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SELECTIVE RECOVERY OF AMYLASE FROM PITAYA (*Hylocereus polyrhizus*) PEEL USING ALCOHOL/SALT AQUEOUS TWO-PHASE SYSTEM

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Abstract

Amylase, which can hydrolyze starch into oligosaccharides, is a significant enzyme with diverse applications in various industries. In comparison to microbial amylases, plants can serve as a rich source of plant-derived amylase for industrial production at lower cost and toxicity. In this study, the ethanol/phosphate aqueous two phase system (ATPS) was adopted to recover the amylase from the red pitaya peel. The effects of several ATPS parameters such as concentrations of ethanol and phosphate, the system pH, and the concentration of sodium chloride (NaCl) on the recovery of amylase were investigated. Based on these results, a central composite design (CCD) was used to optimize the recovery of amylase from the red pitaya peel. Results demonstrated that the optimal conditions for recovery of amylase were observed for the ATPS containing 19% (w/w) ethanol, 26% (w/w) phosphate pH 7.0, and 3.8% (w/w) NaCl. The amylases were recovered to the ethanol-rich top phase with an optimum yield and a purification factor of 92.48% and 5.75, respectively. Therefore, this study demonstrated the feasibility of ethanol/phosphate ATPS as a promising tool for the simultaneous recovery and purification of amylase from the red pitaya peel.

Keywords: Amylase, Aqueous two-phase system, Bio-separation, Protein recovery, Purification

1. Introduction

Amylase is a starch degrading enzyme that helps to break down the carbohydrates into shorter oligosaccharides [1]. With its extensive application in the food, pharmaceutical, textile, detergent, and the paper industries, this class of enzyme accounts for 30% of the worldwide sales of enzyme [2]. Nevertheless, its demand is limited to certain strains of bacteria and fungi for large-scale production, which in turn further limited its application to specific industry only [3, 4]. In view of the great potential of amylase, considerable research interest has drawn towards the exploration of new sources for its production. With the recent advances in plant biotechnology, plants can now serve as a rich source of plant-derived novel enzymes for biotechnological and industrial purposes at a lower cost and toxicity. The peel of pitaya fruit, which amounts to one-third of its total fruit weight, is usually disposed as waste material by the food and beverage industries [5]. However, the pitaya peel is rich in various valuable enzymes, and thus can be re-utilized for the commercial production of natural enzyme.

The enzyme purification strategies employed commonly include the processes such as precipitation and chromatography techniques. The drawbacks of this conventional method are consumption of high amounts of organic solvent, multi-step, expensive, low yields, and time intensive. Moreover, prolonged exposure to undesirable extraction condition often imposes adverse effect on the enzyme activity and stability. Hence, there is a need to develop an alternative cost effective and environmental benign enzyme extraction method. In recent years, the aqueous two-phase system (ATPS) has emerged as an effective separation tool. The mixing of solvents such as polymer and inorganic salts or short-chain alcohol and inorganic salts above a critical concentration lead to the formation of two aqueous phases, which allow the selective recovery of desirable biomolecules to one phase and the separation of impurities to the opposite phase [6]. Through the ATPS, clarification, recovery, and initial purification of desirable biomolecules can be achieved simultaneously in a single step [7, 8].

In this study, ATPS composed of alcohol and salt was employed for the selective recovery of amylase from the pitaya (*Hylocereus polyrhizus*) peel, owing to the numerous process advantages (e.g. less expensive, lower in viscosity, faster settling time, and recyclability) of this system compared to other types of ATPS [9-11]. Ethanol was selected as the phase-forming component for the top phase of the ATPS since the enzyme recovered in the ethanol-rich phase can be easily separated from the ethanol by evaporation [7]. Single factor experiments were performed to evaluate the effect of ATPS parameters, such as phase compositions. pH, and addition of sodium chloride (NaCl), on the separation efficiency of amylase from the pitaya peel using the ethanol/salt ATPS. Subsequently, the ATPS conditions were optimized using response surface methodology (RSM) to achieve optimum yield and extraction efficiency of amylase from the pitaya peel.

2. Materials and Methods

2.1. Materials

Red pitaya fruits (*Hylocereus polyrhizus*) were purchased from Pasar Borong Selayang, Malaysia. The fruits applied in this study were of uniform size, free of visual defects, under the same grade and maturity stage. Absolute ethanol (>99.8%)

was obtained from VWR Chemicals (Fontenay-Sous-Bois, France). Sodium hydroxide was obtained from R & M chemicals (Essex, United Kingdom). The anhydrous sodium acetate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, glacial acetic acid, potassium sodium tartrate tetrahydrate, and soluble starch were sourced from Merck (Darmstadt, Germany). Sodium chloride (NaCl), 3, 5-dinitrosalicylic acid (DNS) and D (+) maltose monohydrate were supplied by Sigma-Aldrich (St. Louis, Missouri, United States of America). The bovine serum albumin (BSA) standard and protein assay kit were obtained from Thermo Scientific (Rockford, United States of America). All reagents and chemicals used in this study were of analytical grade.

2.2. Preparation of crude extract

The pitaya peels were separated from the fruits after thorough rinsing and cleaning using deionised water. The peels were chopped into tiny pieces and immediately blended (Model Takada TK-350, Takada Corporation, Japan) with the sodium acetate buffer (pH 5.0) at a sample to buffer ratio of 1:4 for 2 min. The blended homogenate was filtered through cheesecloth and centrifuged (Model G322511050059, Labogene, Denmark) at 4427 × g for 5 min at 4°C. The crude extract (i.e. supernatant) was collected and kept at 4°C prior to the ATPS partitioning experiment [12].

2.3. ATPS partition experiments

The ethanol-phosphate ATPS with a volume ratio, V_R of 1.0 was prepared in 15 mL centrifuge tube by mixing the ethanol, 50% (w/w) potassium phosphate buffer stock solution at desired pH, 10% (w/w) crude extract and sodium chloride (NaCl) at an appropriate ratio according to the ethanol-phosphate ATPS bimodal curve reported by Ooi et al. [7]. The deionised water was added to a final mass of 5g. The mixture was mixed thoroughly using a vortex mixer (Model BV 1000, Benchmark Scientific, New Jersey, USA) and centrifuged at 1968 × g for 10 min for complete phase separation. The volume of each phase was measured to determine the V_R. Sample was collected from each phase for the determination of amylase activity and total protein content.

2.4. Optimization of extraction parameters

A full-factorial central composite design (CCD) was applied in this study to determine the optimum ATPS operating conditions for the partitioning and the recovery of amylase from the crude extract of pitaya peel. Based on the results of single-factor experiments, three independent variables, namely ethanol concentration (X_1), potassium phosphate at pH 7.0 concentration (X_2), and the NaCl concentration (X_3) were studied at three levels (Table 1). The data analysis and regression optimization were performed using Minitab v.16 statistical package (Minitab Inc., State College, PA, USA).

The experimental data were fitted to the following generalized quadratic polynomial equation, Eq. (1) to explore the response surface model for the response function (Y):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{n} \beta_{ij} X_i X_j \quad (1)$$

where X_i and X_j are the independent variables that influence the predicted response, Y; β_0 is the offset term; β_i, β_{ii}, and β_{ij} are the coefficients for the linear, quadratic, and interaction effects, respectively; while X_i and X_j are the independent variables.

Table 1. Independent variables and levels for three-level full factorial CCD design of amylase partitioning.

Variables	Range and levels		
	-1	**0**	**+1**
Ethanol concentration (X_1, % (w/w))	13	19	25
Potassium phosphate at pH 7.0 concentration (X_2, % (w/w))	22	26	30
NaCl concentration (X_3, % (w/w))	2	5	8

2.5. Analytical methods

2.5.1. Amylase activity assay

The amylase activity of the phase sample was assayed according to the Bernfield method [13]. Briefly, a 200 μL of amylase containing phase sample was mixed with 200 μL of 25% (w/v) soluble starch solution, which prepared using the 10 mM sodium chloride containing sodium acetate buffer (pH 5.0), and incubated at 37°C for 20 min. The enzyme reaction was terminated by the addition of 200 μL of 3,5-dinitrosalicylic acid (DNS) solution and heating the mixture at 100°C for 15 min. The mixture was cooled down and followed by the addition of 900 μL of deionised water. The absorbance of the mixture was measured at 540 nm using a microplate reader (Epoch 2, BioTek, Winnoski, VT, USA). One unit of α-amylase activity (U) was defined as the amount of enzyme that released 1 μmol maltose/min under the assay conditions.

2.5.2. Determination of total protein content

The total protein content in the top and bottom phase were measured by the bicinchoninic acid (BCA) method [12,14] using the Pierce protein assay kit with BSA as the standard protein. The sample solution and working reagent were mixed at a ratio of 1:8 in a microtiter plate for 30 s and incubated at 37°C for 30 min. The absorbance was quantified at 562 nm against the blank phase solution prepared in parallel to eliminate the interference contributed by the presence of ethanol and salt.

2.6. Extraction performance parameters

The partition coefficient (K_e) for amylase from the pitaya peels was calculated according to Eq. (2):

$$K_e = \frac{A_T}{A_B} \tag{2}$$

where A_T and A_B are the amylase activity (U/mL) in the top phase and bottom phase, respectively.

The total protein partition coefficient (K_P) was defined as the ratio of concentration of protein measured in the top phase (P_T) to that in the bottom phase (P_B) as shown in Eq. (3):

$$K_p = \frac{P_T}{P_B} \tag{3}$$

Selectivity was defined as the ratio of the amylase enzyme partition coefficient (K_e) to the total protein partition coefficient (K_P) and calculated using Eq. (4):

$$S = \frac{K_e}{K_P} \tag{4}$$

Specific activity, which defined as the ratio of amylase activity (U/mL) to the total protein concentration (mg/mL) in the phase sample solution, can be estimated using Eq. (5):

$$\text{Specific activity } \left(\frac{U}{mg}\right) = \frac{\text{Amylase activity (U)}}{\text{Amount of total protein (mg)}} \tag{5}$$

Purification factor, PF was determined using Eq. (6) and was defined as the ratio of amylase specific activity in the top phase to the amylase specific activity in the crude extract:

$$\text{PF} = \frac{\text{Specific activity of amylase}}{\text{Specific activity of crude load}} \tag{6}$$

Yield (%) for the amylase recovers in the top phase, Y_T was calculated using Eq. (7):

$$Y_T = \frac{100}{1+\frac{1}{V_R.K_e}} \tag{7}$$

where V_R is the ratio the volume of top phase (V_T) to the volume of the bottom phase (V_B).

3. Results and Discussion

3.1. Effect of ATPS parameters on the extraction efficiency of amylase

3.1.1. Effect of Ethanol/Phosphate ATPS phase compositions

The effect of ethanol/phosphate ATPS phase compositions on the extraction efficiency of amylase from the pitaya peels was investigated. Results in Fig. 1 showed that there was an increase in Ke, S, and PF when the ethanol/phosphate ATPS phase composition increased from 13% (w/w) ethanol and 22% (w/w) phosphate to 19% (w/w) ethanol and 26% (w/w) phosphate. As the salt compositions increases, there is a large amount of salt ions that compete with the proteins for bonding with the water molecules in the salt-rich bottom phase, leading to the dehydration of protein and the salting-out of the protein to the ethanol-rich top phase [15]. Moreover, the decrease in water content following the increase in phase compositions results in greater hydrophobicity differences between the two aqueous phases [16]. These coupled effects eventually enhanced the partition of amylase to the ethanol-rich phase, attaining a maximum K_e of 8.529, S of 3.168, and PF of 5.4 at the 19% (w/w) ethanol/26% (w/w) phosphate ATPS at pH 7.0. A further increase in phase composition to 25% (w/w) ethanol and 30% (w/w) phosphate exhibited a significant decrease in the extraction efficiency. The additional upsurge in ethanol concentrations caused a reduction in the free volume in the ethanol-rich top phase to accommodate the amylase [10]. At high salt concentration, amylase can easily denature and aggregate together due to the poor dissolution of salt [7]. Consequently, the amylase was partitioned to salt-rich

bottom phase or precipitated at the interface, resulting in a minimum Ke, S, and PF of amylase in the 25% (w/w) ethanol/30% (w/w) phosphate ABS.

These results indicate that amylase can be selectively recovered to the top phase using a low concentration of ethanol and moderately high concentration of potassium phosphate. Hence, the 19% (w/w) ethanol/26% (w/w) potassium phosphate ATPS was selected for further study on the effect of other ATPS parameters on the extraction efficiency of amylase.

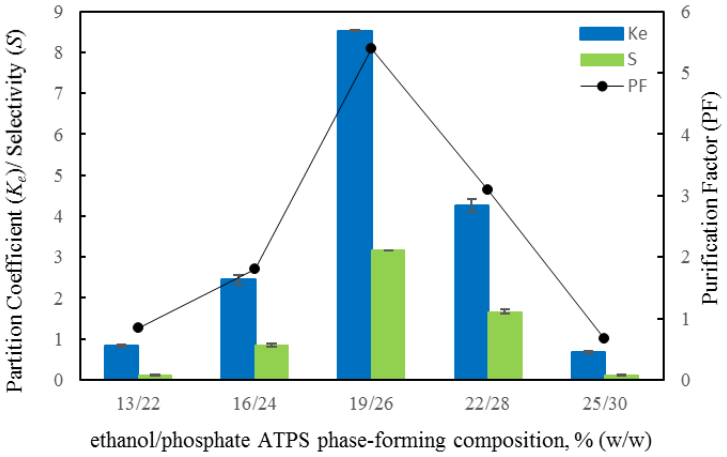

Fig. 1. Effect of the ethanol/phosphate ATPS phase-forming composition on the partition coefficient, selectivity and purification factor of the amylase from pitaya peel. (error bars represent ± standard error).

3.1.2. Effect of pH on Amylase Partitioning

The effect of pH on partition coefficient, purification factor and selectivity on the partitioning of the amylase in the 19% (w/w) ethanol/26% (w/w) phosphate ATPS was studied at different pH, ranging from 7.0 to 9.0 at an interval of 0.5 (Fig. 2). The 19% (w/w) ethanol/26% (w/w) ATPS with system pH below 7.0 was not investigated because two aqueous-phase formation was not visibly noticeable. The system pH often affects the target protein's surface properties, and thus its partitioning behavior [17]. According to Amid et al [5], the amylase has an isoelectric point, pI of 4.7. In theory, the amylase, which is negatively-charged when the system pH is above its pI, tends to partition to the ethanol-rich top phase that possesses higher positive charge density [18, 19].

However, results in Fig. 2 demonstrated that the extraction efficiency decreased significantly when the pH was increased from pH 7.0 to pH 9.0. This phenomenon could be attributed to the change in macromolecular structures and activity of amylase at high system pH [20]. Additionally, the increase in the electrostatic interaction between the impurities and ethanol molecules leads to the unfavourable co-partitioning of impurities to the top phase, thereby resulting in the reduction of PF at increased pH [21]. Hence, these results suggested that selective recovery of amylase from pitaya peel using ethanol/phosphate ATPS with system pH beyond pH 7.0 was not feasible.

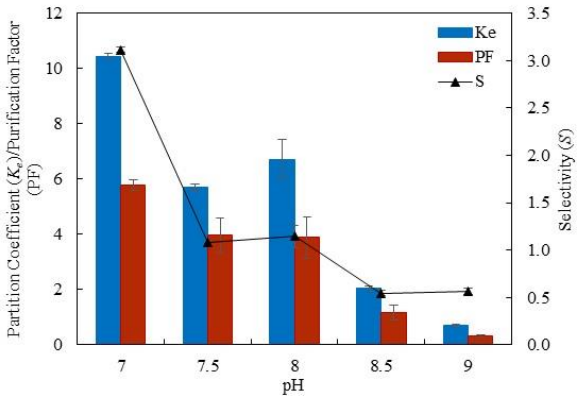

Fig. 2. Effect of pH on the partition coefficient, selectivity and purification factor of the amylase from pitaya peel (error bars represent ± standard error).

3.1.3. Effect of Sodium Chloride (NaCl) on Amylase Partitioning

To improve the selective recovery of amylase from the pitaya peel to the top phase, NaCl was added to the ATPS that contained 19% (w/w) ethanol and 26% (w/w) phosphate at pH 7.0. The effect of NaCl concentration, which ranged from 2.5-10% (w/w), on the extraction efficiency of amylase was shown in Fig. 3. With the addition of neutral and non-toxic NaCl to the ATPS, an electrical potential difference between the two-aqueous phases is generated to steer the selective partitioning of the amylase to the top phase [18, 22]. Besides this, the corresponding changes in ordered water traction with the addition of NaCl will increase the hydrophobicity difference between the two-aqueous phases [7]. As the NaCl concentration increases 5% (w/w), the intensified electrostatic and hydrophobic interaction between the amylase and phase-forming components further promotes the selective recovery of amylase to the top phase. A maximum extraction efficiency was observed at 5 % (w/w) NaCl with an *S* of 4.72, PF of 5.8 and Y_T of 94.96 %. However, a significant drop in extraction efficiency was observed when the NaCl concentration was further increased from 5% (w/w) to 10% (w/w) due to the precipitation and the denaturation of the amylase [23].

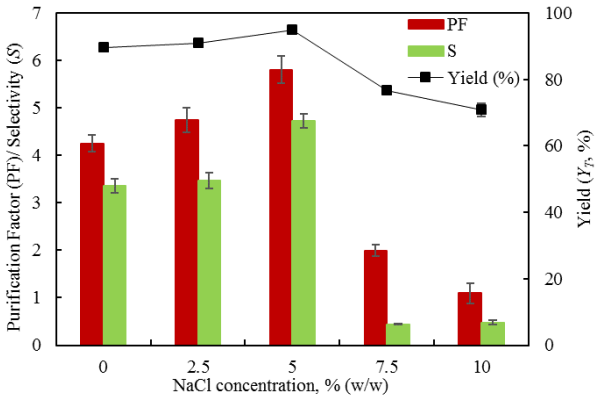

Fig. 3. Effect of NaCl concentration on the selectivity, purification factor and yield of the amylase from pitaya peel (error bars represent ± standard error).

3.2. Optimization of the recovery of amylase in ethanol/potassium phosphate ATPS using response surface methodology

Based on the observation from the single-factor experiments, the concentration of ethanol, phosphate, and NaCl are the key factors that contribute to the enhancement of the extraction efficiency. A three-level CCD design involving three variables, namely ethanol concentration (X_1), phosphate concentration (X_2) and NaCl concentration (X_3), was employed to optimize the recovery of amylase from the pitaya peel crude extract in the ethanol/phosphate ATPS. Table 2 shows the design matrix of the ATPS composition and the experimental responses, which includes the partition coefficient (ln K_e) and the yield of amylase in the top phase (Y_T). The regression analysis shows that the partition coefficient and the yield of amylase in the top phase can be described by Eq. (8) and Eq. (9), respectively:

$$\ln K_e = -53.9834 + 1.0413X_1 + 3.4789X_2 + 0.3501X_3 - 0.0155X_1^2 - 0.0608X_2^2 - 0.0545X_3^2 - 0.0163X_1X_2, \quad R^2 = 0.926 \tag{8}$$

$$Y_T = -967.397 + 20.864X_1 + 61.865X_2 + 21.130X_3 - 0.246X_1^2 - 1.017X_2^2 - 1.408X_3^2 - 0.381X_1X_2 - 0.184X_1X_3 - 0.292X_2X_3, \quad R^2 = 0.985 \tag{9}$$

The ANOVA presented in Table 3 shows that these models are statistically valid ($p < 0.001$) with insignificant lack of fit ($p > 0.05$). The high R^2 values (i.e., > 0.9) indicate a high degree of correlation between the predicted and observed data.

Table 2. Uncoded values of the variables in different experiments of the central composite design (CCD) and the corresponding experimental results.

No.	X_1	X_2	X_3	ln K_e	Y_T (%)
1	13	22	2	-0.47	39.77
2	25	22	2	0.90	71.96
3	13	30	2	0.69	67.86
4	25	30	2	0.56	64.86
5	13	22	8	-0.85	31.14
6	25	22	8	0.01	51.55
7	13	30	8	-0.19	46.64
8	25	30	8	-0.95	28.99
9	13	26	8	0.98	73.78
10	25	26	5	1.54	83.03
11	19	22	5	0.91	72.37
12	19	30	5	0.77	69.61
13	19	26	2	2.41	92.00
14	19	26	8	0.23	57.17
15	19	26	5	2.61	92.67
16	19	26	5	2.05	89.14
17	19	26	5	2.03	88.47
18	19	26	5	1.83	86.73
19	19	26	5	1.81	86.36
20	19	26	5	1.89	87.20

X_1 : ethanol concentration (% w/w); X_2 : phosphate concentration (% w/w); X_3 : NaCl concentration (% w/w).

Table 3. Analysis of variance (ANOVA) for the quadratic models predicted for each response variable.

Response Variable	Source	DF	SS	MS	F value	*p*-value
ln K_e	**Model**	7	20.2287	2.8898	21.40	0.000
	Residual Error	12	1.6203	0.1350		
	Lack of Fit	7	1.1761	0.1680	1.89	0.250
	Pure Error	5	0.4442	0.0888		
	Total	19	21.8490			
Y_T	**Model**	9	7792.83	865.87	72.36	0.000
	Residual Error	10	119.66	11.97		
	Lack of Fit	5	92.52	18.50	3.41	0.102
	Pure Error	5	27.14	5.43		
	Total	19	7912.49			

3.2.1. Partition coefficient

Among the variables, the linear coefficients (X_1, X_2), the quadratic terms (X_1^2, X_2^2, X_3^2), and the interaction coefficient (X_1X_2) significantly ($p < 0.05$) influenced the partition coefficient of amylase in the ethanol/salt ATPS. The quadratic terms, X_3^2 exhibited the significance of X_3, which was highlighted only when the quadratic term was considered. Other interactions were found to be insignificant. The phosphate concentration (X_2) and the ethanol concentration (X_1) exhibited the most prominent effect ($p < 0.005$) on the partition coefficient of amylase, suggesting that the increase in phosphate and ethanol concentration can lead to a favourable amylase partitioning to the ethanol-rich top phase. This was followed in significance by the interaction effect of X_1X_2 ($p < 0.05$). Its response surface plot displayed in Fig. 4(a) shows that the partition coefficient increased gradually with the rise of both ethanol concentration (13-22% (w/w)) and phosphate concentration (19-26% (w/w)). This can be explained by the corresponding increase in the salting-out effect and the hydrophobic interaction between the ethanol and protein molecules, which eventually enhanced the partition of amylase to the ethanol-rich top phase [24]. However, a decline in the partition coefficient was observed with further increase in these compositions. When high ethanol and phosphate are used, the free volume available in the ethanol-rich top phase for the proteins to reside decreases and the latter causes the aggregation of proteins at the ATPS interface, thereby decreasing the partitioning of amylase in the top phase [25]. Hence, the results demonstrated that the optimal partition coefficient of amylase was achieved at 19% (w/w) ethanol, 26% (w/w) phosphate at pH 7.0, and 3.8% (w/w) NaCl through recovery of amylase using ATPS.

3.2.2. Yield

According to the regression analysis, the linear coefficients (X_1, X_2, and X_3), the quadratic terms (X_1^2, X_2^2, X_3^2), and the interaction coefficients (X_1X_2, X_1X_3, and X_2X_3) were significant ($p < 0.05$) for the yield of amylase in the top phase, Y_T. The phosphate concentration and the interaction between ethanol and NaCl concentration had the most ($p < 0.001$) and least significant ($p < 0.05$) effect on the Y_T of the amylase, respectively. Figures 4(b) to 4(d) give the response surface plots for the optimization of the recovery of amylase in the ethanol-rich top phase. The elliptical contours exhibited in Fig. 4(b) was similar to that of Fig. 4(a). Beyond the

optimal recovery conditions which located at the central hump region of the 3D graph, the recovery of the amylase to the top phase decreased drastically. Excess concentrations of ethanol and salt, negatively affected the Y_T due to the reduction of free volume in the top phase and the precipitation of enzyme at the interface by the extreme salting-out effect [25].

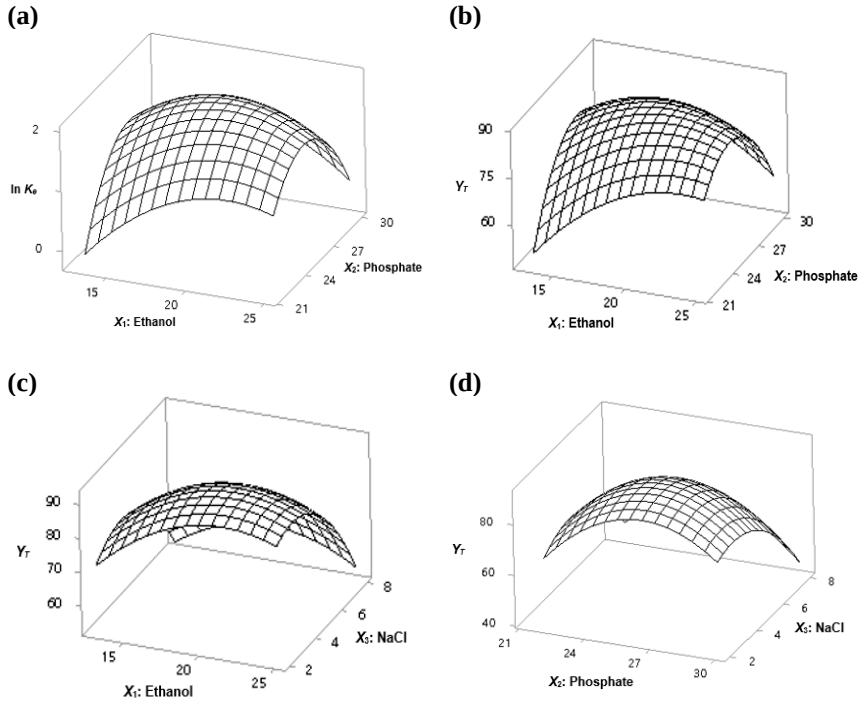

Fig. 4. Response surface plot for the optimization of the recovery amylase from pitaya peels in ATPS. The interaction effects of (a) ethanol and phosphate concentrations in % (w/w) on the partition coefficient of amylase, ln K_e; the interaction effects of (b) ethanol and phosphate concentrations in % (w/w), (c) ethanol and NaCl concentrations in % (w/w), (d) phosphate and NaCl concentrations in % (w/w) on the yield on amylase, Y_T.

On the contrary, the Y_T was lesser at lower concentrations of ethanol and salt as the enzymes are present in a more solubilized state [26]. As observed from Figs. 4(c) and 4(d), the addition of NaCl resulted in a significant yield of amylase since the NaCl has a direct positive effect on the Y_T (Eq. 9). While the phosphate ions help to enhance hydrophobic interaction between the amylase and ethanol, the addition of NaCl helps to generate an electrical potential difference between the two aqueous phases that promote the partitioning of amylase towards the top phase, thereby improving the Y_T [7]. However, high phase compositions and NaCl concentration resulted in a minimum Y_T (Figs. 4(c) and 4(d)), as a greater amount of water molecules are required to solubilize the salt and, consequently denature the protein in the ATPS [23]. An optimal Y_T of was observed at 19% (w/w) ethanol, 26% (w/w) phosphate at pH 7.0, and 3.8% (w/w) NaCl, revealing a fine balance

among the salting-out effect, the hydrophobic and electrostatic interactions between the amylase and the ATPS phase components.

To validate the models, the experimental values obtained from the recovery of amylase from pitaya peels using ATPS, which composed of 19% (w/w) ethanol, 26% (w/w) phosphate at pH 7.0, and 3.8% (w/w) NaCl, were compared with the responses predicted by the final reduced models. Table 4 shows that the experimental values were consistent with the predicted responses, thus confirming the validity of the established models for the recovery of amylase.

Table 4. Validation of the predicted responses and experimental results for the recovery of amylase under optimum ATPS conditions.

ln K_e			Y_T (%)		
Predicted	**Experimental mean**	**Similarity**	**Predicted**	**Experimental mean**	**Similarity (%)**
2.048	2.512 ± 0.09	81.5%	90.83	92.48 ± 0.61	98.2%

4. Conclusions

This study demonstrated the feasibility of using ATPS for the recovery of amylase from the pitaya peels. A central composite design was applied to establish appropriate models for the amylase recovery parameters (ln K_e and Y_T) and the best ATPS conditions for the recovery of amylase. An optimum recovery of amylase from the pitaya peels can be achieved using an ATPS that contains 19% (w/w) ethanol, 26% (w/w) phosphate at pH 7.0, and 3.8% (w/w) NaCl, Under this optimized conditions, 92.48% of amylase was recovered in the ethanol-rich phase with a partition coefficient, ln K_e of 2.512 and a purification factor of 5.75. These results suggest that the ethanol/phosphate ATPS can serve as a potential recovery method for the recovery of amylase from plant sources.

Acknowledgement

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Nomenclatures

A_T	Amylase activity in the top phase, U/mL
A_B	Amylase activity in the bottom phase, U/mL
K_e	Partition coefficient for amylase
K_P	Total protein partition coefficient
P_F	Purification factor
P_T	Total protein concentration in the top phase, mg/mL
P_B	Total protein concentration in the bottom phase, mg/mL
S	Selectivity
V_B	Volume of bottom phase
V_R	Volume ratio
V_T	Volume of top phase
X_1	Ethanol concentration, % (w/w)
X_2	Potassium phosphate at pH 7.0 concentration, % (w/w)
X_3	NaCl concentration, % (w/w)

Y	Predicted response
Y_T	Yield for the amylase recovers in the top phase, %

Greek Symbols

β_0	Offset term
β_i	Coefficients for the linear term
β_{ii}	Coefficients for the quadratic term
β_{ij}	Coefficients for the interactive term

Abbreviations

ATPS	Aqueous two-phase system
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCD	central composite design
DNS	Dinitrosalicylic acid
NaCl	Sodium chloride
pI	Isoelectric point
RSM	Response surface methodology

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