Title: Identification of Protease-producing Bacterial Isolates from Soil and Evaluation of their *Eggshell* and Eggshell Membrane Degrading Capacities

Firaol Zerihun Mamo

A thesis Submitted to the department of Applied Biology,
School of Applied Natural Science
Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Biotechnology

Office of Graduate Studies
Adama Science and Technology University

May, 2024
Adama, Ethiopia

Title: Identification of protease-producing bacterial isolates from soil and evaluation of their *Eggshell* and eggshell membrane degrading capacities

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Declaration

I hereby declare that this Master Thesis entitled Identification of protease producing bacterial isolates from soil and evaluation of their Egg shell and eggshell membrane degrading capacities is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation

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Recommendation

I/we, the advisor(s) of this thesis, hereby certify that I/we have read the revised version of the thesis entitled Identification of protease-producing bacterial isolates from soil and evaluation of their Egg shell and eggshell membrane degrading capacities prepared under my/our guidance by Firaol Zerihun Submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biotechnology.

Therefore, I/we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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Approval of M.Sc. Thesis

I/we, the advisors of the thesis entitled Identification of protease producing bacterial isolates from soil and evaluation of their Egg shell and eggshell membrane degrading capacities and developed by Firaol Zerihun, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Firaol Zerihun have read and evaluated the thesis entitled Identification of protease producing bacterial isolates from soil and evaluation of their Egg shell degrading capacities and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Biotechnology.

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Finally, approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

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List of Acronyms and Abbreviations

AL……..Arable land

CB……..Cake bakery

ESM……...Eggshell membrane

MALDI-TOF MS…… Matrix assisted laser desorption ionization-time of flight mass spectrometry

MSM……..Minimal salt media

PPBI……..Protease producing bacterial isolates

TCA……..Trichloroacetic acid

WES…….Whole eggshell

ABSTRACT

Eggshells and eggshell membranes are non-edible wastes largely disposed of, but these are the reserves of many bioactive compounds. Eggshells are one of the most widely used food processing and manufacturing plant by-products. The disposal of eggshell and underlying membrane waste contributes to abrasiveness, odor, and pollution. Most of the eggshell waste is currently cumulated on-site without any pre-treatment. Proteases are enzymes used in industries such as the production and processing of detergents, food, leather, cosmetics, and waste management. The objective of this study was to isolate and identify protease-producing bacterial isolates from soil and evaluate their eggshell-degrading capacities. Soil samples were collected separately from both the cake bakery waste accumulation site and arable land sampling area. Samples weighing 1 g were serially diluted in double-distilled water and spread onto skim milk agar. A total of 14 protease-producing bacterial isolates were isolated from the study samples. Three bacterial isolates showed high proteolytic activity and were selected for enzymatic study based on their zone of proteolysis. The isolates were identified based on morphological tests, biochemical tests, MALDI TOF MAS (Matrix Assisted Laser Desorption ionization time of flight mass spectrometry), and 16S rRNA sequencing. The sequencing results indicated that the isolated bacteria were Lysinibaccillus sphericus *(99.69%) and two other isolates that were not sequenced. The PPBI (protease producing bacterial isolates) showed promising results for application in degrading whole eggshells and its membrane. The result of the optimization indicated that all three isolates preferred the alkaline condition since their PH was 8. They preferred yeast extract as a nitrogen source, temperature of 37^{o}C for isolate CB1 and 30^{o}C for the left two isolates (AL1 and CB2), and 48 h for the two isolates and 72 h for CB1 of incubation time. Soils from cake bakery waste accumulation sites and arable land in the study area could be good sources from which to isolate whole eggshell and eggshell membrane degrading alkaline protease-producing bacteria. The alkaline protease from PPBI has to be advanced to various sectors of industries, like the leather industries, cosmetics industries, pharmaceutical industries, and waste management systems. Further purification stages and studies should be done for the wide application of the enzyme producing isolates.*

Key words; Alkaline protease, biodegradation, Eggshell, *Lysinibaccillus sphericus*

1: INTRODUCTION

1.1 Background of the study

Waste refers to any unwanted item or material that the owner wishes to get rid of, regardless of whether it can be reused, recycled, recovered, or not (DEA, 2019). Municipal Solid Waste (MSW) mainly consists of household waste; with some commercial waste included (Fischer. et al., 2013). Household waste is produced through various activities within a household, such as cooking, cleaning, gardening, and fuel burning. It is crucial to note that improper management of solid waste can have detrimental effects on aesthetics, human health, and the environment (Alam & Ahmade, 2013) Therefore, in order to effectively control waste management and mitigate these negative impacts, it is essential to have a comprehensive understanding of the effects and risks associated with poorly managed solid waste, which account for approximately 85% of the total (Lestari & Trihadiningrum, 2019).

Eggshells are a common by-product of food processing and manufacturing, with a wide range of uses. They are a key ingredient in many products, including cakes, salad dressings, and fast foods. However, the production of these products generates a significant amount of eggshell waste, leading to high disposal costs worldwide. Approximately 250,000 tons of eggshell waste is produced annually (Verma et al., 2012). The shell itself makes up about 10-11% of the total weight of an egg and is primarily composed of calcium carbonate (96%) and trace elements. Additionally, the eggshell membrane contains collagen types I, V, and X, which have various applications in the cosmetic, food, medical, and pharmaceutical industries (Kingori, 2011). The collagen found in eggshell membrane can serve as an alternative to mammalian collagen and has potential for commercial use in different fields (Elahe et al., 2018)

Egg shells and membranes are non-edible wastes largely disposed, but these are the reserve of many bioactive compounds which can be extracted by efficient separation (Bayraktar et al., 2021). Disposal of eggshell and underlying membrane waste contributes to abrasiveness, odor, and pollution (Kingori 2011). Most of the eggshell wastes are currently cumulated on-site without any pre-treatment. Also given the environmental odor from biodegradation, waste management is not a pleasant function (Waheed et al., 2020). In recent years; a lot of efforts have been made to transform eggshell wastes into a valuable product. These major applications included

a possible bone substitute, the starting material to prepare calcium phosphate bioceramics such as hydroxyapatite (HAp) and bone mineralization and growth, a low-cost adsorbent for removal of ionic pollutants from the aqueous solution or a biodiesel catalyst (Torres-mansilla et al., 2023).

Proteases are a class of large and complex groups of enzymes sometimes known by the name of peptidyl-peptide hydrolases enzymes that catalyze the hydrolysis of peptide bonds from protein molecules into polypeptides or free amino acids. Protease enzymes are found in all living organisms; plants, animals, and microorganisms (Pradeep *et al.*, 2012; Sharma *et al.*, 2014). Acid, neutral, and alkaline are the main classes of protease based on their acid-base behavior. Acid protease is produced mostly by fungi, the optimum PH is 2-5 for activities. Neutral protease has a PH range of 7.0 or around and is mainly from plant sources. The PH of alkaline protease is 8 and above, as stated by Khan et al. in 2011. Among the total world enzyme production, approximately 60% is accounted for by protease enzymes, with the majority being alkaline protease, as mentioned by Chu in 2007, Palsaniya et al. in 2012, and Patel et al. in 2018. Alkaline proteases find applications in various industries such as detergent, pharmaceutical, leather, silk, waste management, photographic, and dairy, as highlighted by Sanathan et al. in 2013. Microbes are preferred as a source for these enzymes due to their rapid growth, minimal space requirements for cultivation, and the ease of genetic manipulation to create new enzymes with desired properties for different applications, as explained by (Malik & Ahlawat, 2019).

Ethiopia has broad microbial diversity; however, protease-producing bacteria for eggshell degradation have not yet been explored. Therefore a current study has been initiated with the objectives of isolating and characterizing bacterial strains from soil by determining their ability to produce protease enzymes and evaluating their potential for degrading eggshell and eggshell membrane wastes.

1.2. Statement of the Problem

Ethiopia is struggling with the ever-increasing issue of solid waste as its urban areas continue to multiply and expand in both size and population. This difficulty of improper waste management is particularly prevalent in low-income countries like Ethiopia, adversely impacting water quality and public health. The problem is further intensified in cities where a high concentration of people results in a significant volume of waste generation (Lestari & Trihadiningrum, 2019). The influx of individuals migrating to urban centers in developing countries like Ethiopia exacerbates this issue. Moreover, densely populated areas are at a heightened risk of health hazards as diseases can rapidly propagate (Ziraba et al., 2016).

The implementations of effective waste management practices has been identified as essential for economic development in low-income countries (Scheinberg, 2010). Waste management in Ethiopia is important due to a small percentage of the country's inhabitants have access to safe drinking water with 21% in rural areas, 84% in urban areas, and 30% country-wide. Additionally, populations in rural areas (7%), urban areas (68%), and people country-wide (15%) have adequate access to latrines and other means of improved human waste disposal options (Hirpe, 2021).

Eggshell is a typical example of product-specific household waste with utilizable parts still considered solid waste (Adeogun, *et.al*., 2018). Global egg production projected increase about 90 million tons by 2030 (Ferraz, *et al*., 2018; Muliwa, *et al*., 2018). As eggshells are considered useless, most of this waste is commonly disposed of in landfills without any transformation into useful products (Cree, & Rutter, 2015; Singh, 2018). However, the management of this waste needs adequate strategies to minimize disposal costs, reduce environmental pollution, avoid the risk of pathogens & unpleasant odors, and reduce the availability of disposal sites (Quina, *et.al*., 2017; Meng, X. & Deng, D,2016; Sarder, 2019). Moreover, according to European Union regulations, eggshell is considered hazardous wastes (Quina *et al.,* 2017; Ummartyotin, & Manuspiya, 2018). Therefore, it is indispensable to find alternative ways to convert eggshells into valuable materials for further applications.

Eggshell and eggshell membrane waste management in Ethiopia is a growing concern due to the rapid expansion of the poultry industry. The country's increasing urbanization and changing consumption patterns have led to a substantial rise in egg consumption, resulting in significant quantities of eggshell and eggshell membrane waste (Haile 2023). Despite their potential as valuable sources of calcium carbonate and protein, respectively, these materials are often discarded inefficiently, contributing to environmental pollution and health hazards. Effective waste management strategies are crucial to mitigate these impacts and harness the potential benefits of eggshell and membrane residues. Composting and bioconversion technologies offer promising avenues to transform these waste materials into valuable resources for agriculture and industry. Implementing such strategies not only addresses waste management challenges but also promotes sustainability and circular economy principles in Ethiopia's poultry sector (Haile 2023).

1.3 Objectives of the study

1.3.1 General objective

- ✓ To identify protease-producing bacteria from soil and evaluation of their egg shell and eggshell membrane degrading capacities.

1.3.2 Specific objectives

- ✓ To identify protease producing bacteria from soil samples by using morphological, biochemical, MALD TOF and 16s rRNA methods bacterial identification
- ✓ To optimize the physical and chemical conditions of protease-producing bacteria for optimum growth condition and good yield of protease enzymes
- ✓ To evaluate the activities of protease producer against egg shell and eggshell membrane

1.4. Significance of the study

Biodegradation of waste can contribute to the country‘s economy by saving costs for waste collection and dumping into landfills. The biological treatment of wastes appears to be the most cost-effective and carries a less negative environmental impact. In terms of socioeconomic consideration, this study will be important because it pointed out some alternative mechanisms to bring household and poultry waste management systems. The study gave some guideline information to policymakers, solid waste managers, and researchers about the preexisting situation of solid waste management. Additionally, the isolation of protease from different environments of soil may lead to the discovery of new proteases with unique physiochemical characteristics. It may also put baseline information to the next work that would like to conduct detailed and comprehensive studies in the area. The study may also contribute to a better understanding of the present status of waste management and handling practices.

Utilizing eggshell waste in various applications offers significant environmental and economic benefits. Eggshells can be repurposed as a biological template for catalysis and antibacterial purposes, as well as for producing food additives, soil conditioners, cosmetics, pure calcium carbonate, and biomaterials.

2. LITERATURE REVIEW

2.1 An overview of solid waste production over the world

The global waste productions from different sources are increasing at alarming state. These wastes are raised to about 70 % if the current situation continued (Cody,2018). It has been sated that almost about 2.01 billion metric tons of house hold and municipal solid wastes are released to our natural ecosystem yearly based. Furthermore, the World Bank estimates that these solid household and other wastes will be accumulated to about 3.40 billion metric tons by the coming2050 (Cody,2018). According to a World Bank report by Hoornweg *et al.,* (2013), global solid waste generation was projected to increase from over 3.5 million tons per day in 2010 to more than 6 million tons per day by 2025. The authors also forecasted a waste peak by 2050 in OECD countries, by 2075 in Asia-Pacific countries, and a continuous rise in Africa until 2100, when solid waste generation is expected to surpass 11 million tons per day. They identified population growth, gross domestic product (GDP), and technological advancements as key influencing factors. Similarly, Karak *et al*., (2011) noted that the exponential growth of population and urbanization, along with improvements in socio-economic standards, led to significant increases in municipal solid waste (MSW) generation worldwide. The researchers highlighted that developed countries produce 521.95-759.2 kg of waste per person per year, while developing countries generate 109.5-525.6 kg per person per year. Interestingly, a considerable amount of household waste is concentrated in smaller geographical regions such as Kuwait and the Caribbean Islands nations. Kuwait, for instance, produces 5.72 kg of waste per capita per day of municipal solid waste, with the lack of proper landfills in the country contributing to the accumulation of trash (Buragohain *et al.,* 1990).

Solid wastes accumulation and its management are the major public health and environmental concern in many developing countries (Guerrero *et al*., 2013). In these developing country, open dumping of solid waste is a common practice, since the bulk of these wastes are not properly collected for managements some country like Thailand (Chiemchaisri *et al*., 2007). The collection of these solid household wastes, the mode of transport, and management are the major concern for sustainable development and environment protection (Cheng *et al.,* 2021). Chemicals which exist within trash can run away into soil and water. Inadequately managed landfills may also cause air and other environmental pollutions across the world (Charlotte, 2012). Waste has a detrimental impact on the environment, resulting in severe hazards for both humans and animals.

These negative effects can lead to disease outbreaks, decreased life expectancy, and an unsafe living environment. Pollution of the air, water, and soil is a direct result of waste. Air pollution can manifest as unpleasant odors, smoke, and dust. The burning of solid waste releases greenhouse gases like carbon dioxide and nitrous oxide, contributing to ozone layer depletion and the greenhouse effect (Bhat *et al.*, 2018).

The types of solid waste generated by households vary according to economic circumstances, seasons, as well as the demographic landscape and location of the areas (Birhanu, Y. and Berisa, G 2015). In higher income areas, more inorganic waste is generated whereas in low-income areas more organic wastes are produced. The population density and socio-cultural, as well as seasonal, factors (e.g., fluctuations in garden waste) also affect waste volumes (Birhanu, Y. and Berisa, G 2015). Tchobanoglous *et al.*, (1993) classified types of solid waste in relation to the source of generation, generation facilities or activities, and locations. However, Charlotte, (2009) Classified solid waste types based on their origin as food waste (60%), rubbish (5%), ashes and residues (25%), demolition and construction (7%), and agriculture waste (3%). On the other hand, Cheremisinoff, (2003) classified solid waste based on their biological characteristics as biodegradable (80%) and the other (20%) is non - biodegradable wastes. Solid wastes could also be classified based on the risk potentials associated with the waste as hazardous waste (16%) and non - hazardous waste (84%) (Zurbrugg, 2003).Types of house hold solid waste includes; a) Organic waste: kitchen waste, vegetables, flowers, leaves, fruits. b) Toxic waste: old medicines, paints, chemicals, bulbs, spray cans, fertilizer and pesticide containers, batteries, shoe polish. c) Recyclable: paper, glass, metals, plastics. d) Soiled: hospital waste such as cloth soiled with blood and other body fluids. (http://edugreen.teri.res.in/explore/solwaste/types.htm, accessed on 22 December 2021).

2.2. Risks and problems associated with solid waste

It is fact that, if solid wastes are not managed properly there are many negative impacts on aesthetic, human health and environment (NEMA, 2007). Therefore, In order to control the management activity in a good manner and have a proactive measure for such negative impact, one must have a good understanding about (85%) the effects and risks that may arise from improperly managed solid wastes (WHO, 1996). The following are some of the most important impacts because of uncontrolled solid waste disposal systems. Flies and Mosquitoes breed in some constituents of solid wastes, and flies are very effective vectors that spread disease (Staniskis,

2005); Waste dumps are good shelter for rats. Rats consume and spoil food, spread disease, damage electrical cables and other materials and uncollected wastes cause blockage of drains, which result in flooding and unsanitary conditions (Jayarama, 2011); Uncollected wastes degrade the urban environment, discouraging efforts to keep the streets and open places in a clean and attractive conditions (Unnisa and Rav, 2013); Dangerous items such as broken glass (23%), razor blades (12%), needles and other healthcare wastes (34%), aerosol cans and potentially explosive containers, (31%) may pose risks of injury or poisoning, particularly to children and people, who sort through waste (Staniskis, 2005); Waste items that are recycled without being cleaned effectively or sterilized can transmit infection to later user (Barr, 2004).

Polluted water (leachate) flowing from waste dumps and disposal sites can cause serious pollution of water supplies (Tasi, 2007). In Africa for instance, solid waste is regarded as the second most important environmental health concern apart from water quality as per the WHO (Zerbock, 2003). The problems caused by solid waste in urban Africa is largely due to the explosive growth rates, particularly in sub-Saharan Africa, which eventually translates into generation of copious amounts of solid waste (Basha, 2017). At the dumpsites, garbage and food leftovers attract animal rodents and insect vectors transmit diseases to the people leaving nearby Flies and mosquitoes breed in the blocked drains and these are vectors that spread diseases such as malaria (Basha, 2017).Emission of bad smell is major other problems they encountered in addition to its health problem Getachew and Habtamu (2015). Waste that is treated or disposed of in unsatisfactory ways can cause a severe aesthetic nuisance in terms of smell and appearance (Zurbrugg, 2003); Fires on disposal sites can cause major air pollution (32%), causing illness (23%) and reducing visibility (4%), making disposal sites dangerously unstable, causing explosions of cans (8%), and other related diseases (32%) possibly spreading to adjacent property (Landreth and Rebers, 1993).

2.3. Waste management

Waste management is the collection, transport, processing, recycling or disposal, managing and monitoring of waste materials. The term usually relates to materials produced by human activity, and is generally undertaken to reduce their effect on health, the environment or aesthetics (Dereje, 2001). It has been reported that waste management used to recover resources from pervious ignored wastes. Waste management can involve solid, liquid, gaseous or radioactive substances, with different methods and field's expertise for each. Waste management practices differ for

developed and developing nations, for urban and rural areas, and residential and industrial producers (Dereje, 2001). Management for non-hazardous waste residential and institutional waste in metropolitan areas is usually the responsibility of local government authorities, while management for non-hazardous commercial and industrial waste is usually the responsibility of the generators (Dereje, 2001).

2.3.1 Solid Waste management practices

Collecting and managing solid waste is an important challenge for countries across the world. This problem is often magnified in cities where a dense concentration of people leads to a substantial amount of waste generation (Contireau, 2004). In developing countries like Ethiopia, this problem is increased by an influx of people moving to urban centers (Dereje, 2001). Densely populated areas are more susceptible to health risks as disease can be spread quickly (Chertow, 2007). The implementation of effective waste management practices has been identified and used for many years as essential for economic development in low income countries in particular (Scheinberg, 2010). Urban centers are usually the hardest hit as efforts to develop and grow lead to an influx of economic opportunities and people (Gilbert, 2013). In the middle ages the Bubonic Plague swept through cities as solid waste was improperly disposed of in the roads (Awetash, 2003). Given the tragic consequences of the past, it is vital that improving waste management practices in the growing cities of Ethiopia be a top priority (Kuma, 2004). Most of them tend to focus on the technical dimensions of the municipal waste management, such as estimating the amount of solid waste composition (i.e. 89.4% around Adama city) (Lemma, 2007). Systems that should be put in place (e.g. new sanitary landfill, transfer stations, composting sites, new trucks and containers, data on waste generation, and waste composition) in order to improve the capacity of the management in the city (Dereje, 2001). The city's suffers from poor solid waste management and refer to lack of data on waste generation, of recycling activities, lack of proper transport schedules, a poor sanitary landfill, and a low level of awareness among the citizens as the main obstacles (Zelke, 2005).

Waste management is complex and, has technical, social, economic, cultural, and political aspects. Some studies have followed the neoliberal discourse, and attempted to explore households' willingness to pay for waste services as well as the role of the private sector in the provision of waste management (Oteng, 2010). As Ethiopia's urban areas increase in number and expand in geographical and population size, solid waste is swiftly emerging as a significant issue in environmental management (NEMA, 2005). Success of any municipal solid waste management

system relies on precise data about the quantity and types of material being generated as a waste. Waste composition studies are also essential to any proper waste management strategy for a need to estimate potential materials recovery, to identify sources of component generation, to facilitate design of processing equipment, to estimate physical, chemical, and thermal properties of the wastes, and to maintain compliance with regulations (Basha Gedefa Bulto, 2017).In most developing countries, however, even the most basic data on waste such as the quantities generated and composition of the waste stream are lacking, making it difficult to organize waste management effectively (Hardoy *et al*., 2001). It is thus, a prerequisite for all program managers of SWM to have detailed information about the nature and quantity of solid waste generated in order to set appropriate management strategies(Basha Gedefa Bulto, 2017).

2.4. Eggshell and eggshell membrane as a waste

Global egg production has reached 5.8 x 10^{10} kg per year, with approximately 167 eggs consumed per person per year (Quina *et al* ., 2017 ; Xiao *et al.,* 2014). Malaysian egg consumption is reported to have increased from 2.2 kg (1961) to 17.9 kg (2017) per person. This consumption surpassed that of Americans in 2017 (15.6 kg per person). In addition, China is not only the second largest economy in the world, but also had the highest egg consumption in 2017 (22.7 kg per person) (UO, 2021). As the consumption of eggs increases worldwide, large amounts of eggshell waste (ES) are generated and the egg industry is often abandoned and the waste sent to landfills. Large volumes of ES waste are not profitable due to limited handling in landfills. In addition, eggshell membranes (ESM) are rich in protein, which attracts rodents and pests. Disposal and management of ES waste is therefore costly (Waheed *et al.,* 2020; Sonenklar, 1999). Currently, there are no estimated costs for ES waste management in landfills in Malaysia, but his ES waste management costs for egg processing plants in the United States are approximately $100,000 per year (Mignardi *et al*., 2020).

Hen eggs constitute one of the most important food resources in the framework of worldwide feeding. They are an important source of essential nutrients to human diet providing proteins, fat-soluble vitamins (A, D, E and K) and trace-minerals like iron and zinc (Pirvutoiu and Popescu, 2012; Roberts *et al.,* 2005). In terms of waste generation in egg breaking operations, eggshell (ES) obtained from processing the raw material (shell eggs) is identified as the main process by-product, regardless of the egg product obtained. In the literature, some data are available to quantify the specific waste index of ES. Russ and Pittroff (2004) for example indicate that ES represents 3 to 12% of the egg mass product obtained, depending on the egg shell properties (size and shell

thickness). In addition, Jewell *et al.*, (1975) have monitored waste and wastewater production in an American egg breaking industry during three days, and again concluded that ES may represent 13% of edible egg product output.

The shell weighs about 11% of the total mass of the egg and consists of calcium carbonate (94%), magnesium carbonate (1%), calcium phosphate (1%) and organic matter (4%) (Wu, S. C. *et al.*, 2013; Stadelman.*et al.*, 2000). Eggshell could be the starting material to prepare hydroxyapatite for the sustainable treatment of water polluted by toxic metals (De Angelis, *et al.*, 2017; Francis, *et al.*, 2016). In addition, the conversion of eggshell waste in to sorbent material for toxic metal remediation may reduce the mining impact on the natural reserves of phosphate rock that is included in the European Commission List of critical raw materials (Mignardi *et al.*, 2020).

2.4.1. Eggshell and eggshell membrane waste in Ethiopia

In Ethiopia, eggshell waste management remains a critical environmental challenge due to its substantial volume and limited recycling infrastructure. Eggshell waste, predominantly generated from poultry farms and households, poses significant disposal issues, contributing to environmental degradation and health risks (Alemu 2020). Despite efforts to promote sustainable waste management practices, including composting and incorporation into agricultural soils, the scale of eggshell waste generation often surpasses the capacity for effective utilization (Alemu 2020). Addressing these challenges requires coordinated efforts among government agencies, private enterprises, and communities to develop comprehensive waste management strategies that prioritize both environmental sustainability and public health. In Ethiopia, the accumulation of eggshell waste presents a significant environmental burden, exacerbated by the country's burgeoning poultry industry. With poultry farming expanding to meet domestic and export demands, the volume of discarded eggshells has surged, straining waste management systems and posing environmental hazards (Alemu 2020). Improper disposal of eggshells not only clogs drainage systems but also contributes to soil and water contamination due to the slow degradation of calcium carbonate residues. Addressing this issue requires urgent attention to develop efficient degradation strategies that can integrate eggshell waste into sustainable agricultural practices (Alemu 2020). Such strategies could include composting, where eggshells can be processed into nutrient-rich fertilizers, or innovative technologies that facilitate their breakdown into bioavailable forms beneficial for soil health. By adopting these approaches, Ethiopia can mitigate the environmental impact of eggshell waste while promoting agricultural sustainability and food security (Alemu 2020).

2.4.2. Structure and composition of eggshell and membrane

Eggshells, which form the outer skin of avian eggs, are naturally porous bioceramics. It has been extensively studied since 1964. The chicken eggshell is a natural porous bioceramic resulting from the sequential deposition of the different layers within the segments of the hen oviduct over a predetermined period. It is a perfectly ordered structure with a polycrystalline organization throughout the calcified shell described in many reviews (Hamilton, 1986; Tullet 1987; Nys *et al.*, 1999, 2001, 2004). The structure of eggshell and egg membrane is better understood today thanks to scanning electron microscopy and microfocus X-rays Scattering techniques (Lammie *et al.*, 2005).However, there is some ambiguity regarding this Configuration still exists. The eggshell which consists of various different layers can be described as a well-organized structure, the formation of which begins at different segments of the hen's oviduct. A set of various proteins (soluble and insoluble) and minerals are deposited during eggshell formation and then consumed by the eggshell Developing embryo. Insoluble proteins have been suggested to function as structural proteins Scaffolds and soluble proteins are embedded in the calcified layer. Of Deposited and mobilized calcium is used in embryonic development and formation skeleton ((Lammie *et al.*, 2005; Stadelman and Cotterill 1996).

The shell of an egg consists mostly of calcium carbonate (95%) and a small amount of organic matrix (3.5%) can be divided into six different layers (from inside to outside) (Nys and Gautron 2007). Inner shell membrane forms the innermost layer (20μm thick) and in direct contact with albumen proteins. The outer membrane which lies just above the inner membrane is approximately 50μm thick and it is located between the inner membrane and the calcified part of the shell. Both, the inner membrane and the outer Membrane are made up of interwoven protein fibers and lie parallel to the egg surface providing structural support to the eggshell as a whole (Lammie *et al.*, 2005; Nys and Gautron 2007). This structure is important in acting as a barrier against microorganism penetration. Their organic composition has been subject to controversy.The eggshell membrane has a great influence on shell strength and also prevents shell damage from Invasion of microorganisms. Shell membrane proteins have been found to do this it contains a lot of arginine, cystine, glutamic acid, histidine, methionine, and proline (Stedelmann and Cotterill, 1996).The identification of desmosine and isodesmosine suggested the presence of elastin-like proteins (Leach, 1982) but did not fit with the low concentration of glycine. Presence of hydroxylysine revealed presence of collagen, which was identified by immunohistochemistry as collagen types I, V, and X (Arias *et al.*, 1997; Wang *et al.*, 1984, 2002). However, the bulk of the amino acid

composition differs from collagen and suggests that collagen is not predominant but that a unique protein containing lysine-derived cross links may be present (Leach, 1982).

The calcified part of the shell (consisting of calcium carbonate crystals) It precedes the adventitia and can be divided into three layers. Breast layer, Palisade and vertical crystalline layers (Lammie *et al.,* 2005).Breast layer forming the innermost layer of calcification (70 µm thick) Part of the eggshell penetrates the outer membrane by numerous carbonates corn. The initiation of formation of calcium carbonate crystals occurs at: Nipples, which are organic nuclei deposited during egg formation (Lammie *et al.,* 2005). The inner calcified layer (cone or mammillary layer) is about 70 µm thick and is composed of basal parts of calcified columns and cones that penetrate the outer eggshell membranes. On the surface of the outer eggshell membranes some organic cores (mammillary knobs) are deposited during the passage of the forming egg in the red isthmus. These organic cores are the seeding sites on which the calcium carbonate crystals deposition is initiated, beginning with a spherulitic deposition of microcrystals of calcite (the calcium carbonate polymorph of the shell) around the mammillary knobs resulting in hemispherical nucleation centers. The palisade layer (200 µm thick) lies above the mammillary layer and forms the major portion of the calcified layer of the eggshell. In this layer the calcite crystals grow perpendicular to the eggshell membranes. It also has a small portion (2-5%) of organic matrix incorporated in the calcite crystals. Pores formed in the palisade layer help in the exchange of gases. The formation of pores takes place when the adjacent crystals fail to fully join each other along their side surfaces, leaving a gap between the crystals. The palisade layer gives way to the vertical crystal layer (Lammie *et al.*, 2005; Nys and Gautron 2007 ; Stadelman and Cotterill 1996).The vertical crystal layer which is about 8µm thick is a very narrow/thin layer and consists of the upper most part of calcite crystals which provides a surface for the formation of the cuticle (Lammie *et al.*, 2005; Nys and Gautron 2007).The palisade layer extends beyond the bases of the cones and ends in the vertical crystal layer. It's a narrow band of vertically oriented crystals that are aligned perpendicular to the shell surface.

The cuticle is the outer most water insoluble layer of the eggshell (10 – 30 µm thick) (Lammie *et al.,* 2005; Nys and Gautron 2007). The layer is largely an organic layer with protein contents as high as 90% and with a high content of cystine, glycine, glutamic acid, lysine and tyrosine. Fructose, galactose, glucose, hexosamines, mannose, and sialic acid have been reported to be present as constituents of the polysaccharides (Stadelman and Cotterill 1996).It contains a thin film of hydroxyapatite crystals in the inner part of the cuticle (Dennis *et al*.,1996). The cuticle clothes the shell and plugs the pores, thus preventing microbial penetration. Using SEM, cuticle has a cracked appearance (Solomon 1991) that is a feature of drying. Cracks are

essential for the exchange of gas via pores. It is of variable thickness and may even be missing, and is composed of glycoprotein, polysaccharides, lipids and inorganic phosphorus including hydrox yapatite crystals (Dennis *et al.,* 1996; Fernandez *et al.,* 2001; Whittow, 2000). This layer, as well as the outer portion of the calcified shell, contains the eggshell pigments responsible for shell color.

2.4.3. Polypeptides and polysaccharides of the eggshell and membrane

Since 1990, numerous efforts have been carried out to identify and characterize the protein components of the calcified shell and the organic membrane. Matrix proteins have been identified using various biochemical and molecular biological techniques. The previous studies in combination with the recent development of the functional genome tools and the sequence of the chicken genome have led to the identification and characterization of a variety of eggshell matrix components (Gautron and Nys, 2007). The chicken eggshell matrix is a complex mixture of interwoven protein fibers and polysaccharides with at least 70% of the matrix being proteins (Gautron and Nys 2007). It was estimated that 11% of the matrix is polysaccharide that contains chondroitin sulphate A and B, dermatan sulphate, hyaluronic acids, keratan sulphate and uronic acids (Gautron and Nys 2007). Ovalbumin, which is an egg white protein, has been observed to be localized in the mammillary knobs of the eggshell. Two other major egg white proteins, the lysozyme and ovatransferrin were identified at the basal parts of the eggshell (eggshell membranes, mammillae) (Gautron and Nys 2007). Various glycoproteins such as osteopontin (a phophorylated glycoprotein) and clusterin (a secretory disulphide-bonded heterodimeric glycoprotein), have been reported to be localized in the different layers of the mineralized and non-mineralized parts of the eggshell (Gautron and Nys 2007).

A number of proteins have been found to be novel and specific to the eggshell. They are only secreted by tissues where eggshell formation takes place and have only been identified in domestic hens. Ovocleidin-17 (OC-17) was the first matrix protein purified to homogeneity (Hincke *et al.,* 1995). It is synthesized in the tubular gland cells of the uterus and secreted into the uterine fluid in relative abundance during the calcification growth phase. It is present throughout the entire calcified part of the shell. Ovocleidin 116 (OC-116) was the first eggshell matrix protein that was cloned (Hincke *et al.,* 1999). It's a 80 ka protein (742 amino acids) that contains two N-glycosylation and two disulphide bonds (Mann *et al.,* 2002). This protein is secreted in the uterine fluid during the active calcification phase and is abundant in the palisade layer of the shell. Ovocalyxin – 32 and 25, Ovocalyxin 36 , are localized in various layers of the calcified shell (Gautron and Nys 2007). High contents of arginine, glutamic acid, methionine, histidine, cystine ,

hydroxyproline, hydroxylysine, and desmosine were found in proteins of the shell membranes (Gautron and Nys 2007).Proteins such as collagen which hold high economic and monetary value have been reported to be present in eggshell membrane (Arias *et al.,* 1990; Wong *et al.*, 1984a).

2.4.4. Potential uses of separated eggshell and membrane

Eggshell which forms the outer crust of an egg is a non-edible product with very limited use & value and is largely disposed of as a waste. There has been an exponential growth in the processed egg industry with 30% of the egg produced in United States today, is consumed by the processed egg industry. According to an estimate by the United States Department of Agriculture, the egg processing industry consumed 25.6 million cases of egg in 1984, to manufacture liquid and dry egg products. In 1997 the same industry consumed about 50 million cases of egg, producing more than 120,000 tons of Unprocessed egg shell waste with disposal costs between $ 25,000 and $ 100,000 per year (MacNeil, 2001).

Keeping in mind the high disposal costs which continue to increase due to increase in landfill taxes and increasing environmental concerns, it is necessary to find an alternative method which would transform the waste eggshells into a valuable item; giving financial benefits to the competitive egg processing industry. Apart from giving manufacturers a new profit stream it would help overcome the high disposal costs and environmental concerns (MacNeil, 2001, 2006).There are many uses of separated eggshell and membrane (MacNeil, 2001) but not many when they are attached. The following section gives a brief review of various potential uses of separated eggshell and membrane.

Collagen: Collagen constitutes 10% of the total protein content of the egg membrane (MacNeil , 2001, 2006). Collagens of type I, V and X have been identified in the eggshell membrane (Arias *et al.,* 1990; Wong *et al.,* 1984a). A lot of emphasis has been given to the presence of collagen in eggshell membrane due to its important economic and monetary value. Collagen finds wide scale usage in the field of biomedical applications, such as skin grafts, tissue replacement products, plastic surgery, cornea repair, prosthetic implants etc (De Vore *et al.,* 2007; Long *et al.,* 2004 ; MacNeil , 2001, 2006). Apart from its biomedical uses it is widely used in food industry for production of gelatin. Keeping in mind the 1997 estimates, 120,000 tons of eggshell waste would yield 110,000 tons of eggshell and 10,000 tons of membrane. Considering that 10% of membrane is collagen, it would yield 1,000 pounds of collagen which is presently priced at $ 1000 per gram or about $ 454,000 per pound (MacNeil, 2001). Collagen is

generally derived from bovine tissues and to lesser extent human collagen is also used. There are a number of issues associated with use of bovine collagen, such as the possible transmission of bovine spongiform encephalopathy (commonly known as the mad cow disease). Though the possibility/ risk of transmission are very low but it calls for the maintenance of a well isolated and expensive herds. Also it is estimated that 2% to 3 % of the population is allergic to bovine collagen. Therefore, extraction of collagen from the eggshell membrane would help to overcome the issues associated with the use of bovine collagen (MacNeil, 2001, 2006).

Lysozyme and Avidin: Lysozyme finds wide scale usage in food and pharmaceutical industry due to its antibacterial properties. Lysozyme has been reported to be present in shell membranes and in the matrix of the calcified shell (Hincke *et al.,* 2000). The main application of lysozyme in the food industry involves the inhibition of clostridium tyrobutyricum during cheese maturation. Its application in pharmaceutical industry, involves the preparation of "aerosols for the treatment of bronchopulmonary diseases and for its prophylactic functions relating to dental caries. It is also used in droplets for nasal tissue protection and various therapeutic creams designed for the protection and topical reparation of certain dystrophic and inflammatory lesions of the skin and soft tissues, e.g., burns, viral diseases such as Herpes and shingles, as well as for the treatment of recurrent aphthous stomatitis" (Lesnierkowsi and Kijowski, 2007). Oral administration of lysozyme has been reported to induce immune stimulation effects in guinea pigs (Namba *et al.,* 1981).Avidin finds wide scale usage in biomedical industry. Due to its high affinity constant for biotin, it is widely used in molecular biology techniques such as Enzyme Linked ImmunoSorbent Assay (ELISA), molecular recognition and labelling, affinity chromatography, cytochemistry and histochemistry (Wilchek and Bayer 1990).

Ovotransferrin (Conalbumin): An iron binding protein present in birds which is widely used for delivering iron to cells and inhibiting and/or controlling bacterial multiplication. Also, the antiviral activity of ovotransferrin towards chicken embryo fibroblast infection by avian herpes virus has been reported. Ovotransferrin was found to be more effective than human and bovine lactoferrins in inhibiting the same (Giansanti *et al.,* 2001). Ovotransferrin is also used as a nutritional ingredient in many iron-fortified products available in the market today such as iron supplements, iron fortified mixes for instant drinks, sport bars and protein supplements and iron-fortified beverages. Ovotransferrin was shown to be effective against acute enteritis in infants (Corda *et al.,* 1983).

Ovalbumin: Ovalbumin is the first egg white protein that was also observed in the shell. It is localized in the mammillae of the eggshell and is secreted in abundance in the uterine fluid at the

initial stage of eggshell formation(Gautron *et al,.* 2010).Therefore, in the hen oviduct it appears likely that ovalbumin is present in the uterine fluid when the proteinaceous cores of the mammillary bodies are formed, and it is incorporated into these structures. Although ovalbumin represents more than 50% of the egg white proteins, a specific function has not yet been defined. One proposed role for ovalbumin is that of metal ion transport and storage and its ability to bind a variety of di- and tri-valent cations, including Ca2+ at a specific site would support this concept. Moreover, ovalbumin may affect calcite mineralization, since it accelerates the nucleation of calcium carbonate in an in vitro precipitation assay(Hincke, 1995). Reported to be present in the mammillae of the eggshell (Gautron and Nys 2007). A purified ovalbumin finds widescale usage in molecular biology techniques, such as Enzyme Linked ImmunoSorbent Assay (ELISA), Western Blotting (used as blocking agent), in SDS- PAGE (Neova Technologies).

Chondroitin sulphate: It forms important structural components of the cartilage largely responsible for giving it the resistance against compression. Along with glucosamine it is now widely regulated as a dietary supplement in many countries including the US (National Centre for Complementary and Alternative Medicine, USA). It also forms an integral component of the alternative medicines used to treat osteoarthritis and along with glucosamine, chondroiton sulphate finds application in veterinary medicine (Forsyth *et al.,* 2006 2014). Chondroitin sulfate has been found to play an essential role in promoting cartilage health and potentially treating osteoarthritis through its impact on chondrocytes and the structure of cartilage(Bierbrauer *et al.,* 2014). In addition, chondroitin sulfate is believed to promote water retention and elasticity in cartilage, as well as exert anti-inflammatory effects by protecting cartilage against the damaging effects of free radicals. Moreover, chondroitin sulfate is also known for its anti-inflammatory and antiapoptotic effects, which contribute to its increasing utilization in the treatment of osteoarthritis (Huang *et al.,* 2015).

Hyaluronic acid: Hyaluronic acid is another substance of high monetary value which is naturally present in and is a constituent of eggshell membrane. The total hyaluronic content of eggshell membrane is estimated to be between 0.5 – 10% (Long *et al.,* 2005). Hyaluronic acid is actually a glycosaminoglycan (GAG) which is found in many body tissues such as cartilage and skin and is responsible for increased resistance to compression in some tissues. Because of high hydration capacity/ability of hyaluronic acid (Long *et al.,* 2005), it finds wide scale usage in cosmetic creams claiming to make the skin appear smoother by hydrating the skin (wikipedia.org). Various studies have reported/ demonstrated hyaluronic acid to be an effective treatment for rheumatoid and osteoarthritis (FDA). Fastening of the wound healing process and reduction in the appearance of old and new scars by the administration of hyaluronic acid has also been

reported. US. Pat. No. 6946551 held by Long *et al.,* (2005) describes a method of preparation/extraction of hyaluronic acid from eggshell membrane.

Amino Acids: The eggshell membrane and the eggshell is known to be rich in arginine, glutamic acid, methionine, histidine, cystine, hydroxyproline, hydroxylysine, desmosine, lysine, leucine, isoleucine, tyrosine, phenylalanine and trytophan which when extracted would find wide scale application in the biomedical, food, cosmetic and pharmaceutical industry (vlad, 2007). The eggshell has proteins like Ovocleidin- 17, Ovocleidin-16, Ovocalyxin – 32 and 25, Ovocalyxin 36 which are novel and unique to eggshell membrane and have new potential applications (Gautron and Nys, 2007).

2.4.5. Therapeutic and cosmetic applications

Much importance has been given to the presence of various therapeutic and cosmetically active components such as collagen, hyaluronic acid, glucosamine, chondroitin sulphate present in eggshell membrane having potential applications in cosmetic and pharmaceutical industries. The following components when extracted from other natural resources demands for significant processing cost due to the presence of these compounds in low quantity or due to the additional costs levied to obtain these compounds in the desired purity. Therefore, the extraction of these compounds from egg membrane, which is typically a waste product, is expected to reduce the cost Considerably. Also, depending upon the targeted application the composition / percentage of the compounds can be altered to serve the purpose (Long *et al.,* 2004).

US patent no. 2007/0178170 held by Devore *et al.,* (2007), discusses the anti-inflammatory properties of eggshell membrane and processed eggshell membrane preparations. Eggshell membrane was reported to be an ideal split-thickness skin graft (STSG) donor site dressing. It exhibited properties of pain relief; wound protection, promotion of healing (Yang *et al.,* 2000). Also, dried non-fibrous egg membrane products assisted and stimulated healing process in damaged mammalian tissues such as the tissues lost or damaged due to cuts, injuries, burns and ulcerations (Neuhauser, 1965).

2.5. Microbial protease; Protease enzyme

Microorganisms are good source of protease. This is because microbes have broad biochemical diversity, susceptibility to genetic manipulation, and possess almost all characteristics desired for their applications in biotechnology. Due to these proteases from microbial sources are preferred

over the enzymes derived from plant and animals. Among microorganisms, Bacteria and fungi are the most prominent source of this enzyme (Abrar, 2017). Among bacteria, most commercial protease are produced by *Bacillus* species such as *B. cereus, B. circulans, B. firmus, B. lentus, B. licheniformis, B. pumilus, B. sphaericus, B. stearothermophilus* and *B. subtilis* . Among fungi, species such as *Aspergillus candidus A. flavus A. fumigatus A. melleus A. niger A. oryzae, A. sojae, A. sydowi, Chrysosporium keratinophilum,Conidiobolus coronatus,Entomophthora coronata, Fusari Rhizopus oligosporu* are tend to produce protease enzymes (Jisha *et al.,* 2013). Microbial proteases can be extracellular or intracellular. Extracellular proteases carry out protein hydrolysis in fermented media and enable the cell to absorb and utilize hydrolytic products. Intracellular proteases have application in various cellular and metabolic processes, such as, sporulation and cell differentiation, protein turn over, enzyme maturation and hormones and also in protoxin activation of Bt-based biopesticides (Jisha *et al.,* 2013). Protease from microbial source highly affected by types of strain, nutritional source and different physiochemical factors including pH, temperature, carbon and nitrogen sources, inorganic salts, agitation, and dissolved oxygen concentration. There is no defined common medium for protease production from different microbial sources and every microorganism or strain has its own kind of requirement for maximum enzyme production (Kasana *et al.,* 2011).

Proteases are class of large and complex group of enzymes which sometimes known by a name of peptidyl-peptide hydrolases, that catalyse the hydrolysis of peptide bond from protein molecule into polypeptides or free amino acids. Protease enzymes are found in all living organisms; plant, animal and microorganisms (Palsaniya *et al.,* 2012; Sharma *et al.,* 2014). Acid, neutral and alkaline are the main class of protease based on their acid -base behaviour. Mostly fungi produce acid protease with optimum PH 2-5. Neutral protease has an optimum pH of 7.0 or around and is obtained mainly from plant source. Alkaline protease has pH of 8 and above (Khan *et al.,* 2011). Protease enzyme accounts for approximately 60% of the total world enzyme production, of which most are alkaline protease (Palsaniya *et al.,* 2012).

Microorganisms are a good source of protease. Proteases are essential constituents of all forms of life on earth, including prokaryotic and eukaryotic organisms. The inability of the plant and animal proteases to meet the current world demand has led to an increased interest in microbial proteases. The proteases available today in the market are derived from microbial sources. They are generally preferred to other organisms for the production of enzymes because of their fast growth, their limited space requirement for cultivation, and the fact that they can easily be genetically

manipulated to produce new enzyme(Josephine *et al.*, 2012). Bacterial proteases have come to represent one of the largest classes of industrial enzymes, accounting for 40 % of the total worldwide sale of enzymes (Rao *et al.*, 1998). Of these, strains of *Bacillus sp*. dominate the industrial sector (Gupta *et al*., 2002b.Proteases have enormous applications in the manufacture of soy products, detergent, dairy industry, tanning, feed processing, baking, textile, photographic , brewing industries and waste management (Singh *et al.*, 2015).

2.5.1. Protease enzyme producing bacteria and Production method

Protease enzymes are crucial for various industries, and bacteria, particularly species like Bacillus, are recognized for their high-level production of these enzymes (Khusro, 2016). Research has demonstrated that bacteria from diverse environments such as marine sediments, soil samples, and leather industry effluent are potent protease producers (Fasiku et al., 2020; Masi et al., 2021; Anab-Atulomah & Nwachukwu, 2021). The ability of bacteria to produce proteases is not restricted to specific habitats; bacteria from extreme environments like hot springs, glacier regions, and soda lakes have also been found to produce proteases (Salwan et al., 2010; Ibrahim et al., 2015; Fachrial et al., 2021). Moreover, the optimization of protease production from bacteria isolated from various sources highlights the potential for utilizing these enzymes in different industries (Balachandran et al., 2021; Jamaludin et al., 2019). The interest in protease-producing bacteria extends to different geographical locations, with studies focusing on bacteria from regions like Indonesia, India, Algeria, and Brazil (Nourine et al., 2023; Murab et al., 2017). The diverse range of bacteria capable of producing protease enzymes underscores their significance in biotechnological applications and industrial processes. Protease enzymes are widely produced by bacteria through various fermentation techniques. One common method involves submerged fermentation, where bacteria are cultured in a liquid medium under controlled conditions of pH, temperature, and oxygenation. Bacterial strains such as *Bacillus subtilis* and *Bacillus licheniformis* are often employed due to their robust enzyme secretion capabilities and adaptability to industrial settings (Adrio & Demain, 2014). Solid-state fermentation is another approach utilized, particularly in low-cost production setups, where bacteria grow on solid substrates like agricultural residues or industrial by-products. Optimization of fermentation parameters, including nutrient composition and culture conditions, plays a crucial role in enhancing protease yield. These methods not only facilitate efficient enzyme production but also support sustainable practices by utilizing renewable resources as substrates.

3. MATERIAL AND METHODS

3.1 Description of study area

The study was carried out in Nekemte town, situated in the East Wollega zone of western Ethiopia. Nekemte is positioned 335 Km west of Addis Ababa, along the main road to Asosa. It is located at 9◦04’ north latitude and 38◦30’ east longitude, with an elevation ranging from 1960 m to 2170 m above sea level. The town experiences a long wet period from late May to early September, with an annual rainfall ranging from 1,500 to 2,200 mm. The average annual temperature in Nekemte ranges from 14◦C to 26◦C. The soil samples were collected from two different sites of the city one from Sada cake bakery waste accumulation site found in the city and urban based arable land found in the city.

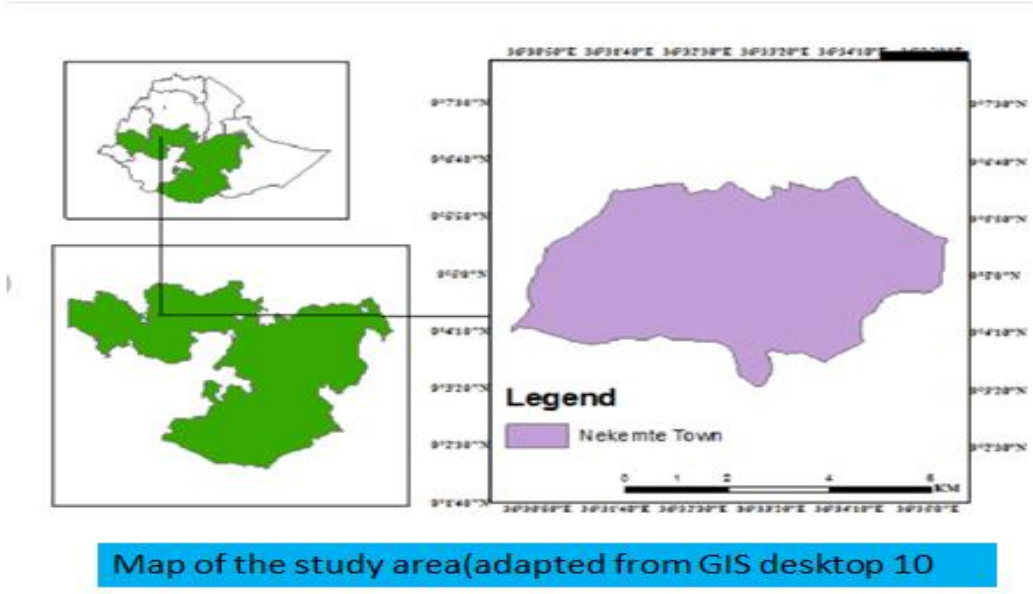

Figure 1. Location of the study area from where the samples were collected

3.2 Soil sample collection

To isolate these Protease-producing bacterial isolates (PPBIs), soil samples were collected from two areas such as arable land present in the city for urban based cultivation and cake bakery waste accumulation site of Sada bread and cake bakery using a sterile spatula from 5-10 cm depth and transferred to different labeled sterile poly bags. The collected soil samples were transported to Wollega University, Department of Biology, and microbiology laboratory. For screening of these PPBIs, 1gm of the waste soil sample for the cake bakery and soil samples for the arable land was weighed and serially diluted up to 10^{-7} in sterile distilled water. About 0.1 ml from the last two dilutions were spread onto a plate on Nutrient agar plates (Peptone 5.0, Yeast extract 1.5, Beef

extract 1.5, Sodium chloride 5.0, and Agar 15.0) and incubated at 30^{o}C for 24 h. Bacterial colonies with distinct characteristics were further transferred on nutrient agar slants and maintained at 4^{o}C.

3.3. Inoculation and screening of protease producing bacteria

3.3.1 Primary screening of protease producing bacteria

Primary screening of bacterial isolates was made to screen protease producers using skim milk agar medium (g/L) (peptic digest of animal tissue, 0.5; beef extract, 0.15; yeast extract, 0.15; NaCl 0.5; milk powder, 1; agar, 1.5, pH 7.0 ± 0.2) (Bhakthavalsalam & Muthusamy, 2018) and well diffusion on gelatin agar plates (Muthusamy, 2018). It was performed by spot inoculation of all the pure cultures onto skim milk agar plates. The clearance zone formed around the bacterial colonies due to proteolysis was measured by ruler after 24-48 h of incubation period at 30^{o} C. The bacterial isolates with large clear zones were selected and subjected to a secondary screening procedure.

3.3.2 Secondary screening of potential protease producing bacteria

The protease enzyme-producing bacterial candidates selected from the primary screening experiments were subjected to a secondary screening. One milliliter of each candidate bacterial inoculum was inoculated to 50 ml of nutrient broth medium and incubated at 30^{o}C on 150 rpm shaking condition. After 24h of incubation, the bacterial cultures were harvested by centrifuging at 10,000 rpm for 15 min at 4°C and the culture supernatant was collected. Skim milk agar plates (2% skim milk) were prepared and wells were made using a 5 mm sterile cork borer. Then 10 µl of culture supernatant of these isolates were loaded in the milk agar wells and incubated at 30°C for 24 h. After the incubation period, the supernatants capable of producing clear zones were screened based on their clear zone sizes. The relevant colonies were then picked and streaked for growing into pure cultures. Thus, pure colonies that showed the significantly larger diameter of clear zones were further grown subsequently on nutrient agar medium. The selected colonies were preserved on nutrient agar slant at 4^{o}C for further study. The maximum protease producing bacterial isolates were selected and identified on the basis of morphological, biochemical characteristics, protein profiles, and molecular techniques

3.4 Characterization of bacterial isolates

The bacterial isolates obtained using primary and secondary screening methods were further characterized and identified through morphological and biochemical analysis as described by Bergey's manual of determinative microbiology. Morphological studies were carried out using Gram staining, motility test and Endospore staining. In addition to cultural characterization of the isolates on agar plates (which include observation for colony morphology as colony shape, size, margin, elevation, opacity, texture and pigmentation). Further characterization of the isolate was done using biochemical tests, which include catalase test, carbohydrate fermentation test, citrate utilization test, H_2S production test, gelatin liquefaction test, methyl red test, starch hydrolysis test , and voges proskauer (VP) test.

3.4.1 Morphological characterization of bacterial isolates

To identify the bacterial isolates that are closely related, screening was executed using morphological characteristics of the bacterial isolates including colony morphology, cell shape, cell structure, motility, endospore staining, and Gram's staining.

Motility test: Semisolid media was prepared by mixing nutrient broth 35%, nutrient agar 65%, and casein 1%. The semisolid media were cooled and inoculated with a straight inoculating needle bursting about 3 cm down into the medium. It was then incubated at 30^{o}C for 24 h. PPBIs were considered as motile based on its dispersed form of growth

Gram staining: Gram staining was performed according to the standard procedure. A smear of bacterial cells was prepared on a clean glass slide following heat fixation. The heat fixed smear was treated with crystal violet solution for 1 min following washing it with running water. The smear was again treated with gram iodine solution for 1 minute following decolorizing it with 95% ethyl alcohol for 10-15 seconds and rinsed with water. Finally, safranin was added as counter stains for 60-80 sec and washed with water, dried and cells were examined under oil immersion microscope.

3.4.2 Biochemical characterization of the best isolates

Catalase test: A drop of 3% hydrogen peroxide (H_2O_2) was used to detect catalase. A drop of 3% H_2O_2 was added to an overnight bacterial culture cell on a clear glass slide and mixed well with the help of sterilized loop. Formation of air bubbles indicated catalase positive and no bubble indicated catalase negative.

Starch hydrolysis: Starch hydrolysis test was carried out using single streak inoculation of the bacterial isolate investigation on to the center of labeled starch agar plates using an inoculating loop. The cultured plate was incubated for 24 h and a zone of hydrolysis of starch was observed around the line of bacterial growth.

Gelatin hydrolysis: Gelatin media were prepared and sterilized using an autoclave at 121°C. Stab inoculation was made using an inoculating loop, from each culture into its appropriately labeled deep tube of nutrient gelatin. The tubes were incubated at 30^0C for 48 hours. After incubation, the tubes were placed in the refrigerator at 4°C for 30minutes. The tubes were later observed for liquefaction.

Oxidase test: The impregnated oxidase disc or strips were placed over a clean sterile petri plate or glass slide which was moisturized with sterile deionized water. A well isolated colony of test bacteria from a fresh 24hour old culture were picked up with sterile inoculating loop and made smear on the oxidase dics/strip. Color changes were observed within time required for change in color up to 60seconds.

Simmons citrate utilization test: Isolates were tested to determine the utilization of citrate as the sole source of carbon for metabolism. Simmons citrate agar slant was prepared and sterilized. After cooling the medium, the bacteria isolates were inoculated into the medium and incubated for 24 h at 30^oC. The color change of the medium from red to green indicates a positive test. The remaining of the medium as red indicates the bacteria cannot utilize citrate. The control was kept without being inoculated

Carbohydrate fermentation test: Carbohydrate fermentation test (test for glucose, sucrose, lactose and mannitol, fermentation) was carried out under anaerobic condition in which a Durham tube was placed in an inverted position to trap the gas bubble formed due to the production of gas. The fermentation broth contains ingredients of nutrient broth, a specific carbohydrate (Lactose, Glucose, Mannitol, and Sucrose separately), and a pH indicator (phenol red), which is red at neutral (pH 7) and turns yellow below a pH of 6.8 due to the production of an organic acid. The isolates showing the color change from pink to yellow with accompanying production of a gas bubble in the Durham's tube indicated that the sugars (glucose, sucrose, and lactose) were fermented

Triple Sugar Iron Agar Test: Isolates were tested to determine the ability to ferment the carbohydrates (Glucose, Sucrose and Lactose). The test organism was inoculated into the slant

media by means of stab and streak inoculation. An uninoculated tube was kept as control. Both tubes were incubated at 30°C for 24 hours and the reaction was observed. Carbohydrate fermentation was indicated by the presence of gas and a visible color change of the pH indicator, phenol red. The formation of a black precipitate that blackens the medium in the butt of the tube indicates production of hydrogen sulphide.

Methyl red test: A sterile MR-VP broth was prepared and 5 ml of the broth was added to sterile test tubes. The broth was then inoculated with pure culture of bacterial isolates using a sterile loop and incubated at 30^{o}C for 48 h. Five or six drops of methyl red were added to the broth and the results were observed without mixing. A positive result was indicated by the formation of a pink to red colour in the reagent layer on top of the medium within 10 -15 sec of adding the reagent (methyl red) while a negative result was indicated by yellow colour.

Voges-proskauer test: Glucose phosphate broth (Voges-Proskauer) was autoclaved and cooled to room temperature. A 24h culture was inoculated with the help of a sterilized loop and incubated at 30^{o}C for 48h. Following this, 1 ml of alpha-naphthol was added with shaking. 0.5 ml of 40% KOH was added to the broth and shaken. Red colour development after the addition of the reagents within one hour was taken as a positive result.

3.4.3. MALDI-TOF MS Analysis for newly isolated protease producing bacterial cells

All MALDI TOF MS characterization in this study was carried out at Wudasie Diagnostic center.The three potential-protease producing bacterial isolates were directly placed on the MALDI target plate as a thin layer and allowed to air dry. Two microliters of 70% formic acid (Merck, Germany) was applied to the sample layer (Ha *et al.,* 2019; Haigh *et al.,* 2011), allowed to dry, and overlaid with 2 µl of matrix solution consisting of 10 mg/ml α-cyano-4-hydroxy-cinnamic acid (Fluka, Switzerland) saturated in 2.5% trifluoroacetic acid (Sigma-Aldrich, USA) and 50% acetonitrile (Honeywell Burdick & Jackson, Fisher Scientific). The matrix and analyte were allowed to co-crystalize, and the target plate was inserted into the MALDI-TOF MS instrument. Mass spectra was automatically acquired with MALDI-TOF MS operating in a linear positive mode by accumulating 1,000 laser shots in the m/z ranges 2,000–20,000 and 500–3,000 using Thinkerbell LT (ASTA Inc., Korea). External calibration of mass spectra was performed using recombinant *E. coli* extracts (ASTA Inc.,) in a protein mass range of 3,000–20,000 Da and Peptide Calibration Standard II (Bruker Daltonics, Germany) in a small molecule mass range of 500–3,000 Da. The resulting spectra were compared with reference spectra by using the Biotyper 3.1 software (Bruker

MALDI Biotyper, UK). The identification score cutoff values were applied to each measurement according to the manufacturer's instructions. Isolates with a score of ≥ 2.0 for a given species were considered High confidence identification to the species level, 1.70–1.99 were regarded as Low confidence identification and 0.00–1.69 were characterized as No organism identification possible as prescribed by Tomazi *et al*,. (2015). *E. coli* ATCC 25922 T was used as a calibration and quality control standard.

3.4.4. Molecular identification of screened isolates

3.4.4.1. Extraction of genomic DNA

All molecular characterization in this study was carried out at Heredity Bioscience, India The *Quick*-DNA™ Fungal/Bacterial Miniprep Kit was designed for the simple, rapid isolation of DNA from tough-to-lyse fungi, including *A. fumigatus, C. albicans, N. crassa, S. cerevisiae, S. pombe*, as well as from mycelium and Gram positive and Gram negative bacteria. The procedure was easy and completed in 15 minutes: AL1 bacterial sample was added directly to a ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) and rapidly and efficiently lysed by bead beating without using organic denaturants or proteinases. The DNA was then isolated and purified using Zymo-Spin™ Technology and it was ideal for downstream molecular-based applications including PCR, array, etc. DNA/RNA Shield™ (R1100-50, R1100-250) was used to stabilize nucleic acids and inactivate infectious agents in our sample, without the need for reagent removal. For rapid, robust, and simple purification of high quality, inhibitor-free DNA from soil the ZymoBIOMICS™ DNA Miniprep Kit (D4300) was used.

For optimal performance, beta-mercapto ethanol was added to the Genomic Lysis Buffer to a final dilution of 0.5 %(v/v) *i.e*., 500 µl per 100 ml. then 50 – 100 mg AL1 bacterial sample that has been resuspended in up to 200 µl of water or isotonic buffer was added to a ZR BashingBead™ Lysis Tube (0.1 mm & 0.5 mm). Then 750 µl BashingBead™ Buffer was added to the tube2. A bead beater fitted with a 2 ml tube holder assembly was secured and processed at maximum speed for ≥ 5 minutes. Next, the Lysis Tube (0.1 & 0.5 mm) was centrifuged in a microcentrifuge at 10,000 x *g* for 1 minute. Then up to 400 µl supernatant was transferred to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuged at 8,000 x *g* for 1 minute. 1,200 µl of Genomic Lysis Buffer was added to the filtrate in the Collection Tube. Eight hundred µl of the mixture from the above step was transferred to a Zymo-Spin™ IICR Column3 in a Collection Tube and centrifuged at 10,000 x *g* for 1 minute. A 200 µl DNA Pre-Wash Buffer was added in a new Collection Tube and centrifuged at 10,000 x *g* for 1 minute and then 500 µl g-DNA Wash Buffer was added to the

Column and centrifuged at 10,000 x *g* for 1 minute. Then the Column was transferred to a clean 1.5 ml microcentrifuge tube and a 100 µl (35 µl minimum) DNA Elution Buffer was added directly to the column matrix, centrifuged at 10,000 x *g* for 30 seconds to elute the DNA, and Ultra-pure DNA was ready to use for 16S rRNA sequencing.

3.4.4.2. Identification of isolated bacteria by 16S rRNA sequencing

DNA isolation was carried out and the amplified DNA was separated by agarose gel electrophoresis (0.8% agarose, w/v) and run in a 1 × TAE buffer at 50 V for 30–45 min till DNA fragments migrated well. The gel was photographed on a gel documentation system. The concentration of DNA samples was then measured by Nanodrop (Biotech instruments, USA). DNA was stored at -80^{o}C for further use. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8–2.0 is generally accepted as "pure" for DNA. After this PCR amplification was performed in a total volume of 25 µl containing 10 pmol each of forward and reverse primers (27F- GAGTTTGATCATGGCTCAG and 1492R- TACGGTTACCTTGTTACGACTT.), 2.5 mM of MgCl2, 200 µM each of the four deoxyribonucleotide triphosphates (dNTPs), 0.5 U of Taq DNA polymerase, 1x concentration of PCR buffer (Invitrogen, Life Technologies, Brazil) and 50–100 ng of isolated bacterial genomic DNA. The template was denatured by heating at a pre-denaturation of 95^{o}C for 5 min. This was followed by 39 cycles of denaturation 30 s at 95^{o}C, 45 s annealing and 1 min elongation at 72^{o}C, with a final extension of 7 min at 72^{o}C. The amplicons were resolved in 1.5% (w/v) agarose gel using 0.5x tris acetate-EDTA (TAE) buffer. The PCR amplicon was subjected to Sanger Sequencing, and 16 SrRNA sequence fragments were sequenced with an automated ABI 3730XL DNA sequencer. The FASTA file of the sequences was uploaded. Consensus sequences that showed a significant match with the maximum identified data on NCBI were submitted to NCBI for obtaining accession numbers.In this method, the 16S rRNA gene sequences were analyzed with the Basic Local Alignment Search Tool (BLAST), and strains were aligned by multiple sequence alignment programs (Clustal W). Finally, a phylogenetic tree was constructed.

3.4.4.3. Phylogenetic analysis of sequences

Phylogenetic analysis and tree reconstruction were performed using Mega 11. The 16S rRNA sequences of the isolate were used for the phylogenetic analysis as they are.

3.5. Determination of protease activities of the selected isolate

3.5.1. Protease assay

Protease activity was assayed by a modified method of Tsuchida *et al.,* (1986) by using casein as substrate. For measuring protease activity, culture supernatant (0.5 mL) was mixed with 2.5 mL of 1% casein solution (in phosphate buffer, pH 7.3) and incubated for 10 min at 30°C. The reaction was terminated by adding 2.5 mL of TCA to the solution. The mixture was vortexed to ensure complete mixing and incubated for further 15 min at room temperature and then centrifuged at 10,000 rpm for 15 min at 4^{o}C.Then 0.5 ml of Folin reagent was immediately added, vortexed and left for 30 min at room temperature. The mixture was used to estimate the amount of free tyrosine released according to Lowry *et al.,* (1951) using tyrosine as a standard. The amount of tyrosine in the solution was measured by reading the absorbance at 660 nm. A tyrosine standard was prepared by dissolving different amounts of tyrosine in TCA solution (Orhan *et al.,* 2005).One unit of enzyme activity is defined as µ mol of tyrosine released from 1ml crude enzyme in 1 h incubation.

Protease activity = Tyrosine liberated (µ mol) × total volume of assay (11 mL) (A × B × C) Where: A is the enzymatic volume used for assay; B is the incubation time required for assay (20 min) and C is the volume used in spectrophotometric determination (1 mL).

3.6. Optimization of culture growth parameter for Production of Protease

Optimization of different growth parameters for the three isolate was performed by one variable at a time

3.6.1. The effect of pH on the biosynthesis of protease

Flask with growth medium containing 1% Casein as a substrate was taken and the pH of the broth was adjusted to different pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) using 1N HCl and 1N NaOH sterilized and incubated at 30^{o}C for 24 h. At the end of incubation period, the cell free culture filtrates were obtained and the protease activities were determined.

3.6.2. The effect of temperature on the biosynthesis of protease

Production medium containing 1% casein as a substrate and its pH was adjusted to the optimum was inoculated with overnight grown selected bacterial strain. The resulting medium was

incubated at different temperatures (i.e. 25, 30, 35, 37, 40 and 45^{o}C for 24 h). At the end of incubation period the cell free culture filtrate was obtained from each and protease activity was determined.

3.6.3. The effect of incubation time on the biosynthesis of protease

To determine the time needed for highest production of protease, the 24 h grown isolate was inoculated into the protease production medium containing 1% Casein as a substrate and incubated at optimum temperature and pH for 24-120 h. Two (2) ml of the sample from the medium was collected every 24 h to determine highest protease activity.

3.6.4. Effect of nitrogen source on protease biosynthesis

Different nitrogen sources like peptone, yeast extract, beef extract and tryptone were examined for their effect on protease production by replacing 1% peptone in the production medium. Each flask containing the medium was inoculated at optimum temperature, pH, and incubation time.

3.7. Application of the isolated protease producers on whole eggshell and eggshell membrane for degradation assay.

3.7.1. Eggshell meal powder preparation and Egg-shell biodegradation test on plate

Eggshell meal powder preparation: The entire eggshell underwent thorough washing and was then boiled at 30-40 psi for duration of 2-3 hours. Subsequently, it was dried in a hot air oven for 4 hours at a temperature of 50 degrees Celsius. The dried eggshells were then pulverized to obtain a powder, which was utilized as an eggshell meal. The PPBI was cultivated on a medium that consisted of finely powdered and highly heat-treated eggshell waste as the sole source of carbon and nitrogen. Additionally, salt media containing 0.2% MgCl2, 0.5% K2PO4, 0.2% MnCl2, and 1% NaCl, along with 1.5% agar, was used. Furthermore, MSM agar, which included high heat-treated eggshell meal powder as the exclusive carbon and nitrogen source, was prepared and sterilized at 121 degrees Celsius and 15 lbs. pressure for 15 minutes. Following sterilization, the MSM agar media was poured into petri dishes and allowed to solidify. Once solidified, fresh 24-hour-old isolates were spot inoculated on the media to assess growth and the formation of clearing zones around the colonies. The media was then incubated at the optimal growth temperature for 24-48 hours to observe the growth of the isolate on eggshells, utilizing eggshell meal powder as the

carbon and nitrogen source. After incubation, the growth of colonies and the formation of clearing zones were examined and documented.

3.7.2. Whole eggshell and its membrane biodegradation test with Submerged Cultivation

The ability of the isolates to degrade native eggshell and eggshell membrane substrate was investigated. The assay consisted of a whole chicken eggshell and separated inner eggshell membrane (to test the speed of degradation separately since the membrane is the protein-containing part and the isolates are protease producers) suspended in 250 ml of minimal medium (pH 8.0) sterilized at 121°C for 15 min and then inoculated with 5% (v/v) inoculum. Different sets were incubated at 150 rpm for 7, 14, and 21 days at optimal temperatures (30±2°C and 37±2°C), respectively. The control experiments were without inoculum i.e. only the media and eggshell. The set-ups were examined daily to determine the level of degradation of the whole eggshell.

Nutrient Agar was prepared and sterilized in an autoclave at 121^{o}C, 15 lbs. for 15 min then the agar is poured into petri plates and left until solidified. After solidifying using the plate streak method the pure isolates are streaked using an inoculating loop to acquire viable fresh colonies. After growth on the agar plates pure colonies of the freshly grown strains are aseptically inoculated into nutrient broth media using an inoculating loop before the start of submerged cultivation until sufficient growth is attained in broth media. The growth of the isolates was compared with McFarland's standard. Minimal salt media in addition to the carbon source (glucose) and optimum nitrogen source (yeast extract) was prepared and sterilized in an autoclave at 121oC, 15 lbs. for 15 min. After sterilization, the MSM media was allowed to cool to 40o C before inoculating with the isolates. After cooling the media was inoculated with 5% (v/v) of the inoculum and the egg shells and egg membranes were added. The flasks were then incubated at room temperature for 5-21 days. After the incubation time the flasks were taken out and the degradation of the samples was investigated visual observation.

3.8. Statistical analysis

Data analysis was performed using SPSS, the graphs were sketched with Origin Pro 8.0 software, and Microsoft office excels 2010 were used for data rearrangement and table formation. All the tests were performed in triplicates and data were expressed as Mean ± standard deviation and the differences were considered to be significant at $p < 0.05$.

4. RESULTS AND DISCUSSION

4.1. Isolation and screening of bacteria for protease production from soil sample

4.1.1. Primary screening of protease producing bacteria

In this study, a total of 45 various bacterial isolates were obtained from the two different soil samples. Previous studies have also focused on isolating and characterizing bacterial isolates, which were employed for protease secretion from waste soil samples (Ravishankar, 2012; Hadush *et al.,* 2017), Ravishankar (2012) successfully isolated six protease-producing bacterial strains from fish waste soil, displaying a remarkable ability to produce this enzyme commercially. Hadush *et al.,* (2017) undertook a more extensive study to isolate 147 isolates from three distinct sampling sites. Among these, 85 isolates demonstrated high effectiveness in protease secretions. The authors attribute the more significant number of positive isolates to larger sample sizes and increased sampling sites. This underscores the importance of thorough exploration when hunting for potent protease-producing bacterial strains, which could applied for industrial applications.

This study aimed to examine the presence of proteolytic activity in different bacterial isolates obtained from diverse environmental sources. Additionally, the isolates were assessed for their potential to produce proteases. Of the 45 isolates screened based on morphological appearance, 14 (31.1%) exhibited positive protease activity based on the appearance of clear zones (table 1and figure 2). The use of skim milk agar for the isolation of protease producing bacteria has earlier been reported by some workers (Hamza & Woldesenbet, 2017). Among these, three isolates demonstrated a notably higher zone of clearance on the skim milk agar plates (Figure 2). A similar investigation conducted by (Patil & Jadhav , 2017) involved the isolation and identification of 28 distinct Bacillus species from soil. The researchers employed morphological and biochemical characterization techniques and further screened the isolates for protease production. Among the 28 isolates, three were designated SP3, SP10, and SP21. These isolates showed clear zones against casein hydrolysis with 15, 18, and 9 mm sizes, respectively, after 24 h of incubation. This emphasizes the potential of these isolates as efficient protease producers (Patil and Jadhav, 2017).

Hamza and Woldesenbet (2017) reported protease-producing bacteria from compost-processing site in Arba Minch town. They identified 22 protease-positive isolates, with five exhibiting higher clear zones on milk agar medium after an 18 h incubation period at 40 °C. These findings highlight the presence and diversity of protease-producing bacteria in such environments. Furthermore, the

effluent of tannery industries in the Hazaribagh area of Dhaka city was examined for protease-producing bacteria. Among the isolates obtained, eight demonstrated a significantly higher clear zone formation, indicating their potential as protease producers (Habib *et al.*, 2012). The detection of protease-producing bacteria in different environmental niches reflects their wide distribution and importance in various industries

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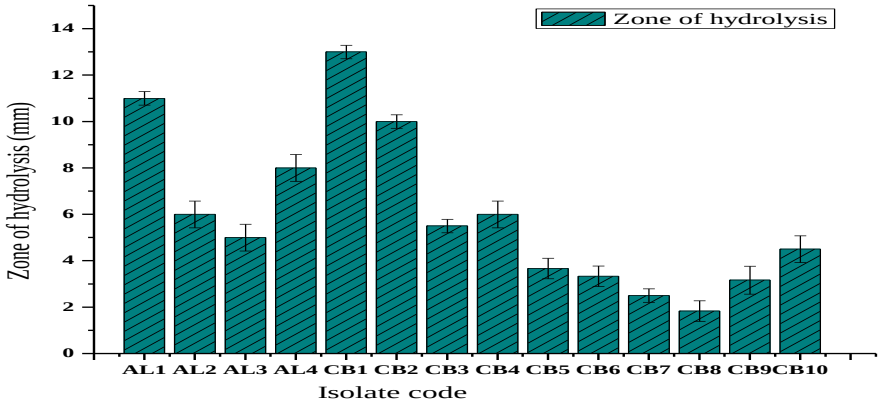

Figure 2: Zone of casein hydrolysis obtained from primary screening result on skim milk agar Shows the clear zone formed after 24h of incubation period

4.1.2. Secondary screening of potential protease producing bacterial isolates

Three isolates were carefully chosen throughout the secondary screening process based on their prominent, clear zone formation in the primary screening. CB2, CB1, and AL1 isolates showed clear zones with 12±0.5, 15±0.5, and 13±0.5mm sizes on the skim milk agar plate (Figure 3), respectively. The increased size of the clear zone indicates a higher efficiency in degrading casein within the skim milk agar medium. The selection of these isolates aligns with the findings of Sidra et al., (2006), who acknowledged the bacterium exhibiting the largest zone of inhibition as the superior producer. The study by Haider & Sanaa (2014) also supports these results, where *Bacillus* spp isolated from soil was identified, specifically *B. licheniformis*, as the most efficient protease producer.

In a similar study by Alnahdi (2012), six bacterial strains were isolated from marine samples. Both qualitative (zone of inhibition) and quantitative (U/ml) protease assays were performed on all six isolates. Among these isolates, No. 2 and No. 3 exhibited significant proteolytic activity during the secondary screening. Furthermore, Verma and Baiswa (2013) isolated twenty-three bacteria from treated tannery effluent, which were found to produce protease on milk agar. Among these isolates, nine isolates revealed a clear zone which is more significant than 20 mm, indicating their exceptional degradative abilities. The selection of isolates CB2, CB1, and AL1 based on their clear

zone formation on the skim milk agar plate exhibits their efficiency in casein degradation. The recent study supported by previous reports, which emphasizing the importance of selecting isolates with the highest protease biosynthesis for various applications.

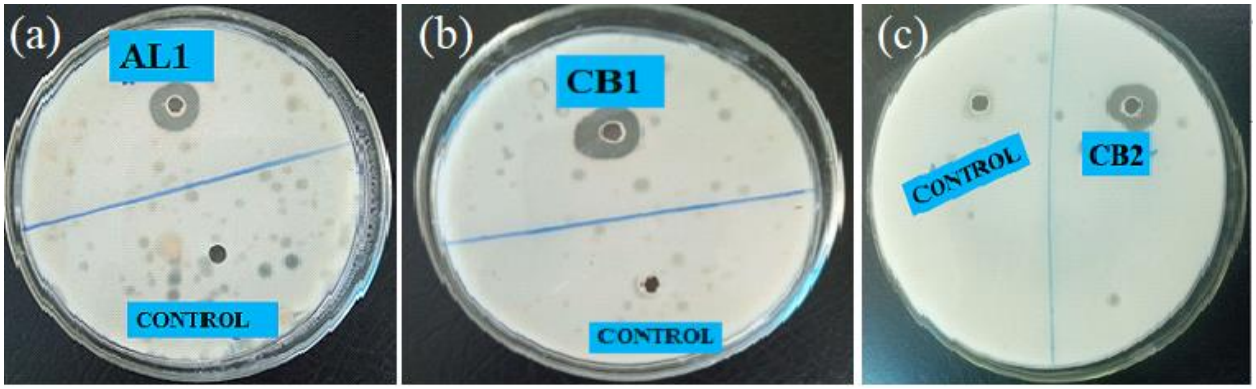

Figure 3: The three isolates showed significant zone of clear hydrolysis. Secondary screening result of the three isolates namely, AL1, CB1 and CB2 which showed higher clear zone on primary screening.

4.2. Culture characterization

4.2.1. Morphological and biochemical characteristics of selected isolates

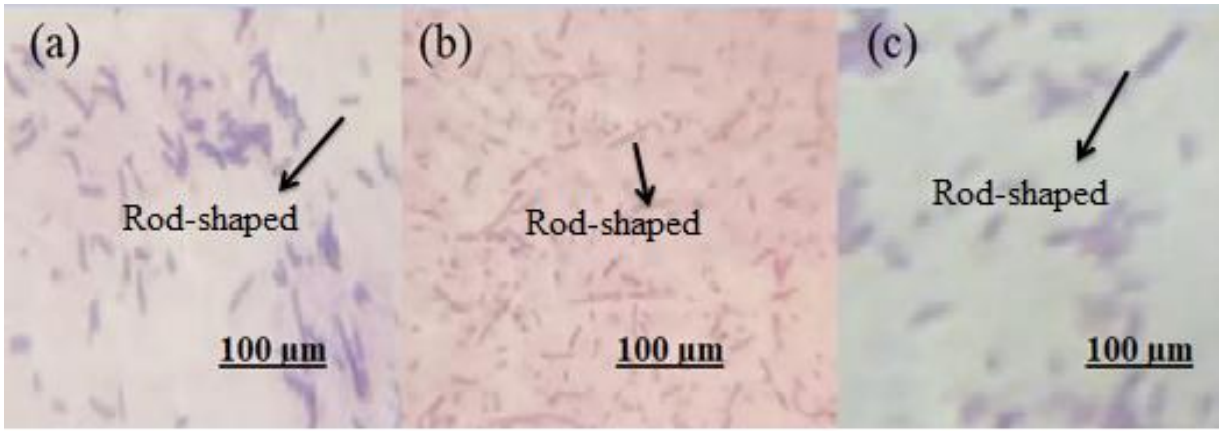

Figure 4: gram staining result. (a) Shows AL1 which was gram positive,(b) shows CB1 which was gram negative and (c) shows CB2 which was gram positive.

Different morphological and biochemical characterization was carried out to identify the bacteria, and the results are summarized in Table 2. The isolate colony morphology, such as shape, colour, elevation and surface margins of pure colonies, were observed on agar plates. As shown in Table 2, the isolates varied in both colony and cellular morphology.

Table2: Morphological characteristics of selected isolate (CB1, CB2 and AL1)

S.N.	Colony morphology	Isolate CB1	Isolate CB2	Isolate AL1
1	Size	Small	Medium	Small
2	Shape	Rod	Rod	Rod
	Color	Opaque	Greyish opaque	Gray
3	Surface	Smooth	Smooth	Smooth
4	Motility	Motile	Motile	Motile
5	Margin	Entire	Entire	Entire
6	Elevation	Convex	Convex	Flat

Furthermore, biochemical tests showed that the three isolates (AL1, CB1 and CB2) do not ferment sucrose and lactose. However, CB1 and CB2 did ferment glucose. Study exhibited that AL1 did ferment glucose. Mannitol found to be fermented by CB2, a protease producing isolate. These three isolates are showed a negative result for Voges proskauer and starch hydrolysis test. However, these are noticed as positive result against catalase, oxidation, and methyle red test. AL1 and CB1 showed negative result for simmon citrate utilization and gelatine liquefaction tests whereas the isolate CB2 showed a negative result for these tests. The isolates AL1 and CB1 displayed a negative result for H_2S test while, CB2 showed a positive result. After Gram staining performed, CB2 isolate found to be medium rod shaped and AL1 and CB1 small and rod shaped. CB2 and AL1 were Gram-positive however; CB1 was Gram-negative for with motility (Appendix 2).

Table 3: Biochemical test of selected isolate CB1, CB2 and isolate AL1

S.N.	Characteristics	CB1	CB2	AL1
1	Catalase test	+	+	+
2	Gram staining	-	+	+
3	Starch hydrolysis	-	-	-
4	Gelatin liquefaction test	-	+	-
5	Simmon citrate utilization	-	+	-
6	Methyl red	+	+	-
7	Vegas-proskauer	-	-	-
8	Glucose fermentation	+	+	+
9	Sucrose fermentation	-	-	-

10	Lactose fermentation	-	-	-
11	Mannitol fermentation	-	+	-
12	H_2S production	-	+	-
13	Acid production	+	+	+
14	Oxidation	+	+	+

4.2.2. MALDI-TOF MS identification of the screened isolates

In this study, these PPBIs such as CB1, CB2 and AL1 were identified based on their protein profiles using MALDI TOF MS techniques as indicated in the Table 4.

Table 4: MALDI-TOF MS analysis for these PPBIs which obtained from soil samples

S.N.	Sample Id.	Species	LSV
1	AL1	*P alcalifaciens*	1.75
2	CB1	*P alcalifaciens*	2.34
3	CB2	*M morganii s*	1.70

CB1, CB2 and AL1 indicate: cake bakery waste accumulation site soil sample first isolate, cake bakery waste accumulation site soil sample secondary and arable land soil Sample primary isolate respectively. The LSV Logarithmic Score Value. Score value of ≥ 2.0 indicates High confidence identification to the species level, 1.70–1.99 - Low confidence identification, and 0.00–1.69 - No organism identification possible

4.2.3. Molecular identification of the screened isolates

4.2.3.1. Protease Producing Bacterial Isolates by 16s rRNA sequence

A new protease-producing bacterial isolate was obtained from two different soil samples. One of the isolates was identified as *Lysinibacillus sp.* Ala1 using 16SrRNA gene sequences. *Lysinibacillus sp.* Ala1 exhibited a considerable number of sequence similarities with *Lysinibacillus capsici* PB300T (PXXX01000046) (98.90%), *L. macroides* DSM 54T (LGCI01000008) (98.04%), *L. xylanilyticus* DSM 23493T (LFXJ01000007) (98.35%), *L. boronitolerans* T-10aT(AB199591)(98.03%), *L. pakistanensis* JCM 18776T(BBDJ01000063) (97.96%), *L. fusiformis* NBRC 15717T(AB271743) (97.66%), *L. cavernae* SYSU K30005T(MN611148)(97.41%), *L. parviboronicapiens* BAM-582T(AB300598)(97.25%), and *L.*

sphaericus KCTC 3346T (AUOZ01000024) (97.17%). As shown in Table 5 and Figure 5, *Lysinibacillus sp.* Ala1, a protease producing bacterial isolate was homologous to *B. ndiopicus* FF3T (HG315675) (95.60%), *Metasolibacillus meyeri* WS 4626T (HE577173) (95.29%), and *Ureibacillus chungkukjangi* 2RL3-2T(JX217747) (95.84%). The highest sequence similarity occurred between *L. capsici* PB300T (PXXX01000046) (98.90%), and the recent protease-producing bacterial isolate. However, the lowest sequence similarity was detected between *Lysinibacillus sp.* Ala1 and *L. alkalisoli* Y2A20T (KX258757)(94.66%). Studies showed that bacterial strains such as L. *odysseyi* 34hs-1T (AF526913), *L. halotolerans* LAM612T (KF443809), *U. xyleni* JC22T (FN179487), *Ureibacillus acetophenoni* JC23T (FN179488), and others (Figure 5, Table 5) allied with *Lysinibacillus sp. Ala1*. A similar study showed that alkaline protease-producing ML12 bacterial isolate obtained from the leather industry effluent was identified as *Bacillus cereus* (MN629232.1) using the same tool. This isolate displays sequence similarity with *B. cereus* strain (KY995152.1) (97.87%) and *B. cereus* (MK968813.1) (97.86%) (Masi et al., 2021). Another novel alkaline protease-producing bacteria isolate identified as *B. firmus* EMBS023 (JN990980) using a 16S rRNA gene sequencing approach from a moist soil sample at a local poultry farm in India (Wishard et al., 2014). The same isolate was reported to be employed as a potential probiotic in poultry feed. In our previous study, Polyethylene degrading (Nedi et al., 2024), PHA producing (Mohammed et al., 2019, 2020; Mohammed & Ray, 2022), and medicinal plant growth promoters (Ebu et al., 2023) were identified using 16sRNA gene sequences analysis.

Table 5: Sequence similarity between *Lysinibacillus* sp. Ala1 and other strains with their accession numbers and type strains

S.N.	Name of allied strains	Stains	Accession number	Pairwise similarity (%)
1	*Lysinibacillus* sp.	Ala1	PP095644	The recent isolate
2	*L. capsici*	PB300^T	PXXX01000046	98.90
3	*L. macrolides*	DSM 54^T	LGCI01000008	98.59
4	*L. xylanilyticus*	DSM 23493^T	LFXJ01000007	98.35
5	*L. boronitolerans*	T-10a^T	AB199591	98.04
6	*L. pakistanensis*	JCM 18776^T	BBDJ01000063	97.96
7	*L. fusiformis*	NBRC 15717^T	AB271743	97.66
8	*L. cavernae*	SYSU K30005^T	MN611148	97.41

9	*L. parviboronicapiens*	BAM-582^{T}	AB300598	97.25
10	*L. sphaericus*	KCTC 3346^{T}	AUOZ01000024	97.17
11	*L. contaminans*	DSM 25560^{T}	LGRV01000001	96.62
12	*L. halotolerans*	LAM612^{T}	KF443809	96.08
13	*L. odyssey*	34hs-1^{T}	AF526913	96.00
14	*L. endophyticus*	DSM 100506^{T}	RBZN01000109	95.99
15	*L. louembei*	NM73^{T}	HG937791	95.92
16	*U. chungkukjangi*	2RL3-2^{T}	JX217747	95.84
17	*U. sinduriensis*	BLB-1^{T}	FJ169465	95.84
18	*U. xylene*	JC22^{T}	FN179487	95.76
19	*L. alkaliphilus*	OMN17^{T}	KF771256	95.68
20	*M. meyeri*	WS 4626^{T}	HE577173	95.68
21	*L. telephonicus*	S5H2222^{T}	KT735049	95.67
22	*B. ndiopicus*	FF3^{T}	HG315675	95.60
23	*L. composti*	NCCP-36^{T}	AB547124	95.60
24	*L. antri*	SYSU K30002^{T}	MK325186	95.53
25	*U. manganicus*	Mn1-7^{T}	JX993821	95.45
26	*L. yapensis*	YLB-03^{T}	QWEI01000026	95.37
27	*M. jejuensis*	N2-5^{T}	HQ392513	95.29
28	*U. acetophenoni*	JC23^{T}	FN179488	95.12
29	*Solibacillus cecembensis*	PN5^{T}	AM773821	95.05
30	*U. massiliensis*	4400831^{T}	AY677116	95.05
31	*Psychrobacillus soli*	NHI-2^{T}	KJ956929	95.02
32	*Solibacillus isronensis*	B3W22^{T}	AMCK01000046	94.74
33	*P. lasiicapitis*	NEAU-3TGS17^{T}	KP219721	94.74
34	*L. alkalisoli*	Y2A20^{T}	KX258757	94.66

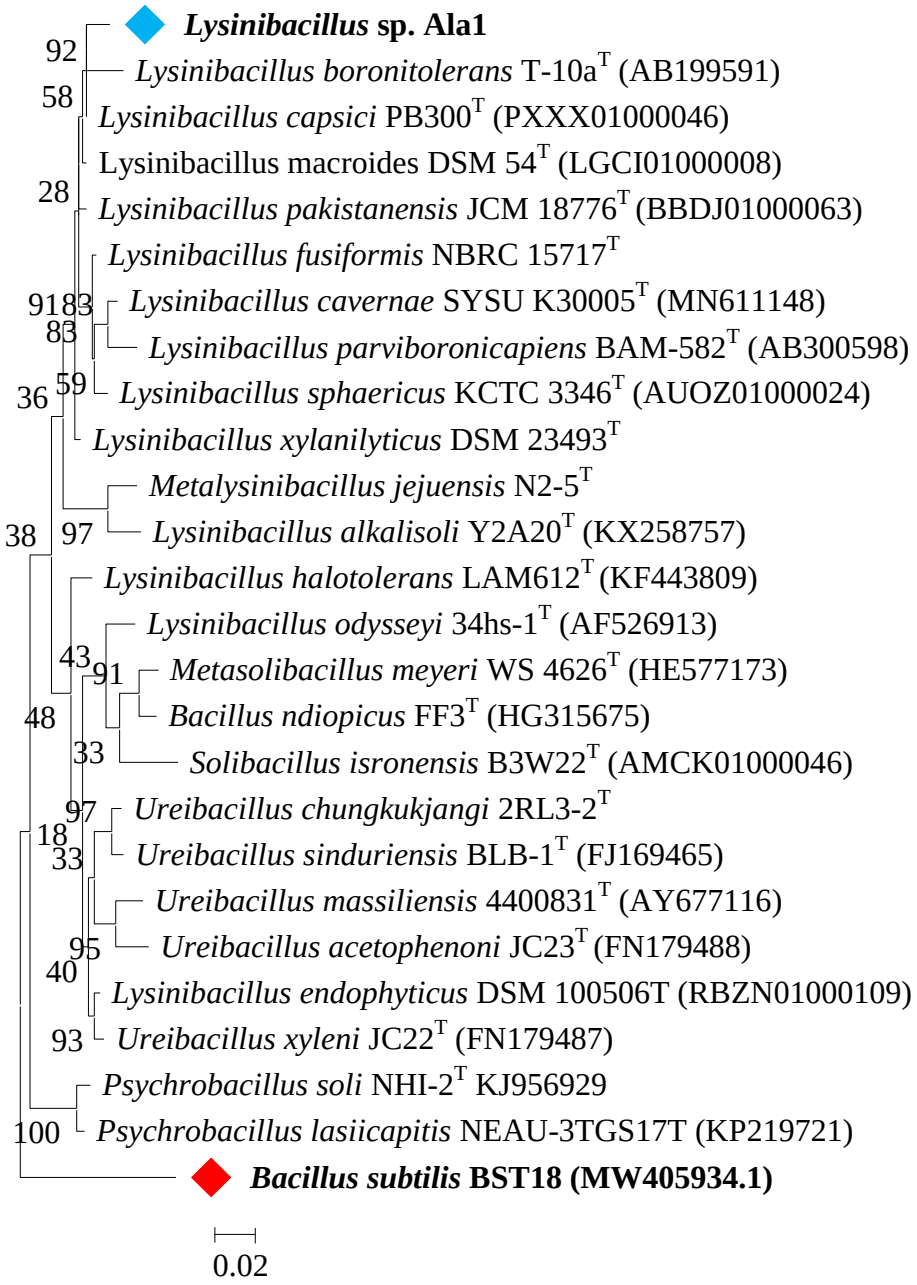

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Figure 5: Evolutionary analysis of PPBI by Maximum Likelihood method: The tree with the highest log likelihood (-5965.42) is shown. The percentage of trees in which the associated taxa clustered is displayed next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site with 1000 bootstraps. This analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

In previous studies, the identification of protease-producing bacteria from lysini*bacillus sphearicus* was done based on physiological, biochemical and molecular characterization. In agreement with this finding different scholars reported protease production by various strains of lysini*bacillus sphearicus*. Singh *et al.,* (2004) reported *that B. sphaericus*, an obligate alkalophile, and overproducers of extracellular alkaline proteases. Similarly, Anbu *et al.,* (2016) have reported a bacterium producing solvent-stable protease was isolated from salt-enriched soil after incubation in benzene and toluene-enriched media and identified as Lysinibacillus sphaericus PAP02 by 16S rRNA sequencing. (Tallur *et al.,* 2016) indicated that *L. sphaericus* is a Gram positive, mesophilic, rod shaped bacterium commonly found in soil. It is spore forming bacteria, tolerant to high temperatures, chemicals, and ultraviolet light, and can remain viable for long periods in soil. *L. sphaericus*' can produce extracellular protease enzymes in pure bacterial cultures, which was carried out by growing the cultures on skim milk agar. *L. sphaericus* is a well known bacterium used in the biological control of mosquitoes. It is exciting to the WHO due to the larvicidal effect of some strains against two mosquito genera (Culex and Anopheles) (Wirth *et al.,* 2014). Additionally, *L. sphaericus* can tolerate and reduce heavy metals; hence, it is commercially important in the leather and detergent industries (Tallur *et al.,* 2016). This bacteria species was effective in detergent and can be used as an additive

4.3. Optimization of media for protease production

4.3.1. Effect of pH against CDW (g/L), OD_{600nm}, and Biosynthesis of protease

In this study, various pH values exhibited significant effects against CDW (g/L) and OD_{600nm}, as illustrated in Figure 6a. The mininum (0.00337±0.00185 g/L at pH 9) and maximum (0.0822±0.0147 g/L at pH8) CDW (g/L) were recorded against CB2 and AL1, respectively. The same isolates gave 0.974± 0.0116 and 1.6797± 0.0031 at the same pH value against OD_{600nm}. These protease-producing bacterial possible isolates gave substantial amounts of CDW (g/L) and OD600nm. For instance, CB2 gave 0.0782± 0.0149 CDW (g/L) at pH8 with 1.665± 0.0186 OD_{600nm}. As indicated in the same Figure, 0.003773±0.00169 to 0.065967±0.0229 CDW (g/L) were obtained for CB1, a protease-producing bacterial isolate at pH 6-7. The same isolate gave considerable amounts of OD_{600nm} (1.726±0.032 to 0.748±0.0175) at the same pH values (Figure 6a).

Table 6: Enzyme activity in U/ml against different PH values

S.N	PH	AL1		CB1		CB2	
		Enzyme activity	Standard deviation	Enzyme activity	Standard deviation	Enzyme activity	Standard deviation
1	4	147.2	0.003	184	0.019	73.5	0.02
2	5	331	0.011	239	0.018	386	0.009
3	6	294	0.006	257.5	0.006	386.3	0.018
4	7	441.5	0.027	294.3	0.049	404.7	0.0108
5	8	450.7	0.003	460	0.032	441.5	0.032
6	9	220.7	0.004	331.2	0.248	312.7	0.015
7	10	110.4	0.006	165.6	0.016	128.8	0.042

Initial pH of production medium is another important factor that significantly influences production of protease (Hamza & Woldesenbet, 2017). It has been noted that microorganisms are dependent on the extra cellular pH for their cell growth and enzyme production (Rao and Narasu (2007). Understanding the influence of pH on OD_{600nm}, enzyme activity and biomass production is crucial for optimizing biological processes. This study investigated three isolates as they were allowed to grow in media with varying pH values ranging from 4.0 to 10.0. The results revealed distinct variations in enzyme activity and biomass production among the isolates at different pH levels. Notably, the optimum pH for all isolates were determined to be pH 8.0, which consistently exhibited the maximum OD_{600nm}, biomass and enzyme activity. In agreement with this result Hamza & Woldesenbet, (2017) reported maximum enzyme production in a medium of pH 8 which was the optimum pH for production of enzyme from Bacillus sp. CAMA14. Isolate AL1 demonstrated an impressive protease production rate of 450.7±0.003 U/ml, accompanied by a maximum biomass production and high OD_{600nm} value. This observation indicates the favorable effect of pH 8.0 on the growth of AL1.

Furthermore, CB1 can thrive in an alkaline environment, exhibiting a substantial alkaline protease activity of 460±0.0032 U/ml (Table 6). Despite a slightly lower biomass production when compared to AL1, CB1 effectively exhibited alkaline pH range. As indicated in Table 6 CB2, the third isolate, exhibited respectable protease activity, although lower than the previous two isolates. It was noticed that a protease activity (441.5±0.02 U/ml) was obtained with pH 8.0. The collective findings affirms that pH 8.0 as an optimal condition for all three isolates, emphasizing its influence on enzyme activity and subsequent CDW (g/L) biosynthesis. The alkaline environment used to enhance protease biosythesis against AL1 , CB2 and CB1 isolates.where as, the experiment of Rajeeva Gaur,*et al,.* (2014) reported the initial pH of the culture strongly affects enzymatic processes and transport of

compounds across the cell membrane. They indicated Maximum protease production ($1234.5 \mathrm{UmL}^{-1}$) with 2.3 g L^{-1} biomass production was achieved at pH-9.0 by Bacillus sp.which was highly greater than the present study. The enzyme production was increased as pH of the medium was increased reaching maximum at $\mathrm{pH} - 9.0$ and above this pH enzyme production was strongly decreased. Results suggest that there is a stimulation of enzyme production at alkaline pH.

Protease biosynthesis increases until it reaches the optimum pH value and decreases afterward. However, all these isolates demonstrated activity at every pH level tested (table 6). It is worth mentioning that most microorganisms that produce alkaline proteases exhibit growth and enzyme biosynthesis under alkaline conditions (Kumar *et al.,* 2012). This finding is further supported by the studies done in 2021 (Padmapriya *et al.,* 2012), who focused on the production and purification of protease from Marine *Bacillus* Sp., and (Geethanjah and Subash 2011), who optimized protease production by *B. subtilis*. Additionally, (Kalaiarasi, and Sunitha, 2009 ; Agrawal *et al.,* 2012 ; Ravishankar *et al.,* 2012) have reported similar results, concluding that pH 8 is the optimal condition for maximum enzyme production.

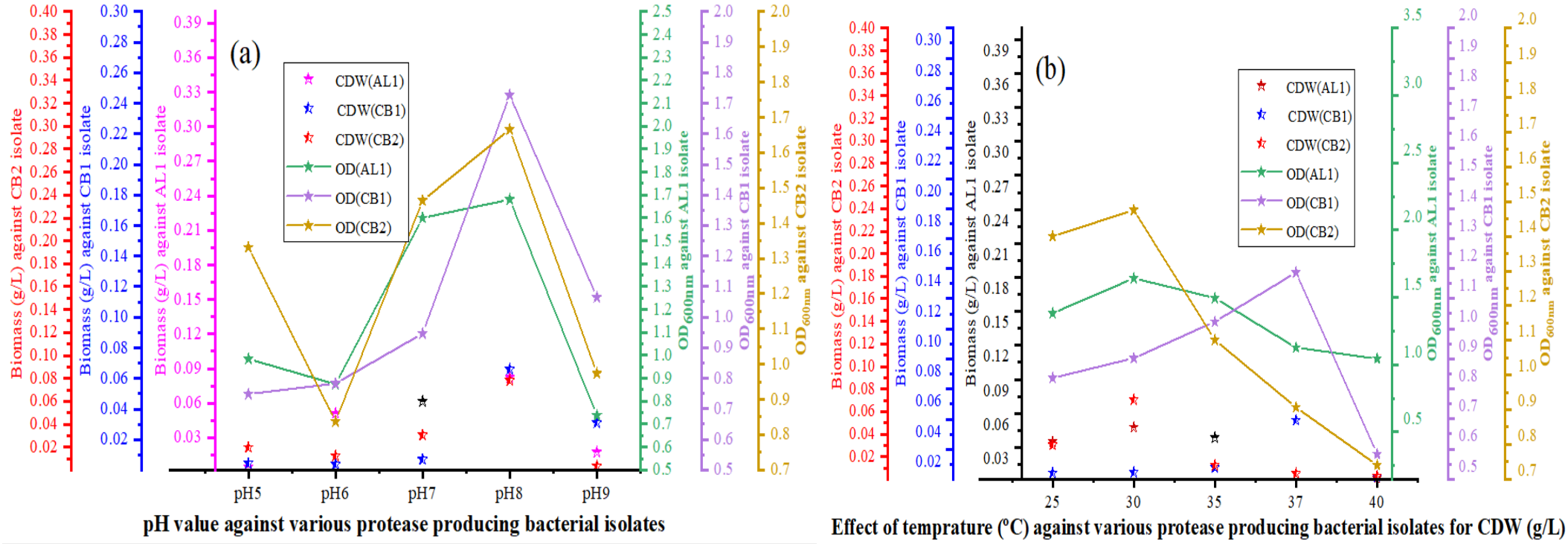

Figure 6: The effect of pH value and temprature agaist AL1, CB1, and CB2, Protease producing bacterial isolates. (a) The effect of different pH (4,5,6,7,8,9 and 10) on PPBI growth. (b) The effect of different incubation temperature (25, 30, 35, 37 and 40°C) on PPBI growth;(A) Enzyme activity obtained from PPBI against Temprature and (B) DB (g/l) obtained from PPBI against temprature. Data represented mean±SD of three replicates. Data represented mean±SD of three replicates.

4.3.2. Effect of temperature on the biosynthesis of protease

In this study, various temperature values exhibited significant effects against CDW (g/L) and OD_{600nm}, as illustrated in Figure 6b. The maximum CDW (0.07± 0.013 g/l) was obtained from CB2 isolate at 30^{o}C, and the minimum (0.0018 ±0.0006 g/l) was recorded for CB1 at 40^{o}C. Considerable amounts of CDW (g/L) and OD_{600nm} were also obtained from these protease-producing bacterial isolates (Figure 6b). For instance, AL1 gave CDW (g/L) (0.0478 ± 0.01) and OD_{600nm} (1.64±0.007) at 30^{o}C. As indicated in Figure 6b, isolate CB1 gave 0.039±0.004 OD_{600nm} and 0.039± 0.004 CDW (g/L) at 37^{o}C, which was the optimum temperature value for the isolate. The same isolates gave 0.0018 ±0.0006 CDW (g/l) and 0.36±0.06 OD_{600nm} at 40^{o}C, which was the minimum OD_{600nm} and CDW (g/) for the isolate (figure 6b). In this study, the two isolates, CB2 and AL1, exhibited maximum CDW (g/L) and OD_{600nm} at 30^{o}C, whereas the isolate CB1 exhibited maximum CDW (g/L) and OD_{600nm} at 37^{o}C.

Table 7: Enzyme activity in U/ml for temperature in (^{0}C)

S.N	Temperature (^{0}C)	AL1		CB1		CB2	
		Enzyme activity	Standard deviation	Enzyme activity	Standard deviation	Enzyme activity	Standard deviation
1	25	404.7	0.045	257.5	0.056	404.5	0.353
2	30	441.5	0.055	292.6	0.084	413.9	0.035
3	35	422.9	0.044	331	0.068	333.3	0.471
4	37	349.5	0.021	367.9	0.026	294.4	0.06
5	40	330.5	0.595	165.6	0.040	228	0.036
6	45	239	0.036	110.3	0.047	115	0.025

Temperature strongly affects the synthesis of protease, either non-specifically by influencing the rates of biochemical reactions or specifically by inducing or repressing their production Abd Rahman *et al.,* (2005). Protease biosynthesis is a crucial process that plays a significant role in various biological activities. This study recorded various protease activities against three isolates at 25 to 45^{o}C. The results showed the optimum temperature for protease production and each isolate's corresponding dry biomass production and OD_{600nm}. These PPBIs, such as AL1, CB1, and CB2, were sown positive for protease activities at 25 to 45^{o}C as indicated in Figure 6b; AL1 exhibited the highest protease production at a temperature of 30^{o}C with 441.5±0.055 U/ml (table7). A similar study conducted by (Haddar *et al.,* 2011) reported that, Protease production was investigated in the temperature range of 25 ~ 45°C. Maximum activity and growth were observed at 37°C. The protease activities were about 612, 483, and 443 U/mL at 30, 40, and 45°C, respectively which were greater than the present study. This suggests that AL1 is particularly efficient in protease

synthesis at this temperature, highlighting its potential for various industrial applications. CB1, on the other hand, demonstrated optimal protease production at a slightly higher temperature of 37^{o}C.The protease production reached 368±0.03U/ml at this temperature, accompanied by a dry biomass production value of 0.039± 0.004 CDW (g/L) (Figure 6b and Table 7). The results indicate that CB1 has adapted to this temperature range and can effectively produce protease under such conditions. Similarly, Table 7 indicates that, isolate CB2 showed significant protease production at 30^{o}C, with a recorded value of 413.9±0.035 U/ml. related studies also reported that protease production by *B. laterosporous* was best at 37°C (Usharani and Muthuraj 2010). In addition, the corresponding dry biomass production was found to be CDW (0.07± 0.013 g/l) (Figure 6b), indicating its favorable response to this specific temperature. It is important to note that each organism possesses specific temperature preferences for optimal growth and metabolism. In contrast to the present study A study was conducted by (Gaur,*et al,*. 2014) to test the effect of various temperatures on the growth and alkaline protease production, different temperatures $(35-80°C)$ were used in the study. Protease production at different temperatures was examined keeping other conditions constant. It was noted that high protease production compared to the present result of ($1020 U mL^{-1}$) with 2.3 g L^{-1} biomass production was achieved at $60°C$ from Bacillus sp. that indicating the thermophilic nature of the enzyme. It was capable of producing protease in the range of $35-75°C$. However, increase in temperature beyond $75°C$ led to decline in enzyme production(Gaur,*et al,*. 2014). Nadeem et al. (2007) also reported that *Bacillus lichniformis* N2 showed maximum protease production at $60°C$.

In this study, the isolates demonstrated their ability to produce protease efficiently under temperatures ranging from 25 to 45^{o}C. This wide temperature range suggests the adaptability and versatility of these isolates in various environments. El-Safey *et al*., (2004) reported the same findings in production, purification, and characterization of protease enzyme from *Bacillus* sp. Moreover, Sinha *et al.*, (2013) found that 25^{o}C was the optimum temperature for protease biosynthesis from *Bacillus* sp. and protease biosynthesis at this temperature was found to be 100.1 U/ml which is much lower than the present study. Many investigators studied the relationship between temperatures and protease production. They found that temperatures range from 2-70 or more depending on the type of organism producing protease, the medium conditions and the type of enzyme.

4.3.3. Effect of incubation time against OD_{600nm}, and Biosynthesis of protease

Understanding of cell growth period in relation to enzyme production could be important factor for identifying the appropriate incubation period for maximal enzyme productivity (Hamza & Woldesenbet, 2017). Enzyme secretion is related to cell growth. It is a correlation between the incubation period and enzyme biosynthesis (Kaur, M *et al.,* 1998). The enzyme biosynthesis varies with incubation time. As depicted in Figure 7, various incubation times ranging from 24-120 h was explored to enhance protease production. The goal was to determine the most favorable incubation period for maximum enzyme yield. In this study, the maximum OD_{600nm} (1.72±0.08) was recorded at 48h for isolate AL1, OD_{600nm} (1.31±0.0095) for isolate CB1 at 72 h (figure 7). It was noticed that isolate CB2 displayed considerable OD_{600nm} (1.42±0.017) against 48h of incubation period, which indicated their optimum incubation period for the three isolates. As indicated in figure 7, A considerable minimum OD_{600nm} were gained for AL1 (0.85 ±0.054), CB1 (0.60±0.03) and CB2 (0.13±0.009) at 120 h of incubation. After analysis had been carried out, it was observed that the peak of enzyme biosynthesis was achieved at 48 h of incubation against AL1 and CB2 isolates, protease-producing strains. The enzyme activities recorded were for these isolates of which 456.2±0.08U/ml and 408.2±0.017 U/ml, respectively (Table 8). In the similar experiment of Rajeeva Gaur,*et al,*. (2014) maximum protease production ($1156 UmL^{-1}$) with 2.2 g L^{-1} biomass production from the Bacillus sp. was observed at 48 h. Similar results also reported by Qadar et al. (2009) from Bacillus sp. showed maximum protease production in 48 h.

Hamza & Woldesenbet, (2017) stated in their study that, highest enzyme production was observed after the *Bacillus sp.* CAMA14 reached around 48 h of cell growth. Periods before and after 48 h were not the convenient harvesting time for protease produced from *Bacillus sp.* CAMA14. For instance, after 48 h of incubation a decrease of enzyme production was observed (Hamza & Woldesenbet, 2017). They suspected that this may occur due to reduced microbial growth and increment of cell death, which is associated with the depletion of available resource, required for the growth of *Bacillus sp.* CAMA14 cells(Hamza & Woldesenbet, 2017). These results were synonymous to studies that were investigated by many researchers (Rao, and Narasu, (2007), Ravishankar, *et al.,*2012, Raj,*et al.,* 2012). On the other hand, a protease-producing CB1 isolate exhibited highest enzyme activity with 386.3± 0.0095 U/ml (Table 8), after 72 h of incubation period. Similar results also reported by Naidue and Devi (2005) and Gibb and Strohl (1988) that *B. subtilis 3411* and *B. subtilis K-30* gave maximum production in 72 h and 96 h, respectively.

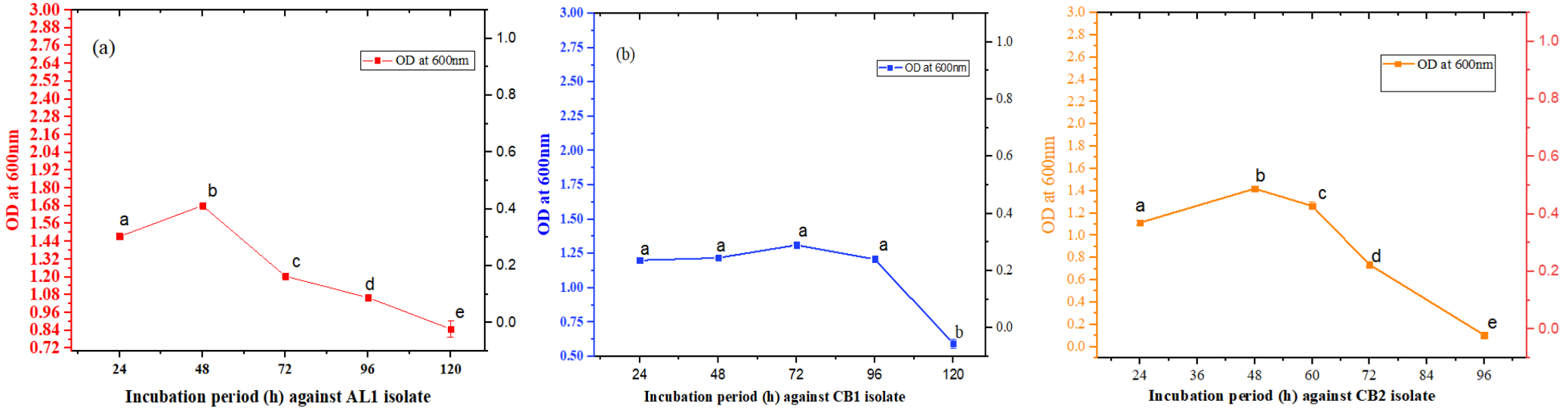

Figure 7: Effect of incubation period against protease-producing bacterial isolates. (g) Effect of OD_{600nm} against AL1 isolate for incubation period. (b) Effect of incubation period with OD_{600nm} against CB1 isolate, which obtained from soil sample. (c) Effect of time course of incubation period on CB1 isolate against OD_{600nm}. Data represented mean ± SD of three replicate (n = 3) against OD 600nm for these proteases producing bacterial isolates, which obtained from soil samples. According to one way ANOVA, the OD (g/L) has significant variation ($p \leq 0.05$) among protease producing bacterial isolates (AL1, CB1, and CB2) having the different latter on their respectivebars.

A study by Johnvesly *et al.,* (2012) displayed that the optimal incubation time for *B. anthraceis* S-44 and *B. cereus* S-98 were recorded with maximum protease activities. Johnvesly *et al.,* (2012) confirmed that the optimum incubation time against *B. anthraceis* S-44 and *B. cereus* S-98 was recorded to be 60 h. During this incubation period, the bacterial strains exhibited significantly increased protease activities. The researchers quantified protease activity in terms of units per milliliter (U/ml), and the results were noteworthy. For *B. anthraceis* S-44, the protease activity was 126.09 U/ml. On the other hand, B. *cereus* S-98 demonstrated a higher protease activity with 240.45 U/ml. It was indicated that *B. cereus* S-98 had a more robust enzymatic activity when compared to *B. anthraceis* S-44. The recent by Durhams *et al.* (1987), Gashaw and Gessesse (1997), and Qadar *et al.* (2009), showed that maximum enzyme biosynthesis was observed during continuous growth of the culture at the late exponential phase and early stationary phase of the development.

Table 8: Enzyme activity in U/ml against incubation period in (h)

S.N	Incubation period (h)	AL1		CB1		CB2	
		Enzyme activity	Standard deviation	Enzyme activity	Standard deviation	Enzyme activity	Standard deviation
1	24	417.6	0.023	367.9	0.064	352.4	0.038
2	48	456.2	0.021	369.6	0.069	408.2	0.049
3	72	366	0.007	386.3	0.076	379.8	0.032
4	96	333	0.006	384.5	0.021	227.5	0.017
5	120	264.9	0.036	148.8	0.031	19.4	0.009

4.3.4. Effect of various nitrogen source against CDW (g/L), OD600nm, and Biosynthesis of protease.

Beef extract, peptone, tryptone, and yeast extract were employed as a nitrogen source to support the growth of these PPBIs. The results show the effect of various nitrogen sources for the growth of PPBIs (AL1, CB1, and CB2). The data was represented in three separate graphs, each showing the biomass concentration (CDW) and optical density (OD_{600nm}) for these isolates when various nitrogen sources (beef extract, peptone, tryptone, and yeast extract) were used (Figure 8). From the results, all these isolates exhibited maximum growth in terms of OD_{600nm} and CDW g/L when yeast extract was used as a nitrogen source. The highest values for both parameters were observed for isolate CB2 against OD_{600nm} (1.965±0.017) and CDW g/L (0.0798±0.013). Substantial amounts of OD_{600nm} (1.89±0.011,) and CDW g/L (0.0757±0.0127) were obtained against AL1 isolate When yeast extract had been supplemented as a sole nitrogen sources, better OD_{600nm} (1.502±0.003) and CDW g/L (0.013±0.002) were also obtained from CB1 isolate, a PPBI (Figure 8).

Additionally, the study indicates that tryptone was the next best nitrogen source for growth of AL1 and CB2 isolates, except for isolate CB1, which showed minimum CDW (0.006±0.001) for tryptone. However, maximum OD_{600nm} (1.254±0.059) and CDW (0.007±0.002) were recorded against peptone after yeast extract (Figure 7). Overall, this study suggests that yeast extract is the most favorable nitrogen source for the growth of these PPBIs, followed by tryptone and peptone. In contrast, beef extract was the least preferred nitrogen source based on the observed growth parameters. The study indicates that there were significant differences ($p>0.05$) in biomass concentration (CDW) among the different nitrogen sources for isolates AL1, CB1, and CB2.

This suggests that the nitrogen source did not significantly impact these isolates' biomass production. Protease production has been determined by various nitrogen sources. It was noticed that certain organisms have unique requirements for specific nitrogen sources, making it a crucial factor in determining optimal protease production. Complex nitrogen sources in the growth medium have been significantly found to affect protease biosynthesis. Among the tested isolates, it was noticed that yeast extract played a pivotal role in achieving optimal enzyme secretion. Additionally, in the absence of yeast extract, tryptone showed potential as an alternative nitrogen source (Table 9).

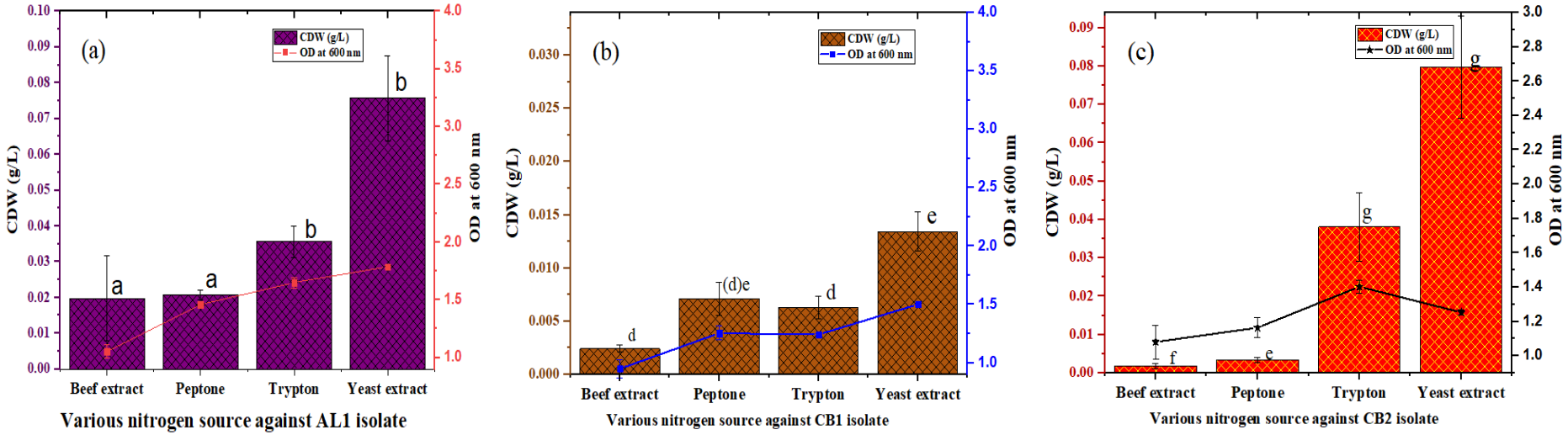

Figure 8 : **Effect of various nitrogen sources against Protease producing bacterial isolates. (a) Effect of various nitrogen sources against CDW (g/L) and OD_{600nm} of AL1 isolate (b) Effect of various nitrogen sources against CDW (g/L) and OD_{600nm} of CB1 isolate. (c) Effect of various nitrogen sources against CDW (g/L) and OD_{600nm} of CB2 isolate. Date represented triplicate (3) (mean ± SD). The same letter on the column bar indicated no significant various ($p>0.05$) among nitrogen sources against CDW (g/L) for these AL1, CB1, and CB2 isolates.**

Yeast extract is also emerged as a highly effective nitrogen source, leading to high protease biosynthesis and dry biomass yield. Notably, variations observed against AL1, CB1, and CB2 isolates, these PPBIs, which indicated that yeast extract significantly increased protease production. The recorded protease levels were ranged between 423-508.64 U/ml for AL1, CB1, and CB2 isolates (Table 9). Furthermore, yeast extract is also exhibited remarkable enzyme activity and biomass production forAL1 (474.61±0.01 U/ml, 0.0756±0.012 g/L) , CB1 (423±0.0025 U/ml, 0.0134±0.002g/L), and CB2 (508.6±0.02 U/ml, 0.0798±0.013g/L) isolates. Based on the results obtained, it is confirmed that the addition of yeast extract used to stimulated protease production. This stimulation resulted in enhanced enzyme activity levels and optimal performance overall. A similar result by Nadeem *et al.,* (2007) also reported maximum enzyme production in the presence of yeast extract followed by peptone. Whereas, In The experiment of Rajeeva Gaur,*et al,*. (2014) maximum protease production of (2020UmL^{-1}) with 2.6 g L^{-1} biomass production was observed in the presence of peptone followed by yeast extract. Similarly, Qadar *et al.,* (20100) also reported that maximum enzyme production was observed in the presence of peptone followed by yeast extract from Bacillus sp.

Numerous studies have shown that selecting an appropriate nitrogen source maximizes protease production by various microbial species. Ashour *et al.,* (1996) emphasized the effectiveness of yeast extract in stimulating the production of protease by various microbial species. This finding underscores the crucial role played by yeast extract in optimizing protease synthesis. Furthermore, Sinha *et al.,* (2013) conducted a study on *Bacillus* sp. and discovered that yeast extract served as an excellent nitrogen source, leading to significantly increased protease production. Using yeast extract as a nitrogen source in microbial cultures has consistently positively impacted protease levels. In another study by Sharma *et al.* (2014), the combined usage of yeast extract and casein as nitrogen sources resulted in a high secretion of protease activities. This research further substantiates the notion that yeast extract, in combination with other nitrogen sources, can effectively boost protease secretion.

Table 9: Enzyme activity in U/ml against different nitrogen sources.

S.N	**Nitrogen source**	**AL1**		**CB1**		**CB2**	
		Enzyme activity	Standard deviation	Enzyme activity	Standard deviation	Enzyme activity	Standard deviation
1	Beef extract	340.4	0.071	303.4	0.118	333.5	0.095
2	Peptone	415.3	0.053	377.9	0.059	361.3	0.044
3	Trypton	450.7	0.047	375.6	0.01	405.3	0.057
4	Yeast extract	474.5	0.049	422.9	0.237	508.3	0.557

4.4. Degradation of eggshell and its membrane with PPBI through fermentation

The disposal of egg shells and their underlying membrane has been identified as a contributor to environmental pollution (Phil and Zhihong, 2009). However, using the calcium carbonate ($CaCO_3$) present in egg shells has shown potential in reducing pollution, acting as a neutralizer in farm fields and as a valuable source of calcium. Making eggshell fertilizer is inexpensive and environmental-friendly, since the process reuses material to promote growth. Ground eggshells are effective liming sources (John and Paul, 2006). The main challenge lies in the insolubility of the eggshell membrane (ESM) in water and organic solvents, limiting its applications. This study explored the solubilization of WES and ESM, presenting their possible uses and finding sustainable environmental solutions. We isolated potentially alkaline protease-producing bacteria from the accumulation site of cake bakery waste and arable land in East Wollega, Nekemte town. After obtaining a total of 14 PPBIs, three of them displayed promising proteolytic activity on skim milk agar medium. These protease enzymes facilitate the hydrolysis of protein (casein) present in the culture media (Skim milk agar) which serve as carbon and nitrogen sources (Fu *et al.,* 2003). The potential of these isolates as a source of alkaline protease-producing bacteria in the study area had yet to be previously reported. This result establishes the accumulation site of cake bakery waste and arable land in East Wollega, Nekemte town, as valuable sources of protease-producing bacteria.

All isolated bacteria demonstrated the ability to hydrolyze casein protein on skim milk agar medium, indicating their capacity to produce the protease enzyme responsible for its degradation. This highlights the potential of the identified sources for alkaline protease biosynthesis. Notably, the eggshell membrane triggered the inducible synthesis of the protease, emphasizing the direct involvement of the produced enzyme in the decomposition of ESM. This result represents the second report describing the production and characterization of an enzyme capable of directly decomposing ESM without additional substances like acid.

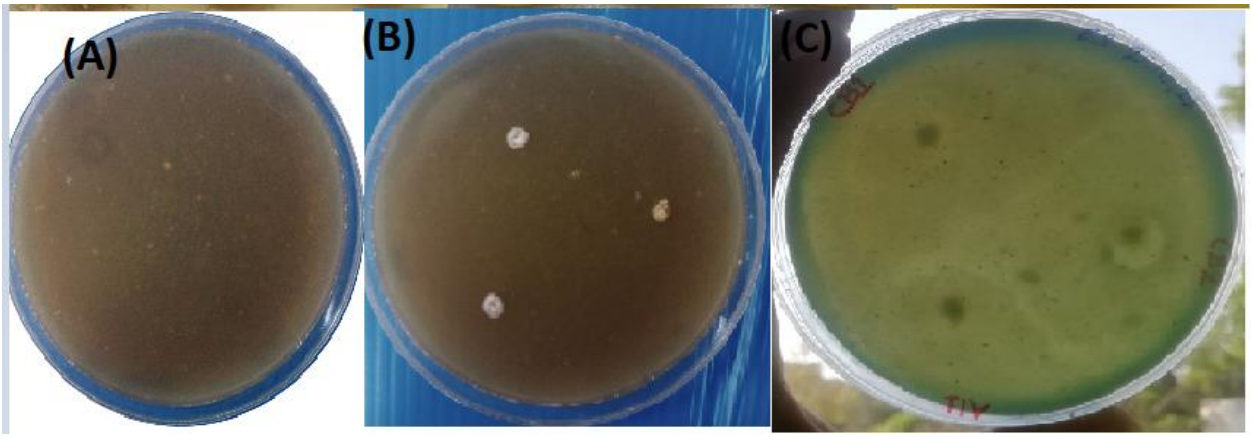

Figure 9: the clear zones in ES meal powder (g/l) agar plates, caused by proteolytic bacteria, are depicted. Three strains, namely AL1, CB1, and CB2, were identified. The microbial culture was spot inoculated on an ES meal agar plate and incubated at the optimal condition. (A) Represents the ESM plate before inoculation. (B) Represents the ESM plate with spot inoculation of the isolate before the formation of hydrolysis zones. (C) Represents the formation of clear zones on the eggshell meal powder.

In the present study, the cultured microorganisms, which derived from the selected study site and their degrading cultures, were found to exhibit significant proteolytic activities. I tried to demonstrate the specific bacterial strains, which was capable of degrading WES and ESM, using eggshell meal powder as the sole carbon and nitrogen source (figure 9). Three bacterial strains, AL1, CB1, and CB2, were selected due to their ability to visually degrade casein protein in skim milk agar under suitable condition. The experiment revealed that a temperature of 37°C was suitable for growth of CB1 isolate, while the others isolates (AL1 and CB2) showed optimal growth at 30°C. Additionally, a pH level of 8 was ideal for all these three isolates. The culture media were prepared for protease biosynthesis, incorporating 1% casein, glucose as the main carbon source and yeast extract as the main nitrogen source. The optimal conditions of incubation times were 48 h for two isolates (AL1 and CB2) and 72 h for CB1, along with a rotation speed of 150 rpm on a rotary shaker. So as shown on the above figure (figure 9) the isolates was grown on the eggshell meal powder and formed a zone of hydrolysis which indicated the isolates produced extracellular protease which can solubilize ESM and WES. A similar previous study showed that (Cheng et al., 2009) *Pseudomonas aeruginosa* ME-4 can display robust growth when ESM was supplied as a main substrate. The strain was found to produce an extracellular protease, which can solubilize ESM.

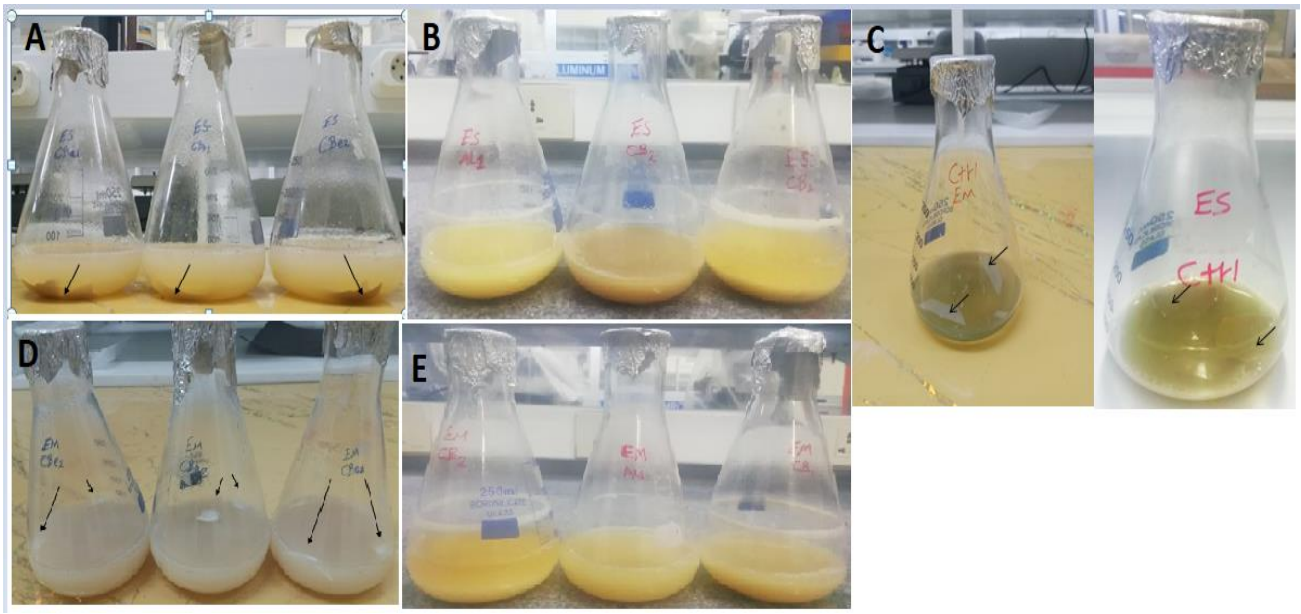

Figure10: the degradation of chicken WES and ESM caused by the bacterial strain obtained from the soil in the study area. The degradation process took place through submerged cultivation at the optimal temperature for the isolates. The figure includes different stages: (A) WES prior to incubation with the PPBI, (B) WES after incubation with the PPBI, (C) ESM and WES incubated without PPBI serving as a control, (D) ESM before incubation with the PPBI, and (E) ESM after incubation with the PPBI.

When ESM was incubated with the supernatant culture of these three isolates, a gradual decomposition of ESM was noticed(figure 10). Over specific days of incubation, large intact ESM particles were visibly broken down into smaller ones (figure 10E). *Lysinibacillus spheraicus*, represented by isolate AL1, achieved ESM full degradation within four days of incubation period. CB2 achieved full degradation within five days, and CB1 demonstrated full degradation within seven days. As depicted on figure 10E, The visibly observed ESM transformed into invisible, smaller particulates throughout these periods. According to Zimmerman (2011), the term "complete solubilization of eggshell membranes" refers to the breakdown of egg shell membranes until no solid is visible or detectable.Still, degradation was not observable by the naked eye due to the presence of calcium carbonate on the outer part of the ES and the growth of the isolates covering it in the case of WES (figure 10B).

Nevertheless, intact whole eggshells were still visible by naked eye (figure 10B). Cheng *et al.*, (2009) reported the egg shell membrane decomposition by a protease producing Pseudomonas sp. High enzyme production was obtained in their study than the previous report. The study of (Cheng *et al.,* 2009) previously purified and characterized an eggshell-membrane-degrading enzyme classified as a metalloprotease from Pseudomonas aeruginosa strain ME-4. The ME-4 protease solubilizes eggshell membrane and is expected to partially hydrolyze this membrane, which is then

more suitable for the production of soluble proteins and/or peptides than chemical hydrolysis. A previous study showed that (Cheng *et al.,* 2009) *Pseudomonas aeruginosa* ME-4 can display robust growth when ESM was supplied as a main substrate. The strain was found to produce an extracellular protease, which can solubilize ESM. Meanwhile, Nagamalli *et al.,* (2017) reported the growth of *Bacillus altitudinis* GVC11 in a fermentation medium containing eggshell and membrane as a main substrate. They observed the degradation/hydrolysis of membrane proteins and analyzed the fermented broth for free amino acids in addition to alkaline protease. Notably, their study recorded higher enzyme production compared to Cheng *et al.,* (2009) report on ESM decomposition by a protease-producing *Pseudomonas* species.

On the other hand there are few studies focusing on the preparation of water-soluble products, including proteins and peptides from ESM using microorganisms and enzymes alone. When microorganisms or enzymes solubilize ESM, specific proteins and peptides are obtained from ESM.one report was published describing the potential of whole eggshell and ESM fermentations using the lactic acid bacteria, *megasphaere cerevisiae* (Rubilar *et al.,* 2006). However, the purification and characterization of the enzymes catalyzing the solubilization of products from whole eggshells and ESM were not reported. When ESM was incubated with the culture supernatant of *p.aeruginosa* strain ME-4,it was gradually decomposed, and after 48 h of incubation, large intact ESM particles were visibly observed to have broken down into smaller ones(Cheng *et al., 2009)* which was more effective than the present study.This study focused on examining the proteolytic activity of protease-produicng bacterial isolates against ESM. The selected bacterial strains demonstrated the ability to degrade ESM effectively in specific incubation conditions. These results contribute to our understanding of the enzymatic degradation of ESM and pave the way for potential applications in various fields.

5: CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The current study was initiated with the objectives of isolating and characterizing bacterial strains from soil samples by determining their ability to produce protease enzymes and evaluating their potential for degrading eggshell and eggshell membrane wastes. Biodegradation of eggshell and eggshell membranes is a promising eco-friendly method whereby eggshell waste is managed with minimum adverse effects on the environment. This study tested 3 isolates collected from cake bakery waste disposal sites and farmland for eggshells and their membrane degrading ability. These isolates were identified by biochemical and MALDI-TOF MS analysis. A potentially new bacterium species was characterized using 16s rRNA and belongs to *Bacillus sp*. It showed the highest similarity with *Lysinibacillus capsici* PB300T (PXXX01000046) (98.90%).

The biodegradation assay conducted demonstrated that *lysinibacillus sphearicus* sp. (AL1), CB1, and CB2 isolates have a great capacity to degrade eggshell and its membrane from the environment, and their degradation ability, which is aided by pre-treatments with only high heat, may improve its degradability. Pre-treatment such as high heat could potentially increase the eggshell and its membrane degradation efficiency of isolates, and the effect of this degradation process was evident in the full degradation of the separated eggshell membrane. Based on the optimized parameters, A temperature of 37^{o}C for isolate CB1 and 30^{o}C for the left two isolates (AL1 and CB2), pH 8 for all three isolates, yeast extract as nitrogen source, 48 h for the two isolates and 72 h for CB1 of incubation time were found to be optimal conditions for degrading eggshell and its membrane with the screened PPBI in the laboratory. This study showed that cake bakery waste dumping sites and arable land soil samples from West Wollega, Nekemte Town could be a great source of eggshell and its membrane-degrading PPBIs.

5.2. Recommendations

Eggshells should not be discarded in a manner that harms the environment, whether they come from hatcheries, fast food industries, or households. In developing nations, it is important to educate communities about the numerous uses of eggshells and shell membranes. Extensive research should be conducted to adapt their utilization to locally developed technologies. In cases where the quantities produced are not economically viable, communities should be encouraged to bring them to designated collection points for further transportation to a central facility where they can be processed into various products. This approach will not only create job opportunities but also enhance the profitability of poultry production, ensure local availability of these products at potentially lower costs, and reduce environmental pollution.

It is imperative to advance this research to the industrial level, particularly in the cosmetics, detergent, and waste management sectors, in order to mitigate the pollution caused by chemical processes. Our thesis does not cover certain aspects of this work. Therefore, individuals interested in this field should focus on the Production, Purification, and characterization of protease, investigating enzyme kinetics, comparing protease activity with other commercially available enzymes, improving protease efficiency, conducting submerged state fermentation, and scaling up protease production. The alkaline protease from PPBI should be introduced to various industries such as leather, cosmetics, pharmaceuticals, and waste management. Further purification steps and research are necessary for the widespread use of the enzyme-producing isolates.

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APPENDIXES

Figure A_1: pure culture of the isolates

Figure A_2: Some Selected Biochemical Results. (A)Voges-proskauer test (All are –ve) (B) Citrate Utilization test (C)methyl red(all isolates are positive) (D) motility (E) Starch hydrolysis test (All are –ve) (F) TSI (G) Oxidase test (All are +ve) (H) catalase Test (All are Positive)

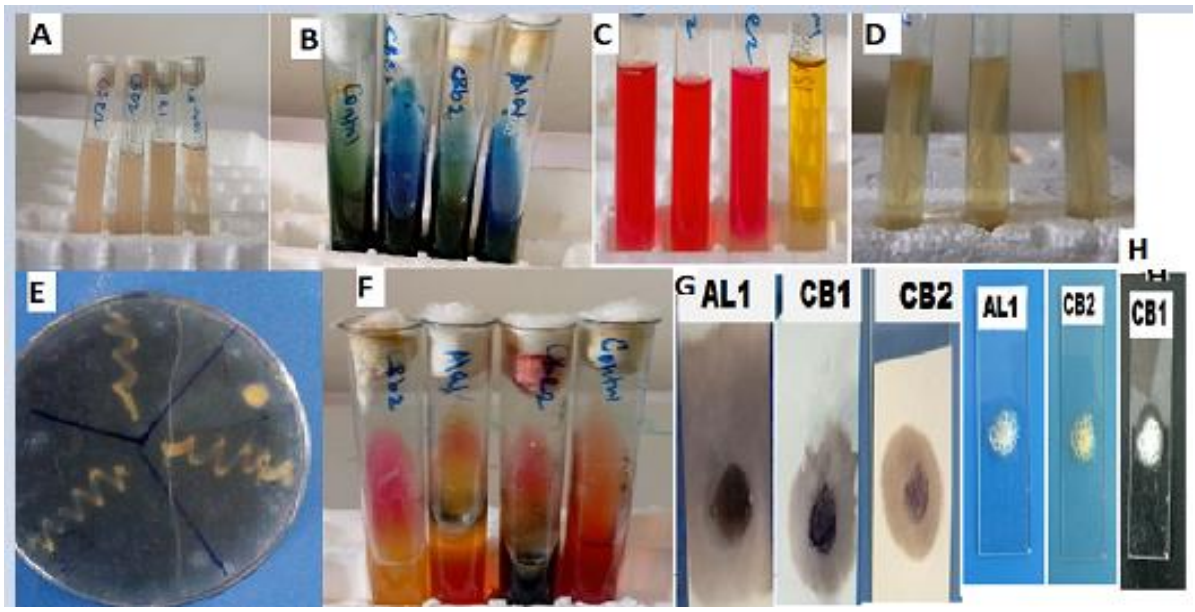
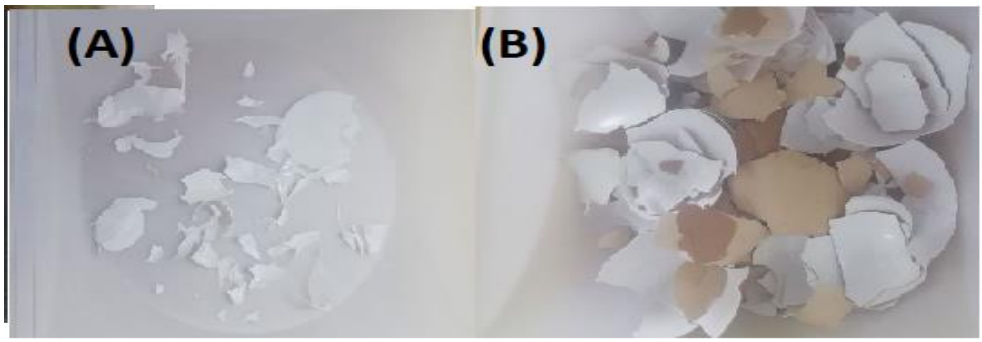


Figure A_3: Egg shell membrane and whole eggshell before degradation.



Appendix >Consensus data > Ala1

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TTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGG
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TGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATG
CCGCGGTGAATACGTTCCCGGGCCTTGGCACACCGTCAGTAAGTCCTAACAAGGTAAC
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Figure A_3. 1.8% Agarose gel showing single 1500 bp of *16s* amplicon.

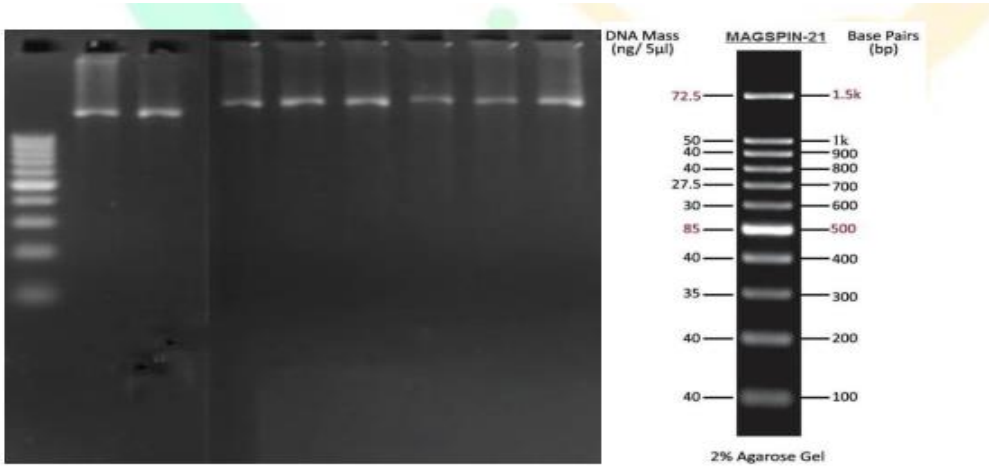


Table A_1: Effect of various temperatures against CDW (g/L) for AL1, CB1, AND CB2, a protease producing bacterial isolates

S.N.	Temperature (^oC)	AL1		CB1		CB2	
		BM	SD	BM	SD	BM	SD
1	25	1.382	0.045	0.836	0.058	1.4	0.0095
2	30	1.642	0.007	0.902	0.017	1.475	0.0324
3	35	1.494	0.014	1.024	0.071	1.101	0.0887
4	37	1.127	0.046	1.187	0.031	0.907	0.0569
5	40	1.045	0.033	0.583	0.023	0.74	0.0383

Table A_2: Effect of various temperatures against OD_{600nm} for AL1, CB1, and CB2, a protease producing bacterial isolates

S.N.	Temperature (^oC)	AL1		CB1		CB2	
		OD	SD	OD	SD	OD	SD
1	25	0.035	0.0199	0.004	0.0007	0.0305	0.0112
2	30	0.048	0.0125	0.005	0.0008	0.0705	0.0137
3	35	0.039	0.0038	0.008	0.0010	0.0125	0.0058
4	37	0.007	0.0017	0.039	0.0038	0.0048	0.0006
5	40	0.003	0.0017	0.002	0.0006	0.0029	0.0009

Table A_3: Effect of various PH against OD_{600nm} FOR AL1, CB1, and CB2, a protease producing bacterial isolates

S.N.	PH (^oC)	AL1		CB1		CB2	
		BM	SD	BM	SD	BM	SD
1	5	0.0049	0.0014	0.0044	0.0013	0.02	0.02
2	6	0.051	0.01	0.0037	0.0016	0.012	0.008
3	7	0.062	0.045	0.0069	0.0024	0.03	0.01
4	8	0.08	0.015	0.066	0.023	0.078	0.012
5	9	0.017	0.002	0.03	0.94	0.0034	0.002

Table A_4: Effect of various PH against CDW (g/L) for AL1, CB1, and CB2, a protease producing bacterial isolates

S.N.	Temperature (oC)	AL1		CB1		CB2	
		BM	SD	BM	SD	BM	SD
1	5	0.984	0.01	0.75	0.017	1.33	0.0091

2	6	0.87	0.006	0.78	0.007	0.84	0.015
3	7	1.6	0.025	0.95	0.07	1.46	0.01
4	8	1.67	0.003	1.72	0.002	1.67	0.02
5	9	0.74	0.004	1.06	0.038	0.97	0.01

Table A_5: Effect of various nitrogen sources against OD_{600nm} for AL1, CB1, and CB2, a protease producing bacterial isolates

S.N.	**Nitrogen sources**	**AL1**		**CB1**		**CB2**	
		OD	SD	OD	SD	OD	SD
1	Beef extract	1.053	0.057	0.95	0.08	1.08	0.09
2	Peptone	1.44	0.012	1.254	0.06	1.164	0.06
3	Trypton	1.65	0.045	1.243	0.01	1.403	0.04
4	Yeast extract	1.79	0.01	1.5	0.0025	1.96	0.02

Table A_6: Effect of various nitrogen sources against CDW (g/l) for AL1, CB1, and CB2, a protease producing bacterial isolates

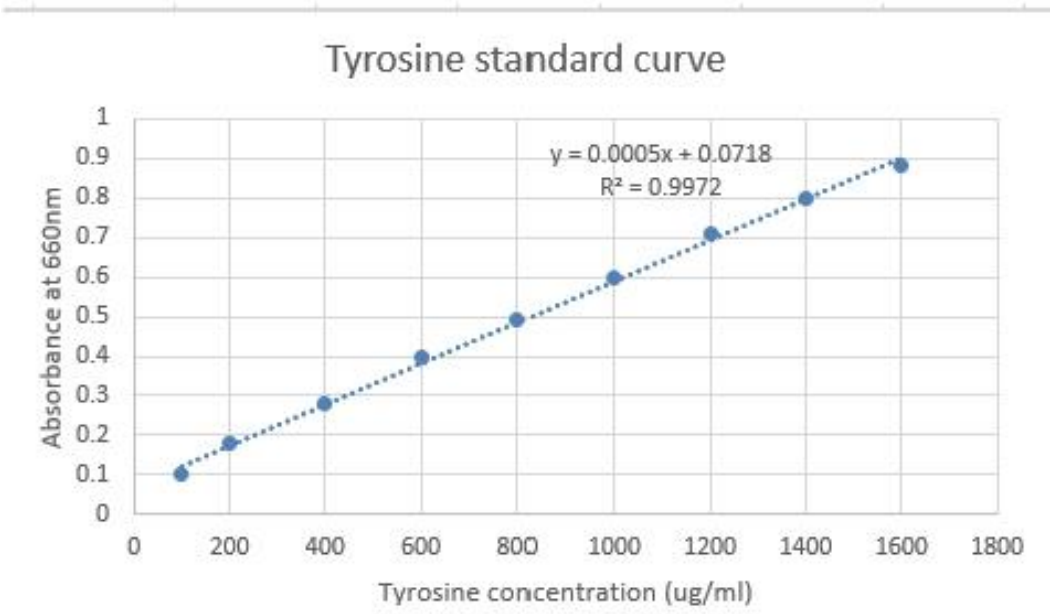
S.N.	**Nitrogen sources**	**AL1**		**CB1**		**CB2**	
		BM	SD	BM	SD	BM	SD
1	Beef extract	0.019	0.02	0.002	0.00035	0.0018	0.00075
2	Peptone	0.02	0.0013	0.007	0.0015	0.0034	0.00078
3	Trypton	0.035	0.004	0.0063	0.001	0.038	0.0088
4	Yeast extract	0.013	0.002	0.013	0.002	0.078	0.013

Table A_7: Effect of various incubation time against OD_{600nm} for AL1, CB1, and CB2, a protease producing bacterial isolates

S.N.	**Incubation (hr)**	**AL1**		**CB1**		**CB2**	
		OD	SD	OD	SD	OD	SD
1	24	1.5	0.02	1.20	0.03	1.12	0.02
2	48	1.7	0.08	1.2	0.009	1.42	0.017
3	72	1.21	0.007	1.3	0.0095	1.265	0.035
4	96	1.06	0.006	1.2	0.02	0.74	0.017
5	120	0.85	0.054	0.6	0.03	0.1	0.009

Preparation of tyrosine standard curve

To prepare the standard curve 0.5M of NaCO3, 50mM of sodium phosphate buffer pH 7.0 diluted 1N Folin reagent and 10 mg/ml of Tyrosine stock solution were used. A required amount of buffer and Tyrosine were added in each test tube except blank. Then 2.5 ml of 0.5 M NaCO3 was added in each test tube including blank. After 5 minutes 500µlof 1N Folin reagent was added in each test tube including blank, the solution was mixed immediately and kept for 30min at room temperature. Finally, the optical density (OD) was measured at 660nm using spectrophotometer and the standard curve was plotted.



Appendix : Tyrosine standard calibration curve for protease assay

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