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ORIGINAL ARTICLE

Use of Aqueous Two-Phase System for Partial Purification and Characterization of α-Amylase from *Burnatia enneandra* Micheli

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Abstract

Alpha-amylase from *Burnatia enneandra* Micheli, a tuber plant from a rural area of Cameroon, extracted under optimal conditions was purified using aqueous two-phase extraction system (ATPS). The separation behaviour was further investigated. Polyethylene glycol (PEG, % w/w), ammonium sulphate (AS, % w/w) and pH were optimized for the purification of B. *enneandra* alpha-amylase. Optimum conditions for purification factor were PEG, 13.1 %, AS, 12 % and pH 4. Highest enzyme recovery was achieved at PEG, 13.0 %, AS, 12 % and pH 5.1. Further, enzyme characterization surveys acknowledged that alpha-amylase formed was just an acidic enzyme. The pH and temperature at which the enzyme was well expressed were 5.5 and 70 °C respectively. Another characteristic of the enzyme was determined like the Michaelis-Menten constant (Km) and Vmax which value obtained was respectively 89.32 µg/mL and 2.99 µg/mL/min.

Practical application

The alpha-amylase obtained from local *Burnatia enneandr*a Micheli tuber can be used for brewing purpose and other field using starch (fruit juice, spirit, sirop etc). This is quite important as none of the amylase enzymes used by factories in Cameroon is from local source.

Keywords: *Burnatia enneandra* Micheli; ATPS; modeling; purification; Km; Vmax.

1. Introduction

It is very well reported that plants are rich wellsprings of enzymes. The possible utilization of α-amylases is gigantic and they are widely used as a part of drinks, child sustenance, therapeutic and pharmaceutical companies. Amylases, classified as E.C.3.2.1.1. endo - 1-4-glucan-4-glucanohydrolase, are extra-cellular enzymes that arbitrarily breakdown the 1,4-d-glucosidic bonds between contiguous glucose units in the straight amylose chain.

The improvement of amylase generation ventures especially partition, purification and formulation are of essential significance because of their effect on the economy. The experimental design was generally used to determine the factors that have a significant impact on the aqueous two-phase system (ATPS) enzyme purification. This methodology was effectively utilized for advancement, displaying and control also (Porto *et al.*, 2007; Zhi *et al.*, 2005). ATPS constitutes an ideal response to the solicitation of

quick, economic and simple processes. From one perspective, this procedure is by all accounts a perfect technology where partial purification, concentration and clarification of the main outcome could be coordinated in one stage (Mohamadi *et al.*, 2007). It is a technique widely used in biotechnology for the separation and purification of biological substances such as enzymes and proteins. Several advantages are to be taken into account such as the simplicity, the speed of the separation with a minimal denaturation of the enzymes (Gupta *et al.*, 2007). The most important part of both phases is water (80 to 90%) and the vast majority of polymers have a stabilizing impact on the protein structure. This technique presents a favourable environment for the extraction and purification of enzymes (Rosa *et al.*, 2007). The adequacy of ATPS subsequent to handling was affirmed on the extraction, partition, concentration and purification of a few compounds including amylase (Biazus *et al.*, 2007; Li & Peebles, 2004; Zhi *et al.*, 2005). Various polymers and salts were utilized for the planning of these bi-stage frameworks. Among them, polyethylene glycol PEG/ammonium sulphate (Mohamadi & Omidinia, 2007) has been utilized in chemical filtration methods. *Burnatia enneandra* Micheli tubers are traditionally used in Cameroon to sweeten the slurry (Taira *et al.*, 2015). Some studies highlighted amylolytic potential in this plant (Taira *et al.*, 2015). The aim of this study is to us response surface methodology (RSM) as a means to optimize the purification parameters of α-amylase from *Burnatia enneandra* Micheli tubers using ATPS and, characterize the α-amylase purified.

2. Materials and Methods

2.1. Material

Burnatia enneandra Micheli tubers from Datcheka (Yagoua, Cameroon) were the plant material from which the α-amylase enzyme was extracted and partially purified. After the cleaning process which consisted of removing dust and sand from the tubers using tap water, they were sliced, dried (in CKA AUF-2000 at 45 °C for 5 days) and milled using a Polymix PX-MFC 90D hammer mill apparatus type (VWR International S.A.S. Le Perigas 201, rue Carnot, 94126 Fontenay-sous-Bois Cedex, France) to obtain a flour ($Ø \leq 250$ µm).

2.2. Extraction of Burnatia enneandra tubers enzymes

Flour (20 g) from *Burnatia enneandra* tubers was measured using a scale (KERN EMB 200-3, Sysmatec Oberdorfstrasse) and introduced into a 500 mL beaker. After that, 200 mL of 200 mM phosphate buffer at pH 4 was added. The beaker was then agitated at 147.3 rpm for 1 h using a laboratory stirrer (model: AM120Z-P, Meditry Instrument Co. Ltd). The mixture was allowed to rest for 15 min and the whole volume of supernatant obtained was transferred into 45 mL tubes and centrifuged at 6500 × g for 10 min at 4 °C, using the Heraeus-Kendro Lab products refrigerated centrifuge (Model: Biofuge primo R, type: D-37520, Germany). The composed supernatant obtained from centrifugation was considered as the enzyme extract to be purified.

2.3. Purification by aqueous two-phase extraction

In a 45 mL centrifuge tubes were added appropriate quantities of polyethylene glycol (PEG), ammonium sulfate, and 2 ml of crude enzyme extract. The mixture was adjusted to 10g by introducing phosphate buffer at required pH. This mass was adequate for the centrifuge tubes.

Then, the mixture was stirred (using a laboratory stirrer, model: AM120Z-P, Meditry Instrument Co. Ltd) and allowed to separate for 1 h at room temperature. To ensure better phase separation, the tubes were centrifuged at 1400 × g for 10 min at 4 °C in Heraeus-Kendro Lab products refrigerated centrifuge (Model: Biofuge primo R, type: D-37520, Germany). Portion of each phase was collected separately to measure the amount of protein and enzyme activity.

2.4. Protein determination

The protein content was estimated applying Lowry's procedure (Frolund *et al.*, 1996; Gerhardt *et al.*, 1994; Lowry *et al.*, 1951). A sample volume of 0.1 mL was mixed with an equal volume of 2 N NaOH. After that, the test tubes were sealed and incubated in a boiling water bath (Memmert) for 10 min and subsequently allowed to cool to 25 °C. A complex-forming reagent solution was prepared by combining, 100:1:1 stock solution (2% (w/v) sodium potassium tartrate; 2% (w/v) Na_2CO_3; 1% (w/v) $CuSO_4$ $5H_2O$) and 1 mL of this reagent was added to the mixture. The reaction occurred at 25 °C in the water bath (Memmert) for 10 min. Hereafter, 0.1 mL of Folin reagent was added and, the mixture allowed to rest in darkness at the same temperature for 60 min. The absorbance was therefore measured at 750 nm (for concentration of protein ≤ 500 µg/mL) and 550 nm (for concentration of protein between 100 µg/mL and 2000 µg/mL) respectively. Standard curve was made using Bovine Serum Albumin as reference protein.

2.5. α-amylase activity measurement

The activity of α-amylase was estimated using Fuwa's colorimetric technique of iodine-starch color reaction (Arpana et *al.*, 2010). A 50 µL of α-amylase solution in a 50 mM phosphate buffer (pH 7.0) was blended with 100 µL of a preboiled 1.1 % (w/v) soluble-starch solution and hatched at 60 °C for 10 min. The reaction was then stopped by adding 250 µL of stopping solution (a mixture of 0.5 N HCl 5:1 ratio and 0.5 N acetic acid), and a 100 µL of the reaction solution was stirred with 1 mL of iodine reagent (0.1 % KI and 0.01 % Iodine). The absorbance was read at 660 nm after incubation for 20 min at 25 °C (room temperature).

One enzymatic (α-amylase) unit was expressed as the measure of enzyme which diminished in 10 min by 0.1, the optical density of 660 nm.

$$A_T = \frac{100 \times d \times (OD_B - OD_S)}{OD_B} \quad (1)$$

The specific activity (A_S) in U/mg was calculated as follows:

$$A_S = \frac{A_T}{P} \quad (2)$$

With: A_T = Total activity (U); d = factor of dilution; OD_B = blank optical density; OD_S = sample optical density; P = quantity of protein (mg).

2.6. Estimation of partition parameters

The partition parameters were all estimated according to the method described by Hamid & Eskander (2008).

The partition coefficient (K_e) was descripted as the proportion between the enzyme activity in the upper phase (A_U) and the enzyme activity in the bottom phase (A_B). It was expressed as:

$$K_e = \frac{A_U}{A_B} \quad (3)$$

The partition coefficient for enzyme concentration (K_p) was interpreted as the ratio amount of protein in the upper phase (P_U) amount of protein in the lower phase (P_B). It was expressed as:

$$K_p = \frac{P_U}{P_B} \quad (4)$$

The selectivity S was characterized as the proportion between K_e and K_p. It was expressed as:

$$S = \frac{K_e}{K_p} \quad (5)$$

2.7. Purification factor (F_P)

This was considered as the ratio of the specific activity of the upper phase (AS_H) and that of the crude extract (AS_E) and, was expressed as:

$$F_P = \frac{AS_H}{AS_E} = \frac{A_H / C_H}{A_e / C_e} \quad (6)$$

With: A_H, enzyme activity in the upper phase; C_H, amount of protein in the upper phase; A_e, activity of the enzyme in the crude extract; C_e, amount of this protein in the crude extract.

2.8. Enzyme recovery

The enzyme recovery (R_a) was considered as the proportion in percentage of remaining purified protein compared with the initial amount. It was expressed by the following formula:

$$R_a = \frac{A_H V_H}{A_e V_e} \times 100 \quad (7)$$

With: V_H and V_e respectively the volumes of the upper phase and the crude extract

2.9. Experimental design, modelling, validation of the model and optimization

The Doehlert experimental design (Goupy & Creighton, 2006) was used to realise the partial purification of the enzyme. This was done to model α-amylase partial purification from *Burnatia enneandra* Micheli. The responses for this study were the purification factor and the enzyme recovery. While, the factors were: PEG concentration (x1), ammonium sulfate concentration (x2) and pH (x3).

For laboratory purposes, the coded-variables from Doehlert experimental design matrix were transformed into real variables using:

$$X_i = X_{0i} + x_i \Delta X_i \quad (8)$$

$$N = k^2 + k + k_0 \quad (9)$$

Where: X_i, real variables; X_{0i}, centre of variable; x_i, coded variable; ΔX_i, increment; k, number of variables; k_0, number of centre points and N, number of experiments.

The second-order equation which resulted from that approach was polynomial multivariable (Ekorong *et al.*, 2015) and was written as follows:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 + \sum \beta_{ij\ldots z} x_i x_j \ldots x_z + e \quad (10)$$

With: Y, is the response; x_i and x_j, assigned level of the factor i and j respectively; $\beta_0, \beta_i, \beta_{ii}, \beta_{ij}$ coefficients of the model.

The models and statistics were performed using Minitab 19 (Minitab, Ltd. Coventry, UK) and, the graphs were plotted using Sigmaplot version 14 (Systat Software, Inc. Wbcuped, GmbH, Germany). In order to validate the models, some formula were applied (Ekorong *et al.*, 2015) :

$$AAD = \frac{\left[\sum_{i=1}^{N}\left(\frac{\left|Y_{i\exp} - Y_{ical}\right|}{Y_{i\exp}}\right)\right]}{N} \quad (11)$$

$$B_f = 10^{\frac{1}{N}\sum_{i=1}^{N}\log\left(\frac{Y_{ical}}{Y_{i\exp}}\right)} \quad (12)$$

$$A_{f1} = 10^{\frac{1}{N}\sum_{i=1}^{N}\left|\log\left(\frac{Y_{ical}}{Y_{i\exp}}\right)\right|} \quad (13)$$

With: $Y_{i,exp}$ and $Y_{i,cal}$; experimental and theoretical (mathematical) responses respectively; N, number of trials; AAD, absolute average deviation; $B_{f,}$ bias factor; A_f, accuracy factor.

Contributions of each variable and interaction were estimated as follows (Ekorong *et al.*, 2015):

Linear,

$$Contribution(\%) = \frac{\left|\beta_i\right|}{\sum_{i=1}^{k}\left|\beta_i\right| + \sum_{i=1}^{k}\left|\beta_{ii}\right| + \sum\sum_{i<j}^{k}\left|\beta_{ij}\right|} \quad (14)$$

Quadratic,

$$Contribution(\%) = \frac{\left|\beta_{ii}\right|}{\sum_{i=1}^{k}\left|\beta_i\right| + \sum_{i=1}^{k}\left|\beta_{ii}\right| + \sum\sum_{i<j}^{k}\left|\beta_{ij}\right|} \quad (15)$$

Interaction,

$$Contribution(\%) = \frac{\left|\beta_{ij}\right|}{\sum_{i=1}^{k}\left|\beta_i\right| + \sum_{i=1}^{k}\left|\beta_{ii}\right| + \sum\sum_{i<j}^{k}\left|\beta_{ij}\right|} \quad (16)$$

The theoretical optimal combination for each response was obtained using Mathcad software v15.0 (build 15.0.0.436 Parametric Technology Corporation, USA). In order to optimize the enzyme purification, the targets were as follows according to the literature (Kammoun *et al.*, 2009; Zhi *et al.*, 2005): purification factor ≥ 3.5 and enzyme recovery ≥ 55 %

The graphs were superimposed using Sigmaplot version 12.5 (Systat Software, Inc. Wbcuped, GmbH, Germany) and, the intersection was marked, to represent the experimental zone which respected the two conditions.

2.10. Characterization of partially Purified enzyme

2.10.1. Effect of temperature and pH on the enzyme activity

This experiment was conducted in order to figure out the optimal temperature of α-amylase. It was done according to Fuwa's colorimetric (Arpana *et al.*, 2010) modified method for determining enzyme activity. The temperatures applied were ranged between 30 and 90 °C (30, 40, 50, 60, 70, 80 and 90 °C) for 10 min during incubation (allowing the enzyme to hydrolyze starch) to investigate the effect of temperature, while the same experiment was done varying the pH from 4 to 9 to estimate the effect of pH. The pH solution was rectified using a phosphate buffer system prepared at specified pH. Citrate-phosphate buffer was used for pH 4-7 and barbital buffer for pH 7-9. The citrate-phosphate buffer was made of citric acid (0.1 M) and

dibasic sodium phosphate (0.2 M), while the barbital buffer was containing sodium barbital (0.2 M) and HCl (0.2M). The resulting activities are expressed as a proportion of the highest activity.

2.10.2. Determination of kinetic parameters

Estimation of the kinetic parameters for α-amylase hydrolysis were determined by introducing the enzyme (50 µL of α-amylase) in variating substrate (0.5 mL) concentration (0, 50, 100, 150, 200, 250 and 300 µg/mL). This permitted to obtain for each starch concentration an initial velocity. After that, the Michaelis-Menten curve was linearized using Lineweaver-Burk plot. The maximum rate values, Vmax (µg/min) and Michaelis-Menten constant Km (µg/mL) were estimated and all the tests were performed at 70 °C and pH 5.5.

3. Results and Discussion

3.1. Choice of purification phase

It was observed in Table 1 that the partition coefficients of the enzyme were for most of the experiments greater than 1.00. This suggests that the activity of the enzyme was concentrated mainly in the upper phase demonstrating its affinity for the upper phase. This result was also consistent with the literature where it was reported that, when using "biphasic aqueous extraction" variant, including polymer/salt, the enzymes are more concentrated in the upper phase (Ratanapongleka, 2010).

Table 1: Partition parameters evaluation

Trial number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Separation coefficient (K_s)	5.99	4.00	3.80	5.38	4.43	4.14	4.29	1.33	5.47	4.56	8.28	1.85	2.39	6.37	8.00	8.01	7.14
Partition coefficient (K_s)	1.61	1.00	1.14	1.13	0.81	0.76	1.13	0.71	1.22	0.25	1.04	1.04	0.67	1.66	1.61	1.64	1.76
Selectivity (S)	3.72	4.00	3.33	4.76	5.46	5.44	3.79	1.87	4.48	18.2	7.96	1.77	3.56	3.83	4.96	4.88	4.05

3.2. Modeling of purification factor and enzyme recovery

As it was discovered that the upper phase is the phase of interest, RSM was used to model and optimize the purification factor and enzyme recovery of that phase of interest. The α-amylase was purified using a 3 variables Doehlert experimental design and in each case, the enzyme recovery and purification factor were acquired (Table 2). The purification was carried out from a crude extract having 1275 µg/mL of protein and an enzymatic activity of 60.35 U/mL.

The models via matrix calculation were accessed using Minitab version 19 software (Minitab, Ltd. Brandon Court Unit E1-E2 Progress Way Coventry CV3 2TE UK). These models were as follows:

$$P_F = 3.63 - 0.30x_1 - 0.40x_2 - 1.82x_3 + 0.72x_1x_2 - 0.42x_1x_3 + 0.19x_2x_3 - 0.97x_1^2 + 0.59x_2^2 - 0.84x_3^2 \quad (17)$$

$$Y = 60.51 - 6.82x_1 - 6.33x_2 - 15.15x_3 + 5.56x_1x_2 + 15.10x_1x_3 - 3.75x_2x_3 - 23.12x_1^2 - 0.79x_2^2 - 41.40x_3^2 \quad (18)$$

Where: P_F, predictive model for purification factor; Y, predictive model for enzyme recovery; x_1, PEG concentration; x_2, $(NH_4)_2SO_4$ concentration and x_3, pH.

The statistical models obtained were 3 variables polynomials equations with R^2 equal 0.991 and 0.923 respectively for purification factor and enzyme recovery. These coefficients, combined to the AAD values of 0.030 and 0.161; to bias factor of 1.000 and 0.995 and to exactitude factors of 1.030 and 1.185, respectively for purification factor and enzyme recovery (Table 2), permitted the validation of the models (Ross, 1996). The variables of the models were of first degree (x_1, x_2 and x_3), second degree (x_1^2, x_2^2 and x_3^2) and interaction (x_1x_2, x_1x_3 and x_2x_3) form. The significance of the variables effect was considered when the probability (P) was ≤ 0.05 (Table 3).

Table 2: Doehlert experimental design and validation parameters

Coded values			Real values				Proteins (µg/mL)	Total activity (U/mL)	Specific activity (U/mg)	Responses			
										Purification factor		Enzyme recovery (%)	
X_1	X_2	X_3	PEG (%)	AS (%)	pH					Exp	Theo	Exp	Theo
0	0	0	14	15	5.5		725±25	125±5.5	172.41±6.8	3.64±0.3	3.63	59.22±0.9	60.51
1	0	0	17	15	5.5		600±14.2	70.27±3.8	117.11±4.9	2.47±0.11	2.36	38.69±1.8	30.57
-1	0	0	11	15	5.5		600±19.2	80.08±4.4	135.13±4.8	2.85±0.14	2.96	36.08±1.72	44.21
0.5	0.866	0	15.5	18	5.5		650±10.0	108±6.2	166.32±5.8	3.51±0.25	3.64	42.86±2.01	47.65
-0.5	-0.866	0	12.5	12	5.5		550±8.2	124.3±5.0	226.04±6.5	4.77±0.26	4.64	70.23±0.8	65.44
0.5	-0.866	0	15.5	12	5.5		500±11.1	92.33±3.1	167.87±7.4	3.54±0.01	3.72	53.84±0.82	53.80
-0.5	0.866	0	12.5	18	5.5		650±20.0	107.8±5.1	165.84±8.2	3.50±0.13	3.32	49.65±1.7	49.66
0.5	0.289	0.816	15.5	16	7		500±5.0	26.63±2.8	53.27±2.8	1.12±0.02	1.10	12.26±1.01	15.58
-0.5	-0.289	-0.816	12.5	14	4		675±7.58	146.8±4.8	217.54±5.4	4.59±0.29	4.61	54.11±0.86	50.78
0.5	-0.289	-0.816	15.5	14	4		225±5.25	48.03±3.0	213.48±6.1	4.51±0.21	4.44	21.88±1.5	30.03
0	0.577	-0.816	14	17	4		625±14	130.1±6.2	208.17±4.8	4.39±0.11	4.43	47.97±2.4	43.15
-0.5	0.286	0.816	12.5	16	7		600±13.5	35.8±1.9	69.67±4.8	1.47±0.14	1.54	16.67±4.01	8.51
0	-0.577	0.816	14	13	7		475±9.26	44.1±6.0	92.85±2.8	1.96±0.01	1.92	20.89±1.45	25.74
0	0	0	14	15	5.5		750±17.8	130.1±8.2	173.47±6.8	3.66±0.09	3.63	60.97±1.85	60.51
0	0	0	14	15	5.5		725±23.5	122.4±4.8	168.9±7.4	3.57±0.07	3.63	60.25±3.01	60.51
0	0	0	14	15	5.5		700±16.1	122.5±3.2	174.93±5.5	3.7±0.21	3.63	58.66±0.58	60.51
0	0	0	14	15	5.5		750±12.4	127.5±5.8	170.07±4.9	3.59±0.05	3.63	63.45±1.01	60.51

Models	R^2	AAD	B_f	A_f
P_F	0.991	0.030	1.000	1.030
Y	0.923	0.161	0.995	1.185

Exp: experimental, Theo: theoretical, x1: PEG concentration (%), x2: AS (ammonium sulfate concentration, %), x3: pH

Table 3: Estimated coefficients impact and contributions

	Purification factor			Enzyme recovery		
	Coefficients	Probabilities	Contributions (%)	Coefficients	Probabilities	Contributions (%)
X_1	-0.30	0.004	4.80	-6.82	0.112	5.78
X_2	-0.40	0.001	6.40	-6.33	0.135	5.36
X_3	-1.82	0.000	29.12	-15.15	0.005	12.84
X_1X_2	0.72	0.004	11.52	5.56	0.541	4.71
X_1X_3	-0.42	0.060	6.72	15.10	0.163	12.79
X_2X_3	0.19	0.334	3.04	-3.75	0.711	3.18
X_1^2	-0.97	0.000	15.52	-23.12	0.008	19.59
X_2^2	0.59	0.002	9.44	-0.79	0.903	0.67
X_3^2	-0.84	0.000	13.44	-41.40	0.000	35.08

3.2.1. Impact of pH on purification factor and enzyme recovery

The effect of the pH (x_3) was significant on the decrease of both purification factor (P = 0.000, Table 3) and enzyme recovery (P = 0.005, Table 3), with respective percentage contribution at 29 % and 13 %. As observed in Figure 1A, the purification factor started from 5.08 at pH 4 and decreased to 2.53 at pH 7. The Figure 1B exhibited an enzyme recovery from 48.38 % at pH 4 then, followed by a non-significant increase to 58.31 % at pH 4.9. After that, the enzyme recovery decreased to 4.31 % at pH 7. As it is well known in this type of purification technic, the partition of proteins (enzymes) is linked to the pI (isoelectric point) (Forciniti *et al.*, 1991). The ATPS technique because of its pH, can affect the properties of surfaces and the solute load, which changes the distribution of the enzyme. This charge becomes negative in the case where the pH is higher pI and positive if this pH is lower than the pI. If there is a tie, the charge becomes zero. It has been stated that the separation of a negatively charged biomolecule in a high pH system increases the partition coefficient and that the biomolecule is found by affinity in the upper phase (Raja *et al.*, 2011). Another way of explaining the decrease in purification factor and enzyme recovery could be linked to the alteration of the charged species ratio in the system (Brockett, 1984). The quadratic level (x_3^2) confirmed that it was useless to increase the pH. Its contribution to the decrease was 14 % and 35 % respectively for purification factor and enzyme recovery.

3.2.2. Impact of PEG concentration on purification factor and enzyme recovery

The variable x_1, corresponding to the PEG concentration, exhibited a significant effect on the purification factor of α-amylase (P = 0.004, Table 3) while, that effect was not significant on enzyme recovery (P = 0.112, Table 3). It contribution was 5 % and 6 % (Table 3) respectively for purification factor and enzyme recovery. As observed in Figure 1C, the purification factor started from 5.08 at 11 % PEG concentration to 5.56 (non-significant increase) at 13.1 % PEG concentration.

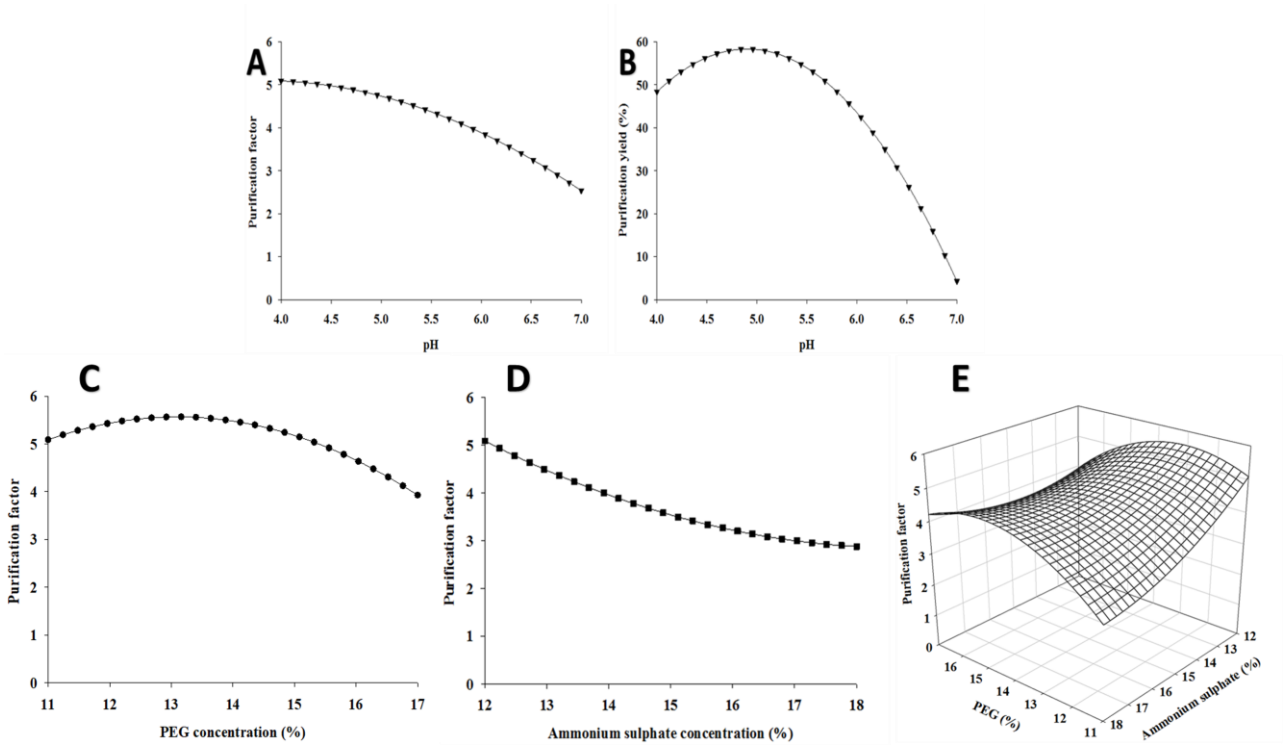

Figure 1: Effect of factors on responses: A- Effect of pH on purification factor; B- Effect of pH on purification yield; C- Effect of PEG concentration on purification factor; D- Effect of Ammonium sulphate concentration on purification factor; E- Effect of PEG/Ammonium sulphate interaction on purification factor

After a significant decrease to 3.95 at 17 % PEG concentration was registered. In the literature the PEG molecular weight has a high impact on separation of biomolecules (Hemavathi & Raghavarao, 2011; Mehrnoush *et al.*, 2012; Yucekan & Onal, 2011). Since the PEG used had high molecular weight (PEG 6000) and low coefficient factor, lower measure of PEG was then required to realize the separation as reported by Raja et *al.* (2011). In fact, an increase of PEG concentration decreases the free volume (Ibarra-Herrera *et al.*, 2011; Priyanka *et al.*, 2012; Priyanka & Rathindra, 2012; Yucekan & Onal, 2011) and therefore enhance the separation in the bottom phase (Hatti-Kaul, 2000). At the quadratic level (x_1^2), the effect of PEG concentration was critical on the decrease of purification factor (P = 0.000, Table 3) and enzyme recovery (P = 0.008, Table 3). It contributed for 16 and 19 % respectively on the reduction of purification factor and enzyme recovery. This confirm the fact that there was no need of expanding the concentration of PEG to get high purification factor and enzyme recovery.

3.2.3. Impact of ammonium sulfate concentration on purification factor and enzyme recovery

The impact of ammonium sulphate (x_2) was significant on the purification factor (P = 0.001, Table 3) but not on enzyme recovery (P = 0.135, Table 3). The contributions were 6 and 5 % respectively (Table 3). From Figure 1D,

purification factor value was 5.08 at 12 % ammonium sulphate concentration and decreased to 2.88 at 18 % ammonium sulphate concentration.

This could be due to an increase in ammonium sulphate that generated the transfer of proteins to the bottom phase, resulting to the decrease of the upper phase volume while increasing that of the bottom phase volume. This was also observed in the literature (Goja *et al.*, 2013).

3.2.4. *Effect of interaction PEG/Ammonium sulphate (x_1x_2) on purification factor and enzyme recovery*

The interaction x_1x_2 had a significant impact on purification factor (P = 0.004, Table 3) and, a non-significant impact of enzyme recovery (P = 0.541, Table 3). As presented in Figure 1E, there was a synergistic effort between the 2 variables on the purification factor.

In that synergistic effort, PEG was contributing to the phase forming system properties (Banks & Tomas-Rodriguez, 2003; Yucekan & Onal, 2011) while, the salt ($(NH_4)_2SO_4$) was contributing to the differential partitioning and enzyme activity recovery (Hemavathi & Raghavarao, 2011). It was also mentioned in the literature that the system PEG/salt was more efficient for better separation and purification (Goja *et al.*, 2013). The contribution of that interaction was estimated at 11 % for purification factor and 5 % for enzyme recovery.

3.2.5. *Impact of interactions PEG/pH (x_1x_3) and pH/Ammonium sulfate (x_2x_3) on purification factor and enzyme recovery*

No significant effects were found with the two interactions on both purification factor and enzyme recovery. In fact, for interaction PEG/pH (x_1x_3), the probabilities obtained were 0.060 for the purification factor and 0.163 for the enzyme recovery, while, for pH/$(NH_4)_2SO_4$ (x_2x_3) interaction, the probabilities were 0.334 and 0.711 respectively.

3.3. *Optimization of partial purification factors*

The optimization of each singular response gave different triplet. The maximization of purification factor gave the triplet 13.1 %, 12.0 %, 4 respectively for PEG concentration, $(NH_4)_2SO_4$ concentration and pH. The maximum purification factor obtained with that triplet was 5.56. Maximizing the enzyme recovery also generated the triplet 13.0 %, 12.0 %, 5.1 respectively for PEG concentration, $(NH_4)_2SO_4$ concentration and pH. The maximum enzyme recovery was therefore evaluated at 68.44 %.

The definition of some specifications provided a compromise between the two responses. The specifications defined for optimization considered the purification factor ≥ 3.5 and enzyme recovery ≥ 55 % as reported in the literature (Kammoun *et al.*, 2009; Zhi *et al.*, 2005). When the pH is fixed at 5, the intersection corresponding to these two specifications was presented in Figure 2.

That intersection was obtained after superimposing the purification factor and the enzyme recovery contour plots. All the combinations taken in the highlighted area permitted to respect the specifications presented before.

3.4. *Characterization of the partially purified enzyme*

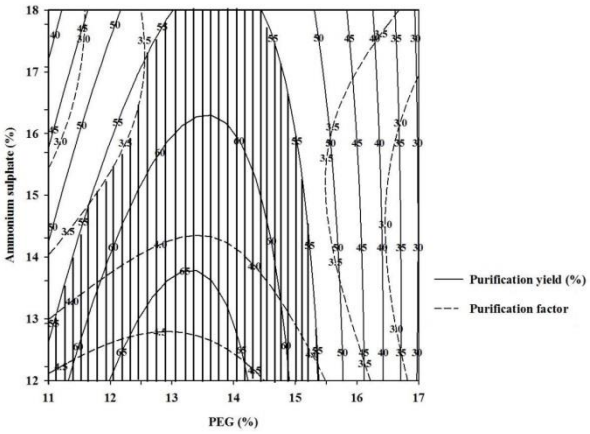

Figure 2: Optimized contour plot area for purification factor and yield

3.4.1. Effect of temperature and pH on the relative enzyme activity

The relative activity determined for a temperature ranged between 30 and 90 °C gave the results presented in Figure 3A. It was observed that, the activity of the enzyme increases with temperature until it reaches a maximum activity at 70 °C and then, suddenly decrease and lost almost half of its activity at 80 °C and; was preserving only about a quarter of its activity at 90 °C. In fact, interactions among all molecules increased with temperature. This could be because of the increase in momentum and kinetic energy that were linked to temperature expansion. With higher momentums, there was lower time for interactions. This resulted in many molecules reaching the activation energy, hence the reaction rate increased. Considering that the molecules were speedy, contacts among enzymes and substrates along increased. The decreasing phase of the graph could be explained by the irreversible degradation (inactivation) of protein (enzyme) appearing from destruction of covalent bonds. The mechanisms of that degradation were therefore increasing at high temperatures. The optimum activity reached at 70 °C confirmed that the α-amylase isolated from the tuber was stable. This thermal stability would suggest the formation of a stable, enzyme-substrate complex at this temperature (70 °C), which protected the enzyme from denaturation by heat (Arpana *et al.*, 2010).

The relative enzyme activity was also determined by varying the pH while maintaining the temperature constant at 70 °C. The results obtained were shown in Figure 3B.

The enzyme activity increased gradually to an optimum pH ranged between 5.5 and 6, and then dropped to 25 % of relative activity (meaning a drop of 75 %) at pH 9. In fact, every enzyme could be efficient in a short pH range. In that range, the enzyme activity expression could be considered as highest at a specific pH called optimal pH. The reduction in activity could be attributed to the breakdown of intra and intermolecular bonds due to the change in pH, the result could be the modification of the enzyme shape and efficiency. The optimum pH reached 5.5 to 6, was suggesting that the α-amylase isolated from the tuber (*Burnatia enneandra*) would be helpful in acidic processes, that was in conformity with optimum pH for plant sources α-amylases (Barbosa *et al.*, 2011; Mohamadi *et al.*, 2007; Ratanapongleka, 2012).

3.4.2. Kinetic parameters determination

Enzyme kinetic parameters were measured by allowing the purified enzyme to react while increasing the concentration of substrates. This permitted to draw the curves representing the product formation with time (Figure 3C). Since initial hydrolysis velocity should be measured in the beginning of the reaction, only the linear parts of the curves were considered to determine

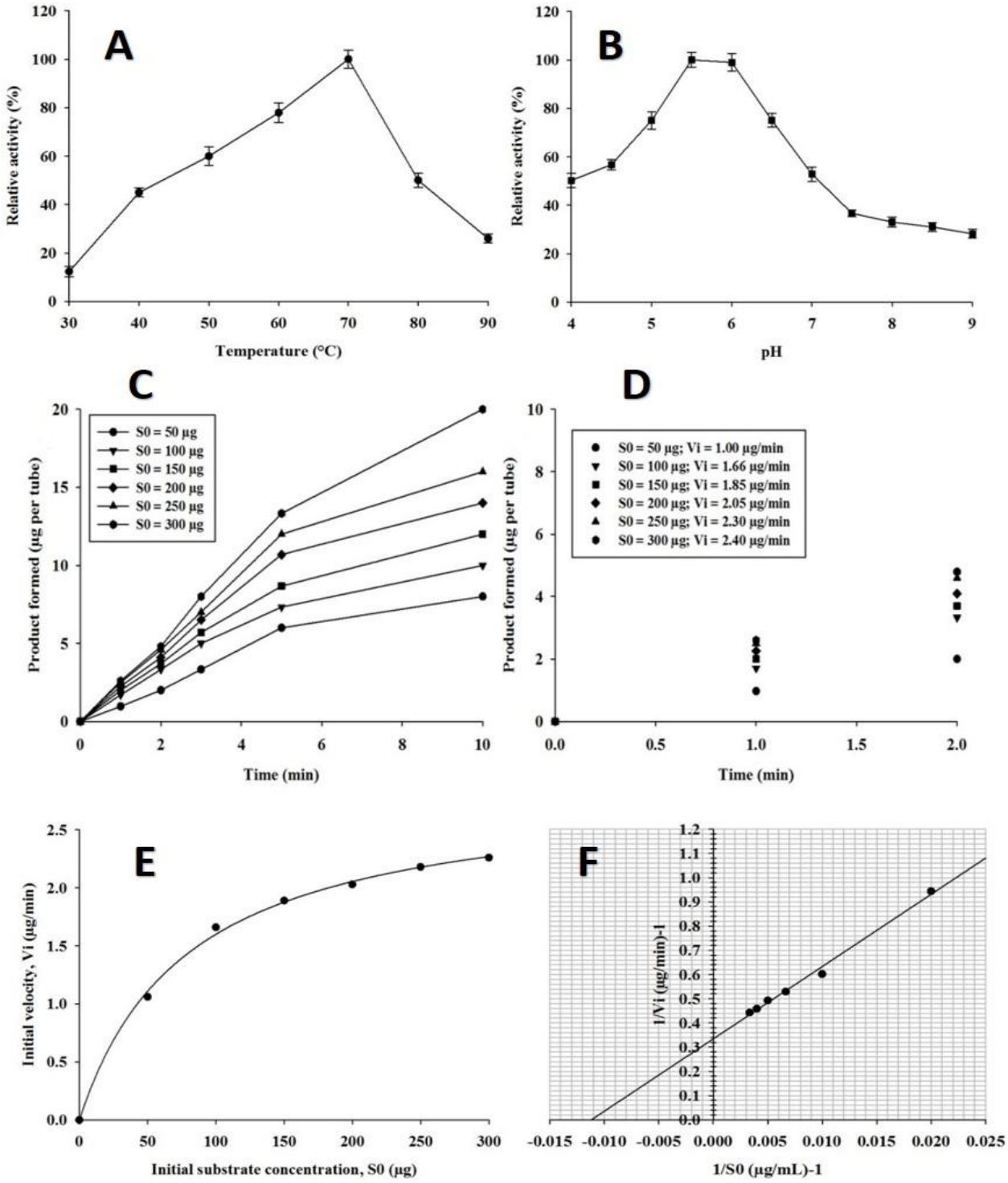

Figure 3: Characterization of the enzyme: A- Effect of temperature on the relative enzyme activity; B- Effect of pH on the relative enzyme activity; C- Evolution of product formed with time; D- Initial velocity: E- Saturation curve; F- Lineweaver–Burk plot

these initial velocities as presented in Figure 3D. Keeping only the linear parts of the curves, the slopes were determined since they were corresponding to the initial velocities. The saturation curve (Figure 3E) was realized to confirm the Michaelis-Menten shape. By using the Lineweaver–Burk plot (Figure 3F), a K_m of 89.32 µg/mL and of V_{max} 2.99 µg/min were obtained. The low K_m value indicated a high affinity of the enzyme for the substrate (Su & Chiang, 2006). The values of K_m and V_{max} obtained were different from the one depicted by Klang *et al*. (2014). This could be due to the extraction conditions and the origin of *Burnatia enneandra* Micheli tubers. Klang et al. (2014) used phosphate buffer 50 mM at pH 6.0 (containing 5 mM of β-mercaptoethanol) and the tubers was coming from Kalfou.

4. Conclusion

At the end of this research, which was based on the partial purification of α -amylase from the raw extract of the *Burnatia enneandra* Micheli tubercle by the ATPS technique, it is retained that this technique is able to concentrate the α-amylase from this tuber as far as we obtain purification factors greater than unity for all the experimental points. The objective which was to purify the enzyme from this tuber is reached. The characterization of the purified enzyme shows that its optimum temperature is 70 ° C and testifies that this enzyme would be thermostable, its optimal pH is located at 5.5 and which indicates stability in the acid conditions. The determination of the enzymatic kinetics parameters gives a maximum velocity (Vmax) and a small Michaelis-Menten constant which reflects a high affinity of the enzyme for the substrate.

Conflict of interest

The authors declare that there are not conflicts of interest.

Ethics

This Study does not involve Human or Animal Testing.

Acknowledgments

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References

Arpana, K., Vinay, K.S., Jörg, F., Tino, P., & Arvind, M.K. (2010). α-amylases from germinating soybean (Glycine max) seeds-Purification and characterization and sequential similarity of conserved and catalytic amino acid residues. *Phytochemistry*, 71, 1657-1666. https://doi.org/10.1016/j.phytochem.2010.06.012

Banks, S.P., & Tomas-Rodriguez, M. (2003). Linear approximation to non-linear dynamical systems with applications to stability and spectral theory. *IMA Journal of Mathematical Control and Information*, 20, 89-103. https://doi.org/10.1093/imamci/20.1.89

Barbosa, J.M.P., Souza, R.L., Fricks, A.T., Zanin, G.M., Soaresa, C.M.F., & Lima, Á.S. (2011). Purification of lipase produced by a new source of Bacillus in submerged fermentation using an aqueous two-phase system. *Journal of Chromatography B*, 879 (32), 3853-3858. https://doi.org/10.1016/j.jchromb.2011.10.035

Biazus, J.P.M., Santana, J.C.C., Souza, R.R., Jord, E., & Tambourgi, E.B. (2007). Continuous extraction of α-and β-amylases from Zea mays malt in a PEG4000/CaCl2 ATPS. *Journal of Chromatography B.*, 858: 227-233. https://doi.org/10.1016/j.jchromb.2007.08.042

Brockett, R.W. (1983). Non-linear control theory and differential geometry. Proc. Int. Congress of Mathematicians, Warsaw. Polish Scientific Publishers. pp. 1357-1368.

Ekorong, A.A.J.F., Zomegni, G., Desobgo, Z.S.C., & Ndjouenkeu, R. (2015). Optimization of drying parameters for mango seed kernels using central composite design. *Bioresources and Bioprocessing*, 2 (8), 1-9.

Forciniti, D., Hall, C.K. & Kula, M.R. (1991). Protein partitioning at the isoelectric point: influence of polymer molecular weight and concentration and protein size. *Biotechnology and Bioengineering*, 38 (9), 986-994. DOI:10.1002/bit.260380905

Frolund, B., Palmgren, R., Keiding, K., & Nielsen, P.H. (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Research*, 30 (8), 1749-1758. https://doi.org/10.1016/0043-1354(95)00323-1

Gerhardt, P., Murray, R.G.E., Wood, W.A., & Krieg, N.R. (1994). Methods for general and molecular bacteriology. ASM, Washington DC.

Goja, A.M., Yang, H., Cui, M., & Li, C. (2013). Aqueous two-phase extraction advances for bioseparation. *Bioprocessing & Biotechniques*, 4 (1), 1-8. http://dx.doi.org/10.4172/2155-9821.1000140

Goupy, J., & Creighton, L. (2006). Introduction aux plans d'expériences. 3 ed. Dunod, Paris.

Gupta, R., Beg, Q.K., Khan, S., & Chauhan, B. (2007). An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Applied Microbiology and Biotechnology*, 60 (4), 381-395. https://doi.org/10.1007/s00253-002-1142-1

Hamid, S.M., & Eskander, O. (2008). Development and application of aqueous two-phase partition for the recovery and separation of recombinant phenylalanine dehydrogenase. *Iranian Journal of Chemistry and Chemical Engineering*, 27(2), 119-127.

Hatti-Kaul, R. (2000). Aqueous Two-Phase Systems. Methods and Protocols. Humana Press, New Jersey.

Hemavathi, A.B., & Raghavarao, K.S.M.S. (2011). Differential partitioning of β-galactosidase and β-glucosidase using aqueous two phase extraction. *Process Biochemistry*, 46, 649-655. https://doi.org/10.1016/j.procbio.2010.11.008

Ibarra-Herrera, C.C., Aguilar, O., & Rito-Palomares, M. (2011). Application of an aqueous two-phase system strategy for the potential recovery of a recombinant protein from alfalfa (Medicago sativa). *Separation and Purification Technology*, 77, 94-98. https://doi.org/10.1016/j.seppur.2010.11.025

Kammoun, R., Chouayekh, I., Abid, H., Naili, B., & Bejar, S. (2009). Purification of CBS 819.72 α-amylases by aqueous two-phase system: Modelling using response surface methodology. *Biochemical engineering journal*, 46, 306-312. https://doi.org/10.1016/j.bej.2009.06.003

Klang, M.J., Talamond, P., Djidimbele, N., Tavea, F., & Ndjouenkeu, R. (2014). Partial purification and characterization of α-amylases from Abrus precatorius, Burnatia enneandra and Cadaba farinosa. *Journal of Enzyme Research*, 5 (1), 66-71.

Li, M., & Peebles, T.L. (2004). Purification of hyperthermophilic archael amylolytic enzyme (MJA1) using thermoseparating aqueous two-phase systems. *Journal of Chromatography B*, 807, 69-74. https://doi.org/10.1016/j.jchromb.2004.03.030

Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275.

Mehrnoush, A., Mustafa, S., Sarker, M.Z., & Yazid, A.M. (2012). Optimization of serine protease purification from mango (*Mangifera indica cv.* Chokanan) peel in polyethylene glycol/dextran aqueous two phase system. *International Journal of Molecular Sciences*, 13 (3), 3636-3649. https://doi.org/10.3390/ijms13033636

Mohamadi, H.S., & Omidinia, E. (2007). Purification of recombinant phenylalanine dehydrogenase by partitioning in aqueous two-phase systems. *Journal of Chromatography B*, 854, 273-278. https://doi.org/10.1016/j.jchromb.2007.04.049

Mohamadi, H.S., Omidinia, E., & Dinarvand, R. (2007). Evaluation of recombinant phenylalanine dehydrogenase behavior in aqueous two-phase partitioning. *Process Biochemistry*, 42 (9), 1296-1301. https://doi.org/10.1016/j.procbio.2007.06.005

Porto, T.S., Pessoa-Filho, P.A., Neto, B.B., Lima-Filho, J.A., Converti, A., Porto-Jr, A.L.F., & Pessoa, A. (2007). Removal of proteases from Clostridium perfringens fermented broth by aqueous two-phase systems (PEG/citrate). *Journal of Industrial Microbiology & Biotechnology*, 34, 547-552. https://doi.org/10.1007/s10295-007-0230-8

Priyanka, B.S., Rastogi, N.K., Raghavarao, K.S.M.S., & Thakur, M.S. (2012). Downstream processing of luciferase from fireflies (Photinus pyralis) using aqueous two-phase extraction. *Process Biochemistry*, 47, 1358-1363. https://doi.org/10.1016/j.procbio.2012.04.022

Priyanka, S., & Rathindra, M., Banik. (2012). Partitioning studies of L-glutaminase production by Bacillus cereus MTCC 1305 in different PEG–salt/dextran. *Bioresource Technology*, 114, 730-734. https://doi.org/10.1016/j.biortech.2012.03.046

Raja, S., Murty, V.R., Thivaharan, V., Rajasekar, V., & Ramesh, V. (2011). Aqueous two phase systems for the recovery of biomolecules – A review. *Science and Technology*, 1 (1), 7-16. DOI: 10.5923/j.scit.20110101.02

Ratanapongleka, K. (2012). Partitioning behavior of laccase from Lentinus polychrous Lev in aqueous two phase systems. *Songklanakarin Journal of Science and Technology*, 34 (1), 69-76.

Ratanapongleka, K. (2010). Recovery of biological products in aqueous two phase system. International journal of chemical engineering and application, 1(2), 191-198.

Rosa, P.A.J., Azevedo, A.M., & Aires-Barros, M.R. (2007). Application of central composite design to the optimisation of aqueous two-phase extraction of human antibodies. *Journal of Chromatography A*, 1141(1), 50-60. https://doi.org/10.1016/j.chroma.2006.11.075

Su, C.K., & Chiang, B.H. (2006). Partitioning and purification of lysozyme from chicken egg white using aqueous two phase system. *Process Biochemistry*, 41(2), 257-263. https://doi.org/10.1016/j.procbio.2005.06.026

Taira, A., Desobgo, Z.S.C., & Nso, E.J. (2015). Partial purification of α-amylase from Burnatia enneandra Micheli. Part 1: Extraction using statistical model. *International Journal of Research in Biotechnology and Biochemistry*, 5 (1), 13-19.

Yucekan, I., & Onal, S. (2011). Partition of invertase from tomato in poly (ethylene glycol)/sodium sulphate aqueous two phase systems. *Process Biochemistry*, 46 (1), 226-232. https://doi.org/10.1016/j.procbio.2010.08.015

Zhi, W., Song, J., Ouyang, F., & Bi, J. (2005). Application of response methodology to the modeling α-amylase purification by aqueous two-phase systems. *Journal of Biotechnology*, 118 (2), 157-165. https://doi.org/10.1016/j.jbiotec.2005.03.017

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