ScienSage

Journal of Advanced Scientific Research

Available online through https://sciensage.info

ISSN
0976-9595
Research Article

# SCREENING AND ISOLATION OF ASPARAGINASE PRODUCERS FROM SOIL SOURCE AND ITS APPLICATION IN FOOD PRODUCTS

**Priya Sundarrajan*[1,2], Joanna D'costa[3], Geeta Ibrahim[3]**

[1]*Department of Life Science and Biochemistry, St. Xavier's College (Autonomous), Mumbai, Maharashtra, India*

[2]*Caius Research Laboratory, St. Xavier's College (Autonomous), Mumbai, Maharashtra, India*

[3]*Department of Food, Nutrition and Dietetics, College of Home Science Nirmala Niketan, Mumbai, Maharashtra, India*

**Corresponding author: priya.s@xaviers.edu*

*Received: 11-11-2021; Revised: 13-03-2022; Accepted: 20-03-2022; Published: 30-04-2022*

 https://doi.org/10.55218/JASR.202213309

## ABSTRACT

Asparaginases have applications in the medical and food industry as part of an anticancer strategy and as a mechanism to reduce acrylamide content in food. Suspension of garden soil sample was plated on nutrient agar plate. Colonies were picked and screened for asparaginase activity on minimal media. Cultures were selected based on qualitative/quantitative assays. Culture characterization and identification was carried out with the help of microscopic and biochemical techniques, checking culture growth parameters on varied pH and temperatures. Application of the crude enzyme obtained from the asparaginase positive isolates was carried out by performing assays using a frozen ready-to-cook food sample - potato smileys. Thin Layer Chromatography (TLC) of untreated and treated food samples was also performed to determine the levels of asparagine in the samples.

Out of a total of 36 isolates, four were selected for the study; which were later identified as *Pseudomonas putida*. The assays showed enzyme activity in all four cultures. Most of the isolates were found to grow at 37°C and pH of 7 and 10. Effect of the enzyme on asparagine content in the potato smiley was also detected. Treated samples showed more absorbance than the untreated sample. TLC method indicated that treated samples showed aspartic acid spots, thus showing effect of the enzyme while untreated sample plate showed no asparagine spots suggesting that low amounts of asparagine are present in the sample, or the raw potato used.

**Keywords:** Asparaginase, Acrylamide, Asparagine, *Pseudomonas putida*, Nessler Reagent, TLC.

## 1. INTRODUCTION

Enzymes are proteins by nature (exception - RNA acting as ribozyme), colloidal and thermolabile by character, and specific in their action. L- asparaginase is a therapeutic enzyme that showed potential in the field of medicine, particularly for its anti-tumour properties [1]. It was first discovered by Lang in 1904 in bovine tissues. Based on the available structural data, enzymes with L-asparaginase activity can be divided into bacterial-type and plant-type. They differ in structure, their substrate affinity and specificity profiles. Bacterial enzymes are then further divided into type I and type II, of which the type II enzyme obtained from *Escherichia coli* [*Escherichia coli* L-asparaginase II (EcAII)] is used clinically [2]. In 2002, Swedish scientists discovered the presence of acrylamide in foods and found out that this chemical may be toxic and carcinogenic to human beings. Following this, a joint conference was held by FAO and WHO that discussed about the general phenomenon of formation of acrylamide in foods. Many research studies were conducted after the findings, and it was discovered that asparagine is a precursor of acrylamide, which would be the link of the solution. Thus, the L- asparaginase that had been studied for many years was then used in various ways to reduce the asparagine content in foods, thereby reducing the acrylamide content [3].

It has also been confirmed that a wide range of foods prepared in high temperatures like fried potatoes, coffee, bread, and biscuit contain high levels of acrylamide. Therefore, it becomes important to reduce the acrylamide levels in such products which are being consumed more, especially by children. Studies have

reported that the use of microbial enzymes like L-asparaginase have negligible effects on the general formation of Maillard reaction products and it also reduces the precursor i.e., it hydrolyses L-asparagine to L-aspartic acid and ammonia which will help in reducing the acrylamide content in foods [4].

This study therefore focuses on extracting the enzyme L-asparaginase from microorganisms and further treating foods that have not been worked upon, to check its potential in reducing the asparagine (precursor to acrylamide) content in them.

## 2. MATERIAL AND METHODS

### 2.1. Material

Garden soil sample was used for this study to obtain asparaginase producers. All the reagents and chemicals used for the study were of analytical grade purchased from local distributors. Food sample used in the application of the enzyme was Potato smileys (frozen snack thawed to room temperature).

### 2.2. Methods

#### *2.2.1. Screening and selection of isolates*

A suspension of garden soil sample with distilled water (1g: 9ml water) was prepared and mixed well. A loopful of the supernatant was plated on Nutrient Agar (NA) plate and incubated overnight at 37°C. A total of 36 isolated colonies were randomly picked up from the NA plate and streaked onto grids of NA plate and Minimal media (M9) plate containing phenol red as indicator. Among the colonies, 10 colonies which showed change in colour of the indicator were then selected for further study and isolated for pure cultures.

#### *2.2.2. Qualitative assay for Asparaginase*

The isolated colonies grown overnight at 37°C, were centrifuged at 14,000 rpm/30mins at 4°C and supernatants were added into wells in the plates containing Asparagine agar with phenol red and observed for pink colouration.

#### *2.2.3. Quantitative assay for Asparaginase*

A loopful of 24 hour grown culture was inoculated in M9 broth. After incubation, absorbance was measured at 560nm. Cultures showing higher readings could be selected for the further study and those who showed less pink coloration and low readings were ruled out so that those showing higher readings could be selected for the further study.

#### *2.2.4. Growth parameters of the isolates*

Determination of growth parameters of the selected isolates was done by determining temperature (5°C, 23°C, 37°C, 50°C, 65°C) and pH tolerance (pH:4,7 and 10). Loopfuls of culture broths were inoculated into the different tubes and results at 24hours and 48hours were checked. Biochemical profiling: included gram staining, growth on MacConkey and XLD agar plates, oxidase test, and culture identification of the isolates (outsourced to Sunflower Laboratories, Malad, Mumbai).

#### *2.2.5. Determination of Asparaginase enzyme*

Asparaginase assay was performed using the culture supernatants as source of enzyme. The standard used was ammonium sulphate solution. Release of ammonia on breakdown of Asparagine to aspartic acid and ammonia was detected using Nessler's reagent. Absorbance was measured at 480 nm using the UV- Visible spectrophotometer. Amount of asparaginase content was calculated. Specific protein estimation using Lowry method was also performed using Bovine Serum Albumin (BSA) as the standard [5].

Reagents used were 0.05 M Tris-HCl buffer (pH 8.6), 0.01 M L-Asparagine in 0.05 M Tris-HCl buffer (pH 8.6), Trichloroacetic acid and Nessler's reagent. Ammonium hydroxide was used as standard.

Enzyme Source used was overnight grown culture at 37°C in Nutrient Broth and further centrifuged at 9000 rpm for 15 minutes (4°C) [6].

*2.2.5.1. Step 1*

The assay tubes were set up as given in Table 1.

**Table 1: Asparaginase assay protocol for enzyme samples**

| Buffer (ml) | Substrate volume (ml) | | Enzyme volume (ml) | Diluent volume (ml) | | Volume of TCA (ml) | |
|---|---|---|---|---|---|---|---|
| 0.2 | 1.7 | Equilibrate at 37°C for 5 minutes | 0.1 | 0.4 | Incubate at 37°C for 10 minutes | 0.1 | Incubate at 37°C for 30 minutes |
| 0.2 | 1.7 | | 0.2 | 0.3 | | 0.1 | |
| 0.2 | 1.7 | | 0.3 | 0.2 | | 0.1 | |
| 0.2 | 1.7 | | 0.4 | 0.1 | | 0.1 | |
| 0.2 | 1.7 | | 0.5 | - | | 0.1 | |

*2.2.5.2. Step 2*

Tubes from step 1 were centrifuged at 9000 rpm for 15 minutes and at 4°C. 0.5 ml of this supernatant was taken; to this 7ml of distilled water and 1ml of Nessler's reagent was added. This was incubated at room temperature for 10 minutes and absorbance at 480 nm was recorded.

Enzyme activity (units/ml) = (µmole of $NH_3$ liberated) (2.5) / (0.5) (10) (0.5)

where, 2.5 - Volume of Step 1, 0.5 - Volume of Step 1 used in Step 2, 10 - Time of assay in minutes, 0.5 - Volume (in millilitres) of enzyme used.

### *2.2.6. Application of the enzyme for treatment of food sample*

The crude enzyme obtained was tested on frozen Potato smileys as this snack is also deep fried and widely consumed.

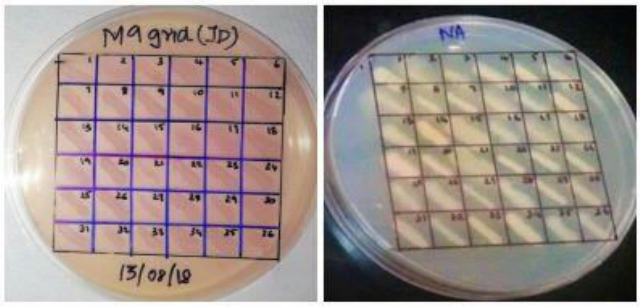
Asparaginase assay using Nessler's method as described above was performed where the food sample (45 mg) was pre-treated with the crude enzyme (0.5 ml) for one hour, mixed with 0.05 M Tris HCl buffer (pH 8.6) and centrifuged at 9000 rpm for 15 minutes. The enzyme substrate supernatant from the above step which was combined with buffer was then incubated for 15 mins, followed by TCA to stop the reaction. This mixture was then incubated for 30 mins and centrifuged at 9000 rpm for 15 minutes. 0.5 ml of supernatant was taken, and the volume was made up to 7.5 ml with distilled water. Nessler's reagent (1ml) was added, incubated for 10 mins and absorbance was measured at 480nm.

TLC was also performed on silica gel plates (EMerck) to identify asparagine in the untreated sample and aspartic acid in the treated sample after the action of the crude enzyme on asparagine. Methanol: Chloroform: 17 % Ammonium hydroxide was used in the ratio (2:2:1) as the solvent system. Ninhydrin solution (0.25%) was used as the staining reagent [7]. Test samples of each culture enzyme were prepared and used as control. 1g of sample was treated with 3 ml of the enzyme for different time periods (5 minutes, 30 minutes, and 60 minutes). The mixture was centrifuged at 8000 rpm for 15 minutes and then used for the chromatography.

## 3. RESULTS AND DISCUSSION

### 3.1. Screening and selection of isolates

Asparaginase positive isolates showed pink coloration on M9 media plates. All the 36 colonies showed pink coloration. Among them, 10 colonies were picked up for further study. The colonies were named -02, 04, 10, 11, 17, 19, 20, 21, 26 and 33 (numbered as per grid numbers) as depicted in Fig. 1.



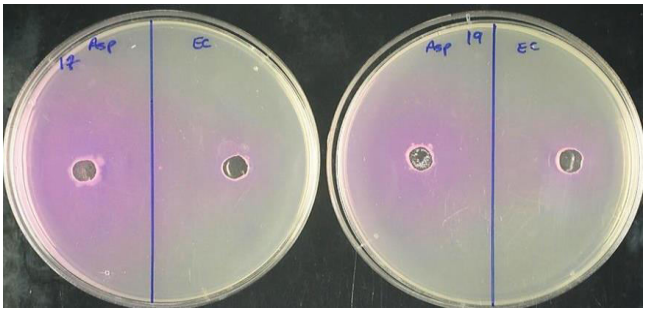

*Minimal media and Nutrient agar grid plates with 36 colonies from the diluted soil sample*

**Fig. 1: Minimal media (M9) and Nutrient Agar (NA) grid plates**

Inoculations of the colonies were done individually on M9 media plates along with Negative Control (Nutrient Broth) to observe asparaginase production.

### *3.1.1. Qualitative asparaginase assay (Supernatant assay)*

The diffusion of the enzyme on the plate showed pink coloration on change of pH because of alkaline condition development, which caused the phenol red to change from yellow to pink. The results shown in Fig. 2 indicates that the isolated colonies showed more colouration than the control (*Escherichia coli*) which indicates that the isolates were better asparaginase producers.




*Supernatant assay plate of colony cultures 17, 19 on the left of the plate and E.coli on the right side of the plate. More pink colouration seen on the left side of the plate shows more enzyme production by the soil sample cultures*

**Fig. 2: Supernatant assay with colony cultures 17, 19 and *E. Coli***

### *3.1.2. Quantitative asparaginase assay*

This assay used the same principle that L-asparaginase producing colonies turn the media broth to pink colour

due to alkaline condition development. The isolates showing the most absorbance at 560nm were selected. The selected cultures were 2b, 4a, 11 and 21a.iii, the results are shown in Table 2.

**Table 2: Quantitative asparaginase assay in the different isolates**

| Culture no. | Absorbance at 560nm |
|---|---|
| 02 a | 1.057 |
| 02 b | 1.065 |
| 04 a | 1.099 |
| 11 | 1.149 |
| 17 | 1.030 |
| 20. b. i | 0.917 |
| 20. b. ii | 0.442 |
| 21. a. ii | 0.406 |
| 21. a. iii | 1.119 |
| 21. b. ii | 1.029 |
| 33 | 0.901 |

### 3.2. Culture characterization

Temperature tolerance tests showed that after 24hours, maximum growth was observed at 37°C (isolate growth: 02b>21aiii>04a>11) for all four isolates followed by growth at 23°C (isolate growth: 21aiii>04a>02b>11) and no growth was observed at 5°C, 50°C and 65°C. as shown in Fig. 3. After 48hours, maximum growth was observed at 23°C (isolate growth: 04a>11>02b>21aiii) unlike the 24hours result, followed by growth at 37°C (isolate growth: 21aiii>02b>04a>11). At 50°C, growth of isolates 04a and 11 were seen and no growth was observed at 5°C and 65°C, as shown in Fig. 4.

The pH tolerance test showed that growth was maximum at pH 7 followed by pH 10 for both 24hours and 48hours. Growth at pH 4 was not seen at 24hours but at 48hours isolates 02b and 04a showed slight growth. At 24hours, growth pattern of isolates at pH 7: 21aiii> 04a>11>02b and growth pattern of isolates at pH 10 was almost the same. At 48hours, growth pattern of isolates at pH 7: 04a>11>21aiii>02b and growth pattern at pH 10: 11>21aiii with 04b and 02a having similar growth pattern. After 48hours, growth was observed at pH 4 as well for two isolates, 02b and 04a, the results being indicated in Table 3.

Biochemical profile of the isolates was carried out. Gram staining showed all the four isolates to be Gram negative coccobacilli. All four isolates showed growth on MacConkey agar plates as shown in Fig. 5, although they did not show pink colouration as compared to *E.coli* (positive control) and were colourless depicting incapability of lactose fermentation.

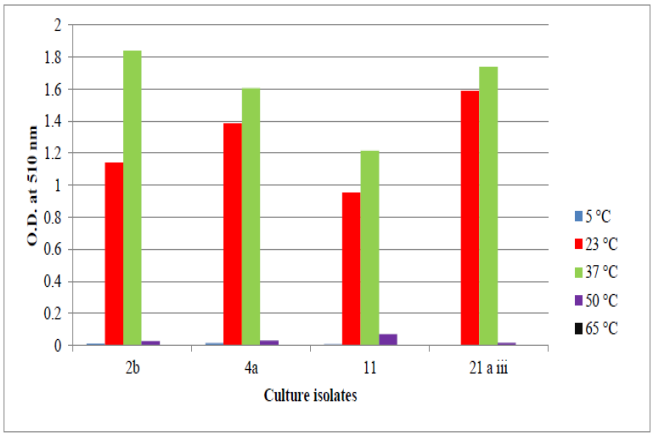



**Fig. 3: 24 hours isolate growth at different temperatures**

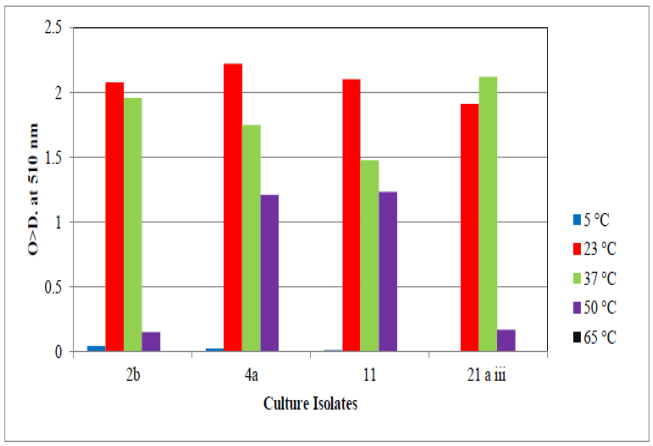
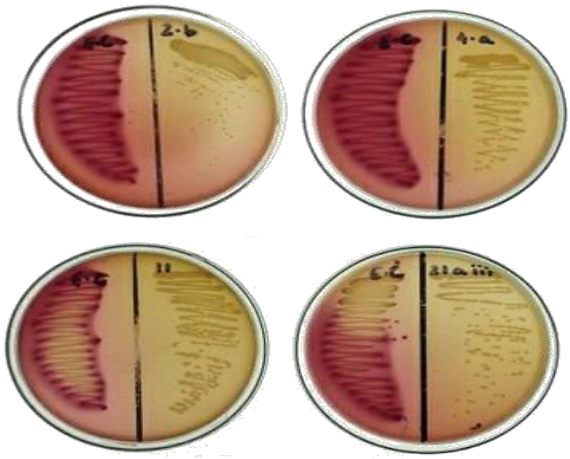


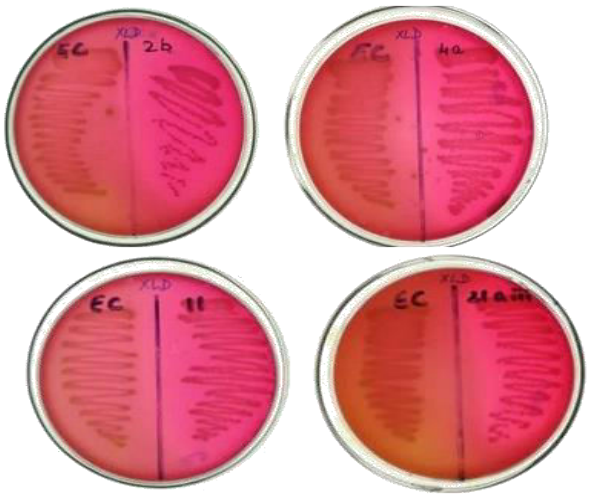

**Fig. 4: 48 hours isolate growth at different temperatures**

All the cultures showed growth on XLD agar plates, as opposed to *E.coli* (positive control) which showed yellow coloration, the colonies showed pink coloration i.e., lysine fermentation capacity, results depicted in Fig. 6. Culture identification tests suggested that all the four isolates were *Pseudomonas putida,* however each of them behaved differently in the enzyme assays, growth at different pH and temperature and the qualitative assay. This could be attributed to a possibility that the subspecies of the organisms are different. The above biochemical tests are not fool proof in terms of their result. To obtain further identification, molecular phylogeny by PCR amplification of 16S rRNA should be performed for the isolates. Oxidase tests were also performed, and all four isolates were oxidase positive.



*MacConkey agar plates of the four isolates with E.coli as positive control (left side of the plates). E.coli showing pink coloration and the four isolates are colorless showing incapability of lactose fermentation*

**Fig. 5: MacConkey agar plate results for isolates 2b, 4a, 11 and 21a iii**



*XLD agar plates of the four isolates with E.coli as positive control (left side of the plates).E.coli showed yellow coloration but the four isolates showed pink coloration suggesting lysine fermentation*

**Fig. 6: XLD agar plate results for isolates 2b, 4a, 11 and 21a iii**

**Table 3: Growth of isolates at different pH conditions**

| pH | Isolate | O.D. (24hours) | Growth | O.D. (48hours) | Growth |
|---|---|---|---|---|---|
| 4 | 2b | 0.010 | - | 0.089 | + |
| | 4a | 0.006 | - | 0.082 | + |
| | 11 | 0.001 | - | 0.025 | - |
| | 21 a iii | 0.009 | - | 0.029 | - |
| 7 | 2b | 0.912 | + | 1.012 | + |
| | 4a | 1.026 | + | 1.269 | + |
| | 11 | 0.956 | + | 1.260 | + |
| | 21 a iii | 1.109 | + | 1.245 | + |
| 10 | 2b | 0.802 | + | 1.017 | + |
| | 4a | 0.806 | + | 1.016 | + |
| | 11 | 0.815 | + | 1.214 | + |
| | 21 a iii | 0.796 | + | 1.113 | + |

### 3.3. Determination of Asparaginase enzyme activity

Highest enzyme activity was shown by isolate 11 (0.396 µmole/ml) followed by isolate 04a (0.308 µmole/ml). Highest specific activity was observed for isolate 02b (274.59 µmole/min/mg) followed by isolate 04b (227.27 µmole/min/mg). As the volume of the enzyme increases, the absorbance at 480nm increases due to increase in release of ammonia because of asparagine breakdown to aspartic acid and ammonia. Ammonia released causes yellow coloration which is measured using the Spectrophotometer.

Isolate 11 showed highest enzyme activity followed by isolate 4a. On treatment of potato smileys sample, untreated sample showed very low absorbance and colour as compared to the treated samples that showed higher absorbance and more colour development.

### 3.4. Application of the crude enzyme in treatment of food sample

After treating the sample with the enzymes, the Nessler's assay was performed, and absorbance was recorded, as shown in Fig. 7. Absorbance of the untreated sample was 0.012. Isolate 04a showed highest absorbance (0.083) and subsequently highest enzyme activity (0.038 µmole/ml) followed by isolate 11 (0.065nm absorbance and 0.028 µmole/ml enzyme activity). The untreated sample showed almost no absorbance (0.012) which shows that there is no ammonia liberated as there was no enzyme action.

Asparagine was detected by yellow spots and aspartic acid was seen as bluish-purple spots. 5 spots were applied on each chromoplate: Standard asparagine (1M), standard aspartic acid (1M), 5 minutes treated supernatant, 10 minutes treated supernatant, 60 minutes treated supernatant. The untreated sample TLC plate showed no presence of asparagine in the supernatant spotting of the food. This may have been due to extremely less amounts of asparagine in the food or no asparagine the food or the species of raw potatoes being used for the manufacture of the food would have had less amounts of asparagine in them. The aspartic acid spots were seen in treated sample TLC plates of 02b and 04a enzyme, as depicted in Fig. 8, which shows the activity of the enzyme on the little amount of asparagine that is present. This also shows that the enzyme from these two cultures show more potential for their use for application in food treatment.

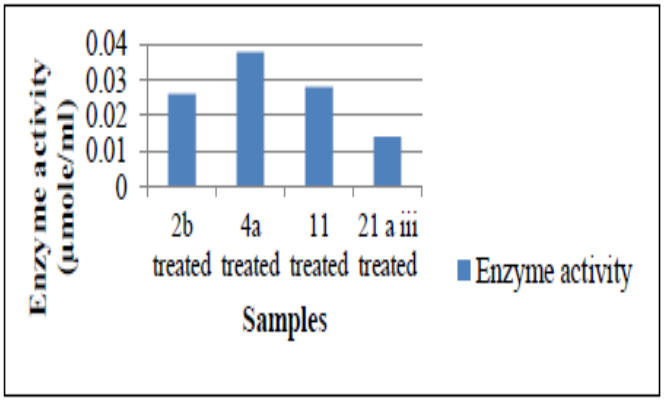
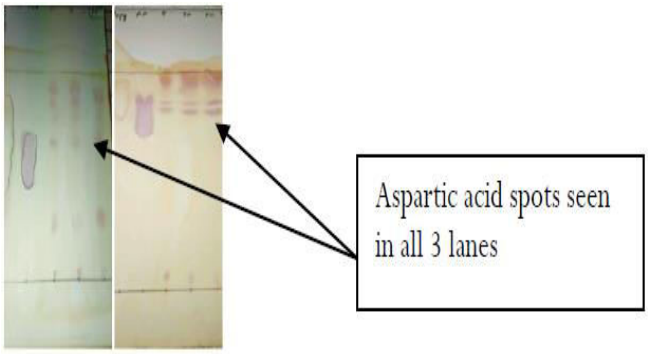



**Fig. 7: Enzyme activity of treated samples**




*TLC plates of isolates 2b and 4a. Key from left: Lane 1- Standard asparagine (1M), Lane 2- Standard aspartic acid (1M), Lane 3: 5 minutes treated supernatant, Lane 4: 10 minutes treated supernatant, Lane 5- 60 minutes treated supernatant*

**Fig. 8: TLC plates of isolates 2b (left) and 4a (right)**

TLC plate results indicate that the raw potatoes used by the manufacturer for preparation of the smileys may contain very less or may not contain asparagine. However, further tests must be conducted to check for the exact amounts and change in the amounts of asparagine before and after treatment with the enzymes. Different potato species must be tested to know which of them contain high and low amounts of asparagine. However, aspartic acid spots show the effect of the Asparaginase enzyme from the cultures.

### 3.5. Asparaginase and its applications

L- asparaginase has come about to be having an important role as an anticancer drug. Acute Lymphoblastic Leukemia (ALL) is one of the common malignancies in adolescents and children. The cure rate for this cancer is around 90% in developed countries which is still considered inadequate as it continues to be one of the leading causes of death. Chemotherapy is extensively used along with radiation therapy and steroids, and chemotherapy involves combining multiple agents. This has led to the use of L-asparaginase for treatment via intravenous injections. L-asparagine is not an essential amino acid and can be produced by the human body, yet cancerous cells are unable to produce this amino acid and depend on the freely available L-asparagine. Continuous supplementation of L-asparaginase can lead to cell cycle arrest in such cancerous cells which makes this enzyme an effective part of treatment [5].

The association of cancers and L-asparaginase also extends to the food industry where food products that are baked, roasted, and fried such as bakery products, coffee, fried potatoes, etc., lead to formation of acrylamide in them due to high temperatures and presence of reducing sugars. Acrylamide is a carcinogenic agent and could also cause neurotoxicity in humans after a certain threshold amount, thus presenting a threat to human health. One of the strategies to reduce acrylamide levels in such food products employs the use of L-asparaginase [5]. In a study by Amrein, Schönbächler, Escher, & Amadò (2004) [8], asparaginase from *E.coli* was added to ginger bread which led to a 55% acrylamide reduction in the product without altering the colour and other organoleptic properties [8]. Commercial asparaginase Acrylaway® was used in a study by Pedreschi, Kaack, and Granby (2008) [9] which helped them achieve a 67% acrylamide reduction in French fries at a temperature of 60°C and pH of 7.0. Acrylaway® along with blanching was used to reduce up to 90% of acrylamide in potatoes. A possible explanation of this

result was that blanching changed the microstructure of the potato tissues which resulted in a more effective interaction of asparagine with asparaginase. *Rhizomucormiehei* was used to extract asparaginase. This extracted enzyme named 'RmAsnase' was cloned and expressed in *E.coli* which proved highly specific to asparagine and resulted in an 80% acrylamide reduction. Asparaginase from *Bacillus subtilis* was applied to potato chips and led to an 80% acrylamide reduction. Similar results were seen in French fries which resulted from asparaginase extracted from *Thermococcus zilligii* [10].

A need for more thermostable enzymes is the future as the food processing industry operates with high temperatures of around 100°C and above. More thermostable asparaginases may prove effective during processes like blanching, steaming and incubation.

## 4. CONCLUSION

This study aimed to show the potential and effect of the enzyme L-asparaginase in reducing L-asparagine, the amino acid which is a precursor to a potential carcinogenic chemical like acrylamide which is present in foods consumed on a day-to-day basis. The study also aimed to isolate Asparaginase producers from which the enzyme could be extracted and used. Microbial sources of asparaginase were given importance as enzymes from microbial sources have been used since many years and have been employed in the food industry; they also help in process optimization, efficiency, and yield. Quite a few microorganisms are used for obtaining this enzyme, especially *Escherichia coli*, Although the amounts obtained are much less than what is required industrially. Hence, this study was conducted to contribute to the amount of research done and the existing available data on asparaginase producers and their activity; and to highlight organisms that can be easily obtained to extract the enzyme to be used commercially in an economic manner.

Asparaginase enzyme assays were also performed to determine the enzyme activity in the microbial sources. The enzyme so obtained, was from the organism *Pseudomonas putida* which was applied to Potato smileys (Frozen snack that is to be deep fried for consumption) to check effectiveness of this enzyme on asparagine. The assays showed the activity of the enzyme in all four cultures. Effect of the enzyme on asparagine content in the potato smiley was also detected. On performing TLC, it was understood that there may be low amounts of asparagine in the food sample and presence of aspartic acid spots in the TLC plate showed that application of the enzyme had broken down the asparagine into aspartic acid. Although further standardization of the TLC method would be required to confirm the findings.

## 5. ACKNOWLEDGEMENT

The authors acknowledge the Department of Life Sciences and Biochemistry and Caius Research Laboratory, St. Xavier's College (Autonomous), Mumbai, India for providing all the assistance with regards to facilities, materials, and equipment for the duration of the research study as well as the Department Food, Nutrition and Dietetics, College of Home Science Nirmala Niketan, Mumbai, India, for all the support in the completion of the study.

### *Conflict of Interest*

The authors claim no conflict of interest

## 6. REFERENCES

1. Batool T, Makky EA, Jalal M, Yusoff MM. *Applied biochemistry and biotechnology*, 2016; **178:**900-923.
2. Michalska K, Jaskolski M. *Acta Biochimica Polonica*, 2006; **53(4):**627-640.
3. FAO/WHO Consultation on Health Implications of Acrylamide in Food, World Health Organization. Food Safety Programme, World Health Organization, World Health Organisation Staff, FAO, WHO, & Food Safety Programme. Health Implications of Acrylamide in Food: Report of a Joint FAO/WHO Consultation, WHO Head-quarters, Geneva, SwitzerlandJune 2002; 25-27. Available at: http://apps.who.int/iris/handle/10665/42563 Accessed February 2, 2019.
4. Waterborg JH. The Lowry method for protein quantitation. In: The protein protocols handbook. (Totowa, NJ); *Humana Press*, 2009; 7-10.
5. Qeshmi FI, Homaei A, Fernandes P, Javadpour S. *Microbiological Research*, 2018; **208**:99-112.
6. Worthington Biochemical Corporation. Asparaginase assay. Available at: http://www.worthington-biochem.com/ASPR/assay.html Accessed February 2, 2019.
7. Bujard EL, MauronJ. *Journal of Chromatography*, 1966; **21**:19-26.
8. Amrein TM, Schönbächler B, Escher F, Amadò R. *Journal of Agricultural and Food Chemistry*, 2004; **52(13)**:4282-4288.
9. Pedreschi F, Kaack K, Granby K. *Food Chem,* 2008 Jul 15; **109(2)**386-392.
10. Xu F, Oruna-Concha MJ, Elmore JS. *Food Chemistry*, 2016; **210**:163-171.