Fermentative Production of Microbial Asparaginase Enzyme and its Application to High Temperature Processed Foods for Acrylamide Mitigation

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THESIS

On

"Fermentative Production of Microbial Asparaginase Enzyme and its Application to High Temperature Processed Foods for Acrylamide Mitigation "

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List of Publications

List of Journal Publications:

- **Mausumi Ray, Dr.Pradyut Kundu and Dr.Sunita Adhikari (Nee Pramanik),** ***"Microbial Production of L-asparaginase by an isolated Bacillus paramycoides MRS4 stain and optimization of process parameters"*** **Indian Journal of Biotechnology.** Vol 20, (242-251), (2021)

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List of Publications in National/ International/Conferences/Workshops

- **Mausumi Ray, Dr.Pradyut Kundu, Dr.Sunita Adhikari (Nee Pramanik),** ***"Statistical Optimization of Process Parameters by Central Composite Design (CCD) for an Enhanced Production of L-asparaginase by an Isolated Bacillusparamycoides MRS4 strain MCCC 1A04098"*** International conference on Health, Energy and Materials (ICHEM'22) Organized by the Department of Chemical Engineering, **Hindustan Institute of Technology and Science, Chennai** in association with IICHE and ABLE during April 28-29th , 2022.

- **Mausumi Ray, Dr.Pradyut Kundu, Dr.Sunita Adhikari (Nee Pramanik),** *"**Purification of L-Asparaginase produced by an isolated Bacillus paramycoides MRS4 and its application in different high temperature processed food for acrylamide mitigation"*** National Conference on "Advancement in Food Technologies (NAF 2K22) Organized by the Department Food Technology, **Bannari Amman Institute of Technology,Tamilnadu,** during December 9-10th , 2022.

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PROFORMA-1

"Statement of Originality"

I, Mausumi Ray, registered on 03.06.02019 do hereby declare that this thesis entitled **"Fermentative Production of Microbial Asparaginase Enzyme and its Application to High Temperature Processed Foods for Acrylamide Mitigation"** contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies.

All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work.

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Mausumi Ray

(Mausumi Ray)

Dedicated to my Parents,Elder Sister and my Research Supervisor(s)

Table of Contents

Objectives of the Present Work

- Isolation of a microbial strain from different food sample (Corn, Oats, Soyabean, Potato etc.) and soil sample capable of producing L-asparaginase.
- Identification of most potent microbial strain capable of producing L-asparaginase.
- Optimization of various process parameters for maximum production of the enzyme L –asparginase in batch scale and using RSM with the selected isolated strain.
- Characterization of the crude enzyme produced in batch scale.
- Optimization of various process parameters for maximum production of the enzyme using RSM in a laboratory fermenter and its characterization.
- Purification of the crude enzyme produced in a laboratory fermenter.
- Application of purified enzyme in acrylamide mitigation of high temperature processed foods.

Scope of Present Work

- Isolation of a suitable bacterial strain capable of producing L-asparaginase enzyme from a suitable source.
- Identification of the most potent bacterial strain capable of production of L-asparaginase
- Optimization of various process parameters like pH, temperature, inoculum concentration, medium volume, time etc. to find the conditions for maximum production of the enzyme.
- Statistical tools like RSM applied for finding optimum parameters for maximum production of the enzyme in batch scale.
- Characterization of the crude enzyme produced in batch scale.
- Optimization of various process parameters for large scale enzyme production in laboratory fermenter.
- Cherecterization of crude enzyme produced by fermentation process in a laboratory fermenter.
- Purification of the enzyme and its application to high temperature processed food.

Methodology Followed

- Isolation of a suitable bacterial strain capable of producing L-asparaginase enzyme from different food sample (Corn, Oats, Soyabean, Potato etc.) and soil sample by serial dilution and plate count method.
- Identification of the most potent bacterial strain capable of producing L-asparaginase
- Optimization of various process parameters like pH, temperature, inoculum concentration, medium volume, time etc. by using batch scale and statistical tools like RSM to find the conditions for maximum production of the enzyme.
- Optimization of various process parameters like medium volume, aeration and agitation rate for maximum production of the enzyme in a laboratory fermenterusing RSM.
- Collection of the crude enzyme produced by batch scale and laboratory fermenter, and salted out with 80% ammonium sulphate.
- Characterization of the crude enzyme produced in batch scale and in a laboratory fermenter.
- Purification of the crude enzyme and application of it in different high temperature processed food samples (French Fry, Nimki, Fried Fish, Fried Chicken).

General Introduction

Asparaginase is an unsaturated and high reactive amide, white crystalline solid ,which is soluble in water, ether, ethanol and chloroform. Asparaginase is an enzyme that is used as a medication and in food manufacturing. As a medication, L-asparaginase (LA) is used to treat acute lymphoblastic leukemia and lymphoblastic lymphoma. In food manufacturing it is used to decrease acrylamide [1-2]. Asparaginase is an enzyme that breaks down the amino acid asparagine, which is one of the key precursors to acrylamide formation. By reducing the amount of asparagine in the food, asparaginase can lower the levels of acrylamide that form during cooking. [3-4].

The Maillard reaction is a complex chemical reaction that occurs between amino acids and reducing sugars at high temperatures, resulting in the characteristic brown color, flavor, and aroma of baked or fried foods. However, it can also produce acrylamide, a potentially carcinogenic compound, which is a concern for food safety [5-6].

The use of asparaginase as a food processing aid can effectively reduce the level of acrylamide in starchy foods, by breaking down asparagine, an amino acid that is a precursor to acrylamide formation, into another amino acid, aspartic acid, and ammonium. This process prevents asparagine from taking part in the Maillard reaction and significantly reduces the formation of acrylamide.

While complete acrylamide removal may not be possible due to other minor formation pathways [7], the use of asparaginase can significantly reduce the level of acrylamide in a range of starchy foods, without affecting the taste or appearance of the end product [8-9].

Overall, the use of asparaginase as a food processing aid is an effective approach to reducing the potential health risks associated with acrylamide formation during cooking, and can help ensure the safety and quality of processed foods [10].

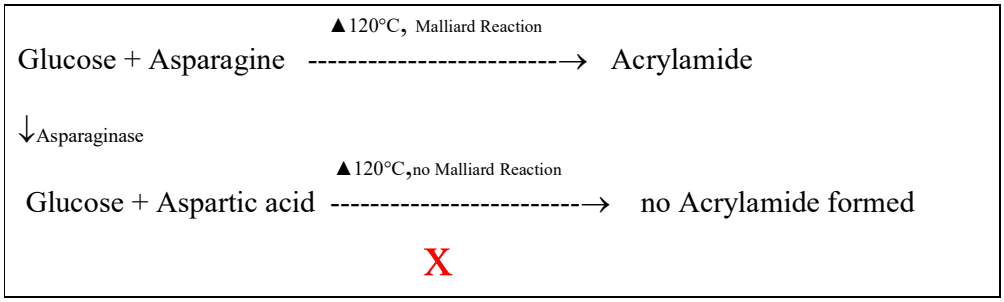

Figure 1: Reaction of Acrylamide formation & Prevented by Asparaginase Enzyme

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Literature Review

L-asparaginase was first described by Clementi in 1922 as an enzyme that hydrolyzes L-asparagine into aspartic acid and [1]. This enzyme has a wide distribution in microbes, plants, and animals, with microbes being the preferred source for industrial production due to their easy upstream and downstream processing. L-asparaginase has recently been the subject of extensive research due to its potential applications in the pharmaceutical and food industries [2]

L-asparaginase is an important drug in the treatment of acute lymphoblastic leukemia (ALL) and lymphosarcoma. This is because these cancers have limited or no production of the L-asparagine synthetase enzyme [3].

In 2002, researchers in Sweden discovered that acrylamide was present in high levels [4] in carbohydrate-rich foods subjected to high temperatures such as frying, baking, and grilling. This was due to the reaction between reducing sugars and L-asparagine through the Maillard reaction [5] when starchy foods are cooked at temperatures above 120°C under low moisture conditions. Acrylamide is formed by the formation of a Schiff base followed by decarboxylation and elimination under heat. Acrylamide is harmful to human health, being neurotoxic, genotoxic, carcinogenic, and toxic to the reproductive system [6]. The International Agency for Research on Cancer classified acrylamide as a probable human carcinogen, and the Scientific Committee on Food reported that it was genotoxic [7]. In response, the European Commissionwere obliged by Regulation 2158/2017 launched new measures to reduce acrylamide content in food in 2017, requiring food businesses in Europe to reduce acrylamide levels in their products [8].

To reduce acrylamide in food, various strategies have been proposed including selection of raw materials, altering product composition, optimizing processing conditions, and using pretreatment procedures [8]. Enzymatic treatment using L-asparaginase is a simple and effective way to lower acrylamide levels in food without affecting its sensory or nutritional properties [9]. L-asparaginase works by hydrolyzing L-asparagine into aspartic acid and ammonia, thereby preventing the Maillard reaction and inhibiting acrylamide formation [10].

Microbial sources of L-asparaginase have been studied for their potential use in reducing acrylamide levels in food. Research has focused on classification, catalytic mechanism, production, purification, and characterization of microbial L-asparaginases, particularly for

their application in mitigating acrylamide in fried potato products, bakery products, and coffee. These studies aim to identify and optimize the most efficient and cost-effective production methods for microbial L-asparaginases, with the goal of reducing acrylamide levels in food products to improve their safety and health impact.

Classification and Catalytic Mechanism of L-asparaginase

Based on their amino acid sequences and structural and functional homologies, L-asparaginases have been divided into three families: bacterial type (type I and type II) L-asparaginases, plant-type (type III) L-asparaginases, and Rhizobial-type L-asparaginases [11,12]. Bacterial type-I L-asparaginases are cytosolic enzymes with relatively low affinity towards L-asparagine (millimolar Km). Bacterial type-II L-asparaginases are periplasmic enzymes that exhibit high specific activity against L-asparagine (micromolar Km) [13]. Plant-type L-asparaginases are the members of the N-terminal nucleophile (Ntn) hydrolases superfamily. These enzymes are expressed as inactive precursors that undergo autocleavage to form α and β subunits. These subunits then form dimers of heterodimers to attain an active conformation. Plant-type L-asparaginases are subdivided into K+-dependent and K+-independent enzymes, depending on their potassium requirement for activation [14]. The third family includes sequences in homology to Rhizobium etli asparaginase II, of which Rhizobium etli (the symbiotic host of leguminous plants) can use L-asparagine as a sole carbon and nitrogen source through the action of two enzymes: asparaginase I and asparaginase II [15]. Despite being extensively studied, the mechanistic details of biochemical reactions catalyzed by L-asparaginase have been ambiguous. The hydrolysis of L-asparagine by L-asparaginases may have diverse mechanisms—the direct displacement or the double-displacement mechanism (Figure 1). The direct displacement mechanism is used by L-asparaginases, in which a water molecule directly attacks the L-asparagine substrate (Figure 2). The double-displacement mechanism, commonly referred to as a ping-pong mechanism, involves an acyl type covalent intermediate, where the hydrolysis of L-asparaginases proceeds with two displacements. Each active site of L-asparaginases consists of two highly conserved catalytic triads (Thr-Tyr-Glu and Thr-Asp-Lys) represented by T16-Y29-E289 and T95-D96-K168, respectively, in Helicobacter pylori L-asparaginase [16,17]. At the beginning of the L-asparaginase reaction, the activated Thr of the first triad initiates a nucleophilic attack on the carbonyl amide in the L-asparagine side chain, resulting in the acyl-intermediate form of the enzyme and ammonia release (first displacement); then, the Thr of the second triad activates water molecules for a nucleophile attack on the same C

atom, which breaks the acyl intermediate and releases aspartic acid (the second displacement) [16,18]. The direct displacement mechanism of catalysis by guinea pig L-asparaginase was supported by the experimental results and computational work [19]. The double-displacement (ping-pong) mechanism of catalysis by E. coli type II L-asparaginase was proven based on the structural and biochemical experiments combined with previously published data [20].

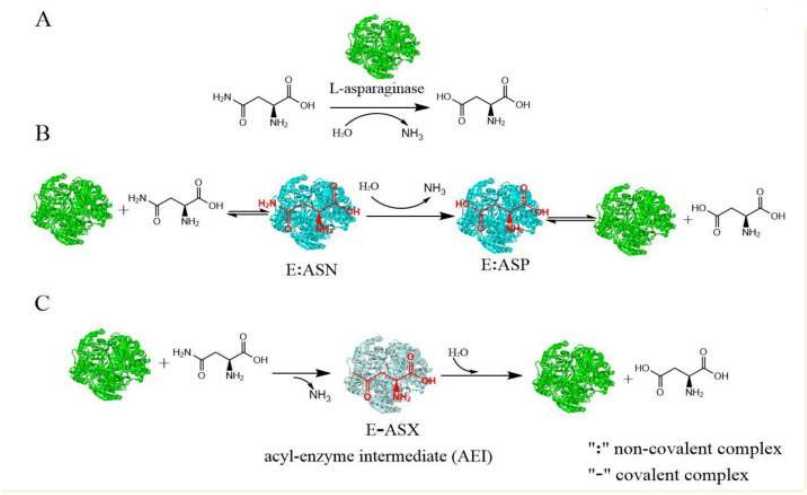

Figure 2: A schematic diagram of the L-asparaginase reaction and its catalytic mechanisms: (A) A schematic diagram of the L-asparaginase reaction. (B) A schematic diagram of the direct displacement mechanism. (C) A schematic diagram of the double-displacement mechanism. Different colors stand for different conformations of the enzyme. (ASN, L-asparagine; ASP, aspartic acid).

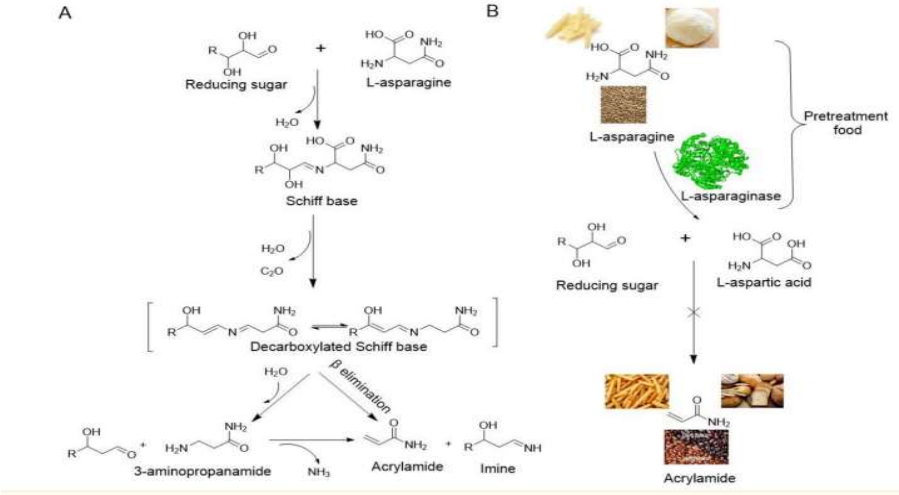

Figure 3: Mechanism of acrylamide formation and acrylamide mitigation through L-asparaginase: (A) Acrylamide formed between reducing sugars and L-asparagine; (B)

Acrylamide mitigation in foods through the conversion of L-asparagine to L-aspartic acid by L-asparaginase.

Sources of L-asparaginase

L-asparaginase is found in a variety of sources including microbes such as archaea, bacteria, actinomycetes, fungi, yeasts, and microalgae. These microbes have been isolated from various environments, including soil, water, marine sediment, river sediment, marine sponges, mosses, and plants. In addition, recombinant L-asparaginases can be produced through protein engineering and recombinant DNA technology, where the L-asparaginase gene is cloned and expressed in E. coli. This provides a versatile and scalable method for producing large amounts of L-asparaginase for use in acrylamide mitigation in food.Bacterial and fungal strains that are important sources of this enzyme have been extensively studied. Some of the recently used L-asparaginase producers, such asbacterial, fungal, yeast, and algae strains, are summarized in Table 1.

Table 1: List of L-asparaginase produced Microorgamisms isolated from Different Sources. Kinetic characteristics of enzyme, comparisons of the characteristics of enzymes obtained from various sources

Microorganism	Strain	Source	Enzyme activity	Kinetic characteristics	Molecular wt	Reference
Bacteria	*Acinetobacter soli Y-3*	cloning gene	42.0 IU mL−1	Km=3.22 mmol L−1 Vmax= 1.55 IU µg−1		Jiao L et al.2020
	Bacillus subtilis WSA3	marine sponges	100 µg/mL	_		Ameen F et al.2020
	Bacillus sonorensis	cloning gene	10 mM	Km=2.004 mM Vmax= 3723 µmol min1-,	36 kDa	Aly N et al.2020
	Bacillus licheniformis	soil	25.2 IU ml−1,	_	_	Abdelrazek N.A et al.2020
	Bacillus sp. SL-1	cloning gene	30 mg/L	Kcat = 23.96s-1 K_m = 10.66 µM	_	Safary A et al.2019
	Bacillus brevis	soil	_	Km=3.5x10^{-2} mM Vmax= 0.77 IU	32 kDa	Azmi W et al. 2017
	Bacillus subtilis KDPS1	soil	47	_	97.4 KDa	Sanghvi

			IU/ml			Get al 2016
Fungi	*Penicillium crustosum NMKA 511*	soil	19.10 U/mL	_	41.3kDa	Khalil N.M et al .2021
	Fusarium equiseti AHMF4	soil	40.78 U mL−1	_	45.7 kDa	El-Gendy M.M.A.A et al .2021
	Aspergillus oryzae CCT 3940	collection	26.0 U mL−1	Km= 5.06 mM Vmax= 588.24 U mL−1	_	Dias F.F.G et al.2019
Yeast	*Leucosporidiummuscorum CRM 1648*	marine sediment	5.12 U.L-1 h-1	_	_	Freire R.K.B et al.2021

Food industry: acrylamide formation

Acrylamide is also known as 2-propenamide, acrylic amide, ethylene carboxamide, propenamide, propanoic acid amide, monomer of acrylamide or acrylic acid amide, presenting 71.08 g/mol of molecular mass.[21] Several studies show that L-asparagine is the main amino acid responsible for acrylamide production in fried and baked foods when reducing sugars are condensed with a carbonyl source. This phenomenon does not occur in boiled food.[22]

Acrylamide formation has been quite studied in the last years. Zyzak et al. (2003)[12] detected that the amide chain present in the acrylamide structure is provided from L-asparagine. Reagents (L-asparagine or reducing sugars) reduction or removal is one of the evaluated strategies for decreasing acrylamide quantity in foods. For L-asparagine reduction, several options have been investigated, such as: selection of vegetal species with lower level of L-asparagine in their composition; deletion of important enzymes for L-asparagine biosynthesis control by suppression of specific genes; acid hydrolysis of L-asparagine leading the formation of aspartic acid and ammonia; and acetylation process of L-asparagine to form N-acetyl-L-asparagine, preventing the formation of acrylamide from intermediate N-glycosides.[23]

In the study of Zyzak et al. (2003),[24] authors confirmed that the use of L-asparaginase enzyme before frying or baking food process could reduce more than 99% acrylamide level in the processed final product. This is because the enzyme reduces more than 88% of the L-

asparagine concentration from the initial feedstock. In last years, other works have dealt with this application of l-asparaginase, that can decrease the negative effects of acrylamide containing foods without impair their characteristics [25, 11, 12, 13,14].

L-asparaginase has been shown to be effective in reducing acrylamide levels in various food products such as fried potato products, bakery products, and coffee. The European Food Safety Authority (EFSA) estimates that acrylamide intake in diets is between 0.3 and 1.9 μg/kg body weight [26], with foods like fried potato products [27], bakery products, and coffee being the primary sources of human-consumed acrylamide. Research on the application of L-asparaginase for acrylamide reduction in these food products is an ongoing field in the food industry. Microbial sources of L-asparaginase have been isolated from various sources, including soil, water, and plants, and recombinant L-asparaginases have also been produced through protein engineering and recombinant DNA technology.

Additionally, cost-effectiveness and stability of the enzyme are also important factors to consider when implementing L-asparaginase in industrial processes [28]. The industrial production of L-asparaginase can be carried out by utilizing microbial fermentation, but optimization of the process is necessary to achieve high yield and stability of the enzyme. The purification and characterization of L-asparaginase produced by microbes also play an important role in the industrial application of the enzyme. In conclusion, the application of L-asparaginase in the food industry as a means to reduce acrylamide formation has great potential, but optimization and further research is needed to effectively transfer the laboratory results to an industrial scale.

Engineering of enzyme to improve properties, various ways to improve thermostability of enzyme.

The fundamentals of thermostability engineering need to be carried out for proteins with low thermal stability to expand their utilization. Thus, comprehension of the thermal stability regulating factors of proteins is needful for the engineering of their thermostability. Protein engineering aims to overcome their natural limitations in tough conditions by refining protein stability and activity. Rational-design approach requires a crystal structure dataset along with the biophysical information, protein function, and sequence-based data, especially consensus sequence that is favourable for the protein folding during natural evolution. It can be attained by either single- or multiple-point mutation, by which amino acids are changed. In fact, these mutation approaches show several benefits. For example, the offered mutations are produced

after an evaluation and design, which raise the chance to acquire favourable mutations. The rational-design engineering can improve the biochemical properties of enzymes, including the kinetic behaviours, substrate specificity, thermostability, and organic solvent tolerance. Moreover, this approach considerably reduces the library size, so less effort and time can be employed. Here, we apply the computational algorithms and programs with experiments to create thermostable enzymes that will be beneficial for future applications [29].

Application of Immobilized L-asparaginase in Food

Most studies of immobilized L-asparaginase have focused on its medical applications, while only a few works have been devoted to the food industry [30]. The stability and reusability of L-asparaginase are both essential from an industrial point of view because of the enzymes subjected to incubation and pretreatment at elevated temperatures. The poor stability and non-recyclability of free L-asparaginase increase the cost of food processing and limit the large-scale usage of the enzyme. Many materials, such as chitosan, aluminum oxide pellets, magnetic nanoparticles, and agarose spheres, have been utilized to immobilize L-asparaginase in order to increase the stability and recyclability of L-asparaginase.

The native properties of the enzymes, such as thermal properties, activity, and substrate affinity, can be enhanced for food applications using immobilization techniques. Moreover, the unique reusability of an immobilized enzyme makes it more suitable for food applications. Alam et al. (2018) used magnetic nanoparticle-immobilized L-asparaginase to reduce acrylamide in a starch–asparagine food model. L-asparaginase from Bacillus aryabhattai was immobilized on magnetic nanoparticles modified with aminopropyl triethoxysilane (APTES) using a cross-linking agent, glutaraldehyde. The immobilized enzyme showed more than a three-fold increase in thermal stability and retained 90% activity after the fifth cycle. The authors also found that the immobilized enzyme had a better affinity towards its substrate. The starch–asparagine food model of 2% (w/v) L-asparagine and 2% starch (w/v) (1:1) was treated with equal dose (18 U) of free or immobilized enzyme for 30 min and then heated at 180 °C for 5 min. The acrylamide formation in starch–asparagine food treated with free enzyme reduced by 60%, whereas no formation of acrylamide treated with immobilized enzyme was observed compared with the control [31].

In another study, Ravi and Gurunathan (2018) used chitosan-immobilized L-asparaginase to reduce acrylamide in fried kochchikesel. L-asparaginase from Aspergillus terreus was covalently immobilized on chitosan by glutaraldehyde. Kochchikesel banana slices were

soaked in 5 U/mL of free or immobilized enzyme suspension at 60 °C for 20 min and then fried at 180 °C for 25 min. The mitigation of acrylamide in fried kochchikesel with treatment by chitosan-immobilized L-asparaginase decreased by approximately 49% compared to treatment with the free enzyme [32]. Immobilized L-asparaginase is more effective for acrylamide mitigation in food than the free enzyme because of its superior properties.

Immobilized L-asparaginase is utilized not only in batch processing but also in continuous production processes for fluidized food components for acrylamide mitigation. Li et al. (2020) used agarose sphere-immobilized L-asparaginase to reduce acrylamide in a fluid food model system. The immobilized L-asparaginase (Aga-ASNase) was obtained from an amidation reaction between L-asparaginase and food-grade agarose spheres activated with N-hydroxysuccinimide esters. The immobilized enzymes exhibited superior storage stability and reusability, with 93.21% and 72.25% of the initial activity retained after six consecutive cycles and storage for 28 days, respectively. The effluent flowing from the continuous catalytic process under optimal conditions (96 U, 35 °C, 1 mL/min) was heated at 180 °C in an oil bath for 10 min. Compared with the amount of acrylamide in the untreated system, the amount of acrylamide was reduced by almost 89% when the fluids flowed through the packed bed reactor, with an average residence time of 12 min [33].

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Chapter I

Isolation and Identification of bacterial stain from different food components capable of producing L- Asparaginase

1.Introduction

Asparaginase enzyme is used in the processing of food industrially and also used as a medicine. In food processing industry asparaginase is used to reduce acrylamide content which forms in starch-based food during high temperature processing like baking, frying and roasting. The compound acrylamide is found having carcinogenic effect on both human and animals. The free amino acid asparagine present in the starch-based food reacts with sugars during high temperature together with low moisture processing condition [1]. The transformation of asparagine to aspartic acid and NH_3 is catalyzed by bacterial L asparaginase enzyme [2]. So, it is used to reduce the acrylamide content high temperature processed starch-based foods. Acrylamide was found in different fried and baked foods like fried potatoes, potato chips, coffee, biscuits, bread, French fries [3]. Inadditionto thatL-Asparaginase is considered as a therapeutic agent for handling of different types of cancer [4]. Numerous types of tumour cells need L-asparagine for synthesis of protein. In presence of L-asparaginase they are lacking an essential growth factor. It causes cytotoxicity of leukemic cells [5]. L-asparaginase produces aspartate thatis aprecursor for the production of ornithine in urea cycle as well as acts as aprecursor for the formation of oxaloacetate to generate glucose through gluconeogenic pathway within the human body [6]. L-Asparaginase could be produced from different plants, animals and various microorganisms [7]like fungi, yeasts, bacteria, algae and actinomycetes [8].Microbial source of L-asparaginase is more popular because of its stability, high productivity, easy extraction and purification. It is known that numerous microorganisms can produce LA mostly by submerged fermentation process. Some examples ofAsparaginase producing microbial species are *Aerobacter, Bacillus, Pseudomonas, Serratia,Xanthomonas, Photobacterium*, *Streptomyces sp.*, *Proteus sp.*, *Vibrio sp.* and *Aspergillus sp.*[9-13].The enzyme is though widely distributed but only few of them are having antineoplastic activity. Most commercially available microbial sources are *Escherichia coli, Erwinia carotovora* and *Serratia marcescens* [14]. The therapeutic enzymes are also having the problem of toxicity [15]. So, there is a continuous search for asparaginase enzyme that haveing therapeutic effect with less or no toxicity. There is a constant interest to isolate organism from diverse sources [16].

2. Materials and Methods

2.1. Isolation and selection of a bacterial strain

Bacterial straincapable to produce asparaginase enzyme were isolated individually from soil, cereals like Corn, Oats, pulses like Soya bean, vegetable like Potato by plate and dilution method [17]. The cereal and pulses were soaked overnight and allowed to ferment for 24 hr. After 24 hr the soaked and fermented cereals were ground and 1g of the ground cereal was diluted in sterile water. The potato sample was cut and kept overnight for fermentation at room temperature. After 24 hr 1g of potato sample was taken into 100 mLsterile distilled water. The diluted sample solutions were transferred to the petri plate along with medium enriched with 1% asparagineas [18]. The medium composition was as follows

Name of the Chemical	**Amount (g/L)**
Potassium dihydrogen phosphate	3.0
Disodium phosphate	6.0,
Sodium chloride	0.5
Magnesium sulfate heptahydrate	0.12
Calcium chloride dihydrate	0.001
Agar	30.0
pH	6.8-7.2

Process for isolating and screening bacteria for their ability to produce lactic acid (LA) as follows:

- **Isolation of bacteria:** The bacteria are first isolated by streaking a sample onto Petri plates containing a suitable growth medium. The plates are then incubated at 35°C for 24 hours to allow the bacteria to grow and form isolated colonies.
- **Transfer to slant:** After 24 hours, the isolated colonies are transferred to slants, which are glass tubes containing a solid growth medium. The slants are then stored at 4°C until further use.
- **Screening for LA production:** To screen the individual slants for the most potent LA-producing bacteria, a spectrophotometric method is used. This involves measuring the absorbance of the bacterial culture at a specific wavelength, which can provide an estimate of the amount of LA produced.
- **Fermentation:** Once the most potent LA-producing bacteria have been identified, they are grown in a larger culture under controlled conditions. The bacteria are grown

in a medium containing nutrients that promote LA production, and the culture is aerated at a rate of 1.5 L/min and agitated at 150 rpm to ensure optimal growth conditions. The fermentation is carried out for 24 hours at 35°C and pH 7.0.

Separation of cell mass and supernatant: After 24 hours of fermentation, the bacterial culture is centrifuged at 4500 rpm for 15 minutes to separate the cell mass from the supernatant. The supernatant, which contains the LA produced by the bacteria, is collected and can be further processed for purification or other applications.

2.2 Estimation of enzyme production capacity of isolated strain /Enzyme assay

Assay of Asparaginase enzyme was performed by calculating the amount of ammonia formed using Nesslerization method and Spectrophotometric analysis [14] at 396 nm. A standard curve was made by measuringthe amount of ammonia liberated from ammonium sulfate in the reaction which is detected spectrophotometrically at 396 nm after adding Nessler's reagent. For the selection of most potent asparaginase producing strain one loop full of organism from individual isolated slant culture was transferred to the fermentation medium having same medium composition as mentioned above (g/L) and kept into the incubator at 35 °C in a shaker incubator for 24 hr. After 24 hr the fermented broth was centrifuged at 4500 rpm for 15 min. 1.0 mL of supernatant was added to0.5 mL of 1% L-asparagine. The mixture was kept in an incubator at 37 °C for 30 min. After 30 min the reaction was ended by adding 0.1 mL of 15 % trichloro acetic acid solution and 0.2mL of that reaction mixture was transferred into a test tube contained 4.3 mL of distilled water. The quantity of the ammonia emitted from the mixed solution by addition of the supernatant was measured by addition of 0.5mL Nessler's reagent. The absorbance was measured in a UV-visible spectrophotometer at 396nm. The quantity of ammonia formed was related to the quantity of the enzyme existing. One IU of L-asparaginase was the quantity of enzyme that under the optimum assay conditions produced 1 μM of NH_3 per minute

2.3. Identification of the selected strain

Identification of the selected strain was carried out according to Bergey's manual of determinative bacteriology [18]. For this purpose different media required for different biochemical tests were prepared [19].

Then morphological, physiological and biochemical characteristics of the selected strain were studied in detail.

2.3.1. Morphological study of the isolated strain

The morphological, cultural, and biochemical characteristics of the isolated strain was studied according to the Bergey's manual of determinative bacteriology [18].

2.3.2. Physiological characterization of theisolates

Bergey's Manual of Systematic Bacteriology , a well-known reference book in microbiology used for the classification and identification of bacterial species. The tests mentioned in the manual were followed to determine the physiological characteristics of the isolated strains, which is important for accurate classification and identification.

2.3.3. Gram Staining Process

Gram staining method, developed by Hans Christian Gram in 1884, used to differentiate between gram-positive and gram-negative bacteria based on the structure of their cell walls. The staining process involves using crystal violet, iodine solution, ethyl alcohol, and safranin, and the final color of the bacteria (violet or red) indicates their gram classification.

2.3.4. Endo spore Staining(Schaeffer-Fulton method)Process

Defferent technique was done by using several reagents as follow

2.3.4.1. Malachite Green

The endospores are surrounded by an impermeable layer, making it difficult for the primary stain (malachite green) to penetrate. Heat treatment was applied to soften the spore coat, allowing the primary stain to penetrate and color both the vegetative cells and spores green. After staining, the next step was decolorization with alcohol or acetone, which removes the stain from the vegetative cells, leaving only the endospores stained green. Finally, the endospores are counterstained with safranin, which gives them a red color, allowing for clear differentiation from the unstained vegetative cells.

2.3.4.2. Decolorizing Agent

After the primary stain (malachite green) has been applied and heat treated, the vegetative cells and endospores are both stained green. The next step was decolorization with alcohol or acetone, which removes the stain from the vegetative cells, leaving only the endospores stained green. The tap water wash does not play a role in removing the primary stain, as the

stain has already been fixed on the endospores and cannot be easily removed. The endospores remain green due to their resistance to the decolorizing agent, while the vegetative cells become colorless. Finally, the endospores are counterstained with safranin, which gives them a red color, allowing for clear differentiation from the unstained vegetative cells.

2.3.4.3. Safranin or Counter Stain

After the decolorization step, the endospores retain their green color from the primary stain (malachite green), while the vegetative cells become colorless. The next step was counterstaining step, in which the colorless vegetative cells were stained with safranin, which gives them a red color. The endospores, which retain the green primary stain, are easily differentiated from the red-stained vegetative cells, allowing for clear visualization and identification of both types of structures in the bacterial sample.

2.3.5. Growth under Aerobic Condition

Luria broth (LB) is a commonly used bacterial growth medium for the cultivation of a wide range of bacteria in aerobic conditions. The composition you mentioned (10 g/L tryptone, 10 g/L sodium chloride, 5 g/L yeast extract) is a standard formula for LB broth. The pH of the broth should be around 7±0.2, which is near neutral. Incubating the bacterial isolates at 37ºC for 24 to 48 hrs provides optimal growth conditions for aerobic bacteria. After incubation, the optical density (OD) of the culture can be measured using a UV-Vis spectrophotometer, such as a Lassany, at 600 nm. This measurement allows for the estimation of bacterial growth in the culture, as the OD is directly proportional to the number of bacteria present in the culture.

2.3.6. Growth under Anaerobic Condition

To cultivate bacteria under anaerobic conditions, the Luria broth medium were used in a closed system to exclude oxygen. The flasks could be flushed with carbon dioxide (CO_2) to create anaerobic conditions and then capped tightly to prevent the entry of oxygen and air. Incubating the bacterial isolates at 37ºC for 7 to 9 days in a standing position provides optimal growth conditions for anaerobic bacteria. After incubation, the optical density (OD) of the culture could be measured using a UV-Vis spectrophotometer at 600 nm to estimate the growth of the anaerobic bacterial culture.

2.3.7. Growth at Different Temperatures

To determine the optimum temperature for bacterial growth, the isolated culture can be grown in Luria-Bertani (LB) medium under shaking conditions at different temperatures (4ºC, 25ºC, 30ºC, 37ºC, and 42ºC) for 24 to 48 hrs. After incubation, the optical density (OD) of the culture could be measured at 600 nm using a spectrophotometer to estimate bacterial growth. The temperature at which the culture shows the highest OD was considered the optimum temperature for growth of that particular bacterium. This information could be useful for understanding the growth conditions and requirements of different bacterial species.

2.3.8.Growth in Different pH

To determine the optimum pH for bacterial growth, the isolated strain was grown in Luria-Bertani (LB) medium at 37ºC temperature for 24 to 48 hrs with shaking conditions. The pH of the LB medium could be adjusted to various values within the acidic and basic range to determine the ideal pH for bacterial growth. The optical density (OD) of the culture was measured at 600 nm against a blank using a UV-Vis spectrophotometer to estimate bacterial growth. The pH at which the culture shows the highest OD was considered the optimum pH for growth of that particular bacterium. This information was useful for understanding the growth conditions and requirements of different bacterial species.

2.3.9. Growth in Different Sodium Chloride Concentrations

To determine the optimum sodium chloride (NaCl) concentration for bacterial growth, the isolate was cultured in Luria-Bertani medium and incubated for 24 to 48 hrs with shaking conditions at 37ºC. The concentration of NaCl in the medium was varied from 0% to 10% in different sets. After incubation, the optical density (OD) of the culture was measured at 600 nm against a blank using a UV-Vis spectrophotometer to estimate bacterial growth. The concentration of NaCl at which the culture shows the highest OD was considered the optimum concentration for growth of that particular bacterium. It was a useful information for understanding the growth conditions and requirements of different bacterial species.

2.3.10. Acid Production from Carbohydrates

Utilization of different sugars and carbohydrates by bacteria was an important aspect for bacterial identification and classification. The production of metabolic byproducts and the

ability to use certain sugars can provide information about the type of bacteria present. pH indicators was also be use to detect changes in the pH of the medium due to the utilization of different sugars, providing further information about the bacteria's metabolism.

The compositions of this test medium (g/L distilled water):
Tryptone; 15, yeast extract; 10, phenol red indicator; 0.05, carbohydrate; 10, pH was maintained around 7. Tryptone is used as an alternative energy source and sugars are used as a carbohydrate source. Sugar is added to detect gas and metabolic acid production from bacterial metabolism of carbohydrate present in different medium. Phenol red is a pH indicator for the detection of organic acids. If the bacteria takeup the sugar present in it and gives off different types of organic acids, the pH indicator will become yellow. Durham tubes are placed in submerged condition in the media to find out if there is any gas produced inside it or not. Durham tubes are special kind of small inverted tubesplaced inside the test tubes in such a way thatits enclosed end at the top and open end reside at the bottom of the test tubes. Small bubble inside this Durham tubes representing trapped gas that has been produced by the bacterial cultures in the process of Substrate utilization and metabolism. There are 4 possible outcomes in this test shown below.

- No Reaction (-) with no Growth: If the broth remains red in color and there is no growth of bacteria, it indicates that no utilization of the carbohydrate present in the media has taken place.
- No Reaction (-) with Growth: If the broth remains red in color but the media becomes foggy or cloudy, it indicates that there is growth of the microorganism, but it is not utilizing carbohydrates. The microorganisms are instead using the alternative energy source (tryptone).
- Acid Production (A): If the broth turns yellow, it indicates that the organism is using the carbohydrate and producing acids that bring down the pH of the media.
- Acid and Gas production (A/G): If the broth becomes yellow and there is a small bubble in the inverted Durham tube, it indicates that the organism is utilizing the carbohydrate present in the media, producing both acids and gas in the process.

The different types of carbohydrates (Mannitol, Fructose, Arabinose, Galactose, Glucose, Lactose, Sucrose etc.) were used to test the metabolic activity of a bacterial strain. A medium was made for each carbohydrate test with a composition of Tryptone (15g), yeast extract

(10g), phenol red indicator (0.05g), and carbohydrate (10g) with a pH around 7. The medium was then divided into two test tubes and a Durham tube was placed in an inverted manner inside each of the test tubes. The tubes were then sterilized at 121°C temperature, 15 psig pressure for 15 minutes and then inoculated with a 24 hr cell-suspension of the bacterial strain in one of the test tubes. The other test tube without inoculum was kept as a control. The tubes were incubated in an incubator at 37°C and observed regularly for 15 days for any changes in color and gas formation.

2.3.11. Catalase Test

It is a description of a catalase test, which was used to identify bacteria based on their ability to produce the catalase enzyme. The test involves adding hydrogen peroxide (H_2O_2) to a bacterial culture and observing the production of oxygen in the form of bubbles, which indicates the presence of the catalase enzyme. The result of the test can depend on the growing conditions and medium used for growing the bacteria.

- If bubbles are produced when H_2O_2 is added to the bacterial culture, the organism is considered to be catalase positive [catalase (+)].
- If no bubbles are produced, the organism is considered to be catalase negative [catalase (-)].

2.3.12. Nitrate Reduction Test

Nitrate reduction test, which was used to determine if a bacterial isolate was able to produce the enzyme nitrate-reductase and nitrite-reductase. The test involves incubating the bacterial isolate in nitrate broth and observing the reduction of nitrate to nitrite and potentially N_2 gas. If nitrite was produced, it was reacted with sulfanilic acid and α-naphthylamine to produce a red-colored precipitate, indicating the presence of nitrite. The absence of a red color indicates the absence of nitrite and suggests that the isolate does not produce the enzymes. The results of the test can provide information about the type of respiration used by the organism and can be used to differentiate between different bacterial species.

- If the nitrate in the media was not reduced, the strain is considered to be nitrate negative [nitrate (-)].
- If the nitrate is reduced to nitrite, which is then reduced to NO, NO_2, or N_2 gas, the strain is considered to be nitrate positive [nitrate (+)].

The lack of reaction with the chemicals that detect nitrite in this case suggests that the nitrite

has been completely reduced to other compounds.

2.3.12. Urease Test

The urease test was used to determine if a microorganism has the ability to produce the enzyme urease, which hydrolyzes urea into ammonia (NH_3) and carbon dioxide (CO_2). The presence of urease in the test medium leads to an increase in alkalinity, resulting in a change in the color of the phenol red indicator from yellow to pink. A uniform mixture of bright pink color after incubation indicates a positive result for urease production.

Medium Composition (g /L of distilled water):

Potassium phosphate, monobasic; 9.1, potassium phosphate, dibasic; 9.5, urea; 20, phenol red; 0.01 and yeast extract; 0.1, pH was kept around, 7.0

The day-old pure culture of the bacterial inoculum was inoculated into the urease test medium and the tube was agitated slowly to keep the bacteria in suspension. Incubation was performed with a cotton plug at around 37°C and the mixture was observed for any change in color at 8th, 12th, 24th, and 48th hrs. A uniform mixture of bright pink color indicates a positive result for the production of urease.

2.3.13. Indole Production

The indole test was used to determine if bacteria have the ability to decompose the amino acid tryptophan and produce indole. The process involves the action of intracellular enzymes called tryptophanase, which decomposes tryptophan into indole, pyruvic acid, and ammonia. The test involves growing the bacteria in a medium containing tryptophan, and then detecting the presence of indole through a reaction with p-dimethylamino benzaldehyde (DMAB) under low pH conditions, which produces a red chemical indicating the presence of indole.

Compositions of tryptone broth medium (g/L of distilled water) :

Tryptone; 10, sodium chloride; 5, pH ~ 7.0

The indole test involves inoculating the isolate into the medium and incubating it for 24 to 48 hr at 37°C. After incubation, 4 to 5 drops of Kovac's reagent were added to the test tube. A positive result was indicated by the formation of a red color in the upper layer within a few seconds of adding the reagent. If no red color was observed, the result was considered indole negative, and the layer remains yellow.

2.3.14. Starch Hydrolysis Test

The starch hydrolysis test was used to differentiate bacteria based on their ability to hydrolyze starch using the enzymes α-amylase or oligo-l, 6-glucosidase. Starch was a large molecule that cannot penetrate the bacterial cell membrane, so it needs to be cleaved into smaller fragments. Bacteria that produce extracellular enzymes such as α-amylase and oligo-l,6-glucosidase have the ability to hydrolyze starch by breaking down the glycosidic bonds between sugar monomers. The test involves growing the bacteria on nutrient agar with added starch, and observing if there was a clearing of the starch around the bacterial growth, indicating starch hydrolysis.

Compositions (g/L of distilled water) are peptone; 5, beef extract; 3, starch; 2, agar; 30, pH was maintained in the range of 7±0.3.

The starch hydrolysis test was used with iodine to detect the presence or absence of starch around the bacterial growth region. Iodine reacts with starch to produce a blue or brown color, so the presence of starch will be indicated by a blue or brown color. However, if the bacteria had hydrolyzed the starch, a clear zone around the bacterial growth will be visible after the colonies were flooded with iodine. The isolated cultures were inoculated in agar and incubated at 37°C for 24 to 48 hrs. After incubation, the results were observed by flooding the petri dishes with iodine and observing the presence or absence of a clear zone around the bacterial growth.

2.3.15. MotilityTest

The motility test was used to determine the ability of bacteria to move spontaneously, referred to as motility. In this test, a soft agar medium is used. Non-motile bacteria will only grow in the area they were inoculated by stabbing, while motile bacteria will grow and spread around the stabbing zone by swimming. The motility of bacteria is thus revealed by observing the growth and spread of bacteria in the soft agar medium.

Compositions for motility test agar medium (g/L of distilled water) are enzymatic digestion of gelatin; 10, beef extract; 3, sodium chloride; 5, and agar; 4, pH; 7±0.2. The motility agar test involves inoculating the isolated culture through the stabbing method in the mid portion of the medium using an inoculation needle to a depth of approximately half of the medium. The inoculated medium was then incubated at 37°C for 5 days and observed for results. The

presence or absence of bacterial growth and spread from the stabbing site was used to determine the motility of the bacteria.

2.3.16. Voges Proskauer(VP)Test

The Voges-Proskauer (VP) test was used to identify bacteria that are capable of fermenting 2,3-butanediol in mixed-acid fermentation. The test was performed using a buffered broth of glucose and peptone. Bacteria that have the ability to ferment glucose will release acid into the medium, causing a decrease in pH. The addition of the coloring agent methyl red in the system will turn red in response to the decrease in pH, indicating a positive result for glucose fermentation and the ability to ferment 2,3-butanediol.

Medium composition for this test: Voges Proskauer (VP) media (g/L of distilled water) are casitone; 3.5, peptone; 3.5, dextrose or glucose; 5, potassium phosphate; 5, pH~7.0. Isolated culture was inoculated in themedia and then the test tubes were kept in incubation at 37°C for a period of 24 to 48 hrs. 5 drops of methyl red was added after this period is over.

Test tubes were also shaken gently and let them to stand for 10 to 15 minutes and it was observed for color change. Positive test is indicated by a pink-red color developing in a few minutes and no color formation is an indicative of a negative result.

2.3.17. Identification of the isolated most potent bacterial strain by 16S rDNA sequence and phylogenetic analysis

DNA was separated from the selected strain.Thes uperiority of this DNA was assessed on 1.2% Agarose Gel where an only band of high-molecular weight DNA has been found.The extracted DNA was enlarged with *16S rRNA* Specific Primer (8Fand 1492R) using Veriti® 96 well Thermal Cycler (Model No. 9902).A soledistinct PCR amplicon band of 1500 bp was found and it was purified enzymaticallyand exposed to Sanger Sequencing next. Two directional DNA sequencing reaction of PCR amplicon was done with 704F and 907R primers using BDT Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus sequence of 1485 bp *16S rDNA* was produced using aligner softwarefrom frontward and opposite sequence data. To conduct BLAST alignment search tool of NCBI Genbank databasethis*16S rDNA* sequence was used. Based on maximum identity score the 1st fifteen sequences were selected and it was associated with ClustalW, a several alignment software program. Distance matrix was produced using RDP database. The Phylogenetic tree was made using MEGA6.

3. Result and discussion:

3.1 Screening of bacterial strains for LA activity

L-asparaginase enzyme producing bacteria were isolated from soil sample and different food sample (Corn, Oats, Soya bean, Potato).From soil sample six, from corn sample twelve, from oat ten, from Soya bean sample twelve and from Potato sample twenty-one isolated colonies were obtained. The enzyme producing activity of different isolates from different sources was measured. The results were shown in Table 1.1 and it was found that among the maximum enzyme producers from different sources, the isolatedstrain MRS4 from fermented soya beanhadthe highest activity for producing L-asparaginaseenzyme that is shown in Table 1.2.

Table 1.1: Isolation of L-asparaginase producing bacteria from different sources

Sample	Organism	Enzyme Produced (IU/mL)
Soil	**MRSO1**	**103.0±0.8**
	MRSO2	21.0±1.3
	MRSO4	15.5±0.5
	MRSO6	4.0±1.1
	MRSO8	4.0±1.5
	MRSO11	2.5±0.9
Corn	MRC1	19.0±2.1
	MRC2	16.0±1.8
	MRC3	86.0±2.8
	MRC4	**99.5±1.2**
	MRC5	86.0±1.5
	MRC6	80.0±0.8
	MRC7	85.5±1.4
	MRC8	95.0±1.7
	MRC9	37.0±1.15
	MRC10	12.5±1.08
	MRC11	85.0±1.35
	MRC12	48.0±1.86
Soybean	MRS1	80.5±1.98
	MRS2	85.5±1.6
	MRS3	91.0±1.45
	MRS4	**105.5±1.34**
	MRS5	97.5±1.21
	MRS6	88.5±1.16
	MRS7	25.0±1.47
	MRS8	23.0±1.63
	MRS9	29.5±1.58

	MRS10	21.0±1.42
	MRS11	88.0±1.49
	MRS12	50.0±1.28
Potato	MRP1	7.0±2.8
	MRP2	34.5±1.9
	MRP3	22.5±1.65
	MRP4	25.0±1.3
	MRP5	2.0±1.11
	MRP6	71.0±1.82
	MRP7	75.5±1.56
	MRP8	18.5±2.91
	MRP9	76.0±1.55
	MRP10	14.0±2.68
	MRP11	72.5±1.33
	MRP12	70.5±1.07
	MRP13	72.5±1.19
	MRP14	60.0±1.04
	MRP15	72.0±1.27
	MRP16	69.5±1.58
	MRP17	69.5±1.04
	MRP18	**96.5±0.95**
	MRP19	62.5±1.87
	MRP20	59.5±1.4
	MRP21	67.5±1.21
Oat	MRO1	68.0±1.28
	MRO2	**100.0±0.87**
	MRO3	75.5±2.23
	MRO4	60.0±2.05
	MRO5	73.0±1.87
	MRO6	14.5±2.49
	MRO7	73.0±0.97
	MRO8	75.0±1.16
	MRO9	39.5±2.17
	MRO10	36.0±1.36

Table 1.2: Maximum L-asparaginase enzyme producer bacteria isolated from different sources

Sl. no	Maximum enzyme producer bacteria (Source)	Enzyme Produced (IU/mL)
1	MRSO1[SOIL]	103.0±0.8
2	MRC4[CORN]	99.5±1.2
3	**MRS4[SOYBEAN]**	**105.5±1.34**
4	MRP18[POTATO]	96.5±0.95
5	MRO2[OATS]	100.0± 0.87

3.2 Morphological study of the isolated strain

The isolate was aerobic, gram positive, rod shaped and showed sub terminal endospore on spore staining. Positive growth of the strain was observed in the medium that contained glucose, arabinose, galactose, lactose and sucrose. The strain had the capability to hydrolyze starch, casein and can use citrate but could not produce hydrogen sulphide (H_2S). Positive urease and catalase activity had been shown by the isolated strain. According to the morphological and biochemical characteristics of the strain MRS4 was identified as *Bacillus sp.* MRS4

Figure 1.1: **Plate Culture of Isolate *Bacillus paramycoides* MRS4 in Minimal agar Media**

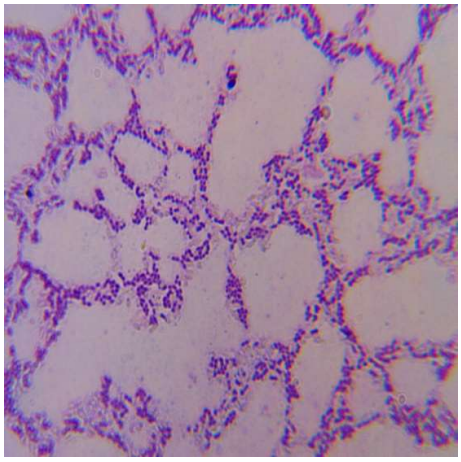


Figure 1.2: Gram-Staining Picture of Isolate *Bacillus paramycoides* MRS4 under Bright Field Microscope

3.3 Identification of the isolated most potent bacterial strain

The isolated bacterial strain MRS4 from fermented soya bean source was found to be aerobic, gram (+) ve and having shape of a rod.On spore staining it showed the presence of sub terminal endospore. The morphological and physiological features of the isolated MRS4 strain wereshown in Table 1.3 and Table 1.4 respectively.

Table 1. 3: Morphological characteristics of the isolate MRS4

Experiment	Observation
i. Morphology of vegetative cells	(a) Cell shape – Rod (b)Cell size – 5.40µm(Length), 2.52µm(Dia)
ii. Arrangement	Single, Double, Multiple
iii. Gram's staining, Spore staining	(+)ve
iv. Growth in Nutrient agar medium in petri plate Colony characteristics : Opacity – Surface growth Edge Consistency Color Pigmentation	 Size – 1.0 – 1.0 mm (Dia) Opaque smooth Entire Good off – white Nil
i. Growth in 50 mL medium taken in 250 mL Erlenmeyer flask	A. Stationary condition (a) After 24 hr : - Poor growth, no ring formation, no pellicle formation, sedimentation at the bottom, upper portion of the broth was clear. (b) After 48 hr : -Same as after 24 hr but the growth was fair.
	B. Shaking condition. (a) Fair growth, turbid, no sedimentation, no pellicle formation, no pigmentation, no ring formation. (b) After 48 hr : - Same as after 24 hr.

Table 1.4: Physiochemical Characteristics of the isolate MRS4

Parameters	Characteristics	
1. Ammonia from Arginine, Arginine used as sole source of energy Nitrate reduction, Catalase reaction, Starch hydrolysis, Growth at anaerobic condition,	Positive	
2. Carbohydrate fermentation	Acidity	Gas formation
(i) Fructose, Glucose, Sucrose, Mannitol, Glycerol	+	-
(ii) Arabinose, Galactose, Xylose, Lactose, Lactose, Raffinose, Maltose, Dextrin, Salicin, Sorbitol, Sorbitol, Inositol	-	-
3. Indole formation, Litmus milk test, Hydrolysis of Urea (Urease test)	Negative	
4. Voges – Proskauer Test (i) pH < 6.0 (ii) pH > 7.0	 Negative Positive	
5. Growth at different temperatures: 5 ^{0} C	Poor growth	
10 ^{0} C	Poor growth	
30 ^{0} C	Vigorous growth	
40 ^{0} C	Vigorous growth	
50 ^{0} C	No Growth	
6. Growth at different NaCl Concentration (i) 2 %	 Vigorous growth	
(ii) 5 %	Vigorous growth	
(iii) 7 %	Poor growth	
(iv) 10 %	Poor growth	
7. Growth at 6.5 % NaCl and pH : - 9.6	Poor growth	
8. Growth at (i) pH – 6.8 (ii) pH – 5.4	 Positive Positive	

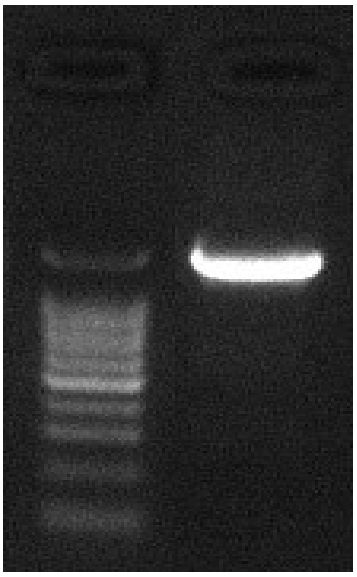
3.4. 16S rDNA sequence and phylogenetic analysis

Thephylogenetic analysis and nucleotide homology were done with 1500 bp 16S rDNA gene arrangement of the isolate strain MRS4 (Gel image of the 16S rDNA amplicon as shown in Figure 1.3) and based on the resultit was found that the isolate *Bacillus sp.* MRS4 strain have a significant similarity with *Bacillus paramycoides*strain MCCC 1A04098 (Gene bank accession no. NR_157734.1). Identity investigation on the EZ taxon server [20] exposed that

the 16S rDNA gene sequence had nearbyresemblance (96.56%) with the gene arrangement of thestrain of *Bacillus paramycoides*refer in Table 1.5. The phylogenetic locationof the isolate R31 have NCBI Genbank accession number NR_157734.1 and it was based on the NJ algorithm.In Figure 1.4 the dendogramhas been shown. Strain MRS4 appeared as a distinguishingphylogenetic line from the cluster that containdifferent strains of *Bacillus*species shown in Table1.5.

Table 1.5: Results of the Identity Analysis of Strain *Bacillus paramycoides* MRS4based on the EzTaxon Server in Relation to the Pairwise similarity with other strains.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_157734.1	***Bacillus paramycoides* strain MCCC 1A04098**	**2446**	**2446**	**99%**	**0.0**	**96.56%**
NR_157729.1	*Bacillus albus* strain MCCC 1A02146	2440	2440	99%	0.0	96.49%
NR_074540.1	*Bacillus cereus* ATCC 14579	2435	2435	99%	0.0	96.42%
NR_115714.1	*Bacillus cereus* strain CCM 2010	2435	2435	99%	0.0	96.42%
NR_114582.1	*Bacillus cereus* ATCC 14579	2435	2435	99%	0.0	96.42%
NR_113266.1	*Bacillus cereus* strain JCM 2152	2433	2433	99%	0.0	96.42%
NR_115526.1	*Bacillus cereus* strain IAM 12605	2433	2433	99%	0.0	96.42%
NR_112630.1	*Bacillus cereus* strain NBRC 15305	2431	2431	99%	0.0	96.42%
NR_157735.1	*Bacillus proteolyticus* strain MCCC 1A00365	2429	2429	99%	0.0	96.36%
NR_152692.1	*Bacillus wiedmannii* strain FSL W8-0169	2423	2423	99%	0.0	96.29%
NR_157728.1	*Bacillus paranthracis* strain MCCC 1A00395	2418	2418	99%	0.0	96.22%
NR_157733.1	*Bacillus pacificus* strain MCCC 1A06182	2412	2412	99%	0.0	96.15%
NR_114581.1	*Bacillus thuringiensis* strain ATCC 10792	2412	2412	99%	0.0	96.15%
NR_113991.1	*Bacillus pseudomycoides* strain NBRC 101232	2410	2410	99%	0.0	96.15%
NR_043403.1	*Bacillus thuringiensis* strain IAM 12077	2410	2410	99%	0.0	96.15%



1 2

Figure 1. 3: Gel Image of 16S rDNA Amplicon of Isolate MRS4
[Lane 1: 100bp DNA Marker and Lane 2: 16S rDNA Amplicon Band]

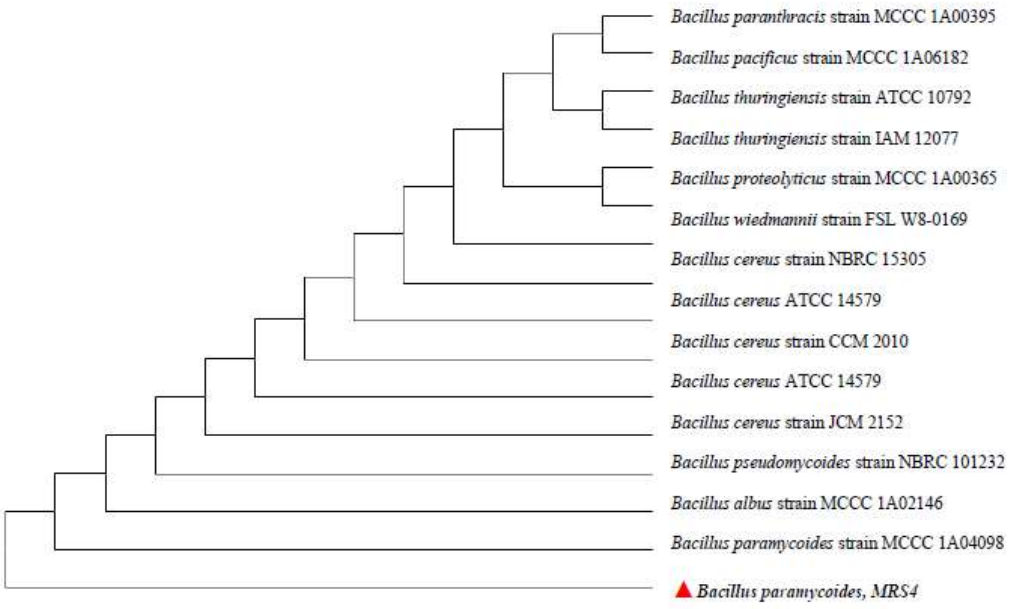


Figure 1. 4: Unrooted Phylogenetic Tree Based on 16S rDNA Sequences obtained by the Neighbor-Joining (NJ) Method Showing the Position of Strain *Bacillus paramycoides* MRS4 among its Phylogenetic Neighbors. NCBI Accession Numbers are provided in Parentheses.

4. Conclusion

In the current study six organisms were isolated from soil, twelve from corn, ten from oat, twelve from soya bean and twenty-one from Potato sample. After screening it was found that the strain marked as MRS4 which was isolated from fermented soya bean was producing the maximum amount of enzyme. The strain MRS4 has maximum similarity with *Bacillus paramycoides* strain MCCC 1A04098 on the basis of nucleotide homology and phylogenetic assay.

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Chapter II

Optimization of process parameters and characterization for maximum enzyme production by Isolated *Bacillus paramycoides* MRS4 in Batch Scale and via Response Surface Methodology (RSM)

1. Introduction

The L-asparaginase enzyme is widely produced by different microorganisms [1], with most commercially available sources being *Escherichia coli*, *Erwinia carotovora*, and *Serratia marcescens*[2-7]. However, the therapeutic enzymes have a problem of toxicity[8], leading to a continuous search for less toxic L-asparaginase enzymes from diverse sources[9].

RSM is a statistical technique used to optimize the response of a system or process by identifying the relationship between the input variables and the output response.

In the context of food and herbal plants extraction, RSM can be used to optimize various extraction parameters such as time, temperature, solvent type, and concentration, among others. By using RSM, researchers can determine the optimal conditions for maximum extraction yield or quality of the target compound.

Central composite design (CCD) and Box-Behnken design are two commonly used designs in RSM that allow for efficient exploration of the response surface and identification of the optimal conditions.

ANOVA is used to analyze the results obtained from the RSM experimental design and determine the significance of the relationship between the variables and the response. This information is then used to develop a mathematical model that describes the relationship between the variables and the response.

The RSM model can be used to predict the response at any given set of input variables, and can also be used to optimize the extraction process by identifying the optimal conditions for maximum yield or quality of the target compound.

Overall, RSM is a powerful tool for optimizing the extraction process in food and herbal plants extraction studies, allowing for efficient exploration of the response surface and identification of the optimal conditions for maximum yield or quality.

2. Methods and material

2.1. Medium preparation and culture mantainance

Selected culture was maintained by making subcultureand stored it in the refrigerated condition. Medium Composition and methods followed were same as described in previous chapter I

2.2. Ezyame Eassy

Methods followed were same as described in previous chapter I

2.3. Optimization of process parameters for maximum enzyme production in batch scale

The optimization study of process parameter i.e. pH, temperature, fermentation period, inoculum percentage, age of inoculum and medium volumefor maximum production of enzyme by the isolated strain was studied. The isolated strain was grown in the same fermentation mediummentioned in methods and materials. Except the medium volume optimization experiment all the other experiments were conducted in 50 mL fermentation medium taken in 250 mL Erlenmeyer flask. The amount of the enzyme produced was measured by the enzyme assay method mentioned also in methods and materials. All the experiments were done in triplicate.

2.3.1. Optimization of pH

The selected bacterial strain was grown in a fermentation medium at varying pH levels (4.0-8.0) at 35°C for 24 hrs. After 24 hrs, the cell mass was separated from the medium by centrifugation and the clear supernatant was used to measure the enzyme activity.

2.3.2. Optimization of Temperature

The isolated organism was grown in the fermentation medium at different temperatures (25-45°C) for 24 hrs, with the pH of the medium maintained at 7.0 throughout the experiment. After 24 hrs, the cell mass was separated from the medium by centrifugation and the clear supernatant was used to measure the enzyme activity.

2.3.3. Optimization of Fermentation period

The most potent bacterial strain was grown in the fermentation medium at 35°C and pH 7.0 for different fermentation times (18, 20, 24, 28, and 32 hrs). After the fermentation was completed, the fermented broth was centrifuged and the clear supernatant was used to measure the enzyme activity.

2.3.4. Optimization of Inoculum percentage

The most potent isolate was grown in the fermentation medium with different percentages of inoculum (1%, 2%, 3%, 4%, and 5%) and the pH of the medium was kept constant at 7.0. The medium was incubated at 35°C for 24 hrs. After the fermentation was complete, the fermented broth was centrifuged and the clear supernatant was used to measure the enzyme activity.

2.3.5. Optimization of Age of Inoculum

The isolated bacterial strain was grown in the fermentation medium and inoculated with 1% inoculum of different ages (16 hrs, 20 hrs, 24 hrs, 28 hrs, and 32 hrs) at 35°C and pH 7. The inoculum was incubated for 24 hrs. After the fermentation was complete, the fermented broth was centrifuged and the clear supernatant was used to measure the enzyme activity.

2.3.6. Optimization of Medium volume

In this experiment, the isolated most potent bacterial strain was grown in different volumes of fermentation medium (40, 50, 60, 70, and 80 mL) and inoculated with 1% inoculum. The incubation conditions were maintained at 35°C and pH 7 for 24 hrs. After the fermentation was complete, the fermented broth was centrifuged to obtain the cell-free supernatant, which was then used for enzymatic assay to determine the Asparaginase activity.

2.4. Determining the characteristics of enzyme produced by *Bacillus paramycoides* MRS4 in batch scale

2.4. 1. Effect of different carbon source in different concentrations in the production of enzyme L-Asparaginase and weight of cell mass production

Carbon source(Sucrose, Glucose, Fructose, Lactose, Maltose, Mannose, Galactose, Glycerol, Starch, Sodium acetate);were introduced into the nutrient medium in different concentration 1%,2% and 3% .After sterilization of the medium 2% culture was inoculated and placed inside the shaker and incubated for 24 hrs at 35◦C. The flasks were then taken out and centrifuged for 10 mins at 4500rpm. The supernatant and the cell mass were collected. The tubes were then placed inside the hot air oven for 24 hrs and the difference was calculated with respect to the dry weights. The absorbance was measured at 396nm.

2.4.2. Effect of different nitrogen source in different concentrations in the production of enzymeL-asparaginase and weight of cell mass production

Nitrogen source (Peptone, Beef extract, Yeast, Malt extract, Tryptone, Urea, Ammonium Chloride, Ammonium sulphate, Ammonium oxalate)were introduced into the nutrient medium in different concentration 1%,2% and 3% .After sterilization of the medium 2% culture was then inoculated and placed inside the shaker and incubated for 24 hrs at 35◦C. The flasks were then taken out and centrifuged for 10 mins at 4500rpm. The supernatant and the cell mass were collected. The tubes were then placed inside the hot air oven for 24 hours and the difference was calculated with respect to the dry weights. The absorbance was measured at 396nm.

2.4.3. Effect of different minerals/metal ions source in different concentrations in the production of enzyme L-Asparaginase and weight of cell mass production

Metal ions/ minerals(Di-Potassium phosphate, Mono-Potassium phosphate, Sodium chloride, Calcium Chloride, Magnesium chloride, Magnesium Sulphate, Disodium Hydrogen Phosphate)were introduced into the nutrient medium in different concentration 1%,2% and 3% .After sterilization of the medium 2% culture was inoculatedand placed inside the shaker and incubated for 24 hrs at 35◦C. The flasks were then taken out and centrifuged for 10 mins at 4500rpm. The supernatant and the cell mass were collected. The tubes were then placed inside the hot air oven for 24 hrs and the difference was calculated with respect to the dry weights. The absorbance was measured at 396nm.

2.5. Determining the nature of L-Asparaginase

Nutrient media was prepared then after sterilization 2% culture was inoculated in two diffetent flask and placed inside the shaker and incubated for 24 hrs. The flasks were then taken out and centrifuged for 10 mins at 4500rpm. After centrifugation **Sample 1**: the medium broth was collected and the absorbance was measured at 396nm, on the other hand **Sample 2:** cell mass was taken and mixed with sand in a mortar press and then transferred into a sterilized nutrient media. It was then placed for 10 mins in a Shaker at 35˚C. Then it was centrifuged and the absorbance was measured at 396nm.

3. Results and Discussion

3.1. Optimization of process parameters

3.1.1. Optimization of pH

The effect of pH shown in Figure 2.1 on the growth and enzyme production of *Bacillus paramycoides* MRS4 strain in fermentation medium. The strain was grown at various pH levels within 4.0 to 8.0 and the results showed that the maximum amount of enzyme was produced at pH 7.0. The results suggest that the optimal growth of the organism was at pH 7.0, leading to maximum enzyme production. At pH levels below or above 7.0, the growth of the organism and enzyme production decreased. The decrease in both cell mass and enzyme production was more pronounced at higher pH levels.

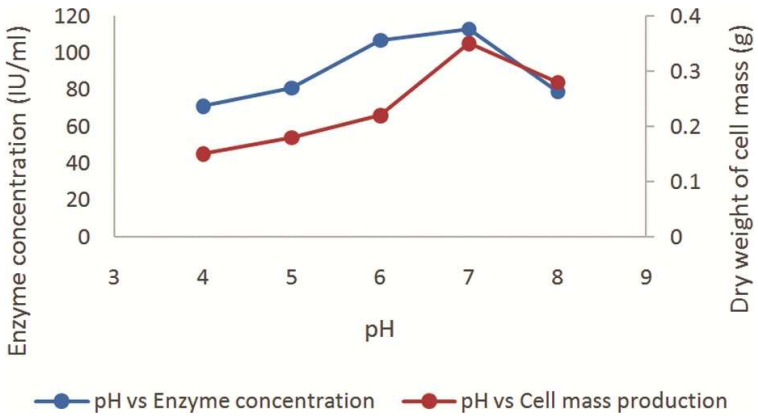


Figure 2.1: Optimization of pH for maximum enzyme production by the isolated strain and cell mass production

3.1.2. Optimization of temperature

The results of the temperature optimization experiment shown in Figure 2.2that the maximum enzyme production by the *Bacillus paramycoides* MRS4 strain was obtained at 35°C, while the pH of the medium was kept constant at 7.0. The maximum enzyme production of 111 IU/mL was achieved at this temperature as the growth of the organism was also maximum at 35°C. At temperatures below or above 35°C, both the growth of the organism and the amount of enzyme produced decreased. asparaginase enzyme production with *Stenotrophomonas maltophilia* variant. With increase in temperature enzyme production increased and maximum [10].

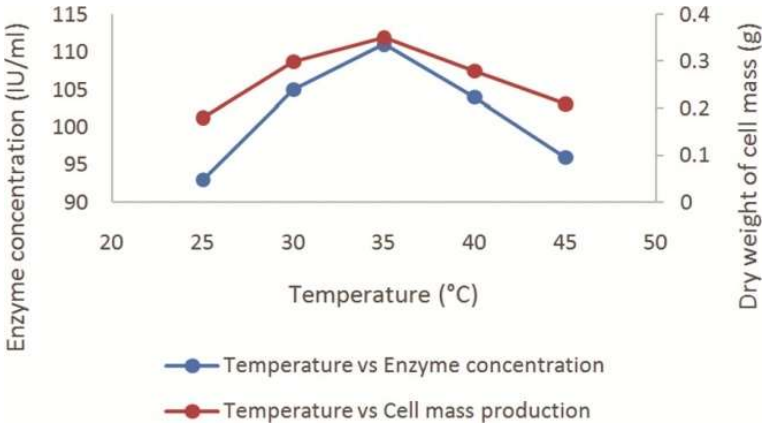


Figure 2.2: Optimization of temperature for maximumenzyme production by the isolated strain and cell mass production

3.1.3. Optimization of fermentation period

For the optimization of fermentation period the isolated *Bacillus paramycoides* MRS4 strain was grown in fermentation medium for different fermentation timei.e. 18, 20, 24, 28 and 32 hrs at 35°C, pH 7.0, agitation speed of 150 rpm, aeration rate of 0.25 L/min. Figure 2.3 showed that both enzyme production and cell mass production was maximum at 24 hrs fermentation time. The reason might be as the growth of the organism was maximum at that time so enzyme produced was also maximum and it was 113 IU/mL. With further increase in fermentation period the organism enters to its death phase so amount of enzyme produced was also decreased. Microbial asparaginase enzyme production reduced beyond optimum fermentation period and probable reason for it was agglomeration of poisonous end products, lack of moisture and pH change of the medium [11].

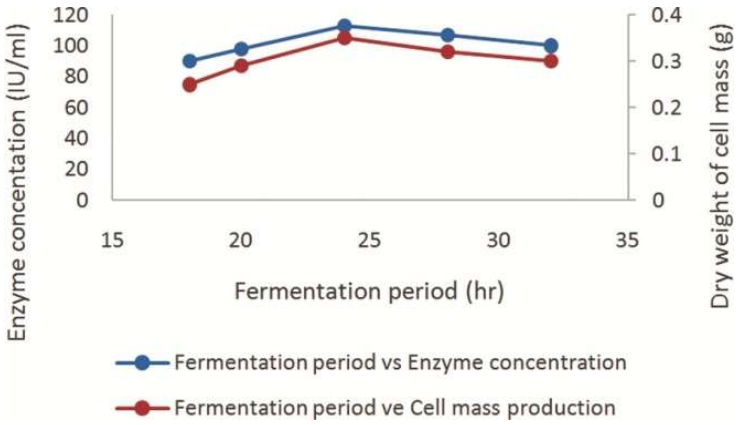


Figure 2.3: Optimization of fermentation time for maximum enzyme production by the isolated strainand cell mass production

3.1.4. Optimization of inoculum percentage

The isolated most potent organism was grown in fermentation medium and the medium was inoculated with different percentage of inoculum within 1 - 5%. The pH of the medium was kept constant at 7.0. The inoculated medium was placed in the incubator at 35°C, agitation speed of 150 rpm, aerationrateof 0.25 L/min for 24 hrs. The amount of enzyme producedwas dependent on the amount of biomass produced. Figure 2.4 showed that enzyme production was maximum 117 IU/mL at inoculum percentage of 1.0% but with further increasein inoculums concentration biomass production was limited due to limiting amoun tof the nutrient present in the medium. The amount of cell mass produced at different inoculums concentration is also shown in Figure 2.4. Similar results showed that asparaginase enzyme production by *Bacillus* sp.WF67 [12]. The enzyme production was increased with the increase in inoculum volume initially and it was maximum at 2 mL (% v/v) inoculum volume but with further increase in inoculums volume enzyme production was reduced.

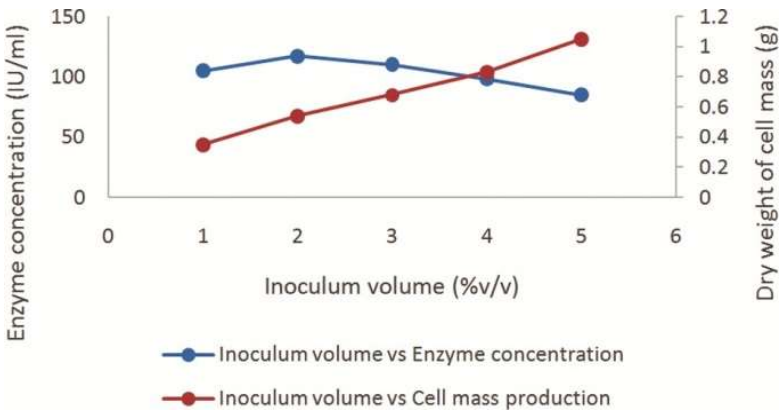


Figure 2.4: Optimization of inoculum percentage for maximum enzyme production by the isolated strain and cell mass production

3.1.5. Optimization of age of inoculum

The isolated bacterial strain was grown in 50 mL fermentation medium and inoculated with 1% inoculum of different age as 16, 20, 24, 28, 32 hrs at35°C and pH 7, agitation speed of 150 rpm, aeration rate of 0.25L/min for 24hr. Figure 2.5 showed that enzyme production increased in itially up to 20hrs inoculums age but after further increasein age of noculum the amount of enzyme produced was reduced. The amount of enzyme produced was related to the amount of biomass produced.The biomass produced was also dependent on the inoculum. So, It might be possible that 20hr inoculums produced maximum amount of cellas found in Figure 2.5. Thus enzyme produced was also maximum for 20hrs inoculumage. During the α-amylaseenzyme production by the bacteria *Bacillus licheniformis* produced maximum enzyme with the inoculums age of 24hrs [13]. They concluded that it might be as the bacteria were in their activestate of growth. With further increase in inoculums age enzyme productivity reduced due to accumulation of secondary and tertiary metabolitesor due to proteolysis.

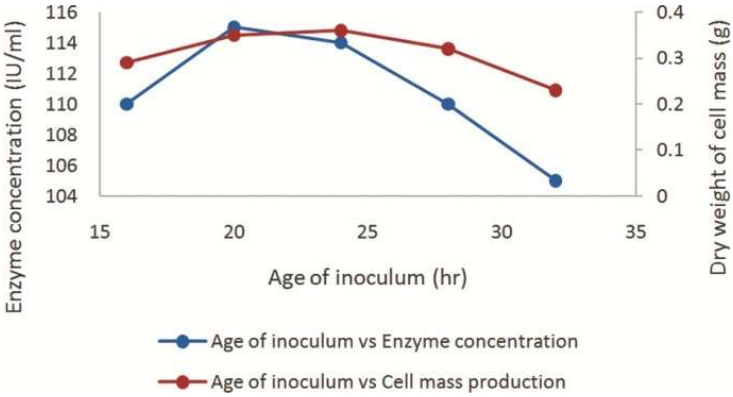


Figure 2.5: Optimization of age of inoculum for maximum enzyme production by the isolated strain and cell mass production

3.1.6. Optimization of medium volume

The selected bacterial strain was inoculated in thefermentation medium of different volume i.e. 40, 50,60, 70 and 80 mL in a 250 mL Erlenmeyer flask and inoculated with 1% inoculum, incubated at 35°C, pH7, agitation speed of 150 rpm, aeration rate of 0.25L/min for 24 hrs. Figure 2.6 showed that production of enzyme by the strain was increased from 40 mL andmaximum production of 117IU/mL was occurred in 50mL medium volume.

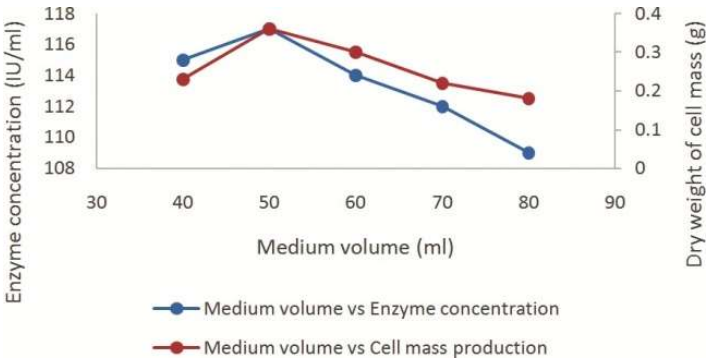


Figure 2.6: Optimization of medium volume for maximum enzyme production by the isolated strain and cell mass production

3.1.7. Optimization of agitation rate

The selected bacterial strain was cultivated in thefermentation medium with 1% inoculum at differentagitation rate i.e. 100, 125, 150, 175 and 200 rpm andkept in incubator at 35°C, pH 7, aeration rate of 0.25L/min for 24 hrs. Figure 2.7 showed that maximum 118IU/mL of

enzyme was produced at agitation rate of 150rpm.Agitation helps in cellmass production. Thestudy also supports that with increase in agitation ratecell mass production increased but too much agitation will hamper cell mass production. So after 150 rpm ofagitation rate cell mass production was decreased so enzyme production was also reduced. Asparaginase enzyme production by *Stenotrophomonas maltophilia* variant increased in itially with increaseinag it at ion rate and it was maximum at 180 rpm but enzyme production reduced with further increase in agitation rate [10].

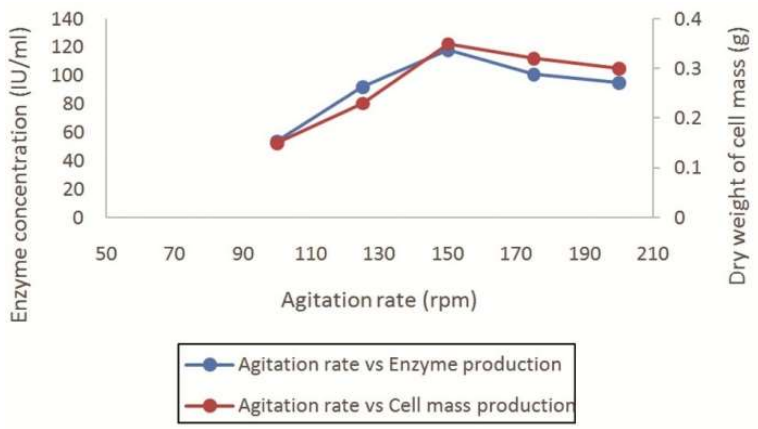


Figure 2.7: Optimization of agitation rate for maximum enzyme production by the isolated strain and cell mass production

3. 1.8. Optimization of aeration rate

The isolated most potent bacterial strain was cultivated in the fermentation medium with 1% inoculums at different aeration rate i.e. 0.15, 0.25, 0.35, 0.45, 0.55 L/min and kept in incubator at 35°C, pH 7, agitation speed of 150 rpm for 24hrs. Figure 2.8 showed that at beginning enzyme production increased with increase in aeration rate and it was maximum at 0.25 L/min but too much aeration can reduce cell mass production. From this study it was clear that cell growth was reduced with further increase in aeration rate as a result enzyme production was also reduced. During α-amylase enzyme production by *Bacillus licheniformis* AZ2 the enzyme and cell mass production both increased with initial increase in aeration rate up to optimum level. With further increase in aeration rate both enzyme and cell mass production reduced [14].

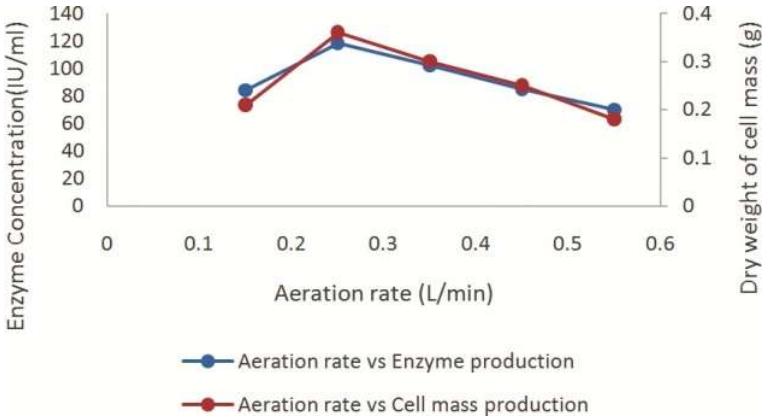


Figure 2.8: Optimization of aeration rate for maximum enzyme production by the isolated strain and cell mass production.

3.2. Determining the characteristics of enzyme produced by *Bacillus paramycoides* MRS4 in batch scale

3.2.1. Effect of different carbon source in different concentrations in the production of enzyme L-Asparaginase and weight of cell mass production

It was found that among all carbon Source 3% of glucose produce maximum enzyme production about 112.76 IU/mL shown in Figure 2.9 and it also produce maximum weight of cell mass about 0.171gm shown in Figure 2.10

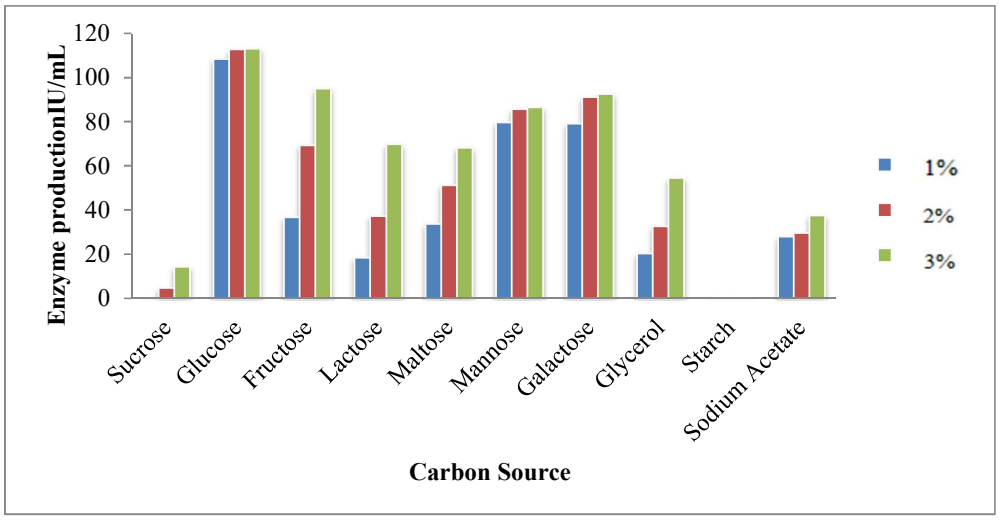


Figure 2.9: Effect of different carbon source in different concentrations in the production of enzyme L- Asparaginase by *Bacillus paramycoides* MRS4

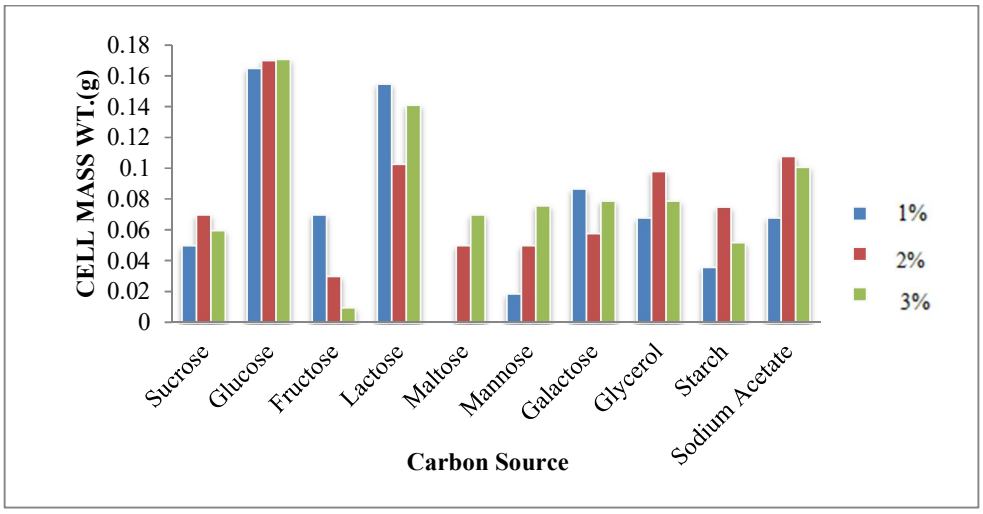


Figure 2.10 : Effect of different carbon source in different concentrations in the production of cell mass

3.2.2. Effect of different nitrogen source in different concentrations in the production of enzyme L-asparaginase and weight of cell mass production

It was found that among all nitrogen Source 3% and 2% of peptone produce maximum enzyme production about 108.56 IU/mL shown in Figure 2.11 and maximum weight of cell mass about 0.343gm shown in Figure 2.12 respectively.

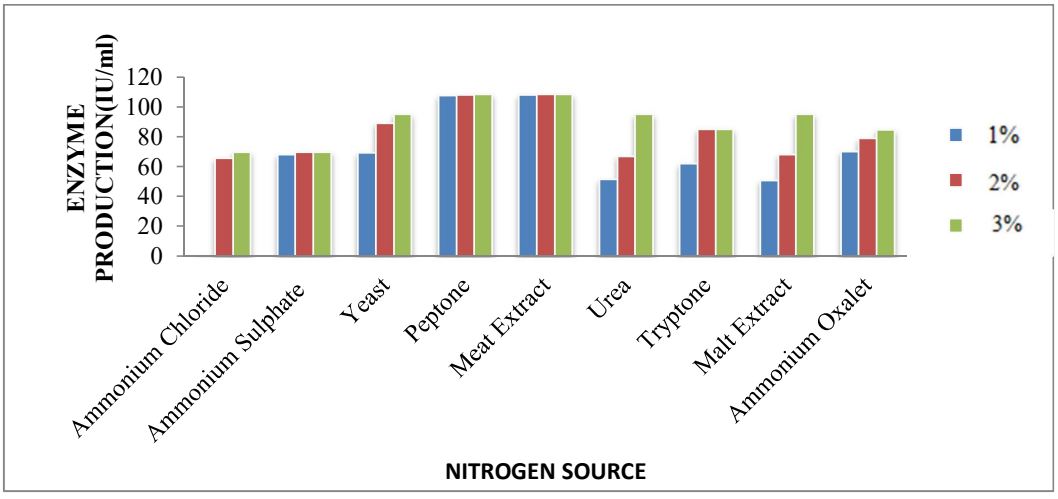


Figure 2.11: Effect of different nitrogen source in different concentrations in the production of enzyme L- Asparaginase by *Bacillus paramycoides* MRS4

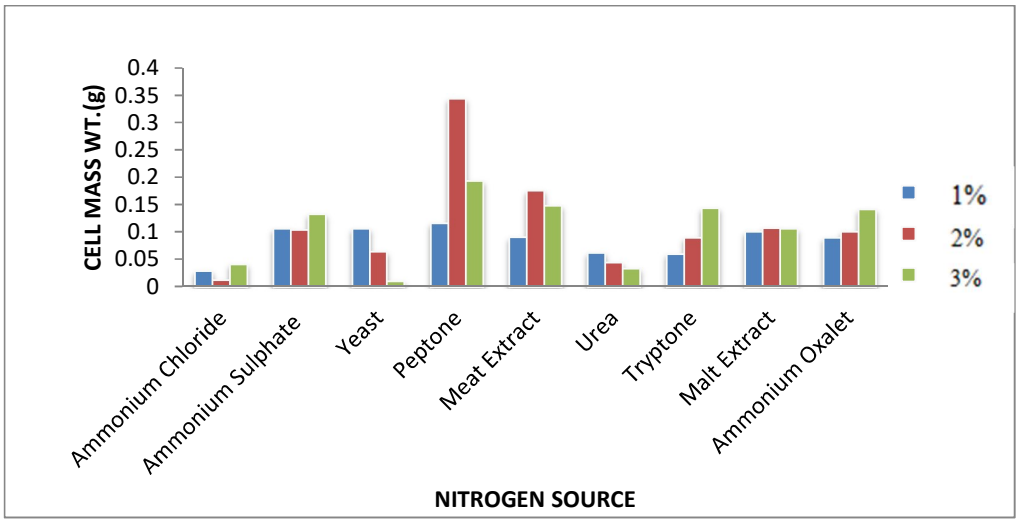


Figure 2.12: Effect of different nitrogen source in different concentrations in the production of cell mass

3.2.3. Effect of different minerals/metal ions source in different concentrations in the production of enzyme L-Asparaginase and weight of cell mass production

It was found that among all minerals/metal ions source 3% of Di-Potassium phosphate produce maximum enzyme production about 103.9 IU/mL shown in Figure 2.13 and maximum cell mass about 0.18gm shown in Figure 2.14.

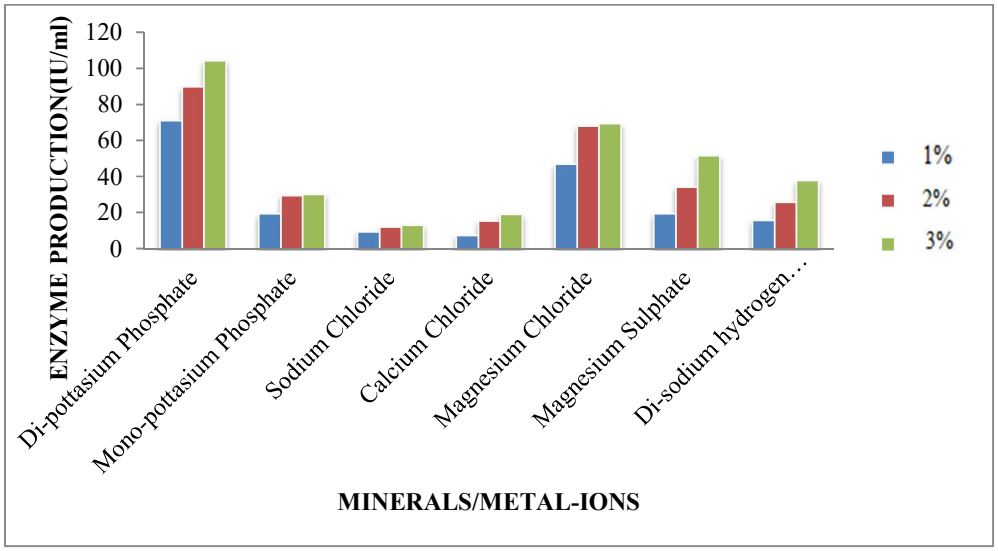


Figure 2.13: Effect of different minerals/metal ions source in different concentrations in the production of enzyme L-Asparaginaseby *Bacillus paramycoides* MRS4

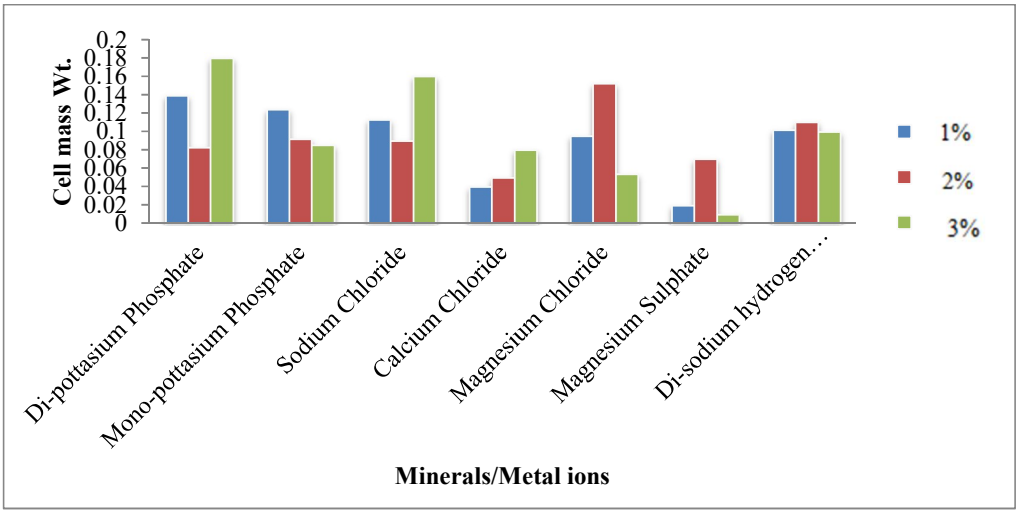


Figure 2.14: Effect of different minerals/metal ions source in different concentrations in the production of cell mass.

3.3. Determining the nature of L-Asparaginase production by isolated *Bacillus paramycoides* MRS4

Sample 1: The absorbance was measured which produce 110 IU/ml enzyme.

Sample 2: The absorbance was zero.

The enzyme was extra cellular.

4. Response Surface Methodology (RSM)

4.1. Materials and Methods

4.1.1. Design Experiment

The use of CCD second order design and Design-Expert software13.0.5.0 in this experimental design provides a comprehensive and efficient method for understanding the relationships between the factors and the response, and for optimizing the response. Five different independent variables as pH (X_1): 6-8; Temperature (X_2):25°C -45°C; Fermentation Time (X_3): 15-35hrs; Inoculum % (X_4): 1-3%; Medium Volume (X_5):40-60mL was coded at five levels between -1 and +1 based on preliminary studies as shown in **Table 2**. In this experimental design, the use of five factors and five replicates at the design center is a common approach to increase the precision of the estimation of the pure error. The pure error represents the measurement variability that is not explained by the factors, and it is an important component of the total variability in the response. By including replicates at the

design center, the researchers can obtain a more accurate estimate of the pure error and better understand the sources of variability in the response.

The randomization of the order of the experiments is a good practice to reduce the risk of systematic effects and to ensure that the results are not biased.

Quadratic models are commonly used in response surface methodology to describe the relationships between the factors and the response. These models take into account the linear and second-order effects of the factors, and allow for a more accurate representation of the complex relationships between the factors and the response. By fitting a quadratic model, the researchers can determine the optimal factor levels for the response of interest, and make predictions about the response for new sets of factor levels.

Table 2.1: Four-factor, five level CCD including coded value.

Name	Units	Low	High	-1	+1	Coded value
pH		6	8	6	8	X1
Temperature	°C	25	45	25	45	X2
Fermentation Time	hr	15	35	15	35	X3
Inoculum	%	1	3	1	3	X4
Medium Volume	mL	40	60	40	60	X5

The behaviour of the process is explained by the following quadraticequation

$$Z=\alpha_0+\sum_{a=1a=1a=1}\alpha_a y_a+\sum_{b=1}\alpha_{aa} y_b^2+\sum\sum\alpha_{ab} y_a y_b+£$$

where Z represents the process response or output, and the independent variables (factors) are represented by the index numbers a and b. The model used to describe the relationships between the factors and the response is a polynomial equation, which includes various terms that represent the main effects and interactions of the factors.

The intercept term, α_0, represents the free or offset term, and represents the response when all factors are at their average values. The first-order (linear) main effect, α_b, represents the effect of each factor on the response on a linear scale. The quadratic (squared) effect, α_{aa}, represents the effect of each factor on the response on a non-linear scale. The interaction effect, α_{ab}, represents the combined effect of two or more factors on the response.

The random error, £, represents the difference between the predicted and measured values of the response [in Table 1]. This error is an important component of the total variability in the response, and it represents the measurement variability that is not explained by the factors.

Analysis of Variance (ANOVA) is a statistical technique used to determine the significance of the various terms in the polynomial model, and to assess the interaction between the factors and the response. The quality of the fit of the polynomial model is expressed by the coefficient of determination, R^2, which represents the proportion of the total variance in the response that is explained by the model. The adjusted R^2 is a modified version of R^2 that takes into account the number of parameters in the model and the sample size.

The statistical significance of the model is tested by the F-test, which compares the explained variance in the model to the unexplained variance in the response. The desired goals in this study are to maximize the production of enzyme, and the experimental design is focused on understanding the relationships between the factors and the response, and on finding the optimal factor levels for maximizing the production of enzyme.

The use of 3D surfaces and 2D contour plots in this experimental design provides a visual representation of the relationships between the factors and the response, and helps to understand the effects of changes in the factor levels on the response. The experiments were carried out to validate the statistical models and to determine the optimal factor levels for maximum production of enzyme.

4.2. Result and discussion

4.2.1. Model fitting and ANOVA

50 different no of experiments set were conducted by design matrix and the results such as Production of enzyme (Y) was measured and given in Table 1. Second-order polynomial equations were linear and fitted to the experimental result to obtain the regression equations.

The sequential model sum of squares and model summary statistics were used to determine the best fit model for the production of enzyme. The model summary statistics provide information about the statistical significance of the regression coefficients and the goodness of fit of the model. The F-test is often used to test the significance of the coefficients, and the R^2 and adjusted R^2 values are used to measure the goodness of fit of the model.

The sequential model sum of squares is a method for comparing different regression models by comparing their residual sum of squares (RSS). The goal is to find the model that provides the best fit to the data while still being parsimonious and avoiding overfitting.

By comparing the results of the sequential model sum of squares and model summary statistics, the best fit model can be chosen based on its statistical significance, goodness of fit, and parsimony. In this case, the results presented in Table 2.3 .The quadratic model provides the best fit to the data based on the F-value 58.74, predicted R^2 value 0.9162 and adjusted R^2 value 0.9593.

This equation can described as following:

$$\mathbf{Y=113.31}-0.4556X_1-2.96X_2+2.22X_3+4.28X_4+4.25X_5+0.3153X_1X_2-0.1491X_1X_3-1.91X_1X_4+2.21X_1X_5+1.03X_2X_3+0.1091X_2X_4+0.4516X_2X_5+1.92X_3X_4+1.47X_3X_5-0.5884X_4X_5-23.34X_1^2-14.17X_2^2-16.88X_3^2-21.88X_4^2-0.8645X_5^2$$

Table 2.2: Five factors, five level CCD observed, predicted and residual of enzyme production

Run	Independent coded variables					Enzyme production		
	X_1	X_2	X_3	X_4	X_5	Observed	Predicted	Error
1	6	25	15	3	60	31.51	40.27	-8.76
2	8	25	15	3	40	42.35	35.72	6.63
3	7	35	25	2	50	118	113.31	4.69
4	7	35	25	2	50	118	113.31	4.69
5	8	25	15	1	60	48.26	40.11	8.15
6	6	45	35	3	60	50.21	50.34	-0.1285
7	7	35	25	3	50	19.24	95.71	-0.4682
8	7	35	25	2	50	118	113.31	4.69
9	8	25	15	1	40	30.35	33.86	-3.51
10	8	25	15	3	40	42.35	34.7	-8.17
11	6	25	15	3	40	54.21	45.2	9.01
12	6	35	25	2	50	82.31	90.43	-8.12
13	8	45	35	3	40	38.52	30.77	7.75
14	7	35	25	2	40	105.26	108.2	-2.94
15	8	25	15	3	60	34.21	39.61	-5.4
16	6	25	35	1	60	42.35	38.9	3.45
17	6	45	15	1	40	24.52	25.96	-1.44
18	6	45	15	1	60	28.26	25.19	3.32
19	7	35	25	1	50	78.24	87.15	-8.91
20	6	45	15	3	60	32.52	32.79	-0.2676
21	8	35	25	2	50	88.25	89.51	-1.26

22	7	35	25	2	50	118	113.31	4.69
23	8	45	34	3	60	51.21	50.34	0.8964
24	8	45	35	1	40	24.56	20.82	3.74
25	6	25	35	3	40	38.54	44.78	-6.24
26	6	25	35	3	60	62.51	53.72	8.79
27	8	25	35	1	60	38.52	45.3	-6.78
28	7	35	25	2	60	110.25	116.69	-6.44
29	7	35	25	2	50	118	113.31	4.69
30	6	45	35	3	40	32.54	39.59	-7.05
31	8	45	15	1	60	38.65	33.45	6.2
32	6	45	35	1	60	34.21	35.08	-0.8702
33	8	45	35	1	60	36.21	4274	-6.53
34	6	45	15	3	40	42.51	35.91	6.6
35	7	45	25	2	50	9.25	96.18	-5.93
36	8	45	15	1	40	22.54	25.4	-2.86
37	7	25	25	2	50	98.65	102.1	-3.45
38	8	45	15	3	40	18.52	27.69	-9.17
39	7	35	25	2	50	118	113.31	4.69
40	8	45	15	3	60	35.62	33.39	2.23
41	7	35	35	2	50	95.24	98.65	-2.41
42	8	25	35	1	40	30.52	25.18	5.34
43	8	25	34	3	60	56.21	52.46	3.75
44	7	35	25	2	50	118	113.31	4.69
45	6	24	14	1	40	32.54	35.69	-3.15
46	7	35	25	2	50	118	113.31	4.69
47	6	25	15	1	60	32.51	33.11	-0.6022
48	6	45	35	1	40	26.51	2198	4.53
49	6	25	35	1	40	28.54	27.6	0.9358
50	7	35	15	2	50	88.24	94.21	-5.97

The analysis of variance (ANOVA) results in Table 2.4 provide important information about the regression model for the production of enzyme. The F-value of 58.74 indicates that the model is statistically significant and that the independent variables have a significant effect on the production of enzyme.

The Adeq Precision value of 20.984 indicates that there is an adequate signal-to-noise ratio in the model, meaning that the signal (production of enzyme) is strong relative to the noise (random error). A value greater than 4 is desirable for this ratio, so the high Adeq Precision value indicates that the model is reliable for predicting the production of enzyme.

The ANOVA results in Table 2.4 provide support for using the quadratic model for the production of enzyme, as it accurately describes the relationship between the independent

variables and the response variable and can be used to navigate the design space. The results of the ANOVA test provide valuable information for further optimization of the process, as it helps identify the most important factors that affect the production of enzyme and the nature of the relationship between these factors and the response variable.

Table 2: 3: Reliability and Usefulness of the Models Tested for the Production of Enzyme

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	1.852E+05	1	1.852E+05			
Linear vs Mean	1708.14	5	341.63	0.2586	0.9331	
2FI vs Linear	830.83	10	83.08	0.0493	1.0000	
Quadratic vs 2FI	55850.76	5	11170.15	224.74	< 0.0001	Suggested
Cubic vs Quadratic	863.72	15	57.58	1.40	0.2694	Aliased
Residual	577.67	14	41.26			
Total	2.450E+05	50	4900.51			

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	36.35	0.0285	-0.0818	-0.1631	69587.39	
2FI	41.05	0.0424	-0.3800	-0.8956	1.134E+05	
Quadratic	7.05	0.9759	0.9593	0.9162	5010.97	Suggested
Cubic	6.42	0.9903	0.9662	-0.2975	77628.02	Aliased

Table 2.4: ANNOVA of the second order polynomial equation for enzyme production:

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	58389.73	20	2919.49	58.74	< 0.0001	significant
X_1-pH	7.06	1	7.06	0.1420	0.7091	Insignificant
X_2-Temperature	298.07	1	298.07	6.00	0.0206	Insignificant
X_3-Fermentation Time	167.17	1	167.17	3.36	0.0769	Insignificant
X_4-Inoculum	622.23	1	622.23	12.52	0.0014	significant
X_5-Medium Volume	613.62	1	613.62	12.35	0.0015	significant
X_1 X_2	3.18	1	3.18	0.0640	0.8021	Insignificant
X_1 X_3	0.7110	1	0.7110	0.0143	0.9056	Insignificant
X_1 X_4	117.31	1	117.31	2.36	0.1353	Insignificant
X_1 X_5	155.81	1	155.81	3.13	0.0872	Insignificant
X_2 X_3	33.68	1	33.68	0.6777	0.4171	Insignificant
X_2 X_4	0.3806	1	0.3806	0.0077	0.9309	Insignificant
X_2 X_5	6.53	1	6.53	0.1313	0.7197	Insignificant
X_3 X_4	117.47	1	117.47	2.36	0.1351	Insignificant
X_3 X_5	384.68	1	384.68	7.74	0.0094	Significant
X_4 X_5	11.08	1	11.08	0.2229	0.6404	Insignificant
X_1^2	1347.31	1	1347.31	27.11	< 0.0001	significant
X_2^2	496.59	1	496.59	9.99	0.0037	significant
X_3^2	704.70	1	704.70	14.18	0.0008	significant
X_4^2	1184.02	1	1184.02	23.82	< 0.0001	significant
X_5^2	1.85	1	1.85	0.0372	0.8484	Insignificant
Residual	1441.39	29	49.70			

Lack of Fit	1441.39	22	65.52			
Pure Error	0.0000	7	0.0000			
Cor Total	59831.12	49				

Negative coefficient for the model X1 , X2,X3, X1 X2,X1 X3,X1 X4,X1 X5,X2 X3,X2 X4,X2 X5,X3 X4,X3 X5 indicated unfavourable effects on enzyme production. Whereas, positive coefficients for X4,X5, $X1^2$,$X2^2$,$X3^2$,$X4^2$ indicate favourable effects on the enzyme production. All terms with positive coefficients greater than zero,so all the terms having impact output variable.

The ANOVA analysis and the calculation of the Fisher's exact test provide important information about the statistical significance of the model and its coefficients as shown in Equations (2). A high F-value of 224.74 for the model and a low p-value indicate that the model is statistically significant and that the coefficients of the independent variables have a significant effect on the response variable (production of enzyme in this case).

Additionally, the calculation of the 95% confidence interval (CI) helps determine the range of values within which the true population parameter is likely to fall, with a 95% degree of certainty. A narrow CI implies a high degree of precision in the estimates of the parameters, while a wide CI indicates that the estimates are less precise.

The application of ANOVA with 95% CI provides a comprehensive evaluation of the statistical significance of the model and its coefficients, and helps ensure the validity and reliability of the results.

The p-value is used to determine the statistical significance of the model terms, and a low p-value (less than 0.05) indicates that the term is statistically significant and contributes to the explanation of the variation in the response variable. On the other hand, a high p-value (greater than 0.10) indicates that the term is not statistically significant, and has little effect on the response variable.

In the case of $X1^2$ and $X4^2$, if the quadratic effect is not significant, it means that the optimal levels of these parameters are likely to be found in the extremes of the experimental region. This suggests that the response variable is not significantly affected by the change in these parameters within the intermediate levels, but that the response may change significantly as the parameters approach the extreme levels.

The coefficient of determination (R^2) and the adjusted R2 are two measures of how well the model fits the data. The R^2 value ranges from 0 to 1, with a higher value indicating a better fit of the model to the data. An R^2 value of 0.9593 for the production of enzyme suggests that the model explains approximately 96% of the variability in the response. The adjusted R^2 takes into account the number of predictor variables and adjusts the R^2 value accordingly. A high adjusted R^2 value indicates a good fit of the model to the data, even when the number of predictor variables is high.

The lack of fit p-value is greater than 0.05, which indicates that the model fits the data well and there is no significant lack of fit. The results suggest that the quadratic model accurately represents the relationship between the independent variables and the dependent variable, the production of enzyme. The results obtained from the analysis provide a useful tool for process optimization and for developing a predictive model for the production of enzyme was found to 20.984.

A normal probability plot was also performed to check the normality of residuals. If the residuals are normally distributed, the plot should form a straight line, which means that the quadratic models are normally distributed in Figure 2.15. If the residuals are not normally distributed, the plot should not form a straight line, and the models may need to be improved or transformed. Hence, the normality assumption was confirmed by the normal probability plot.

The quadratic model for the production of enzyme was determined to be the best-fit model based on the statistical analysis and the results of the model evaluation. The F-value of 58.74, high R2 and adjusted R2 values of 0.9593 and 0.9162, respectively, and adequate precision value of 20.984 showed in Figure 2.16 that the model was significant and could be used to navigate the CCD design space. Additionally, the normal distribution of the residuals and the constant variance of the data confirmed the validity of the model predictions [11].

The correlation between the observed and predicted values showed in Figure 2.17 for a good agreement, which indicated that the quadratic model predictions for production of enzyme were satisfactory.

Actual and Predicted Enzyme Production:

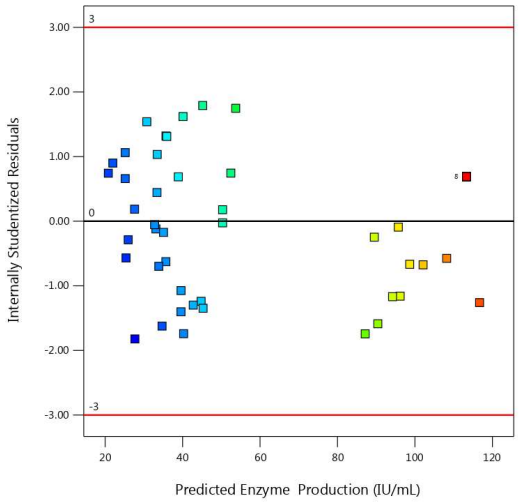


Figure 2.15: Internally studentized residuals versus for production of enzyme

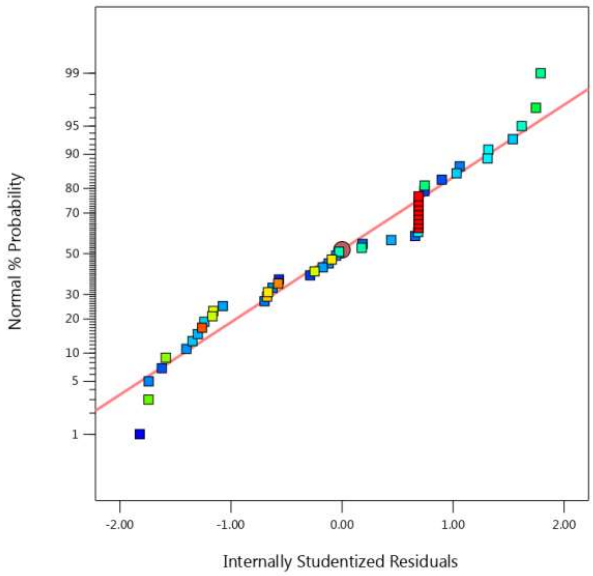


Figure 2.16: Internally studentized residuals versus normal probability for production of enzyme

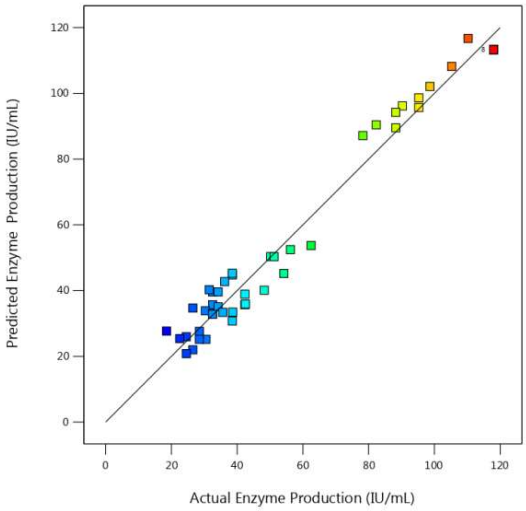


Figure 2.17: Observed experimental data versus predicted values for production of enzyme

3.2. Effects of Model Parameters by both Individual Interaction and Cross-Factor Interaction

The results of a statistical analysis show that all five independent variables, influent pH (X_1), temperature (X_2), fermentation time (X_3), inoculum (X_4), and medium volume (X_5), have a significant effect on the response of enzyme production. This was concluded based on the fact that the p-values for each of these factors were less than 0.05. In addition, some of the cross-factor and square term interactions of the model parameters, such as the interaction between fermentation time and medium volume (X_3X_5), and the square terms of X_1^2 X_2^2, X_3^2, and X_4^2, were found to be significant as well, as their p-values were also less than 0.05 shown in **Figure 2.20 (b)**.

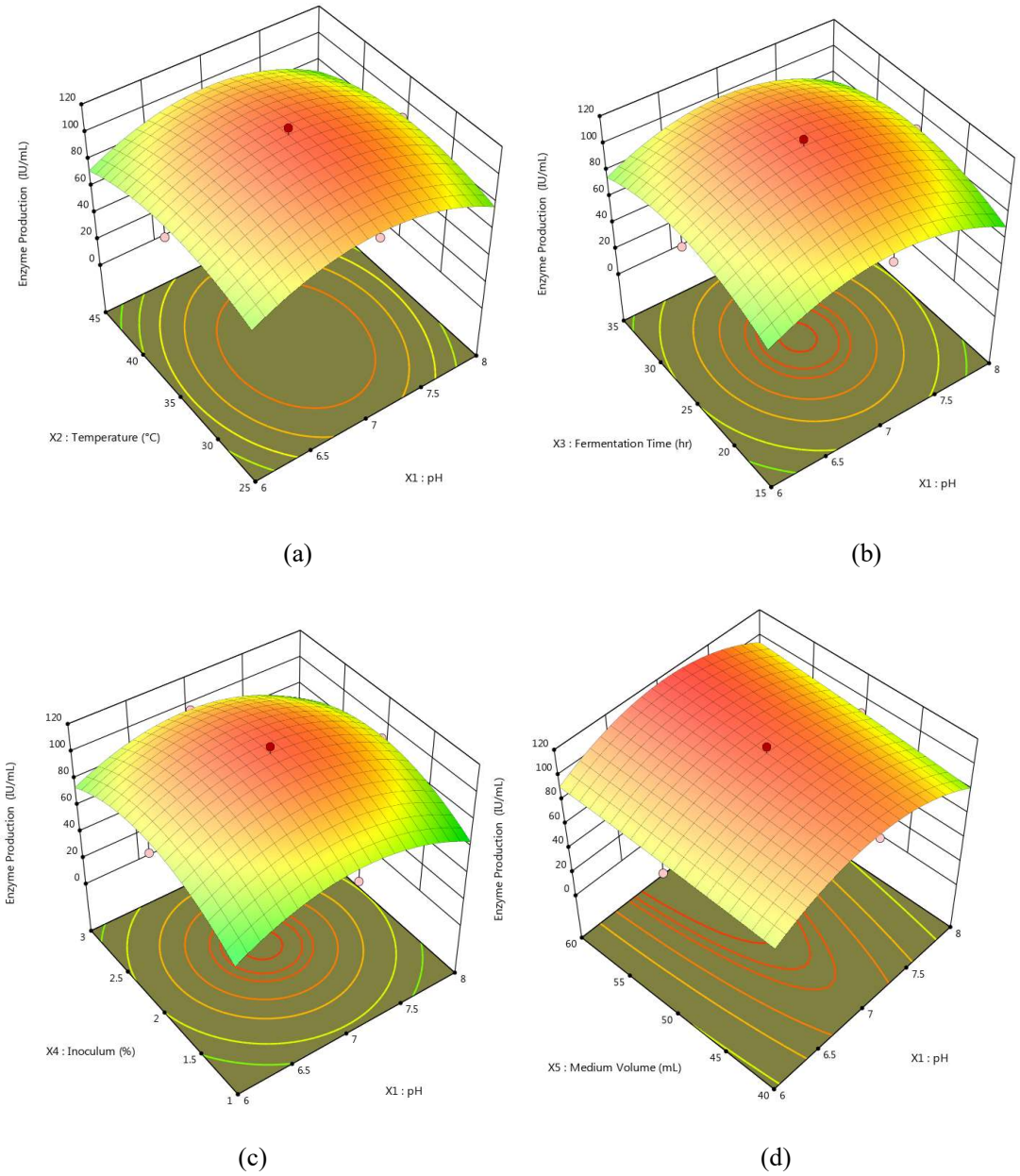
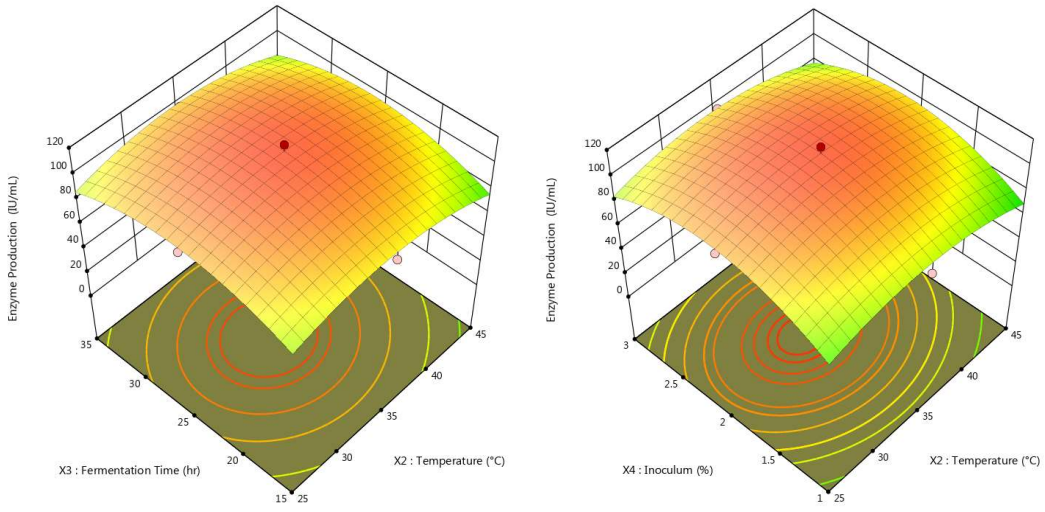
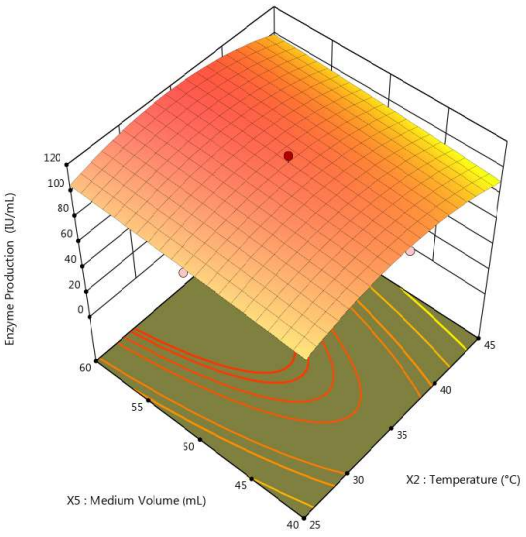


Figure 2.18:3D surfaces and 2D plots of the interaction effects of (a) pH (X_1) &Temperature (X_2),(b) pH (X_1) &Fermentation Time (X_3), (c) pH (X_1) &Inoculum (X_4),(d) pH (X_1) & Medium Volume (X_5).



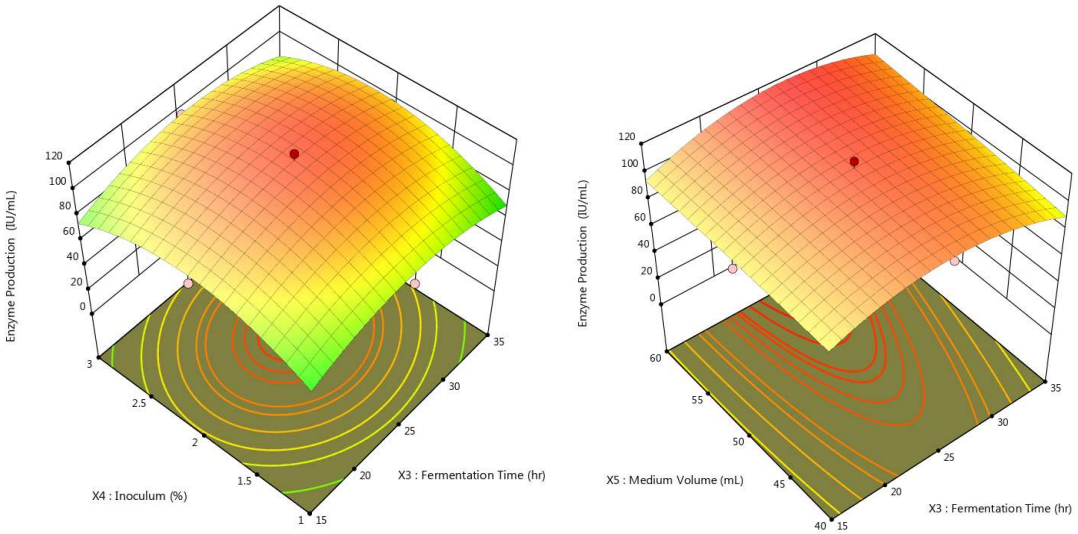
(a)

(b)



(c)

Figure 2.19: 3D surfaces and 2D plots of the interaction effects of (a) Temperature (X_2) &Fermentation Time (X_3), (b) Temperature (X_2) &Inoculum (X_4),(c) Temperature (X_2) &Medium Volume (X_5).



(a) (b)

Figure 2.20:3D surfaces and 2D plots of the interaction effects of (a) Fermentation Time (X_3) &Inoculum (X_4),(b) Fermentation Time (X_3) &Medium Volume (X_5).

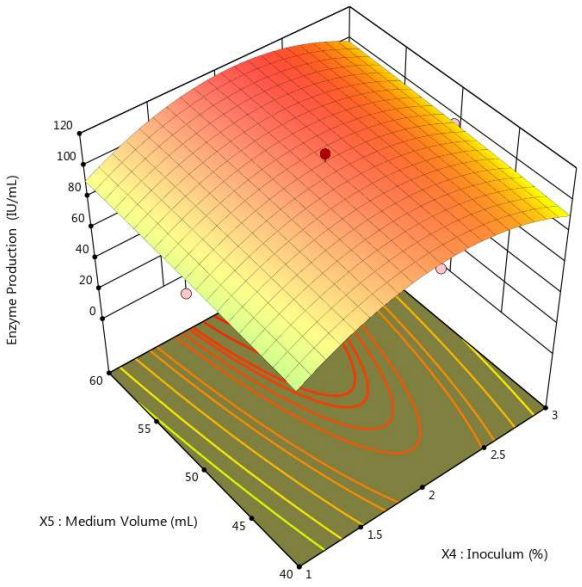


Figure 2.21: 3D surfaces and 2D plots of the interaction effects of Inoculum % (X_4) & Medium Volume (X_5).

5. Conclusion

Optimization of various process parameters were made to find different fermentation conditions to produce the enzyme by the isolated strain in maximum amount. It was found after process optimization that the strain *Bacillus paramycoides* MRS4 was able to produce almost 118 IU/mL of enzyme in the selected medium with 2% inoculum of 25 hr time in 50mL medium taken in a 250 mL Erlenmeyer flask and incubated at 35 °C, pH 7 for 24 hrs in 150rpm and 0.25 L/min in aeration rate. Effect of different carbon, nitrogen and minerals/metal ions in different concentrations for production of enzyme of L- Asparaginase by *Bacillus paramycoides* MRS4 is shown and the result was among all carbon Source 3% of glucose produce maximum enzyme production about112.76IU/mL , among all nitrogen Source 3% of peptone produce maximum enzyme production about 108.56 IU/mL and among all minerals/metal ions source 3% of Di-Potassium phosphate produce activity of enzyme was maximum 71.17 IU/mL.So, using these parameters the enzyme can be produced in laboratory fermenter and then purified. Properties of purified enzyme will be studied further to find its application either as an antitumor or an acrylamide mitigationagent. The interaction effects of the influent pH (X_1), Temperature (X_2), Fermentation Time (X_3), Inoculum (X_4), Medium Volume (X_5) had a significant effect on% of enzyme production. Optimum conditions were found for each variable to achieve maximum production of enzyme. The mathematical models developed in this study have thoroughly investigated the interactive effects of the independent variables on the response of enzyme production. The models, which explain the percentage of enzyme production in a batch production system, are considered to be useful for further research in the areas of reactor design, modeling, and scaling up of the production process. These models can provide valuable insights into the relationship between the independent variables and the response, which can be used to optimize the enzyme production process and improve its efficiency.

6. References

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Chapter III

Statistical optimization of process parameters by Central Composite Design (CCD) in laboratory scale fermenter for maximum enzyme production of L- Asparaginase produced by *Bacillus paramycoides* MRS4 MCCC 1A04098

1. Introduction

Acrylamide is acarcinogenic chemical [1] that has been linked to an increased riskof certain types of cancer such as ovarian, endometrial, breast, and kidney cancer [2-3] . It is formed in food during baking and frying processes and is related to the Maillard reaction [4-8] and asparagine. To reduce the formation of acrylamide, longer baking times [9-10]and adjustments to ingredients, such as substituting ammonium bicarbonate with inverted sugar or adding inorganic salts and organic acids, can be used[11-12]. However, these changes may also impact the flavor, texture, browning [13] and appearance of the food [14], which may result in decreased consumer acceptance. Additionally, the changes may affect the technology of the process [15].

It has been found that asparagine is a major contributor to acrylamide formation in cereal products. High levels of asparagine in cereals can result in high acrylamide levels. To reduce acrylamide in baked and fried products, the amount of free asparagine can be reduced using the enzyme asparaginase, which breaks down asparagine into aspartic acid and ammonia. Research has shown that treatment with asparaginase can reduce acrylamide levels by over 90% in products such as mashed potatoes, potato flakes, rye flour, and wheat flour [8,11].

Fermenters with controlled culture conditions, such as temperature, pH, air flow, agitation, and medium volume, are necessary for large-scale expression of cells. Different types of fermenters may be required depending on the specific cell culture being used. [16]
Microorganisms are used in industry to produce a range of chemical compounds, enzymes, and drugs because they have the ability to produce large quantities of desired substances through efficient and controlled metabolic processes. Microbes are also capable of synthesizing complex compounds that are difficult to produce using chemical methods as follows -

- Microorganisms are small and can be easily grown in large quantities in fermenters, making them economical for industrial-scale production. They can be cultured in either solid or liquid media, depending on the type of microbe and the desired product. The controlled environment in fermenters allows for precise manipulation of growth conditions, including temperature, pH, oxygen levels, and nutrient availability, to optimize production yields.

- They can carry out complex biochemical reactions, like the production of specific enzymes and chemicals, which can be hard to synthesize through other means.
- They are able to grow and produce product in controlled conditions, leading to high quality and consistency of the end product.
- They can be manipulated genetically to optimize the production process and increase yields.
- Microorganisms can be genetically modified by techniques such as recombinant DNA technology or CRISPR-Cas9, which allows for the introduction of new genes or modification of existing genes, resulting in the production of non-natural products of interest, such as enzymes, drugs, and chemicals. This makes them a valuable tool in the industrial production of a variety of compounds.
- They can be easily manipulated and controlled to optimize production conditions.

Having controlled culture conditions in a fermenter is important for maximizing the production of a desired enzyme. The temperature, pH value, air flow, and agitation all play a role in the growth and expression of the microorganisms and adjusting these conditions can optimize the production yield. Additionally, having a consistent medium volume is important for ensuring uniform growth and consistent production of the enzyme.

Figure 3.1: Laboratory scale fermenter

RSM is a statistical approach to optimize the extraction process by examining the relationship between multiple variables such as Aeration, Agitation, and Medium volume. It involves conducting experiments using central composite design or Box-Behnken method to create a mathematical model that describes the response of the extraction process to changes in these variables. The model is then evaluated using analysis of variance (ANOVA) to determine its accuracy. By optimizing these variables, RSM helps to identify the best conditions for extraction and can be used to predict the response under different conditions. This can lead to improved efficiency and yield of the extraction process.

2. Meterials and Methods

2.1. Organism preparation

Selected culture was maintained by making subculture and stored it in the refrigerated condition. Culture sample was prepared and 2% of culture was injected to the fermenter. Composition and methods were described in previous chapter I

2.2. Ezyame Eassy

Methods were described in previous chapter I

2.3. Design of the experiment

The CCD is a type of second-order design, and it was applied in this study using Design-Expert software version 13.0.5.0 . Five independent variables, aeration (X1)): 0.5 – 2.5 L/min, agitation speed (X2): 100-200 rpm, medium volume (X3): 3.0-5.0 L , were coded at three levels between -1 and +1 based on preliminary studies, as shown in **Table A**. The experimental design consisted of five factor design experiments augmented with five replicates at the design center to evaluate the pure error. The experiments were carried out in a randomized order, which is a common requirement in many experimental design procedures. The responses of the experiment were related to the chosen factors through quadratic models.

By the following quadratic equation the behaviour of the process was explained:

$$Z=\alpha_0 + \sum_{a=1}\alpha_a y_a + \sum_{a=1}\alpha_{aa} y_b{}^2 + \sum_{a=1}\sum_{b=1}\alpha_{ab}\, y_a y_b + £$$

Where ,

Z = process response or output (dependent variable),

k = number of the patterns,

a and b = index numbers for pattern,

α_0 = free or offset term called intercept term,

α_b = first-order (linear) main effect,

α_{aa} = quadratic (squared) effect,

α_{ab} = interaction effect,

£ =random error between predicted and measured values [in Table 3.3].

ANOVA was used to investigate the interaction between the process variables and the response variable, which was the enzyme production. The coefficient of determination R2 and adjusted R2 were used to evaluate the quality of the fit of the polynomial model developed using response surface methodology. These values indicate how well the model fits the data, with higher values indicating a better fit.

The statistical significance of the model was tested using the F-test, which compares the variation between groups to the variation within groups. If the F-test indicates that there is a significant difference between the groups, this suggests that the model is statistically significant and can be used to make predictions about the response variable.

Three-dimensional (3D) surfaces and two-dimensional (2D) contour plots were obtained to visualize the relationship between the process variables and the response variable. These plots were created by holding one factor constant while varying the other factors, and they provide a graphical representation of the behavior of the response variable under different conditions.

The 3D surface plot shows the response variable as a function of two independent variables, and it can be used to identify the optimal values of the process variables that maximize the response. The contour plot, on the other hand, shows the response variable as a function of two independent variables, with contour lines representing constant values of the response variable. The contour plot can be used to identify the regions of the process variable space that produce the desired response.

Experiments were carried out to validate the statistical models developed using response surface methodology. These experiments were designed to test the predictions of the model and to determine the accuracy of the model in predicting the response variable under different process conditions. By comparing the experimental results to the predictions of the model, the researchers can assess the validity of the model and identify any areas where the model may need to be refined or improved. This helps to ensure that the optimal process conditions identified through the use of the model can be achieved reliably in practice.

Table 3.1: Independent variables with coded levels based on a five -factor, three level CCD.

Sl.No.	Name	Units	Low	High	-1	+1	Coded value
1	Aeration	L/min	0.5000	2.50	0.50	2.50	X1
2	Agitation	rpm	100.00	200.00	100.00	200.00	X2
3	Medium volume	L	3.00	5.00	3.00	5.00	X3

3. Result and discussion

Table 3.2: Model fitting andANOVA

			Factor 1	Factor 2	Factor 3	Response 1
Std	Run	Space Type	X_1:Aeration	X_2:Agitation	X_3:Medium volume	Enzyme Production
			L/min	rpm	L	IU/mL
1	10	Factorial	0.5	100	3	79
2	3	Factorial	2.5	100	3	71
3	7	Factorial	0.5	200	3	72
4	17	Factorial	2.5	200	3	69
5	5	Factorial	0.5	100	5	84
6	2	Factorial	2.5	100	5	72
7	4	Factorial	0.5	200	5	77
8	19	Factorial	2.5	200	5	76
9	14	Axial	0.5	150	4	81
10	12	Axial	2.5	150	4	85
11	13	Axial	1.5	100	4	104
12	20	Axial	1.5	200	4	92
13	8	Axial	1.5	150	3	109
14	11	Axial	1.5	150	5	111

15	1	Center	1.5	150	4	117
16	9	Center	1.5	150	4	117
17	6	Center	1.5	150	4	117
18	18	Center	1.5	150	4	117
19	16	Center	1.5	150	4	117
20	15	Center	1.5	150	4	117

Table 3.3: Adequacy of the Models Tested for The Production of Enzyme

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	1.775E+05	1	1.775E+05			
Linear vs Mean	137.60	3	45.87	0.1054	0.9557	
2FI vs Linear	37.00	3	12.33	0.0231	0.9950	
Quadratic vs 2FI	**6705.82**	**3**	**2235.27**	**101.24**	**< 0.0001**	**Suggested**
Cubic vs Quadratic	79.40	4	19.85	0.8424	0.5459	Aliased
Residual	141.38	6	23.56			
Total	1.846E+05	20	9228.70			

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	20.86	0.0194	-0.1645	-0.6520	11731.04	
2FI	23.08	0.0246	-0.4256	-5.1780	43871.41	
Quadratic	**4.70**	**0.9689**	**0.9409**	**0.8202**	**1276.86**	**Suggested**
Cubic	4.85	0.9801	0.9370	-23.4630	1.737E+05	Aliased

A set of 20 experiments were conducted and the production of enzyme was measured. The data obtained was used to develop a design matrix, and linear and second-order polynomial equations were fitted to the experimental data to obtain the regression equations. To decide on the suitable model, sequential model sum of squares and model summary statistics were tested. The results of these tests are presented in Table 3.4. The F value for the quadratic model was found to be 34.63, which indicates that the quadratic model is statistically significant. Additionally, the adjusted R-squared value and predicted R-squared value were found to be the highest for the quadratic model, at 0.9409 and 0.8202 respectively. This indicates that the quadratic model provides the best fit for the data and is the most suitable model to use for predicting the production of enzyme.Therefore the quadratic model was chosen for further analysis of Production of enzyme and it can be described as following equation:

$\mathbf{Y}$=114.38-2.00X_1-2.40X_2+2.00X_3+2.00X_1X_2-0.2500X_1X_3+0.7500X_2X_3-27.45X_1^2-12.45X_2^2-0.4545X_3^2

Table 3.4: ANNOVA of the second order polynomial equation for enzyme production

Run	Independent coded variables			Enzyme production		
	X_1	X_2	X_3	Observed	Predicted	Error
1	1.5	150	4	117	114.38	2.62
2	2.5	100	5	72	73.42	-1.42
3	2.5	100	3	71	71.42	-0.4182
4	0.5	200	5	77	74.62	2.38
5	0.5	100	5	84	81.92	2.08
6	1.5	150	4	117	114.38	2.62
7	0.5	200	3	72	68.62	3.38
8	1.5	150	3	109	111.93	-2.93
9	1.5	150	4	117	114.38	2.62
10	1.5	100	3	79	78.92	0.0818
11	1.5	150	5	111	115.93	-4.93
12	2.5	150	4	85	84.93	0.0727
13	1.5	100	4	104	104.33	-0.3273
14	0.5	150	4	81	88.93	-7.93
15	1.5	150	4	117	114.38	2.62
16	1.5	150	4	117	114.38	2.62
17	2.5	200	3	69	69.12	-0.1182
18	1.5	150	4	117	114.38	2.62
19	2.5	200	5	76	74.12	1.88
20	1.5	200	4	92	99.53	-7.53

Table 3.5: ANNOVA of the second order polynomial equation for enzyme production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6880.42	9	764.49	34.63	< 0.0001	significant
X_1-Aeration	40.00	1	40.00	1.81	0.2080	Insignificant
X_2-Agitation	57.60	1	57.60	2.61	0.1373	Insignificant
X_3-Medium volume	40.00	1	40.00	1.81	0.2080	Insignificant
X_1 X_2	32.00	1	32.00	1.45	0.2563	Insignificant
X_1 X_3	0.5000	1	0.5000	0.0226	0.8834	Insignificant
X_2 X_3	4.50	1	4.50	0.2038	0.6613	Insignificant
X_1^2	2072.82	1	2072.82	93.89	< 0.0001	significant

X_2^2	426.57	1	426.57	19.32	0.0013	Insignificant
X_3^2	0.5682	1	0.5682	0.0257	0.8757	Insignificant
Residual	220.78	10	22.08			
Lack of Fit	220.78	5	44.16			
Pure Error	0.0000	5	0.0000			
Cor Total	7101.20	19				

ANOVA is a statistical technique used to analyze the variation in a set of data and determine the significance of the variables being studied. In this case, ANOVA was used to analyze the production of enzyme and the results indicated that the model was statistically significant with an F-value of 34.63. The "Adeq Precision" measures the signal-to-noise ratio, which indicates the adequacy of the model to navigate the design space. A ratio greater than 4 is desirable, and in this case, the signal-to-noise ratio of 14.239 indicates an adequate signal for production of enzyme. Therefore, this model can be used to optimize the production of enzyme in future experiments.

Negative coefficient for the model X_1,X_2,X_3,X_1 X_2,X_1 X_3, X_2 X_3, X_2^2,X_3^2 indicate unfavourable effects on enzyme production.Where as positive coefficients for X_1^2, indicate favourable effects on the enzyme production. All terms with positive coefficients greater than zero ,so all the terms having impact output variable.

ANOVA with 95% CI is a statistical technique used to evaluate the significance of the developed quadratic models. In this technique, Fisher's (F) exact test is used to compare probability (p) values greater than F, and small probability values ($p < 0.05$) indicate significant model terms, which confirm the accuracy of the developed models to predict the response functions. The model F-values of 224.74 for production of enzyme imply that the models are significant, and the p-values > 0.10 indicate that X_1^2 for production of enzyme is not significant. If the quadratic effect is not significant, then the optimal levels of the parameter are in the extremes of the experimental region.

The high values of R^2 and adjusted R^2 (0.9409) suggest that the quadratic model fits the experimental data well and can be used to accurately predict the production of enzyme. The adequate precision value of 14.239 indicates that the signal-to-noise ratio is high enough to navigate the design space using the model. However, the lack of fit p-value of 0.0500 suggests that there may be some lack of fit in the model and further investigation may be needed to improve its accuracy.

The assumption of constant variance was verified by plotting the internally studentized production versus predicted values, as shown in **Figure 3.4**. The studentized production was calculated by dividing the production values by their corresponding standard deviations. The plot shows a randomly scattered pattern within the outlier detection limits of -3 and +3. This indicates that the model predictions, as described in Equations (1), are satisfactory for both the production of enzyme. It is important to verify assumptions such as constant variance to ensure that the model is reliable and can accurately predict future outcomes.

In this case, the normal probability plot shown in **Figure 3.3** of residuals for production of enzyme showed a straight line pattern followed by the points on the plot, indicating that the residuals are normally distributed. Therefore, a transformation of the response variable is not required in order to improve the normality of the residuals [11].

The quadratic model predictions for production of enzyme responses are satisfactory. The correlation between the observed and predicted values shown in **Figure 3.2** indicates a good agreement between the two. A straight line trend with minor discrepancies suggests that the model predictions are close to the observed values, and the model is able to capture the variation in the data adequately. This is a positive sign for the accuracy and reliability of the model, and further supports its use in predicting the response values for the given experimental factors.

Actual and Predicted Enzyme Production:

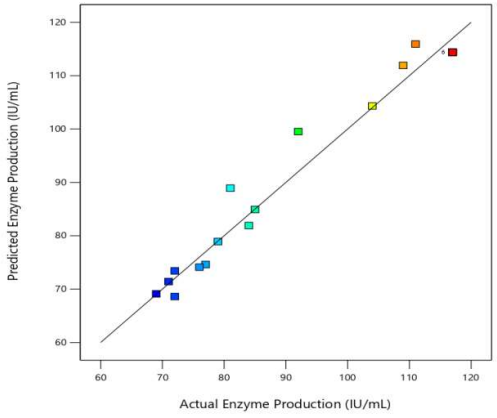


Figure 3.2: Actual and Predicted Enzyme Production versus normal probability for production of enzyme

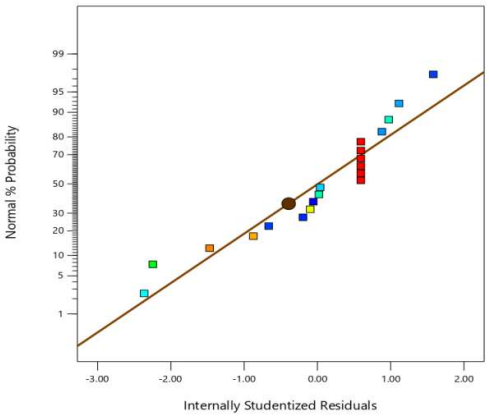


Figure 3.3: Internally studentized residuals

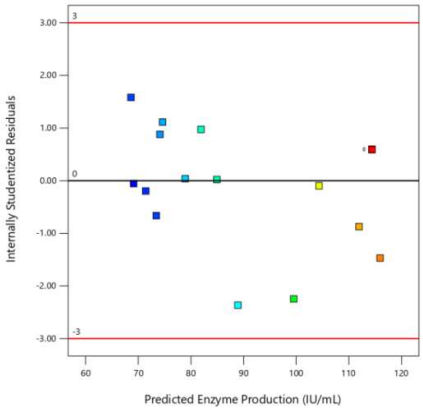
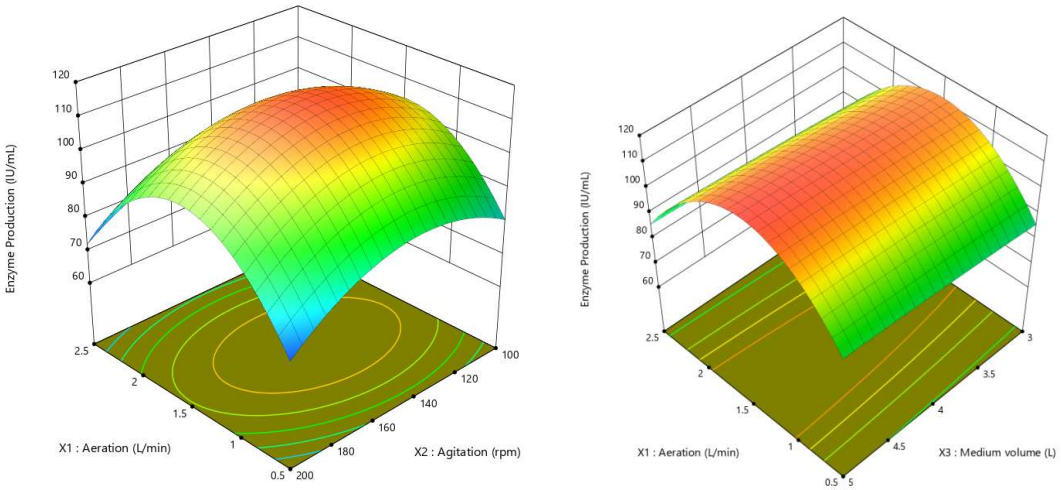


Figure 3.4: Observed experimental data versus predicted values for production of enzyme

Individual and Cross-Factor Interaction Effects of Model Parameters:

The F-exact test and p-values were used to evaluate the significance of each factor in the model, including the linear, quadratic, and cross-factor interaction terms. This was done to determine which variables have a significant effect on the response variable. Five independent variables including influent namely Aeration (X_1),Agitation speed (X_2), Medium Volume (X_3) have a significant effect on response % of enzyme production since their *p*-values are lower than 0.05. Besides, the cross-factor and square term interactions of some model parameters, including the X_1, X_1^2 are significant as their *p*-values < 0.05 are illustrated in **Figure 3.5(a)**



(a) (b)

Figure 3.5: 3D surfaces and 2D plots of the interaction effects of(a) Aeration (X_1)&Agitation speed (X_2);(b) Aeration (X_1)& Medium Volume (X_3)

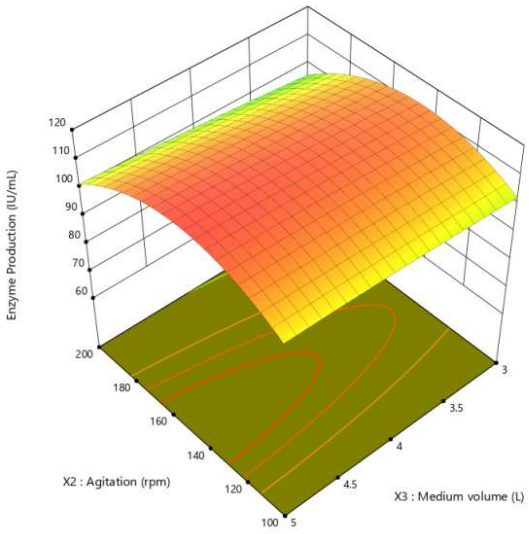


Figure 3.6: 3D surfaces and 2D plots of the interaction effects of Agitation speed (X_2) & Medium Volume (X_3)

4. Conclusion:

The interaction effects of the influent Aeration (X_1); Agitation speed (X_2); Medium Volume (X_3) had a significant effect on percentage of enzyme production. Optimum conditions were found for each variable to achieve maximum production of enzyme. The quadratic models allow for a more comprehensive exploration of the cross-factor interactive effects of the independent variables on the responses, which can provide valuable insights into the process and help optimize the conditions for maximum response.The proposed models explaining the percentage of enzyme production by using the batch production were found suitable for future studies on reactor design, modeling, and scale-up.

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Chapter IV

Separation, purification and characterization of the crude enzyme produced by *Bacillus paramycoides* MCCC 1A04098 MRS4 in the laboratory fermenter

1. Introduction

L-asparaginase is widely used in combination therapy for the treatment of acute lymphoblastic leukemia in children [1] and is also showing potential for industrial and clinical applications [2] in the future. It is considered a key drug for the treatment of extranodal NK/T-cell lymphoma [3].

The purification of L-asparaginase is crucial for both its characterization and its safe therapeutic and industrial use. Impurities, such as associated L-glutaminase activity and bacterial endotoxins, can cause fatal allergenic reactions [4], which is why research groups have been focused on producing and purifying L-asparaginase to minimize these impurities [5].

The genetic nature of the microbial strain used for producing L-asparaginase can affect its biochemical and kinetic properties, leading to variations in the enzyme's activity and stability. This highlights the need for further research to identify other sources of L-asparaginase [8].

Most microbial L-asparaginase is intracellular, but there are a few that are secreted outside the cell [9]. Extracellular L-asparaginase is more desirable because it accumulates in higher amounts in culture broth, is easier to extract and process [10-11] and is biologically active and free of contaminants like endotoxins. Secretion also allows for proper protein folding, especially those requiring disulfide bridge formation, due to the favorable redox potential in the periplasmic space.

2. Materials and Methods

2.1. Separation of the Enzyme

2% of asparaginase producing strain*Bacillus paramycoides* MRS4 was injected to the fermentar, medium having same medium composition as mentioned above chapter I and kept at 35 °C and pH 7 in the condition of aeration 1.5L/min, agitation150 rpm and 4L medium volume for 24 hrs. After 24 hr the fermented broth was centrifuged at 4500 rpm for 15 min and the cell mass separated, the supernatant was collected.

2.2. Partial purification

As medium broth of enzyme, the supernatant was used.20mL medium broth were placed in separate 100mL beakers and the amount of ammonium sulphate required to achieve different

degrees of saturation for salting out. (30-90%) of the samples at 4◦C were measured and added to the respective beakers. Each beaker was kept at 4◦C for 2 hours. The suspension was then centrifuged at 10,000 rpm for 10 mins. The precipitate was collected.

2.3. Purification of crud enzyme by Dialysis of the ammonium sulphate fraction

The ammonium sulfate fraction showing maximum specific activity was dialyzed at 16°C for 16 hrs against 250 mL .0021M disodium hydrogen phosphatebuffer (pH7) using the magnetic stirrer and changing the buffer solution townies during dialysis. 12kDa molecular weight cut off dialysis sac was used. The dialyzed enzyme was centrifuged and clear supernatant was was suspended in 8mLdisodium hydrogen phosphatebuffer (pH7) and resulting solution was assayed for the enzyme activity and applied in different food sample.

Purification method of enzyme using HPLC

The elution pattern that worked well consisted of HPLC grade water: 40% (v/v), hydrogen phosphatebuffer (pH7) 60% (v/v), and formic acid: 0.10% (v/v). The eluent resulted in highly resolved chromatograms. the study provides important information on how to purify L-Asparaginase by using HPLC, including the optimal column temperature and elution pattern. The use of 0.1% formic acid in the eluent helped in reducing noise and undesirable peaks in the chromatogram. The flow rate was kept at 0.8 mL/min to ensure a consistent elution rate and sufficient separation of the analytes. Using a UV-Vis detector the detection was performed at 210 nm. The developed method was found to be simple, fast, and accurate with high recovery rates of L- Asparaginase.

3. Determining the characterization of crude and purified enzyme produced by *Bacillus paramycoides* MCCC 1A04098 MRS4 in laboratory scale fermenter

3.1.Effect of different minerals/metal ions in different concentrations in the pure and purified enzyme

Metal ions/ minerals (Di-Potassium phosphate, Mono-Potassium phosphate, Sodium chloride, Calcium Chloride, Magnesium chloride, Magnesium Sulphate, Disodium Hydrogen Phosphate) were introduced in different concentration 1%, 2% and 3% intopurified L-Asparaginase.The absorbance was measured at 396nm.

3.2. Effect of pH on purified enyme

The effect of Purified L- Asparaginase by*Bacillus paramycoides*MRS4in different pH within 4.0 - 8.0 at 35 °C for 24 hrs was determined.

3.3. Effect of temperature of purified enzyme

The effects of purified enyme in different temperatures from 25-45°C for 24 hrs. During the experiment pH of the medium was reserved persistent at 7.0.

4. Determination of kinetic parameters of purified enzyme produced by *Bacillus paramycoides* MRS4

The method to determine the V_{max} and K_m of purified enzyme. By testing different substrate concentrations, can analyze the enzyme's activity and determine the maximum velocity and Michaelis-Menten constant, which are important parameters for understanding the kinetics of the enzyme and its substrate interaction.

4.1. Determination of the kinetic parameters K_m and V_{max}

The Michaelis-Menten constant (Km) and maximal velocity (Vmax) of purified enzyme can be determined using the Michaelis-Menten equation and by testing different substrate concentrations of L-asparaginase (0.5-2.5mM). These kinetic parameters are important for understanding the enzymatic activity and substrate interaction of L-asparaginase.
Michaelis-Menten equation:

$$\nu = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

Whereas: initial reaction velocity (V), substrate concentration (S).

5. Thermodynamic study of purified enzyme

The thermal stability of purified enzyme can be studied by incubating the purified enzyme at different temperatures from 20° to 60° C and measuring its activity at 10 minutes intervals. This can give information about the temperature range at which the enzyme remains active and stable, and can help in optimizing conditions for its use in various applications. A first order plot was done according to the equation-

ln P= kt+c …….(i)

where, P= product formation, k = inactivation rate constant (min $^{-1}$) , t = time of the reaction (min).

The half-life of the purified enzyme was calculation from equation

t ½ = ln 2/k ……(ii)

The D value (Decimal reduction Time) was calculated-

D- value = ln 10/ k ……..(iii)

The z value (rise in temperature for one log cycle reduction in D- value) was calculated from the slope of log D vs temperature plot as-

Slope = - 1/z ……. (iv)

The activation energy of L- Asparaginase (E_a) was obtained from the Aeehenius plot of ln k vs reciprocal of temperature in Kelvin scale (1/T) using the equation

Slope =- E_a/R……... (v)
Where R= Universal gas constant = 8.314 J mol^{-1} K^{-1}

Further analysis of thermodynamic data allowed us to evaluation some other paramiters like enthalpy (▲H) in kJ mole $^{-1}$, Gibbs free energy (▲G)in kJ mole $^{-1}$and enthalpy (▲S) in kJ mole $^{-1}$K^{-1} using the following equation –

▲H = E_a– RT…….. (vi)

▲G = - RT ln (kh/ k_BT)…….. (vii)

▲S = (▲H - ▲G)/T……... (viii)
Where,

h = Plank's constant (11.04 x 10 $^{-36}$ J min)

kB = Boltzmann constant (1.38 x 10 $^{-23}$ J K^{-1})

T= Absolute Temperature (K)

6. Detection of Moleculor weight of purified L- Asparaginase

6.1. ESI-MS process

Electro Spray Ionization Mass Spectrometry (ESI-MS) is a useful tool for protein analysis and characterization. The parameters described in your statement, such as the instrument used, the mode of operation, the scan range of m/z 50-1800, capillary voltage were set to 30-3kV respectively. The source temperature was 100 °C and dissolving temperature was 250 °C. The nebulising gas pressure was 3000L/hr (N_2) and auxiliary gas pressure was 50L/hr (Ar).The sample was diluted 10 folds with 0.1% formic acid in LC-MS grade water. Sample were directed infused into the ESI source at slow rate 5µL/min and acquisition time was given for 1min. Additionally, the use of lock masses and calibrants helps ensure accurate mass determination and reduces the impact of instrument drift. The product ion MS analysis can be performed using software such as MassLynx.

6.2. SDS –Gel electrophoresis

6.2.1. Chemical preparation:

6.2.1.1. **8% Gel for 1 plate**

40%Acrylamide	1mL
Mili Q water	2.125mL
1M Tris.pH 8.8	1.850 mL
20% SDS	25mL
10%APS	30mL
TEMED	2.5mL

Preparation of 40% acrylamide:

380g acrylamide + 20 g bisacrylamide in 600mL,slight heating during stirringthen filtered it.

6.2.1.2. Stacking gel:

4% gel	**for 1 plate**
40% acrylamide	0.3 mL
Mili Q water	2.295mL
1M Tris.pH6.8	0.375mL
20% SDS	20mL
10%APS	20mL

TEMED 3.0mL

6.2.1.3. Running Buffer:

For 1 Litter: 1gm SDS + 3gm Tris + 14.4g glysin (stirring until desolves)

6.2.1.4. Preparation of stain:

0.2% coomassie blue G

10% acetic acid glacial

20% Methanol

Volume were makeup with mili Q water and filtered it with Wattman Filter paper

7. Results and Discussion

7.1. Salting out of ammonium sulphate:-

Ammonium sulphate concentration varied from 30-90% for precipitation of protein present from crude enzyme mixtrure.Amount of enzyme was increases up to 80% and 133 IU/mL/mit enzyme was resulted maximum at 80% and then decreased at 90%(Table 4.1).A study conducted by Bedaiwy 2019 has shown that 80% ammonium sulphate fraction was selected for purification of L-Asparaginase produced by *Pleurotus ostreatus* MRS4

Table 4.1: Effect of Ammonium sulphate saturation concentration on the precipitation of L- Asparaginase by *Bacillus paramycoides* MRS4

Fraction having Ammonium saturation (%)	Enzyme (IU/mL/mit)
30	106
40	112
50	116
60	121
70	125
80	133
90	127

7.2. Determining the characterization of crude and purified enzyme produced by *Bacillus paramycoides* MRS4 in laboratory scale fermenter

7.2.1. Effect of different minerals/metal ions in different concentrations of crude enzyme

It was found that among all minerals/metal ions source in 3% of Di-Potassium phosphate produce activity of enzyme was maximum about 70.247IU/mL.

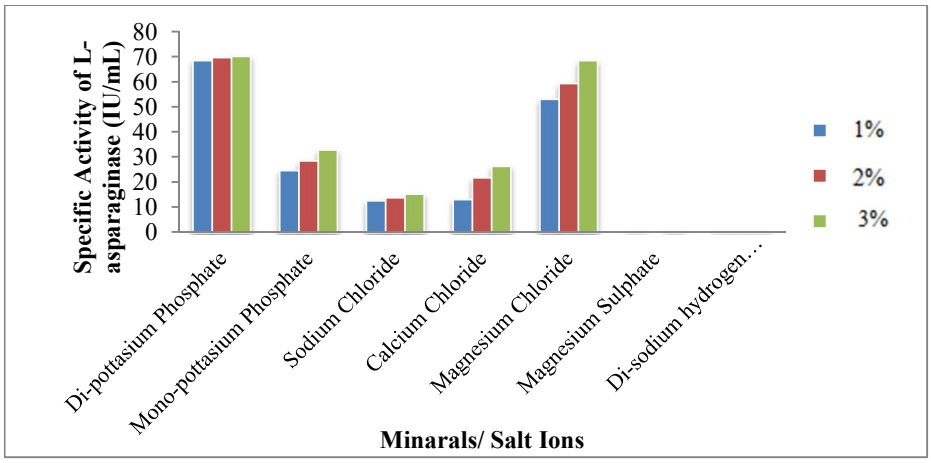


Figure 4.1: Effect of different minerals/metal ions in different concentrations of crude enzyme of L- Asparaginase by *Bacillus paramycoides* MRS4

7.2.2. Effect of different minerals/metal ions in different concentrations of purified enzyme

It was found that among all minerals/metal ions source in 3% of Di-Potassium phosphate produce activity of enzyme was maximum about 69.954 IU/mL.

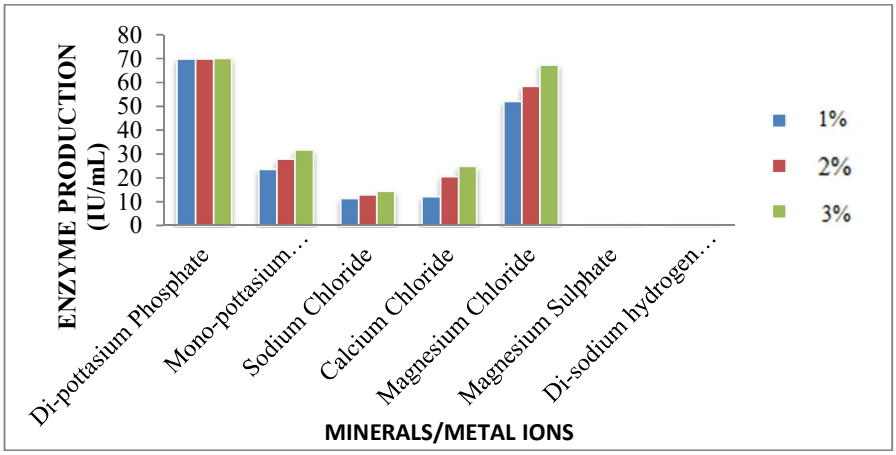


Figure 4.2: Effect of different minerals/metal ions in different concentrations of purified enzyme

7.2.3. Effect of pH on purified L- Asparaginase by *Bacillus paramycoides* MRS4

The result is shown in Figure 4.3 and it shows that the amount of enzyme produced is maximum at pH 7.0 the enzyme activity at that pH was also maximum117.2 IU/mL With the decrease or increase of pH activity of purified L- Asparaginase by *Bacillus paramycoides* MRS4 became less so enzyme production were also low at pH below 7.0 or above 7.0.This results coincide with that of L-asparaginase, purified from *Streptomyces acrimycini* NGP, [12] and *Corynebacterium glutamicum* [13].

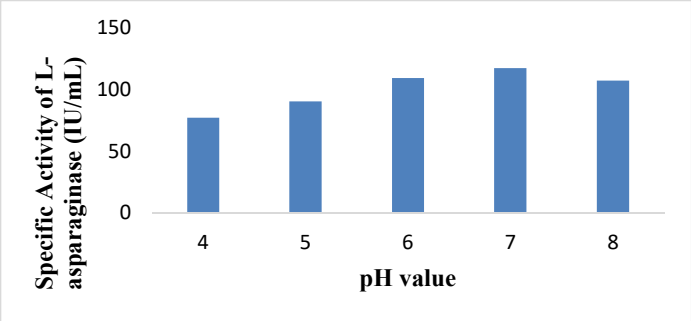


Figure 4.3: Effect of pH of purified L- Asparaginase by *Bacillus paramycoides* MRS4

7.2.4. Effect of temperature on purified L- Asparaginase by *Bacillus paramycoides* MRS4

Effect of temperature on purified L- Asparaginase by *Bacillus paramycoides* MRS4 was detected at temperatures within 25- 45°C for 24 hr while pH of the medium was reserved constant at 7.0. The result of the experiment is shown in Figure 4.4.The figure shows that enzyme production was maximum at35ºC the enzyme activity at that temperature was also maximum96.4 IU/ml but below or above temperature 35ºC growth of the organism was less.This result coincide with that the enzyme activity obtained from *Pseudomonas stutzeri MB-405* [14] and *Erwinia sp* [15].

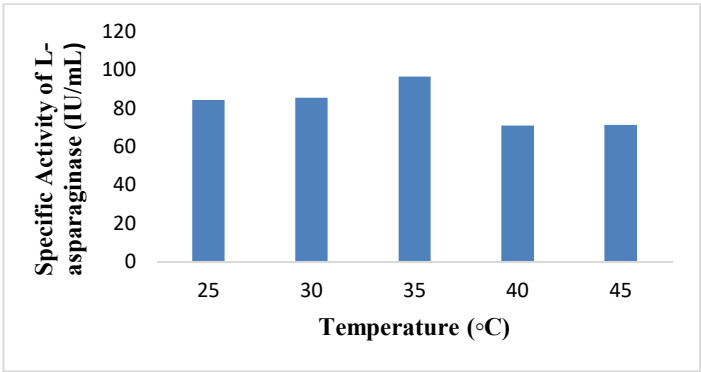


Figure 4.4: Effect of temperature of purified L- Asparaginase by *Bacillus paramycoides* MRS4

7.3. Determination of kinetic parameter of purified L- Asparaginase by *Bacillus paramycoides* MRS4*:*

Beyond a certain concentration ranging from 0.5 to 2.5 mM, the enzyme activity does not increase significantly, indicating that the substrate has reached its saturation point. Therefore, the optimum substrate concentration for L-asparaginase activity (Figure 4.5) can be determined as the concentration at which the enzyme activity reaches its maximum value and remains constant or only slightly increases with further increases in substrate concentration.

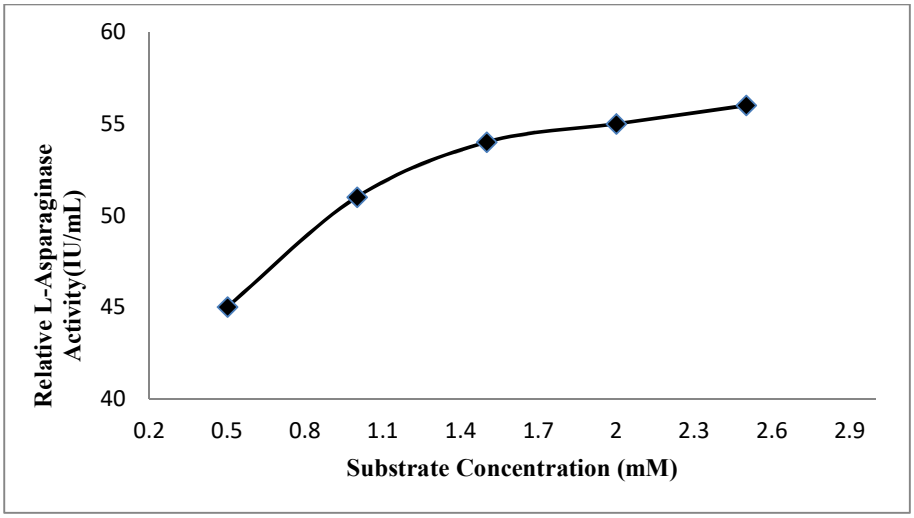


Figure 4.5: Effect of the substrate concentration of the reaction on L-asparaginase activity.

The Michaelis-Menten relationship between the substrate concentrations and the initial velocity of the reaction, for the L-asparaginase enzyme. The Michaelis-Menten plot in Figure 4.6 shows the Km and Vmax values for the hydrolysis of L-asparagine, with a Km value of 0.22666 µmole and Vmax of 66.66 µmole/min/Ml. Km is a measure of the affinity of an enzyme for its substrate, and represents the concentration of substrate at which the enzyme reaction reaches half of its maximum velocity [16]. A lower Km value indicates a higher affinity of the enzyme for the substrate, while a higher Km value indicates a lower affinity [17]. In this case, the lower Km value of 0.22666 µmole indicates that the L-asparaginase enzyme has a strong binding ability with its substrate.Vmax, on the other hand, is a measure of the catalytic activity of the enzyme and represents the maximum rate at which the reaction can proceed when substrate is present in large excess. It is influenced by various factors such

as the type of enzyme, its different forms, changes in enzyme conditions, source of the enzyme, type of substrate used, and the assay procedures [18].

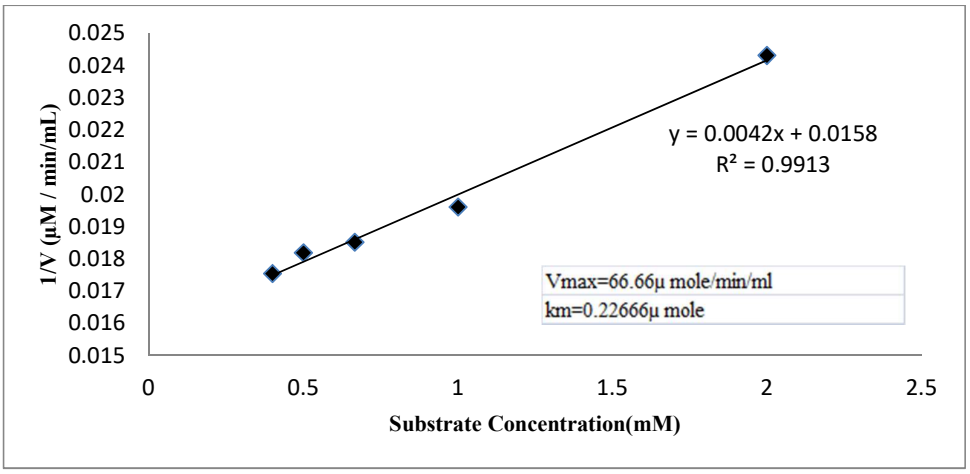
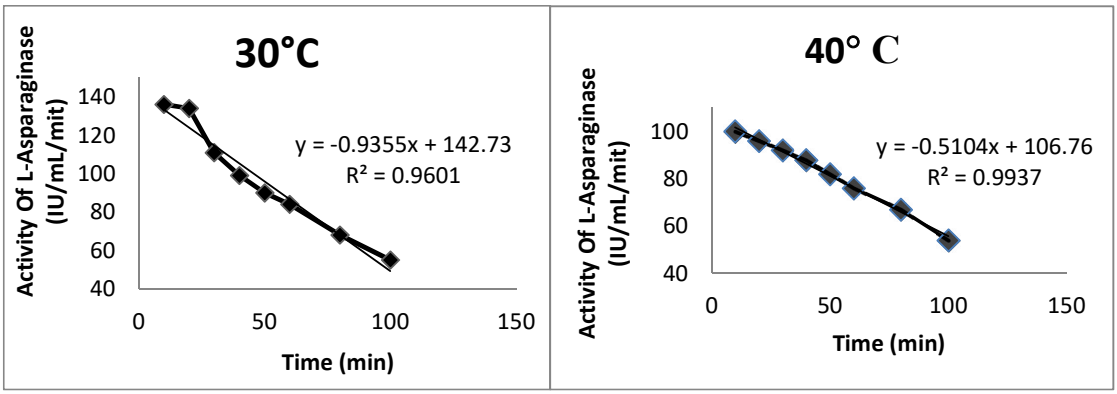


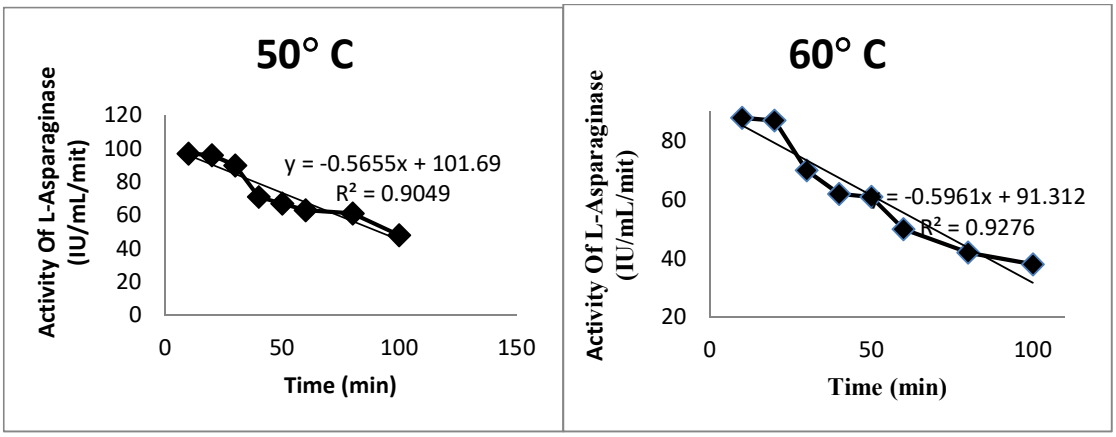
Figure 4.6: Michaelis-Menten plot for L-asparaginase produced by *Bacillus paramycoides* MRS4

7.4. Thermodynamic study of purified L-Asparaginase

Table 4.2: Thermodynamic parameters for thermal deactivation of purified L-Asparaginase

Sl no.	Temperature (K)	k (mit^{-1})	$t_{1/2}$ (min)	D –Value (min)	▲G kJ $mole^{-1}$
2	313.15	-0.510	- 1.3588	-4.515	1.75×10^{-2}
3	323.15	-0.565	-1.226	-4.076	1.53×10^{-2}
4	333.15	-0.596	-1.162	-3.864	1.43×10^{-2}





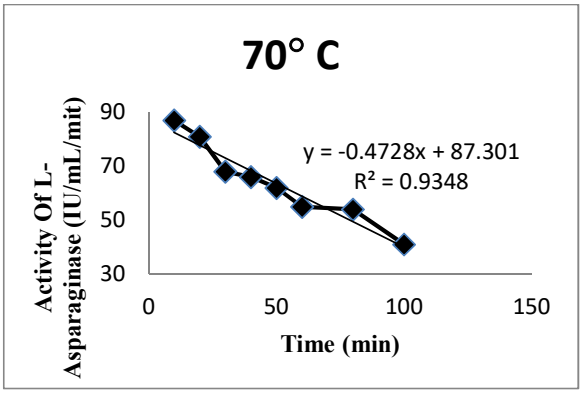


Figure 4.7: First order Thermal deactivation of L –asparaginase

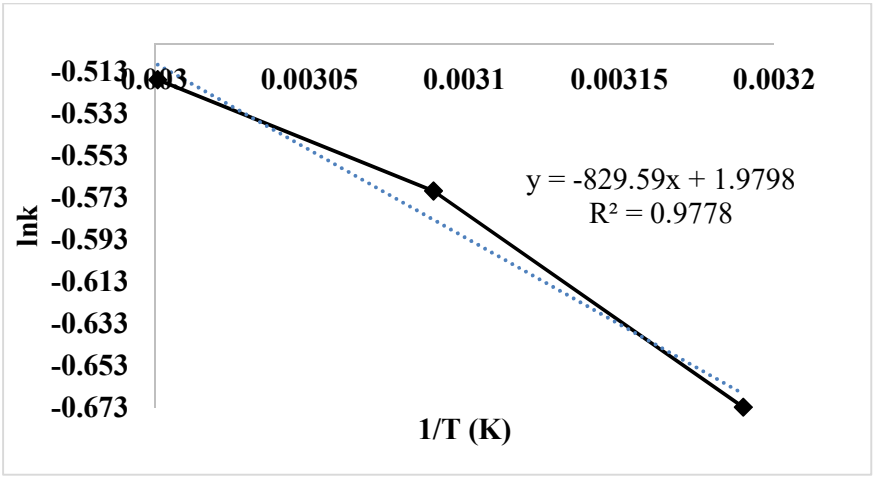


Figure 4.8: Deactivation energy for Arrhenius plot

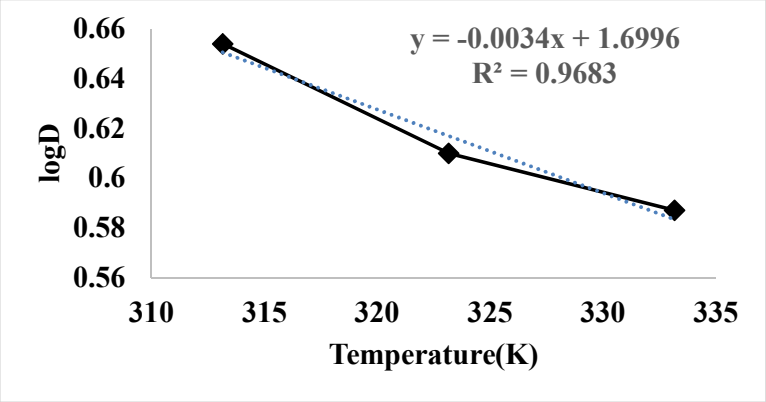


Figure 4.9: Plot log D vs temperature (K) in case of detection of z value of purified L – asparagines

7.5. Detection of Moleculor weight of purified L- Asparaginase produced by *Bacillus paramycoides* MRS4

The molecular weight of the purified L-asparaginase was determined in comparison with standard molecular weight markers (molecular mass range: 9–178 kDa).Moleculor weight of purified L- Asparaginase by *Bacillus paramycoides* MRS4 were detected in two ways ESI – MS and SDS –Gel electrophoresis and the moleculor weight is 48.756 kDa (Figure 4.14 and Figure 4.15). L-asparaginase is known to form a homotetramer, which is composed of four subunits. The active sites of the enzyme are located at the interface between two subunits, forming an intimate dimer [9-11, 19-20].

(i)ESI-MS process

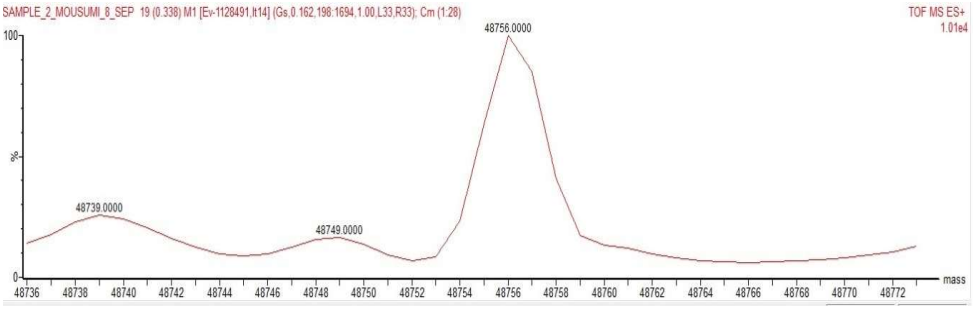
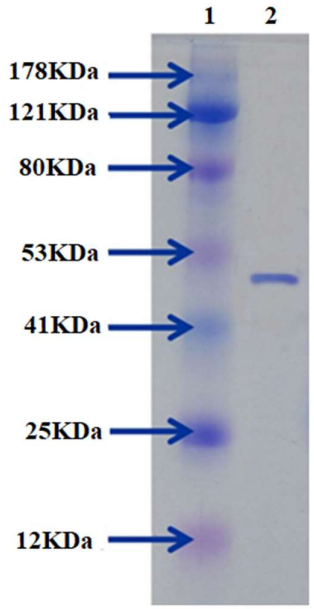


Figure 4.10: Detection of Moleculor weight of purified L- Asparaginase by *Bacillus paramycoides* MRS4 by ESI-MS process

(ii)SDS –Gel electrophoresis



Lane 1: Protein marker; **Lane 2**: Purified L-asparaginase

Figure 4.11: Detection of Moleculor weight of purified L- Asparaginase by *Bacillus paramycoides* MRS4by SDS –Gel electrophoresis

8. Conclusion

Purification of L-asparaginase with 80% ammonium sulphate fraction followed by dialysis and after that again purify by HPLC. Effect of different minerals/metal ions in different concentrations of crude and purified enzyme of L- Asparaginase by *Bacillus paramycoides* MRS4 is shown and the result was 3% of Di-Potassium phosphate produce activity of enzyme was maximum 70.247 IU/mL. & 69.954 IU/mL respectively. Effect of pH and temperature was remain same of the purified enzyme as crude enzyme about pH-7 and 35°C.Maximum Velocity(Vmax) and Michealis Menten constant(K_m) of purified L-Asparaginase were calculate by using Lineweaver-Burk plot and the value comes out to be 66,66µmole /mint/mL and 0.22666µ mole respectively. Molecular weight 48.756 kDa was measured by SDS-PAGE and LC-MS .

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Chapter V

Application of purified L-Asparaginase produced by *Bacillus paramycoides MCCC 1A04098 MRS4* on different type of high temperature processed food to study it's effect onacrylamide formation

1. Introduction

Heating of certain foods can lead to the formation of acrylamide, a toxicant that can be harmful to human health if consumed in high amounts. To minimize the formation of acrylamide, it is important to follow recommended cooking times and temperatures, and to choose a variety of cooking methods, such as baking, boiling, and steaming, to reduce exposure to high heat [1].It's also important to store and handle food properly to reduce the formation of acrylamide. For example, storing potatoes in a cool, dark place before cooking can help to reduce the formation of acrylamide. Other strategies, such as using a variety of ingredients and spices to add flavor to food, can also help to reduce the need for high-heat cooking methods that can lead to acrylamide formation[2].The International Agency for Research on Cancer (IARC) has classified acrylamide as a Group 2A carcinogen, which means that it is a "probably carcinogenic to humans." This classification is based on the evidence that acrylamide can cause cancer in laboratory animals and the limited evidence of human exposure to acrylamide through diet. It's important to note that the level of acrylamide in food can vary depending on factors such as cooking method and food preparation, and more research is needed to fully understand the health effects of acrylamide exposure. Nevertheless, it is always a good idea to follow recommended cooking and storage methods to minimize exposure to acrylamide and other potential toxicants in foods[3, 4, 5]. Acrylamide has been shown to cause neurotoxicity, specifically paralysis of the central nervous system, in laboratory animals, and it is thought to be the result of the conversion of acrylamide to glycidamide (GA) in the body. GA is a highly reactive molecule that can form adducts (molecular complexes) with cellular proteins, including DNA, which can cause mutations and other types of DNA damage. These mutations can potentially lead to the development of cancer and other health problems over time. It's important to minimize exposure to acrylamide and other potential toxicants in food to reduce the risk of these health effects [6]. The European Union (EU) has acknowledged the toxic properties of acrylamide and has taken steps to minimize human exposure to this substance. For example, the EU has established a framework for monitoring and reducing the levels of acrylamide in food, and it provides guidelines for food manufacturers, processors, and suppliers on how to minimize the formation of acrylamide during food production and cooking. Additionally, the EU provides information to consumers on how to reduce their exposure to acrylamide in food, including recommendations on cooking methods and food choices. By keeping human exposure to acrylamide as low as possible, the EU aims to reduce the risk of harmful health

effectsassociated with this substance[5].The No Significant Risk Level (NSRL) for acrylamide, established by the US government, is set at 2.6 micrograms per kilogram of body weight per day to avoid the risk of cancer. This is equivalent to a tolerable daily intake (TDI) of 182 micrograms for a 70 kg human. To minimize the formation of acrylamide during the heating process, various preventive strategies are employed, such as optimizing cooking conditions, using low-heat cooking methods, and adding certain ingredients to the food. Additionally, there are also interventions aimed at removing or decomposing already formed acrylamide molecules in the finished food product, such as using certain chemical treatments or processing techniques. By implementing these strategies, the goal is to reduce human exposure to acrylamide and minimize the associated risk of health effects[7]. The food industry faces several challenges in mitigating dietary acrylamide, as reducing acrylamide formation during food preparation and processing can require changes to established processes and product parameters. This can pose a challenge because the changes needed to reduce acrylamide formation may also impact the taste, texture, and appearance of the food, which are important factors that can affect consumer acceptance and sales. To overcome these challenges, food manufacturers, processors, and suppliers are experimenting with different techniques and ingredients to minimize acrylamide formation while still producing high-quality food that meets consumer expectations. This can involve a combination of changes to cooking methods, food ingredients, and processing techniques, as well as ongoing monitoring and research to identify and address new challenges as they arise [8-9]. As shown in the Figure.1,The formation of acrylamide during high-heat cooking of food is primarily due to the Maillard reaction, which occurs between naturally occurring asparagine and reducing sugars (such as glucose and fructose) in food. This reaction is responsible for the desirable browning and flavor development that occurs during cooking, but it can also lead to the formation of acrylamide. To mitigate acrylamide formation, one approach is to use asparaginase, an enzyme that hydrolyzes asparagine into aspartic acid. By reducing the amount of asparagine available for the Maillard reaction, asparaginase can reduce acrylamide formation while still preserving the sensory characteristics of the food product, such as taste, aroma, and appearance. This approach has the potential to be a useful tool for food manufacturers, processors, and suppliers to minimize acrylamide exposure while maintaining the quality of their products [10-11]. The use of L-asparaginase from Bacillus paramycoides, in combination with physical techniques, has been shown to effectively reduce the acrylamide content in food items. The treatment was tested in a range of food products, including French fries, nimki, fried fish, and chicken pakoda, and resulted in a decline of

nearly 93% in the acrylamide content when analyzed using High Performance Liquid Chromatography (HPLC). This approach has the potential to be an affordable and effective way to mitigate acrylamide exposure in food products and may have broad applications in the food industry. However, it is important to note that the results of these studies should be interpreted with caution and more research is needed to fully understand the impact of asparaginase treatment on the sensory characteristics, nutritional value, and overall quality of the food products.

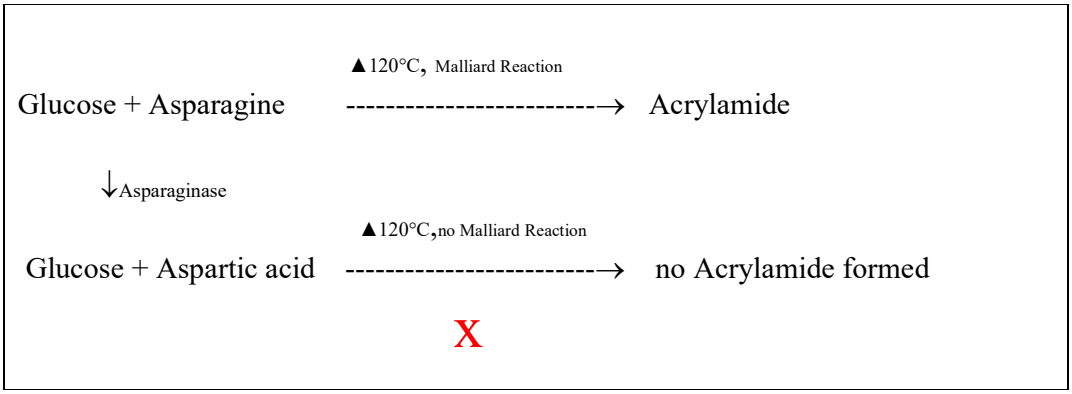

Figure 5.1: Modeofaction of aspraginase in acrylamide formation during food processing

Acrylamide is not intentionally added to food but is naturally formed when food is subjected to high heat and low moisture conditions, such as during frying, roasting, and baking. The formation of acrylamide starts at temperatures above 120°C and increases drastically towards the end of the cooking process at temperatures higher than 170-180°C. This is why it is important to control the cooking temperature and time, as well as the moisture content of food, to minimize acrylamide formation and reduce exposure to this potentially harm ful substance [12]. The main route for the formation of acrylamide in fried potato products is due to the presence of high concentrations of asparagine, the precursor for acrylamide, in potatoes. The processing conditions, such as frying temperature and time, also play a key role in acrylamide formation. To minimize the formation of acrylamide, it is important to control these factors and choose cooking methods that reduce exposure to high heat and low moisture conditions. This can help reduce the risk of exposure to acrylamide and its potential health effects. [13].The formation of acrylamide in food has two major pathways: the Maillard reaction and the asparagine-carbohydrate reaction. During the Maillard reaction, the reaction of asparagine with reducing sugars leads to the formation of acrylamide through a series of intermediate compounds, including a Schiff base, which is further

decomposed into acrylamide and other carbonyl compounds. In the asparagine-carbohydrate reaction, asparagine reacts with carbohydrates to form a N-glycosyl conjugate, which may also lead to acrylamide formation. These reactions occur at high temperatures and low moisture conditions, such as during frying, roasting, or baking. To minimize acrylamide formation, it is important to control these factors and choose cooking methods that reduce exposure to high heat and low moisture conditions [14]. In addition to asparagine, other amino acids such as glutamine, cysteine and aspartic acid can also contribute to the formation of acrylamide, but in lower amounts compared to asparagine. It's important to consider all factors that contribute to acrylamide formation in order to minimize its presence in food products [15]. Studies have shown that asparagine is the main precursor for the formation of acrylamide in potato- and cereal-based foods, and the use of asparaginase, a substrate-selective enzyme, effectively reduces acrylamide formation. The minor route through other amino acids is not significant compared to the major route through asparagine and its contribution to acrylamide formation in foods might be limited [16].

By the US Environmental Protection Agency (EPA), carcinogen risk assessment involves a systematic evaluation of the available scientific evidence to determine the likelihood that exposure to a substance may cause cancer. This process typically includes an evaluation of the substance's toxicity and exposure data, as well as any available epidemiological studies or animal studies. Based on this information, a risk assessment will be conducted to determine the potential health risks associated with exposure to the substance, and to identify any necessary risk management strategies to protect public health [17], acrylamide has also been found to cause genotoxicity, neurotoxicity and developmental toxicity in animal studies. These effects raise concerns about the potential for similar effects in humans. Based on these findings, it is important to reduce human exposure to acrylamide as much as possible [18]. After consumption, acrylamide is rapidly absorbed and widely distributed in the body. In the liver, it is metabolized to an epoxide glycidamide by the CYP2E1 enzyme, which can lead to the formation of covalent, adducts with DNA and cause mutations and chromosomal aberrations. These effects have been observed in both mice and rats [19]. It is important to note that although animal studies and some observational studies suggest a link between acrylamide exposure and increased cancer risk, the evidence is not yet strong enough to establish a definitive causal relationship. Further research is needed to better understand the potential health effects of acrylamide in humans and to determine if there is indeed a causal relationship between acrylamide

consumption and cancer risk. Additionally, acrylamide levels in food can vary greatly depending on factors such as the type of food, processing conditions, and cooking methods, so it is difficult to estimate the amount of acrylamide that individuals are exposed to through their diets [20] and there is limited evidence for the association between dietary acrylamide and other cancers such as kidney, breast, lung and prostate cancers, but more research is needed to confirm these findings. Despite the evidence of potential harm, it is important to remember that acrylamide is just one of many factors that contribute to an individual's overall risk of developing cancer and that a balanced diet and lifestyle are crucial for maintaining good health [21]. It is important to note that the exact extent of the link between acrylamide and different types of cancer i.c bladder, prostate, renal cancers brain cancers, lung cancer, or ovarian cancer [22-25].

2. Materials and Methods

2.1. Chemicals Needed

Acrylamide (>99%) was obtained from Sigma (Deisenhofen,Germany). Methanol, acetic acid, acetonitrile and acetone were of analytical grade and potassium hexa cyano ferrate (Carrez I) and zinc sulfate (CarrezII) were procured from Merck (Darmstadt, Germany). Throughout the experiments distilled, deionized and 0.20µm filtered water was used.The preparation process of stock and working solutions of acrylamide, as well as the preparation of Carrez I and Carrez II solutions as follows:

- **Preparation of stock solution:** A stock solution of acrylamide is prepared by dissolving a known amount of acrylamide (1 mg) in a known volume of distilled water (1 mL). This results in a stock solution with a concentration of 1 mg/mL.
- **Preparation of working solutions:** Working solutions for the calibration curve are prepared by diluting the stock solution of acrylamide to concentrations of 0.5, 1.0, 3.0, 5.0, and 8.0 µg/mL with distilled water. This is done by taking a known volume of the stock solution (e.g., 0.5 mL) and diluting it with a known volume of distilled water (e.g., 4.5 mL), resulting in a working solution with a concentration of 0.5 µg/mL.
- **Preparation of Carrez I solution:** Carrez I solution is prepared by dissolving 15 g of potassium hexacyanoferrate in 100 mL of water. This solution is used to precipitate proteins in the sample, which can interfere with the analysis of acrylamide.

- **Preparation of Carrez II solution:** Carrez II solution is prepared by dissolving 30 g of zinc sulfate in 100 mL of water. This solution is used to further precipitate proteins in the sample.

2.2. Aplication of Enzyme in High Temperature Processed Food

2.2.1. Sample Preparation

Different Food samples like French fry, Nimki, Fried fish and Fried Chicken was prepared as without and with applying L-asparaginase enzyme. Purified L-asparaginase and pure L-asparaginase from Sigma (Deisenhofen,Germany) was used in different concentration of dose like 50IU & 100IU and dipping different time as 15 minutes, 30 minutes, 60 minutes.

2.2.2. Extraction of Acrylamide and its Estimation

- **Sample extraction:** Different Food samples like French fry, Nimki, Fried fish, Fried Chicken without and with applying L-asparaginase was taken and smashed in a mortar pestle. Finely mashed food samples were weighted (1g)and putinto15mL centrifuge tube. The samples mixed with acetone and spiked with acrylamide were subjected to ultrasonication to ensure proper mixing. After that, the mixture was centrifuged for 10 minutes at a speed of 10000 rpm. This process was likely done to separate the acrylamide and any other substances present in the samples. The purpose of spiking the samples with acrylamide at different levels was to calculate the percentage relative recovery, which provides information on the accuracy and efficiency of the acrylamide extraction process. It is important to discard the solid residues from the supernatant well and if needed the sample was centrifuged again. This will purify in the acetone phase from the water-soluble co-extractive components.
- **Liquid-liquid extraction:** The extract is treated with Carrez I and II (100 µLeach) solutions, which are used to isolate co-extractives. The mixture is then centrifuged to separate the two phases, and the upper phase (which contains the acrylamide) is transferred to a conical flask.
- **Evaporation:** The sample is evaporated to dryness in order to remove the solvent and concentrate the acrylamide. The presence of trace water from the Carrez solutions helps to prevent loss of acrylamide during the evaporation process.

- **Reconstitution:** The residue is dissolved in a small volume (4 mL) of acidic water (pH 3) using vortex mixing. This step helps to solubilize the acrylamide while minimizing the presence of lipids and other co-extractives that could interfere with the analysis.
- **Filtration:** The solution is filtered through a 0.22 μm syringe filter to remove any remaining particulate matter or other impurities.
- **Injection:** A small volume (10 μL) of the filtered solution is injected into the HPLC system for analysis.

2.3. Estimation of the Acrylamide content

2.3.1. HPLC Analysis of the Sample

Different eluent compositions and column temperatures were evaluated to optimize the HPLC method for separating acrylamide from other interferents. It was found that a mixture of acetonitrile and formic acid with a certain ratio worked well for separating acrylamide from interferents, and a negative association was observed between the column temperature and retention time and. By optimizing the eluent composition and column temperature, the study was able to achieve satisfactory separation of the analytes. This optimization of the chromatography conditions is necessary to obtain the desired separation of the components and enhance the accuracy and precision of the analysis. When mixed with acetonitrile and formic acid, the resolution improved and acrylamide was well separated from the other peaks. It was concluded that the optimal eluent composition was a mixture of acetonitrile and formic acid with a certain ratio. It appears that the study investigated the effect of column temperature on the retention time of acrylamide and interferents using HPLC. The study found that a negative association existed between retention time and column temperature between 35 and 45 °C. Based on this finding, a column temperature of 30°C was chosen. The study also investigated the elution pattern that could distinct acrylamide from interferents at a practical retention time: 1.6 minutes. The elution pattern that worked well consisted of HPLC grade water: 40% (v/v), acetonitrile: 60% (v/v), and formic acid: 0.10% (v/v). The eluent resulted in highly resolved chromatograms. the study provides important information on how to separate acrylamide from interferents using HPLC, including the optimal column temperature and elution pattern. The use of 0.1% formic acid in the eluent helped in reducing noise and undesirable peaks in the chromatogram. The flow rate was kept at 0.8 mL/min to ensure a

consistent elution rate and sufficient separation of the analytes. Using a UV-Vis detector the detection was performed at 210 nm, with an injection volume of 10 µL. This detection wavelength was chosen as it is specific to the absorption spectrum of acrylamide, making it possible to accurately detect and quantify the analyte. The developed method was found to be simple, fast, and accurate with high recovery rates of acrylamide. The method was validated by analyzing the spiked food samples and it was found to be effective in quantifying acrylamide levels in real food samples with a good precision and accuracy. The method can be useful in monitoring the acrylamide levels in different food products and ensuring the safety of food consumed by the general population [26, 27].

3. Result and Discussion

An over viewofthereportedmethodologiesforthedetermination of acrylamide in food samples was shown inTable 5.1 that without applying sample L-asparaginase formation of acrylamide is higher,for French fry-75.23µg/kg, nimki -47.35µg/kg,fried fish-50.05µg/kg, Friedchicken -45.05µg/kg. After applying Pure L-Asparaginase from Sigma (Deisenhofen,Germany) and Purified L-Asparaginase produced by *Bacillus paramycoides* MRS4 in different dose and time i.c 50IU for 15min and 100IU for 30min & 60 min ,the reduction is maximum shown in 100IU dose for 60min for Pure L-Asparaginase about 0.5237µg/kg, 0.5183µg/kg, 0.4735µg/kg, 0.5494µg/kg and forpurified L-Asparaginase produced by *Bacillus paramycoides* MRS4a bout 0.8327 µg/kg, 0.8197 µg/kg, 0.7175 µg/kg, 0.8184 µg/kg in French fry, nimki, fried fish, Friedchicken respectively shown in Table 5.2.

Table 5.1: Acrylamide content in different food samples

Sl.no	Sample	Without Appling Asparaginase (µg/kg)	Time-15 minute Dose-0.5mL (µg/kg)	Time- 30 minute Dose-1.0 mL (µg/kg)	Time- 60 minute Dose-1.0 mL (µg/kg)
1	French Fry	75.23	71.68	0.9752	0.8327
2	Nimki	47.35	44.09	0.8611	0.8197
3	Fried Fish	50.05	47.61	0.83277	0.7175
4	Fried Chicken	45.05	39.80	0.8839	0.8184

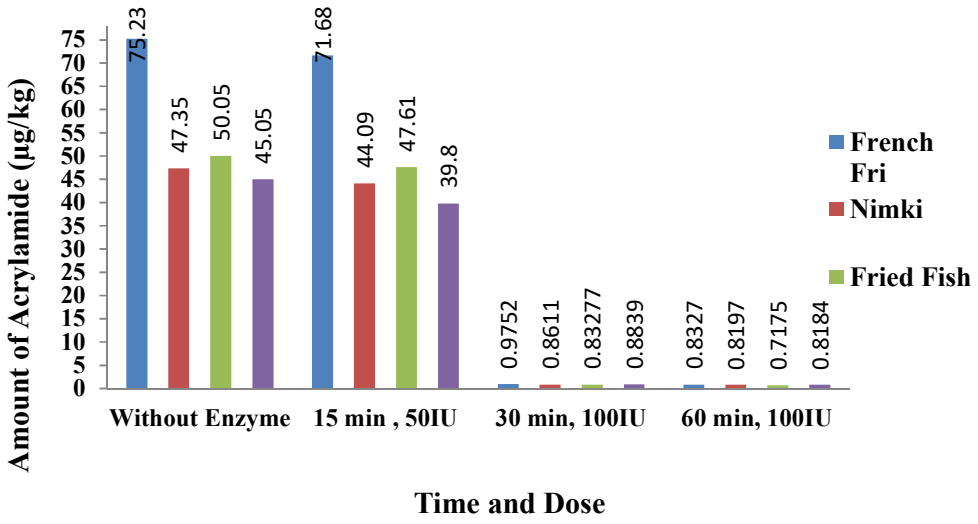

Figure 5.2: Comparison graphs for Acrylamide Contain in Different Food samples

Table 5.2:- Acrylamide Content in Different Food samples by applying Pure L-Asparaginase and L-Asparaginase produced by *Bacillus paramycoides* MRS4

Sl.no	Sample	Pure L-Asparaginase Time-15 minute Dose-50IU (µg/kg)	Pure L-Asparaginase Time- 30 minute Dose-100 IU (µg/kg)	Pure L-Asparaginase Time- 60 minute Dose-100 IU (µg/kg)	L-Asparaginase produced by MRS4 Bacillus paramycoides Time-15 minute Dose-50 IU (µg/kg)	L-Asparaginase produced by MRS4 Bacillus paramycoides Time- 30 minute Dose-100 IU (µg/kg)	L-Asparaginase produced by MRS4 Bacillus paramycoides Time- 60 minute Dose-100 IU (µg/kg)
1	French Fry	71.35	0.6602	0.5237	71.68	0.9752	0.8327
2	Nimki	43.78	0.5601	0.5183	44.09	0.8611	0.8197
3	Fried Fish	47.29	0.5274	0.4735	47.61	0.83277	0.7175
4	Fried Chicken	39.48	0.5897	0.5494	39.80	0.8839	0.8184

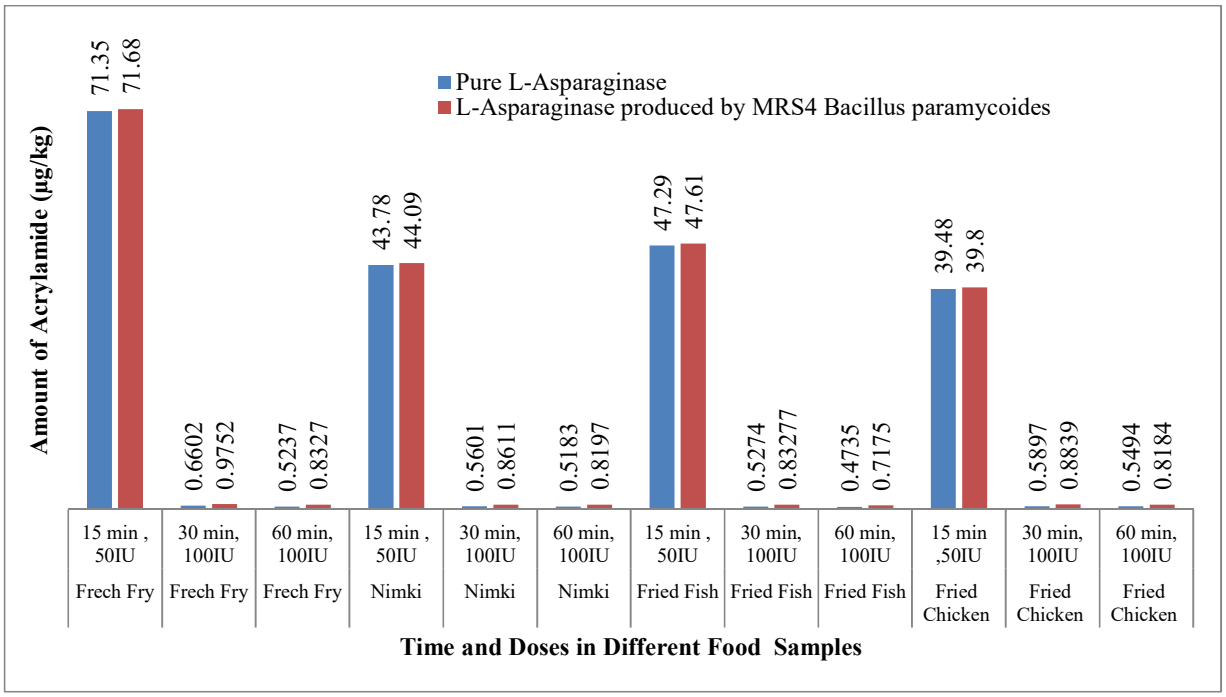

Figure 5.3: Comparison graphs for Acrylamide Contain in Different Food samples by applying Pure L-Asparaginase and L-Asparaginase produced by *Bacillus paramycoides* MRS4

4. Conclusion

The establishment of a tolerable daily intake (TDI) for acrylamide is important in ensuring that exposure to this chemical is minimized and that potential health risks are reduced. The TDI is the quantity of a substance that can be consumed daily over a lifetime without producing adverse health effects. The new toxicology study from the US suggests that a TDI of 2.6 micrograms of acrylamide per kilogram of body weight should be set to avoid the risk of cancer. For a person weighing 70 kg, this would translate to a tolerable daily intake of 182 micrograms of acrylamide. It is important to note that the establishment of a TDI is based on available scientific data and is subject to change as new information becomes available. It is also important for individuals to be aware of the sources of acrylamide in their diet and to take steps to minimize their exposure to this chemical

LA treatment has shown promise in reducing acrylamide levels in processed foods and can be a potential solution to mitigate the cancer risk associated with acrylamide. However, more research is needed to optimize the effectiveness of LA and to address any potential impact on the sensory characteristics of foods. The introduction of new LA will depend on further studies and approvals from regulatory agencies.

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Annexure I

List of frequently used Abbreviations/Symbols:

Abbreviations:

Standard Abbreviated Terms:

ANOVA : Analysis of Variance
ATP: Adenosine Triphosphate
APS: Ammonium Per Sulfate
Ca: Calcium
DMSO: Dimethyl Sulfoxide
D-Value: Decimal Reduction Time
EDTA:Ethylene Diamine Tetraacetic Acid
G: Gibbs free energy
g: Gram
H: Enthalpy
HCL: Hydrochloric Acid
hr: Hour
IU: International Unit
k: Maximum Substrate utilization rate
kDa: Kilo Dalton
kJ: Kilo Joule
LA: L-Asparaginase
L: Liter
M: Molarity
mg: Milligram
mL: Milliliter
mM: Millimole
mm: Millimeter
N:Normality
NaOH: Sodium Hydroxide
OD: Optical Density
P-Value: Probability Value
p.s.i.g: Pound per Square Inch(Gauge)
PAGE: Poly-acrylamide Gel Electrophoresis
rDNA: Ribosomal DNA
rpm: Rotation Per Minute
S: Concentration of Substrate
SDS: Sodium dodecyl-sulfate
TCA: Tri-chloro Acetic acid
TEMED: Tetra-methyl ethylene di-amine
U: Specific Substrate utilization rate
UV: Ultraviolate
Y: Yield Coefficient

Special Abbreviated Terms used in Thesis:

FTBE: Food Technology and Biochemical Engineering
JU: Jadavpur University

Symbols:

°C : Degree Centigrade
%: Percentage
µg: Microgram
µm: Micrometer

μmole: Micromole
v/v:Volume Per Volume
w/v: Weight per Volume
K_s: Half Saturation Constant
K_d: Death/Decay coefficient
∑: Summation

Annexure II

Sacnned Image of Publised Papers

Isolation and Characterization of Microbial Asparaginase to Mitigate Acrylamide Formation in Food

13

Mausumi Ray, Sunita Adhikari (Nee Pramanik), and Pradyut Kundu

Abstract

Asparaginase is an enzyme which is used in food processing industry and also used as a medicine. It is used to treat acute lymphoblastic leukemia, acute myeloid leukemia and non-Hodgkin's lymphoma. In food manufacturing it is used to decrease the acrylamide which is occurring in some starch-based foods during baking, frying and roasting. Acrylamide has carcinogenic effect on animals and human. The free amino acid asparagine reacts with sugars like glucose and fructose during Maillard reaction under high temperature and low moisture condition. To reduce acrylamide in food products, bacterial LA (L-asparaginase) is used. LA catalyzes the conversion reaction of L-asparagine to L-aspartic acid and ammonia. In present investigation, characterization of an extracellular LA from an isolated *Bacillus* sp. strain M6 was carried out in batch scale fermentation process. The effect of pH, temperature and incubation time was measured and the highest asparaginase activity (47 IU/ml) was achieved at pH 7.0, temperature 30 °C.

Keywords

Acrylamide · L-asparaginase · *Bacillus* sp

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13.1 Introduction

Acrylamide is a chemical compound which has carcinogenic effects on human as well as on animals [1]. In 2008 a research work was conducted on acrylamide and it was found that different types of human cancer such as ovarian, endometrial, breast and kidney cancer are linked with high exposure to acrylamide [2, 3]. Acrylamide is linked with asparagine and Maillard reaction in foods [4–8]. Many processes are used to reduce the formation of acrylamide in foodstuffs. Acrylamide formation during baking and frying depends on moisture content and baking temperature/time. Longer baking times are required to minimize the formation of acrylamide if final products are prepared to the same final moisture but with less color development [9, 10]. The acrylamide load can be reduced by substituting ammonium bicarbonate with inverted sugar and raising agents or by spraying with glycine or by adding inorganic salts and organic acids [11, 12]. Alteration of the sensorial properties like flavor, texture, browning [13] and formation of other undesirable compounds [14] may lead to less consumer acceptance. The changes may even affect the technology of the process like reduction of yeast fermentation properties in bread [15].

It has been also found that asparagine is the major ingredient for the formation of acrylamide in cereal food products. Increased amount of asparagine in various cereals can enhance the production of acrylamide. To reduce acrylamide in different baked and fried products, the free asparagine content needs to be reduced by the enzyme asparaginase that hydrolyzes asparagine to aspartic acid and ammonia. Over 90% of acrylamide content is reduced when asparaginase-treated mashed potato, potato flakes, rye flour, and wheat flour are incubated [8, 11, 16].

The objective of the preseat work is to isolate a potent bacterial strain capable of producing asparaginase enzyme and optimize various process parameters for maximum production of the desired enzyme.

13.2 Materials and Methods

13.2.1 Isolation of a Potent Bacterial Strain

For the isolation of a potent bacterial strain, 10 g of soil was transferred to conical flask containing 100 ml of sterile modified M9 medium having the following composition (g/L): KH_2PO_4, 3.0; Na_2HPO_4, 6.0; NaCl, 0.5; $MgSO_4.7H_2O$, 0.12; and $CaCl_2.2H_2O$, 0.001. The medium was enriched with 1% asparagine. Then the conical flask was kept in incubator for 24 h. The diluted soil sample was added to the modified M9 agar medium enriched with 1% asparagine and 0.005% phenol red. Plates were incubated at 37 °C for 24 h. The asparaginase enzyme production was accompanied by an increase in pH of the medium; as a result there will be formation of pink zone around the colonies that help in identification of asparaginase production. The selected colonies were isolated and maintained on nutrient agar slant at 4 °C.

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Use of Microbial Asparaginase to Mitigate Acrylamide Formation in Fried Food

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Review Article

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Abstract

Acrylamide or 2-propenamide is an industrial chemical formed in some foods particularly starchy foods during heating process such as baking, frying and roasting. Acrylamide is present in significant quantities in carbohydrate rich foods such as Potato chips, French fries and Bakery products up to 7000 ppb followed by protein rich foods up to 400 ppb. In general, deep fat fried potato products, roasted coffee beans and bakery products are the most important sources of acrylamide. 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 possible strategies of acrylamide reduction were grouped into four categories i.e., selection of raw materials, changing formulation and product composition without affecting the taste and preferences of consumers, pre-treatment procedures and optimized processing conditions. The use of microbial L-asparaginase (LA) is one of the alternative approaches for acrylamide reduction in food stuffs as it catalyzes the conversion of L-asparagine to L-aspartic acid and ammonia.

Keywords: Acrylamide; Asparagine; Glycine

Introduction

Asparaginase, an enzyme is used in food processing industry and also used as a medicine. It is used to treat acute lymphoblastic leukemia, acute myeloid leukemia and non-Hodgkin's lymphoma. In food manufacturing it is used to decrease the acrylamide which is occurred in some starch based foods during baking, frying and roasting. Acrylamide has carcinogenic effect on animals and human. The free amino acid Asparagine reacts with sugars like glucose and fructose during Maillard reaction under high temperature and low moisture condition. To reduce acrylamide in food products bacterial LA (L-asparaginase) is used. Acrylamide has carcinogenic effects on human as well as animals [1]. Recent research work conducted in 2008 on acrylamide has shown different type of human cancer such as human ovarian, endometrial, breast and kidney cancer are linked with high exposure to acrylamide [2, 3]. Acrylamide is linked with Maillard reaction and particularly asparagine in

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Microbial production of L-asparaginase by an isolated *Bacillus paramycoides* MRS4 strain and optimization of process parameters

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L-asparaginase, a hydrolytic enzyme which breakdowns L-asparagine, a non-essential aminoacid to L-aspartic acid with the release of ammonia. In this research work various sources like soil, cereals: corn and oat, pulses: soya bean, vegetable: potato were used for isolation of L-asparaginase producing bacterial strain. It was found that among the all isolated bacterial strain, a strain isolated from soya bean produced maximum amount of enzyme. The isolated strain MRS4 was found having significant similarity with *Bacillus paramycoides* strain MCCC 1A04098 based on nucleotide homology and phylogenetic analysis. After optimization of various process parameters, it was found the strain was able to produce almost 118 IU/ml of enzyme in the selected medium when inoculated with 1% inoculums of 20 hr age in 50 ml medium taken in a 250 ml Erlenmeyer flask and incubated at 35°C, pH 7, agitation speed of 150 rpm, aeration rate of 0.25 L/min for 24 hrs.

Keywords: L-asparaginase, bacterial strain, nucleotide homology, phylogenetic analysis, *Bacillus paramycoides*

Introduction

Asparaginase, an enzyme that is used in the processing of food industrially and also used as a medicine. In food processing industry asparaginase is used to reduce acrylamide content which forms in starch-based food during high temperature processing like baking, frying and roasting. The compound acrylamide is found having carcinogenic effect on both human and animals. The free amino acid asparagine present in the starch-based food reacts with sugars during high temperature together with low moisture processing condition[1]. The transformation of asparagine to aspartic acid and NH_3 is catalyzed by bacterial L asparaginase enzyme[2]. So, it is used to reduce the acrylamide content high temperature processed starch-based foods. Acrylamide was found in different fried and baked foods like fried potatoes, potato chips, coffee, biscuits, bread french fries[3]. In addition to that L-asparaginase is considered as a therapeutic agent for handling of different types of cancer[4]. Numerous types of tumour cells need L-asparagine for synthesis of protein. In presence of L-asparaginase they are lacking an essential growth factor. It causes cytotoxicity of leukemic cells[5]. L-asparaginase produces aspartate that is a precursor for the production of ornithine in urea cycle as well as acts as a precursor for the formation of oxaloacetate to generate glucose through gluconeogenic pathway within the human body[6].

L-asparaginase could be produced from different plants, animals and various microorganism like fungi, yeasts, bacteria, algae and actinomycetes[7-8]. Microbial source of L-asparaginase is more popular because of its stability, high productivity, easy extraction and purification. It is known that numerous microorganisms can produce L-asparaginase mostly by submerged fermentation process. Some examples of asparaginase producing microbial species are *Aerobacter, Bacillus, Pseudomonas, Serratia, Xanthomonas, Photobacterium*[9], *Streptomyces* sp.[10], *Proteus* sp.[11], *Vibrio* sp.[12] and *Aspergillus* sp.[13]. The enzyme is though widely distributed but only few of them are having anti-neoplastic activity. Most commercially available microbial sources are *Escherichia coli, Erwinia carotovora* and *Serratia marcescens*[14]. The therapeutic enzymes are also having the problem of toxicity[15]. So, there is a continuous search for asparaginase enzyme that have therapeutic effect with less or no toxicity. There is a constant interest to isolate organism from diverse sources[16].

The objective of the current study is to isolate asparaginase producing bacterial source from some different sources which are not reported yet, identify it

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STATISTICAL OPTIMIZATION OF PROCESS PARAMETERS BY CENTRAL COMPOSITE DESIGN (CCD) FOR AN ENHANCED PRODUCTION OF L-ASPARAGINASE BY AN ISOLATED *BACILLUS PARAMYCOIDES* STRAIN MCCC 1A04098

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ABSTRACT :

Asparaginase is an enzyme which is used in food processing industry and also used as a medicine. It is used to treat acute lymphoblastic leukemia , acute myeloid leukemia and non-Hodgkin's lymphoma. In food manufacturing it is used to decrease the acrylamide which is occurred in some starch-based foods during baking, frying and roasting. Acrylamide has carcinogenic effect on animals and human. The free amino acid Asparagine reacts with sugars like glucose and fructose during Maillard reaction under high temperature and low moisture condition. To reduce acrylamide in food products bacterial LA (L-asparaginase) is used. LA catalyzes the conversion reaction of L-Asparagine to L-Aspartic acid and ammonia. In present study fermentative production of bacterial LA (L-asparaginase) was carried out in a laboratory scale fermenter using an isolated bacterial strain having significant similarity with *Bacillus paramycoides* strain MCCC 1A04098 obtained from nucleotide homology and phylogenetic analysis.

The present research aimed to develop the efficient technique for the production of bacterial LA (L-asparaginase) by optimizing the operating parameters with the help of response surface methodology. The effect of three independent variables such as aeration rate (0.5 – 2.5 L/min), agitation speed (100-200 rpm), and medium volume (3.0-5.0 L) for maximum production of bacterial LA (L-asparaginase) was investigated. A quadratic model was proposed to correlate the independent variables for maximum bacterial LA (L-asparaginase) production at optimum process condition such as constant medium pH of 7.0, constant medium temperature of 35 °C and constant inoculum concentration of 2% by using central composite design (CCD) method. The performance of the RSM model was statistically evaluated in terms of coefficient of determination, R^2 through regression analysis of quadratic models. The value of predicted R^2 of 0.8202 is in reasonable agreement with the Adjusted R^2 of 0.9409; that is the difference is less than 0.2. Analysis of variance (ANOVA) results revealed that the strain was able to produce almost 117 IU/mL of enzyme in the selected medium composition at optimum process condition and shows the highest asparaginase activity at aeration rate of 1.5 L/min, agitation speed of 150 rpm and medium volume of 4 L. ANOVA result also shows F-value of 34.63 and P-values less than 0.0500 which indicate model terms are significant.

Keywords: L- Asparaginase production , *Bacillus paramycoides*, Fermenter, Optimization of process parameter

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