CHARACTERIZATION AND APPLICATION OF AMYLASE AND XYLANASE ENZYMES FROM *BACILLUS CEREUS* IN ENHANCING MANGO JUICE CLARIFICATION

A Dissertation Submitted to the
Department of Microbiology,
Central Campus of Technology,
Tribhuvan University, Dharan, Nepal,
in Partial Fulfillment of the Requirements for the Award of
Degree of Master of Science in Microbiology (Food)

BY:
Priyanka Thakur
Roll No: MB 1178/074
TU Regd. No: 5-2-589-169-2012

RECOMMENDATION

This is to certify that **Mrs. Priyanka Thakur** has completed this dissertation work entitled **"Characterization and Application of Amylase and Xylanase Enzymes from *Bacillus cereus* in Enhancing Mango Juice Clarification"** as a partial fulfillment of the requirement of M.Sc. degree in Microbiology **(Food)** under my supervision. To my knowledge, this work has not been submitted for any other degree.

……

Mr. Shiv Nandan Sah

Assistant professor

Thesis Supervisor

Central Campus of Technology

Tribhuvan University

Hattisar, Dharan, Nepal

Date: ...../……/…...

CERTIFICATE OF APPROVAL

On the recommendation of Asst. Professor **Mr. Shiv Nandan Sah** this dissertation work of **Mrs. Priyanka Thakur** entitled **"Characterization and Application of Amylase and Xylanase Enzymes from *Bacillus cereus* in Enhancing Mango Juice Clarification"** has been approved for the examination and is submitted for the Tribhuvan University in Partial fulfillment of the requirements for M. Sc degree in Microbiology (Food).

……..

Mr. Dhiren Subba Limbu

Program Coordinator

M.Sc. Microbiology

Central Campus of Technology

Dharan, Nepal

Date: ...../...../.....

BOARD OF EXAMINERS

Recommended by:

……
Mr. Shiv Nandan Sah
(Supervisor)
Assistant professor
Department of Microbiology
Central Campus of Technology
Hattisar, Dharan, Nepal

Approved by:

…….
Mr. Dhiren Subba Limbu
Program Coordinator
M.Sc. Microbiology
Central Campus of Technology
Hattisar, Dharan, Nepal

……..
Mrs. Kamana Bantawa
(Assistant Campus Chief)
Assistant professor
Department of Microbiology
Central Campus of Technology
Hattisar, Dharan, Nepal

Examined by:

……
Mrs. Babita Adhikari
(Internal Examiner)
Assistant professor
Central Campus of Technology
Hattisar, Dharan, Nepal

……..…….....
Prof. Dr. Dhan Bahadur Karki
(External Examiner)
Dharan Multiple Campus
Dharan-16, Sunsari, Nepal

Date: ...../...../.....

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my esteemed supervisor, **Assistant Professor Mr. Shiv Nandan Sah,** for his unwavering support, guidance, and encouragement throughout my research journey. Without his invaluable assistance, consistent mentoring, and supervision, the successful completion of this dissertation would not have been possible.

I am deeply indebted to our Campus Chief, **Associate Prof. Dr. Dil Kumar Limbu**, Program Coordinator (M.Sc. Microbiology), **Mr. Dhiren Subba Limbu** and the Head of Department (Department of Microbiology)/ Assistant Campus Chief, **Mrs. Kamana Bantawa** at Central Campus of Technology, Dharan for their generous provision of facilities and invaluable guidance during the dissertation process.

In addition, I extend my profound appreciation to the entire teaching faculty, laboratory staff and library staff of the Department of Microbiology at Central Campus of Technology. Your expertise, guidance, and unwavering support have been instrumental in shaping this research. Furthermore, I wish to thank my dedicated classmates, whose assistance and support were instrumental in my academic endeavors.

I would like to express my sincere appreciation to my loving husband Er. Gajendra Kumar Jha and our beloved daughter, Shravya Jha for their unwavering support, understanding, and patience during this research.

Lastly, I extend my sincere thanks to my family members for their unwavering motivation and support during the challenging phases of this thesis.
This research has been a collaborative effort, and I am thankful to everyone who contributed to its success.

Mrs. Priyanka Thakur

Date: ...../...../.....

ABSTRACT

Enzymes with potential industrial applications, including the food industry, include amylase and xylanase. Fruit juice contains a variety of sugars that must be clarified without compromising the juice's quality; therefore, appropriate, high-quality enzymes are crucial for this process. The purpose of this study was to separate and characterize bacteria that produce xylanase and amylase from soil and fruit peel samples that were gathered in the Kathmandu Valley. The amylase was produced in a conical flask with nutritional broth containing 1% starch at 37 °C in a rotatory water shaker bath at 120 rpm. After 24 hours, the mixture was centrifuged at 10,000 for 5 minutes. The amylase activity in the supernatant was measured using the DNSA technique. After isolating 17 amylase-producing bacteria, one (6%) of them produced xylanase and was recognized as *Bacillus cereus* by means of extensive characterization techniques such as colony morphology, staining, biochemical testing, and 16S rRNA sequencing. The ideal pH and temperature for B. cereus amylase production were found to be 7.0 and 40°C, respectively. Mango juice was successfully clarified using *B. cereus*-extracted enzymes, indicating their possible use in industrial operations. Additionally, analyses of the isolated enzymes' physicochemical characteristics and structure predictions were carried out. The *B. cereus* strain K21 has shown promise as a source of amylase and xylanase enzymes, and it is very adept at clarifying fruit juice. These results demonstrate the enzymes' industrial potential, especially in the food processing industry.

Keywords: Amylase, Xylanase, *Bacillus cereus,* Mango juice, Clarification,16S rRNA

Table of Contents

List of Table

List of Figure

List of Photographs

List of Appendices

ABBREVIATIONS

AI	=	Amylolysis Index
B.	=	Bacillus
BBD	=	Box-Behnken Design
BLAST	=	Basic Local Alignment Search Tool
DNA	=	Deoxyribonucleic Acid
EC	=	Enzyme Commission
GRAVY	=	Grand Average of Hydropathicity Index
II	=	Instability Index
MLST	=	Multilocus Sequence Typing
N.	=	Nitrogen
PCR	=	Polymerase Chain Reaction
PBD	=	Plackett-Burman Design
PDBsum	=	Protein Data Bank Summary
PE	=	Pectinesterase
PG	=	Polygalacturonase
pH	=	Potential of Hydrogen
PI	=	Percentage Identity
PL	=	Pectinlyase
PROCHECK	=	PROtein CHECKer
PSIPRED	=	Protein Structure Prediction Server
Qc	=	Query Coverage
rRNA	=	Ribosomal Ribonucleic Acid
RSM	=	Response Surface Methodology
S	=	Svedberg
SNP	=	Single Nucleotide Polymorphism
SOPMA	=	Self-Optimized Prediction Method with Alignment
sp	=	Species

CHAPTER-I

INTRODUCTION AND OBJECTIVES

1.1Background

The use of presses, such as the traditional rack and cloth press, screw presses, Bucher-Guyer horizontal presses, and belt presses, is the most conventional way of juice extraction. Diffusion extraction, decanter centrifuge, screw type juice extractor, and fruit pulper are further methods for extracting juice. By combining these juice extraction procedures with other pre-treatments, such as cold, hot, and enzymatic extraction, the yield of juice can be raised. A considerable improvement in juice recovery over cold and hot extraction is provided by enzymatic treatment (Sharma *et al.*, 2017).

Fruit juices are clarified using a variety of enzymes, such as pectinases, amylases, cellulases, arabanases, fructozymes, dextranases, proteases, and amyloglucosidase. The commercial pectinase products used to digest fruit often include cellulases, amylases, and a combination of the enzymes pectinesterase (PE), polygalacturonase (PG), and pectinlyase (PL) (Singh & Singh, 2015).

Dr. Jokichi Takamine (1894,1914) was a pioneer in recognizing the industrial potential of cultured enzymes, primarily focusing on fungi, while Boidin and Effront later contributed to bacterial enzyme development. Technological advancements have rapidly shifted the role of cultured microbial enzymes, substituting animal or plant-based enzymes in various applications. For instance, bacterial amylase now replaces pancreatin or malt in textile desizing. Although only a few microbial enzymes are commercially used presently, this field's growth indicates a broader scope for future applications (Uwajima, 1987).

Xylanase enzymes offer a sustainable alternative in converting xylan into essential industrial products like paper, textiles, food, and biofuels. Integrating xylanases into production processes for these items enhances overall process economics. There's room for advancing current techniques or innovating new methods to optimize xylanase production for better efficacy and economic

feasibility (Bhardwaj *et al.*, 2019).

Bacillus (B.) cereus is a highly mobile bacterium that often appears as single cells but can occasionally form longer threads. It quickly liquefies gelatin when it comes into contact with it. It was first discovered in air in a cowshed (Frankland *et al.*, 1887). *B. cereus* has more recently come to be understood as the aetiological cause of both systemic infections and localized wound and ocular infections (Bottone, 2010).

A straightforward, widely used technique for identifying bacteria is the analysis of 16S rRNA sequences (Amann *et al.*, 1995). Early research, however, using a small number of isolates from the Bacillus cereus group, showed that species within this group's 16S rRNA sequences exhibited up to a 99–100% similarity (Ash *et al.*, 1991).

The starch business uses amylases the most frequently among commercially significant enzymes. Amylases make between 25–30% of the global market for catalyst (Deb *et al.*, 2013). High temperatures are used in a lot of industrial operations, but amylase has a limited range of uses because of its low stability. The stability issue is solved, and the catalytic activity and reaction rate are also improved by the discovery of thermostable amylases. Thus, the usage of thermostable amylase decreases potential microbiological contaminations and medium viscosity, which supports the starch processing industry at high temperatures (Homaei *et al.*, 2016).

Since it is expensive to produce enzymes, a low-cost growth medium is needed to support microbial growth and the synthesis of enzymes in order to meet industrial demand. The two possible cultivation techniques for microbial xylanase production are submerged fermentation and solid-state fermentation (Walia *et al.*, 2017). Short production times allow for great yields and cheap cost with submerged fermentation technology. Production of xylanase is significantly influenced by the nutrient medium's nutrient composition and the culture's environmental factors. The following physical and chemical variables are known to affect the production of xylanase: temperature, pH, incubation time, carbon and nitrogen sources and concentration, and agitation rate (Kereh & Mubarik, 2018). The growth of the organism (mesophilic, psychrophilic, or

thermophilic) has a major impact on temperature impacts on enzyme synthesis. Due to the sensitivity of microorganisms to the concentration of hydrogen ions in the medium, pH is one of the most crucial parameters influencing their growth (Jain & Pundir, 2011).

On the other side, bioinformatics is the application of computational and analytical techniques to gather and understand biological data (Webster *et al.*, 2002). Numerous software programs or programmers have been developed throughout the years and are currently being utilized for the simple examination of gene and protein sequences in a new branch of molecular biology known as bioinformatics or computational biology (Wormwood *et al.*, 2014).

Bioinformatics tools offer efficient and cost-effective means to analyze nucleotide and protein sequences, providing accurate results and streamlining the characterization process. These computational methods aid in diverse in silico analyses, spanning from identifying introns to predicting restriction enzyme cleavage sites in genes. They facilitate determinations such as molecular weights, isoelectric points (pI), subcellular localization, 3D protein structures, conserved domains, and hydrophobicity with precision and reduced time and cost (Blum *et al.*, 2009).

Enzyme technology, an evolving field within biochemical science, is gaining prominence in global industrialization. Leveraging biological agents, biotechnology applies scientific principles to manufacture products, offering potential for new energy-efficient industrial processes using renewable resources. It presents advantages over chemical catalysts, particularly in mild environmental conditions, with superior efficiency and selectivity in both industrial and analytical domains (Osho, 2018).

Advancements in recombinant DNA technology have revolutionized food preparation by tailoring new enzymes suitable for specific conditions, enhancing flavor and texture. Microorganisms, like bacteria and yeast, engineered through protein engineering or sampled from varied habitats, yield key enzymes (e.g., amylase, lipases). These high-performing enzymes, a product of modern biotechnology, optimize food processing settings,

significantly impacting businesses' efficiency and product quality (PANDIT, 2023).

Clarifying juices is a crucial step in the process of getting them ready for commercialization and meeting the high standards needed by consumers. The best juice clarity, the highest yield, and the creation of a high-quality product that consumers will want to purchase all depend on the use of enzymes (Bhat, 2000).

Clarifying juices is regarded as a standard and crucial procedure in the fruit juice industry with the goal of increasing consumer acceptance. The primary goal of this clarification process is to make the juice more appealing to consumers by enhancing its visual and color qualities. Juice clarity, however, goes beyond purely cosmetic benefits and contributes to the end product's nutritional value, quality, and safety (Conidi *et al.*, 2018).

1.2 Objectives

1.2.1 General Objectives

To evaluate mango juice clarification by using amylase and xylanase enzymes produced by *Bacillus cereus*

1.2.2 Specific Objectives

- To Isolate and identify amylase producing bacteria from soil and fruit peel samples
- To detect xylanase production among amylase producers
- To optimize amylase production parameter.
- To predict and evaluate the tertiary structure of amylase and xylanase Enzymes

1.3 Limitation

- Using 16S rRNA sequencing might pose limitations in identifying closely related bacterial strains accurately.
- While temperature and pH were optimized for Bacillus cereus growth, variations in these conditions might affect enzyme production differently.

- The stability and shelf life of the isolated enzymes under industrial conditions could be a potential limitation.
- The study focused primarily on amylase and xylanase enzymes; other potentially beneficial enzymes from Bacillus cereus might remain unexplored.

1.4 Statement of problem

Despite the wide industrial applications of enzymes like amylase and xylanase, there remains a dearth of comprehensive studies focused on indigenous microbial sources in Kathmandu, Nepal. This lack of exploration into the enzymatic potential of local bacterial strains, specifically *Bacillus cereus*, from soil and fruit peel samples, presents a critical knowledge gap. Understanding the enzymatic capacity of these local strains and their suitability for various industrial processes is essential. This study aims to isolate, identify, and characterize *Bacillus cereus* strains, elucidating their enzymatic profiles and exploring their viability for industrial applications. The lack of such localized studies limits our understanding of the biotechnological significance and industrial potential of these enzymes in the context of Kathmandu, Nepal.

1.5 Significance of the study

- **Industrial Application:** Discovering Bacillus cereus enzymes offers potential for eco-friendly industrial processes, benefiting sectors like food, textiles, and biotechnology.
- **Fruit Juice Quality:** Enzyme efficacy in fruit juice clarification, particularly in mango juice, can enhance its quality, impacting the beverage industry positively.
- **Microbial Diversity:** Exploration of enzyme-producing bacteria from diverse sources expands our knowledge of microbial diversity and their potential biotechnological roles.
- **Production Optimization:** Insights into optimal conditions for enzyme synthesis pave the way for cost-effective, large-scale production, benefiting enzyme-dependent industries.

CHAPTER-II

LITERATURE REVIEW

2.1 Enzymes in Fruit Juice Processing

In fact, enzymes are employed in many different industries, such as pulp & paper, pharmaceuticals, food, textiles, and chemicals (Kumar, 2015). According to the reaction they catalyze, enzymes are divided into six categories and assigned an Enzyme Commission (EC) number. The following are these groups: Oxidoreductases (EC1), Transferases (EC2), Hydrolases (EC3), Lyases (EC4), Isomerase (EC5), and Ligases (EC6). The first, second, third, and fourth digits of these numbers, in that order, represent the kind of bond that is involved in the reaction, the category of the enzyme, and the specificity of the bond (Basheer *et al.*, 2021).

Pulp is mixed with water in a variety of ratios. Other studies indicate that the enhanced tissue breakdown brought on by the repeated freezing and thawing of whole fruit, along with the pectinase enzyme treatment of fruit macerate, resulted in higher solids (McLELLAN *et al.*, 1985). Apple pomace enzyme extraction using hot water and a mixture of pectinases and cellulases yields a higher yield. Date fruit pulp was combined with three times the amount of water before adding enzyme to extract juice (Al-Hooti *et al.*, 2002).

Traditional juice extraction methods often fall short in both quantity and quality, while enzymatic extraction, primarily employing pectinase, cellulase, and hemicellulase, significantly enhances yield. Enzymatic clarification further enhances juice appearance, reducing turbidity, viscosity, and improving filterability. They also prevent browning and bitterness in citrus juices. Enzyme-processed juices offer improved nutraceutical content and sensory attributes, mitigating bitterness and preventing darkening (Kumar, 2015).

Amylases are enzymes that break down starch. They are abundantly found throughout the kingdoms of microbes, plants, and animals. They break down starch and similar polymers to produce byproducts that are unique to each

amylolytic enzyme. The name "amylase" was first used to refer to enzymes that could hydrolyze the glucosidic linkages in amylose, amylopectin, glycogen, and their breakdown products (Bernfield, 1955 ; Aiyer, 2005).

An enzyme called amylase helps break down starch into sugars. Human saliva contains amylase, which kicks off the chemical process of digesting. In addition to bacteria, fungus can also produce amylase. All amylases are glycoside hydrolyses that operate on a-1,4-glycosidic bonds. Amylases are commonly utilized in the production of processed foods, such as bread, beer, digestive aids, cakes, fruit juices, and starch syrups. Clarity of fruit by amylase (Couto & Sanromán, 2006).

Digestive enzymes called amylases dissolve the glycoside bonds in starch. One of the most crucial enzymes is amylase, which is important for biotechnology. They supply around 25% of the enzymes sold globally (Kulkarni *et al.*, 1999). Endoamylases and exoamylases are the two subtypes of amylases. Exoamylases function sequentially from the non-reducing end, producing short end products, as opposed to endoamylases, which randomly split the center of the starch molecule. There are three different categories of enzymes: Alpha (α) -amylase, Beta (β)-amylase, Gamma (γ)-amylase. One of the most well-known and significant types of industrial amylases is α-amylase (Gupta *et al.*, 2003).

Due to their simplicity in manufacture, substrate specificity, and environmentally friendly chemistry, enzymes are frequently used in industrial operations. In secondary plant cell walls, xylan, the main renewable hemicellulosic polysaccharide, is made up of a β-1,4-linked D-xylopyranose backbone that has been predominately substituted with L-arabinofuranosyl, acetyl, and glucuronosyl residues (Shallom & Shoham, 2003). The coordinated action of multiple enzymes, including xylanase, β-xylosidase, and enzymes that cleave groups in the side chains, is necessary for its full hydrolysis. The hydrolytic cleavage of glycosidic linkages in the xylan backbone by xylanase (endo-β-1,4-D-xylan xylanohydrolase; EC 3.2.1.8) (Dhiman *et al.* 2008; Morosoli *et al.* 1986). For industrial purposes, xylanase is frequently produced by bacteria and fungi. Yeast, bacteria, and filamentous

fungi all produce xylanase in the extracellular space. The fungus Trichoderma, Aspergillus, Fusarium, and Pichia are excellent xylanase producers. Xylanases have been found in *Bacillus, Streptomyces,* and other organisms. (Motta *et al.*, 2013).

The transformation of the linear polysaccharide β-1,4-xylan into xylose is catalyzed by xylanase enzymes. By using solid-state fermentation, Bacillus megaterium, which was isolated from rice paddy soil, was used to produce xylanase. After that, crude xylanase was purified with a 40–60% saturated ammonium sulfate solution. When applied to untreated sugarcane bagasse, it yielded more reducing sugars (3200 g/ml) compared to acid- and alkali-treated bagasse (1460 g/ml and 1760 g/ml). Xylanase efficiently clarified apple (20%), tomato (16%), and pineapple (44%) juices, rich in hemicelluloses (Manju Phadke, 2015).

2.2 Bacterial Enzyme Production

There is no end to the supply of microbial enzymes thanks to the advancement of fermentation techniques. Microbial cells from the fungal, bacterial, or yeast families are chosen for the manufacture of industrial enzymes. These days, a lot of protease, glucamylase, alpha-amylase, and glucose isomerase are created (Volesky *et al.*, 1984).

All living cells, including microbes, contain enzymes. Different strains of organisms produce various metabolic enzymes, which can be hydrolyzing, oxidizing, or reducing agents. Enzyme synthesis rates, however, differ between species and even strains of the same species. By choosing strains that can manufacture the desired enzymes in the greatest amounts, one can maximize the production of commercial enzymes (Underkofler *et al.*, 1958).

In addition to preventing congealing in chocolate and licorice syrups, recovering sugars from starch-rich candy residues, processing cereal products to create food dextrin and sugar blends, and boosting the formation of clear pectin, microbial amylases have a variety of uses. Additionally, they facilitate the removal of starch for the processing of flavoring extracts, fruit extracts,

and juices as well as the modification of starch in vegetable purees for canning (Mishra & Behera, 2008).

Xylanase is widely found in nature, spanning marine and terrestrial bacteria, fungi, crustaceans, insects, algae, plants, and seeds. Industrial xylanase production predominantly utilizes fungi and bacteria. Notably, *Bacillus species (sp.)* like *B. halodurans, B. pumilus, B. subtilis, B. amyloliquefaciens, B. circulans,* and *B. stearothermophilus* are known for their robust xylanolytic enzyme production. Xylanase has been isolated and purified from a variety of bacteria living in harsh conditions that are high-temperature stable, acid/alkali resilient, and cold tolerant (Bhardwaj *et al.*, 2019).

2.3 Bacillus cereus as an Enzyme Producer

Aspergillus niger, Penicillium species (sp), Cephalosporium species (sp), Rhizopus species (sp), and Neurospora species are among the fungi that make amylase enzymes, as are *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Bacillus licheniformis, and Bacillus amyloliquefaciens*. Bacillus-produced -amylase is active at high temperatures (80-90 °C), has a stable molecular weight of 50 kDa, and functions well in the pH range of 5.5 to 9.0. Its biosynthesis takes place as a result of substrate induction and catabolic suppression as the cell enters the stationary phase after exponential development. Gram-positive, rod-shaped *Bacillus sp*. is motile by means of peritric and sporogenic flagella (Welker & Campbell, 1967).

Microscopic analysis confirmed the bacteria as *B. cereus*. The Amylolysis Index (AI), calculated as the ratio of amylolysis halo diameters to colony diameters, was 3.3, indicating strong amylolytic enzyme production capability. The successful experiments demonstrated that *Bacillus cereus* can produce potent amylase enzymes suitable for various industrial applications. To maximize microbial amylase production, growth parameters such as culture media, pH, temperature, rpm, time, growth factors, amylase inducers, and downstream technologies must be optimized. (Mario *et al.*, 2021).

Microbial xylanases offer promising biotechnological potential. This study focused on Bacillus cereus BSA1's xylanase synthesis control. Xylan presence boosted synthesis, further enhanced by xylan addition (0.5% to 6.02 U/ml) and low xylose and arabinose concentrations. Glucose (approx. 0.1%) in xylan-supplemented media inhibited synthesis, as did higher xylose and arabinose levels (>0.1%). Cyclic adenosine monophosphate partially alleviated glucose-mediated suppression, and chemicals like 2-4-dinitrophenol, disrupting ATP formation, hindered xylanase synthesis, highlighting its energy-dependent process (Mandal *et al.*, 2012)

2.4 Molecular Identification and Characterization

We analyzed 183 16S rRNA and 74 23S rRNA sequences across *Bacillus cereus* group species to determine if rRNA sequence variations could differentiate group members. The group was divided into seven subgroups, including the following: Anthracis, Cereus A and B, Thuringiensis A and B, and Mycoides A and B. These subgroups were then divided into two separate clusters based on 16S and 23S rRNA sequence variants. (Bavykin *et al.*, 2004).

Bacillus species, in particular *B. cereus*, have a genetic makeup that is varied and ambiguous. Universal 16S primers are unable to distinguish between bacterial species that are closely related. Therefore, for precise identification of *Bacillus* species, including *Bacillus cereus*, *B. anthrax*, and *B. thuringiensis*, we created and validated *Bacillu*s species-specific 16S rRNA primers. To identify the genetic diversity within the Korean-isolated wild-type *Bacillus* species, we constructed a phylogenetic tree and carried out genetic analysis (Rahman *et al.*, 2022)

16S rRNA gene sequencing has been limited in clinical microbiology labs due to cost, technical expertise, and the absence of user-friendly analysis software and validated databases (Clarridge, 2004). Currently, GenBank (NCBI) contains more than 29,000,000 entries for 16S sequences of varying lengths and quality derived from a variety of bacteria recovered from various clinical and environmental sources; while many of these entries only contain 16S

sequences, an increasing number of partial or entire genomes containing complete 16S sequences are also being deposited. (Clarridge *et al.*, 2001).

The primer sets 27-F (5′-FAM) and 1492-R were used to target the bacterial 16S rDNA genes. A 50-l reaction mixture including 60 nanogram (ng) of pooled triple-extraction DNA template, 1 ReadyMix from KAPA Biosystems in Cape Town, South Africa, and 0.5 M of each primer from Inqaba Biotec in Pretoria, South Africa was used for the Polymerase Chain Reaction (PCR). Without adding pH indicator to Buffer PB, the PCR products were purified using the QIAquick® PCR Purification Kit from Qiagen in Hilden, Germany. Within 50 l of distilled water, DNA was eluted (Claassen *et al.*, 2013).

The Infectious Bursal Disease Virus (IBDV) strain was isolated in 2014 from a flock of commercial broilers who were 27 days old and had respiratory issues and elevated mortality. Using the TRIzol reagent (Invitrogen), viral RNA was extracted from bursae tissue. Random hexamers (Thermo Scientific) were used to create first-strand cDNA. Real-time PCR was used to determine the IBDV's low virulence. Reverse transcription PCR with overlapped consensus primers and direct sequencing were used to get the whole genome sequence of this isolate. Macrogen Inc. (Seoul, Republic of Korea) sequenced purified goods in both directions. The SeqMan software from Lasergene was used to construct and modify sequences. Phylogenetic trees were created using PhyML, and multisequence alignments were carried out with MEGA5 (Tomás *et al.*, 2015).

The number of allele differences is used for genome comparison, much as the number of Single Nucleotide Polymorphism (SNP) differences, and each gene or allele sequence is converted to a number. The gathered short reads are compared using the Basic Local Alignment Search Tool (BLAST) to the Multilocus Sequence Typing (MLST) scheme reference allele database, which includes all known allelic variations for each locus allocated for a specific species. The kind of MLST is established by this comparison. SNPs, indels (insertions and deletions), and recombination are three different variants that might affect the same gene and are classified as having a single allele difference (Jagadeesan *et al.*, 2019).

Galaxy and Konstanz Information Miner (KNIME) are popular software tools for distributing complex data analysis tasks across grids or clouds. Cloud-

based bioinformatics applications include BLAST for sequence alignment, HBLAST (a high-performance BLAST implementation), Trans-Proteomic Pipeline (TPP) for proteomic analysis, HIPPIE for promoter analysis via Amazon Machine Image, and BG7 for bacterial genome annotation on Amazon Web Services (Alkema *et al.*, 2016).

Thermo ScientificTM SureTectTM Listeria monocytogenes test is a new real-time PCR test for the detection of Listeria monocytogenes in food and environmental samples. The following foods and food contact surfaces were used in the Performance Tested MethodsSM program of the AOAC Research Institute (AOAC-RI) to validate the assay in comparison to the reference method described in International Organization for Standardization 11290-1:1996, including Amendment 1:2004: smoked salmon, processed cheese, fresh bagged spinach, fresh cantaloupe, cooked prawns (chilled product), cooked sliced turkey meat (chilled product), ice cream, and pork frankfur Each matrix was evaluated by Thermo Fisher Scientific's Microbiology Division in Basingstoke, UK (Cloke *et al.*, 2014).

2.5 Optimization of Enzyme Production

This study utilized statistical modeling techniques such as Plackett-Burman Design (PBD) and Box-Behnken Design (BBD) to successfully optimize the production of xylanase by a *T. harzianum* strain in submerged fermentation. A 4.16-fold increase in enzyme activity with BBD compared to One-Factor-at-a-Time (OFAT) and a 2.24-fold increase with PBD were achieved as a result of significant independent factors being found and improved. PBD facilitated considering multiple factors, preventing information loss essential for process improvement. Experimental data validated the mathematical models, showing strong enzyme activity at high pH levels, indicating versatile applications. Purified and recovered acidic-thermostable xylanase with 2.52-fold purification and 10.42% recovery has potential in animal feed (Dhaver *et al.*, 2022).

The goal of the study was to evaluate Neurospora intermedia, an edible fungus utilized in biorefineries, for its ability to synthesize amylase and xylanase. Utilizing synthetic medium and a waste stream made up of thin stillage and

wheat bran, enzyme synthesis was studied by submerged fermentation. Yeast extract and NaNO3 were the best nitrogen source combinations found to produce extracellular enzymes. In order to best display their outstanding capacity, nitrogen (N.) intermedia enzymes showed their peak activity at 65°C and a pH of about 5. At 65 °C, the N. intermedia amylase displayed increased stability and substrate affinity, which is consistent with effective hydrolysis procedures. Both enzymes retained over 50% activity at pH 4-7 for 4 hours, indicating their suitability for wheat-based biorefinery enzyme production in thin stillage and wheat bran medium (Shahryari *et al.*, 2019).

Halotolerant and halophilic bacteria-derived extracellular enzymes maintain structural stability and catalytic activity across various salinities. Halomonas meridiana VITSVRP14 was utilized for simultaneous amylase, agarase, and xylanase production via submerged fermentation, with optimized conditions of pH 8, 1.5% inoculum, 24 hours at 40°C, 8% sodium chloride, 1% lactose, and sodium nitrate. Plackett-Burman design and Response Surface Methodology (RSM) assessed pH, temperature, and lactose effects and interactions on enzyme synthesis. Post-optimization, xylanase, amylase, and agarase activity increased by 3.29, 1.81, and 2.08-fold, respectively. The enzyme mixture achieved significant saccharification rates of 38.96% (rice bran), 49.85% (wheat bran), 61.2% (cassava bagasse), and 57.82% (corn cob) within 24 hours of enzymatic treatment (Veerakumar & Manian, 2022).

Fish gut-isolated *Bacillus licheniformis* was submerged-fermented with grapefruit peels to produce amylase while being optimized for this purpose. With grapefruit peels as the carbon source, ammonium nitrate as the nitrogen source, and magnesium sulphate as the mineral salt, nutritional characteristics were gradually improved. Central composite design in response surface methodology fine-tuned these factors and the initial medium pH. The optimal conditions for maximum amylase production were determined as pH 7, with 5% grapefruit peels, 0.9% ammonium nitrate, and 0.6% magnesium sulphate. Amylase exhibited its highest activity at pH 9 and 80°C, highlighting its suitability for high-temperature industrial applications like paper and textiles (Iram *et al.*, 2021).

2.6 Bioinformatics Analysis of Enzymes

Exons, which are used to code for proteins, and introns, which are used to make other types of nucleotides, make up genes in their native forms (de Conti *et al.*, 2013). However, exons that code for the production of functional proteins remain left after introns are spliced during posttranslational modifications (De Conti *et al.*, 2013). This study found that the Aspergillus niger CSA35 -amylase gene (CDF Amyl) has five exons and four introns. The gene is predicted to encode a protein with 222 amino acids and a partial nucleotide sequence of 926 base pairs. Most fungi have been shown to include fungus-type, intron-rich -amylase genes (Da Lage *et al.*, 2013). The same level of knowledge of enzyme function is used to predict the as-yet-unidentified functions of protein structures (Hermann *et al.*, 2007). If a good database is available that contains near evolutionary homologues of the query sequence, which are typically predicted to share a comparable function, Sequence-based function prediction can also produce highly accurate results. (De Ferrari *et al.*, 2012). However, there is a worry that the high throughput and challenging curation of automated function prediction would spread numerous incorrect annotations throughout the bioinformatics databases (Furnham *et al.*, 2009).

According to analysis, the extracellular alpha amylase from Aspergillus Niger CSA35 has a molecular weight (Mw) of 25.13 kDa and a pI of 4.17. Amylases generally range in Mw from 10-210 kDa, with most falling within 50-60 kDa, while the extremes are found in Bacillus caldolyticus (10 kDa) and Chloroflexus aurantiacus (210 kDa) amylases (R. Gupta *et al.*, 2003). The pI values, such as the one observed here, provide insights into how enzymes respond to purification techniques across various pH levels, with amylase pI typically spanning from 3.25 to 10.1 (Silva, 2013).

Many industrial operations are based on amylases' depolymerization of starches (Richardson *et al.*, 2002). used the reduced extent approach and the thermo kinetics concept to examine the thermo kinetic activity of α-amylase in the process of starch hydrolysis under diverse temperature, pH, and metal ion circumstances (Jeon *et al.*, 2014). A new maltose-forming α-amylase of the family GH57 was studied bioinformatically and biochemically in the

hyperthermophilic archaeon *Thermococcus* sp. CL1-CL1. A member of the GH57 family of glycoside hydrolases, maltose-forming α-amylase is unique in that it has dual hydrolysis activity against α-1, 4- and α-1, 6-glycosidic bonds while exclusively recognizing maltose. Prior to now, *Pyrococcus* sp. IS 04 was the only known species that contained this enzyme (Li *et al.*, 2014).

Haloarcula sp. HS's amylases, active in high salinity and high temperatures, hold industrial promise for their stability with surfactants. Proteomic analysis identified three enzymes, including one in the extracellular milieu, bearing α-amylase family consensus domains and residues (Gómez-villegas *et al.*, 2021).

The hydrophobicity value of a peptide is represented by the grand average of hydropathicity index (GRAVY), which calculates the sum of the hydropathy values of all the amino acids divided by the length of the sequence. The hydropathy values from Kyte and Doolittle were used to calculate GRAVY. GRAVY scores are positive for hydrophobic and negative for hydrophilic. The ExPASy website (http://www.expasy.org) provides access to all of these physicochemical values (Chang & Yang, 2013).

The Self-Optimized Prediction Method with Alignment (SOPMA) server was used to predict the hypothetical protein's secondary structure. The accuracy of the SOPMA results was additionally ensured using the Protein Structure Prediction Server (PSIPRED) server. When the HP was evaluated by SOPMA, alpha helices were discovered to be the most prevalent structure (69.87 percent). The prolonged strand was observed at 5.65 percent, followed by the random coil at 19.67 percent. Beta-turn was also discovered to be 4.81 percent. PSIPRED was used to cross-check the results, and a similar outcome was found (Mazumder *et al.*, 2022).

Based on the protein's amino acid sequence, the beta sheets, alpha helices, and coils were predicted using the SOPMA and PSIPRED servers. SOPMA results showed 64.81% random coils, 24.69% extended strands, 8.02% beta turns, and 2.47% alpha helices in the intended protein's secondary structure. PSIPRED also highlighted coil regions as predominant, followed by strand and helix structures, aligning with the anticipated secondary structure composition of 64.81% random coil, 24.69% extended strand, 8.02% beta turn, and 2.47% alpha helix (Shams *et al.*, 2021).

The backbone conformation of the rough model was inspected using the Phi/Psi. Using the PROtein CHECKer (PROCHECK) server, a Ramachandran plot was acquired (http://nihserver.mbi.ucla.edu/SAVES_3/saves.php). Loop refinement and energy minimization have both been applied to the rough model. Plot calculations were performed on the cold active lipase 3D model and a Ramachandran plot of the enzyme was constructed. In the model's Ramachandran plot, the residue distribution was as follows: 90.6% in the core region, 8.8% in the authorized regions, and 0.6%, or one residue, in the forbidden region (G. N. Gupta *et al.*, 2015).

Protein structures are thoroughly assessed using PROCHECK34 in the Protein Data Bank Summary (PDBsum), identifying atypical or potentially incorrect sections. The Ramachandran plot reveals "disallowed" conformations, assessing overall structure quality. Although, The Research Collaboratory for Structural Bioinformatics (RCSB) and Protein Data Bank in Europe (PDBe) servers have largely replaced the dated PDBsum, it still offers unique features. Users can upload their structures for a comprehensive analysis, including PROCHECK results, interaction graphs, and domain architecture networks, making it a valuable resource accessible for free at http://www.ebi.ac.uk/pdbsum (Laskowski *et al.*, 2018).

2.7 Industrial Applications of Enzymes

Industrial biocatalysts play a transformative role in global biotechnology applications. Extremophilic microorganisms, equipped with diverse molecular adaptations for harsh environments, offer unique potential. These taxonomically diverse extremophiles can manufacture starch hydrolyzing enzymes with protein stability, opening up new biotechnological possibilities. For these enzymes to be used in industry, it is crucial to emphasize their thermostability (Osho, 2018).

One of the biggest industries in the world is the enzyme sector, and there is a sizable market for enzymes. Enzymes are used in a wide range of industrial products and processes thanks to improvements achieved in modern biotechnology, and new applications are constantly being developed. Microorganisms provide an incredible number of catalysts in a variety of

industries, including those involving food, animal feed, technical industries, paper, fine chemicals, and pharmaceuticals. Due to the unique properties of enzymes, such as their high selectivity, rapid action, and biodegradability, enzyme-assisted industrial processes can function with better yields and produce less waste under softer reaction conditions (Liu & Kokare, 2023)

Xylanases are commercially produced through liquid or solid culture and hydrolyze xylans. They are used alongside other enzymes like -amylase in bread production, breaking down hemicellulose in wheat flour to improve dough texture and delay crumb development. Xylanase is recommended for biscuit preparation to lighten cream crackers, enhance texture, and improve taste (Polizeli *et al.,* 2005). Xylanases reduce oligosaccharides and beer viscosity by hydrolyzing arabinoxylans. They enhance juice production, stabilize fruit pulp, and recover essential compounds. Xylanases, in combination with other enzymes, improve juice yield by reducing viscosity and breaking down obstructive compounds. These enzymes also assist in separating gluten from wheat flour starch and find applications in coffee bean mucilage processing. Their excellent stability and optimal activity at acidic pH levels are highly desired traits (Christopher & Kumbalwar, 2015).

The sources of amylase are fungus and bacteria, mainly members of the Bacillus, Pseudomonas, and Clostridium families. It is known as the first starch-degrading enzyme and is one of the most important enzymes in the food business. Starch, beer, bread, and sugar are among the industries it works in. It was identified and contained in 1811 by Kirchhoff (R. Gupta *et al.* 2003; Abada 2018). It has been utilized in bakery products when it is extracted from fungus or malted cereals because α-amylase may be added to flour and dough preparation to speed up fermentation and decrease the viscosity of the dough, which enhances the volume and texture of the finished product. The development of more fermentable sugars is also aided, which improves the bread's color, flavor, quality, and crust, among other features. The manufacturing of maltose syrups, however, which are used in the food, brewing, and pharmaceutical industries, uses β-amylase. (Ray *et al.* 2017; Zhang *et al.* 2018).

2.8 Fruit Juice Clarification Techniques

The major goal of the clarification process used in industrial juice processing is to eliminate the colloids that cause the turbidity and cloudiness in fruit juices, including metals, proteins, tannin, and polysaccharides (pectin, cellulose, hemicellulose, lignin, and starch). Pectin, a sophisticated fiber-like polymer, must be thoroughly degraded by enzymes in order to produce clear and stable fruit juice. Fining agents including bentonite, gelatin, and silica sol have been successful in assisting in the removal of these elements from juice for the best clarity (Uçan *et al.*, 2014).

The most frequent method of nonenzymatic clearing involves applying heat to break up the emulsion. Gelatin, casein, and mixtures of tannic acid and proteins are further methods (Kilara & Van Buren, 1989). Additionally, it has been discovered that the use of honey and treatments combining honey and pectinase are efficient clarifying agents. When honey and pectinase are used together, a synergistic effect is thought to be caused by the honey's proteinaceous component (McLELLAN *et al.*, 1985).

Modern juice extraction and clarification primarily rely on enzyme treatment, resulting in increased yield, higher levels of reducing sugars, soluble dry matter, galacturonic acid content, and titrable acidity through the hydrolysis of cell walls. This process also reduces waste pomace and lowers pulp viscosity. Enzymatic breakdown depends on factors like enzyme type, incubation period, temperature, concentration, pH, and the use of enzyme combinations (Sharma *et al.*, 2017).

Juice clearing is necessary because substances that generate haze and sediment damage the quality of fruit juice while it is being stored (Dıblan & Özkan, 2021). The most often used pectinolytic enzymes in this area are pectinesterases, polygalacturonases, pectin lyases, and protopectinases (Ramadan, 2018).

Apple, pear, orange, and peach juices are just a few of the liquids that enzymes can improve in clarity and output. When pectin or the cell wall are

broken down by enzymes, more juice can be extracted from a ton of fruit. In exceptional cases, the addition of an enzyme complex can boost juice yield and color extraction, resulting in the production of a premium product (Santin & Di Luccio, 2004).

In the study conducted by (De Oliveira *et al.*, 2006) with a 19 bp difference in favor of the enzymatic process, processing apple juice by liquefaction yielded 83.5% of the juice with 16.5% of bagasse, compared to processing by pressing, which yielded 64.5% of the juice with 35.5% of bagasse. This difference shows that the enzymatic liquefaction process has advantages over the extraction by pressing process in terms of practicality. During the production of both white grape and red grape juice, enzymes are essential for depectination, yield enhancement, and clarity. Utilizing an enzymatic preparation for red grapes, especially Concord grapes (V. labrusca), serves as a supplement to the procedure to obtain the maximum extraction of the pigment that is naturally present in the fruit (Kumar, 2015).

Yamasaki & Yasui, (1964) demonstrated that apple juice clarity can be achieved with a mix of polygalacturonase and polymethylesterase, without apple contamination enzymes. However, clarified apple juice, especially when refrigerated, may develop late sediment due to high-temperature treatment, polyphenol polymerization, and proanthocyanin oxidation during processing. Unlike highly methylated apple pectin, orange pectin has fewer methoxy groups due to abundant pectin esterase in orange juice. When pectinases are used to extract orange juice, the output of sugar and soluble solids rises, resulting in more juice being produced with less viscosity.

Sin *et al.*, (2006) employed Sapodilla juice in his study and pectinase enzyme to treat the juice at various incubation durations (30–120 min), temperatures (30–50^{o}C), and enzyme concentrations (0.03-0.10%). The effects of these three variables on turbidity, clarity, viscosity, and color were assessed. It was advised to use a 0.1% enzyme concentration at 40 ^{o}C for 120 minutes for enzyme clarity. Juice clarity increases after enzymatic treatment. A UV-visible spectrophotometer can be used to measure the absorbance and transmittance of juice at 660 nm. An increase in enzyme concentration accelerates the

clarifying process by allowing these particles to aggregate into larger particles and finally settle out by exposing some of the negatively charged protein underlying and lowering electrostatic interaction between cloud particles.

2.9 Challenges and Future Prospects

Peels, seeds, straws, and other waste products from the food processing industry contribute significantly to environmental contamination because they are rarely put to commercial use. It's critical to alter attitudes toward agro-waste residues since they can be used as carbon and nitrogen sources for the creation of the amylase enzyme, which enables the transformation of waste into useful goods. This strategy lowers production costs, encourages recycling of waste, and improves environmental sustainability. There are several uses for developing starch-based amylases and nano-structured metal-oxide amylase sensors in biotechnology and bioanalytical sectors, particularly in the food, pharmaceutical, and starch-based industries (Naik *et al.*, 2023).

Today, enzymes are a necessity for the beverage industries. The type of strain is one of several variables that will affect enzyme output. It is necessary to find strains that are suitable for the continuous procedure. Depending on the substrate found in the fruit juice, an appropriate enzyme must be added. Enzyme kinetics has a lot of possible applications in the future (Singh & Singh, 2015).

The food industry seeks improved enzymes for flavor, texture, and shelf life enhancement. Enhancements in stability, reduced dependency on metal ions and inhibitors, and increased catalytic efficiency are sought. Reusing enzymes through immobilization methods remains an underexplored area, along with the challenge of enzyme specificity limited to natural substrates. Developing artificial enzymes capable of interacting with both natural and synthetic substrates could greatly benefit food processing (Sindhu *et al.*, 2020).

CHAPTER-III

MATERIAL AND METHODS

3.1 Research Design

The research design refers to the overall strategy that integrate the different components of the study in a coherent and logical way, ensuring that it will effectively address the research problem it constitutes the blueprint for the collection and analysis of data. The materials, equipment, media and reagents used in this study are systematically listed in Appendix A.

3.2 Research Methodology

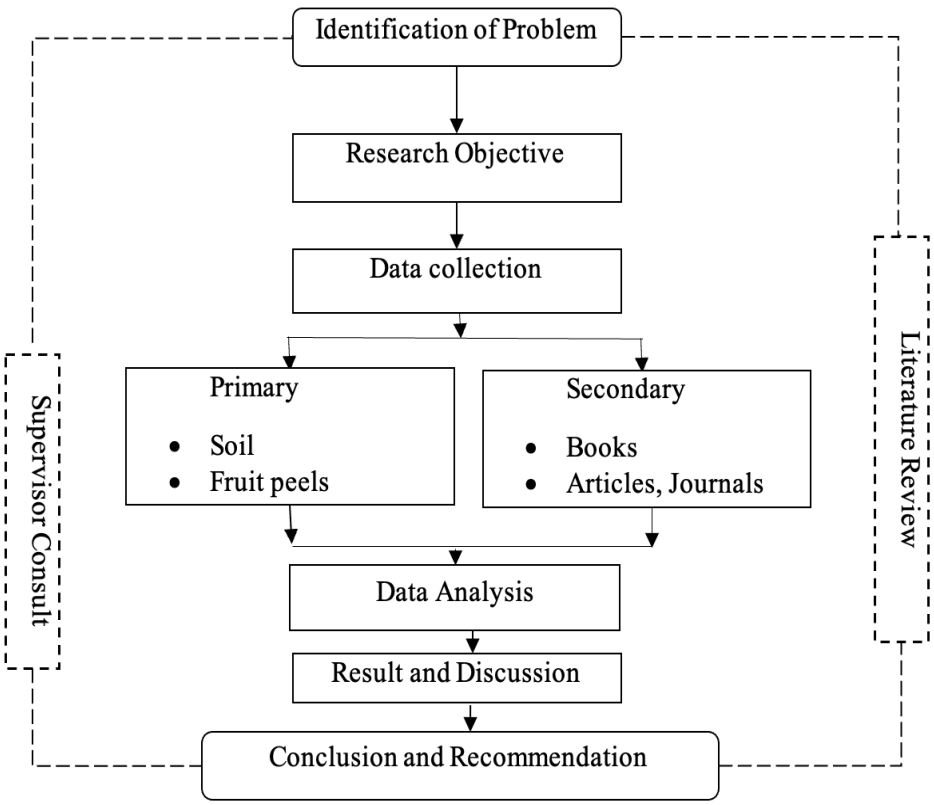

Figure 1 Flow Chart of Research Methodology

3.2.1 Study design

This research design was basically qualitative in nature. The research work was primarily focused on mango juice clarity by generating screening of amylase

and xylanase producing bacteria from samples (soil and fruit peel) collected from Kathmandu valley.

3.2.2 Study duration

The study was conducted for (January 2023 - July 2023).

3.2.3 Laboratory setting

All the laboratory works were performed in Microbiology laboratory, Annapurna Research Centre Kathmandu.

3.2.4 Sample size

For the gathering of the sample, simple random sampling was used. 12 Soil and 12 fruit peels samples were collected from different places of Kathmandu valley.

3.2.5 Sample collection

For isolation of amylase and xylanase producing bacteria, about 10-gram of each sample were collected in sterile plastic bags and transported to the laboratory for further processing.

3.2.6 Isolation of amylase producing Bacteria

1 gm of soil sample was weighed and added to 9 ml of sterile distilled water. Sterile dilutions were prepared up to 10-9 dilutions and 0.1 ml of each dilution was added using spread plate method to starch agar plates. For 24 hours, the starch agar plates were incubated at 37°C.

3.2.7 Selection of the amylase producing bacteria

On starch agar medium, bacterial colonies producing a clear zone around them when flooded with gram's iodine was considered as amylase producer. The bacteria forming the largest halo zone were further used for studies.

3.2.8 Selection of xylanase producing bacteria among amylase producer

Selection of xylanase enzyme producing bacteria was carried out by using sub culturing on Nakamura and Horikoshi basal medium. The xylanase producer showed clear zone around the colonies(Yadav *et al.*, 2018).

3.2.9 Identification of the microorganisms

Various morphological characteristics, cultural properties were observed as well as grams staining and biochemical test were performed for the

identification of bacteria.

3.2.10 Identification of the bacteria

Identification of potential isolate was done on the basis of biochemical test and molecular techniques.

3.2.11 Biochemical test and microscopy

Various morphological characteristics, cultural properties were observed as well as grams staining and biochemical test were performed for the identification of bacteria.

3.2.12Molecular identification of the bacteria

3.2.12.1 PCR Amplification and sequencing of 16S rRNA gene

The isolate that displayed amylase and xylanase activity was submitted to additional molecular analysis. The isolate bacteria's colony PCR was carried out utilizing universal primers (27F AGAGTTTGATCMTGGCTCAG and 1492R CGGTTACCTTGTTACGACTT). Using a sterile toothpick, a single colony of newly formed culture was harvested. It was then combined with 50 µL of autoclaved distilled water and heated for 20 minutes. For one minute, the sample was centrifuged at 10,000 Xg to remove the cell debris. Supernatant was put into an autoclaved microcentrifuge tube, and 2 µL of it served as the template for a typical 20 µl PCR reaction.

Table 1 Universal forward and reverse primers for 16S rRNA of Bacteria

Primer	Sequence	Reference
27F	AGAGTTTGATCMTGGCTCAG	(Miller *et al.*, 2013)
1492R	CGGTTACCTTGTTACGACTT	

Table 2 Optimized PCR reagents

Reagent	**Concentration**	**Volume (µL)**
PCR buffer (10X)	1X	2
Mgcl2	2.5 mM	0.6
dNTPs Mix	200 µM	1
Forward Primer	500 nM	1
Reverse primer	500 nM	1
Template	-	2

Taq polymerase	1 U	1
Distilled water		11.8
Total volume		**20**

Table 3 Optimized PCR reactions

Initial denaturation	**Temperature (ºC)**	**Time**
Initial denaturation	94	3 min
Denaturation Annealing Extension	94 50 72	30 sec 45 sec 2 min
Final extension	72	8 min
Hold temperature	4	1 h

The thermal cycler (Thermo Fisher) program was used to accomplish the PCR amplification as follows: 94 ºC for 3 min as the initial denaturation phase, followed by 30 cycles of 94 ºC for 30 sec, 50 ºC for 45 sec, 72 ºC for 2 min, and finally 72 ºC for 8 min as the final extension. The PCR results were examined using gel electrophoresis on 1% agarose (Qiagen, Germany) and contrasted with a 1 kb DNA ladder (Thermo Fisher). PCR products from high yield isolates were purified using a PCR purification kit (Qiagen, Germany) in accordance with the manufacturer's instructions. MacroGen Co. (Seoul, Korea), which purified the products, sequenced them. The determined sequences were also compared with sequences from various Bacillus species' 16S rDNA genes using the BLAST software. Utilizing Mega11 software, a phylogenetic tree was created.

3.2.13 Culture conditions and amylase production

The bacteria were cultivated aerobically at 37 °C in starch broth medium (nutrient broth containing 1% starch). Amylase production was conducted in a volume of 20 ml in a 100 ml conical flask inoculated with loopfuls of cells from a 24-hour-old slant culture and was maintained at 37 °C in a rotatory water shaker bath at 120 rpm. The contents were centrifuged at 10,000 rpm for five minutes to eliminate the bacterial cell after being incubated for 24 hours. Amylase activity was measured using the supernatant.

3.2.14 Extraction of crude enzyme (amylase) enzyme

Bacteria grown in the respective medium was centrifuged at 10,000 rpm for 5 min to remove cells. The supernatant was collected as crude enzyme.

3.2.15 Confirmation of amylase production

The recovered supernatant was utilized to verify the synthesis of the amylase enzyme. The supernatant was produced, and then it was added to the Wells that had been created in the starch agar media. 48 hours were spent incubating the media. The plates were flooded with gram's iodine following the incubation, and the hydrolysis zone was checked for.

3.2.16 Determination of α-amylase activity

3.2.16.1 Determination Amylase activity according to DNSA method.

The reaction mixture, consisting of 1 ml of soluble starch solution and 1 ml of potassium phosphate buffer (pH 6.9), was combined with 0.1 ml of the crude enzyme source from 1 of the labeled conical flasks and left to sit for 15 minutes at room temperature. Following the incubation, 2 ml of the DNS reagent was added, and the reaction mixture was stopped by submerging the tube in boiling water (100 ^{o}C) for 10 min. the blank was made as described above without incubation, and the absorbance was measured at 540 nm. The amount of enzyme that releases 1μ mole of reducing sugar (maltose equivalents) every minute under the assay conditions is considered one unit of " α- amylase activity."

3.2.17 Optimization of production parameters

3.2.17.1 Effect of temperature on amylase production

The optimum temperature for the amylase production was determined by incubating fermentation media at different temperature (20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C) for 24 hours for Bacillus amylase. Utilizing DNS technology, the enzyme assay was conducted.

3.2.17.2 Effect of pH on amylase production

The optimum pH for amylase production was determined by incubating fermentation media, or basal media, at various pH levels (6, 6.5, 7, 7.5 and 8). The Bacillus amylase enzyme assay was conducted after a 24-hour period.

The enzyme assay was carried out after 24 hours for Bacillus amylase.

3.2.18 Bioinformatic analysis of amylase and xylanase enzyme

3.2.18.1 Retrieval of enzyme sequence

Amylase and xylulose protein sequence were downloaded in the form of FASTA format from the NCBI database.

3.2.18.2 Physico-chemical analysis

Using the ProtParam tool, physico-chemical analysis of vaccine candidate proteins was carried out, including molecular weight, theoretical pI, instability index, aliphatic index, amino acid composition and grand average of hydropathicity (GRAVY).

3.2.18.3 Secondary structure prediction

PSIPRED server was used for the secondary structural components of proteins.

3.2.18.4 Tertiary structure prediction

Protein tertiary structures were forecast using Phyre2.

3.2.18.5 Clarification of fruit by using enzyme produced by the bacteria

Fresh fruit juices were made, fruit pulps were removed from juices by centrifuging at 4500 rpm for 30 min, and then supernatants were used for the enzymatic tests. The crude enzyme was mixed with diluted juices (15:85; juice: distilled water) and incubated at 50 ^{o}C and the results were observed for clarification fruit juice.

3.2.18.6 Data Analysis

The collected data were maintained in Microsoft Excel. Data collection, entry and validation organization, and monitoring approaches were used to generate quality of generated data. A backup of every data (both hard and soft copy) was maintained. Moreover, results especially for laboratory experiments such as source data, photographs etc. were maintained. Data maintained in the computer sheets were organized and analyzed by using SPSS software for Windows (version 16). A value of $\alpha \leq 0.05$ were assumed wherever applicable and 95% confidence intervals along with the exact p-values was presented.

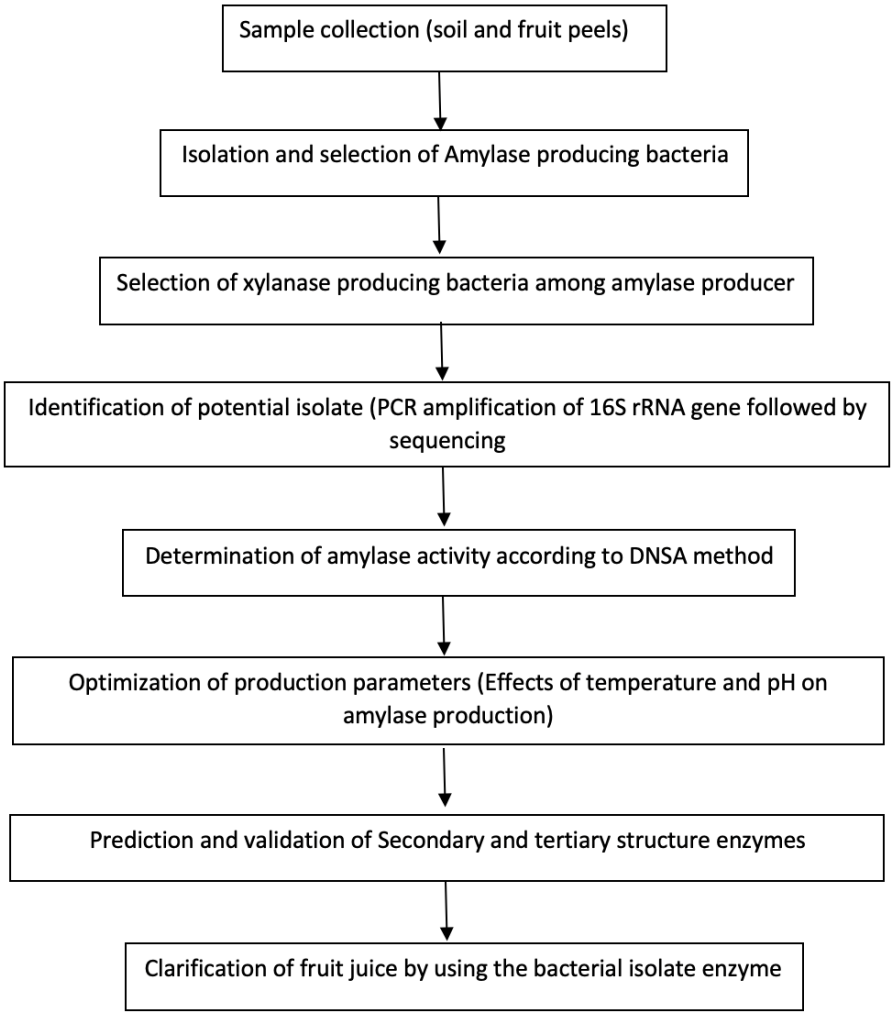

Figure 2 Flow Chart of Data Analysis

CHAPTER-IV

RESULTS

4.1 Sample collection (soil and fruit peel sample)

All together 24 samples (12 soil samples and 12 fruit peels sample) were collected from various part of the Kathmandu valley in sterile plastic bags.

4.2 Isolation of amylase producing bacteria

Twelve soil samples and twelve fruit peel samples were taken from various locations throughout the Kathmandu valley, and a total of 17 amylase-producing bacteria were isolated. Only one strain (K21) out of 17 isolates was discovered to generate xylanase. Among the 17 amylase-producing isolates, none produced pectinase.

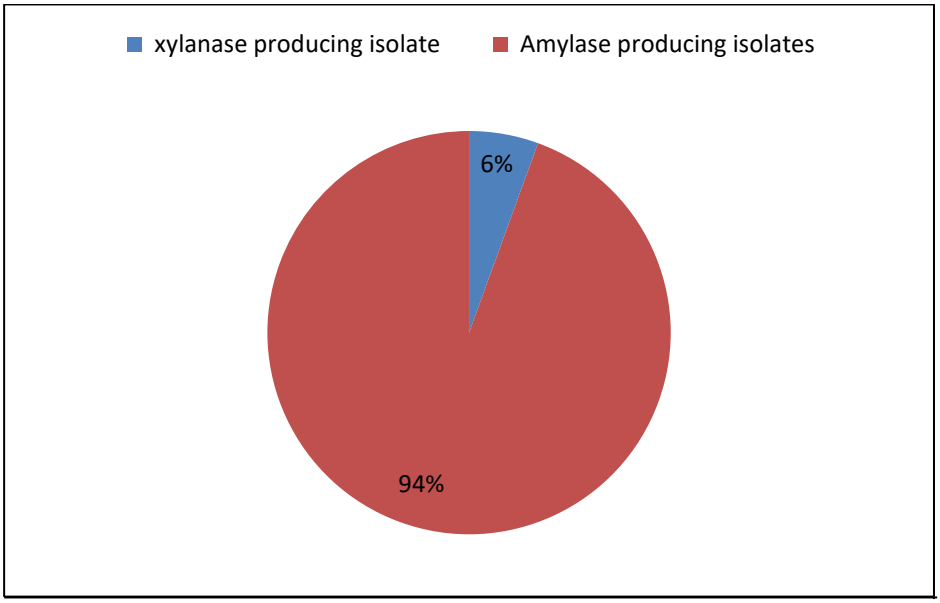

Figure 3 Chart showing the xylanase producer among amylase producers

4.3 Identification of bacterial isolate

A generating isolate of xylanase and amylase was chosen for additional research. Based on biochemical tests and molecular characterization by 16S rRNA sequencing, this isolate was identified.

4.3.1 Colonial Morphology

The isolate showed off-white colonies with circular, entire, umbonate, and larger in size. The colonies were opaque with no pigmentation. According to the colony morphology (Table 4), the isolate was identified as presumable identified as *Bacillus* spp.

Table 4 Colony morphology of the isolate K21

S.N.	Colony Characteristics	Remarks
1	Colour	Off white
2	Form	Circular
3	Margin	Entire
4	Elevation	Umbonate
5	Texture	Matt
6	Size	Large
7	Pigmentation	Opaque with no pigmentation

4.3.2 Biochemical test

On the basis of Microscopy, a colony morphology and biochemical test (Table 5), the isolate was identified as be *Bacillus* spp.

Table 5 Biochemical test of the isolate (K21)

S.N.	Tests	Results
1	Gram staining	+Ve, rod, spore forming
2	Catalase	+ve
3	Oxidase	-ve
4	Indole	-ve
5	Methyl red (MR) test	-ve
6	Voges-Proskauer (VP) test	+ve
7	Citrate utilization test	+ve
8	Urease	-Ve
9	Gelatin liquefaction	-ve
10	Starch hydrolysis	+ve
11	Xylan hydrolysis	+ve
12	Motility	Motile

4.4 Molecular characterization

4.4.1 Amplification of 16srRNA gene and its sequencing

Using the forward and reverse primers 27F and 1492R, respectively, and the

PCR reagents at the specific conditions described in the protocol, the 16S rRNA gene was amplified by colony PCR.

Using gel electrophoresis on 0.8% agarose (Qiagen, Germany) and a DNA ladder (Thermo Fisher Scientific), the PCR results were seen. The confirmed 16S rRNA gene measured 1399 nucleotides in length.

High yield isolates' PCR products were purified using a PCR purification kit from Qiagen in Germany in accordance with the manufacturer's instructions. MacroGen Inc. (Seoul, Korea) sequenced the products after they had been purified. The determined sequences were further compared with sequences of other active Bacillus species that were stored in NCBI GenBank using the BLAST software. Isolated bacteria were positively identified as Bacillus cereus by 16S rRNA gene sequence analysis (Appendix C). Forward sequence Blast in NCBI server showed 98% and 96.55% query cover and Percentage identity respectively with *B. cereus.* At the same time, reverse sequence Blast in NCBI server showed 74% and 98.33% query cover and Percentage identity respectively with *B. cereus.*

4.5 Phylogenetic tree of the isolate on the basis of sequence of 16S rRNA

According to phylogenetic tree composed by Mega11, K21 was found to be very close to other strains of *B. cereus* (Figure 2).

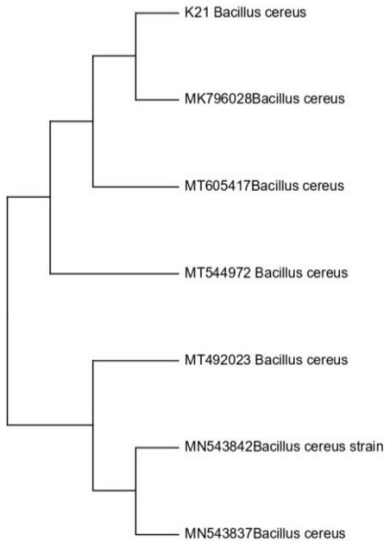

Figure 4 Phylogenetic tree of the isolate K21

4.6 Bioinformatics analysis of Amylase enzyme of *B. cereus*

4.6.1 Amylase enzyme of *B. cereus*

Amylase enzyme of *B. cereus* contains a total 645 amino acid sequence with molecular weight 76.13 KDa. Its theoretical PI value was 5.62. Instability index and Aliphatic index were found to be 44.13 and 67.07. Grand average of hydropathicity (Gravy) was found to be -0.229 (Table 6).

Table 6 Retrieved Amylase sequence from NCBI

>sp|C1EX54|GLGB_BACC3 1,4-alpha-glucan branching enzyme GlgB OS=Bacillus cereus (strain 03BB102) OX=572264 GN=glgB PE=3 SV=1

MSVINCEEVKRDEFHTEKYYESYNIFGAHIVTEDEMRGVRFTVWAPHAKAMSVVGDFNEW
DYEQHKMLQVTEEGIWSLFIPHIEEREIYKYAIETMAGDVIFKADPYAVYAEVRPNTASV
VFDIKGYEWNDKNWSRKKKKSVYKEAMTVYELHFGSWKKKEDGTLYSYREMAEELIPYV
VEHQFTHIEIMPLVEHPYDRSWGYQGTGYYAATSRFGTPYDLMHFVDECHKYGIGVILDW
VPGHFCKDAHGLYLFDGTPTYEYKDKDVQENPVWGTVNFDLGKREVRNFLISNALFWMRY
FHIDGFRVDAVANMLYWNKEGQEQSNEHAVSFLRELNEAVFAEDEDFLMTAEDSTAWPLV
TAPTYEGGLGFNYKWNMGWMNDVLKYMECAPEYRKYIHDKMTFSLLYTYSENFILPLSHD
EVVHGKKSLLNKMPGDYWDKFAQLRLLYGYFFTHPGKKLLFMGGEFGQFDEWKDLEDLDW
NLHDFEMHRYMHDYFKELIALYKRSKPLWQLDHSREGFQWIDANNNEQSIFSFIRQGDKQ
EDALVVVCNFTKATYENYKVGVPDFEYYNEILNSDAEQYGGSGQVNKKRLKTIQEPYHNQ
TAHVEITIPPFGVSILRPVKTRKGSKKQDGSKTKVRSNVTSRGKR

4.6.2 Secondary structure of Amylase

Amylase's random coils are dominated by its alpha helix, which has 159 amino acids, extended strand, which has 157 amino acids, beta turn, which has 57 amino acids, and random coil, which has 272 amino acids, as seen below.

```
MSVINCEEVKRDEFHTEKYYESYNIFGAHIVTEDEMRGVRFTVWAPHAKAMSVVGDFNEWDYE
QHKMLQV

eeecccccccheeecttcchhhhhhhtcceecctttttteeeeectteeeeeeeccccccccc
cchhhc

TEEGIWSLFIPHIEEREIYKYAIETMAGDVIFKADPYAVYAEVRPNTASVVFDIKGYEWNDKN
WSRKKKK

ccttceeeeettcettceeeeeeeecttteeeccccccehccccccheeeehttccccchh
hhhccc
```

KSVYKEAMTVYELHFGSWKKKEDGTLYSYREMAEELIPYVVEHQFTHIEIMPLVEHPYDRSWG
YQGTGYY

ccccccccceeeeeeecccccttcceehhhhhhhhhhhhhhhttcceeeeecccccccccccc
ceeeeee

AATSRFGTPYDLMHFVDECHKYGIGVILDWVPGHFCKDAHGLYLFDGTPTYEYKDKDVQENPV
WGTVNFD

cccccccccthhhhhhhhhhtttceeeeeecccccccccceeeeettcceecccccccccccc
cceeeee

LGKREVRNFLISNALFWMRYFHIDGFRVDAVANMLYWNKEGQEQSNEHAVSFLRELNEAVFAE
DEDFLMT

ccchhhhhhhhhhhhhhheccttcchhhhhhheeeetcccccccchhhhhhhhhhhhhtc
cttteeee

AEDSTAWPLVTAPTYEGGLGFNYKWNMGWMNDVLKYMECAPEYRKYIHDKMTFSLLYTYSENF
ILPLSHD

ehccccceecccccttteeeeeechhhhhhhhhhhtcccccccccchheeeeeehhcttte
eeeecct

EVVHGKKSLLNKMPGDYWDKFAQLRLLYGYFFTHPGKKLLFMGGEFGQFDEWKDLEDLDWNLH
DFEMHRY

heehtchhhhhhccccchhhhhhhhhhhhheecttceeeeetcccchhhhhhhhhhhhhhee
hccchh

MHDYFKELIALYKRSKPLWQLDHSREGFQWIDANNNEQSIFSFIRQGDKQEDALVVVCNFTKA
TYENYKV

hhhhhhhhhhhhhcccchhhhhcccctteeeecccccccheeeeehtcccccceeeeeetcccc
cccceee

GVPDFEYYNEILNSDAEQYGGSGQVNKKRLKTIQEPYHNQTAHVEITIPPFGVSILRPVKTRK
GSKKQDG

ecccccceeeeectteeeetcccccccccceeecccccccccceeeeeccccceeeeecccccc
ccccccc

SKTKVRSNVTSRGKR

ccccccccchhhhcc

Secondary structure prediction of amylase

4.6.3 Tertiary structure of Amylase

Amylase enzyme sequence was downloaded from NCBI server and tertiary structure was constructed by I-TASSER server (Figure 3) and validation of structures by Ramachandran plot.

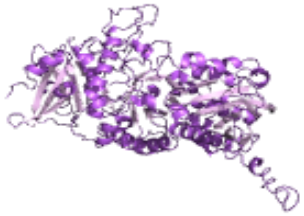

Figure 5 Tertiary structure of Amylase of *B. cereus* as predicted by I-TASSER

4.6.4 Validation of tertiary structure of Amylase

The tertiary structure of the Amylase was validated by Ramachadran plot (Table 7; figure 5) prepared using PROCHECK server in PDB-sum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html)

Table 7 Validation of tertiary structure of Amylase

Regions	**(%)**
Most favored regions [A, B, L]	74.0
Additional allowed regions [a, b, l, p]	20.8
Generously allowed regions [~a, ~b, ~l, ~p]	2.6
Disallowed regions	2.6
Total	100

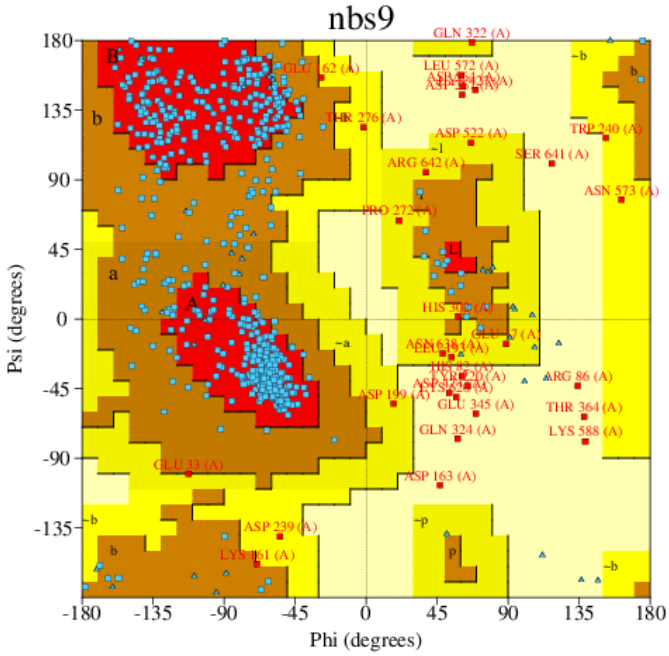

Figure 6 Validation of tertiary structure of Amylase by Ramachandran Plot using PROCHECK

4.7 Bioinformatics analysis of *Xylanase enzyme of B. cereus*

4.7.1 Xylanase enzyme of *B. cereus*

Xylanase enzyme of *B. cereus* contains a total 254 amino acid sequence with molecular weight 28.57 KDa. Its theoretical PI value was 9.46. Instability index and Aliphatic index were found to be 22.68 and 92.13. Grand average of

hydropathicity (Gravy) was found to be -0.229 (Table 8).

Table 8 Retrieved Xylanase sequence from NCBI

>tr|Q63H51|Q63H51_BACCZ Probable xylanase/chitin deacetylase OS=**Bacillus cereus (strain ZK / E33L)** OX=288681 GN=cda1 PE=4 SV=1

MFFFFITSKRNFKHISLIVILSLFTAWLLFLKTYSHESAFSTATGPKVIYKGDTSKKQVA
FTFDISWGDKKAIPILDTLKERDIKNATFFLSAAWAERHPDVVERIIKDGHEIGSMGYN
Y
TSYTSLETNEIRRDLMRAQDVFTKLGVKQIKLLRPPSGDFNKATLKIAESLGYTVVHW
SN
NSNDWKNPGVNNIVSTVSNNLKGGDIVLLHASDSALQTNKALPLLLQKLKSDGYEQI
SVS
QLISNTSTKSKDVQ

4.7.2 Secondary structure of Xylanase

Alpha helix has 92 amino acids, extended strand has 53 amino acids, beta turn has 20 amino acids, and random coil has 89 amino acids, according to the secondary structure of xylanase as shown below:

```
        MFFFFITSKRNFKHISLIVILSLFTAWLLFLKTYSHESAFSTATGPKVIYKGDTS
KKQVAFTFDISWGDK

        heeeeeettttcchheeeehhhhhhhheeehcccceeeeecccccceeeecccc
ccceeeeeeecccccc

        KAIPILDTLKERDIKNATFFLSAAWAERHPDVVERIIKDGHEIGSMGYNYTSYTS
LETNEIRRDLMRAQD

        chhhhhhhhhhttcchheeeeeecchhcchhhhhhhhhttcccccccccccccc
cchhhhhhhhhhhhh

        VFTKLGVKQIKLLRPPSGDFNKATLKIAESLGYTVVHWSNNSNDWKNPGVNNIVS
TVSNNLKGGDIVLLH

        hhhhhtcccceeecccccccchhhhhhhhhttceeeeeecccccccttchhhhh
hhhhtccttceeeee

        ASDSALQTNKALPLLLQKLKSDGYEQISVSQLISNTSTKSKDVQ

        cccccchhhhhhhhhhhhhhttttcceeeehhhhhtcccccchhh
```

Secondary structure prediction by SOPMA.

4.7.3 Tertiary structure of xylanase

Tertiary structure of of xylanase was developed by I-TASSER server (Figure 5).

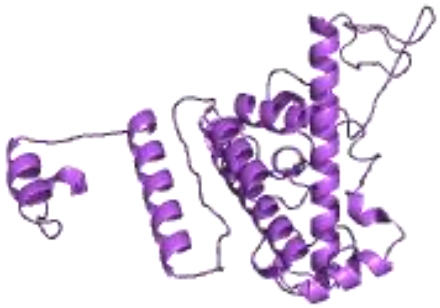

Figure 7 Tertiary structure of
Xylanase of B. cereus

4.7.4 Validation of tertiary structure of xylanase

The tertiary structure of the xylanase was validated by Ramachandran plot (Table 9; Figure 6) prepared using PROCHECK server in PDB-sum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html)

Table 9 Validation of tertiary structure of Xylanase

Regions	**(%)**
Most favored regions [A, B, L]	87.5
Additional allowed regions [a, b, l, p]	9.9
Generously allowed regions [~a, ~b, ~l, ~p]	2.2
Disallowed regions	0.4
Total	100

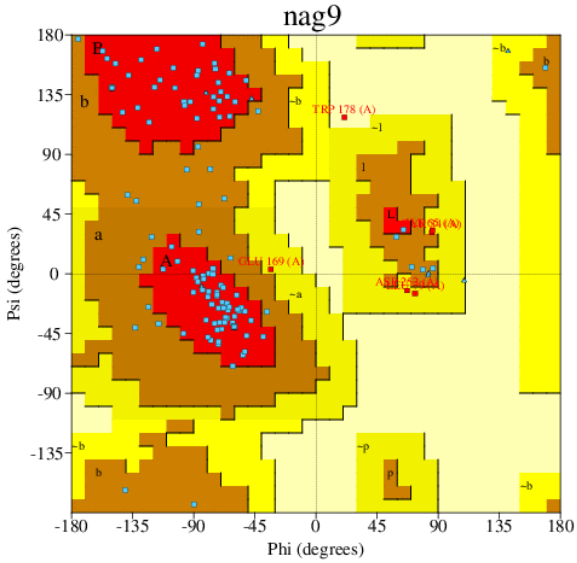

Figure 8 Validation of tertiary structure of xylanase by Ramachandran Plot using PROCHECK

4.8 Characteristic of amylase enzyme

4.8.1Analysis of amylase activity

Amylase activity was measured by comparing the crude enzyme's absorbance at 540 nm to a blank solution. The amount of the enzyme that releases 1 µmole of reducing sugar (maltose equivalents) every minute under the assay conditions is considered one unit of α-amylase activity.

Table 10 Analysis of Amylase activity

	Crude Enzyme	Absorbance (540 nm)
	K21	0.27

4.8.2 Effect of temperature on enzyme production

In order to produce enzymes, the impact of temperature was investigated at different temperature (20-45ºC) for 24 hours for *Bacillus cereus*. It was found that the amylase production was low at 20ºC which increased with raising temperature giving optimum production at 40ºC (Figure 7).

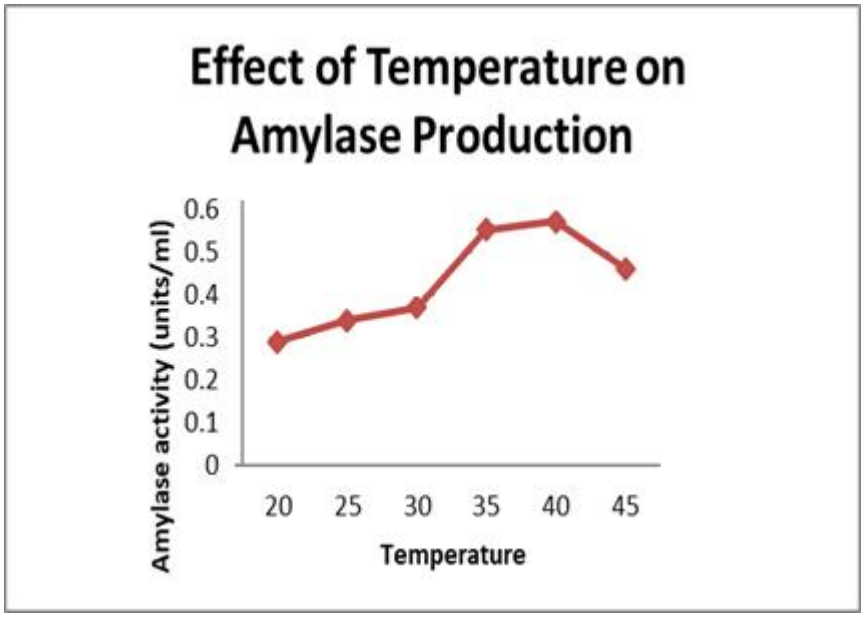

Figure 9 Effect of temperature on production of enzyme

4.8.3 Effect of pH on enzyme production

The enzymes Production in relation to the medium's pH was studied for

Bacillus cereus. Bacillus cereus produced amylase at its highest level, according to the study, at pH 6.5. (Figure 8)

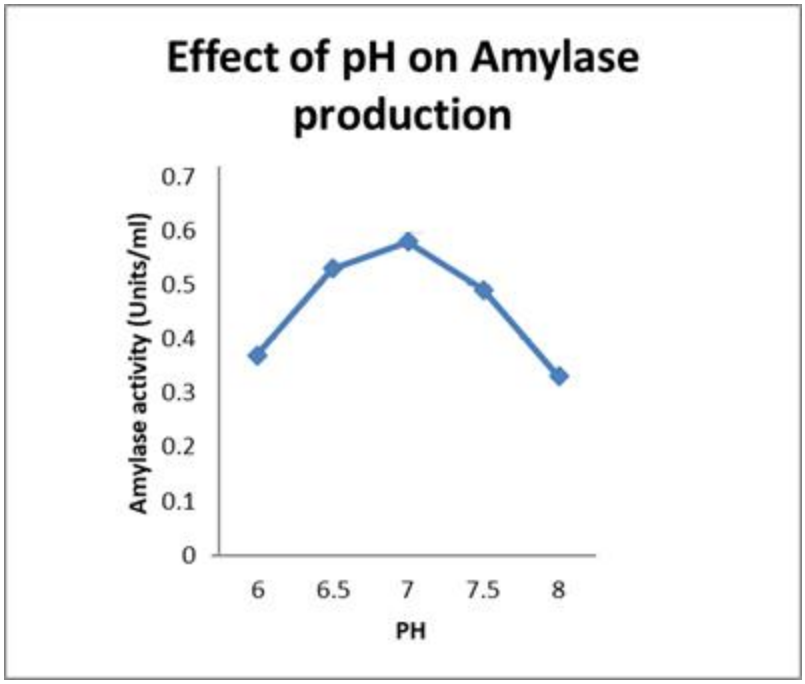

Figure 10 Effect of pH on production of enzyme

4.9 Fruit juice clarification

After incubating at 50^{o} C for 60 mins, all test tubes containing mango fruit juice and enzyme together showed a positive clarification. Xylanase was observed more effective than amylase in mango fruit juice clarification. Effective clarification was observed by using both the enzyme together than anyone single.

CHAPTER-V
DISCUSSION

Enzymes are protein-based biological catalysts that live cells produce to speed up specific biochemical processes. They often form an integral element of a cell's metabolic process. They operate on substrates with great specificity (Sivaramakrishnan *et al.*, n.d.).

Particularly intriguing is the application of certain enzymes as organic catalysts in a range of industrial processes. The remarkable nature of microbial enzymes, which are obtained from a variety of bacteria, is well known, notably for their use in commercial operations. Although the enzymes originated from microorganisms and were only discovered in the 20th century, research on their isolation, characterization of their properties, manufacturing on bench to pilot scales, and their application in the bioindustry have all advanced over time. The body of knowledge has also frequently been updated. Enzymes from microorganisms are already used in a wide range of commercial processes. Globally, several microorganisms, including bacteria, fungi, and yeasts, have been studied for their capacity to produce a variety of commercially useful enzyme formulations through biosynthesis (Nigam, 2013).

In this study, 17 amylase producing bacteria were isolated from 24 (12 soil and 12 fruit peel) samples collected from Kathmandu valley. A solitary xylanase-producing bacterium (K21) was found in this study among 17 amylase-producing bacteria. The soil was chosen as the source of microorganisms since it is home to a variety of microorganisms. Fruit peels are used as a sample to identify bacteria that produce the amylase since fruits contain starch and xylan as well. The soil sample was gathered from various locations inside Kathmandu valley. A total of 12 soil samples and 12 fruit peels samples were collected and studied for amylase producing bacteria initially. Among 17 isolates, all of them were *Bacillus* sp.

The cost-effective synthesis of microbial enzymes has led to an increase in their utilization in industrial settings, ability to immobilize substances that cannot be dissolved in water, and long-lasting application in biotechnological

processes. *Bacillus* species are among the microbes that are effective in secreting extracellular enzymes like amylase, arabinase, cellulase, lipase, protease, and xylanase, which are crucial to many biotechnological processes (Khodayari *et al.*, 2014)

In the expectation that certain bacteria might be able to biosynthesize distinctive enzymes, researchers have isolated certain bacteria from harsh habitats and under intense growing conditions. Various bio-industries require enzymes with special characteristics to process substrates and raw materials. Microbial enzymes work as bio-catalysts to carry out reactions in bio-processes in an economical and environmentally friendly manner, as opposed to employing chemical catalysts. Enzymes' special qualities—including thermotolerance, thermophilicity, tolerance to a wide pH range, stability of enzyme activity over a range of temperature and pH, and other harsh reaction conditions—are exploited for their economic value and industrial applications. These enzymes have shown effective in bioindustries such as animal feed, food, leather, textiles, and bioconversions and bioremediation (Nigam, 2013). Isolating a high-amylase-producing strain is essential since amylases for industrial manufacturing are often isolated from microorganisms. The bacteria that produce the amylase enzyme were isolated, identified, and characterized in this work. Amylase and xylanase from the same isolate K21 used for fruit juice clarification which showed positive results.

By soaking the starch agar plates in gram's iodine, the amylase production was verified. Starch and iodine combine to produce the blue color, whereas amylase-producing microbes use the starch as their only source of carbon to create the translucent zone. On the basis of clear zone formation on iodine-flooded starch agar plates, the initial selection of amylase-producing microbes was carried out. The clear zones developed because there was no starch to be digested by the bacterially produced enzyme (Lal & Cheeptham, 2012).

The isolate k21 was selected for amylase production and xylanase production. Colonies forming the largest clear zone were further identified by microscopy and biochemical testing and further by using molecular technique such as sequencing analysis of 16S rRNA and used for the production of the amylase enzyme on submerged fermentation.

The isolate K21 having capacity of producing both enzymes amylase and

xylanase was identified on the basis of their colonial characteristics, gram staining, biochemical test and molecular technique (16S rRNA) identified as *Bacillus cereus*.

The study of 21 isolates' 16S rRNA gene showed 98% similarity to Bacillus cereus. The function of the 16S rRNA gene has not changed over time, and its size is suitable for use in informatics (Patel, 2001). This study determined the size of strain K21's 16S rRNA gene to be 1399 kb. The 16S rRNA sequencing method used for identifying bacterial species molecularly has several limitations, though. Sequencing of the 16S rRNA gene, rather than closely related strains of bacteria, can only tell apart bacteria that are distantly related (Guo *et al.*, 2008). Multiple copies of the 16S rRNA, according to some studies, are uncommon and unlikely to have an impact on the phylogenetic analysis of the species because, for the most part, their sequences are completely or nearly completely identical (Engene & Gerwick, 2011). However, other research has shown that when the number of multiple copies of 16S rRNA increases, the sequence variation also increases (Větrovský & Baldrian, 2013). Therefore, it seems that sequencing multiple copies of 16S rRNA is not useful for identifying species (Khadayat *et al.*, 2020).

Since many years ago, α-amylase has been widely used in starch-based industries. Even though there are many microbiological sources for producing this enzyme effectively, only a few numbers of carefully selected fungi and bacteria strains are acceptable for industrial production. A constant effort is made to find new microbes that can be employed to produce amylase.(de Souza & e Magalhães, 2010)

Earlier research by Singh & Rani, (2014) examined 14 distinct soil samples for amylase-producing bacteria. Of these, 2 spp. of Bacillus were recovered and identified as *Bacillus subtilis* and *Bacillus brevis,* with *Bacillus subtilis* displaying the highest amylolytic activity. It was noted that Bacillus subtilis was the most frequently found amylolytic bacteria, followed by *Bacillus cereus* and *Bacillus brevis.* However, the amount and types of amylases generating organisms on soil vary on the types of soil.

The α-1,4-glycosidic and α-1,6-glycosidic links that hold the starch polymer together undergo enzymatic hydrolysis. The starch's α-1,4-glycosidic bonds are hydrolyzed by alpha-amylase. As a result of its attack on polysaccharide

chains' interior, a mixture of fragments with 5 to 9 units of the alpha configuration is created. Amylase fully divides amylose into glucose molecular components. In addition to α-1,4-glycosidic connections in starch, oligo α-1,6-glucosidase also affects α-1,6-branch points. It produces linear or branching dextrin and maltose by cleaving glucose units from the polysaccharide starch's nonreducing ends. Specific internal enzymes hydrolyze dextrin and maltose after being delivered into the bacteria (Lal & Cheeptham, 2012).

In this investigation, submerged fermentation was used to create the crude amylase enzyme in a rotating water shaker bath. The NB with 1% starch was incubated at 37°C for 24 hours with 1 ml of the Bacillus cereus culture that had been growing for the previous 24 hours in order to produce bacterial amylase. The supernatant from the centrifuged fermented broths was utilized to validate the formation of amylase. Starch hydrolysis testing on starch agar plates was used for confirmation. Bacteria often prefer submerged fermentation because it needs a lot of moisture. Additionally, it is employed due to the ease of recovering the substance (Subramaniyan and Vimala 2012). After that, the supernatant, or crude enzyme, was further analyzed using the DNS method, and the absorbance at 540 nm was measured. The DNS method is one of the most straightforward and popular ways to gauge the quantity of reducing sugar generated, which serves as a proxy for enzyme activity (el-fallal *et al.*, 2012).

Amylase converts starch into glucose to create lowering sugar. Therefore, a higher glucose concentration denotes the highest activity and intensity of starch breakdown. For the study, the isolate with the highest degree of activity was chosen. Numerous amylase production-related characteristics were examined in the medium used for amylase production in order to increase amylase production. Temperature and pH of the growth media are crucial physical factors that affect amylase production because they cause morphological changes in the organism and promote enzyme release. Therefore, in this study, the optimization of production parameters based on pH and temperature C was recorded for. *Bacillus cereus* produces amylase maximum at a temperature of 40^{o}C. The amylase production was low at the starting temperature and rises as the temperature rises, giving C. Similar

results were found in the investigation of maximum production at 40°C by Rai, (2022). Ikram-ul-Haq *et al.*, (2005) also reported maximum yield at 40°C for *Bacillus licheniformis*. Whereas the optimum C was recorded on the study of (Shanmugasundaram *et al.*, 2015) temperature of 45°C where strain of B. subtilis SUS3 was used for enzyme production. In contrast to the C was recorded, the optimum temperature of 35°C was recorded in the study of (Pokhrel *et al.*, 2013 ; Singh & Kumari, 2016) where alpha amylase producing bacteria was isolated from sewage enriched soil.

The ideal pH for Bacillus cereus to produce alpha amylase was found to be pH 6.5 in the current investigation as well. At the initial pH, amylase production was found to be minimal, peaking at pH 6.5. Bacillus sp., which was isolated from sewage in a study by (Pokhrel *et al.*, 2013) exhibited its greatest production at pH 7. Similar findings on the optimal pH of 7 were made in study by (Singh & Kumari, 2016) and similarly 7.5 by (Verma *et al.*, 2011). In contrast, (Shanmugasundaram *et al.*, 2015) found that Bacillus subtilis SUS3 thrived best at a pH of 6. Varied organisms have varied pH optimal ranges, and pH changes on either side of the ideal range have a negative or positive impact on microbial growth (Nelson 1942-, 2005). By using ammonium sulphate precipitation, the crude enzyme was partially purified. The most popular salt for enzyme precipitation is ammonium sulphate. For the partial purification of the enzyme, it is done at different saturation percentages. Ammonium sulphate was used at 65% in this investigation. Due to its high solubility, low density, availability, low cost, and ability to maintain the protein structure, it is helpful as a precipitant.

Bacillus was discovered to be a possible source of xylanases among bacteria, and several bacilli, including *B. circulans, B. stearothermophilus, B. amyloliquefaciens, B. subtilis, B. pumilus, and B. halodurans* have been reported to have significant xylanolytic activity (Gupta *et al.*, 2015)

Hemicellulose, along with cellulose, lignin, and pectin, is one of the essential components of agricultural waste and plants (Singh Nee Nigam & Pandey, 2009). The main element of hemicellulose, xylan, is made up of D-xylopyranosyl residues that are β-1,4-linked. Endo-β-1,4-xylanase and β-D-xylosidase are among the hydrolytic enzymes that are used to hydrolyze xylan

in plant materials (Polizeli *et al.*, 2005). The importance of xylanase has increased significantly as a result of its biotechnological applications for manufacturing pentose, clarifying fruit juice, improving rumen digestion, and bioconverting lignocellulosic agricultural leftovers to fuels and chemicals (Singh Nee Nigam & Pandey, 2009). Collins *et al.*, (2005) have thoroughly investigated the xylanase enzyme, its families, and the unique xylanases with extremophilic properties**.** In addition to using agro-industrial leftovers for manufacture of ethanol and animal feed, xylanases have established usage in the food, pulp, paper, and textile industries (Garg *et al.*, 1998).

After incubating at 50^{o} C for 60 mins, all test tubes containing mango fruit juice and enzyme together showed a positive clarification. Xylanase was observed more effective than amylase in mango fruit juice clarification. Effective clarification was observed by using both the enzyme together than anyone single.

The fruits contain significant amounts of different kinds of carbohydrates; historically, the extraction of fruit juice has resulted in it having a cloudy appearance and a high viscosity (Haile *et al.*, 2022). So, using the appropriate enzyme can help the fruit juice become clearer. The amount of a certain carbohydrate in the fruit juice affects how clear it becomes.

Only orange juice is eaten more frequently worldwide than clarified apple juice (Kahle *et al.*, 2005). Because it is turbid, brown in color, extremely viscous, and tends to settle during storage, raw apple juice needs to be cleared before being sold. Polysaccharides (including pectin, cellulose, hemicellulose, and starch), proteins, tannins, metals, and microorganisms are the main contributors to the turbidity in apple juice. Conventional clarification procedures aim to remove the insoluble solids and destroy pectic substances by dissolving pectin and starch with specific enzymes, flocculating cloudiness with clarifying agents (bentonite, gelatine, and/or silicasol), and filtering through plate and frame or vacuum Oliver-type filters (Grampp, 1976).

The NCBI database was used to retrieve the *B. cereus* amylase and xylanase sequences, and several servers were employed to ascertain their physicochemical properties. The B. cereus amylase enzyme has a total amino acid count of 645 and a molecular weight of 76.13 KDa. Its PI value in theory was 5.62. The stability index and arithmetic index were discovered to be 44.13

and 67.07, respectively. The hydropathycity (Gravy) global average was discovered to be -0.229. The *B. cereus* amylase enzyme was discovered to be effective, stable, and soluble. SOPMA and the I-TASSER service, respectively, predicted its secondary and tertiary structure. B. cereus amylase was not found to be less stable than other enzymes because the protein stability prediction index is greater than 40. Protein thermostability is explained by the aliphatic index value (>60). The hydrophilic or hydrophobic properties of proteins is explained by GRAVY (Chakdar *et al.*, 2016). Because more than 90% of the amino acids were in the Ramachandran plot's preferred regions, the amylase tertiary structure was valid. (Pražnikar *et al.*, 2019)

Similarly, Xylanase enzyme of B. cereus contains a total 254 amino acid sequence with molecular weight 28.57 KDa. Its theoretical PI value was 9.46. Instability index and Aliphatic index were found to be 22.68 and 92.13. Grand average of hydropathycity (Gravy) was found to be -0.229. Hence the enzyme was found to be stable, thermostable and soluble.

CHAPTER-VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Amylase and xylanase are enzymes that have wide spread application in industries due to their substrates hydrolysis activity. The search of new microorganisms for the production of amylase and xylanase is a continuous process due to its wide spread use in various industries. soil samples and fruit peel samples taken from several locations throughout the Kathmandu valley were used to isolate bacteria that produce amylase. Among the amylase producers, K21, the only Xylanase producer, was eliminated. Colony morphology, microscopy, biochemical assays, and 16S rRNA sequencing all contributed to the isolate's identification as *B. cereus.* Both the xylanase and amylase enzymes, which can clear mango fruit juice, were produced by the *B. cereus* strain. Xylanase was more effective than amylase enzyme in mango fruit juice clarification. Hence other enzyme including amylase and xylanase can be used in effective fruit juice clarification.

6.2 Recommendations

- Isolation of potential amylase xylanase producing bacteria are essential for better enzyme capacity and yield.
- Further optimize the production of amylase and xylanase enzymes from *Bacillus cereus* are necessary to enhance yield, stability, and efficiency, making them more suitable for industrial applications.
- Investigate the broader microbial diversity in soil and fruit peel samples using metagenomic approaches, enabling a comprehensive understanding of microbial ecosystems and potential biotechnological resources.
- Search various habitats for pectinase-producing microorganisms to find strains suitable for the food processing industry and related fields.
- Evaluate the practical feasibility of applying *Bacillus cereus*-derived enzymes in various industries, with a focus on bioremediation, agriculture, and sustainable production processes.

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APPENDICES

APPENDIX A

MATERIALS AND EQUIPMENTS

1. **Equipment**

   Micropipette, Pipette, Microscope, Thermocycler (PCR machine), Electrophoresis unit, Gel documentation system, Incubator, Refrigerator, Digital Balance, Hot Plate, Cabinet Dryer, Water Bath Shaker, Autoclave, Spectrophotometer, Hot air Oven.

2. **Materials**

   Beakers, Conical flasks, Volumetric Flask, measuring cylinder, Glass slides, cubett Coverslips, Petri plates, Inoculating loop, Spatula, Cuvette, Wire gauge, Test tubes, Screwcap tubes, Pipettes, Glass rods, Filter paper, Funnel etc.

3. **Chemicals**

   Starch, Methanol, Ethanol, DNSA, Ammonium-Sulphate, Sodium hydroxide, Gram's iodine, Crystal violet, Safranin, Conc.H_2SO_4, Conc. HCl, etc.

4. **Media**

   Nutrient Broth (NB), Nutrient Agar (NA), HK medium,

APPENDIX B

METHODOLOGY OF BIOCHEMICAL TEST FOR THE IDENTIFICATION OF BACTERIA

A. Methyl Red test

This test is intended to identify the fermentation pathway that an organism uses to consume glucose. Lactic, acetic, succinic, and formic acids are among the organic acids produced during the fermentation of glucose in the mixed acid pathway. A pH below 4.4 will occur from the steady creation of sufficient acid to overcome the phosphate buffer. When methyl red, a pH indicator, is introduced to an aliquot of culture broth and the pH is below 4.4, a red color will show up. If the MR turns yellow, the pH is more than 6.0 and the mixed acid fermentation pathway has not been used.

Procedure

Inoculating a pure colony of the organism into 2 ml of MRVP broth and incubating it there for 24 hours at 37 °C. Five drops of the methyl red reagent were added and thoroughly mixed after incubation. The appearance of a vivid red color, a sign of acidity, signified a positive test.

B. Voges-Proskauer (VP)test

The voges-proskauer test looks for acetoin, which is converted to 2,3-butanediol during the fermentation of carbohydrates. The culture will turn "brownish-red to pink" if it tests positive for acetoin. The culture will change from "brownish-green to yellow" if it does not contain any acetoin.

C. Indole production test

The enzyme tryptophanase, which can change the amino acid tryptophan into indole, can be produced by bacteria. Red color is created by the reaction of indole and another Kovac reagent. The deamination reaction, which targets the tryptophan molecule's side chain and produces indole in the process, is catalyzed by the enzyme tryptophanase.

Procedure

A sterile stab wire was used to stab a pure bacterial colony onto SIM (Sulphide Indole Motility) media, which was then incubated at 37°C for 24 hours. 2-3 drops of the Kovacs reagent were added after the 24-hour incubation period. When the top of the medium turns red, an indole test result has been successful.

D. Citrate Utilization test

This test is done to identify organisms that use citrate as their only source of carbon. The enzyme citrate hydrolyzes citrate into oxaloacetic acid and acetic acid if an organism is able to use it as a carbon source. The subsequent hydrolysis of the oxaloacetic acid yields pyruvic acid and CO2. In the event that CO2 is created, it combines with the elements of the medium to form an alkaline compound, such as Na2CO3. The pH indicator (bromthymolblue) changes from green to blue in an alkaline pH.

Procedure

A loopful of the test organism was streaked over the citrate agar's slant region, and it was left there for 24 hours at 37°C. The development of organisms and the media's transformation from green to blue as a result of the alkaline reaction were indicators of a successful outcome.

E. Catalase test

The purpose of this test is to locate the organisms that make the catalase enzyme, which detoxifies hydrogen peroxide by converting it to water and oxygen gas. The creation of bubbles as a result of the released oxygen gas indicates a successful outcome.

Procedure

With the use of an inoculating loop, a small portion of a 24 hour-old culture sample from NA was placed on a spotless, grease-free slide. On its surface, 2 to 3 drops of 3% H2O2 were applied. Production of active oxygen gas bubbling served as a sign of successful outcomes.

F. Oxidase test

This test is used to find microorganisms that have the crucial electron transport chain enzyme cytochrome oxidase. The enzyme cytochrome oxidase lowers oxygen to water by transferring electrons from the electron transport chain to oxygen. The oxidase test includes the synthetic electron sources and acceptors. The electron donor turns dark purple as a result of cytochrome oxidase's oxidation.

Procedure

A few drops of the oxidase reagent, which is 1% tetra methyl-p-phenylene-diamine dihydrochloride, were applied to a piece of Whatman's No. 1 filter paper. The filter paper was coated with a little quantity of test organisms. The presence of blue-purple tint quickly signals a positive test result.

G. Motility test

It is determined by this test whether the test organism was motile or not. A single stab of culture is used to inoculate SIM tubes all the way to the bottom. If an organism is mobile, the growth will extend from the stab site and discolor the entire tube. While non-motile bacteria grow along the stab line, migratory species migrate from the stab line and disperse into the medium to cause turbidity.

H. Carbohydrate fermentation test

This test evaluates an organism's capacity to ferment the sugar glucose as well as its capacity to transform the glycolysis byproduct pyruvic acid into gaseous byproducts. This test is frequently used to detect microorganisms that digest glucose and release gas.

Additionally, the medium contains phenol red, a pH indicator. Thus, acidic byproducts are produced and the pH indicator turns yellow when an organism is able to ferment the sugar glucose.

Pyruvate is the byproduct of glycolysis. By using the enzyme formic hydrogenlyase, organisms that can convert pyruvate into formic acid and

formic acid into H2(g) and CO2(g) release gas. The Durham tube, where this gas is imprisoned, causes a bubble to form at the top of the tubes.

APPENDIX C

A. Sequence based on forward primer of 16 S rRNA of *B. cereus*

Isolate	Sequence based on forward primer
K21	>H220124-010_A01_S1_27F.ab1 1322 GTTGCCTGCGGCGTGCCTATACATGCAGTCGAGCGAATGGATTAAGAGCT TGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG CCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACA TTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTAT GGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG GCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGG TCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGG CACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAA GCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACC GTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGG AATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTG GCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT GCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAA GCACTCCGCCTGGGGAGTACGGCCGCACGGCTGAGACTCAAAGGAATTGA CGGGGGCACGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG TAGAACTTACCAGGTCTTGACATCATCTGCAAACGCTAGAGATAGGGCTT CTCCCTCGGGAGCAAATGGACAGTCCGTGCAAGGGTGTCAATCAGCTCGT GTCGTGAGATGTTCGGCTCAGATCCAGCAACGAAAGCAACGCGTGAATCT TACCTGACATCCGGGAGGTTGGGCCCTTTAAGGTGTCTTGTCGAGGAAAA Blast result: B. cereus Qc=98% PI=96.55

B. Sequence based on forward primer of 16 S rRNA of *B. cereus*

Isolate	Sequence based on Reverse primer
K21	**>H220124-010_C01_S1_1492R.ab1 1453** TCCACATCTGTCACTTAGGCGGCTGGCTCCAAAAGGTTACC CCACCGACT TCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTA CAAGGCCCG GGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGC GATTCCAGC TTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAAC GGTTTTATG AGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTC CATTGTAG CACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGAC GTCATCCCC ACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCC CAACTTAAT GATGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTT AACCCAACA TCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCA CTCTGCTCC CGAAGGAGAAGCCCTATCTCTAGGGTTTTCAGAGGATGTCA AGACCTGGT AAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCAC CGCTTGTGC GGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGT ACTCCCCAG GCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGA AACCCTCTA ACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTA TCTAATCCT GTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGAC CAGAAAGT CGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCA CCGCTACA CATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGT

	TTCCAATG ACCCTCCACGGTTGAGCCGTGCGCTTTCACATCAGACTTAA GAGACCACC TGCGCGCGCTTTACGCCCAATAAGTCCGGATAACGCTTGCC ACCTACGTA TTACCGCGGCTGCTGGCACGTAATTAGCCCGTGGCTTTCTG GTTAGGTAC CGTCAAGGTGGCAGGCTTATTTCACCTAGCACGTGGTTCTT TCCCTAAAC AGCAGAGTTTTTACGACCCCAAAAGCCATTCATTAATTCAA AGCGGGCGA TTACTCCCGCCAAAAATTTTCCGCCCCCTTTGTGTAAATAAT ACCCTTAT TTGTGTGCCTTTCGGGAAAAAAAAATCTTGGGGGCCCGGGG TCCCCAAGT CCCAAAGGGAGGGGGCGGAATACCCCCCTTTCTTCAAGGG GGCCAGGAAA CACACCCACCCCCTTTCCCCCCCTGTGGAAAAACCCCCCTT ATCCCTCCC CTGATGTTGATGAAGAAAAGAAGGCGCGAACGGCGCGGGA GACACACTAA CAACAAGAGAAAAGAAGAGGCGGGGGGGGAGGCCCCCTCT TTCTTCTATT TATAAAAGAGACCCCGGGCGCTTGTTTAAATAATTTTTTTTC CGCGCAGA TAA • Blast results: B. cereus • Qc=74% • PI 98.34

Trimmed and corrected 16S rRNA gene sequence of isolate K21 (FASTA format)

TGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGG
GTGAGTAACACGTG
GGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGG
ATAACATTTTGAACC
GCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCG
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GGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGA
TCGGCCACACTGGGA
CTGAGACACGGCCCAGATTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
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CGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTT
AGGGAAGAACAAGT
GCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAA
CTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGTGGTTTCT
TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG
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CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCT
TTAGTGCTGAAGTTA
ACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGG
AATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
GGTCTTGACATCCT
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CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTT
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CAAGACCGCGAGGT

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ACATGAAGCTGGAAT
CGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC
ACACCGCCCGTCACA CCACGAGAGTTTGTAACACCCGAAGTCGGTGGGG