ISSN : 0974 - 7435 Volume 9 Issue 5

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 9(5), 2014 [188-194]

# Statistical optimization of ATPi system and extraction of papain in desktop ENZextractor

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## ABSTRACT

ATPi- A concept of process integration which is meant for single step purification process in Bioseparation industry is designed for the Industrial enzyme extraction. Integrating protein affinity groups i.e., ligands to one of the phase forming polymer in ATP system is termed as integrated aqueous two phase system (ATPi) in this work. The ATPi system is composed of PEG+ 1-butyl phosponium bromide as ligand and Ammonium sulphate. RSM through CCD analysis was used to identify optimal values of extraction parameters for papain separation from carica papaya fruit latex. The optimized parameters from laboratory scale was applied in a 3L pilot scale, Laboratory designed ENZextractor with a working volume of 2L. It was proved that 96% accuracy of model and 92% recovery was achieved through the ENZextractor.


## KEYWORDS

Papain;
ATPi;
Ionic liquid;
Partition coefficient;
Scale up;
ENZextractor.

## INTRODUCTION

Aqueous two phase extraction (ATP) is the potential tool for separation of enzyme and highly active bio molecules. It's less energy consumption and ease of maintenance gives consistent platform for bioprocess industry[1]. Biological molecules, particularly enzyme kind of macro-molecules need high biocompatible environment when it is in isolated forms[2]. Recent trends have developed process integration which engages aqueous two phase extraction systems for the single step purification protocols[3]. In this aspect, many works has been reported for enzyme purification. ATP system has many attractions like bio compatibility, economic, ease of maintenance, scalability and good selectivity[4-6]. Because of these salient features, ATP has been identified as a potential and promising tool in protein industry. Integrated aqueous two phase (ATPi) extraction system is one of the recently reported tools, based on process integration aspect[3,7,8]. The design step that converts the conventional ATP extraction system in to highly selective ATPi system is by integrating protein affinity groups to one of its phase forming polymer. Theoretically the ligands are protein affinity groups used in liquid chromatography (FPLC) techniques, also applied in ATP system for this integration. Very few protocols have been reported in this aspect and many in pipeline. By integrating these kinds of protein affinity ligands to ATP system the number of steps in this protocol can be reduced appreciably and economic too[9].

Papain (EC 3.4.4.10) is one of the frequently used proteolytic enzymes, industrially and medicinally, having random application in textile, food and medical industry[10]. Originally papain can be purified in many ways like spray dried crude papain, two step precipitation, ATP system and liquid chromatographic methods[11]. It belongs to cysteine-proteinase family, originated in latex of *Carica Papaya* fruit[12]. In those protocols, atleast five to six downstream processing steps have been followed to purify the high pure papain with good yield[12].

Studies of papain extraction using ATP system based on ionic liquid has been emerging technique in Bioseparation. The present work is about to develop single step purification of papain using ionic liquid ligand in ATPi system for high scale. Response surface methodology by central composite design tool has been used to optimize the extraction parameters[13,14]. The optimal parameters from the small scale have been implemented in the laboratory customized design extractor named as ENZextractor. The ENZextractor facilitates the mixing and temperature monitoring through online mode. The effect of parameters for the papain extraction was also studied for the high scale by varying the process conditions.

## MATERIALS

Poly ethylene glycol (PEG), Ammonium sulphate, Casein, Tri-Chloro Acetic acid (TCA), Ionic liquid 1-Butyl Phosponium chloride were purchased from Sigma,India. ENZextractor & Sensors are locally designed. All the chemicals involved in this work were of Analytical grade.

## METHODOLOGY

### Crude sample preparation

Crude sample was prepared by collecting the fresh latex of papaya. Latex was gathered in a cold container by making thin longitudinal incisions on the papain fruit. The collected latex was mixed in phosphate buffer with pH 7 till the concentrations were optimized. Clear latex was collected through centrifugation at 6000 rpm at 20°C.

### ATPi Extraction

An aqueous two phase system was formed by the addition of different concentrations (10,15,20%w/v) of PEG at various molecular mass (4000,6000, 8000) to 10,12, 14%w/v of ammonium sulphate in aqueous medium. Based on free liganding mechanism, an integrated aqueous two phase system was formed. Ionic liquid-(4-10 mg/ml) 1-Butyl phosphonium bromide Ionic liquid was applied as ligand to the ATPi system. The crude enzyme sample was added to the ATPi system and centrifuged at 9000 rpm for 5 minutes to separate the two phases.

### Experimental design by central composite design:

Response surface methodology is an effective alternate mathematical approach applied for the optimization of various parameters in papain extraction. Central composite design involves the process model development in this challenging separation procedure through selective runs for the optimization of process conditions. PEG molecular mass, PEG concentration, Ionic liquid concentration, and pH of the system are considered as the four factors for the response of partition coefficient of papain that help in determining the optimum extraction values were shown in TABLE 1.

CCD analyses the process parameter interrelationship that influences the responses using the graphical approach. Design expert 8.0.7.1 software was used for this optimization work through a four factor Central composite design. This CCD approach is selected for its process step reduction that directly helps to decrease the overall cost. Through random basis, 30 experimental values were arranged for a complete design.

**TABLE 1 : Design table Central composite design.**

| Variable | Factors | Range Studied | Level -1 | 0 | 1 |
|---|---|---|---|---|---|
| A | PEGmm | 4000-6000 | 4000 | 6000 | 8000 |
| B | PEG concentration | 10-20%w/v | 10 | 15 | 20 |
| C | IL concentration | 4-10mg/ml | 4 | 7 | 10 |
| D | pH | 4-10 | 4 | 7 | 10 |

### Regression analysis: statistical

CCD approach equation from response surface methodology examines the statistical values of the ex-

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perimental design given below.

$$Y_i = \beta_o + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j \quad (1)$$

Where $Y_i$ indicates the predicted output, $\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ are the coefficients of regression for the intercept, linear, quadratic and interaction coefficients respectively, $X_i$ and $X_j$ are the coded autonomous variables.

Design expert 8.0.7.1 is a software package where the ANNOVA (Analysis of Variance) analyse the statistical parameters. The experimental data give a second order polynomial equation and a three dimensional plot was visualized through the parameter interrelationship and the responses. In order to get maximized response, a number of optimization steps were carried out for every factor.

### ENZextractor (Enzyme –extractor) studies and purification

The scale up studies was carried out in ENZextractor in a 3L vessel with view glass. It was connected to a lead plate equipped with a motor shaft impeller. The temperature and pH values influence the phase separation. The ENZextractor should be in a sterile environment at 150°C for 10 minutes. Compressed air was blown into the vessel to ensure the sterility. All parameters like pH, temperature, enzyme separation were supervised periodically through a microcontroller. A working volume of 2L was used in the ENZextractor to provide much space for the bioseparation process which in turn gives high yield. The resulting fractions from the both scales were anlaysed for purity in Sephadex G 100,Gel Filtration chromatography (GFC) column in AKTA Prime Plus system, GE.

### Analytical methods

By the Lowry's method, with BSA as standard, the total protein estimation was estimated[15]. Casein Digestive assay validates the papain activity of the crude sample. Liberation of 1µg of tyrosine from papain enzyme after completing one minute of digestion at 37°C, pH 7 from casein substrate solution as standard maintained at pH 7.0. GFC column was used for both purification and desalting purposes. The fraction from the forward extraction of ATPi step then proceeded to PEG recovery in backward extraction. The resulting fraction highly salted with NaCl by desalting. The GFC column previously buffered with pH 7 phosphate solutions and then the sample was loaded for desalting. The simultaneous desalting and enzyme purification occurs. The partition coefficient (K) is termed as the ratio of enzyme concentration in the PEG rich upper phase to enzyme concentration in the lower phase.

## RESULT AND DISCUSSION

### Optimization of ATPi parameters

The design matrix value of the ATPi system from CCD approach was presented in TABLE 2. Fisher's F-test was applied for the analysis of the RSM using CCD approach, where F-value of 10.54 assures the significances of the system. PEG molecular mass, PEG concentration, Ionic liquid concentration and salt pH of the system are the four factors notated as A, B, C and D respectively. PEG molecular mass (4000,6000,8000), PEG concentration (10%,15%,20%), Ionic liquid concentration (4-10 mg/ml), Ammonium sulphate salt (10,12,14%) concentration are the ranges selected for the optimization studies of the factors involved in the CCD analysis. The $R^2$ (Coefficient of determination) value was found to be 0.9078, that assures an ambient variance of the quadratic model.

The interaction between the factors and maximum enzyme production was highlighted through the three dimensional response surface plots. They were plotted to ensure the optimized value of factors considered from the experimental values. The model terms B, $A^2$, $B^2$, $C^2$ and $D^2$ were significant as the prob>F is less than 0.0500.

The results of the three dimensional plot through abundant number of experimental data clearly assures that the optimized value of the factors involved in the ATPi formation were found to be at PEG molecular mass of 6000, 15% w/v of PEG concentration, Ionic liquid concentration 7 mg/ml and pH 7.

Regression analysis was used for developing model equations for the response. The responses were analyzed from the design and the results were tabulated. The partition coefficient equation that is assessed for the ATPi system is termed below.

**Partition Coefficient, K =+11.33+0.34 * A+0.88 * B+0.22 * C+0.61 * D+0.32 * A * B-0.063 * A * C+0.45 * A * D+0.093 * B * C+0.28 * B * D-.19 * C * D-2.58 * A^2-1.11 * B^2-2.27 * C^2-3.06* D^2** (2)

where 'K' represents the partition coefficient of the ATPi

TABLE 2 : Design Matrix: ATPi experimental design

| Run | Factor 1 A:PEGmm | Factor 2 B:C_PEG | Factor 3 C:Me2+ | Factor 4 D:pH | Response 1 Partition Coefficient |
|---|---|---|---|---|---|
| 1 | 6000 | 15 | 7 | 7 | 11.32 |
| 2 | 4000 | 10 | 10 | 4 | 0.96 |
| 3 | 6000 | 15 | 7 | 7 | 11.34 |
| 4 | 6000 | 15 | 7 | 7 | 11.33 |
| 5 | 6000 | 25 | 7 | 7 | 12.42 |
| 6 | 6000 | 5 | 7 | 7 | 3.24 |
| 7 | 4000 | 20 | 10 | 10 | 1.14 |
| 8 | 6000 | 15 | 1 | 7 | 1.22 |
| 9 | 8000 | 10 | 10 | 10 | 1.45 |
| 10 | 6000 | 15 | 13 | 7 | 5.1 |
| 11 | 4000 | 20 | 4 | 10 | 1.98 |
| 12 | 6000 | 15 | 7 | 7 | 11.32 |
| 13 | 2000 | 15 | 7 | 7 | 1.66 |
| 14 | 8000 | 10 | 4 | 10 | 3.46 |
| 15 | 8000 | 10 | 4 | 4 | 1.17 |
| 16 | 8000 | 20 | 4 | 4 | 0.55 |
| 17 | 4000 | 20 | 10 | 4 | 0.96 |
| 18 | 6000 | 15 | 7 | 1 | 0.01 |
| 19 | 4000 | 20 | 4 | 4 | 0.94 |
| 20 | 4000 | 10 | 4 | 10 | 2.14 |
| 21 | 4000 | 10 | 10 | 10 | 2.21 |
| 22 | 10000 | 15 | 7 | 7 | 2.23 |
| 23 | 6000 | 15 | 7 | 7 | 11.33 |
| 24 | 6000 | 15 | 7 | 13 | 0.03 |
| 25 | 8000 | 20 | 10 | 4 | 0.86 |
| 26 | 8000 | 20 | 4 | 10 | 4.84 |
| 27 | 6000 | 15 | 7 | 7 | 11.325 |
| 28 | 8000 | 10 | 10 | 4 | 1.11 |
| 29 | 4000 | 10 | 4 | 4 | 0.94 |
| 30 | 8000 | 20 | 10 | 10 | 4.86 |

system.

## 3D response plot of ATPi extraction of papain in lab scale:

The three dimensional plot investigate the process parameters with the dependent values. Figure2a and b show the relationship of PEG molecular mass and PEG concentration over the partition coefficient values. PEG molecular mass of 6000 was found to be the maximised value that gives high partition coefficient of 12.42 Figure2 c depicts the interrelationship exist between PEG concentration, Ionic liquid concentration and partition coefficient. The 3-D graph highlights the optimized value of 15% w/v PEG concentration giving 11.34 partition coefficient values. Figure 2 d plots the IL concentration, PEG molecular mass towards the partition coefficient value that reveals the 7 mg/ml value of IL concentration as an optimized one at 11.325 partition coefficient. The optimal conditions for all the factors were derived through the three dimensional plot.

## ATPi in ENZextractor 3L scale

The optimized value of PEG 6000 (15% w/v) at 7 mg/ml Ionic liquid concentration were applied for the ATPi system formation and the forward and backward extractions were carried out for the partition coefficient and purity analysis. The partition coefficient value increases till the 15% w/v of PEG 6000 and start to decline further. In the IL concentration ranging from 4 to 10 mg/ml, till 7 mg/ml the partition coefficient value shows a progressive result and beyond that it starts to decrease. The optimized values shown in TABLE 3 derived from the lab scale were used to carry out the studies on the *ENZextractor* is shown in Figure1. A 2L of classified enzyme solution with 15% w/v PEG 6000, 7 mg/ml of Ionic liquid concentration and defined amount of ammonium sulphate was loaded within the *ENZextractor* vessel having a working volume of 3L

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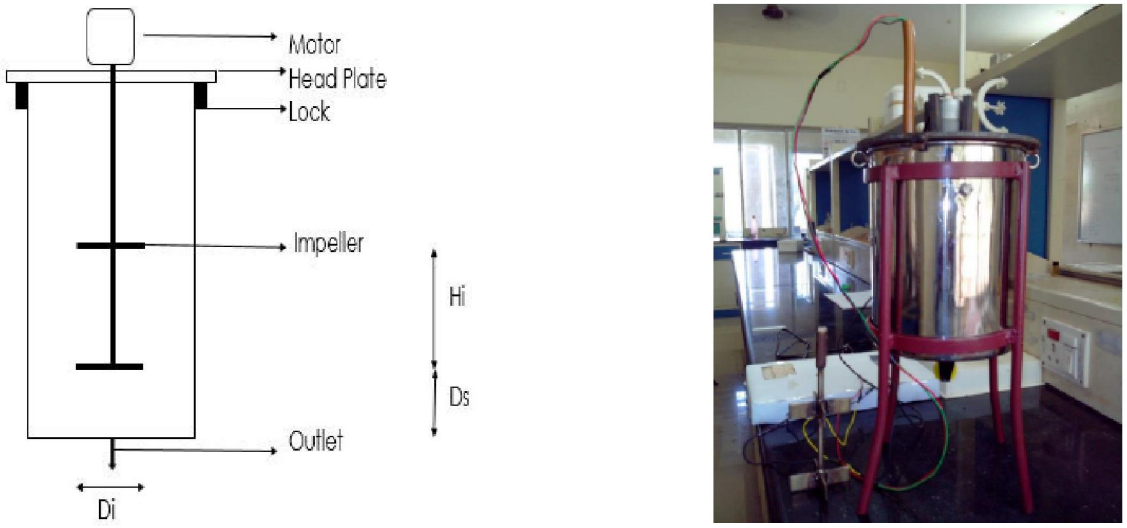


**Figure 1: ENZextractor: Schematic and Desktop 3L Scale extractor**

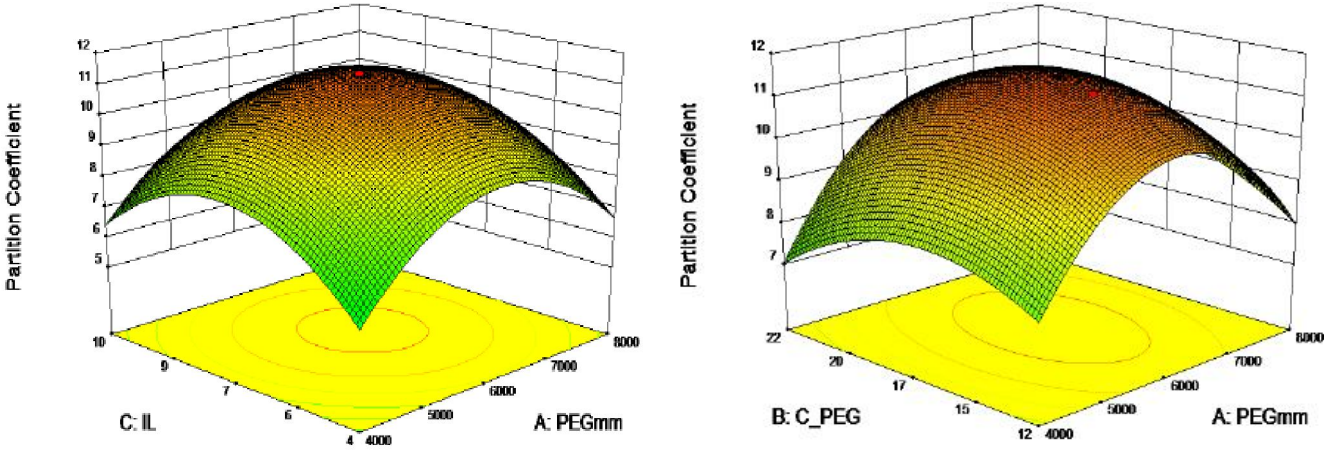


**Figure 2 a, b : 3D surface plot for ATPi extraction- PEG molecular mass, PEG concentration and IL concentration**

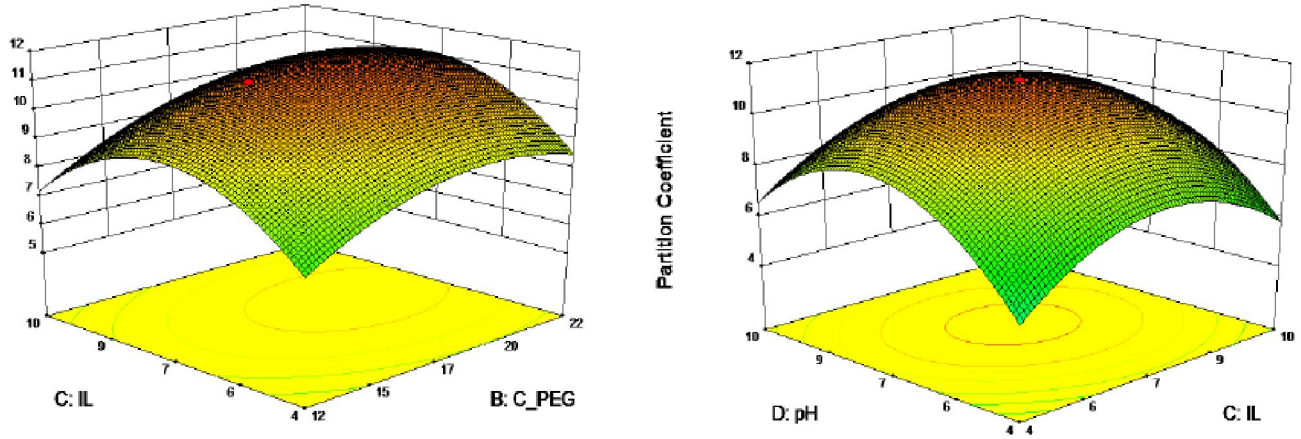


**Figure 2 c, d : 3D surface plot for ATPi extraction- PEG concentration, pH and IL concentration**

and centrifuged at 150 rpm for 90 minutes in order to activate the phase separations. The papain extracted about 15400U/ml in lab scale, purity factor of 22.62 with 68% recovery shown in TABLE 4. But in ENZextractor papain was extracted about 18440U/ml with 78% recovery is shown in TABLE 5. This is comparatively higher than the lab scale where adequate mixing and large extraction area provides high enzyme par-

**TABLE 3 : Optimal parameters by RSM from 5ml lab scale**

| PARAMETERS | RANGE OF VALUES | OPTIMUM VALUE |
|---|---|---|
| Molecular weight of PEG | 4000, 6000, 8000 | 6000 |
| Concentration of PEG(W/V) | 10%, 15%, 20% | 15% |
| IL ligand concentration | 3-10 mg/mL | 7mg/L |
| pH | 2-12 | 6.5 |
| Temperature | 20-80 °C | 30°C |
| Agitator speed | 50-200rpm | 150rpm |

**TABLE 4 : ATPi extraction of Papain in 5ml scale**

| Stages | Specific Enzyme activity, U/mg | % Yield | Purity factor |
|---|---|---|---|
| Crude | 680 | 100 | 1 |
| PEG6000_IL+AmS(5ml) | 11440 | 74.5 | 16.8 |
| GFC | 15400 | 68.2 | 22.65 |

**TABLE 5 : ATPi extraction of Papain in ENZEXTRACTOR 3L scale**

| Stages | Specific Enzyme activity, U/mg | % Yield | Purity factor |
|---|---|---|---|
| Crude | 680 | 100 | 1 |
| PEG6000_IL+AmS (2L ENZextractor) | 12442 | 81 | 18.3 |
| GFC | 18440 | 78 | 27 |

tition. Regular monitoring of pH and mixing speed favours papain recovery and the accommodating compounds settled in the bottom phase. The free liganding mechanism also improves the purity factor, the high purity fold possible here.

## CONCLUSION

The papain purification was found to be 78% recovery with 18440 U in lab scale and 68% recovery with 15440U in the ENZextractor. The high mixing area and more interaction of phase separation compounds increases the protein separation appreciably in ENZextractor. The main driving force for extraction is influenced highly by the temperature and pH. By continuous monitoring and regulation of these extraction parameters in high volume extractors, it is possible to achieve high output. Since the extractions condition was maintained as such developed in lab scale papain was purified almost 83% of lab results. With little modification and additional arrangements, the same extractor can also be used for ATP conversion based process.

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