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Evaluation of factors affecting L-asparaginase activity using experimental design

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Abstract

The present study aims for the purification and characterization of L-asparaginaseproduced by *Fusarium solani*. Microbial L-Asparaginase has attracted considerable attention, owing to the cost effective and eco-friendly nature. The most common use of asparaginases is as a processing aid in the manufacture of food. L-asparaginase used to reduce the formation of acrylamide, a suspected carcinogen, in starchy food products such as baked or fried snacks and biscuits. Extracellular L-asparaginase was produced by solid-state fermentation (SSF) using sequential statistical strategy as optimization for some vital factors; wheat bran concentration, fermentation time, moisture content, inoculum size, temperature, culture age and asparagine concentration which were adjusted by the sequential two design of response surface methodology. *Fusarium solani* produced the highest L-asparaginase level (254 I/U) using Placket-Burman design andimprovement up to 290 U/gds upon applying the Box–Behnken design. Partial purification of the enzyme using acetone was done, the specific activity increased to 709.24/mg in the fraction of 40-60 % acetoneThe optimum temperature and pH of the enzyme were 40ºC and 7, respectively. The K_m and V_{max} of the partially purified enzyme were 9.1×10^{-2} mμ and 20 U/mg proteins respectively. The impact of L-asparaginase on the acrylamide content reduction after high heat treatment in a model system as well as in potato based material was investigated.

Keywords : *Fusarium solani* -L-Asparaginase - Solid State Fermentation- partially purified enzyme- sequential statistical strategy.

Introduction

Therapeutic enzymes have a wide variety of applications as replacement for metabolic deficiencies and as anticoagulants and anti-tumor. In the field of medicine, L-asparaginase is considered as an effective antitumor agent (Baskar and Renganathan, 2009). It is used in the treatment of lymphoblastic leukemia. An enzyme called asparagine synthetase in human is responsible for the synthesis of L-asparaginase from central metabolic pathway intermediates inside the cells. L-asparaginase helps in the hydrolysis of L-asparagine into L-aspartic acid and ammonia (Jha *et al*., 2012). The normal cells are able to synthesize their own asparagine while the leukemic cells need a great amount of it to keep up with their rapid malignant growth, L-asparaginase (ASNase) triggers metabolic reprogramming of leukemic cells which is part of the adaptation process to stress caused by amino acid depletion (Hermanova *et al.*, 2016). Also, L-asparaginase is known for its importance in food application, this enzyme is used in food industry to prevent the acrylamide formation when foods are processed in high temperatures. This use is important because acrylamide is a neurotoxin classified as potentially carcinogenic to humans (Cachumba *et al*., 2016). Previously, bacteria are considered as a great source for producing L-asparaginase and a series of preclinical and

clinical tests. Nowadays, fungi, actinomycetes, plants and animals are different sources for the enzyme. L-asparaginasefrom fungal sources is preferred to that of bacterial sources as they don't cause allergic reactions and anaphylaxis (Kumar *et al*., 2012).

The submerged fermentation technique (SF) is the most adopted method used throughout the world for L- asparaginase production. This method has some limitations which can be overcome by solid state fermentation technique (SSF). The latter method has many advantages such as low energy used, high product, low cost and water use in addition to simple fermentation media used (Chavez-Gonzalez *et al*., 2011).

Agricultural wastes can be used as a source of nutrients; e.g. agro-wastes from leguminous crops (Mishra, 2006), rice bran (Venil *et al*., 2009), soybean meal (Hosamani and Kaliwal, 2011).These wastes are cost effective and environment friendly (Couto and Sanroman, 2005).

The objectives of this work were to study the various effects that influence the production of L-asparaginase using *F. solani* from different agro-wastes using solid state fermentation and analyze the mutual interactions among the variables in a statistically valid manner using Placket-Burman design. Partial purification of the enzyme and its kinetic properties also application of the partially purified in food industry were also examined.

MATERIALS AND METHODS

Chemicals : All chemicals used in this study were of analytical grade.

Microorganism and inoculum preparation : The fungus used through this study was *Fusarium solani* provided by culture collection Centre of the National Research Center, Cairo, Egypt. The culture was maintained on the modified Czapek-dox agar medium supplemented with L-asparagine 1.5 % (w /v), incubated at 30ºC for 3 days, the stock culture was preserved at –80°C in 50% (v/v) glycerol with regular monthly transfer. Fungal suspension was prepared from freshly raised seven days old culture of *Fusarium solani* on Czapek-dox agar slants by suspending in 10 ml of o.85% sterile saline.

Fermentation medium : Wheat bran obtained from local market was used as the substrate for the production of L-asparaginase by *F. solani*. One gram of the substrate was taken in 250 ml Erlenmeyer flasks and moistened by modified Czapek medium under solid state fermentation (SSF). The flasks were autoclaved at 121ºC for 20 min, then cooled and followed by inoculation with 2 ml of spore suspension. The flasks were mixed thoroughly and incubated at room temperature for different incubation periods. Afterwards, the moldy substrate was analyzed for L-asparaginase production.

Extraction of L-asparaginase in SSF : Samples were withdrawn after different time intervals of fermentation and L-asparaginase activity was tested. A solution of NaCl (1 %), Trition X-100 (1 %) was used to transfer the solid media to liquid one so that the enzyme could be extracted by incubating the solution in a shaker at 180 rpm for 2 h at 30ºC (Vaseghi *et al*., 2013).

Qualitative estimation of L-asparaginase : L-asparaginase was assayed calorimetrically according to (Usha *et al.*, 2011). A standard curve was prepared with ammonium sulphate. One L-asparaginase (1U) is defined as the amount of enzyme that librates 1µmol of ammonia per minute under optimal assay conditions.

Partial purification of L-asparaginase : The purification was carried out using crude enzyme extract according to the following steps at 40ºC (Shafei *et al*., 2015).

Acetone precipitation : The enzyme was precipitated in a sequential manner using different acetone concentrations (v/v %). The mixture was centrifuged at 6000 rpm at 4ºC for 30 min and the precipitate was collected and stored at 4ºC.

Characterization of the partially purified L-asparaginase : Determination of optimum pH and temperature : Optimum pH and temperature

were determined by changing individually the condition of the reaction mixture assay: pH from 5.0 to 7.0 using citrate-phosphate buffer and 8.0 to 9.0 using tris buffer while temperature varied from 30º to 50º C.

Thermal stability of the partially purified enzyme : The thermal stability of the enzyme was evaluated by measuring the residual activity. Samples were incubated at different temperatures from 40º-60ºC in tris buffer (1M, pH 8.0) for different time intervals.

Substrate specificity and determination of V_{max}&K_m : Identical reaction mixtures containing the same amount of enzyme preparation were made each received different concentration of L-asparagine (0.02M-0.12M).The maximum reaction velocity (Vmax), Michaelis–Menten constant (Km) - defined as the substrate concentration at half the maximum velocity- of the partially purified enzyme were measured according to Lineweaver-Burk plots relating 1/v to 1/s**.**

Statistical optimization

Placket-Burman design : Seven different fermentation variables were prepared in two levels (-1) for the low level and (+1) for the high level according to Placket-Burman design (1969). The design is practical especially when there are large numbers of factors and implemented in the setting that produce optimal or near optimum responses (Strobel and Sullivan, 1999).Table ² showed the factors under investigations as well as the levels of each factor used in the experimental design; the Placket-Burman experimental design is based on the first order model:

$$Y=â_0+ © â_1xi$$

Where Y is the responses, $â_0$ is the model intercept and $â_1$ is the variables estimates. This model evaluates the important factors that affect the production of L-asparaginase using SSF. The effect of each variable was determined by the following equation:

$$E(x) =© M^+ - M^- /N$$

where E(x) is the effect of the tested variable, M^+ and M^- represent L-asparaginase activity from the trials where (x) measured was at the high and low concentration respectively and N is the number of trials.

Box-Behnken design for modified Czapekdox media under SSF: Table IIpresents the design matrix of 13 trials using this design (Box and Behnken, 1960), factors of the highest main effect were prescribed into three coded levels (-1, 0, +1) for low, middle and high concentrations respectively). For predicting the optimal point, a second-order polynomial function was fitted to correlate the relationship between variables and responses (L-asparaginase activity). For the three factors, the equation is as follows:

$$Y=â_0+ â_1x_1+â_2x_2+â_3X_3+â_{12}x_1x_2+â_{13}x_1x_3+â_{23}x_2x_3+â_{11}x_1^2+â_{22}x_2^2+â_{33}xâ_3^2.$$

Where Y is the predicted response; $â_0$ in the model constant; X_1, X_2 and X_3 are the independent variables; $â_1$, $â_2$ and$â_3$ are the linear coefficients, $â_{12}$, $â_{13}$ and$â_{23}$ are the cross product coefficients and $â_{11}$, $â_{22}$and$â_{33}$are the quadratic coefficients. The values of the coefficients were calculated and the optimum concentrations were predicted using JMP software. The quality of the fit of the polynomial model equation was expressed by R^2 (regression coefficient). The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. All experimental designs were randomized to exclude any bias. Experiments were carried out in duplicates and mean values were given. The SE of the concentration effect was the square root of the variance of an effect, and the significant level (P-value) of each concentration effect was determined using student's t-test.

$$T(x) = E(x)/SE$$

Where E(x) is the effect of the variable x

Validation model : The statistical model was validated with respect to L-asparaginase production under the conditions predicted by the model. Samples were withdrawn at the desired

intervals and L-asparaginase assay was carried out.

Effect of moisture level in SSF : The solid substrate i.e. wheat bran was moistened using modified Czapek medium the composition of which was reported previously. Different levels of moisture were tested 3%, 4% and 5% ml to determine the optimum moisture level for enzyme production. The enzyme was the extracted and assayed.

Application of L-asparaginase in food : Potato chips were washed, peeled and cut into 2mm thick chips using a slicer. The chips were soaked for 10 min with 5 ml of 0.5 % (w/v) glucose solution. Five ml of partially purified enzyme solution and 15% (w/v) of tri-chloroacetic acid were incubated in Tris-buffer (1M, pH 8 at 37°C. The chips were then fried in oil for 8min at 190°C. The quantification of acrylamide was performed by an Agilent 1100 model HPLC system (Waldbrann, Germany). The chromatographic separations were performed in Zorbax ODS column using the mobile phase (7% v/v) methanol in 0.025 mol/L sodium dihydrogen phosphate) at a flow rate of 1ml/ min. The acrylamide was detected at 215nm with continuous monitoring the peak spectra within the range of 190-350nm. Control samples were also prepared, using untreated potato chips (Ahn *et al*., 2002).

RESULTS AND DISCUSSION

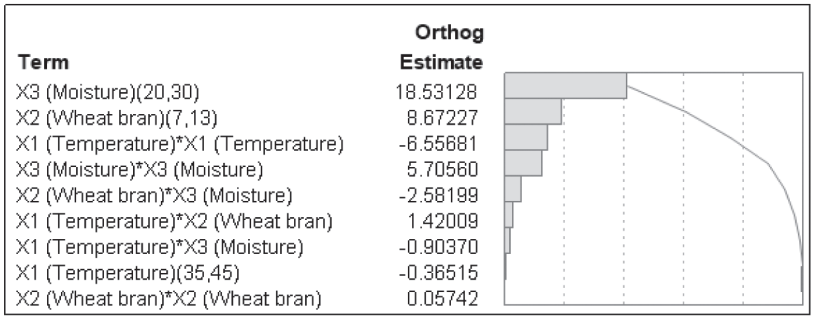
Evaluation of medium composition and operating condition affecting L-asparaginase production by Placket-Burman design: In solid state fermentation, seven factors namely; wheat bran concentration, fermentation time, moisture content, inoculum size, temperature, culture age and asparagine concentration were tested using Placket- Burman design (1946) for the enzyme production. Variations of enzyme activity ranging from 65 to 254 U/ml in the nine trials were observed (Table I). This variation shows that both medium composition and the operating conditions have great influence on the enzyme activity. The main effects of the tested variables on L-asparaginase production were calculated and represented in Figure 1. The enzyme activity was positively affected by wheat bran concentration, temperature, culture age and inoculum size while the other three factors time, moisture level and asparagine concentration showed negative effect on L-asparaginase activity. In order to approach the optimum response of L-asparaginase activity, the effective independent variables, including the temperature (X_1), wheat bran concentration (X_2) and moisture level (X_3), were further investigated each at three levels according to the Box and Behnken design (Table II). These results obtained were used for ANOVA analysis (Table III). Mathematical model equation 3 represents relationship between L-asparaginase activity (Y) and temperature (X_1), wheat bran (X_2) and moisture level (X_3) in coded levels was as follows:

$$\mathbf{Y = 250-0.5X_1+11.875X_2+ 25.375X_3+ 2.75X_1X_2-1.75X_1X_3-5X_2X_3-12.25X_1^2+X_2^2+11.5X_3^3}$$

The variables X_1X_2, X_1X_3 and X_2X_3 are interaction effects of temperature-wheat bran concentration, temperature-moisture level and wheat bran concentration-moisture level respectively. The significance of each coefficient was determined by the F and P-values.

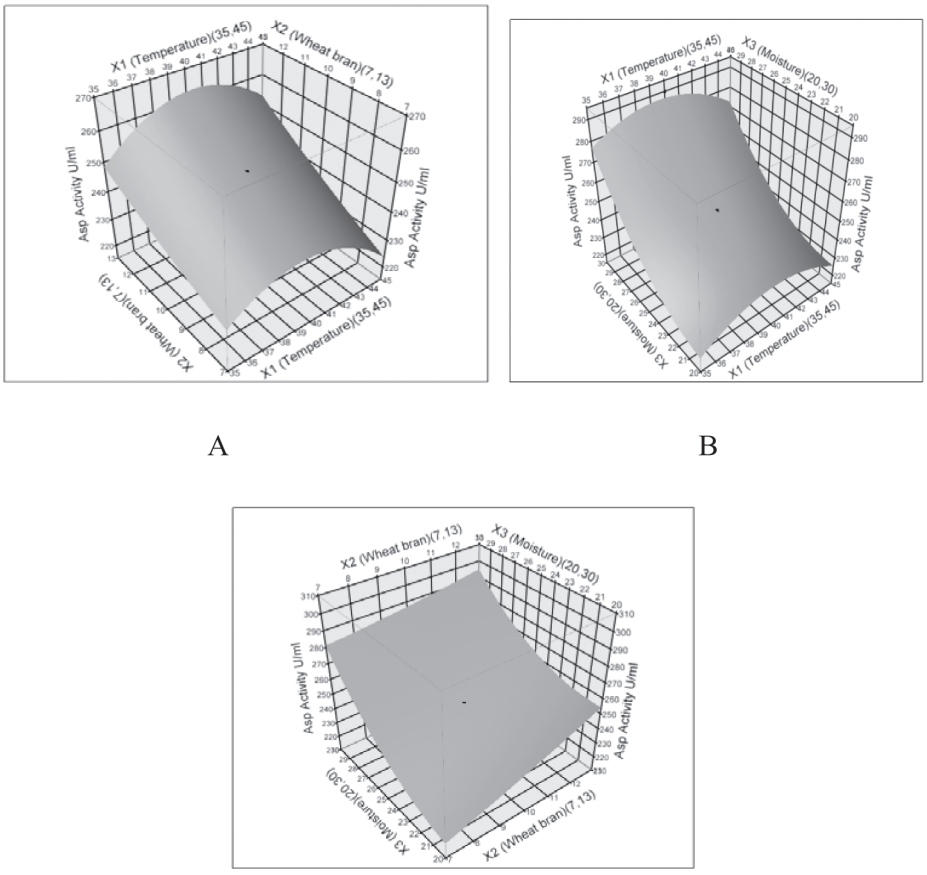
Low P-values of the linear terms for wheat concentration and moisture level and low P-values for quadratic terms for temperature and moisture showed high linear and quadratic effects of this parameter on enzyme production. Temperature showed high P-value (0.7584) for linear term and high quadratic term for wheat bran concentration which indicated insignificant linear and quadratic effect of these variables. It should be noted that the interaction effect of these variables were significant. From the ANOVA, the high F-value (44.3095) and low P-values ($P<0.0001$) indicate that the regression model were valid.

Lack of fitness more than 0.05 indicate that the model is significant for enzyme production. Also (R^2) which is multiple correlation coefficients indicate the correctness of the model also the predicted value is very close to the actual value.



Term	Orthog Estimate
X3 (Moisture)(20,30)	18.53128
X2 (Wheat bran)(7,13)	8.67227
X1 (Temperature)*X1 (Temperature)	-6.55681
X3 (Moisture)*X3 (Moisture)	5.70560
X2 (Wheat bran)*X3 (Moisture)	-2.58199
X1 (Temperature)*X2 (Wheat bran)	1.42009
X1 (Temperature)*X3 (Moisture)	-0.90370
X1 (Temperature)(35,45)	-0.36515
X2 (Wheat bran)*X2 (Wheat bran)	0.05742

Fig 1Pareto chart for Box behnken design under SSF



A B C

Fig. 2: Response surface plots showing the effects of the most significant independent variables on L-asparaginase production. (A) Wheat bran and temperature (B) Temperature and moisture content (C) Wheat bran and moisture content

R^2 value is 0.987617 which shows that the predicted value is very close to 1.0 and also very close to the actual one.The surface plot and the pareto chart (Figure 2) showed that near to moderate levels of wheat bran concentration, temperature and moisture level supported high L-asparaginase activity.

In each 3D curve (Figure 3 a, b, c), the effect of two factors on enzyme activity are shown, keeping other variables constant at zero level. From the statistical analysis, the moisture content had the most significant effect on the enzyme activity. Results obtained are in accordance with other findings where it was reported that high initial moisture content is accompanied by a decrease in enzyme production due to decrease in substrate porosity, gas volume and fungal growth in addition to change in structure of substrate particles. On the contrary, low moisture content decrease the nutrients solubility, substrate swelling and water retention by the substrate. All these factors influence the fungal growth and consequently the enzyme production (Baysal *et al*., 2003; Hosamani and Kaliwal, (2011); Beniwal *et al*., (2013).

Validation of the model: The maximum experimental response for L-asparaginase activity was in strong agreement with the predicted values.

Partial purification of L-asparaginase : The partial purification of the enzyme crude extract was carried out using acetone precipitation where the most active fraction was (40-60 %).The enzyme activity increased with every step of purification (Table IV). The partial purification steps were rapid and cost-effective; the fractions were collected and examined for enzyme activity and protein content. The total protein decreased from 62, 8 to 21.9 mg for the crude extract and the final preparation, respectively. The specific activity increased to709.24/mg in the fraction of 40-60 % acetone. The purification fold increase to 3.64.Similar results were obtained by (Thakur *et al*., 2014) on the purification of extracellular L-asparaginase from *Mucor hiemalis* by acetone, also (Bora and Bora, 2012)who reported that fewer steps of purification are more preferred as a loss of about 10% enzyme yield is recorded at each step of purification.

Kinetic properties of the purified L-asparaginase : Some properties of L-asparaginase from *F.solani* were investigated as

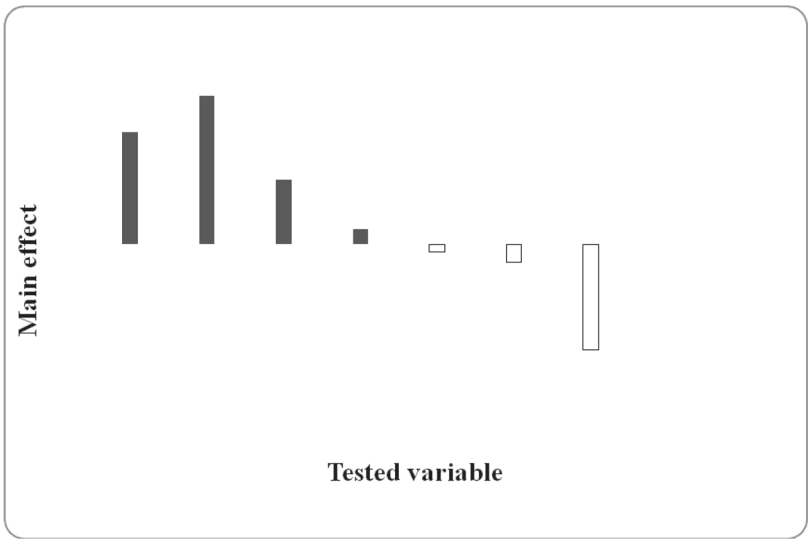


Fig (3): Main effect of variables affecting L-asparaginase activity under solid state

shown in Table V . The enzyme was active between pH values 5.0-9.0 with an optimum activity at pH 7.0, at higher pH values, L-asparaginase activity decreased. Nearly similar results were obtained by using *Penicillium sp* where the enzyme showed maximum activity at pH 7.0. (Patro and Goupta, 2012).(Amruthae*t al*., 2014) recorded the pH profile of L-asparaginase from *Fusarium sp* not only showed optima at pH 9.0 but also was active in the acidic pH that was in accordance with the studies of Sahue*t al*., 2007 who indicated that L-asparaginase is pH-dependent.

A temperature profile showed that the enzyme was measured at various ranges from 25-50°C. Maximum activity was obtained at 40°C. At higher temperatures, the reaction rate declined gradually until it lost nearly 57.3 %. Nearly similar results were shown by the purified L-asparaginase from *Penicillium brevicompactum* NRC 829 which was active at a wide range of temperature from 30-75°C with maximum activity at 37°C (El shafei *et al*., 2012).

The temperature tolerance of the enzyme showed it was stable at 40°C for 15 min, 30 min and 1 h and may be quite stable at 45°C (FIGURE IV). There was a progressive loss in enzyme activity at higher temperatures. L-asparaginase lost about 23.4 % of its activity after incubation at 50°C for 15 min while a rapid decrease (65 %) was observed after incubation at 55°C for 15 min. Kirshna and Gupta (2012) reported near results for the temperature stability of L-asparaginase at 37°C from *Penicillium sp.* On the contrary, enzyme activity from *Aspergillus terreus* KLS_2 showed stability at temperature 70°C for 30 and 60 min (Shafei *et al*., 2015).

L-asparaginase of different microorganisms expresses a great variety of substrate affinities and consequently plays ecophysiological roles in the enzyme activities. The effect of L-asparagine substrate concentrations (0.02-0.12 mμ/mol) on the partially purified enzyme was studied. The K_m and V_{max} of the partially purified enzyme were 9.1×10^{-2} mμ and 20 U/mg proteins respectively. The affinity of the enzyme to its substrate shows the degree of

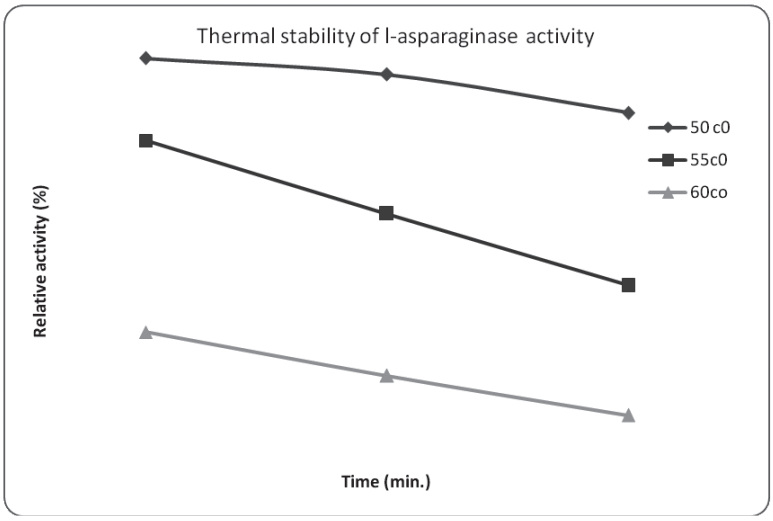


Fig. 4 : Thermal stability L-asparaginase produced by *F. solani*

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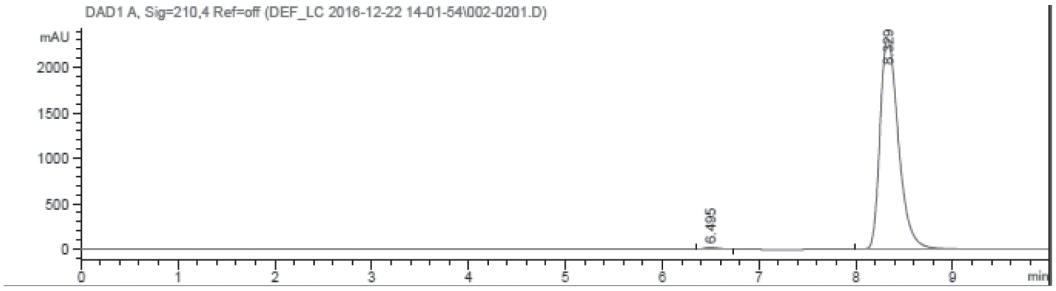
its effectiveness towards tumors. L-asparaginase of different microorganisms has different substrate affinities and probably plays different physiological roles in the enzyme activity. The linearity of the Linweaver-burk double reciprocal plot also indicates that our enzyme followed Michaelis-Menten kinetics (Gaffes, 1975). Also Km and Vmax values of L-asparaginase from Pseudomonas aeruginosa 50071 were 0.147 mM and 35.7 IU, respectively (El-Bessoumy *et al.*, 2004).Lower K_m values were reported for other microorganisms like *Vibrio succinogens* (Prabhu and Chandrasekaran, 2000).



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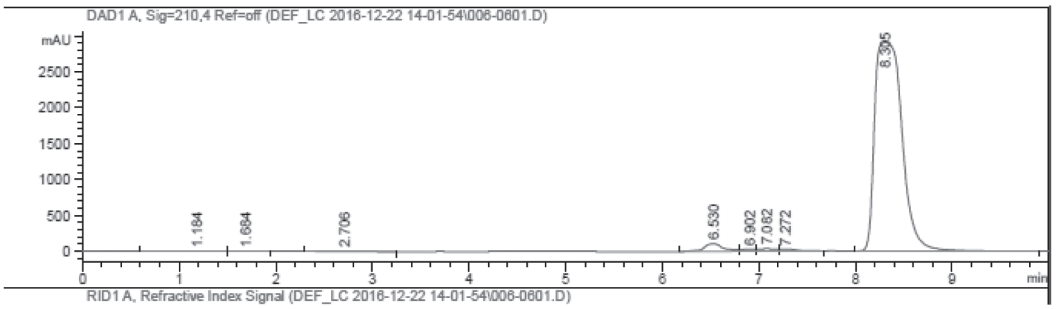

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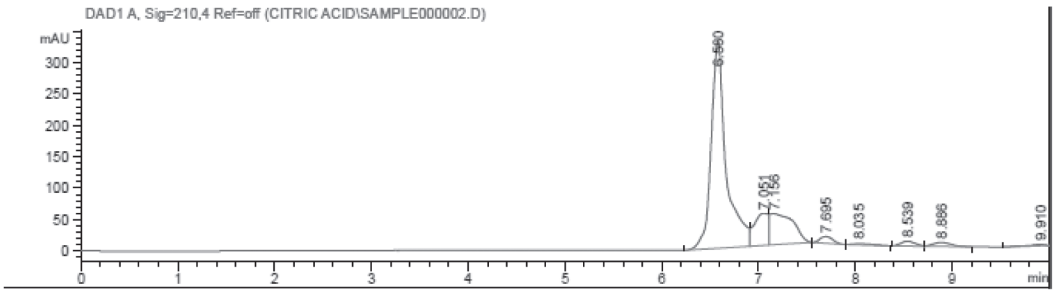

Fig (5): HPLC chart of (A) Standard acrylamide (B) the untreated fried potato chips (C) the enzyme (partial purified) treated fried potato chips

Table (1): Plackett-Burman experimental design for evaluation of factors (Coded levels and real values) affecting L-asparaginase activity under SSF

Trial	Wheat bran (g) x_1	Time (h)x_2	Moisture (%)x_3	Inoculum size (ml) x_4	Temp°Cx_5	Age of culture (day) x_6	Asparagine conc (g) x_7	Asp. Activity U/ml
1	-(3)	-(8)	-(30)	+(6)	+(35)	+(10)	-(0.3)	254
2	+(7)	-(8)	-(30)	-(4)	-(25)	+(10)	-(0.3)	222
3	-(3)	+(12)	-(30)	-(4)	-(25)	-(4)	+(0.5)	208
4	+(7)	+(12)	-(30)	+(6)	+(35)	-(4)	-(0.3)	205
5	-(3)	-(8)	+(50)	+(6)	-(25)	-(4)	+(0.5)	105
6	+(7)	-(8)	+(50)	-(4)	+(35)	-(4)	+(0.5)	222
7	-(3)	+(12)	+(50)	-(4)	-(25)	+(10)	-(0.3)	131
8	+(7)	+(12)	+(50)	+(6)	+(35)	+(10)	+(0.5)	245
9	0(5)	0(10)	0(40)	0(5)	0(30)	0(7)	0(0.4)	65

Table (2): Box-Behnken factorial design for optimization for L-asparaginase production using SSF

Trial	Independent Variables			Asp. ActivityU/ml
	Temperature°CX_1	Wheat bran(g)X_2	Moisture level%X_3	
1	- (35)	-(7)	0(25)	228
2	+(45)	-(7)	0(25)	222
3	-(35)	+(13)	0(25)	250
4	+(45)	+(13)	0(25)	255
5	-(35)	0(10)	-(20)	220
6	+(45)	0(10)	-(20)	222
7	-(35)	0(10)	+(30)	280
8	+(45)	0(10)	+(30)	275
9	0(40)	-(7)	-(20)	225
10	0(40)	+(13)	-(20)	255
11	0(40)	-(7)	+(30)	280
12	0(40)	+(13)	+(30)	290
13	0(40)	0(10)	0(25)	250

Table (3): Analysis of variance for L-asparaginase production by *F. solani* under SSF

Term	Coefficient estimate	DF	SE	SS	t-value	F-ratio	P-value
Corrected Model	-	9	-	7556.9833	-	44.3095	0.0003*
Intercept	250	1	2.513298	-	99.47	-	<.0001*
X1(Temperature)	-0.5	1	1.539074	2.0000	-0.32	0.1055	0.7584
X2 (Wheat bran)	11.875	1	1.539074	1128.1250	7.72	59.5317	0.0006*
X3 (Moisture)	25.375	1	1.539074	5151.1250	16.49	271.8272	<.0001*
X_1X_2	2.75	1	2.17658	30.2500	1.26	1.5963	0.2621
X_1X_3	-1.75	1	2.17658	12.2500	-0.80	0.6464	0.4579
X_2X_3	-5	1	2.17658	100.0000	-2.30	5.2770	0.0700
X_1^2	-12.25	1	2.265456	554.0769	-5.41	29.2389	0.0029*
X_2^2	1	1	2.265456	3.6923	0.44	0.1948	0.6773
X_3^2	11.5	1	2.265456	488.3077	5.08	25.7682	0.0038*

SS: Sum of squares.DF: Degrees of freedomSE: Standard error
R^2 =R Squared = 0.987617 (Adjusted R Squared = 0.965328)*Significant at 5% level.

Table (4): Summary of steps employed in partial purification of L-asparaginase produced by*F. solani*

Acetone Concentration	Total activity (u/F)	Recovered activity%	Total protein of fraction (mg/F)	Recovered Protein(%)	S.E.A (U/mg)	Purification fold
Crude enzyme	9825	100	62.8	100	156.45	0.80
0-20	1313	13.36	8.45	13.45	155.40	0.79
20-40	655	6.66	3.625	5.77	180.68	0.93
40-60	4213.3	42.88	5.941	9.46	709.20	3.64
60-80	615	6.26	3.885	6.18	158.30	0.51
Total	6796.3	69.17	21.90	25.6	194.90	1

Specific activity= total activity of fraction /protein of fraction
Culture filtrate means before precipitation
mg/f= milligram / fraction
U/f= unit/ fraction
U/mg protein= unit/ milligram protein

Table (5) Some properties of partially pure L-asparaginase produced by *F. solani*

Properties	Relative activity (%)
pH	
5.0	90.9
6.0	100.6
7.0	143.1
8.0	100
8.5	75.9
9.0	50.5
Temperature °C	
25	55.6
30	64.7
35	79.5
40	100
45	70.6
50	42.7
Substrate concentration mM/ml	
0.02 M	54.1
0.04 M	100
0.06 M	46.2
0.08 M	31.2
0.1 M	11.6
0.12 M	5.6

Application of L-asparaginase from *F. solani* in potatoes : The partially purified enzyme was tested for its impact in potato chips. The released ammonia confirmed the conversion L-asparaginase present in potato to L-aspartic acid. Upon frying the potato chips treated with L-asparaginase from *F. solani*, the acrylamide formed was approximately 99.3405 % lower than that with the untreated potatoes (FIGURE V). This is due to reduction of L-asparaginase in the external layer of the potato chips that could be reached by the enzyme. Thus, indicating the formation of L-aspartic acid and ammonia and accordingly preventing the formation of Millare reaction product and reduction in the high L-asparagine content in potato (Shafei*et al*., 2015).

CONCLUSION

Extracellular*Fusariumsolani* L-asparaginase was produced by solid-state fermentation (SSF)using sequential statistical strategy as optimization for some vital factors; wheat bran concentration, fermentation time, moisture content, inoculum size, temperature, culture age and asparagine concentration. Partial purification of the enzyme using acetone was done, the specific activity increased to709.24/mg in the fraction of 40-60 % acetone The optimum temperature and pH of the enzyme were 40ºC and 7, respectively. The Km and Vmax of the partially purified enzyme were 9.1×10-2 mμ and 20 U/mg proteins respectively. The impact of L-asparaginase on the acrylamide content reduction after high heat treatment in a model system as well as in potato based material was investigated.

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