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Optimization of *Borassus flabellifer* Amylase Extraction Procedure Using Box-Behnken Design and Development of Simple Affinity Chromatographic Technique for Purification of Amylases

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Authors' contributions

This work was carried out in collaboration between all authors. RKB performed experiments PMA conceived idea, designed experiments, analyzed data and prepared manuscript RT, SRS designed experiments. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

This work is aimed to study the optimal conditions of extraction and development of novel purification process for α-Amylase from pericarp of *Borassus flabellifer* fruit. Optimization was done by Response Surface Methodology (RSM) using Box-Behnken design. The variables chosen for this purpose are pH (X1), temperature (X2, °C) and $CaCl_2$ concentration (X3, ppm). A total of 9 runs were conducted, varying each factor while others were kept constant. The contribution of each factor was established giving raise to significant t-values and p-values. This revealed that the three variables are essential for activity of the enzyme. Based on the obtained optimal conditions from RSM studies, enzyme formation was successfully achieved with an activity of 28.8 U and concentration of 1.4 mg/ml for the 90% ammonium sulfate precipitated dialyzed enzyme sample. A novel affinity chromatographic process was developed for purification process and by this

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process the enzyme concentration was found to be 0.032mg/ml and activity was determined as 4.76 U.

Keywords: Amylase; Borassus; partial purification; RSM; Box-Behnken; affinity chromatography.

1. INTRODUCTION

Extraction, purification and optimization of parameters for certain enzymes are important for the industry and became one of the significant fields of research. Amylase is one such enzyme which is most widely focused, because of its economic and industrial importance. These α-amylases catalyze the hydrolysis of internal α-1, 4-O-glycisidic bonds in polysaccharides which helps in starch de-polymerization. This property made amylases as a significant enzyme for food and processing industry (Prasanna, 2005). These are used especially in Conversion of starch into sugar, bio-ethanol, syrups and dextrin's preparation. Amylases are obtained from many types of sources like animals, plants, bacteria and fungus. Various methods are available for extraction and purification of these enzymes. This paper focuses on one such process aimed at isolation and purification of α-Amylases from pericarp of *Borassus flabellifer* fruit. *Borassus flabellifer* is a palm crop found abundantly along coastal belt of India. The pericarp of its fruit is made up of a brittle sensitive fibrous tissue that is assumed to have some digestive activity and found to be abundantly embedded with α-amylases (Srinivasa Rao et al., 2005). The extraction and purification steps marked for large scale production of such enzymes must be highly economical. Therefore competitive advantage in commercialization will depend not only on bimolecular production but also on innovation and optimization of process parameters (Jose, 2007). Response surface methodology (RSM) is a tool highly used for this. It uses mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables (Ravi Kumar et al., 2009). A catalogue of independent variables (pH, temperature, $CaCl_2$ concentration, buffer) affecting the α-Amylase activity has been identified (Ashraf Muhammad et al., 2008). Of the above mentioned parameters, pH, temperature and $CaCl_2$ concentration are selected for optimization by RSM. Besides this, downstream process also plays a key role in industry, nearly 80% role in overall production costs. New trends and techniques have been emerging in this field to minimize the economy of production. Affinity chromatography is one such process extensively used in present day scenario. It is constantly subjected to many modifications to reduce the cost effectiveness involved in ligand used and time consumed. Amylose which is a component of starch is extensively degraded by Amylases at α-1,4-O-glycisidic bonds which helps in starch de-polymerization (Usha et al., 2011) and are found to have high affinity for starch, rapid purification methods exploiting affinity of higher plant amylases to its substrate starch came into light (Azad et al., 2009) (Subbaramaiah and Sharma, 1988). In this context we have developed a novel affinity chromatography process for purification of Amylases from plant extract (Pericarp of *Borassus flabellfer* fruit), using starch flakes as affinity adsorbents for amylases.

1.1 Response Surface Methodology (RSM)

RSM combines statistical experimental designs and empirical model building by regression for the purpose of process optimization. The relationships among the variables were expressed mathematically in the form of a quadratic polynomial model, which gave the response as a function of relevant variables. This work was based on the Box-Behnken design (Box and Behnken, 1960) utilized to obtain the experimental data, which would fit an empirical, full second-order polynomial model representing the response surface over a relatively broad range of parameters. RSM had not only been used for optimization of culture parameters in the fermentation process but also for studying the combined effects of media components. The product concentration Y is related to

$$\mathbf{Y = f\ (X_1,\ X_2,\ X_3,\ X_4,\ldots\ldots,\ X_k)} \quad (1)$$

The true relationship between Y and X_k may be complicated and, in most cases, it is unknown; however a second-degree quadratic polynomial can be used to represent the function in the range interest (Annadurai and Sheeja, 1998).

$$Y = \sum_{i=1}^{k} R_i X_i + \sum_{i=1}^{k} R_{ii} X_i^2 + \sum_{\substack{i=1}}^{k-1} \sum_{\substack{j=2 \\ i<j}}^{k} R_{ij} X_i X_j + \epsilon \quad (2)$$

Where X_1, X_2, X_3… X_k are the independent variables which affect the response Y, R_0, R_i, R_{ii} and R_{ij} (i=1-k, j=1-k) are the known parameters, ϵ is the random error. A second-order model is designed such that variance of Y is constant for all points equidistant from the center of the design. The Box-Behnken design helps in investigating linear, quadratic and cross-product effects of these factors each varied at these levels and also includes three center points for replication. It is suitable for exploration of quadratic response surfaces and constructs a surface order polynomial model, thus helping in optimizing a process using a small number of experimental runs (Anupama et al., 2008). The design is performed because relations for experimental combination of the variables are adequate to estimate potentially complex response functions. The 'STATISTICA' software was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface plots.

1.2 Affinity Chromatography

Classical enzyme purification methods often used for amylase purification from crude extracts include gel filtration using Sephadex beads, ion exchange chromatography using DEAE-cellulose and affinity chromatography (Lalit et al., 2010; Nagwa et al., 2011). These are modified by new approaches using where starch is used as binding agent for amylases were developed for replacing classical chemical ligands and some expensive natural beads (Wolfgang and Jorg, 1996*)*. This has a remarkable change in the downstream processing for reducing expenses in large scale production of amylases. Considering the economy and feasible downstream processing as main objective a novel approach is developed here by using starch flakes as ligands.

2. MATERIALS AND METHODS

2.1 Experimental Design

Parameters influencing α-amylase stability and activity, pH (X1), temperature (X2, ºC) and $CaCl_2$ concentration (X3, ppm) were studied by Box-Behnken design (Anupama et al., 2008; Box and Behnken, 1960). The effect of each variable is studied by estimating amylase activity. The three process variables and their range dosage range are mentioned in Table 1. A fractional factorial design having 9 runs with a single centre point were generated (Table 2). A second-degree polynomial was calculated with STASTICA to estimate the response of dependent variables.

$$\mathbf{Y=}\ b0 + b1\ \mathbf{X1} + b2\ \mathbf{X2} + b3\ \mathbf{X3} + b11\ \mathbf{X12} + b22\ \mathbf{X22} + b33\ \mathbf{X32} + b12\ \mathbf{X1\ X2} + b23\ \mathbf{X2\ X3} + b13\ \mathbf{X1\ X3} \quad (3)$$

where Y is the predicted response, X1, X2 and X3 are the independent variables, b0 is the offset term, b1, b2, b3 are the linear effects, b12, b23, b13 are the interaction terms.

Table 1. Process variables and levels

Factors	Lower limit (-)	Central point 0	Upper point (+)
pH	2.5	4.5	6.5
Temperature(ºC)	5	20	35
$CaCl_2$(ppm)	0.005	0.02	0.035

Table 2. Box-Behnken three variable fractional factorial designs

Sl. No	Coded variables			Natural variables			Amylase activity (Units)*
	pH	Temperature (ºC)	$CaCl_2$ (ppm)	pH	Temperature (ºC)	$CaCl_2$ (ppm)	
1	-1	-1	-1	2.500000	5.00000	0.005000	13
2	-1	0	+1	2.500000	20.00000	0.035000	20
3	-1	+1	0	2.500000	35.00000	0.020000	13
4	0	-1	+1	4.500000	5.00000	0.035000	19
5	0	0	0	4.500000	20.00000	0.020000	28
6	0	+1	-1	4.500000	35.00000	0.005000	12
7	+1	-1	0	6.500000	5.00000	0.020000	20
8	+1	0	-1	6.500000	20.00000	0.005000	19
9	+1	+1	+1	6.500000	35.00000	0.035000	14
10	0	0	0	4.500000	20.00000	0.020000	27.5

*Box-Behnken design 3** (3-1) fractional factorial design, 1 block, 9 runs (Spreadsheet1) + 1 center points per block.*

**1 unit of enzyme activity is the amount of enzyme required to release 1.0mg of maltose in 1 h.*

2.1.1 Enzyme extraction

Fresh pericarp was pealed into a beaker from the *Borassus* fruit initially and stored at a temperature of 4ºC. A 20% crude extracts (10 numbers) was prepared using cold 0.025M

Sodium acetate buffer containing $CaCl_2$ as a stabilizing agent of pH as given in the table for the experimental design. 20gm of the pericarp was weighed and ground carefully using a motor and pestle with intermittent addition of buffer which is maintained at the temperatures mentioned in Table 2 for each of the set of experiments. After getting a thick paste of pericarp it is carefully suspended in 100ml of buffer, mixed well and then filtered using cheese cloth. The temperature was kept cold to ensure enzyme stability and was retrieved before use. Filtrate obtained is stored in sterile flask in a refrigerator until further use. For rest of the procedures after optimization of the extraction conditions, optimal values obtained were maintained.

2.1.2 Ammonium sulfate ($(NH_4)_2SO_4$) precipitation

Stock solutions of Ammonium sulfate were prepared and were added to test tubes containing crude extract of pericarp amylase to obtain a salt concentration from 50 to 90% (w/v). The tubes were allowed to stand in cold for 30minutes to get enough precipitation. The protein precipitate was obtained by centrifuging the samples in a Cooling centrifuge (REMI-C24) at 11200g for 10minutes. Supernatant was discarded and protein pellets were solubilized in extraction buffer. These fractions were analyzed for their amylase activity and used for further dialysis. Dialysis was carried out against the same extraction buffer using membranes having 20KDa cutoff capacity.

2.1.3 Starch flakes preparation

These are thin polymer layers made from starch. 12% w/v of viscous starch solution was prepared and heated. This hot solution is poured, such that it has a thickness of 2mm, on glass plates and kept for drying in oven. A thin semi transparent film of starch was observed on the glass plates. This film was carefully peeled off from the plates and made into square pieces of 1 cm length.

2.1.4 Column preparation

A glass column with 10 cm long and 2 cm diameter was taken for this purpose and filled with 1 gram of starch flakes. These Starch flakes are thoroughly washed with distilled H_2O and then equilibrated with cold (4ºC) sodium acetate buffer containing $CaCl_2$.

After equilibration 5ml of dialysate was added to the column and left for 1 h incubation. After 1 hr the column was washed with buffer and eluted with acidic sodium acetate buffer (pH 2.5) without $CaCl_2$. This reduces the affinity of amylase towards starch flakes. Fractions were collected at the end of the column while eluting with acidic buffer and are re-suspended in sodium acetate buffer to regain the activity of the enzyme. These eluted fractions were taken for analysis of enzyme activity and concentration.

2.2 Determination of Enzyme Activity

Amylase activity (Fischer, 1961) was determined by adding 1 ml of the enzyme solution to 1 ml of starch solution (Starch-Analytical grade, Merck) and incubating at 37ºC for 3 min. After incubation, 2 ml of 3, 5 di nitro salicylic acids (DNS- Analytical grade, Merck) was added to stop the reaction. Color due to reducing sugar was developed by heating the reactants in boiling water bath for 5 min then rapidly cooling to room temperature. The extinction value (absorbance) was determined at 550 nm against standard maltose curve (Maltose-

Analytical grade, Merck). One unit of enzyme activity is the amount of enzyme that liberates reducing sugar equivalent to 1.0 mg maltose hydrate under specific assay conditions.

2.3 Protein Estimation

Protein estimation was done by using Lowry method (Oliver H. Lowry et al., 1951). The Chemicals and Tyrosine used are of Analytical grade (Merck).

3. RESULTS AND DISCUSSION

The experimental values were taken to conduct the experiment as per the design and the results, i.e., α-Amylase activity obtained for each run is listed in Table 3. Multiple regression analysis was applied for the experimental data obtained and the result was incorporated into 2nd order polynomial, which is as follows

$$\mathbf{Y_{amylase}=}\ 14.76042 + 1.16667.\ \mathbf{X1} + 1.60937.\ \mathbf{X2} - 2.16667.\ \mathbf{X3} + 3.60938.\ \mathbf{X11} - 2.71875.\ \mathbf{X22} + 4.21875.\ \mathbf{X33}$$

The predicted values for the enzyme were obtained using the above equation. The coefficients of regression model for the enzyme and the linear quadratic terms are listed in Table 3. The significance of each coefficient was determined by student's t-test and the p-values are given in Table 3. The larger the magnitude of the t-value and smaller the p-value, the more significant is the corresponding coefficient (Akhnazarova and Kafarrov, 1982; Khuri and Cornell, 1987). Using a 5% significance level a factor is considered to affect the response if the coefficients differ from zero significantly and the p-value < 0.050 (Morgan, 1991).

Table 3. Model co-efficient estimate by multiple linear regressions

S. No	Coefficient	Std. error	t-value	p-value
Intercept	14.76042	0.564821	26.13292	0.000123*
X1	1.16667	0.403615	2.89054	0.062985
X2	1.60937	0.326966	4.92215	0.016068*
X3	-2.16667	0.403615	-5.36815	0.012654*
$X1^2$	3.60938	0.326966	11.03901	0.001592*
$X2^2$	-2.71875	0.768460	-3.53792	0.038421*
$X3^2$	4.21875	0.653931	6.45137	0.007554*

Pareto chart (Fig. 1) and regression analysis (Table 3) results indicate that temperature is the second most influencing variable among the chosen parameters as indicated by the p values. The linear effect of pH was found to have a p-value > 0.05 indicating the broad range effect of the variable on amylase activity. $CaCl_2$ also has good influence on amylase activity influencing its stability. Square values of the variables are used to indicate their quadratic effects so as to get the curvature in the response surface graphs and to get the optimal value for each variable. The fit of the model was checked by the determination of coefficient (R^2), which is 0.9902 and this revealed that 99.02% of the sample variation in amylase production is attributed to independent variables. The critical values for optimal α-Amylases formation given by the software are as follows: pH, 4.86246; temperature, 17.74892ºC; and $CaCl_2$ concentration, 0.02267 ppm. The influential effect of input variables on amylase activity was represented using response surface plots.

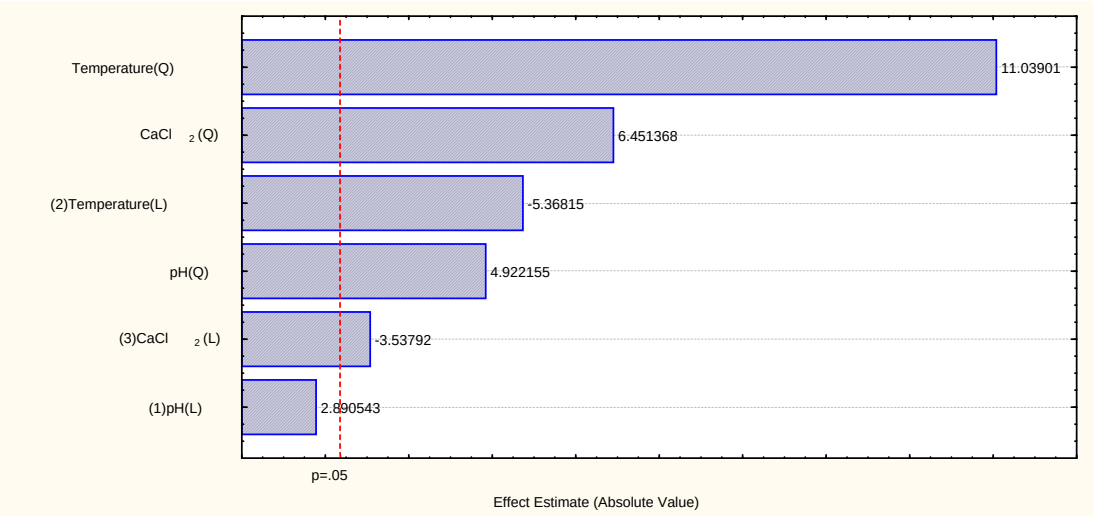

Fig. 1. Pareto chart of standardized effects of α-amylase activity

Note- L values are linear values of p values and Q are quadratic values of the variables. Q values helps to get the optimal values of each of the vairbles and the negative values are represented as positive values.

Fig. 2 indicates the effect of pH and temperature on amylase activity maintaining the third variable at its centre point. With the increase in pH and temperature an initial increase in amylase activity (Y-axis) was observed indicating that the enzyme requires optimal physical conditions to perform its activity. Further increase in variable levels has deleterious effects on enzyme activity as pH is required for maintain the enzyme in its active conformation while increase in temperature leads to denaturation of enzymes.

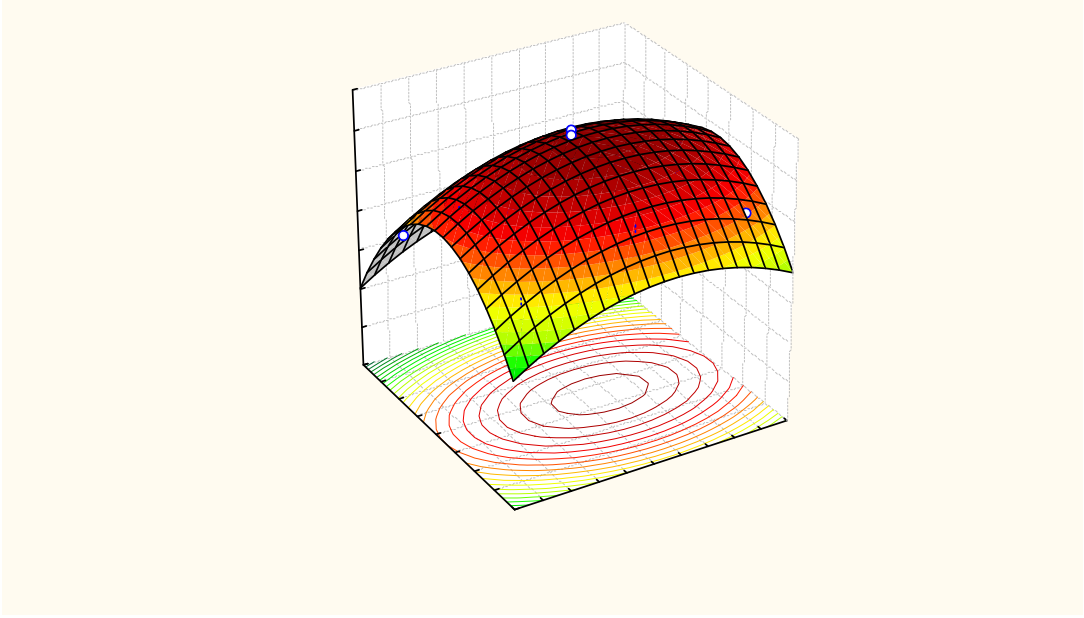

Fig. 2. Effect of pH and temperature on amylase activity

Fig. 3 indicates the effect of a stabilizing agent ($CaCl_2$) and temperature on the enzyme activity keeping pH value at centre point. Both the parameters were found to have significant effect. Increase in $CaCl_2$ concentration along with increase in temperature has increased the activity of enzyme to certain extent. There was a steep descent as variable levels was increased, showing reduced enzyme activity sharply in the graph, than in other variable combinations.

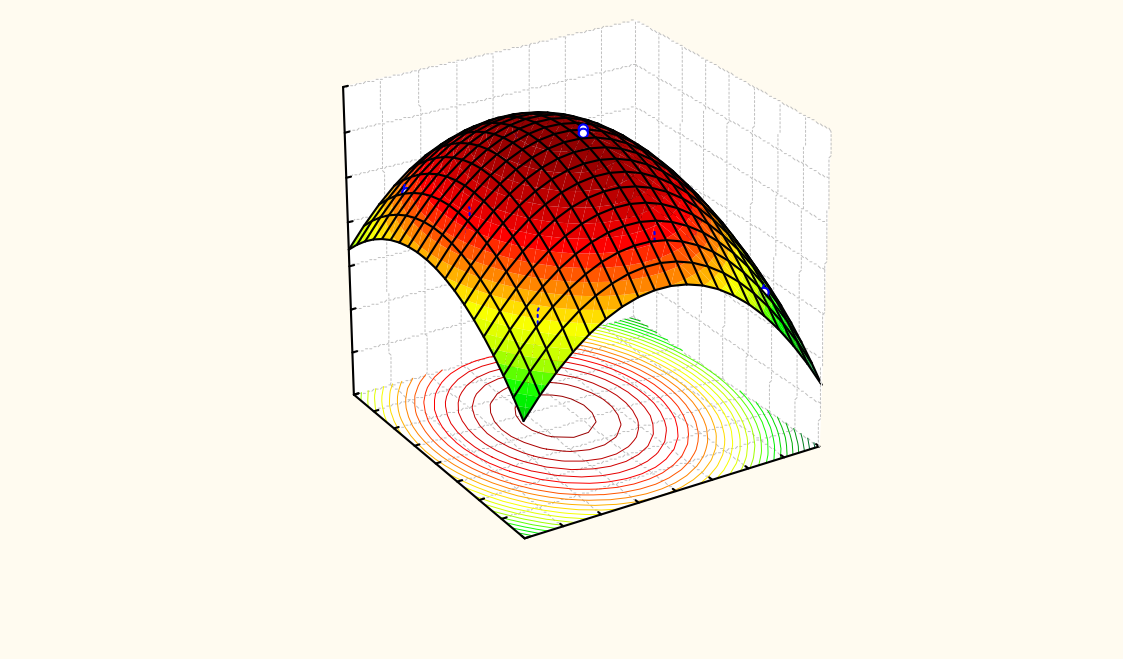

Fig. 3. Effect of Calcium Chloride and Temperature on amylase activity

Fig. 4 indicates the effect of $CaCl_2$ concentration and pH on enzyme activity keeping the third variable at its temperature at 20ºC. The initial enzyme activity for the lowest levels of the two parameters was more than that for the other parameter combinations. The enzyme activity was observed to increase gradually as the variable levels increased up to a maximum extent and then a gradual decrease in enzyme activity was observed.

3.1 Ammonium Sulfate Precipitation

With obtained optimal conditions, i.e., pH, 4.86; temperature, 17.75ºC; and $CaCl_2$ concentration, 0.023 ppm crude extracts were prepared and subjected to salt precipitation. Maximum amylase activity was observed in 80% and 90% salt precipitates. The 90% salt precipitate upon re-solubilization resulted in amylase activity 33.2 U, with an enzyme concentration of 1.26 mg/ml. This is higher as compared to the blank that was prepared in distilled water and done at room temperature and pH 7. Dialysis of 90% salt precipitated sample against distilled H_2O has resulted in activity of 28.8U and with an enzyme concentration of 1.4 mg/ml.

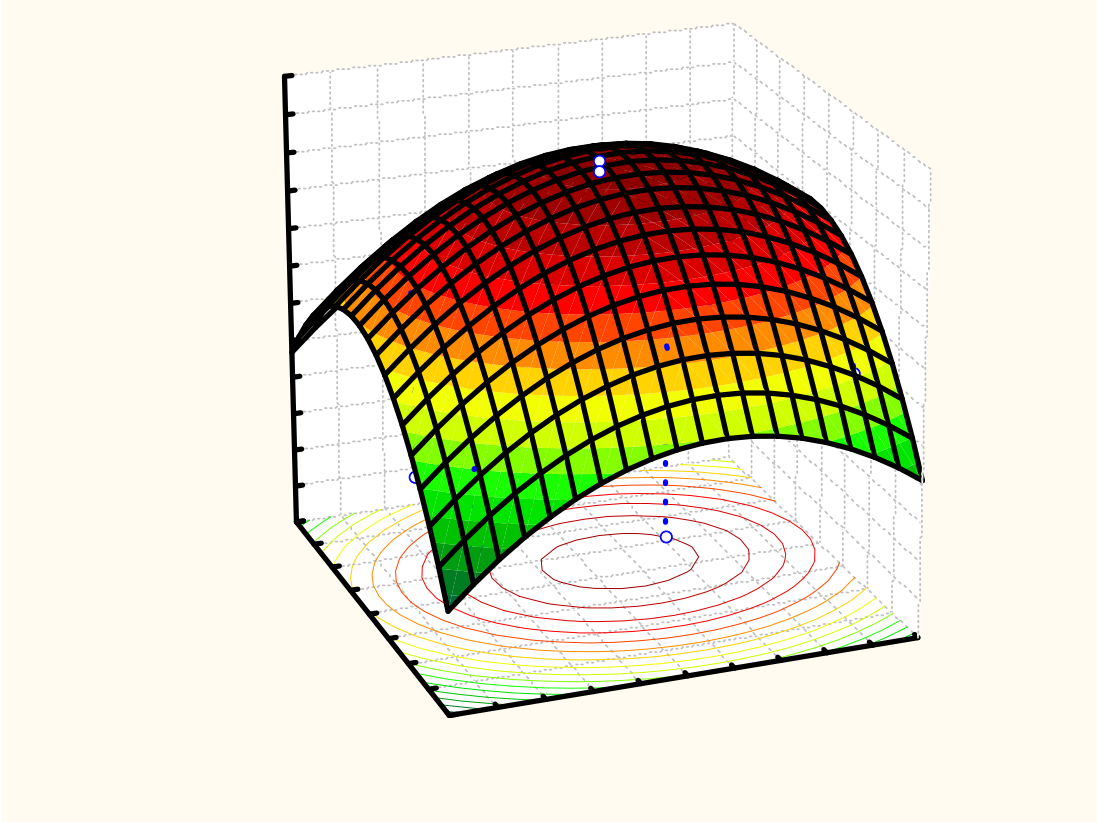

Fig. 4. Effect of Calcium Chloride and pH on amylase activity

3.2 Affinity Chromatography

The sample was tested for the presence of amylases by starch hydrolysis method initially. The samples showed no change in color of starch solution to iodine test when compared with blank, this conforms the presence of amylases in the eluted samples. The enzyme concentration was found to be 0.032mg/ml and activity was determined as 4.76 U.

4. CONCLUSION

The optimal values of the parameters that are essential for the preparation of α-amylases extract from pericarp of *Borassus flabellifer* fruit are successfully determined by the response surface methodology (RSM). The predicted amylase activity was 27.74538 U per ml of extract at the optimal conditions (pH, 4.86246; temperature, 17.74892ºC; and $CaCl_2$ concentration, 0.02267 ppm). When the model was checked for the fitness the coefficient (R^2) was determined as 0.9902. This fitness reveals that 99.02% of sample variation in α-amylase production is attributed to independent variables. Based on the optimal values obtained by RSM, the α-amylase production was successfully done and its concentration and activity were determined as 1.4 mg/ml, 28.8 U for the 90% dialyzed enzyme sample. Novel affinity chromatographic technique designed by us has showed good results. An economical affinity chromatographic process was successfully designed and results of enzyme concentration 0.032mg/ml and activity as 4.76 U, depicts that this method has no reduced effect on enzyme activity and has good affinity for amylases by starch flakes. It has

better affinity towards amylases driving new way towards downstream processing with reduced costs.

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COMPETING INTERESTS

The studies were carried out using a seasonally available fruit that has a digestive enzyme in its pericarp. Owing to the applications of amylases an alternative enzyme source with good activity and which could be purified using an inexpensive method was indicated. No competing interest exists.

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