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STUDY OF L-ASPARAGINASE FROM *RHIZOBIUM PUSENSE* STRAIN SS1 AND ITS APPLICATION IN MITIGATION OF ACRYLAMIDE FROM FRIED POTATO CHIPS

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ABSTRACT

Acrylamide (carcinogen, neurotoxin) is formed in starchy and carbohydrate rich foods because of Maillard reaction wherein L-asparagine and reducing sugar present in high amount react with each other. In this study, L-asparaginase enzyme was employed to lower acrylamide content from potato chips before frying. L-asparaginase producing bacteria (*Rhizobium pusense Strain SS1*, GenBank accession no. MH611376.1) was isolated from garden soil. Enzyme production media was optimized with 2% starch, 0.5% L-asparagine, pH 6.5 - 7.5, temperature of 28°C and agitation of 150 rpm, which gave 2200 unit/ml. The enzyme was partially purified with Ammonium Sulphate precipitation and dialysis to obtain enzyme activity of 1466 units/ml and specific activity of 36.60 units/mg. Molecular weight of the enzyme was found to be 37.5 kDa by SDS-PAGE. Enzyme kinetic studies showed the enzyme optimally active at pH 8.6, with temperature of 37°C and maximum substrate concentration of 190mM of L-Asparagine.Sliced potato chips were treated with partially purified enzyme. After frying the Acrylamide content was analysed with HPLC. There was 99.92% reduction in acrylamide content in enzyme treated potato chips sample. This study reports L-Asparaginase enzyme production by *Rhizobium pusense Strain SS1*for the first time and suggests a potential agent in mitigating acrylamide content from carbohydrate rich foods to be used in food industry.

Keywords: Acrylamide, L-Asparaginase, Potato chips, French fries, *Rhizobium pusense*, HPLC.

1. INTRODUCTION

The Swedish National Food Administration reported in 2002 the presence of relevant amounts of acrylamide or 2-propenamide (C_3H_5NO), a toxic and potentially cancer-causing chemical in several carbohydrates rich foods baked at high temperatures [1]. Soon after its discovery in heat-processed foods, scientists reported that acrylamide was formed during the Maillard reaction. Acrylamide can be released by the thermal treatment of certain amino acids (asparagine and methionine, for example), particularly in combination with reducing sugars [2]. Other mechanisms of formation is *via* acrylic acid which may be derived from the degradation of lipid, carbohydrates, or free amino acids; formation *via* the dehydration/decarboxylation of organic acids (malic acid, lactic acid, and citric acid); and direct formation from amino acids [3]. Mean concentrations of acrylamide in major foods were found to range from 399 to 1202 µg/kg for potato chips; 159 to 963 µg/kg for french fries; 169 to 518 µg/kg for cookies; 87 to 459 µg/kg for crisp bread and crackers; and 3 to 68 µg/l for coffee (ready to drink) [4]. The daily intake of acrylamide in human diets was estimated to be 0.3 to 0.8 µg/kg body weight. The maximum recommended daily intake of acrylamide is 195µg/75kg body weight [5].

Potato products are strongly susceptible to acrylamide formation. This food commodity contains the acrylamide precursors (asparagine and reducing sugars) and the traditional baking conditions applied (temperatures > 120°C), such as frying and roasting favour the Maillard reaction to occur, which is linked to acrylamide formation [6]. Thus, all potential strategies to prevent acrylamide formation may be resumed in two major approaches, (i) removal of the acrylamide precursors and (ii) interference with the Maillard reaction [7]. But the non-enzymatic, maillard browning reaction also influences several aspects of food quality such as, flavour, colour and aroma formation [8] and hence mitigation procedures that modify the Maillard reaction may negatively affect flavour and colour. Therefore the use of asparaginase to convert asparagine

to aspartic acid and hence not allowing the reaction of reducing sugars with this amino acid may provide a means to reduce acrylamide formation, while maintaining sensory quality [9,10]. Food and Agriculture Organization of the United Nations also recommends asparaginase treatment as one of the acrylamide mitigation measures to industry for the manufacture of potato products (*e.g.* French fries, potato crisps, potato snacks) in their publication on Prevention and Reduction of Food and Feed Contamination [11].

2. MATERIAL AND METHODS

All the chemicals were purchased from Merck and potatoes and frying oil from local market.

2.1. L-asparaginase source

Rhizobium pusense SS1 (GenBank *accession no.* MH6113 76.1) isolated from garden soil was used for the L-asparaginase production. The partially purified enzyme was used to study enzyme kinetics and use in mitigation of acrylamide from fried potato chips.

2.2. L-asparaginase assay

To quantify L-asparaginase enzyme, protocol of Mashburn L. T., 1963was used which recommends nesslerization where the rate of hydrolysis of asparagine is determined by measuring released ammonia. 3mM standard ammonium sulphate stock was used to prepare range of standards from 0.5-3.0 mM using distilled water as diluent, keeping system volume to 1ml. For enzyme assay, to 1 ml of 50mMTris buffer (pH8.6), 0.1 ml of 189mM L-asparagine solution was added with 0.9 ml of diluent. To this solution 0.1 ml of crude enzyme from the broth supernatant was added and incubated at 37°C for 30 min. After completion of incubation, 0.1 ml of 1.5M Trichloroacetic acid was added to stop the enzyme reaction. The mixture was centrifuged at 5000 rpm for 10 min and 0.2 ml of supernatant was taken and added to 4.3 ml of distilled water. 0.5 ml of Nessler's reagent is added. For blank, inactivated crude enzyme was used. One unit releases one micromole of ammonia per minute at 37°C at pH 8.6. The colour developed was recorded at 436 nm using the UV-Visible spectrophotometer (Systronics) [12].

2.3. Effect of various physical and chemical media parameters on production of enzyme

Modified M9 media was used as a basal media with incubation time of 48 hours. Physical parameters like pH (3, 4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9) [13, 14], temperature (4°C, 28°C, 37°C, 45°C, 55°C) [14], static and shaker (100rpm, 120rpm, 150rpm and > 200rpm) [15]; chemical parameters like carbon source (2% Mannitol, 2%Starch, 2%Sucrose, 2% Maltose, 2% Lactose, 2%Glucose) [13, 16] and nitrogen source (0.5% L-Asparagine, 0.5% Urea, 0.5% Ammonium sulphate, 0.5% Ammonium chloride, 0.5% Ammonium nitrate, 0.5% Sodium nitrate, 0.5% Ammonium sulphate) [16] were employed to determine optimum enzyme production.

2.4. Purification and characterization of L-asparaginase:

The production broth *i.e.* modified M9 medium [17] was inoculated with *R. pusense* SS1 and after 48 hrs of incubation was centrifuged at 10,000g for 10 min [18]. The supernatant acting as crude enzyme source was precipitated using 85% ammonium sulphate. The precipitate was separated from the broth by centrifugation at 10,000g for 15 min and resuspended in cold 0.05M Tris-HCl buffer of pH 8.60. The sample was subjected to dialysis overnight using the Tris-HCl buffer with membrane of 12-14 kDa cut off limit (Hi-Media) [19, 20]. SDS-PAGE was performed to identify the molecular weight of the partially purified enzyme [21].

2.5. Enzyme kinetic studies

The partially purified L-asparaginase enzyme stability was studied by incubating the enzyme with buffers of pH (3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 8.6, 9.0) [21, 22], incubation temperatures of (25°C, 30°C, 37°C, 45°C and 55°C) [23], substrate concentration (150mM, 170mM, 190mM, 210mM and 250mM of L-Asparagine monohydrate solution).

2.6. Acrylamide mitigation from potato chips

Potatoes were washed with distilled water, peeled and cut into slices. The slices were rinsed immediately after cutting for 1 min in distilled water to eliminate starchy material adhering to the surface prior to frying. For control, the rinsed slices were not given enzyme treatment and deep-fried for 4 min in sunflower oil. The rinsed slices to be used as test were incubated with the crude enzyme extract at 50°C for 30 minutes (20g of sliced potatoes in 20 ml of extracted crude enzyme with known assay unit) [24].

2.7. Analysis of Acrylamide in food

The fried chips were cooled and finely chopped and homogenized, weighed (4g) and transferred to a closed

flask. 10 ml of hexane was added to defatten with continuous shaking for 5 minute and dried under vacuum after decanting, 20 ml of acetone was added to the samples. The flasks were placed in an ultrasonic bath at 4°C for 20 minutes. 10 ml of the filtrate was evaporated under vacuum to dryness. 2ml of HPLC grade water was added and shaken vigorously to dissolve the residue [25].

2.8. Preparation of standard solution of acrylamide

Stock solution of acrylamide (1000µg/ml) was prepared in HPLC grade water and further 2µg/ml of working stock was prepared. Working standard solutions of acrylamide viz. 160, 320 and 400 Nano gram/ml were prepared and stored at 4°C [25].

2.9. Acrylamide measurement using High Performance Liquid Chromatography (HPLC)

The aqueous solution was filtered through a 0.2 µm membrane filter and injected to the column using a 20 µl injection loop. The column used was C18-5µm, 4.6×250 mm (Shimadzu). Flow rate of 1ml/min was adjusted and the column temperature was kept constant at 40°C. The analysis was performed at 202 nm with a UV detector. The retention time for acrylamide in above condition was 2 min. Application of standard acrylamide and acrylamide extracted from the treated potato chips was done at the same time. Chromatograms were generated with post run analysis tool and were analyzed for their intensity of peak responses at 202nm [24].

3. RESULTS AND DISCUSSION

3.1. Effect of physical and chemical media parameters on enzyme production

Table 1 describes the best physical and chemical media parameters, which resulted in optimum enzyme production. 2200 unit/ml of enzyme was produced for each of the best parameters considered. In case of nitrogen source, L-asparagine in combination with ammonium salts gave a higher units/ml of enzyme, but this could be due to unutilized ammonium in the broth reacting with Nessler's reagent and giving a false positive higher values. Hence those were not considered.

3.2. Purification of L-asparaginase

Enzyme was partially purified by performing Ammonium Sulphate precipitation with 85% saturation by referring to the standard ammonium precipitation chart [26] followed by dialysis using Tris-HCl buffer. The enzyme produced was tested for enzyme activity using L-asparaginase assay and the protein content of the purified enzyme by Folin Lowry method [27]. Specific activity was thus calculated and mentioned in Table 2.

On performing SDS-PAGE a band of 37.5 kDa [21, 22] was observed against a corresponding 42.6 KDa band of Egg albumin used as control as seen in Fig. 1.

Table 1: Effect of Physical and Chemical Parameters of medium on Enzyme Production

Parameters	Optimum	Unit/ml of enzyme
pH	6.5 – 7.0	2200
Temperature	28 ^{0}C	2200
Shaker	150 rpm	2200
Carbon source	2% Starch or 2 % Glucose	2200
Nitrogen source	0.5 % L-asparagine	2200

Table 2: Specific activity of enzyme before and after purification

	Before Purification	After Purification
Protein (µg/ml)	100	40
Enzyme (U/ml)	2200	1466
Specific activity (U/µg)	2.2	36.6

Fig. 1: SDS PAGE of partially purified Asparaginase enzyme with Egg albumin as control

3.3. Enzyme Kinetic Studies

The enzyme was found to be stable over a wide range of pH from 5.0 to 10, with maximum activity at pH 8.6. Similarly, enzyme was stable for temperatures from

37°C-55°C, with maximum activity at 37°C. The optimum substrate concentration for 0.1 ml of enzyme was found to be 190mM of L-Asparagine. Higher concentration of substrate did not alter the activity of enzyme. It may be thus inferred that, the substrate does not inhibit the enzyme and the enzyme shows a good activity at concentration above 190mM of L-asparagine till 250mM as found experimentally. Optimum volume of enzyme was found to be 0.1 ml for 0.1 ml of 189mM of L-Asparagine, substrate at 37°C.

3.4. HPLC Analysis

The content of Acrylamide in Control Potato chips was too high having a Peak height and Peak area of 1,44,391 and 15,65,500 respectively in chromatogram represented by Fig. 2. The peak for acrylamide of L-asparaginase treated chips, was having height and peak area of 229 and 1236 respectively represented in Fig. 3. Acrylamide concentration from potato chips reduced to almost 99.92% after treatment with Asparaginase enzyme obtained from *Rhizobium pusense*SS1 as mentioned in Table 3.

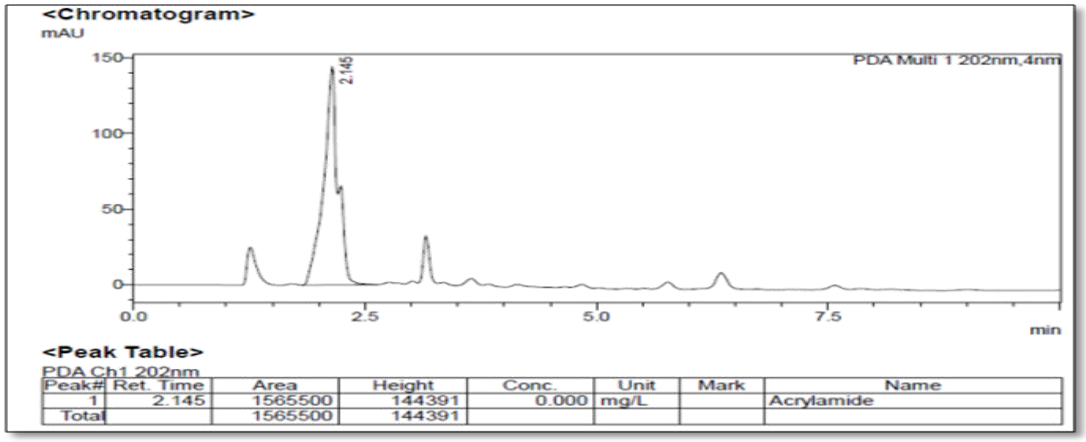

Fig. 2: Chromatogram of Untreated Potato slice

Table 3: Retention time, peak heights and peak area of standard acrylamide, untreated and treated potato chips

Treatments	Retention time of Acrylamide peak in min	Peak Height	Peak Area
Control-Untreated potato slice	2.145 min	1,44,391	15,65,500
Standard Acrylamide - 320 ng/ml	1.913 min	6515	57,777
L-asparaginase (2200 U/ml) treated potato slice	2.111 min	229	1,236

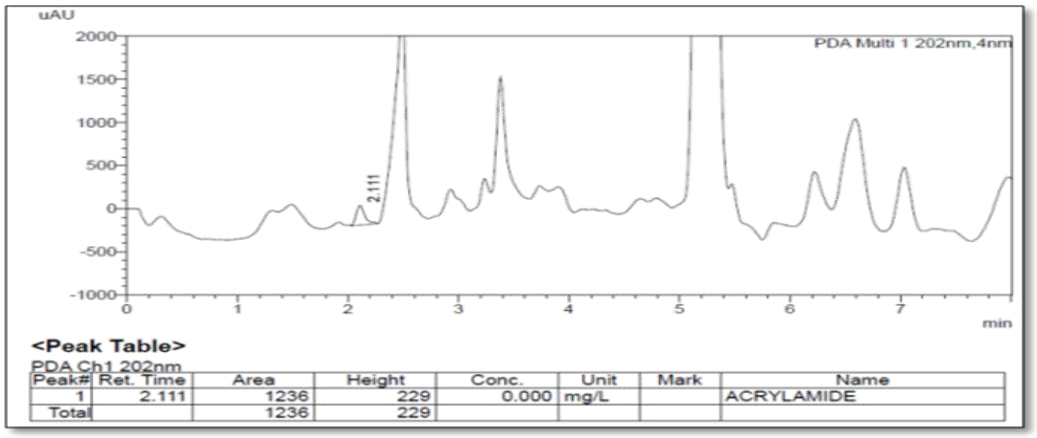

Fig 3: Chromatogram of treated Potato slice with L-asparaginase (2200U/ml) from *Rhizobium pusense SS1*

4. CONCLUSION

People are exposed to different amounts of acrylamide mainly through the diet. The sedentary and unhealthy life involves consumption of starchy, fried, baked foods as part of daily diet of many. Therefore mitigating the content of acrylamide from starchy food-stuffs is essential. To help lower the content of acrylamide in potato chips, L-asparaginase enzyme from *Rhizobium*-pusense SS1 was used. This is the first study which reports the use of *R. pusense* in L-asparaginase production. The L-asparaginase enzyme was partially purified by Ammonium sulphate precipitation method and dialyzed subsequently to remove the salt. Specific activity of enzyme was found to be 36.6 units/mg protein. The molecular weight of this enzyme was 37.5 kDa as observed with SDS-PAGE which is close to those cited in literature [21]. The wide range of pH and temperature stability as seen from the enzyme kinetic results is important since the enzyme can be used in food processing industry to mitigate the acrylamide produced during frying process.Partially purified L-asparaginase was employed to reduce L-asparagine content from Potato slices. About 99.92% reduction in acrylamide was observed after treating the potato slices with L-asparaginase [28] have reported reduction in acrylamide 62% in French fries and 80 % in potato crisps respectively whereas [29] have reported 65-85% reduction of acrylamide in French fries. In accordance with the other studies, enzyme isolated from *Rhizobium pusense* is prospective candidate for commercial asparaginase production.

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