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

Project report submitted in partial fulfilment of the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

Submitted by:

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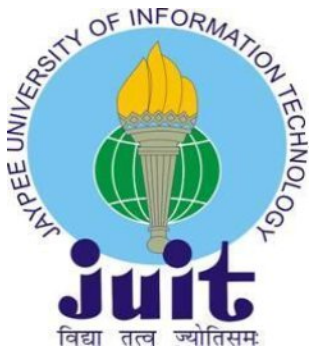

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY WAKNAGHAT

WAKNAGHAT– 173234, HP, INDIA

Month- June, Year- 2016

CERTIFICATE

This is to certify that the work titled "**Development of L-asparaginase to reduce the acrylamide content in fried food products"** submitted by **Sanjana Khosla (121561)** in partial fulfilment for the award of degree of B. Tech Biotechnology 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.

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DECLARATION

I, **Sanjana Khosla**, student of B.Tech Biotechnology, Jaypee University of Information Technology, Solan, Himachal Pradesh do here by declare that the project entitled "**Development of L-Asparaginase to reduce the acrylamide content in fried food products"** submitted towards partial fulfilment for the award of degree of **Bachelor of Technology in Biotechnology** of Jaypee University of Information Technology is based on the results of studies carried out under the guidance and supervision of **Dr. Saurabh Bansal**. This project or no part of this has been submitted elsewhere for the award of any degree or diploma.

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LIST OF ABBREVIATIONS

BHI	Brain Heart Infusion
DNA	Deoxyribonucleic Acid
EDTA	Tris Ethylenediaminetetraacetic Acid Tris
EtBr	Ethidium Bromide
SDS	Sodium dodecyl sulphate
SET	Sodium (SDS) EDTA
TNE	Tris Sodium (Na) EDTA
Fig	Figure
min	Minutes
OD	optical density
pH	Potential of hydrogen
rpm	Revolution per minute
ml	Millilitre
nm	Nanometer
g	Gram
mg	Milligram
µl	Microliter
ºC	Degree celsius
%	Percentage

ABSTRACT

L-asparaginase is one of the important commercial enzymes having several applications in food and pharmaceutical industry. The most common use of asparaginases is as a processing aid in the manufacture of food. Marketed under the brand names Acrylaway and PreventASe, asparaginases are used as a food processing aid to reduce the formation of acrylamide, a suspected carcinogen, in starchy food products such as snacks and biscuits. Based on its importance, the present research was aimed to isolate and purify L-asparaginase from *Streptococcus thermophilus*. The Bacteria was screened for L-asparaginase production qualitative and quantitative manner. L-asparaginase activity of bacteria was detected on the basis of formation of pink colour around the colony. L-asparaginase producers were characterized by morphological and biochemical studies. Further quantitative assay was done to check the activity of the enzyme. The activity was found to be 0.380 IU/ml.

CHAPTER 1

INTRODUCTION

REVIEW OF LITERATURE

Acrylamide (C3 H5 NO; 2-propenamide), is a colourless, non-volatile crystalline solid, soluble in water and has a molecular weight of 71.08 kDa. It is produced commercially by hydrolyzing acrylonitrile using nitrile hydrase. Acrylamide is a toxic and probably human carcinogen molecule (IARC, 1994) that can form in heated foods as a consequence of the reaction between asparagine and a carbonyl source via Maillard-type reactions (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002; Zyzak et al., 2003; Becalski, Lau, Lewis, & Seaman, 2003; Yaylayan & Stadler, 2005).

Important acrylamide dietary sources are staple foods such as potato derivatives, cereal products and coffee (IRMM, 2005; FDA, 2006). Many potential routes have been identified to reduce acrylamide levels in foods. These are relevant to agronomical and technological strategies (CIAA, 2009). Among the latter 60 are interventions based on precursor consumption, i.e. fermentation and asparaginase 61 pre-treatments. 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.

1.1 Uses of acrylamide

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.

1.2 Occurrence and Dietary Intake

People are exposed to different amounts of acrylamide mainly through the diet. Acrylamide occurrence in foods is being studied intensively since the original report of high levels of acrylamide found in food that are subjected to high temperature. Acrylamide primarily found in plant based foods; heat treated starchy foods such as potato, cereal and bakery products contains high levels of acrylamide. Acrylamide is not found in foods that are not fried or baked such as boiling or microwaving (Eriksson, 2005; Törnqvist, 2005) and found very low levels in animal based food products such as meat and fish. To date, there is no permissible limits have been set worldwide for acrylamide consumption in diet. To analyse the acrylamide presence in diet, different dietary exposure databases consist of different food groups have been prepared by the European Union's acrylamide monitoring database, the United States Food and Drug Administration's acrylamide survey data and the WHO's summary information and Global Health Trends database.

Foods with high acrylamide levels contribute to 38% of daily calories, 36% of fibre and greater than 25 % of micronutrients. Generally, darker the colour of food product, higher the acrylamide content. Acrylamide formation increases drastically towards the end of the frying process. The amounts of acrylamide present in different food and food products are shown in the figure 1.

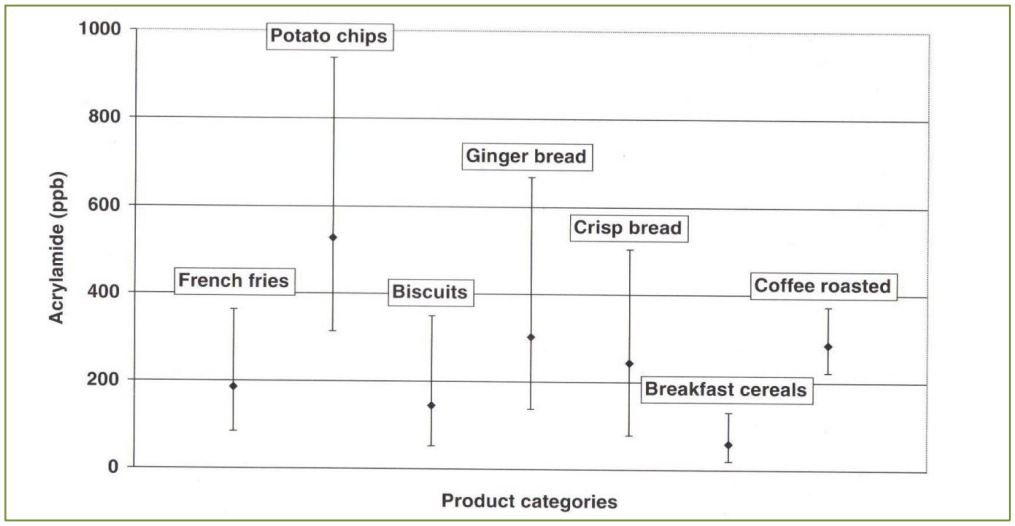

Fig 1. Acrylamide content of several typical food products [36]

Estimation of acrylamide occurrence in food commodities is a great concern in many countries. Moreover, the predictions of dietary acrylamide intake have been made for populations in many countries consist of different dietary records. These studies found that the amount of acrylamide was extremely higher in fried potato products (such as French fries and potato chips) followed by cereals, crisp breads, biscuits and other bakery products. Concentration and dietary intake of food have significant variations, which depends upon cooking methods. Factors such as difference in food composition, high temperature (more than 120°C), and high carbohydrate, free asparagine, reducing sugars, pH, water content, ammonium bicarbonate and high concentration of competing amino acids could be the sources for variation in acrylamide level. Dietary acrylamide intake may increase the risks of kidney and breast cancer. The daily intake of acrylamide in human diets was estimated to be 0.3 to 0.8 µg per kg body weight. At normal conditions, the average total daily intake of acrylamide is about 0.85 µg per kg body weight. The total macro and micro nutrient composition of the diet is mainly obtained from acrylamide containing foods. Foods that are contributing most dietary intake of acrylamide differ from country to country.

1.3 Mechanism of formation

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 byproduct of the Maillard reaction.

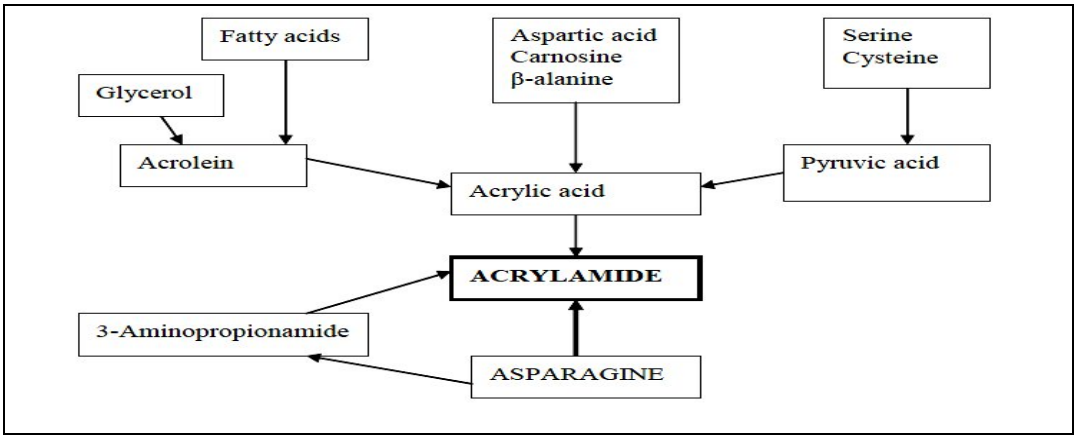

Fig2. The basic formation routes of acrylamide in foods [20]

The Maillard reaction 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.

1.3.1 Formation via Asparagine Route

The major pathway leading for acrylamide formation in foods is a part of the Maillard reaction with free amino acid (asparagine) and reducing sugars (mainly glucose and fructose) Maillard reaction is a non-enzymatic browning reaction occurring in foods during baking or frying. This happens at proper combination of carbohydrates, lipids and proteins for desirable color, flavour and aroma. Asparagine, is the free amino acid present in potatoes in high level (93.6 mg per 100 g), needs carbohydrates to form acrylamide. The potential of acrylamide formation is strongly related to glucose and fructose content. Free asparagine concentration to be the main determinant of acrylamide formation in rye varieties and in cooked flours and doughs (mainly rye and wheat). Research has shown that the reducing sugars are the major limiting factors in potatoes, while asparagine (mainly in the cereal bran) is the major limiting factors in cereal products.

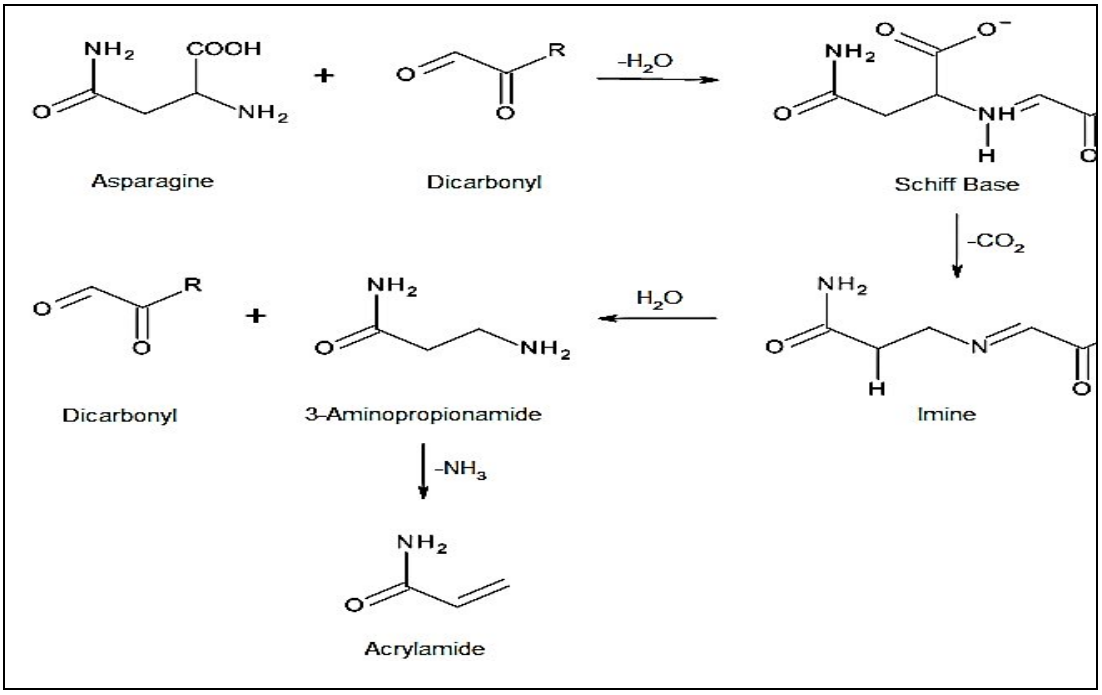

Fig 3. Proposed mechanism for formation of the acrylamide in heat treated foods[26]

1.3.2 Formation via Alternative Routes

Although formation of acrylamide in foods has its major routes through asparagine and reducing sugars, several other formation routes suggested via. acrolein and ammonia. In the absence of asparagine, acrolein and ammonia play a role in lipid rich foods in the formation of acrylamide. It is known that acrolein and acrylic acid are produced by degradation of lipids (triglycerides) in subject to high temperature.

Degradation of amino acids with ammonia can give rise to acrylamide formation by thermal decomposition. Amino acids such as glutamine, cysteine and aspartic acid have also been found to produce low amounts of acrylamide. However, stated that this mechanism might be irrelevant for acrylamide formation in foods.

Based on current stage of knowledge, acrylamide is a natural byproduct 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

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. Median 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.

Acrylamide is classified as probably carcinogenic to humans and its occurrence in food products has therefore caused intensive debate concerning the potential health risk through dietary exposure.

1.5 Mitigation Strategies for Acrylamide

Significant efforts have been undertaken in order to develop appropriate strategies that reduce acrylamide in foods. Reducing acrylamide content in foods at household and industrial level can help the public not only from food hazards but also to create perception about the food safety. A number of mitigation strategies have been focused so far regarding acrylamide formation at different stages of food production.

1.6 L- asparaginase

L-asparaginases **(E.C.3.5.1.1**) are enzymes expressed and produced by microorganisms It catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia (Capizzi et al., 1971). 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 or slurried 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.

1.6.1 General Classification of L-asparaginase:

Based on biochemical and crystallographic data, the known asparaginase sequences can be divided into three families. The first family corresponds to bacterial-type asparaginases, the second to plant-type asparaginases and the third one to enzymes similar to Rhizobium asparaginase. The enzymes included in this classification catalyze a number of different enzymatic reactions; including reactions connect with protein biosynthesis (Oinonen et al., 1995).

1.6.2 Mechanism of action

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 asparaginase prior 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.

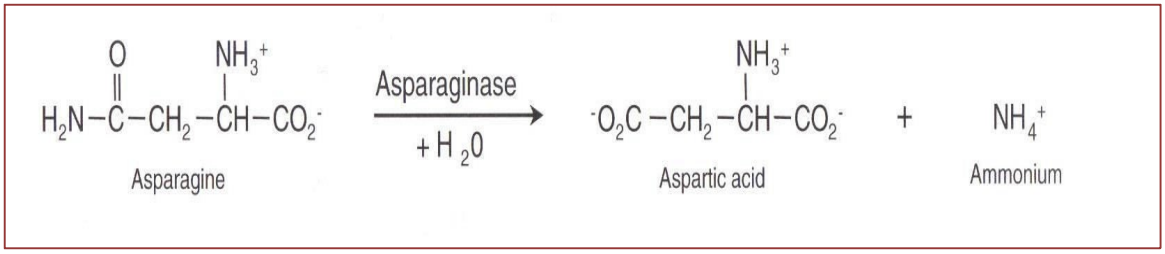

Fig4. Mechanism of action of L-asparaginase

The first documented application of using L-asparaginase for generating asparagine and concomitant acrylamide reduction in potato matrix that of ZYZAK et al. Furthermore, the effect of asparaginase was examined in gingerbread, resulting in a decrease of 75% of free asparagine, 55% of acrylamide content and no negative effect on either taste or colour.

It has been reported by Yohei et al.(2015) that acrylamide, a potential carcinogen, is formed from the reaction of L-asparagine and reducing sugars contained in foods during heating processes and free asparagine is a limiting factor for acrylamide formation. It has been reported that potato products 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/g potato), 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 enzymatic treatment, sliced potatoes were freeze-thawed ,dried at decompressed condition, resulting in the hydrolysis of approximately 90 % of L-asparaginase to L-Asp. 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.

Studies have shown that asparaginase activity is affected by enzyme dose, reaction time, temperature and pH at which the reaction occurs (Hendriksen, Kornbrust, Østergaard, & Stringer, 2009). Furthermore, the enzyme activity is influenced by the contact with the

substrate. In fact, a limited mobility of substrate and enzyme would be responsible for incomplete hydrolysis of asparagine and only partial reduction in acrylamide formation.

In fact, great reductions of acrylamide content could be achieved by using relatively low asparaginase concentrations in formulated foods (up to 1000 U/kg), such as bakery products and potato-based snacks, as a good enzyme distribution in the system can be reached. On the contrary, very high concentrations of asparaginase are necessary to obtain a significant reduction of acrylamide in fried potatoes (Pedreschi, Kaack, & Granby, 2008). However, also in this case, any technological operation which favours the substrate diffusion and its contact with the enzyme can lead to a greater reduction of acrylamide levels. This is the case of potato pieces treated with asparaginase after blanching (Hendriksen et al., 2009). In fact, blanching reduces the integrity of the potato, weakening the cell wall and membrane thereby improving the substrate-enzyme contact. Besides, especially when asparaginase is added to food formulations, the water content should be sufficiently high for mobility of reactants and facilitate the contact between enzyme and substrate (Amrein, Schoenbaechler, Escher, & Amadò, 2004; Hendriksen et al., 2009). For this reason the enzyme resulted more effective when added in the aqueous phase of the dough preparation instead of in the mixture.

1.6.3 Determination of L-Asparaginase Activity

There are different methods for determination the activity of L-asparaginase enzyme, which differ from each other in susceptibility and complexity, which are either quantitative or qualitative methods L-asparaginase activity can be measured by:

- Disappearance of alternative substrate (Jackson and Handschumacher, 1970)
- Direct nesslerization of ammonia (Wriston, 1970)
- Determination of aspartic acid (Howard and Carpenter, 1972)
- Determination of disappearance of asparagine (Howard and Carpenter, 1972)

Direct Nesslerization Assay is the most common method to estimate the asparaginase enzyme, due to its simplicity and qualification, this method depends on the estimation of ammonia which produced from the reaction of L-asparaginase with Asparagine as product, the direct Nesslerization assay include incubating of asparaginase enzyme with Asparagine at 37ºC for a period of time, the reaction is stopped by addition of Trichloroacetic acid (TCA), the mixture is centrifuged to rid the proteins, the

supernatant is treated with particular volume from Nessler's reagent, in the presence of ammonia the colour of solution converts from colourless to brown in relation to ammonia concentration.

And thus the ammonia concentration is estimated by measuring the absorbance at wave lengths vary from (425-500nm) according to the conditions of the experiment (Manning and Campbell, 1957). Direct nesslerization is the most suitable method as it is highly sensitive and can detect enzyme concentrations as low as 0.1 IU/ml.

1.6.4 Physical and Chemical properties of L-Asparaginase

Size and shape of *E.coli* enzyme have been determined through physical studies. Crystallographic studies provide information on its tetrameric structure (Epp et al., 1971). Furthermore, the subunits are structurally similar. Swain et al., 1993 identified the crystal structure of both *E.coli* L-asparaginase as well as *Erwinia chrysanthemi* L-asparaginase. Both these enzymes are active in their homotetrameric form; each monomer containing 330 amino acids.

1.6.5 Microbial production of L-asparaginase

Production of L-asparaginase by *Streptomyces albidoflavus* under submerged fermentation and maltose as carbon source increased the level of L- asparaginase in different culture media. The optimum pH and temperature for enzyme production was found at 7.5 and 350C respectively. Media for screening of L-asparaginase production in *E.coli* with different carbon sources and modified M9 medium ingredients has been optimized, and a semi quantitative plate assay is also reported for L-asparaginase production by *E.coli*. The determination of L-asparaginase activity in the human plasma based on the HPLC quantitation of the L-aspartic acid produced during the enzyme incubation has been developed. Screening and optimization of the nutrients by Plackett-Burman design for the production of L-asparaginase from *Bacillus cereus* MNTG-7 in submerged fermentation was achieved. Starch, L-asparaginase, ammonium oxalate, gelatine and calcium carbonate were identified as most effective in terms of product yield among 67 nutrients. The production of intracellular L-asparaginase from *Erwinia carotovora* and optimization of the various parameters using response surface methodology has been reported. The production and characterization of Lasparaginase by solid substrate fermentation using carob pod as substrate at pH 8 and temperature

70ºC for 30 to 60 minutes retaining 100% activity of the enzyme was reported from *Aspergillus terreus* KLS2. High levels of L-asparaginase were produced using solid-state fermentation than submerged fermentation from marine actinomycetes and *Aspergillus terreus* MTCC 1782 using artificial neural network linked genetic algorithm with optimized media.

L-asparaginase can be produced by both submerged as well as solid state fermentation.

1.6.5.1 Production of L-asparaginase through submerged fermentation

Submerged fermentation is mostly preferred for bacterial species. Growth of fungi is not preferred in submerged fermentation (SmF) as their natural form of mycelia causes viscosity in the media in which they are growing, causing problems in mixing and aeration.

The following is a list of the workers who've reported L-asparaginase production through SmF.

Heinemann and Howard, 1969 reported the production of L-asparaginase by submerged growth of *Serratia marcescens* ATCC 60. Five strains of *S. marcescens* were screened in shake-flask studies and 48 hrs after inoculation were found to produce 0.8 to 3.7 IU/ml. The requirements for asparaginase production with *S. marcescens* ATCC 60, the highest producing strain, included the following: an incubation temperature of 26 degree Celsius, 4% autolyzed yeast extract medium (initial pH 5.0), and limited aeration to maintain a zero level of dissolved oxygen (DO) during the fermentation. Yields were not enhanced by addition of various carbohydrates to the fermentation medium. Highest enzyme yields were found when the *p*H of the fermentation cycle rose to approximately 8.5. Yields of asparaginase/ml (4 IU) of cell suspension have been obtained consistently in 40 to 42 hr from 10-liter volumes (500 ml/4-liter bottle) produced on a reciprocating shaker. Also yields of 3.1 IU/ml were obtained by scale-up to a 60-liter fermenter in 35 hr. Maria et al., 2004 have investigated the filamentous fungi *Aspergillus tamarii* and *Aspergillus terreus* for L-asparaginase production by SmF. Nitrogen source affect its growth rate.

Karamitros and Labrou, 2014 performed extracellular expression and purification of L-asparaginase from *E. chrysanthemi* and *E. coli*. The strain *E. coli* Rosetta (DE3)

exhibited the highest extracellular expression levels among all the strains tested and it was chosen for further optimization and the development of purification protocol.

Upadhyay et al., 2014 reported the refolding and purification of recombinant L-asparaginase from inclusion bodies (IBs) of *E. coli* into active tetrameric protein. Protection of the existing native-like protein structure during solubilization of IB aggregates with 4M urea improved the propensity of monomer units to form oligomeric structure. Bioactive tetrameric form of L-asparaginase was efficiently recovered by retaining the native-like structures through their mild solubilization technique.

1.6.5.2 Production of L-asparaginase from Solid state fermentation

Solid state fermentation (SSF) is when a microorganism is allowed to grow on a solid matrix in absence or near absence of free water. Moisture level is optimised for such processes. SSF started initially as Koji fermentation and has gained importance in the recent years for the production of enzymes due to high yields.

Komathi et al., 2013 isolated, produced and partially purified L-asparaginase from *Pseudomonas aeruginosa* by SSF. The isolated strain was subjected to SSF techniques along with Soya bean meal maker as substrate. The high amount of L- asparaginase production was observed in 30 mins incubation on optimal medium. Further, the maximum enzyme activity was recorded at 40ºC.

Abha Mishra, 2006 reported the production of L-asparaginase from a new isolate of *Aspergillus niger* in SSF using agro wastes from three leguminous crops. *Glycine max* as a substrate is economically attractive as it is a cheap and readily available raw material in agriculture-based industries and therefore asparaginase production process should be developed on the basis of bran of *G.max.*

1.7 Applications of L-asparaginase

Asparaginases can be used for different industrial and pharmaceutical purposes. *E. coli* strains are the main source of medical asparaginase. . L-asparaginase has a significant role in food industry. Acrylamide, reported as a significant toxic agent and cause neurotoxicity in humans, is present in ample amount in food items which are heat-derived and contain some reducing sugars. Formation of acrylamide

is the result of Maillard reaction between the free amino acid asparagine and carbonyl group of reducing sugars like glucose. Maillard reaction is heat-induced reaction. Hence, food industry like baking food industry got an application of L-asparaginase. The enzyme helps in hydrolysing the asparagine which significantly reduces the formation of acrylamide. The reported reduction in acrylamide content is about 90% (Mario Sanches et al. 2007 3).

The enzyme L-asparaginase has the chemotherapeutic property against the tumor cells. It is an effective curable agent against the treatment of acute lymphoblastic leukemia and lymphosarcoma. The enzyme catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia. The principle behind the use of asparaginase as an anti-tumor agent is that it takes advantage of the fact that all leukemic cells are unable to synthesize the non-essential amino acid asparagines their own, which is very essential for the growth of the tumor cells, whereas normal cells can synthesize their own asparagine; thus leukemic cells require high amount of asparagine.

1.8 Organism used for study

Streptococcus salivarius subsp. thermophilus (previous name *Streptococcus thermophilus*) is a Gram-positive bacterium and a homofermentative facultative anaerobe, of the viridans group. It tests negative for cytochrome, oxidase, and catalase, and positive for alpha-hemolytic activity. It is not motile and does not form endospores.

It is also classified as a lactic acid bacterium. *S. thermophilus* is found in fermented milk products, and is generally used in the production of yogurt, alongside *Lactobacillus delbrueckii* subsp. *bulgaricus*. The two species are synergistic, and *S. thermophilus* probably provides L. d. *bulgaricus* with folic acid and formic acid which it uses for purine synthesis.

S. thermophilus is one of the most widely used bacteria in the dairy industry. USDA statistics from 1998 showed that more than 1.02 billion kilograms of mozzarella cheese and 621 million kilograms of yogurt were produced from *S. thermophilus*. Although its genus, includes some pathogenic species, food industries consider *S. thermophilus* a safer bacterium than many other*Streptococcus* species. In fact, yogurt and cheese that contain live cultures of *S. thermophilus* are thought to be beneficial to health. Live cultures of *S. thermophilus* make it easier for people who are lactose intolerance to

digest dairy products. The bacteria break down lactose, the sugar in milk,that lactose-intolerant people find difficult to digest.

1.9 Objectives of the study

The present study has been undertaken to utilize bacteria (*Streptococcus thermophilus)* source for production of the enzymes L-asparginase with the following objectives:

1. Isolation and identification of bacterial species from a capsule. The specie undertaken for the study is *Streptococcus thermophilus*
2. Screening for enzymatic activity by performing qualitative and quantitative assay.
3. Optimization and production of enzyme L-asparaginase from *Streptococcus thermophilus.*
4. Isolation of bacterial DNA.

CHAPTER- 2

MATERIALS AND METHODS

2.1Materials and Chemicals

L-asparagine was obtained from LobaChemie (Mumbai). All the other chemicals used were analytical grade and obtained from Merck (Mumbai) and SRL (Mumbai). Nessler's reagent used for assay was procured from CDH (Central Drug House), New Delhi present already in the lab.

2.2 Equipments used

Autoclave

Weighing balance

Spectrophotometer;

Water bath

Centrifuge

Microscope

Sonicator

2.3 Media preparation

Composition of M9 salt(500ml) **:-**

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

Agar solution(500ml)

Agar	50g
Distilled water	500ml

Cool them to 50°C and combine them aseptically.

Filter sterilized solution to be added

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

2.4 Preparation of reagents

2.4.1 Reagents for L-asparginase estimation

Nessler's reagent was used for the assay of the enzymes.

2.4.1.1 Tris-HCl buffer

0.5M Tris-HCl buffer was prepared in distilled water (pH 8.6).

2.4.1.2 L-asparagine solution

L-asparagine solution was prepared at a concentration of 0.01mM each in distilled water.

2.4.1.3 TCA solution

1.5M (10% w/v) TCA solution was prepared in distilled water.

2.4.2 Reagents and chemicals for isolation of DNA

2.4.2.1 Extraction of DNA

TNE Buffer

Composition	**Concentration**
Tris HCl (pH 7.4)	50mM
NaCl	100mM
EDTA	0.1mM

SET Buffer/Lysis Buffer

Composition	Concentration
Tris HCl (pH 8)	100mM
SDS	1 % w/v
EDTA	50mM

T.E Buffer

Composition	Concentration
Tris HCl (pH 8)	10mM
EDTA	0.5M

Chemicals

4.4 M Ammonium Acetate

0.5 M Ethylene Diamine Tetraacetic Acid (EDTA)

Phenol:Chloroform (2:1;volume:volume)

4M Sodium Chloride (NaCl)

10% Sodium dodecyl Sulphate (SDS)

2.4.2.2 Buffer for agrose gel electrophoresis

SB buffer

Composition	Quantity Stock (20X)
Boric acid	45 g
NaOH	8 g

Loading Dye

Composition	Concentration
Glycerol (30%)	3ml
Bromophenol blue (0.25%)	25 mg
dH_2O	10 ml

2.5 Methodology

2.5.1 Isolation of bacterial species

100 ml of brain heart infusion broth (*Streptococcus thermophillus*) was prepared and weas inoculated with the desired microorganisms to prepare the seed culture. *Streptococcus thermophillus* culture was incubated at 50°C for 24 hours. Four-fold serial dilution of 100µl inoculum was done in 900µl of N-Saline. Brain Heart Infusion Agar media was prepared and was poured in petri plates.100 µl of dilution was spread on respective media. Incubation of *Streptococcus thermophillus* at 50°C for 24 hours was done.

2.5.2 Identification of bacteria

2.5.2.1 Gram Staining

Streptococcus thermophilus are Gram positive bacteria and the Gram status of the isolated bacteria was determined by the microscopic examination of Gram-stained isolate. 1 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 resuspended 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.

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 safranine for 30 sec.

• Washing under tap water.

• Drying with cotton towels gently.

Gram positive bacteria became blue-purple after gram staining; however gram negative bacteria became pink-red.

2.5.2.2 Catalase activity

This test detects the presence of catalase, which converts hydrogen peroxide to water. This test is useful in differ*entiating the given strain is Streptococcus (-)* from that of the *Streptococcus (+)*. Hydrogen peroxide when present in the organisms is harmful, in the process of evolution organism have developed a unique mechanism to break the hydrogen peroxide into water and oxygen thus reducing its toxicity activity. Few drops of 3% hydrogen peroxide were added to culture and looked for the release of oxygen as a result of hydrogen peroxide breakdown and appeared as foaming.Oxygen gas is produced, if the baceria is catalase positive. The evolution of gas causes bubbles to form and is indicative of a positive test.

2.5.3 Long term preservation of the isolate

The isolated bacteria were stored in a -80ºC deep freezer in BHI medium in 20% (v/v) glycerolfor preservation. The glycerol stock was prepared by mixing 500µl of overnight grown culture with 500µl of fresh, sterile BHI medium with 40% glycerol in eppendorf tubes. Hence the resulting suspension included 20% glycerol.

2.5.4 Ammonia Standard Curve

Standard graph was prepared by treating 1ml of 0.25, 0.5, 0.75 and 1mM ammonium sulphate with trichloroacetic acid (TCA), NaOH and Nessler‟s reagent.

2.5.5 Screening of bacteria for L-asparaginase production

The isolates were screened for asparaginase activity using the rapid plate 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 were incubated for 48 hours at 37ºC. L-asparaginase activity was identified by formation of a pink zone around colonies.

2.5.6 Enzyme assay

The enzyme activity was determined in culture broth after 24 h by asparaginase assay (Imada et al, 1973) using Nessler's reagent. To check whether L-asparaginase activity in *streptococcus thermophiles* is extracellular or intracellular, both the fractions were checked for the presence of L-asparaginase activity as follows:

2.5.6.1 Extracellular fraction

Streptococcus thermophilus was grown in 10 ml BHI broth for 24 h and 1 ml of this culture was inoculated in 100 ml BHI broth. Erlenmeyer flasks containing the inoculated medium were kept on a shaker at 37ºC. After 24 h, 1 ml of broth was collected from the flasks aseptically and centrifuged at 10,000 x g to sediment the cells. The supernatant thus obtained was used as crude enzyme.

2.5.6.2 Intracellular fraction

1 ml of inoculum was used for inoculation in 500 ml flasks containing 100 ml of BHI broth. The inoculated media in Erlenmeyer flasks were kept on a shaker at 37ºC for 24 h. The cells thus grown were harvested by centrifugation at 7,800 x g (4°C). Cells were washed once with sodium phosphate buffer, pH 8.0 and again centrifuged at 7,800 x g for 10 min at 4ºC. The supernatant was discarded and cells were resuspended in 10 ml of the same buffer, (pH 8.0) to make a cell suspension. Cells were disrupted using sonicator. The cell lysate thus obtained was centrifuged at 7,800 x g for 15 min at 4 ºC. Pellets were discarded and the cell free supernatant was subjected to enzyme assay. The supernatant was then used as a crude enzyme and checked for presence of asparaginase activity.

2.5.6.3 Determination of L-Asparaginase activity

The L-Asparaginase activity was determined according to the method of Mashburn L and Wriston J. The reaction mixture contained 0.1ml of enzyme extract, 0.2ml of 0.05M Tris-HCl buffer (pH 8.6), and 1.7ml of 0.01 M L-Asparaginase . The mixture was incubated at 37°C for 10 min.

The reaction was stopped by the addition of 0.5 ml of 1.5 M TCA. Precipitated proteins were removed by centrifugation, and the liberated ammonia was determined by

nesslerization. The ammonia released in the supernatant was determined spectophotometrically at 450nm. One international unit (IU) of L-asparaginase is the amount of enzyme that liberates one micromole of ammonia in 1 min at 37°C.

2.5.7 Isolation of DNA

2.5.7.1 Extraction of DNA

- 1.5 ml of overnight grown culture was taken and was centrifuged at 15000 rpm for 5 min.
- Pellet of sample was collected and resuspended in 900 µl TNE buffer and cells were collected by centrifuging at 15000rpm for 5 min.
- Pellet of sample was collected and resuspended in 70% ethanol (ice cold) and placed on ice for 20 min.
- Suspension was centrifuged at 12000 rpm for 5 min.
- Pellet was resuspended in 480 µl of SET buffer; tubes were kept at -20 for 20 min and immediately transferred to a water bath at 68 ºC for 10 min.
- Phenol:Chloroform:Isoamyl (25:24:1) were added and mixed by gentle inversion.
- Centrifuged at 5000 rpm for 10 min.
- 100µl of 3M sodium acetate and equal amount of absolute alcohol was added to each tube and mixed gently and was centrifuged at 5000rpm for 10 min.
- Pellet was washed with (70%) ethanol at 5000 rpm.
- Pellet was resuspended in 100ul TE Buffer and stored at 4°C.

2.5.7.2 Visualization of DNA by Gel Electrophoresis

- Gel was prepared using SB buffer 0.8 g of agarose was added in 50ml of SB buffer and heated till the agarose dissolves.
- Gel was cooled down till 45-55^0 C and then 2.5µl EtBr was added to the gel.
- Agrose gel was poured slowly in the casting tray with comb on and was left for solidification.
- After solidification of gel, comb was removed and gel apparatus was flooded with SB buffer till it covered the surface and the gel got dipped.

- 5 µl of samples were mixed with the 2 µl of loading dye respectively and loaded into the wells.
- Gel was run for 60 mins at 100 V voltages.
- Gel after run was slowly removed from the apparatus and visualized under Gel doc system.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Isolation of bacteria

Streptococcus thermophilus was successfully isolated without any contamination and the isolated colonies were further sub cultured and cross checked for their contamination.

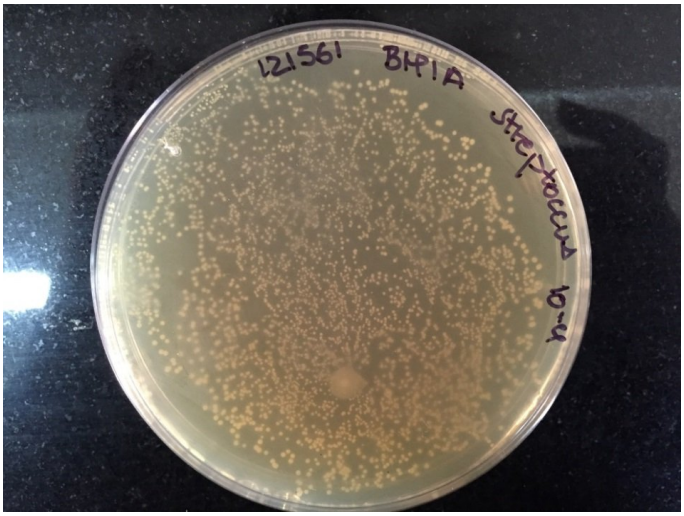

Fig1. Plate of isolated *streptococcus thermophilus* colonies

3.2 Identification of bacteria

3.2.1 Gram staining

The isolated colonies were subjected to Gram staining and they were examined under light microscope. The strain gave blue- purple color with staining; hence it was Gram positive bacteria. The isolates were all cocci with spherical or ovoid morphology. They appeared mostly as pairs or forming chains.

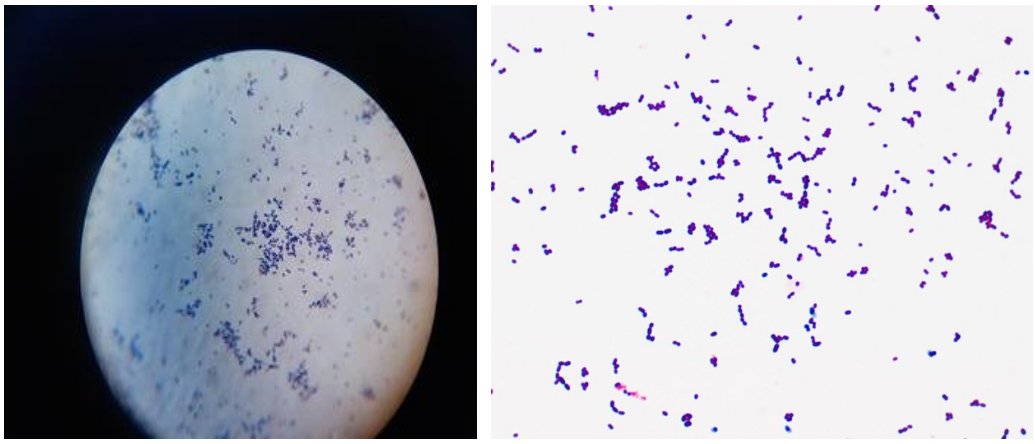

Fig 2. Visualization of *streptococcus thermophilus* under microscope

3.2.2 Catalase activity

No bubble formation was there which indicated that there was no evolution of gas (oxygen). Hence the isolated strain was catalase negative.

3.3 Screening of bacteria for L-asparaginase production

The release of ammonia from aspargine in M9 agar plates led to increase in local pH and hence the reddish appearance of plates harbouring positive cultures was identified. It was due to the presence of phenol red which was used as an indicator in the media.

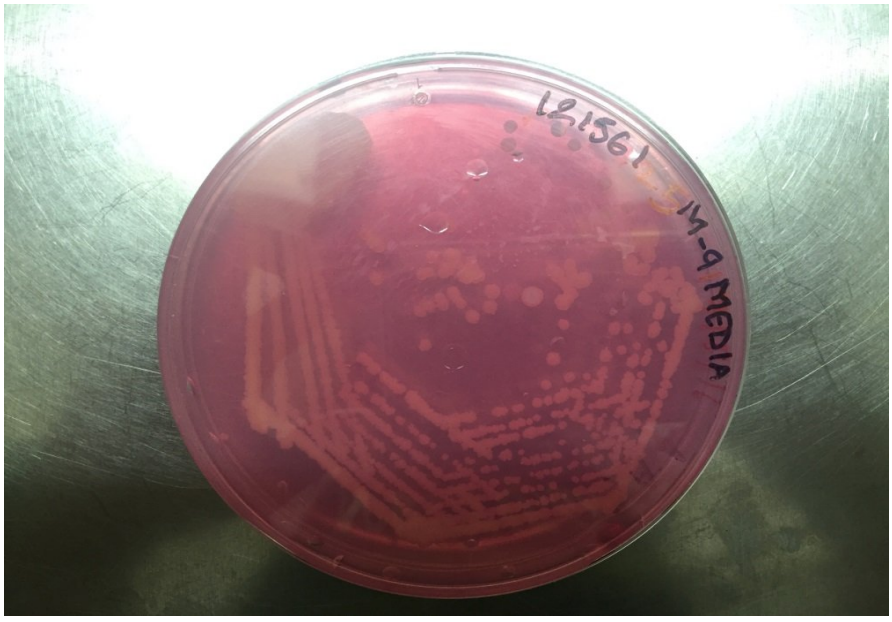

Fig 3. Plate showing l-asparaginase positive bacteria

3.4 L-asparaginase activity in extracellular and intracellular fraction

When both the intra and extracellular fractions were checked for asparaginase activity, it was observed that there was visible activity in the intracellular fraction. On the other hand, the extracellular fraction showed negligible activity. This confirmed that the L-asparaginase enzyme in *streptococcus thermophilus* is intracellular, not extracellular. The present study adds L-asparaginase to the list of intracellular enzymes of *streptococcus thermophilus*

3.5 Standard curve for ammonia

In order to determine the L-ASNase and activity, a standard curve was plotted for determining the concentration of ammonia using Nessler's reagent.

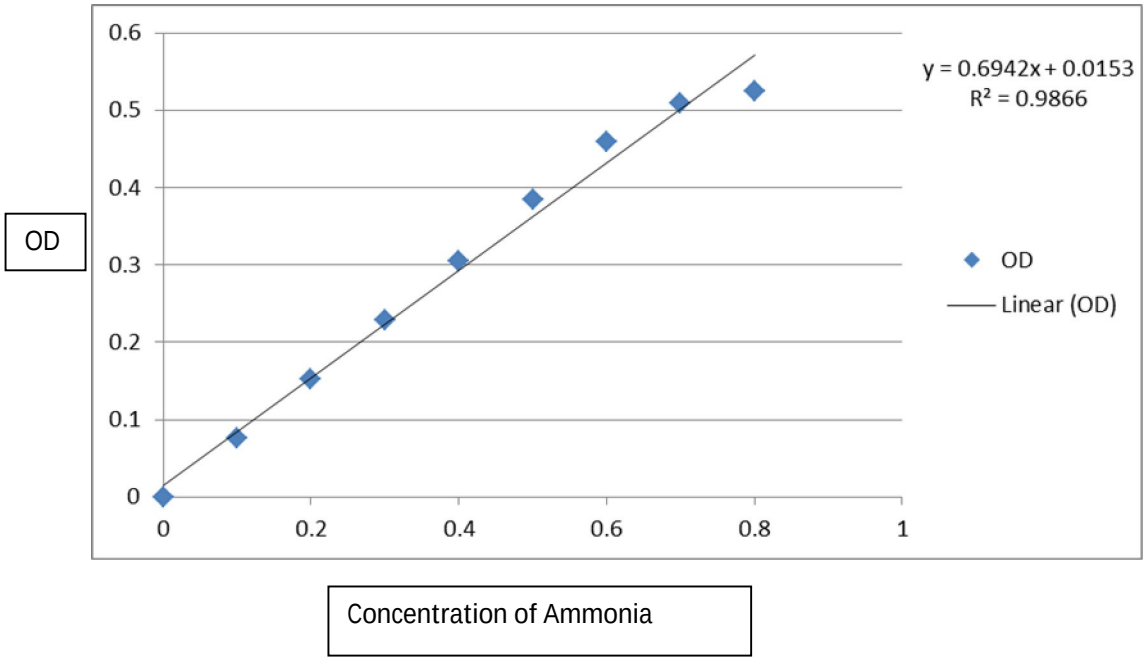

Fig 4. Standard curve of ammonia

A standard curve was constructed by taking ammonium sulphate (µM/ml) on X-axis and corresponding optical density on Y- axis.

3.6 Isolation of DNA

DNA of *streptococcus thermophilus* was isolated successfully. DNA was visualized using Gel Doc system and image was taken as shown in fig 5.

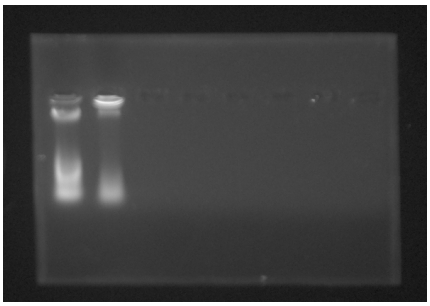

Fig 5. Gel image for isolation of DNA

CONCLUSION AND FUTURE PROSPECTS

L-asparaginase is an important anti-cancer enzymes and continued research is being carried out on it. Present study showed production of L-asparaginase with the help of bacteria *Streptococcus thermophilus*. Qualitative analysis which was done using M9-medium (containing L-asparagine) confirmed the production of enzyme by the bacteria. Further quantitative assay done to check the activity of the enzyme which was found to be 0.380 IU/ml.

Further studies such as purification and optimization of parameters can be carried out on the crude extract of the enzyme obtained. Research on L-asparaginase from bacteria *streptococcus thermophilus* can be exploited therefore as a novel source.

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