# Optimizing Bromelain Extration by Reversed Micelles from Pineapple Fruit

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The present work aimed at optimizing the conditions for bromelain extraction by reversed micelles from pineapple juice (Ananas comosus). A micro-column with pulsed caps was employed in the continuous extraction. The cationic micellar solution was composed of BDBAC as surfactant, isooctane as solvent and hexanol as co-solvent. Assays of enzyme activity and total protein were performed to samples of the heavy aqueous phase from the backward extraction. It was found that the maximum purification factor obtained was about 3 and the best values of the independent variables - surfactant agent, co-solvent and salt concentrations, pH of the back and forward extractions – were, respectively: 100 mM, 10% v/v, 1 M, 3.5 and 8. For the continuous extractor, the effects over the purification factor and the productivity were analysed. The independent variables optimal point was determined: ratio between light phase flow and total flow rate equal to 0.67 and 1 s for the time interval between the pulses. This optimal point led to a productivity of 1.29 mL/min and a purification factor of 4.96.

## 1. Introduction

In many biotechnological industries, including food and pharmaceutical ones, the selective separation of a protein out of fermentation broths or vegetable sources has been a primary research interest for downstream processing operations. It is difficult and expensive to selectively recover a targeted protein from a broth due to the low protein concentration and the similarity of the physical properties between proteins present in the same solution.

The liquid-liquid extraction process consists of transferring a substance from a liquid mixture to another immiscible (or partially miscible) liquid phase by putting them in contact. In order to avoid the protein denaturation, it has been employed the liquid-liquid extraction by reversed micelles. Reversed micelles are aggregates of surfactant molecules containing an inner core of water molecules, dispersed in a continuous organic solvent medium. These reversed micelles are capable of selectively solubilizing polar compounds in an apolar solvent and is useful for recovering a specific biomolecule from aqueous solutions, such as fermentation broths or cell culture media. Kilikian et al. (2000) points out the reversed micelles extraction as a versatile and useful process to purifying proteins.

The present work aimed at optimizing the conditions for bromelain extraction. Bromelain is the name of a group of powerful protein-digesting, or proteolytic, enzymes that are found in the pineapple plant (Ananas comosus). Experimental batch and continuous liquid-liquid extraction processes were studied. A micro-column with pulsed caps was used in the continuous extraction. A design of experiments methodology (Box et al., 1978) was employed to determine the best concentrations of surfactant agent, co-solvent and salt, and the pH of the back extraction and the pH of the forward extraction. A central composite design was also carried out to the study of the continuous operating conditions. The independent variables were: the ratio between light phase flow rate and total flow rate; and the time interval between the pulses. The effects over the purification factor (PF) and the productivity (Pr) were analysed.

## 2. Material and Methods

### 2.1 Bromelain sample preparation

Fruit bromelain (EC 3.4.22.5) was obtained from fruit extract from pineapple, species Perola. Pineapple fruit was triturated and filtered. The filtrate, named as pineapple juice, contained the enzyme bromelain. Samples were frozen at -5oC.

### 2.2 Micellar solution

The cationic micellar solution was composed of BDBAC as surfactant, isooctane as solvent and hexanol as co-solvent. Concentrations will be presented in the design of experiments item.

### 2.3 Backward extraction solution

The bromelain-rich phase (raffinated) was treated with a buffered phosphate solution (citric acid/sodium phosphate) and sodium chloride, which was added until conditions of DOE were reached.

### 2.4 Pulsed caps micro-column

This column was made in glass, 19 cm high and 2.54 cm internal diameter (Figure 1). Three perforated caps were mounted on a central stainless steel stem, at a distance of 4 cm. A pulse frequency controller drove the movements of the stem, 2.8 cm up and down. This way, part of the light phase was pulverized and a uniform dispersion was formed. Another portion of the light phase was retained beneath the cap, increasing the contact between the phases. The inlet flow rates of the dispersed and continuous phases were maintained constant by using previously calibrated peristaltic pumps.

### 2.5 Experimental Procedures

*Batch extraction*

Samples of the pineapple juice were used to the enzyme activity and total protein determinations. Pineapple juice and micellar solution were evenly mixed (5 mL each). The mixture was stirred in a glass tube until getting homogenous aspect (emulsion). The separation of the phases was performed by centrifugating the mixture at 8000 rpm for 5 minutes. The light phase (micellar) was taken for the backward extraction of the bromelain.

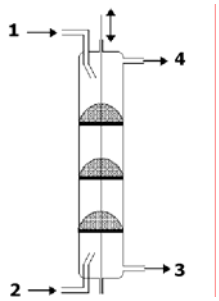


*Figure 1. Extraction micro-column with pulsed caps: (1) Pineapple juice inlet (continuous phase); (2) Micellar solution inlet (dispersed); (3) Heavy phase outlet (extracted); (4) Light phase outlet (raffinated).*

*Continuous extraction*

The column was filled with the pineapple juice. The feeding pumps were started up in such a way that the total flow rate (pineapple juice plus micellar solution streams) was set to 8mL/min (constant) and the ratio between light phase flow rate and total flow rate was set to 0.5. Afterwards, the pulse frequency (i.e., the time interval between the pulses) was adjusted to 4 seconds. After the steady state was reached (about 15 minutes), the operating conditions were changed according to the DOE. A new stationary point was found. Samples were collected from the raffinated phase (micellar phase outlet) at every steady state achieved.

*Backward Extraction*

The protein rich-phase (micellar) obtained from the extraction (batch or continuous operation) was mixed to the same volume of the backward extraction solution. During 3 minutes, the tube was stirred in vortex. In order to split the phases, the mixture was centrifuged at the same conditions used in the batch extraction - 8000 rpm during 5 min. The light phase (micellar) was rejected. Assays of enzyme activity and total protein were performed to samples of the heavy aqueous phase.

**2.6 Enzyme activity assay**

Enzyme activity was determined by enzymatic hydrolysis of casein 2% (w/v) at pH 7.5, temperature 37$^{o}$C, during 10 min. Tricloroacetic acid (TCA) was employed in the precipitation of the non-hydrolyzed product. The amount of soluble peptides in TCA was determined by measuring the absorbance at 280 nm. The method defines one unity of enzyme activity as the amount of enzyme that modifies in 1.0 the absorbance value.

**2.7 Total protein assay**

Total protein was determined according to Lowry method (1951), using BSA as standard.

**2.8 Performance indexes**

a) Total protein yield (TP): $$TP(\%) = (P_2 / P_1).100 \quad (1)$$

where P1 is the total protein concentration (g/L) from the pineapple juice and P2 is the total protein of the backward extraction solution.

b) Enzyme activity yield (EA): $$EA(\%) = (A_2 / A_1).100 \quad (2)$$

where A1 is the enzyme activity measurement (in units per liter) from the pineapple juice and A2 is the enzyme activity of the backward extraction solution.

c) Purification factor (PF), which represents the increase of the bromelain purity:

$$PF = \frac{A_2 / P_2}{A_1 / P_1} \quad (3)$$

d) Productivity (Pr): $$\Pr = (raffinated\ phase\ flow\ rate).TP \quad (4)$$

**2.9 Design of Experiments (DOE)**

*Batch extraction*

A $2^{5-1}$ factorial design was carried out to verify the effects and interactions of the surfactant agent, the co-solvent and the salt concentrations, besides the pH of the back and forward extractions on the purification factor (PF) of the studied process.

Because the bromelain enzyme has positive charges distribution (i.e., up to the Isoeletric Point) it was employed the extraction pH range of 6 to 8. The pH upper bound to avoid enzyme denaturation is pH=10.3 (Murachi, 1973). The range of concentrations was determined based on the work of Hasmann (2000). The Table 1 shows the levels and the experimental values of the factorial design variables.

Table 1. Levels and experimental values of the $2^{5-1}$ fractional factorial design.

| Independent Variable | Level (-1) | (+1) |
|---|---|---|
| pH | 6 | 8 |
| [BDBAC] mM | 100 | 200 |
| % v/v hexanol | 5 | 10 |
| pH of back extraction | 3.5 | 6 |
| [NaCl] M | 1 | 2 |

*Continuous extraction*

A central composite design was carried out to the study of the continuous operating conditions. The independent variables were: the ratio between light phase flow rate and total flow rate; and the time interval between the pulses. The effects over the purification factor (PF) and the productivity (Pr) were analysed.

Table 2. Levels and experimental values of the central composite design.

| | Level | | |
|---|---|---|---|
| | (-1) | (0) | (+1) |
| Light phase flow rate/ Total flow rate | 0.3 | 0.5 | 0.7 |
| Time interval between the pulses (s) | 6 | 4 | 2 |

## 3. Results

### 3.1 Batch extraction

The range and levels of the independent variables used in the experiments are given in Table 1. The results show that only the salt concentration in the backward extraction solution did not present significant effect over the purification factor. The buffering solution seemed to be responsible for the enzyme expulsion from the micelles, independent of the salt concentration. The best conditions were found for the bromelain recovery by reversed micelles from pineapple juice (see Table 3).

Table 3. Best conditions for the bromelain recovery by reversed micelles from pineapple juice.

| Factor | Best conditions |
|---|---|
| pH | 8 |
| [BDBAC] mM | 100 |
| % v/v hexanol | 10 |
| Backward extraction pH | 3.5 |
| [NaCl] M | 1 |

### 3.2 Continuous extraction

The best conditions determined through the analysis of the results of the batch extraction factorial design (Table 3) were used. It was observed that the purification factor (PF) increased with pulse frequency within the operating range studied. The frequency must be high enough to promote good contact between light and heavy phases, but it should not be extremely high since it may cause denaturation of the enzyme.

The experiments also show that the time interval between the pulses did not affect the productivity (Pr). Because the productivity is directly proportional to the light phase flow rate (Eq. 4) and because the operating range guaranteed the presence of total proteins in the backward extraction solution (P2), the light phase flow rate must be kept in the upper bound of the studied range.

The optimal conditions obtained were: light phase flow rate/total flow rate equal to 0.67 and time interval between pulses equal to 1 second, corroborating the previous observations. This point led to a productivity of 1.29 mL/min and a purification factor

of 4.96. According to Rabelo et al. (2004), the value 1.25 was the maximum purification factor obtained for the bromelain recovery from pineapple fruit via two-phase aqueous extraction. This, in fact, shows that reversed micelles method is a promising way for enzymes purification.

## 4. Conclusions

Fruit bromelain (EC 3.4.22.5) was obtained from fruit extract from pineapple, species Perola. In order to avoid the enzyme denaturation in the purification process, the present work was concerned about optimizing the conditions for the liquid-liquid extraction by reversed micelles.

From the $2^{5-1}$ fractional factorial design of experiments (batch runs), it was found that the maximum purification factor obtained was about 3 and the best values of the independent variables - surfactant agent, co-solvent and salt concentrations, pH of the back and forward extractions – were, respectively: 100 mM, 10% v/v, 1 M, 3.5 and 8. These conditions were then employed to the continuous runs.

A central composite design was carried out to the study of the continuous extraction. The effects over the purification factor and the productivity were analysed. The independent variables optimal point was determined: ratio between light phase flow and total flow rate equal to 0.67 and 1 second for the time interval between the pulses. This optimal point led to a productivity of 1.29 mL/min and a purification factor of 4.96.

In terms of the purification factor, the results showed the great efficiency of the pulsed micro-column equipment when compared to the batch extraction and to the two-phase aqueous extraction of the bromelain.

## 5. References


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