-systems

Phase systems were constructed according to Antov and Perićin (12), by mixing thoroughly PEG (molecular masses: PEG 400, PEG 600, PEG 1500, and PEG 4000) and sodium dihydrogen phosphate salt with the culture filtrate of \textit{P. squamosus}. The mixture was equilibrated at room temperature and mixed for 5 minutes on a shaker. The total mass of the two-phase system was 10 g. The two phases were allowed to separate for 12 h before sampling, and then the upper phase was carefully removed with a pipette, leaving a small amount at the interface. The lower phase was then sampled through the interface. Samples of each phase were analyzed for enzyme activities.

Enzyme assays

The endo-p activity was determined by measuring the decrease of the specific viscosity of the reaction mixture using 0.25\% apple pectin in 0.1 M citrate buffer, pH 4.5, according to Perićin \textit{et al}. (2). One unit was defined as the amount of enzyme that reduced the initial specific viscosity of the reaction mixture by 20\% in 1 min.

The partition coefficients for endo-p in the ATP-systems were defined as:
According to Pan et al. (8), the distribution coefficient \( G \) gives the ratio of the amount of enzyme in the top and in the bottom phase as:

\[
G = R_v K
\]

where \( R_v \) is the volume ratio of \( V_t \) and \( V_b \); \( V_t \) and \( V_b \) are volumes of the top and bottom phase, respectively. The yield in the top phase was defined by Pen et al.

\[
Y_t(\%) = \frac{100 \left( \sqrt{K} \frac{V_t}{V_b} \right)}{1 + \sqrt{K} \frac{V_t}{V_b}}
\]

The tie-line length (TLL) was defined according to Furuya et al. (14) as:

\[
Tie \text{- line length} = \left[ \left( w_{TOP}^{TOP} - w_{BOT}^{BOT} \right)^2 + \left( w_{TOP}^{TOP} - w_{BOT}^{BOT} \right)^2 \right]^{1/2}
\]

where \( w_{TOP}^{TOP} \) and \( w_{BOT}^{BOT} \) represent the weight percentages of phase-forming components in the top and bottom phases, respectively.

The results are the mean value of at least three measurements of activity (the accuracy is considered to be ± 5%) on a minimum of three replicates for every partition experimental point.

Miscellaneous

The concentration of polyethylene glycol was measured by the method of Skoog (15).

RESULTS AND DISCUSSION

Investigation and optimization of a suitable system for the separation of endo-p into the top PEG-rich phase from the culture filtrate of mushroom \( P. squamosus \) included: (i) Investigation of the effect of molecular mass of PEG on construction of phase diagrams; (ii) Effect of molecular mass of PEG on partitioning of endo-p; (iii) Optimization of the suitable ATP-system PEG 400/sodium dihydrogen phosphate for partitioning of endo-p into the top phase – the PEG-rich phase.

Investigation of the effect of molecular mass of PEG on the construction of phase diagrams

The selection of the molecular mass of PEG, employed in the ATP-systems, is one of the key points of this technique. To ensure the efficiency of extraction, four different mo-
lecular masses of PEG were applied for this purpose. The phase diagram data for any aque-ous two-phase extraction process are needed. The phase diagrams (binodial curves) of the systems made of different molecular masses of PEG (400, 600, 1500 and 4000) and sodium phosphate were determined as shown in Figure 1. The shape and position of binodial curves are dependent on the molecular mass of PEG. The binodial curves of low molecular mass PEG (400 and 600) are formed at a higher level in the phase diagram, and two phases are formed at high polymer and sodium phosphate concentrations. The binodial curves of higher molecular mass PEG (1500 and 4000) are formed at a lower level of phase diagram, with a lower concentration of the polymers and salt. Phase composition indicated by point A in Figure 1 (25% PEG; 20% NaH₂PO₄), represents the ATP-system used to study the effect of molecular mass of PEG on partitioning of endo-p from the culture filtrate of *P. squamosus*.

**Fig. 1.** Binodial curves for the two-phase PEG/phosphate systems. Phase composition indicated by point A (25% PEG, 20% NaH₂PO₄), represents the ATP-system used for the study of the effect of molecular mass of PEG on partitioning of endo-p from the culture filtrate of *P. squamosus*

**Effect of the PEG molecular mass on partitioning of endo-p**

Investigation of the effect of PEG molecular mass on the partitioning of endo-p into the PEG-rich top phase was carried out in two types of PEG/sodium dihydrogen phosphate ATP-systems. In the first ATP-system, the pH was the same as in the culture filtrate pH (pH = 3.8) and in the second, the pH was kept at the optimum of endo-p activity (pH = 5.0).

The values of partitioning coefficients as a function of the molecular mass of PEG are given in Figures 2. It shows the partitioning behavior of endo-p at pH 3.8 and pH 5.0. It was found that in both types of ATP-systems, the enzymatic activity showed a strong tendency to concentrate in the upper PEG-rich phase of low molecular mass PEG (400 and 600). This implied that the enzyme was strongly hydrophobic, since the hydrophobicity of the top PEG-rich phase is higher than that of the bottom salt-rich phase. A similar behavior was observed by Lima *et al.* (16) in the partitioning of a commercial pec-
tinase (Pectinex-3XL). Lima and coworkers have shown that most of the pectinase activity present in the commercial enzyme preparation had a higher affinity for the PEG-rich phase, especially when PEG of low molecular mass was utilized.

![Fig. 2. The influence of the molecular mass of PEG (Mr) on the partitioning coefficient (K) of endo-p from the culture filtrate of P.squamosus. The phase ratio of ATP-systems is approximately 1.1 at 25°C.](image)

On the other hand, increasing of molecular mass of PEG, decreases the K of endo-p; the enzyme is concentrated in the bottom salt-rich phase. This phenomenon can be attributed to the so-called excluded volume mechanism, where a large volume occupied by PEG molecules squeezes proteins out of the solution into the salt-rich bottom phase. The results of our study agree with those of many authors that have demonstrated that an increase in the molecular mass of phase forming polymer, in this instance PEG, generally leads to a decrease in K (7,9,17).

**Optimisation of the suitable PEG 400/sodium dihydrogen phosphate ATP-system**

The best result for the partitioning of endo-p into the top PEG-rich phase was obtained with PEG 400/phosphate ATP-systems, when pH was 5.0; the coefficient K for endo-p was 5.14. We have evaluated the effect of the TLL on the partitioning coefficient of endo-p in ATP-systems at pH 3.8, which was the pH of the filtrate. The results are shown in Figures 3 and 4. The increase in the length of the tie-line favored the partitioning of endo-p into the top phase, and was characterized by the increase in the partitioning coefficient to K = 8.0.

These results are in agreement with the findings of Wu et al. (11). Our experimental results indicate that the application of the PEG 400/sodium dihydrogen phosphate system (in a volume ratio 1:1 and tie-line 78.9 % (w/w)) is optimal for the recovery of endo-p in the top phase at pH 3.8.

The partitioning behavior is also influenced by the enzymes net charge. The isoelectric points (pI) of endo-p from P. squamosus is 3.0 (unpublished data), so that the net charge of endo-p in the ATP-systems used (pH 3.8, 5.0, and 6.0) should be negative. The highest
value for $K$ was 8.00 at pH 6.0 (Figure 5). Since the pI was lower than the pH of ATP-systems it was found in all types of the investigated ATP-systems that the enzymes showed a strong tendency to concentrate in the PEG-rich phase at low molecular mass PEG. These results were in agreement with the findings of Kula (7). According this author the charge effects are also involved and an increased negative net charge ($pI < pH$ of the systems) has been shown to favor the partitioning into the PEG-rich top phase.

![Fig. 3. Phase diagram for the PEG 400/phosphate system and three working tie-lines for ATP-systems. The phase ratio of ATP-systems is approximately 1:1](image)

On the other hand, increase in the pH of ATP-systems (from 3.8 to 6.0), increases the yield of endo-p and slightly decreases the yield of proteins (Figure 6).

It is probable that endo-p partitioning in ATP-systems (PEG/sodium dihydrogen phosphate) is a complex function of a number of different factors, including, pH, tie-line length, endo-p size, its surface properties, and the net charge.

![Fig. 4. The effect of the tie-line length (TLL) on the partitioning coefficient of endo-p in the PEG 400/phosphate system at 25°C and pH 3.8. The phase ratio of the ATP-system is approximately 1:1](image)
Fig. 5. Influence of pH on the partitioning coefficient of endo-\textit{p} at 25°C in the top phase of the PEG-400/phosphate system, with the tie-length of 51% (w/w). The phase ratio of the ATP-system is approximately 1:1.

Fig. 6. Influence of pH on the yield of endo-\textit{p} and proteins in the top phase of the PEG-400/phosphate system at 25°C, with a tie-length 51% (w/w). The phase ratio of ATPS is approximately 1:1. (●) Ye : (■) Yp

CONCLUSIONS

The partitioning of endo-pectinase from \textit{P. squamosus} from the culture filtrates in PEG/sodium dihydrogen phosphate ATP-systems ($R_v = 1:1$) was investigated, and the influencing factors such as PEG molecular mass, the tie-line length, and pH were examined. Endo-\textit{p} activity and total proteins in the culture filtrate were predominantly distributed in the top
phase of PEG 400/sodium dihydrogen phosphate, and the majority of the partitioning coefficient data for endo-p were higher than 3. Based on the results presented in this study, it can be concluded that the optimised PEG 400/sodium dihydrogen phosphate systems for the separation of endo-p looks promising for the application in downstream processing as an industrial unit operation substituting micro-filtration.

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ЕКСТРАКЦИЈА АКТИВНОСТИ ЕНДО-ПОЛИГАЛАКТУРОНАЗЕ ИЗ ФИЛТРАТА ФЕРМЕНТАЦИОНЕ ЧОРБЕ ГЉИВЕ *Polyporus squamosus* У ВОДЕНОМ ДВОФАЗНОМ СИСТЕМУ: ПОЛИЕТИЛЕН-ГЛИКОЛ МАЛИХ МОЛСКИХ МАСА/НАТРИЈУМ ДИХИДРОГЕН ФОСФАТ

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Издвајање ендо-полигалактуназе из филтрационе јестиве гљиве *Polyporus squamosus* - соj MMOL76, помоћу воденог двофазног система полиетилен гликол/натријум дихидроген фосфат, представља прву фазу "down-stream" процесне технике. Најбољи резултати коефицијента расподеле и приноса у горњој фази су постигнути применом двофазног система полиетилен гликол 400/натријум дихидроген фосфата на дужини везне линије 78.92% при pH 3.8 (K = 8) са приносом од 90%.

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