DEVELOPMENT OF L-ASPARAGINASE TO REDUCE THE ACRYLAMIDE CONTENT IN FRIED FOOD PRODUCTS

Dissertation submitted in partial fulfilment of the requirements for the Degree of

BACHELOR OF TECHNOLOGY

Under the Supervision of

Dr. Saurabh Bansal

By

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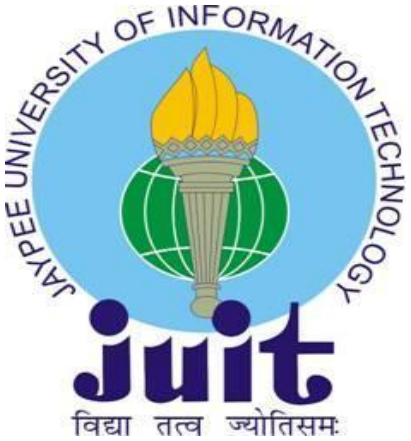

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

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DECLARATION

I hereby declare that the work reported in the B-Tech, thesis entitled **"DEVELOPMENT OF L-ASPARAGINASE TO REDUCE THE ACRYLAMIDE CONTENT IN FRIED FOOD PRODUCTS"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Saurabh Bansal**. I have not submitted this work elsewhere for any other degree or diploma.

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CERTIFICATE

This is to certify that the work titled "**Development of L-Asparaginase to reduce the acrylamide content in fried food products"** submitted by **Richa Rathore** in partial fulfilment for the award of degree of B. Tech of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. Saurabh Bansal

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ACKNOWLEDGEMENT

All praise belongs to the almighty lord to whom I thank for the strength, courage and perseverance bestowed upon to me to undertake the course of the study.

I hereby acknowledge with deep gratitude the cooperation and help given by all members of Jaypee University in helping with my project.

With proud privilege and profound sense of gratitude, I acknowledge my indebtedness to my guide **Dr. Saurabh Bansal** for his valuable guidance, suggestions, constant encouragement and cooperation.

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Richa Rathore

Date:

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ABSTRACT

Acrylamide has been found in certain foods that have been cooked or processed at high temperatures. The levels of acrylamide appear to increase with the time of heating. Acrylamide appears to be formed as a by-product of the Maillard reaction. Acrylamide is cancerous and efforts are being done to reduce its content in fried food products. L-Asparaginase is an enzyme that coverts free asparagine which is the source of acrylamide to aspartic acid and ammonia.

In this project, the strains of *Streptococcus thermophilus* and *Lactobacillus acidophilus* were successfully isolated and identified by performing different biochemical identification tests like gram staining, catalase test. The qualitative assay suggested that the enzyme is intracellular in nature. The enzymatic assay showed the activity of L-Asparaginase by colour change i.e. from colourless to orange.

The DNA of *Streptococcus thermophilus* was successfully isolated.

CHAPTER-1

INTRODUCTION

1.1 Acrylamide

Acrylamide is a versatile organic compound that finds its way into many products in our everyday life. Acrylamide exists in two forms: a monomer (single unit) and a polymer (multiple units joined together by chemical bonds). The single unit form of acrylamide is toxic to the nervous system, a carcinogen in laboratory animals, and a suspected carcinogen in humans. The multiple unit or polymeric form is not known to be toxic.

1.2 Acrylamide Uses

The monomeric form of acrylamide is primarily used in research laboratories for gel preparation. The acrylamide gel is used for electrophoresis, a technique for protein separation. It is also used to produce grout, dyes, contact lenses, and in the construction of dams, tunnels, and sewers.

Acrylamide polymers are used as additives for water treatment, flocculants, paper making aids, thickeners, soil conditioning agents, textiles (permanent-press fabrics), production of organic chemicals, and ore and crude oil processing. Although the polymer polyacrylamide is not toxic, a small amount of the acrylamide monomer may leach from the polymer.

1.3 Formation of acrylamide in foods

Acrylamide has been found in certain foods that have been cooked or processed at high temperatures. The levels of acrylamide appear to increase with the time of heating. However, the mechanism of acrylamide formation in foods is not well understood. Acrylamide appears to be formed as a by-product of the Maillard reaction. The Maillardreaction is best known as a reaction that produces the tasty crust and golden colour in fried and baked foods. This reaction can occur during baking or frying when there is a proper combination of carbohydrates, lipids, and proteins in foods. Millard

reaction is a chemical reaction between an amino acid and reducing sugar that gives browned food its desirable flavour. The reaction is a form of non-enzymatic browning which typically proceeds rapidly from around 140 to 165 °C (284 to 329 °F).

Because the asparagine content of foods within a certain category (e.g., potatoes) varies greatly, this asparagine-dependent reaction may explain the tremendous variability in acrylamide levels even within one food category. There may be more than one way that acrylamide forms in foods. An understanding of how acrylamide forms in various foods may lead to the development of methods to prevent or limit its formation.

Based on current stage of knowledge, acrylamide is a natural by-product that forms when certain carbohydrate-rich foods are fried, baked, or roasted at high temperatures above 120°. Acrylamide can cause cancer in laboratory animals at high doses, although it is not clear whether it causes cancer at the much lower levels found in food.

1.4 Concern about acrylamide in fried and baked foods

A group of Swedish researchers found that some fried or baked high-carbohydrate foods such as potato chips and french fries contain high levels of acrylamide. Because these foods are widely consumed in significant amounts, much interest and concern was generated from the report published in April 2002.

The researchers reported moderate levels of acrylamide (5-50 ppb) in heated protein-rich foods and higher contents (150-4000 ppb) in carbohydrate-rich foods such as potato, beetroot, and selected commercial potato products. Medium levels of acrylamide were found at 1,200 ppb in potato chips, 450 ppb in french fries, and 410 ppb in biscuits and crackers. The same group of researchers also stated that acrylamide was not found in the same foods in the raw state or in foods prepared by boiling. The researchers examined the heated foodstuffs for acrylamide because they have consistently found acrylamide in red blood cells of some Swedish adults who apparently had no known sources of exposure to acrylamide.

Based on these findings, the Swedish National Food Administration and the researchers at Stockholm University estimated an average intake of acrylamide from food in Sweden to be 35-40 micrograms per day.

1.5 ASPARAGINASE-Therapeutic Enzyme

Enzymes are proteinaceous in nature. Each enzyme is programmed to carryout one special task. Like a key in a lock each enzyme fits together with one specific substrate modifying it in one proper way. The manufacture or processing of enzymes for use as drugs is an important facet of today's pharmaceutical industry. Attempts to capitalize on the advantages of enzymes as drugs are now being made at virtually every pharmaceutical research centre in the world.

Therapeutic enzymes have a broad variety of specific uses: as oncolytics, thrombolytics or anticoagulants and as replacements for metabolic deficiencies. Additionally there is a growing group of miscellaneous enzymes of diverse function. Proteolytic enzymes have been widely used as anti-inflammatory agents.

A major potential therapeutic application of enzymes is in the treatment of cancer. Asparagianse has proved to be particularly promising for the treatment of acute lymphocytic leukaemia. Its action depends upon the fact that tumour cells are deficient in aspartate-ammonia ligase activity, which restricts their ability to synthesise the normally non-essential amino acid, L-asparagine.

Therefore, they are forced to extract it from body fluids. The action of Asparaginase does not affect the functioning of normal cells, which are able to synthesise enough for their own requirements, but reduce the free exogenous concentration, and so induce a state of fatal starvation in the susceptible tumour cells. A 60% incidence of complete remission has been reported in a study of almost 6,000 cases of acute lymphocytic leukaemia. This enzyme is administered intravenously.

1.6 Role of Asparaginase

Addition of the enzyme asparaginase has been shown to reduce asparagine and thus acrylamide levels in fried food products.Asparaginase may be best suited for food products manufactured from liquidised materials. In practice,asparaginase can functionally reduce acrylamide in prefabricated crisps and fries however the amount of asparagines in the raw product is generally so high that in order to achieve a meaningful reduction in acrylamide a large amount of asparaginase must be added. This may preclude the use of the enzyme for some products.

Asparaginases are enzymes expressed and produced by microorganisms.A very promising way of eliminating acrylamide is reduction of free asparagine by asparaginase, which hydrolyses asparagine to aspartic acid and ammonia. Patents related to the use of asparaginase have been filed by several companies (e.g. Frito Lay, Procter & Gamble, DSM, Novozymes) nevertheless, experiments conducted so far have been restricted to laboratory and bench-scale trials. Incubation of mashed potato, potato flakes, rye flour, and wheat flour with asparaginaseprior to heating decreased the acrylamide content in model systems by over 90%. Asparaginase is a promising method of acrylamide reduction especially in biscuits and crisp bread. Much effort has been put into lowering of acrylamide levels by the enzyme's producers, although a legal mandate for use in food is still needed because the enzyme is not yet licensed for food use in all countries.

1.7 Mechanism of Action

Acrylamide is often formed in the cooking of starchy foods.During heating the amino acid asparagine naturally present in starchy foods, undergoes a process called Maillard reaction is responsible for giving baked or fried foods their brown colour, crust, and toasted flavour. Suspected carcinogens such as acrylamide and some heterocyclic amines are also generated in the Maillard reaction. By adding asparaginase before baking or frying the food, asparagine is converted into another common amino acid, aspartic acid. As a result, asparagine cannot take part in the Maillard reaction, and therefore the formation of acrylamide is significantly reduced. Complete acrylamide removal is probably not possible due to other, minor asparagine-independent formation pathways.

As a food processing aid, asparaginases can effectively reduce the level of acrylamide up to 90% in a range of starchy foods without changing the taste and appearance of the end product.

CHAPTER 2

LITERATURE REVIEW

2.1 L-ASPARAGINASE EFFECTIVE TREATMENT

It has been reported by Yohei et al.(2015) that acrylamide, a potential carcinogen, is formed from the reaction of L-asparagine (L-Asn) and reducing sugars contained in foods during heating processes and free asparagine is a limiting factor for acrylamide formation. It has been reported that potatoproducts such as potato chips, which are made through heating processes, contain high levels of acrylamide. To decrease the amount of L-asparagine in potatoes using L-asparaginase,effective treatment conditions of sliced potatoes with the enzyme have been investigated. By treating sliced potatoes with *Bacillus subtilis* L-asparaginase II (BAsnase;4 U/gpotato), approximately 40 % of L-Asparagine in the sliced potatoes was converted into L-aspartic acid (L-Asp).To make this enzyme more effective, prior to enzymatictreatment, sliced potatoes were freeze-thawed,dried atdecompressed condition, resulting in the hydrolysis of approximately 90 % of L-asparagine to L-aspartic. The acrylamide content of BAsnase-treated fried potato chips decreased to below 20 % of that of BAsnase-untreated fried potato chips. Treatment conditions examined in this study were found to be effective to suppress the formation of acrylamide in fried potato chips.

2.2 ACRYLAMIDE IN BAKERY

Krishna Kumar and Visvanathan (2014) reported that acrylamide or 2-propenamide an industrial chemical formed in some foods particularly starchy foods during heating process such as baking, frying and roasting. Acrylamide is proven to be carcinogenic in animals and a probable human carcinogen mainly formed in foods by the reaction of asparagine (free amino acid) with reducing sugars (glucose and fructose) as part of the Maillard reaction during heating under high temperature and low moisture conditions. The main aim of this review is to summarize the results of academic and industrial research on occurrence, dietary exposure, formation mechanism and mitigation measures of acrylamide in bakery, cereal and potato food products.

2.3 THE FOOD DRINK EUROPE "TOOLBOX"

The FoodDrinkEurope "Toolbox" (2011) reflects the results of >8 years of cooperation between the food industry and national authorities of the European Union to investigate pathways of formation of acrylamide and potential intervention steps to reduce exposure. The aim of the Toolbox is to provide national and local authorities, manufacturers (including small and medium size enterprises, SMEs) and other relevant bodies, with brief descriptions of intervention steps which may prevent and reduce formation of acrylamide in specific manufacturing processes and products. It is in particular intended to assist individual manufacturers, including SMEs with limited R&D resources, to assess and evaluate which of the intervention steps identified so far may be helpful to reduce acrylamide formation in their specific manufacturing processes and products. During the latest revision of the Toolbox, it has been restructured around the three main product categories with higher risk of acrylamide formation, namely: potatoes, cereals, and coffee. These are then sub-divided into compartments and the individual tools.

2.4 ACRYLAMIDE REDUCTION

Acrylamide (2-propeamide, CAS No. 79-06-1) is a well-known colourless and odourless crystalline compound (2011) used for more than fifty years to synthesize polyacrylamides with numerous applications in papermaking, textile, cosmetics and as flocculants to clarify drinking water. This small compound is soluble in water, acetone and ethanol, has a high mobility in soil and groundwater and it is biodegradable (Zhang and Zhang, 2007).

The studies showed that heat, light and outdoor environmental conditions promoted the de-polymerization of polyacrylamide to acrylamide, demonstrating strong adverse health effects on workers exposed to high acrylamide levels. Since 1994, despite the fact that acrylamide exposure was considered as potentially carcinogenic(Friedman et al., 1995) due to its presence in tobacco smoke, many concerns were only recently raised on acrylamide presence in foods. As a result, in April 2002, the Swedish National Food Agency and the University of Stockholm published the first report on the high content of acrylamide levels in carbohydrate-rich foods treated at relatively high temperatures (SNFA, 2002). The report demonstrated that it was formed as a result of a

reaction between amino acids, namely asparagines and reducing sugars, particularly glucose and fructose as part of the Maillard reaction (Mottram et al., 2002; Stadler et al., 2002). It was also reported that dietary acrylamide intake may increase the risks of kidney and breast cancer (Olesen et al., 2008; Hogervorst et al., 2008) and further tests on animals proved that acrylamide has genotoxic and neurotoxic effects, causing gene mutation and DNA damage, and it may represent a health hazard for humans (Mojska et al., 2010).

2.5 Isolation and Production of clinical and Food Grade L-Asparaginase enzyme from fungi

The study by Lynette Lincoln and Sunil S. deals with the isolation and optimization of an extracellular L-asparaginase from a fungal microorganism screened from marine soil. At pH 6.5 and temperature 37 degree celcius maximum enzyme production took place and optimization and production of *Trichodermaviride* species was performed by submerged fermentation. Further enhanced production took place when the optimization of the various other carbon and nitrogen source supplements as nutritive additives was carried out. Production also increased when 0.5% substrate was supplemented with 0.5% peptone and 0.6% maltose. Good scavenging property and the ability to lower the acrylamide levels in food stuffs makes L-asparaginase a valuable enzyme in pharmaceutical and food industries.

2.6 A critical review on properties and applications of microbial l-Asparaginases

This review is an attempt to compile information on the properties of l-asparaginases obtained from different microorganisms. The study by KrishnapuraPR,Belur PD,Subramanya S. showed that L-Asparaginase is one of the main drugs used in the treatment of Acute Lymphoblastic Leukemia (ALL), a commonly diagnosed paediatric cancer. Although several microorganisms are found to produce L-asparaginase, only the purified enzymes from *E. coli* and *Erwiniachrysanthemi* are used in the clinical and therapeutic applications in humans. However, some evidence of hypersensitivity and other toxic side effects have occurred. L-Asparaginase is also of prospective use in food industry to reduce the formation of acrylamide in fried, roasted or baked food products. The review is also an attempt to compile the complications involved with the

therapeutic use of the currently available l-asparaginases, and the enzyme's potential application as a food processing aid to mitigate acrylamide formation. Further, search for alternate sources of l-asparaginase have been discussed in this review article, highlighting the prospects of endophytic microorganisms as a possible source of l-asparaginases with varied biochemical and pharmacological properties.

2.7 Award-winning Novozymes solution enables acrylamide mitigation in even more product categories

The Food and Beverage Journal on 19th November 2013 stated this latest enzyme technology targets the reduction of acrylamide formation in foods usually processed at high temperatures. Novozymes launched the ACRYLAWAY HIGH T, the first thermo stable solution that reduces the acrylamide levels in breakfast cereals and other products processed at high temperatures. Novozyme first introduced the ASPARAGINASE ACRYLAWAY in 2007 and today the solution is used in more than 30 countries in a wide range of consumer products like biscuits, French fries, chips, snacks and coffee. ACRYLAWAY HIGH T, majorly modifies asparagine the other amino acids and sugars remain active to contribute maillard reaction. This means the great taste and appearance of the final product is preserved.

2.8 CHARACTERIZATION AND IDENTIFICATION OF LACTOBACILLUS ACIDOPHILUS USING BIOLOGRAPID IDENTIFICATION SYSTEM

HASSAN PYAR AND K.K PEH carried out a study to isolate and identify *Lactobacillus acidophilus*and to compare the effect of inoculation methods and cultivation conditions on the yield of the bacteria. Probiotic *Lactobacillus* strain was isolated from a commercial yogurt and the characterization of the bacteria was performed using gram staining, motility test, catalase test, biochemical tests and morphological features were confirmed using scanning electron microscope (SEM). Finally the identification was confirmed by BioLog system. Effect of inoculation methods and cultivation conditions on the growth and yield of the bacteria were studied and the results were as follows, the isolated strain was Gram positive, non-motile and catalase negative. It is able to ferment maltose, lactose, sucrose and glucose, but unable to ferment arabinose and sorbitol. The scanning electron microscope examination displayed the ranging of the cells varied from 2.02 - 5.49 × 0.50 - 0.59 µm. From all the results it was confirmed that the species was *Lactobacillus acidophilus*. In addition,

BioLog rapid identification system revealed the presence of *Lactobacillus acidophilus* in the prepared samples. Further pour plates and spread plates were prepared and pour plate provided a relatively higher viable count than the spread plate, while no significant differences were observed between aerobic and anaerobic conditions. The bacterial strain was successfully isolated after series of purification. It was identified as *Lactobacillus acidophilus*. To conclude, carbon utilizationmicroplate assay system developed by BioLog, has the potential to simplify the identification scheme of lactic acid bacteria to the genus level.

2.9 Impact of 16SrRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases

Jill E.Clarridge conducted the study on the traditional identification of bacteria on the basis of phenotypic characteristics which generally is not as accurate as identification based on genotypic methods. The bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. 16S rRNA gene sequence analysis is a technique which can better identify poorly described, rarely isolated, or phenotypically aberrant strains. It can routinely be used for identification of mycobacteria, and can lead to the recognition of novel pathogens and non-cultured bacteria. The problem identified was that the sequences in some databases were not accurate. Despite its accuracy, 16S rRNA gene sequence analysis lacks widespread use because of technical and cost considerations. Thus, a future challenge is to translate information from 16S rRNA gene sequencing into convenient biochemical testing schemes, making the accuracy of the genotypic identification available to the smaller and routine clinical microbiology laboratories.

2.10 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls

J. Michael Janda and Sharon L. Abbott studied the use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy which have been by far the most common housekeeping genetic markers used for a number of reasons. The reasons identified were as follows, firstly its presence in almost all bacteria, often existing as a multi-gene family secondly the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time and

thirdly the 16S rRNA gene (1,500 bp) is large enough for informatics purpose. DNA-DNA hybridization is no doubt the “gold standard” for proposed new species and for the definitive assignment of a strain with ambiguous properties to the correct taxonomic unit.. DNA hybridization assays are not without their shortcomings, however, being time-consuming, labour intensive, and expensive to perform. Today, fewer and fewer laboratories worldwide perform such assay and many studies describing new species are solely based upon small subunit (SSU) sequences or other polyphasic data.

CHAPTER 3

MATERIALS AND METHODS

3.1 METHODOLOGY

3.1.1 Isolation of *Streptococcus thermophilus* and *Lactobacillus acidophilus*

Inoculation from the capsule

100ml of Lactobacillus MRS(*Lactobacillus acidophilus*) and brain heart infusion broth(*Streptococcus thermophilus*) were prepared and were inoculated with the desired microorganisms to prepare the seed culture. *Lactobacillus acidophilus* culture was incubated at 37°C for 48 hours and *Streptococcus thermophilus culture* 50°C for 24 hours.

0.9% N-Saline was prepared

Four-fold serial dilution of 100 µl inoculum was added in 900 micro litre of N-Saline.

Isolation

Lactobacillus MRS Agar and Brain Heart infusion Agar media were prepared and were poured in petri plates.100 micro litre of dilution was spread on respective media.Incubation of *Lactobacillus acidophilus* at 37° for 48 hours and *Streptococcus thermophilus* at 50° for 24 hours was done.

Long Term Preservation of the Isolates

The purified bacteria with homogeneous cell morphology were stored in a -80°C deep freezer in MRS medium in 20% (v/v) glycerol for preservation.

PROTOCOL

The glycerol stocks were prepared by mixing 0.5 ml of active cultures with 0.5 ml of fresh, sterile MRS medium with 40% glycerol in eppendorftubes. Hence, the resulting suspension included 20% glycerol. The frozen stocks were prepared.

3.1.2 Characterization

Biochemical identification

The genus *Streptococcus* includes Gram positive bacteria with similar metabolic properties but they live in different habitats and have many physiological differences. In the past two decades, several important *Streptococcus* species have been reclassified as members of recently named genera *Enterococcus* and *Lactococcus.* The only dairy *streptococcus* remained is *S. thermophiles*.

Gram Staining

Streptococcus thermophilus are Gram positive bacteria and the Gram status of the isolated bacteria were determined by the microscopic examination of Gram-stained isolates. One ml of overnight activated broth culture was aliquoted into a centrifuge tube and centrifuged at 6.000 rpm for 5 min. Supernatant was removed and cells were re-suspended in sterile water. 10 µl of cell suspension was pipetted to a glass slide and they were Gram stained after drying and fixation by exposure to a flame.

PROTOCOL

The main steps in Gram Staining procedure were as follows:

• Crystal violet staining for 1 min.

• Removing the excess stain by washing under tap water.

• Staining with Gram's iodine mordant for 1 min.

• Washing under tap water.

• Fixation with 95% alcohol for 15 sec.

• Counter staining with safranin for 30 sec.

• Washing under tap water.

• Drying with cotton towels gently.

Catalase Test

Catalase enzyme breaks down hydrogen peroxide into oxygen and water molecules ($2H_2O_2 \rightarrow 2H_2O + O_2$) and oxygen production is observed by the generation of O_2 bubbles. The generation of gas bubbles indicates the presence of the enzyme, hence the catalase positive nature of the bacterium. *Streptococcus thermophilus* is catalase negative and no O_2 production (gas bubbles)was observed when 3% H_2O_2 solution was dropped on top of the colonies grown overnight on agar medium.

Growth at Different Temperatures

For the classification of isolates, the growth temperatures of 10°C and 45°C were used. *Streptococcusthermophilus* cannot grow at 10°C, but grow well at 45°C.In order to determine the growth at given temperatures, brain heart infusion media was used. Basically, all ingredients were the same, except for Bromocresol purple. Fifty microlitres of overnight activated cultures were inoculated into 5 ml test media and the incubation period at the given temperatures was observed for 7 days to determine the growth.

Growth at Different Sodium Chloride Concentration

Unlike other cocci, *Streptococcus thermophilus* are highly sensitive to sodium chloride. *Streptococcus thermophilus* does not grow even at 2% sodium chloride concentration. The most frequently used sodium chloride concentrations for the identification are 4% and 6.5% salt concentrations. Hence growth at 2% and 4% sodium chloride concentrations were used for isolates in sodium chloride test medium. 50 microlitres of overnight activated cultures were inoculated into 5ml sodium chloride test media and the incubation at 42°C was observed for 7 days to determine the growth.

3.1.3 Qualitative plate assay for screening of L-asparaginase production

PROTOCOL

The isolates were screened for asparaginase activity using the method of Gulati et al. The fully grown cultures were plated in standard petri plates aseptically. The medium used is modified M9 medium (pH 5.9) and incorporated with a pH indicator (phenol red). The plates incubated for 48 hours at 37ºC.L-asparaginase activity identified by formation of a pink zone around colonies.

3.1.4 Enzymatic Analysis

Nesslerization

L-Asparaginase activity was measured by direct Nesslerization of ammonia. The activity of L-asparaginasewas measured employing the modified method. It catalyzesL-asparagine to L-aspartic acid and ammonia and the latter react with the Nessler's reagent to produce an orange coloured product.

PROTOCOL

- The enzyme assay mixture consisted of 100 µL of freshly prepared L-asparagine (189 mmol/L) in Tris-HCl buffer (pH 8.6) and 100 µL of crude extract of the enzyme.
- The reaction mixture was incubated at 37 °C for 30 min and the reaction was stopped by adding 100 µL of 15% trichloroacetic acid (TCA).
- The reaction mixture was centrifuged at 6 000×g for 5 min at 4 °C to remove the precipitates.
- The ammonia released in the supernatant was determined using colorimetric technique by adding 500 µL Nessler's reagent into the sample containing 200 µL supernatant and 4.3 mL distilled water.

3.1.5 Isolation of DNA

PROTOCOL

- 1.5 ml of culture was taken and centrifuged at 15000 rpm for 5 minutes.
- Pellet of each sample was collected and re-suspended in 900 µl TNE buffer and cells were collected by centrifuging at 15000rpm for 5 min.
- Pellet of each sample was collected and re-suspended in 70% ethanol(ice cold) and placed on ice for 20 minutes.
- Suspension was centrifuged at 12000 rpm for 5 minutes.
- Pellet was re-suspended in 480 µl of SETbuffer, tubes were kept at -20 for 20 minutes and immediately transferred to a water bath at 68 ºC for 10 minutes.
- Phenol:Chloroform:Isoamyl(25:24:1) was added and mixed by gentle inversion.
- Centrifuged at 5000 rpm for 10 minutes.
- 100µl of 3M sodium acetate and equal amount of absolute alcohol was added to each tube and mixed gently.

- Centrifuged at 5000rpm for 10 min.
- Pellet was washed with (70%) ethanol at 5000 rpm.
- Pellet was re-suspended in 100ul TE Buffer and stored at 4°C.

Buffer Preparation

PROTOCOL

EthyleneDiamineTetraaceticAcid(EDTA)(pH 8.0)

- Added 18.6g EDTA disodium salt (m.w372.24) to 80ml deionized or distilled water
- Adjusted to pH 8.0
- Mixed vigorously by hand. EDTA will only dissolve after pH has reached 8.0 or higher
- Volume made was 100ml
- Stored at room temperature

4M Sodium Chloride

- Dissolved 116.9g of sodium chloride (m.w58.44) in 250ml of deionized or distilled water
- Added deionized or distilled water to make a total volume of 500ml of solution
- Stored at room temperature

10% Sodium Dodecyl Sulphate(SDS)

- Dissolved 10g electrophoresis-grade SDS (m.w288.37)in 80ml deionized water
- Added deionized or distilled water to make 100ml total solution
- Stored at room temperature

1M TRIS(pH 8.0)

- Dissolved 12.1g Tris base in 70ml distilled water
- Adjusted pH
- Added distilled water to make 100ml total solution

TRIS/EDTA(TE)Buffer

- Mixed in a 200ml beaker
- 99ml of deionized or distilled water
- 1ml of 1M Tris pH 8.0
- 200ul of 0.5M EDTA

Agarose Gel Electrophoresis

PROTOCOL

50x Tris/Acetate/EDTA Electrophoresis Buffer(1 litre)

- 242g of Tris Base was added in about 600ml distilled water.
- 57.1ml of Glacial Acetic Acid was added.
- 100 ml 0.5M EDTA was added to the solution.
- The volume was raised to 750ml.

1x TAE Buffer

Mixed 20ml of TAE in 100ml distilled water

Preparation of Agarose Gel

1g of agarose was dissolved in 100ml 1x TAE buffer by boiling. After boiling, it was cooled to 45°C. 15µl ethidium bromide solution (10mg/ml) was added and stirred. The agarose gel was poured into the gel casting stand and the combs were placed.

Loading of Agarose Gel

Five microlitres of products were mixed with 2µl of gel loading dye. The samples were loaded into the wells, starting from the second well on the gel. Electrophoresis of the products was electrophoresed at 80V for 30 min. Amplification products were visualized in a gel documentation system.

3.1.6 Finding the Concentration of DNA Using a NanoDrop

The NanoDrop micro volume sample retention system (Thermo Scientific NanoDrop Products) functions by combining fibre optic technology and natural surface tension properties to capture and retain minute amounts of sample independent of traditional containment apparatus such as cuvettes or capillaries.

PROTOCOL

- A NanoDrop plate was taken (16 well plate)
- 2 micro litre control was taken and placed in one well
- 2 micro litre sample was taken and placed in another well

3.2 MATERIALS

Disodium phosphate	12.8 g
Monopotassium phosphate	3 g
Sodium chloride	0.5 g
Ammonium Chloride	1 g
Distilled water	478 ml

Table 1: Composition of M9 salts used for the Qualitative assay

Dissolve and autoclave at 121°C

Agar	50g
Distilled water	500ml

Table 2: Agar solution

Cool them to 50°C and combine them aseptically.

Glucose(20%solution)	20ml
1 M Magnesium sulphate solution	2ml
1 M Calcium chloride solution	0.1ml

Table 3:Filter sterilized solution

3.2.1 DNAISOLATION

- 4.4 M Ammonium Acetate
- 0.5 M Ethylene DiamineTetraaceticacid(EDTA)
- Phenol:Chloroform(2:1volume:volume)
- 4 M Sodium Chloride(NaCl)
- 10% Sodium dodecyl Sulphate(SDS)

- 1 M Tris (pH 8.0)
- Tris/EDTA Buffer(TE) Buffer

3.2.2 BUFFERPREPERATION

- 1.0% Agarose
- 1ug/ml Ethidium Bromide Staining Solution
- 0.2% Methylene Blue Stock Solution
- 0.025% Methylene Blue Staining Solution
- 50x Tris/Acetate/EDTA Buffer(TAE) Electrophoresis Buffer
- 1xTris/Acetate/EDTA Buffer(TAE) Electrophoresis Buffer

3.2.3 INSTRUMENTSUSED

- Digital pH meter
- Incubator
- Centrifuge
- Autoclave
- Laminar Air Flow Cabinet
- Water Bath

CHAPTER-4

RESULTS AND DISCUSSION

4.1 Isolation of *Streptococcus thermophilus* and *Lactobacillus acidophilus*

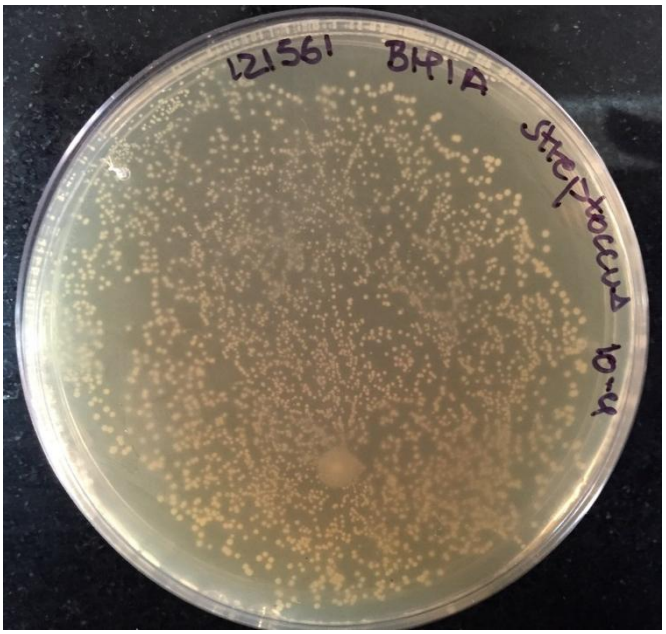

Figure1:*Streptococcusthermophilus*

Characterization

• Gram positive, non-motile coccus

• Spherical/ovoid cells of 0.7-0.9µm diameter

• Occurs in pairs or in long chains of 10-20 cells

• Homofermentative,L(+) lactic acid as the major end product

• Facultative anaerobe

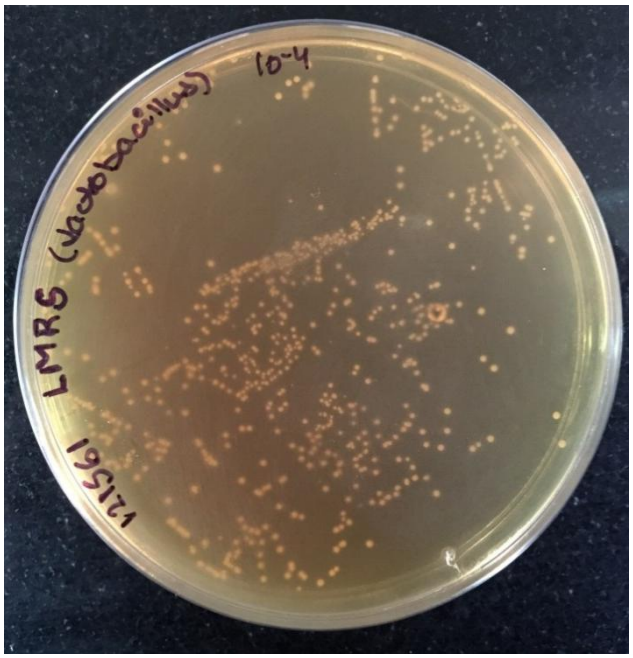

Figure 2*: Lactobacillus acidophilus*

Characterization

- Gram positive, rod shaped coccobacilli, occurring singly or in chains.
- The gram staining results indicated that the isolated bacteria could be identified as lacto bacilli.

4.2 Biochemical Identification

All of the isolates were subjected to Gram staining and they were examined under light microscope. All the strains gave blue- purple colour with staining; hence they all were Gram positive bacteria. The isolates coming from MRS plates were all bacilli with long and rounded ends. They appeared mostly as a chain of 3-4 cells or single. The isolates coming from Brain heart infusion medium were all cocci with spherical or ovoid morphology. They appeared mostly as pairs or forming chains.

Catalase test

All of the isolates were tested for catalase activity. None of them showed catalase activity. *S. thermophilus* is catalase negative and no O_2 production took place.

Growth Conditions

Regarding the ability to grow at different NaCl concentrations, there was no stated record for the ability of growth even at 2% NaCl concentration for *S. thermophilus*. Ability to grow at 2% and 4% NaCl concentrations were tested for all cocci isolates and they were found to be resistant to 2% NaCl concentration but sensitive to 4% concentration.

For bacilli isolates the most frequently used NaCl concentration for classification was 4% and 6.5%.

Temperature conditions

Streptococcus thermophiles cannot grow at 10°C, but grow well at 45°C. All of the isolates showed the expected results. All of them were able to grow at 45°C.

4.3 Isolated DNA of *Streptococcus thermophilus*

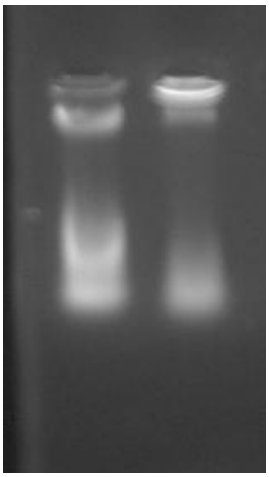

Figure 3: DNA of *Streptococcus thermophilus*

4.4Concentration of the isolated DNA

Concentration:-690ng/µl

260/280=2.28

260/230=2.18

CONCLUSION

The overall objective of the current study was to extract L-asparaginase enzyme from *Streptococcus thermophilus* bacteria which can be used to reduce the content of acrylamide from fried food products. Acrylamide is a substance that is produced naturally in foods as a result of hightemperature cooking, e.g., baking, grilling, or frying. Acrylamide can cause cancer in animals and experts believe it can probably cause cancer in humans. Although acrylamide has probably been part of our diet since man first started cooking, because of concerns over safety, world experts have recommended that we reduce the levels of acrylamide in foods.

The colonies of *Streptococcus thermophilus*and *Lactobacillus acidophilus* were successfully isolated, identified and characterized. Biochemical identification was carried out. Qualitative Assay and Enzymatic Assay were carried out. No pink colony formation in the Qualitative Assay suggested that the enzyme is intracellular. The enzymatic Assay showed the activity of L-Asparaginase by colour change i.e. from colourless to orange.

The DNA of *Streptococcus thermophilus*was successfully isolated.

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