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Research Article

L-ASPARAGINASE FROM *BACILLUS* SP. RKS-20: PROCESS OPTIMIZATION AND APPLICATION IN THE INHIBITION OF ACRYLAMIDE FORMATION IN FRIED FOODS

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Abstract: Reports of presence of acrylamide in wide range of fried and baked foods, most notably potato chips and French fries, by Swedish researchers in 2002 has raised a worldwide concern. However, the enzyme L-asparaginase reduces the formation of acrylamide in fried foods by pre-amidohydrolase of L-asparagine present. In this context, we report the hyper production of L-asparaginase from *Bacillus* sp. RKS-20, by process optimization involving statistical modeling approach. A maximum of 15.10 IU/ml of L-asparaginase were obtained in 18h under statistically optimized conditions wherein KH_2PO_4 (3.0 g/L), NaCl (1.0 g/L), L-asparagine (14.0 g/L) and glucose (2.0 g/L) were the influential factors. This was an approximately 10-fold increase as compared to the initial un-optimized activity of 1.50 IU/ml. The potential of this enzyme for the inhibition of acrylamide formation was confirmed when the potato slices treated with L-asparaginase (40 IU/mg of dry potatoes), showed reduction of 69.80% in acrylamide formation upon frying as compared to untreated potato slices. Hence, this enzyme is potential candidate for healthier production of food.

Keywords: L-asparaginase; L-asparagine; Response Surface Methodology; Acrylamide inhibition.

Introduction

Presence of acrylamide in a wide range of fried and oven-cooked foods have been found to be associated with the problems of neurotoxicity, reproductive toxicity, genotoxicity, clastogenicity and carcinogenicity (Friedman, 2003; Dybing, 2005). L-asparaginase (E.C.3.5.1.1) is an amidohydrolase of L-asparagine, which is being used for inhibition of acrylamide formation in fried or heated food products (Ciesarova *et al.*, 2006). The acrylamide formation in heated foods is mainly attributed to the reaction of free asparagine and reducing sugars (Ciesarova *et al.*, 2006). The enzyme interrupts the interaction of L-asparagine with reducing sugars thus preventing the acrylamide formation (Ciesarova *et al.*, 2006). L-asparaginases has also been known as potential candidate for treatment of certain types of cancer including acute lymphoblastic leukemia (ALL) and other human cancers (Jayam and Kannan, 2014).

L-asparaginase is widely distributed among plants and animals (Wriston Jr. and Yellin, 1973). Apart from these sources, number of microorganisms are known to produce L-asparaginase such as *E. coli*, *Erwinia carotovora, Enterobacter aerogenes*, *Corynebacterium glutamicum, Pseudomonas aeruginosa*, *Candida utilis*, *Aspergillus tamari*, *Aspergillus terreus*, *Staphylococcus aureus* and *Thermus thermophillus* (Mahajan *et al.*, 2012).*Bacillus* spp. has also been a well known producer of L-asparaginase and has been studied

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by different researchers. However, there are very scarce reports for over expression L-asparaginse from *Bacillus* spp.

It is essential to work on the economics while developing a bioprocess for industrial purposes (Joo *et al*., 2002). Optimization studies by one variable at a time approach is extensive but time consuming and neglects the interaction amongst the significant variables (Babu *et al*., 2009). Hence, statistically planned experiments effectively tackle the problem, which involves the specific design of experiments, which minimizes the error in determining the effect of parameters, and the results are achieved in an economic manner (Bari *et al*., 2009; Kwak *et al*., 2006; Abdel-Fattah *et al*., 2002a). Statistically based experimental design was applied for optimization of solid-state fermentation for the production of L-asparaginase by *Pseudomonas aeruginosa* 50071 (El-Bessoumy *et al*., 2004).

In the present investigation, optimization of L-asparaginase production by *Bacillus* sp. RKS-20 using statistical design of experiments by Face centered central composite design (FCCCD) was carried out. Further the application of L-asparaginase in inhibition of acrylamide formation in fried foods was also performed.

Materials and Methods

Chemicals: Nessler's Reagent was purchased from Fluka (Buchs, Switzerland). L-asparagine was purchased from Spectrochem (India). Other chemicals used were of analytical grade from Hi-Media (India).

Culture conditions: L-asparaginase production by *Bacillus* sp. RKS-20 was carried out in modified minimal synthetic medium with composition: Na_2HPO_4, 6 g/L; KH_2PO_4, 3 g/L; NaCl, 0.5 g/L; L-asparagine, 5 g/L; glucose, 2 g/L; $MgSO_4.7H_20$, 0.5 g/L; $CaCl_2.2H_2O$, 0.005 g/L. Flasks containing production medium (50ml in 250ml flask) were inoculated with 2% inoculums (v/v) (O.D. 4.0). Incubation was carried out at 37°C at 200 rpm for 24 h. All the experiments were carried out in triplicates and the mean values with standard deviations were calculated.

Assay for L-asparaginase: Assay of enzyme was carried out as per the nesslerization procedure given by Shirfrin *et al*. (1974), using 189 mM L-asparagine as substrate in 50 mM Tris buffer (pH 8.6), reading the absorbance at 436 nm. Cell free extract was used as the source of crude enzyme. The enzyme activity was expressed in International Unit (IU). One International Unit (IU) of asparaginase activity is defined as the amount of enzyme required to release one µmole of ammonia per ml per minute at pH 8.6 at 37°C.

Process Optimization for L-asparaginase production by one variable at a time method: The effect of different concentrations of various medium components ranging from 4-8 g/L for Na_2HPO_4; 1-5 g/L for KH_2PO_4; 0.25-1.25 g/L NaCl; 0.25-1.25 g/L for $MgSO_4.7H_2O$, 3-15 g/L for L-asparagine and 1-4 g/L glucose on enzyme production was evaluated by varying one factor at a time. The effect of physiological parameters like, temperature (30-50°C), pH (5-8), agitation rate (0-250 rpm), inoculum size (1.0-5.0% v/v) and incubation period (0-30 h) were also evaluated for their effect on L-asparaginase production. The factors showing predominant effect on the production of the enzyme were further carried over for RSM studies.

L-asparaginase optimization by RSM: The levels of four variables *viz*. L-asparagine (A), KH_2PO_4 (B), NaCl (C), and glucose (D) were obtained as the most influential parameters after optimization by one variable at a time method. These factors were selected for RSM studies (Table 1). Face centered central composite design (FCCCD) was employed to study the interaction of these parameters as it incorporates replication of the medial point (000). The ranges of these variables were decided according to the ranges determined by one variable at a time method with other variables at optimum level. Other variables were set at their optimum levels as obtained by one variable at a time (pH 6.0, agitation 200 rpm and incubation time 18 h). Experiments were performed in triplicates to minimize the experimental error.

The statistical software package 'Design–Expert ® 6.0, Stat-Ease Inc., (Minneapolis, USA) was used to analyze the experimental design. A set of 30 experiments were generated (Table 1). The design matrix with different variables was set at five levels ($-\alpha$, -1, 0, +1, $+\alpha$). All the variables were taken at a central coded value considered

Table 1
FCCCD Design for Optimization of L-asparaginase

Run	*Factor 1 A:Asparagine g/L*	*Factor 2 B:KH_2PO_4 g/L*	*Factor 3 C:NaCl g/L*	*Factor 4 D:Glucose g/L*	*Response 1 Activity U/ml*	*Predicted Activity IU/ml*
1	14.00	5.00	0.50	4.00	2.463	2.17
2	14.00	3.00	1.00	4.00	3.5	3.62
3	12.00	4.00	0.75	3.00	8.16	8.69
4	10.00	3.00	1.00	4.00	4.5	4.02
5	14.00	3.00	1.00	2.00	15.1	14.71
6	10.00	5.00	1.00	2.00	1.759	1.16
7	12.00	4.00	0.75	3.00	8.4	8.69
8	10.00	3.00	1.00	2.00	7.1	7.22
9	14.00	5.00	1.00	2.00	3.2	3.48
10	10.00	5.00	0.50	2.00	1.407	1.12
11	10.00	5.00	0.50	4.00	9.2	9.44
12	10.00	3.00	0.50	4.00	3.4	2.96
13	14.00	5.00	0.50	2.00	1.407	1.73
14	12.00	4.00	0.75	3.00	8.62	8.69
15	14.00	5.00	1.00	4.00	2.1	1.61
16	10.00	5.00	1.00	4.00	6.8	7.18
17	14.00	3.00	0.50	2.00	10.2	9.65
18	10.00	3.00	0.50	2.00	3.519	3.85
19	14.00	3.00	0.50	4.00	0.42	0.86
20	12.00	4.00	0.75	3.00	8.3	8.69
21	12.00	4.00	1.25	3.00	4.8	5.16
22	12.00	2.00	0.75	3.00	5.6	5.82
23	12.00	4.00	0.75	3.00	8.3	7.77
24	8.00	4.00	0.75	3.00	3.5	3.70
25	12.00	4.00	0.75	1.00	5.1	5.32
26	12.00	4.00	0.75	5.00	2.463	2.56
27	12.00	6.00	0.75	3.00	1.055	1.11
28	12.00	4.00	0.75	3.00	8.53	7.77
29	12.00	4.00	0.25	3.00	2.4	2.36
30	16.00	4.00	0.75	3.00	3.8	3.92

as zero. The minimum and maximum ranges of variables were investigated.

Application: The effect of purified L-asparaginase from *Bacillus* sp. RKS-20 on acrylamide inhibition in fried potato slices was studied. Potatoes were cut to very thin slices, oven dried and immersed in different concentration of enzyme from 10-50 IU/mg of dry potatoes. These samples were incubated at 37°C and were subjected to frying at 160-180 °C for 20 min. After frying, all the contents were grinded, mashed and extracted in 5ml of ethanol. The chloroform extract was concentrated by vacuum evaporation and analyzed by gas chromatography (GC) for acrylamide using a stable wax DA column with FID detector and helium gas as carrier. Potato samples not treated with enzyme were considered as control.

Results and Discussion

Process optimization for L-asparaginase production by one variable at a time approach: The optimization for the production of L-asparaginase by one variable at a time approach

(data not shown) showed that increasing or decreasing the concentration of $MgSO_4.7H_2O$ and Na_2HPO_4 had no predominant effect on the production. The results also showed that increasing the concentration of L-asparagine from 5 g/L to 12 g/L and NaCl from 0.5 g/L to 0.75 g/L resulted in increase in the production. However, 3 g/L of KH_2PO_4 and 2 g/L of glucose were found to be optimum, whereof further increase or decrease led to decrease in L-asparaginase production. The results also showed that production was feasible within the pH range of 5.5 to 7.5 with maximum production at pH 6.0. In contrast, pH of 7.5 was found to be the best for maximum enzyme production by *Staphylococcus* sp. (Prakasham *et al*., 2007). The temperature of 37°C was found to be the optimum though production was feasible from 35°C to 45°C. There was no production observed in cultures incubated below 30°C. Moorthy *et al*. (2010) have also reported 37°C as the optimum temperature for asparaginase production from *Bacillus* sp. However, optimum temperature for *E.coli* and *Erwini acarotovora* was found to be 30°C (Warangkar and Khobragade, 2008). The agitation rate of 200 rpm was found to be optimum for enzyme production. Decreasing the agitation rates (100 rpm and 150 rpm) led to decrease in the production of L-asparaginase whereas increase in agitation rates above 200 rpm (250 rpm) had no effect on the enzyme production. Optimum condition for production of enzyme by *Staphylococcus* sp. is 100 rpm (Prakasham *et al*., 2007) and for *Bacillus*, *E. coli* and *Erwinia* sp. is 200 rpm (Warangkar and Khobragade, 2008). Therefore, a medium containing Na_2HPO_4 at 6 g/L; KH_2PO_4 at 3 g/L; $MgSO_4.7H_2O$ at 0.5 g/L; NaCl at 0.75 g/L; L-asparagine at 12 g/L; glucose at 2 g/L and $CaCl_2.2H_2O$ at 0.005 g/L was found to be optimum for L-asparaginase production at pH 6.0, incubation temperature of 37°C and agitation at 200 rpm. Using these optimized conditions L-asparaginase activity of 6.38 IU/ml was achieved in 18 h which did not increase on further incubation.

Application of Face Centered Central Composite Design and data analysis: A four factor RSM using L-asparagine, KH_2PO_4, NaCl and glucose was carried out. FCCCD model was used using the optimal values obtained in OVAT. L-asparaginase production (response) of the experiment for each individual run along with the predicted responses are presented in Table 1 where maximum L-asparaginase titers of 15.10 IU/ml was achieved in 18 h of incubation in medium containing 14 g/L, L-asparagine; 3 g/L, KH_2PO_4; 1 g/L, NaCl and 2 g/L, glucose. There was approximately 10-fold increase in the L-asparaginase titers as against the yield obtained initially in un-optimized conditions which was 1.50 IU/ml. The result obtained after FCCCD was then analyzed by Standard Analysis of Variance (ANOVA), which gave the following regression equation:

$$*Y = +8.23 + 0.54\ A - 1.19\ B + 0.70\ C - 0.69\ D - 0.99\ A^2 - 1.07\ B^2 - 1.00\ C^2 - 0.96\ D^2 - 1.29\ AB + 0.42\ AC - 1.97\ AD - 0.83\ BC + 2.31\ BD - 0.58\ CD$$

Where Y: L-asparaginase activity, A: L-asparagine, B: KH_2PO_4, C: NaCl, D: Glucose

The regression equation obtained from ANOVA showed that AB, AC, AD, BC, BD, CD were significant model terms. The R^2 value 0.9877 for L-asparaginase production, point to the accuracy of the model. The ANOVA for the responses (Table 2) indicated that the model was significant. The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value should be between 0 and 1. The closer the R^2 value is to 1, stronger the model is and the better is the predicted response (Haaland, 1989). Sample variation of 98.77% for L-asparaginase production was attributed to the independent variables and only 1.23% of the total variation was not explained by the model. This ensured a satisfactory fitting of the quadratic model to the experimental data. An adequate precision of 35.089 for L-asparaginase activity indicated an

Table 2
ANOVA Analysis

R^2	0.9877
Adj R^2	0.9754
Pred R^2	0.9406
Adeq Precision	35.089
Model F-value	328.10
Lack of fit-value	03.95

adequate signal (Mahajan *et al*., 2012). The *Pred* R^2 of 0.9406 indicated a good agreement between the experimental and predicted values for L-asparaginse production (Table 2). The *adjusted* R^2 value is very close to the R^2 value. The *Lack of fit F-value* of 3.95 for L-asparaginase activity implied that the lack of fit is insignificant and the model is adequate.

Hence, optimum condition for L-asparaginase production by *Bacillus* sp. RKS-20 was found to be Na_2HPO_4, 6 g/L; KH_2PO_4, 3 g/L; NaCl, 1 g/L; L-asparagine, 14 g/L; glucose, 2 g/L; $MgSO_4.7H_20$, 0.5 g/L; $CaCl_2.2H_2O$, 0.005 g/L at pH 6 at 200 rpm at 37°C with the maximum activity of 15.10 IU/ml in 18 h. In this respect, the enzyme activity was 2.68 times more than that obtained by Siddalingeshwara *et al.* from *Aspergillus terreus* in 72 h (Gurubasappa and Kattimani, 2010). The reported yield of enzyme is 27.88 IU/ml from *Pectobacterium carotovorum* MTCC 1428 (Kumar *et al*., 2009). Ghosh *et al.* (2013) reported the maximum L-asparaginase activity of 5.86 U/gds by *Serratia marcescens* (NCIM 2919) under solid state fermentation using coconut oil cake. Venil *et* al., (2009) also reported the maximum L-asparaginase production from *Serratia marcescens* SB08 optimized by RSM. The enzyme yields reported were only 3.27 IU/ml from *E. coli* and even lesser from *Erwinia* sp. (Warangkar and Khobragade, 2008); hence yields reported by us is significantly important and economical from industrial view point.

The response surface curves were plotted to understand the interaction of the medium components and its concentration required for optimum L-asparaginase production. The interaction of two variables *viz.* glucose and NaCl at constant L-asparagines (14 g/L) and KH_2PO_4 (3 g/L) after 18 h of incubation is presented in Figure 1. It can be inferred that maximum L-asparaginase activity of 15.10 IU/ml was predicted at the concentration of 2 g/L of glucose and 1 g/L of NaCl. Further increase or decrease in the respective concentrations lead to the decrease in the enzyme production. The interaction amongst different NaCl and L-asparagine concentrations is presented in Figure 2. Again, the maximum activity of 15.10 IU/ml was predicted at concentrations *viz.* L-

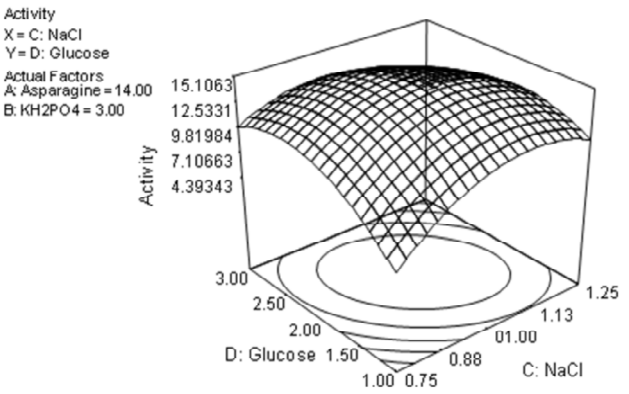

Figure 1: **Response surface curve of L-asparaginase activity by *Bacillus* sp. RKS-20 showing interaction between glucose and NaCl.**

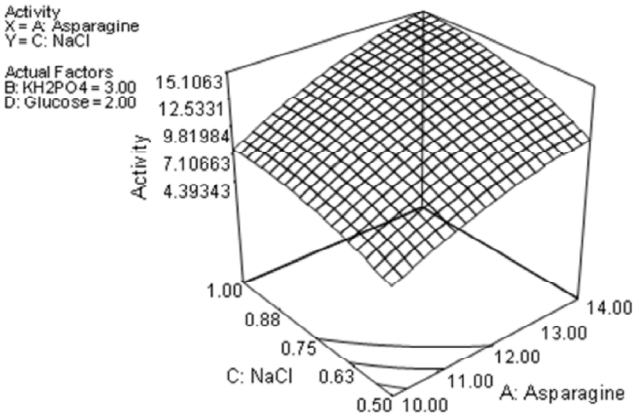

Figure 2: **Response surface curve of L-asparaginase activity by *Bacillus* sp. RKS-20 showing interaction between NaCl and L-asparagine.**

asparaginase (14 g/L) and NaCl (1 g/L) keeping KH_2PO_4 (3g/L) and glucose (2 g/L) concentrations constant.

Validation of the model: In order to determine the accuracy of the model, 10 sets of experiments were performed on the same four factors *viz*- L-asparagine (A), K_2HPO_4 (B), NaCl (C), and glucose (D) which were selected for RSM designs. Table 3 presents the L-asparaginase yield of each individual experiment along with the predicted response. The results verify the previous model that L-asparagine at 14 g/L, KH_2PO_4 at 3 g/L, NaCl at 1 g/L, and glucose at 2 g/L as the best combination for obtaining the maximum L-asparaginase production. The maximum yield of 15.07 IU/ml was obtained experimentally and this was closer to the predicted value 15.10 IU/ml.

Table 3
Validation model of FCCCD

Run	*Factor 1 A:Asparagine g/L*	*Factor 2 B:KH_2PO_4 g/L*	*Factor 3 C:NaCl g/L*	*Factor 4 D:Glucose g/L*	*Response 1 Activity IU/ml*	*Predicted Activity IU/ml*
1	13.51	2.91	1.17	2.07	12.87	13.03
2	11.66	2.77	1.62	3.24	10.86	11.02
3	10.70	1.02	1.12	2.25	11.06	10.90
4	12.18	2.67	1.75	3.49	7.34	7.10
5	12.72	2.91	1.56	3.13	9.18	8.94
6	13.26	2.48	1.49	2.98	9.17	9.10
7	10.14	2.47	1.83	3.65	10.98	11.10
8	12.94	2.98	1.36	2.73	9.08	9.21
9	13.39	2.90	1.23	2.46	8.20	8.08
10	14.00	3.00	1.00	2.00	15.07	15.10

***Application*:** Application of L-asparaginase was tested for inhibition of acrylamide formation in fried foods. The potato slices were treated with different enzyme and some left untreated. These samples were incubated at 37°C and fried in olive oil for 20 min at high temperature (160°C-180°C). The inhibition of acrylamide formation at different enzyme concentrations is presented in Figure 3. The potato slices which were treated by L-asparaginase at 40 IU/mg showed maximum inhibition of acrylamide concentration of 69.80%. Those samples which were treated by L-asparaginase at 10, 20, 30 and 50 IU/mg showed 14%, 26.50%, 41% and 57% inhibition, respectively. The chromatogram formed by standard sample, enzyme treated sample and

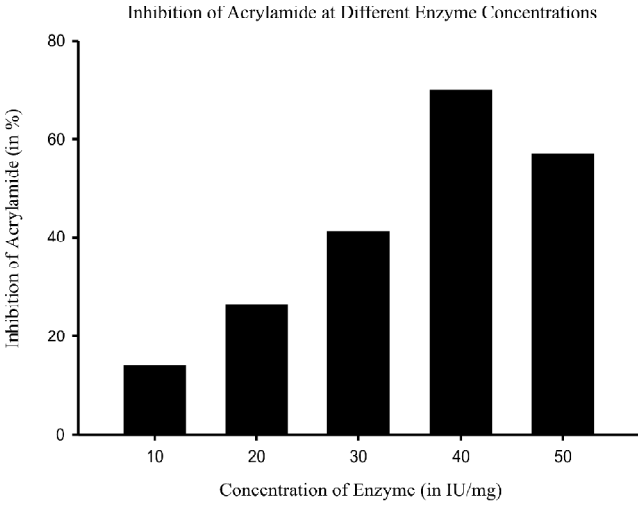

Figure 3: **Inhibition of acrylamide formation at different L-asparaginase concentrations.**

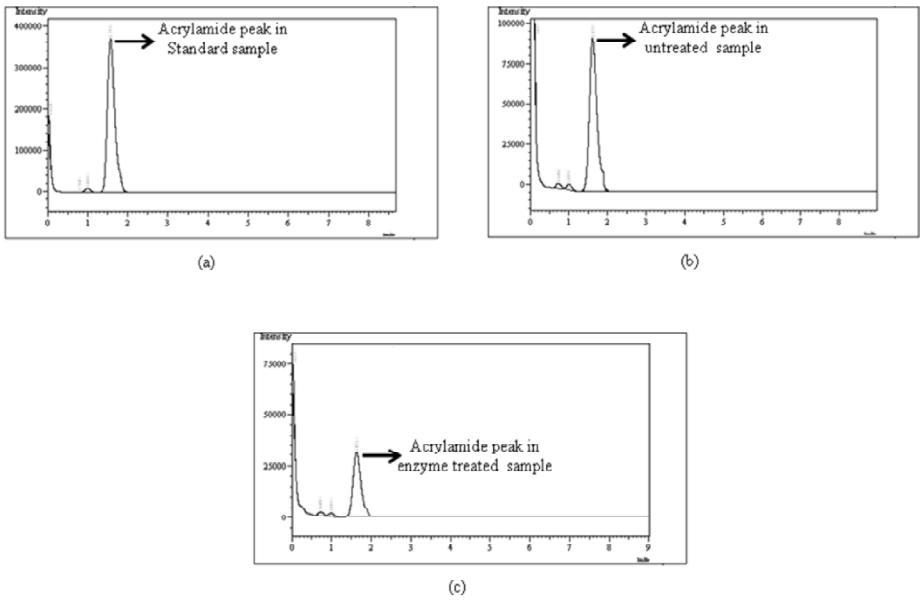

Figure 4: **Gas chromatograms of acrylamide (a) standard sample, (b) without L asparaginase treated sample and (c) L-asparaginase treated sample.**

without enzyme treated sample in gas chromatography is presented in Figure 4. It is evident that L-asparaginase treated sample showed 69.80% inhibition of acrylamide concentration as compared to the untreated sample. Hence, this study showed the immense importance of L-asparaginase in inhibition of acrylamide from fried foods. Therefore, L-asparaginase is an important tool in production of acrylamide free foods.

Conclusion

In the present work on process optimization, different concentrations of L-asparagine, KH_2PO_4, NaCl and glucose were selected and optimized to produce L-asparaginase. Design Expert from Stat-Ease was used to develop design of experiments. The activity of L-asparaginase enzyme produced by *Bacillus* sp. RKS-20 was enhanced to 15.10 IU/ml after 18 h with an increase of approximately 10-fold as compared to the initial un-optimized enzyme activity. The application of L-asparaginase in inhibition of acrylamide formation was successfully studied and it was found that the potato slices treated with L-asparaginase (40 IU/mg) showed maximum inhibition in acrylamide formation as compared to untreated potato slices.

Acknowledgement

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Abbreviations

IU, international unit; h, hours; min, minutes; °C, degree Celsius; v/v, volume per volume; O.D., optical density; ml, milliliter; g/L, gram per liter; rpm, rotation per minute.

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