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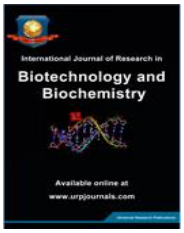
International Journal of Research in Biotechnology and Biochemistry



ISSN: 2277-3827

Original article

Partial purification of α-amylase from *Burnatia enneandra* Micheli. Part 1: Extraction using statistical model

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Received 13 September 2015; accepted 31 December 2015

Abstract

The impact of pH, stirring speed and centrifugation time was tested on α-amylase specific activity of *Burnatia enneandra* tubers from Cameroon. The response surface methodology using Doehlert experimental design as instrument, was employed to establish, validate and optimize the mathematical model conducive to understand the influence of extraction factors (pH, stirring speed and centrifugation time) either singular or in interactions. It was exhibited that pH as singular factor and, in its first degree (X_1) provided 12 % of the specific activity of α-amylase although, stirring speed (X_2) and centrifugation time (X_3) provided 3 % and 10 % respectively. It was also observed that, the raise of pH reduced specific activity of α-amylase while, an increase of centrifugation time increased the specific activity of α-amylase. The combined effort (interaction) of pH / centrifugation time and stirring speed / centrifugation time was significant with respective contribution of 25 % and 9 % respectively. For optimization, a theoretical triplet (pH, 4.02; stirring speed, 147.3 rpm and centrifugation time, 10 min) was exhibited and, gave 49.33±1.9 U/mg as maximal α-amylase specific activity from *Burnatia enneandra*.

Keywords: *Burnatia enneandra* tubers, extraction, Doehlert experimental design, Modelling, Optimization.

1. Introduction

Burnatia enneandra Micheli is an herbaceous plant of the Sudano-Sahelian region, belonging to the family of *Alismataceae*. In Cameroon this plant grows mainly in swampy areas of the north and the far north. Common names of this plant is known among Tupuri as "*tigna'ri"* [1, 2] and among Fulani as "*Anjakooje"* [3]. These *Burnatia enneandra* Micheli tubers dried and crushed are used to "sweeten" the slurry during the grazing due to the remoteness of grazing areas. Others ate the tubers after blown. Finally, the white latex in it is commonly used to treat injuries and some intestinal parasites of children [1]. With the traditional use of this tuber, scientific works have been done [1, 2]. This work has shown that these tubers had a very low simple sugar content, this assumes that the *Burnatia enneandra* tuber is not sweet itself and the fact of sweeten porridge would not be due to its high simple sugar content but rather due to substances with amylolytic potential [2]. This suggests the presence in the tuber of amylolytic enzymes. Other preliminary works related to the brewing area has focused on the characterization of worts obtained by saccharification of sorghum mash by extracts of *Burnatia enneandra* Micheli tuber. These studies showed that the enzyme extracts obtained from these tubers were capable of hydrolyzing sorghum starch [4].

The general objective is set to extract the α-amylase from the crude extract of *Burnatia enneandra* Micheli tuber. More specifically it will be to model the extraction process of α-amylase from the tuber and, optimize extraction conditions.

Abbreviations

ANOVA	Analysis of variance
AAD	Average absolute deviation

A_f	Accuracy factor
B_f	Bias factor
k	Number of variables
k_0	Number of center points
N	Number of experiments
P	Probability level
R^2	Coefficient of determination
Res	Residue
RSM	Response surface methodology
x_i	Coded variables given by the Doehlert table
X_i	Real variables
X_{0i}	Centre of variable
ΔX_i	Increment
Y	Response
$Y_{SpecAct}$	Mathematical model for α-amylase specific activity of crude extract of *Burnatia enneandra*
Y_{ical}	Theoretical response
$Y_{i\exp}$	Experimental response

Greek symbols

β_{ij}	Coefficient of the interactions terms
β_{ii}	Coefficient of the quadratic terms
β_i	Coefficient of the linear terms
β_0	Constant

2. Materials and methods

2.1. Material

Plant material utilised for this study consisted mainly of *Burnatia enneandra* Micheli tubers. These tubers were harvested in the region of the far north precisely Datcheka (located at about 70 Km of Yagoua, Cameroon). Once harvested they were sorted and the film or the protective cover was removed. Bare tubers were then cleaned in tap water, and cut into thin slices (about 2 to 3 mm) and finally dried (in drier CKA AUF-2000) at 45 °C for 5 days. The dried discs were ground and the obtained powder was sieved to select only a smaller particle size (Ø ≤ 250 µm).

2.2 Chemicals

All chemicals used mainly consists of analytical grade reagents provided by Sigma-Aldrich. It mainly: Sodium phosphate, soda pellets, potassium iodide, concentrated sulphuric acid, bovine serum albumin (BSA), soluble starch and the Folin-Ciocalteu reagent, polyethylene glycol molecular mass 6000 (PEG-6000) and ammonium sulfate.

2.3 Experimental design, modelling and validation of the model

Doehlert experimental design [5-7] was implemented to make experiments in favour of building and optimizing statistical model for α-amylase extraction and partial purification from *Burnatia enneandra* Micheli. The independent factors were obtained from preliminary studies, while the response was the specific activity.

Matrix from the coded variables were transformed into real values for experiments, using the formulas below:

$$X_i = X_{0i} + x_i \Delta X_i \quad (1)$$

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

Where: X_i, real variables; X_{0i}, centre of variable; x_i, coded factors (variables) given by Doehlert matrix; ΔX_i, increment; k, number of variables; k_0, number of centre points and N, number of experiments.

Mathematical models of second-order equation were developed [8]. Design-based experimental data were builtusing to the second-order polynomial model as follows:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 + \sum \beta_{ij\ldots z} x_i x_j \ldots x_z + e \quad (3)$$

With: Y, is the response. It is measured during the experiment and is obtained with a given accuracy; x_i and x_j, represent the level assigned to the factor i and j respectively by the experimenter to perform a test. This value is well known. It is even assumed that this level is determined without error (classical hypothesis of regression); β_0, β_i,, β_{ii}, β_{ij} ,are the coefficients of the adopted mathematical model. They are not known and must be calculated from the results of experiments.

Statistical models and analysis of variance (ANOVA) were obtained using Minitab version 16 (Minitab, Ltd. Coventry, UK). Sigma plot version 12.5 (Systat Software, Inc. Wbcuped, GmbH, Germany) was used to plot the curves.

Models validation was achieved by calculating some validation parameters as mentioned in the literature [9-11]. The formulas were as follows:

$$AAD = \frac{\left[\sum_{i=1}^{N}\left(\frac{\left|Y_{i\exp} - Y_{ical}\right|}{Y_{i\exp}}\right)\right]}{N} \quad (4)$$

$$B_f = 10^{\frac{1}{N}\sum_{i=1}^{N}\log\left(\frac{Y_{ical}}{Y_{i\exp}}\right)} \quad (5)$$

$$A_{f1} = 10^{\frac{1}{N}\sum_{i=1}^{N}\left|\log\left(\frac{Y_{ical}}{Y_{i\exp}}\right)\right|} \quad (6)$$

With: $Y_{i,exp}$ and $Y_{i,cal}$; respectively data-based (experimental) and mathematical responses; N, trials number; AAD, absolute average deviation; B_f, bias factor; A_f, accuracy factor.

Linear, interaction and quadratic contributions of each variable were calculated as follows [11]:

Linear,
$$Contribution(\%) = \frac{|\beta_i|}{\sum_{i=1}^{k}|\beta_i| + \sum_{i=1}^{k}|\beta_{ii}| + \sum\sum_{i<j}^{k}|\beta_{ij}|} \quad (7)$$

Quadratic,
$$Contribution(\%) = \frac{|\beta_{ii}|}{\sum_{i=1}^{k}|\beta_i| + \sum_{i=1}^{k}|\beta_{ii}| + \sum\sum_{i<j}^{k}|\beta_{ij}|} \quad (8)$$

Interaction,
$$Contribution(\%) = \frac{|\beta_{ij}|}{\sum_{i=1}^{k}|\beta_i| + \sum_{i=1}^{k}|\beta_{ii}| + \sum\sum_{i<j}^{k}|\beta_{ij}|} \quad (9)$$

Optimization was the last stage of the study and, was performed using Mathcad software v15.0 (build 15.0.0.436 Parametric Technology Corporation, USA). The conditions to maximize the specific activity of α – amylase during extraction was fixed. A test was done at that theoretical

optimum to confirm it.

2.4 Factors ranges and responses.

Preliminary studies permitted to identify three main factors that could have greater influence on α-amylase extraction. These factors and their ranges were as follows: pH, 4 to 7, because as found in the literature, that amylases catalyse better within this range [12]; Stirring speed, 100 to 250 rpm, to minimize enzyme denaturation, and centrifugation time, 10 to 30 min at 6500 × g. Doehlert experimental design was used to carry out extraction.

2.5 Extraction of enzymes of *Burnatia enneandra* tubers

A 20 g of the sieved powder of *Burnatia enneandra* tubers was weighed using analytical balance (KERN EMB 200-3, Sysmatec Oberdorfstrasse). To that mass was added 200 mL of phosphate buffer (200 mM) at pH between 4 to 7. The mixture was stirred for one hour at precise speed according to experimental design (100 to 250 rpm) using laboratory overhead stirrer (model: AM120Z-P, Meditry Instrument Co. Ltd), and then permitted to settle down for 15 min. The supernatant was recovered and introduced into a 45 mL centrifuge tubes and, centrifuged at 6500 × g for 10 to 30 minutes at 4 °C using refrigerated Heraeus centrifuge. The collected supernatant was then filtered and the filtrate obtained was the enzyme extract containing the enzyme to be purified.

2.6 Protein quantitation

The protein amount was obtained using Lowry's method [13-15]. A volume of sample or standard (0.1 mL) and NaOH (0.1 mL, 2 N) was mixed. The mixture was boiled at 100 °C for 10 min in a boiling water bath and, was chilled to 25 °C. A was prepared by associating, 100:1:1 stock solutions (2% (w/v) Na_2CO_3; 1% (w/v) $CuSO_4$ $5H_2O$; 2% (w/v) sodium potassium tartrate). After that, 1mL of that complex-forming reagent was added to the mixture. The solution was left for 10 min at 25 °C. Folin reagent (0.1 mL) was then added, and the mixture leftfor 30-60 min at 25 °C. The absorbance was read at 750 nm for protein concentration less than 500 µg/mL, or at 550 nm for protein concentration at interval between 100 and 2000 µg/mL. Standard curve was performed for protein concentrations (P) determination in the samples.

2.7 Measurement of enzyme activity

α-amylase activity was carried using Fuwa's colorimetric method [16, 17] with some modifications. A diluted enzyme (50 µL) in phosphate buffer pH 7 (50 mM) was mixed with soluble-starch (100 µL, 1.1%) and, the mixture was incubated for 10 min at 50 °C. After that incubation, the reaction was interrupted by incorporating 250 µL of stopping solution (made of 5: 1 ratio of 0.5 N acetic acid and 0.5 N HCl). A volume (100 µL) of the previous reaction was mixed with 1 mL iodine (0.01% I_2 dissolved in 0.1% KI) reagent. The colour reduction was obtained by measuring at 660 nm against the reference (control), which was prepared using the same method but, by adding the stopping solution before the enzyme. One α-amylase activity unit was considered as the measure of enzyme whichreducedby 0.1 in 10 min, the absorbance of 660 nm.

$$A_T = \frac{d \times (DO_B - DO_S)}{DO_B} \times 100 \qquad (10)$$

The specific activity (A_S) in U/mg was obtained using the formula:

$$A_S = \frac{A_T}{P} \qquad (11)$$

With: A_T, Total activity (U); d, dilution factor; DO_B, optical density of the blank; DO_S, sample optical density; P, protein quantity (mg).

3 Results and discussion

Modelling the impact of pH, agitation speed and centrifugation time, on α-amylase extraction from *Burnatia enneandra* Micheli was carried out using Doehlert experimental design with specific activity as response (Table 1). The mathematical model obtained was as follows:

$$\begin{aligned} Y_{SpecAct}(x_1,x_2,x_3) &= 36.378 - 10.757x_1 - 2.825x_2 \\ &+ 8.470x_3 - 2.069x_1x_2 + 22.258x_1x_3 + 7.580x_2x_3 \\ &- 14.304x_1^2 - 10.892x_2^2 + 8.891x_3^2 \end{aligned} \qquad (12)$$

With $Y_{SpecAct}(x_1, x_2, x_3)$ representing the mathematical model for α-amylase specific activity of crude extract; x_1, pH; x_2, Stirring speed and x_3, centrifugation time.

The mathematical model was polynomial and validated according to the method described by [9] as shown in Table 2. The factors of the models were of first degree (x_1, x_2 and x_3), of second degree (x_1^2, x_2^2 and x_3^2), and of interaction (x_1x_2, x_1x_3 and x_2x_3) form. They were statistically significant or not if the probability (P) was ≤0.05 or ≥0.05, respectively.

3.1. Effect of pH

The pH effect on α-amylase specific activity of the crude extract is shown in Fig. 1a. Specific α-amylase activity decreased significantly from 47.84 U/mg at pH 4 to 0 U/mg

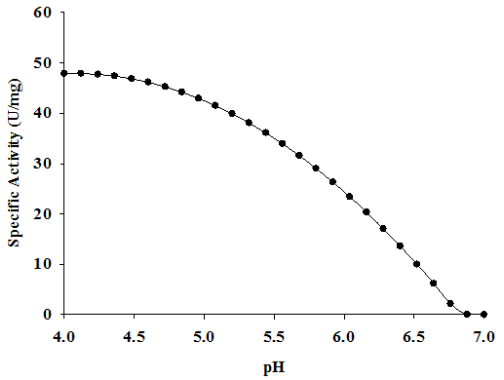

Fig. 1a Effect of pH as the singular factor on the specific activity of α-amylase from *Burnatia enneandra* (fixing stirring speed at 100 rpm and centrifugation time at 10 min)

At pH 7, with a probability P = 0.000, its contribution was 12 % (Table 3). It was observed when fixing stirring speed at 100 rpm and centrifugation time at 10 min. It was also obtained that, the quadratic effect of pH (x_1^2) contributed for 16 % with a significant probability of 0.000 (Table 3). This optimal pH of 4 is in accordance with the α-amylase pH of malt [18] and, could be explained by the

Table 1: Doehlert experimental design: coded variables, real variables and response

N°	Factors						Protein (µg)	Total activity (U/mL)	Response		
	Coded variables			Real variables					Specific activity (U/mg)		
	x_1	x_2	x_3	X_1	X_2	X_3			Exp	Cal	Res
1	0	0	0	5.5	175	20	1340.90±5.70	48.60±0.46	36.25±1.24	36.37	-0.12
2	1	0	0	7	175	20	1497.72±3.00	15.20±0.63	10.15±0.99	11.31	-1.16
3	-1	0	0	4	175	20	429.54±3.21	14.60±0.52	34.00±1.88	32.83	1.17
4	0.5	0.866	0	6.25	250	20	952.27±3.20	18.90±1.86	19.85±1.23	15.91	3.94
5	-0.5	-0.866	0	4.75	100	20	663.63±19.24	21.21±2.57	31.95±2.81	31.56	0.39
6	0.5	-0.866	0	6.25	100	20	1090.9±7.90	26.52±0.27	24.30±1.90	22.59	1.71
7	-0.5	0.866	0	4.75	250	20	447.72±3.22	13.73±1.10	30.60±1.40	28.46	2.14
8	0.5	0.289	0.816	6.25	200	30	1570.45±3.23	68.72±1.05	43.75±1.28	49.09	-5.34
9	-0.5	-0.289	-0.816	4.75	150	10	852.27±3.26	38.52±2.25	45.20±1.92	47.66	-2.46
10	0.5	-0.289	-0.816	6.25	150	10	1204.54±11.01	25.34±0.52	20.92±1.51	19.34	1.58
11	0	0.577	-0.816	5.5	225	10	618.18±6.44	16.50±2.99	26.69±1.21	26.56	0.13
12	-0.5	0.286	0.816	4.75	200	30	831.81±4.80	32.80±0.94	39.43±1.34	42.29	-2.86
13	0	-0.577	0.816	5.5	125	30	750.00±6.42	32.63±0.96	43.47±1.43	43.64	-0.17
14	0	0	0	5.5	175	20	1343.18±3.21	48.81±0.60	36.33±2.25	36.37	-0.04
15	0	0	0	5.5	175	20	1338.63±3.22	48.71±0.35	36.38±1.31	36.37	0.01
16	0	0	0	5.5	175	20	1340.90±6.20	49.00±0.50	36.54±2.42	36.37	0.17
17	0	0	0	5.5	175	20	1341.00±6.42	48.80±0.63	36.39±1.70	36.37	0.02

Table 2: Model validation

Models	R^2	AAD	B_f	A_f
$Y_{SpecAct}(x_1, x_2, x_3)$	0.990	0.041	0.987	1.043

Table 3: Estimated coefficients impact and contributions to enzyme specific activity

Effects	Specific activity		
	Coefficients	Probability	Contribution (%)
Constant	36.378	0.000	
x_1	-10.757	0.000	12
x_2	-2.825	0.009	3
x_3	8.470	0.000	10
x_1^2	-14.304	0.000	16
x_2^2	-10.892	0.000	13
x_3^2	8.891	0.001	10
x_1x_2	-2.069	0.292	2
x_2x_3	7.580	0.000	9
x_1x_3	22.258	0.019	25

fact that, the side chains of amino acids in the active site of the enzyme could act as weak acids and bases according to the ionization state. They could play an essential role in the interactions that maintain the structure of proteins. Eliminating a proton from an enzyme could discard an ionic interaction, crucial for stabilizing the active conformation of the enzyme [19]. That decrease could also probably due to synchronous denaturation in the structure of the enzyme protein induced by pH variation [20].

3.2 Effect of Stirring speed

The effect of stirring speed on the specific activity of α-amylase crude extract is shown in Fig. 1b. Specific α-amylase activity was 47.84 U/mg at 100 rpm then increased non-significantly to 51.10 U/mg at 147.40 rpm. After that a significant decrease (P = 0.009, Table 3) was observed till 35.82 U/mg at 250 rpm. Its contribution was 3% (Table 3). This was observed when fixing pH at 4 and centrifugation time at 10 min. The quadratic effect of stirring speed (x_2^2) contributed for 13% and was significant (P = 0.000, Table 3). The decrease could be explained by the fact that, the higher the stirring speed, the higher the destruction of hydrogen bonds and some London forces, inducing protein denaturation. When a protein is denatured, its secondary and tertiary conformations are modified but, the peptide bonds of the primary structure between the amino acids are

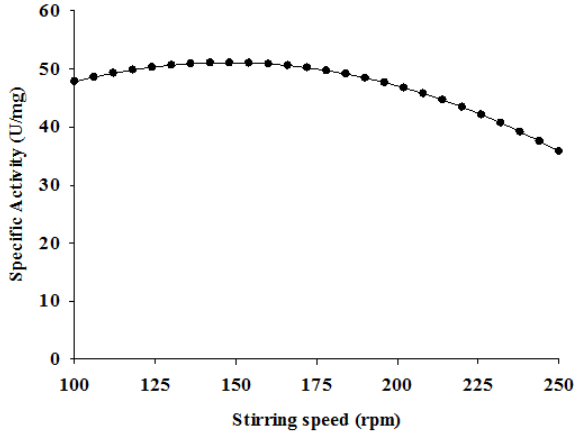

Fig. 1b Effect of stirring speed as the singular factor on the specific activity of α-amylase from *Burnatia enneandra* (fixing pH at 4 and centrifugation time at 10 min)

maintained intact. Seeing that all structural levels of the protein dictate its function, the protein can no longer achieve its role once it has been deteriorated. An enzyme depletes its catalytic activity when it is denaturized [21, 22].

3.3 Effect of centrifugation time

The effect of centrifugation time on the specific activity of α-amylase crude extract is shown in Fig.1c. α-amylase specific activity started with 35.38 U/mg after 10 min of centrifugation (at 6500 × g) then decreased non-significantly to 34.36 U/mg at 14.16 min. After that it increased significantly (P = 0.000, Table 3) to 49.21 U/mg at after 30 min of centrifugation. Its contribution was 10 % (Table 3). It was observed when fixing pH at 5.5 and stirring speed at 175 rpm. The quadratic effect of centrifugation time (x_3^2) had a significant probability of 0.001 with a contribution of 10 % (Table 3). This could be explain by the fact that, at the beginning (10 min to 15 min), the liquid phase was still charged with suspension and the consequence was a lower specific activity. When increasing the centrifugation time, the liquid phase became much free of suspended materials [23] and therefore inducing higher specific activity.

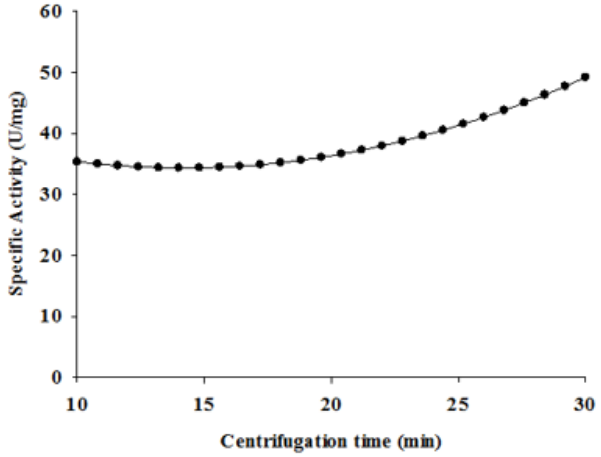

Fig. 1c Effect of centrifugation time as the singular factor on the specific activity of α-amylase from *Burnatia enneandra* (fixing pH at 5.5 and stirring speed at 175 rpm)

3.4 Effect of interaction pH/centrifugation time

The pH / Centrifugation time interaction had significant (P =0.000, Table 3) impact on α-amylase specific activity. This was obtained when fixing the stirring speed at 100 rpm. Its contribution was 25 % (Table 3). In fact, when decreasing the pH from 7 to 5.5, whatever the centrifugation time, it was observed an increase of specific activity. While, when decreasing the pH from 5.5 to 4, an increase of specific activity was observed when decreasing centrifugation time (Fig. 2a). Fractional precipitation was achieved by varying the pH of the medium. At the isoelectric point, the charge of the protein was not clear. This imposed a reduced solubility because it was unable to interact with the environment and precipitated [24]. The centrifugation time permitted to settle them down with also secondary metabolite and, at the same time increased the purity of amylase [23]. This confirm that amylase is stable at acidic pH [25-27].

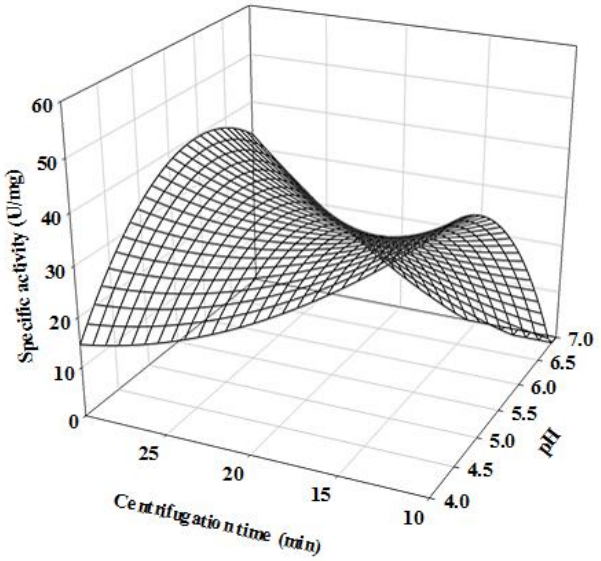

Fig. 2a Effect of interaction pH/centrifugation time on the specific activity of α-amylase from *Burnatia enneandra* (fixing the stirring speed at 100 rpm)

3.5 Effect of interaction stirring speed / centrifugation time

The effect of Stirring speed / Centrifugation time interaction had significant (P = 0.019, Table 3) impact on α-amylase specific activity. Its contribution was 9 % (Table 3). It was observed that, whatever the stirring speed, when decreasing the centrifugation time, the α-amylase specific activity increased significantly. It was also observed that, when increasing the stirring speed, the enzyme activity increased to reach a maximum and decrease afterward (Fig. 2b). Since in plant tissues cell are covered with strong cell walls, the increase of stirring speed (mechanical agitation) permitted the disruption of the cell walls [28] and at the same time the release of enzyme in the medium. The higher the enzyme concentration resulting from that disruption induced higher activity. Also when the stirring was executed, the better homogenization level in the liquid phase was obtained when increasing rates [29] thus, increasing amylase extraction and activity. At higher

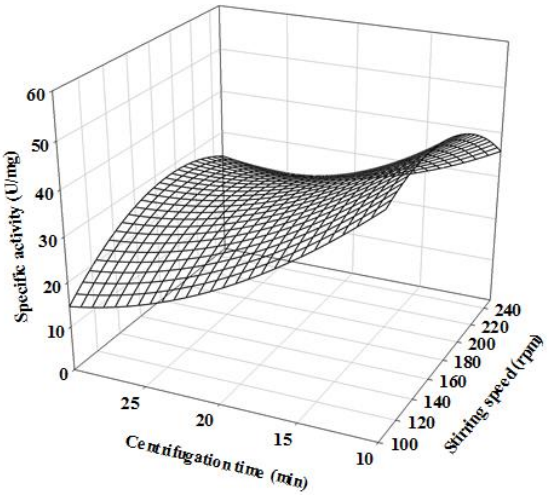

Fig. 2b Effect of interaction stirring speed/centrifugation time on the specific activity of α-amylase from *Burnatia enneandra* (fixing the pH at 4)

stirring speed, extracted enzymes undergone deactivation which explained the decrease of enzyme activity. This was also observed in the literature [30, 31].

3.6 Optimization

The result obtained for the action of extraction parameters (pH, stirring speed and centrifugation time) on α-amylase specific activity on the basis of the model, was optimized to determine the best condition for α-amylase extraction, in order to go to the next step which is partial purification. After using Mathcad 15.0, theoretical optimum was obtained and was as follows: pH, 4.02; stirring speed, 147.3 rpm and centrifugation time, 10 min. These values permitted to obtain a theoretical α-amylase specific activity at 51.11 U/mg. That combination (pH, 4.02; stirring speed, 147.3 rpm and centrifugation time, 10 min) was tested in the laboratory to verify the theoretical result and, 49.33±1.9 U/mg was obtained.

4 Conclusion

The impact of extraction parameters (pH, stirring speed and centrifugation time) on the specific activity of α-amylase from *Burnatia enneandra* Micheli were studied. The pH was the singular parameter which was influencing more the specific activity of α-amylase, followed by the centrifugation time. Concerning interactions, the pH / Centrifugation time was very important, meaning there was a high synergetic action between the two factors, compare to stirring speed / centrifugation time while, pH / stirring speed interaction was not significant. The theoretical optimization showed interesting results which was confirmed by the laboratory test. This could permit to use the same modelling approach to purify the α-amylase from *Burnatia enneandra* in order to valorise that herbaceous plant.

Acknowledgements

The authors gratefully acknowledge the Department of Process Engineering and Food Science and Nutrition of the National School of Agro- Industrial Sciences (ENSAI) and, The University of Ngaoundere (Cameroon) for providing necessary facilities for the successful completion of this research work.

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Source of support: Nil; Conflict of interest: None declared