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A N I N T E R N A T I O N A L J O U R N A L

Recovery of Ascorbic Oxidoreductase from Crude Extract with an Aqueous Two-phase System in a Perforated Rotating Disc Contactor

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ABSTRACT

A continuous perforated rotating disc contactor was used to extract the enzyme ascorbic oxidoreductase (E.C.1.10.3.3) from crude extract of Curcubita maxima *with an aqueous two-phase system of poly (ethylene glycol) and phosphate salts. The effect of dispersed phase velocity on either protein mass transfer coefficients or separation efficiency at 1, 2 and 3 mL/min was studied. An increase of the mass transfer coefficients was observed with the dispersed phase velocity, while the separation efficiency showed a small decrease with the increase of this parameter. The experimental results obtained during continuous extraction showed that the ascorbic oxidoreductase activity was partitioned preferentially into the salt-rich phase in all conditions studied. The best recovery of enzyme activity was 236%, with a purification factor of 34 in flow rates of 1 mL/min for dispersed phase.*

Key words: Efficiency, mass transfer coefficient, Perforated Rotating Disc contactor, aqueous two-phase system, ascorbic oxidoreductase

INTRODUCTION

The extraction and purification of biological products is an important area in biotechnological process. Among the techniques used for protein recovery and purification, liquid-liquid extraction using aqueous two-phase systems (ATPS) offers the advantages of gentle environment, favorable processing time and easy scale-up (Albertsson, 1986; Oliveira et al., 2002).

Methods of conventional extraction, such as spray column, can be conveniently applied to aqueous two-phase systems (Sawant and Sikdar, 1990; Jafarabad et al., 1992a and b; Pawar et al., 1997). Another tool that has been recently successfully applied for liquid-liquid extraction with aqueous two-phase systems is the perforated rotating disc contactor (PRDC). This type of extraction apparatus shows greater efficiency and better operational flexibility than the more conventional types.

In this extraction column, which has a rotating element, a better contact between the two phases can be achieved (Vermijs and Kramers, 1954). Porto et al. (1997, 2000) have reported the extraction of cytochrome b5 in a PRDC using

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PEG-phosphate aqueous two-phase systems. More recently, Sarubbo et al. (2003) have shown the characterization of Bovine Serum Albumin mass transfer in a PRDC using a polymer-polymer aqueous two-phase system. However, no data about continuous extraction of enzyme using a PRDC have been found in literature.

This work describes the recovery of ascorbic oxidoreductase (E.C.1.10.3.3) from crude extract of *Curcubita maxima* with ATPS using a laboratory PRDC. Protein transfer and separation efficiency related to operational conditions as well as enzyme purification factor and enzyme activity recovery are discussed.

MATERIALS AND METHODS

Materials

Ascorbic oxidoreductase (E.C.1.10.3.3) from crude extract of *Curcubita maxima* L. was used. This enzyme has a molecular weight of 150 KDa. The enzyme was extracted following the procedure of Carvalho et al. (1981). Poly (ethylene glycol) (PEG) 550 was obtained from Sigma Chemical Co (St. Louis, Mo, USA). All other chemicals were analytical grade.

Experimental apparatus

The perforated rotating disc contactor was made of Perspex tube 32-mm internal diameter and 160-mm high. Three perforated discs equally separated were mounted on a central shaft, which was rotated at 220 rpm. The perforated discs were 30 mm in diameter and drilled with six holes of 1.5-mm diameter (disc free area for flow 20%). the column was maintained at room temperature (25º C±2).

Preparation of phase systems

Phase systems (600g) were prepared by weighing quantities of stock solutions of the polymer in water, 50% (w/w) PEG and appropriate quantities of phosphate salts in order to obtain a tie-line length of 16.7% (w/w) PEG 550 and 14.8% (w/w) phosphate salts (pH 6.0). The mixture of the components was stirred for 1 hour to equilibrate. The final concentration of the crude extract of ascorbic oxidoreductase in the PEG-rich phase was 0.06 mg/ml.

Experimental procedure

Mass transfer coefficients, separation efficiencies, enzyme activities recovery and purification factors were measured in a perforated rotating disc contactor (PRDC) for different dispersed phase velocities. The column was operated in a continuous mode. The flow rates of dispersed, continuous, raffinated and extracted phases were maintained constant by using two multi channel peristaltic pumps with a flow of 2.0 ml/min for the continuous phase while the dispersed phase velocity varied for values of 1.0, 2.0 and 3.0 ml/min. Samples were collected from the extracted and raffinated phases at 10, 20, 30, 40, 50, 60 and 70 minutes. The partition experiments were performed at room temperature (25°C ± 2).

Enzyme activity

Total ascorbic oxidoreductase activity in both phases was assayed as described by Carvalho et al (1981). The enzyme activity recovery in both phases was compared with the initial enzyme activity (crude extract) in the system.

Protein determination

The amount of total protein in both phases was determinated by the Bradford method (1976). The samples of the phases were diluted at least 1/10 with water before the addition of the dye to eliminate the interference of PEG 550 and phosphates on the protein assay. A blank of each phase was prepared without protein extract and diluted in the same way. The protein partition coefficient (K) was defined as the ratio between total protein concentration in the top and bottom phase, respectively.

Purification factor

The purification factor was defined as the ratio between the specific activity of ascorbic oxidoreductase after partial purification and specific activity of ascorbic oxidoreductase in the crude extract.

Mass transfer determination

To determine mass transfer coefficients, the transfer rate of protein from the PEG-rich (dispersed) phase to the salt-rich (continuous) phase needed to be measured. This was done by monitoring the concentration of solute at the inlet and outlet of the column. Samples of the phases were collected and analysed for protein content. The mass transfer coefficient was expressed in

terms of the concentrations by following a simple material balance:

$$K_{Da} = V_D/V\ [(\ln C_{ui} - KC) / (C_{uo} - KC)]$$

Where V_D is the dispersed (PEG) phase velocity (mL/min), V is the dispersion volume (mL), K_{Da} is the dispersed phase volumetric mass transfer coefficient (min^{-1}), C_{ui} and C_{uo} are the protein concentrations in the dispersed PEG-rich phase at the column inlet and outlet (raffinated) (mg/mL), respectively; C is the protein concentration in the continuous salt-rich phase (extracted) (mg/mL) and K is the protein partition coefficient.

Separation efficiency determination

The separation efficiency (E) was calculated by Kawase´s method (1990) as follow:

$$E = C_{ui} - C_{uo} / C_{ui}$$

The result obtained is better defined as solute recovering rate.

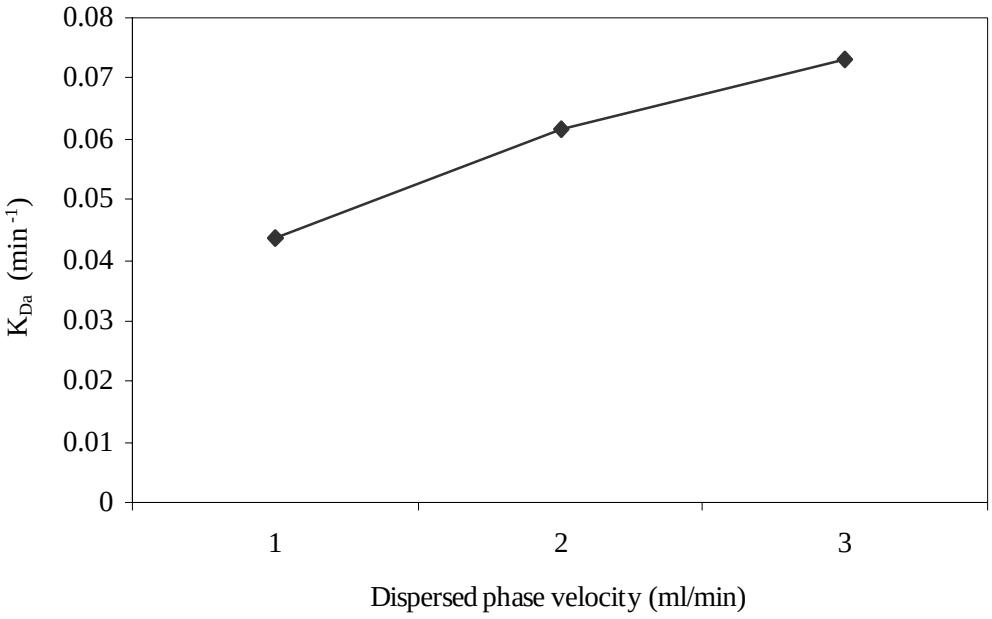

Figure 1 - Influence of the dispersed phase velocity on the mass transfer coefficients in PRDC for 16.7% (w/w) PEG 550 - 14.8% (w/w) phosphate salts (pH 6.0) system

RESULTS AND DISCUSSION

Influence of the dispersed phase velocity on the mass transfer coefficient

Fig. 1 shows the experimental results where the mass transfer has been plotted versus the dispersed phase velocity for values of 1, 2 and 3 mL/min. The results obtained with PRDC showed an increase of the mass transfer coefficients with the dispersed phase velocity. Similar results were obtained by Sarubbo et al. (2003) studying bovine serum albumin transfer using a polymer-polymer biphasic system in a PRDC. Investigations with Spray columns using aqueous two-phase systems also demonstrated an increase of the mass transfer with the increase of dispersed phase velocity, once the drop size at higher velocities is minor and then produces high areas of mass transference (Sawant and Sikdar, 1990; Jafarabad et al., 1992a and b). Studies with a similar PRDC using a PEG-phosphate salts systems showed an independence of the mass transfer coefficient with the increase of dispersed velocity.

Investigations with York-Sheibel columns (Raghav Rao et al., 1991) using ATPS of PEG 4000 and potassium phosphate salts and sodium sulfate salts showed mass transfer coefficients three times higher than the ones obtained with spray columns (Jafarabad et al., 1992a and b), while the mass transfer coefficients for the PRDC using PEG-phosphate systems (Porto et al., 1997; 2000) were 20 times higher than the ones for Spray and York-Sheibel columns. This proportion was also observed in values obtained in this investigation. This information is very important for the scale-up of extraction processes using this kind of equipment.

Influence of the dispersed phase velocity on separation efficiency

Fig. 2 shows the behaviour of separation efficiency influenced by the dispersed phase velocity. It was expected that an increase in the values of dispersed phase velocity should produce higher values of the efficiency. Results showed the inversion of this tendency and were in accordance with results obtained by Tambourgi and Pereira (1993) studying the separation efficiency of a PRDC using an aqueous-organic system. This behavior should be due the fact that high flow rates, associated to the flow resistance imposed by the rotating discs, could promote entrainement of the continuous phase explaining such inversion in our results. Rabelo et al. (1997) showed a direct relationship between the efficiency and the feed ratio in a rotating Blade extractor column using the acetic acid-water-butanol system.

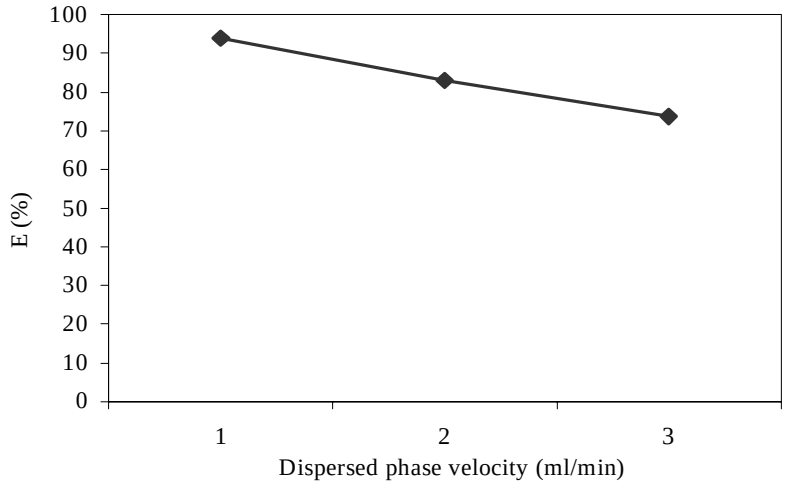

Figure 2 - Influence of the dispersed phase velocity on the separation efficiency in PRDC for 16.7% (w/w) PEG 550 - 14.8% (w/w) phosphate salts (pH 6.0) system

Influence of the dispersed phase velocity on the purification factor

The results obtained in this investigation showed the tendency of ascorbic oxidoreductase activity to be concentrated in the phosphate-rich phase during the time of experiments. The influence of the dispersed phase velocity on the enzyme purification factor and on the enzyme activity recovery is shown in Figs. 3 and 4, respectively. It can be observed, for the conditions studied in this work that an extraction corresponding of 236% of activity and a relative ascorbic oxidureductase purification factor of 34.3 were achieved for dispersed phase velocity of 1 ml/min. The increase in activity (from 2.44 U/ml to 5.76 U/ml) must be consequence of a higher enzymatic structural stability promoted by the polymer (PEG), as described by Albertsson (1986) and due to

elimination of contaminants during extraction. the As there are no data in the literature regarding enzyme continuous extraction using a PRDC, it is difficult to discuss the results found in this investigation.

The results obtained in this investigation suggest that this type of extractor can be successfully applied as a continuous extraction equipment for protein and enzyme using aqueous two-phase systems.

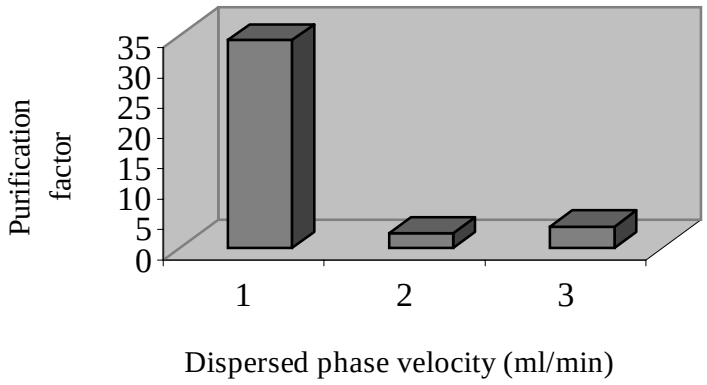

Figure 3 - Influence of the dispersed phase velocity on the enzyme purification factor in PRDC for 16.7% (w/w) PEG 550 - 14.8% (w/w) phosphate salts (pH 6.0) system

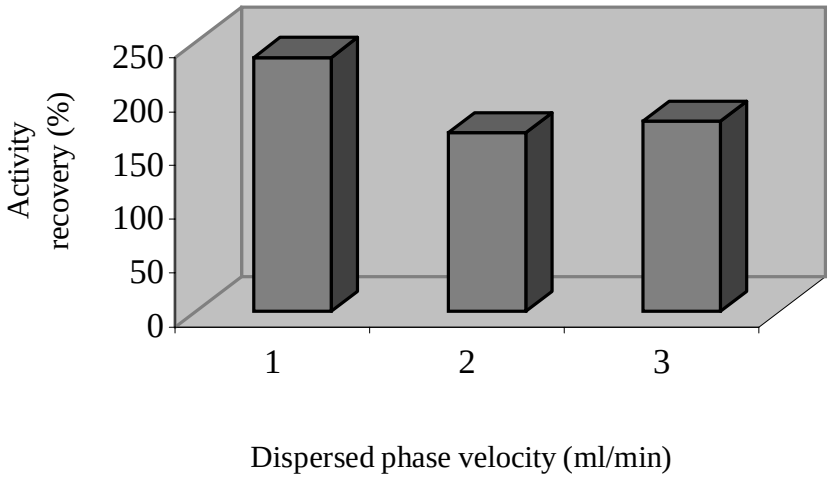

Figure 4 - Influence of the dispersed phase velocity on the enzyme activity recovery in PRDC for 16.7% (w/w) PEG 550 - 14.8% (w/w) phosphate salts (pH 6.0) system

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RESUMO

Uma coluna de discos perfurados rotativos foi utilizada na extração da enzima ascorbato oxidorredutase (E.C.1.10.3.3), obtida do extrato bruto de *Curcubita maxima*, através da utilização do sistema bifásico aquoso Polietilenoglicol-sais de fosfato. Os efeitos da velocidade da fase dispersa nos coeficientes de transferência de massa e na eficiência de separação para valores de 1, 2 e 3 mL/min foram estudados. Observou-se um aumento da transferência de massa com a velocidade da fase dispersa, enquanto que a eficiência de separação demonstrou uma ligeira redução com o aumento deste parâmetro. Os resultados experimentais obtidos durante a extração contínua demonstraram que a atividade da ascorbato oxidorredutase se concentrou preferencialmente na fase rica em sal para todas as condições estudadas. A maior recuperação da atividade enzimática foi de 236%, com um fator de purificação de 34 para o valor de 1 mL/min para a fase dispersa.

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