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College of Natural and Computational Science

Screening of Endophytic Bacteria Isolated from Selected Plants in Hawassa for Production and Characterization Amylase

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Declaration

I hereby declare that the thesis I have submitted for the Master of Science (MSc) degree in the Department of Microbial, Cellular, and Molecular Biology (Applied Microbiology) at the School of Graduate Studies, Addis Ababa University, titled "Amylase Production, Purification, and Characterization from Selected Endophytic Bacteria in Hawassa," is my own original work. This thesis has not been submitted elsewhere by me or anyone else. It includes proper acknowledgments for all contributions made by others.

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List of abbreviations

CFS	Cell Free Supernatant
OD	Optical Density
RDS	Rapidly Digestible Starch
rpm	Revolution Per Minute
RS	Resistant Starch
SDS	Slowly Digestible Starch
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
U/mL	Unit Per Milliliter
w/v	Weight by Volume

Abstract

Amylases are crucial enzymes in the global industry, constituting 25% of enzyme production. Endophytes, known for producing bioactive compounds and enzymes, offer promise for industrial applications. The aim of the study was to screen amylase producing endophytic bacteria, to produce amylase under controlled laboratory conditions and characterize the enzyme. Purposive sampling was used to select the study areas as well as the plants screened for amylase producing endophytic bacteria. After collection the plant samples were washed and surface sterilized before isolation of the endophytic bacteria. The endophytic bacteria were isolated then screened for amylase production on starch agar media and purified. The pure isolates were characterized using morphological and biochemical test. Amylase was produced through submerged fermentation, and its production was optimized using different carbon and nitrogen sources, pH and temperatures. Ammonium sulphate precipitation and dialysis were used for partial purification and the partially purified enzyme was assayed for its activity using the dinitrosalicylic acid (DNS) method. Out of 60 endophytic bacteria isolated from the plants, 32 demonstrated amylase production. From these, 16 pure isolates were found to be the most efficient amylase producers and 7 best amylase producing isolates based on submerged fermentation. Among these top 16 producers, 12 were Gram-positive bacteria and 4 were Gram-negative bacteria.The 7 isolates were identified to species level by rRNA gene sequence analysis. Then 3 isolates were selected for further purification and characterization. Diameter (Mean ± SD) of clear zone on starch agar ranged from 4.48 ± 0.54 for isolate I10L2 from Enset to 11.53 ± 0.34 for isolate D10F2 from Datura. The highest ($p < 0.05$) amylase production was recorded for isolate D10L2 when glucose or starch was used as carbon source. The highest amylase production was recorded for all isolates in the presence of ammonium sulfate and the lowest in the presence of tryptone as nitrogen source. The optimum temperature for production of amylase was 30 ^{O}C, but differences in the optimum pH ranging from pH 5 to pH 8 were recorded based on the isolates preferences. After partial purification the highest enzyme activity was recorded at pH 7 and pH 9, 50 ^{O}C, and 0.5% and 1% calcium ion (Ca^{2+}) concentrations. The maximum activity in the presence of 1mM Ca^{2+} was about 16.5 U/mL. These findings highlight the

potential of endophytic bacteria for amylase production. With further optimization and scale up, the amylase can be produced in large quantities and for specific applications.

Key words and phrases: Amylase, Endophyte, enzyme activity, starch, submerged fermentation

1. Introduction

1.1 Background

Endophytes are symbiotic organisms that live within plants without creating any symptoms, at least throughout some stages of their life cycle. The organisms mostly encompass bacteria, fungus, and actinomycetes. Endophytes can inhabit numerous sections of a plant, such as the stem, roots, petioles, leaf segments, weed inflorescence, fruit, buds, seeds, and dead or hollow hyaline plant cells. The number of endophytes within a plant species can differ greatly, determined by factors such as the host species, developmental stage, inoculum density, and conditions in the environment (Khan *et al.,* 2017). They have evolved into useful suppliers of unique metabolites, essential industrial enzymes, and stress alleviation for host plants. The multitude of and continuous interactions that individual endophytes have with the host plant and other microbes in the plant's micro-biome determine their respective roles. Comprehending and incorporating these intricate connections can be accomplished by regarding plant micro-biome as a holistic system (Kaul *et al.,* 2016). Endophytes have long been recognized for their ability to produce new, bio-active secondary metabolites with a variety of diverse functions as well as enzymes, which are important for industrial applications. From these sources, several modern and novel enzymes have been found, and their potential for industrial application has been identified (Zaferanloo *et al.,* 2013) Many microorganisms, such as bacteria, actinomycetes, fungi, and yeast, create a broad variety of flexible and interesting enzymes that demonstrate a wide range of structural variants and practical applications. Extracellular production yields a wide variety of microbial enzymes, including as cellulases, lipases, xylanases, pectinases, amylases, and proteases. On the other hand, some enzymes are present within cells, such as catalase from *Saccharomyces cerevisiae* and *Aspergillus niger* (Ravindran *et al.,* 2017).

Although starch can also be hydrolyzed with acids or enzymes, amylases are essential for breaking down starch. Currently, enzymatic hydrolysis is used in almost all starch processing companies. Important enzymes for this usage are glucose isomerases, beta amylases, glucoamylases, thermostable amylases, and debranching enzymes (Gessese *et al.,* 2011). A quarter of the world market for enzymes is made up of commercial enzymes in the amylase class. These are some of the most significant enzymes with wide-ranging biotechnological uses.

Amylases can be obtained from different sources, including plants, animals, and microbes. Nowadays, a wide variety of commercially accessible microbial amylases have nearly completely supplanted chemical starch hydrolysis in the starch processing industry (De Souza *et al.,* 2010). The best source of industrial enzymes is microorganisms since they can produce large amounts of enzymes quickly and have shorter generation times. Additionally, bacterial cells are more amenable to genetic alterations that can improve the production of enzymes (Anbu *et al.,* 2015).

Alpha amylase is a type of amylase enzyme that operates as a hydrolase, facilitating the hydrolysis of internal α-1,4-glycosidic bonds in starch to create glucose and maltose. Because of its classification as a calcium metalloenzyme, calcium is required for the enzyme's action to occur (Neidleman and Geiduschek, 1966). There have been numerous investigations on the production of this enzyme in Ethiopia from a variety of sources, including plants, soil, water, agricultural wastes, and a few endophytes. This research aims to screen endophytic bacteria isolated from the selected plants (*Datura*, potato, inset, coffee) For production of amylase, and to characterize the amylase.

1.2 Statement of the problem

Ethiopia has a diverse range of habitats, temperatures, geographies, and environmental conditions, which contributes to its high biodiversity, particularly in terms of microbial diversity. In both terrestrial and aquatic settings, it is known to have a great diversity of microorganisms. A novel microorganism that can produce amylolytic enzyme with potential application for starch hydrolysis is expected to develop from the isolation of fungal and bacterial strains from these various habitats.

It is well known that the majority of Ethiopian enterprises are dependent on importing different chemicals, enzymes, and most processed materials; as a result, their products have occasionally grown increasingly expensive. Every commodity imported now costs a lot more money, especially with the rising value of other currencies. Industries are for-profit businesses, hence they assign a cost-based value to their products. The reason for their greater price is that they import resources rather than produce the basic ingredients themselves. Inflation follows, which makes it one of Ethiopia's biggest problems. Producing this enzyme in Ethiopia by employing the raw materials and resources available has not only economic benefit but also scientific value

in order to address the issue of inflation and to boost the production rate of industries like food, pharmaceutical, detergents, paper, and bio fuels. This topic has been the subject of numerous research, but none of them have reached the level of manufacturing and application; as a result, this study will be a pioneer in resolving this issue.

1.3 Objectives

General objective

- To screen endophytic bacteria isolated from selected plants in Hawassa for amylase production and its characterization.

Specific objectives

- To identify amylase producing endophytic bacteria
- To produce of amylase under optimal submerged fermentation
- To characterize of amylase from fermentation broths

2. Literature review

2.1 Source of endophytes

Plants are habitat for varieties of microorganisms which have potential to produce different essential compounds, antibiotics and industrially important enzymes (Trivedi *et al.,* 2020). Datura, potato, coffee and enset are samples collected for this study to obtain a potential endophytes to produce an alpha amylase enzyme.

The enormous trumpet-shaped blooms of Datura species set them apart as herbaceous, sprawling annuals or short-lived perennials. The fragrant white, pink, purple, or yellow blossoms give way to a spiny capsule that contains several seeds that becomes the fruit. Basic leaves with wavy, lobed, or whole margins that are placed alternately (Sharma *et al.,* 2021). The plants contain strong alkaloids like scopolamine, hyoscyamine, and atropine, which make them lethal if swallowed. According to Masuura *et al.,* (2015), some *Datura* species are gathered for their medicinal properties, while others are grown for their eye-catching blooms. Commonly found growing alongside roadsides and other disturbed habitats, many are regarded as weeds in warmer regions of the world. The plant used for this study is jimsonweed, or *Datura stramonium*, sometimes known as the devil's snare or thorn apple.

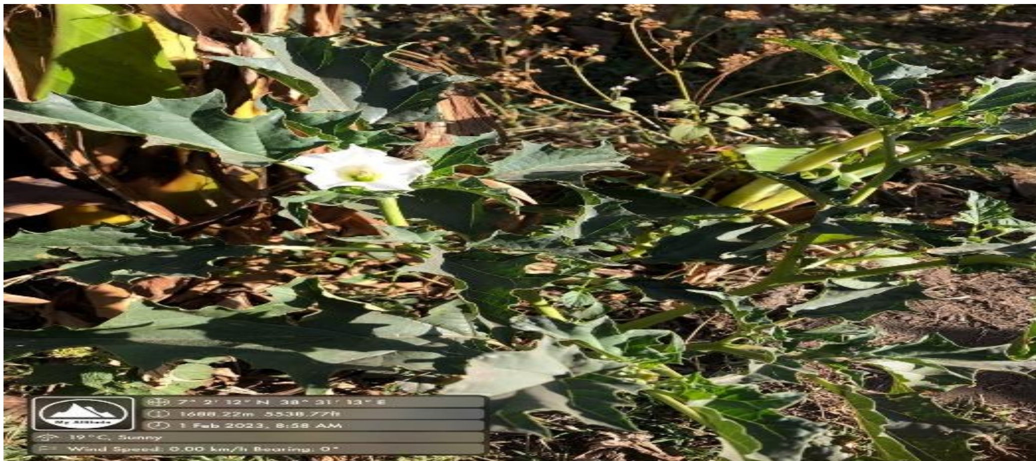

Figure 1. Picture of *Datura stramonium from Chefe (Photo taken by the investigator)*

There are 150 species in the genus *Solanum* that yield tubers, which are the swollen tips of subterranean stems. The potato is among these species. Apart from the terminal leaflet, each of the 20–30 cm (about 8–12 inch) long compound leaves has two to four pairs of leaflets. Spirally

organized are the compound leaves (Reddy *et al.,* 2018). The white, lavender, or purple flowers have five fused petals with yellow stamens. The fruit is made up of many seeds and small, potentially harmful berries. Underneath the stems are structures called stolons. A few to up to twenty tubers of varying sizes and shapes may be formed by the ends of the stolons growing significantly (Struik Paul, 2023). These tubers usually weigh up to 300 grams (10 ounces), although on rare occasions, they may weigh up to 1.5 kilograms (3.3 pounds).

A member of the Solanaceae family, *Solanum tuberosum* is an annual nightshade plant that is grown for its starchy, eatable potatoes. Native to the Peruvian and Bolivian Andes, potatoes are among the world's principal food crops. Vegetables are often served whole or mashed after preparation. Potato flour is another product that is made from potatoes and is used to thicken sauces and baked items. The tubers are very easily digested and high in protein, thiamine, niacin, and vitamin C.

Figure 2. *Solanum tuberosum, taken from Chefe (Photo taken by the investigator)*

Ensete ventricosum, sometimes referred to as pseudo banana, is a large, perennial herb that is monocarpic and evergreen; it is not a tree. Big and not woody, that is. In Ethiopia in particular, Enset is an essential native food source. The United Nations Food and Agriculture Organization

states that enset provides more food per unit area than most cereals (Borrell *et al.,* 2019). In Ethiopia, enset (*E. ventricosum*) is the most important root crop and has long been a staple in the heavily populated southwest and south of the country. The first person to recognize its importance for the food and economics of the Gurage and Sidama people in the seventeenth century was Jerónimo Lobo (Karlsson *et al.,* 2016). Enset also has a high starch content (Yemata Getahun, 2020).

Figure 3. *Ensete ventricosum, taken from Loke (Photo taken by the investigator)*

The glossy green foliage and restricted growth pattern of the coffee plant make it a lovely tiny plant. In a pot, it makes an unexpectedly good indoor plant. Ethiopia is the natural home of the coffee plant, *Coffea arabica* (Ferreira *et al.,* 2019). It has half-inch berries that gradually change from green to blackish pods after blooming with tiny white flowers in the spring. Each of these fruits has two seeds, which eventually grow into the coffee beans that are brewed (Senbeta *et al.,* 2013).

Figure 4. *Coffea arabica, taken from Atote (Photo taken by the investigator)*

2.2 Starch

Starch is an essential component of the human diet and is processed chemically and enzymatically to create a range of products used in the food business, including glucose syrups, fructose, starch hydrolysates, and derivatives of maltodextrin. These sugars can also be fermented to make ethanol. Despite the ability of numerous plants to generate starch, only a handful are crucial for commercial starch production. The principal industrial sources are maize, tapioca, potato, and wheat. However, their usage in many food industry applications is limited due to concerns like weak shear resistance, poor temperature resistance, a strong tendency to retrograde, and poor thermal breakdown (De Souza *et al.,* 2010).

The two main components of starch—amylopectin (70–75%) and amylose (25–30%)—are utilized by alpha-amylase as its principal substrate. Amylose has a molecular weight range of 1x 10^5 to 1 x10^6 Da and contains glucose monomers connected to one another by (1-4) glycosidic bands. The other polymer is called amylopectin, and it has a molecular weight between 1x 10^7 and 1x 10^9 Da. It is polymerized by (1-4) glycosidic bands and is branched by (1-6) glycosidic

bands .Rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) are the three primary categories of starch based on their digestion characteristics. The most recent RDS are employed in the gelatinized waxes and meals (Far *et al.,* 2020).

2.3. Endophytic enzymes

Microorganisms known as endophytes (fungi or bacteria) develop intracellularly or between cells and reside in higher plant tissues for at least a portion of their life cycle without exhibiting any symptoms. Endophytic bacteria, found in all known plant species, have been detected in many plant components, including leaves, stems, flowers, roots, and seeds. These endophytes dwell within plant tissues, offering protection from environmental stressors and microbial competition. Consequently, they boost the health of the host plant by directly obtaining nutrients and indirectly battling soil-borne diseases or moderating environmental stressors (Bhutani *et al.,* 2021). Endophytic bacterial communities are known as key providers of extracellular enzymes. Various endophytic bacterial strains have been isolated and characterized from a range of plants, including pea (*Pisum sativum*), tomato (*Lycopersicum esculentum*), corn (*Zea mays*), wheat (*Triticum aestivum*), oat (*Avena sativa*), canola (*Brassica napus*), barley (*Hordeum vulgare*), radish (*Raphanus sativus*), soybean (*Glycine max*), potato *(Solanum tuberosum*), lettuce *(Lactuca serriola*), and cucumber (*Cucumis sativa*) (Khan *et al.,* 2017).

Fungi have the potential to colonize either the intercellular or intracellular regions within plants, however full and widespread colonization is more typically detected in the roots rather than in the aerial leaves or stems. Colonization in aerial organs primarily relies on the host's apoplastic fluid as a nutrition supply to sustain the proper reproduction of fungal endophytes in these organs (Alam *et al.,* 2021). In the past 20 years or more, endophytic fungi isolated from various plant sources have been found to produce a variety of extracellular enzymes. The majority of endophytic fungi known to produce extracellular enzymes have been found on medicinal plants. According to reports, *Aspergillus terreus, Hymenoscyphus ericae,* and *Alternaria alternate* are among the endophytic fungi that manufacture xylanase. Similar research revealed that the endophyte *Periconia* sp. produces $^{\beta}$ glucosidase, while other studies revealed that the endophytes of *Acremonium* species produce cellulases and hemicellulases (Khan *et al.,* 2017).

Endophytic cultures are injected into wells made on quarter-strength LB agar medium with 1% substrate in order to perform enzyme screening. Enzymes such as protease, cellulase, amylase, and lipase use casein, carboxymethyl cellulose, starch, and tributyrin as substrates. Congo Red

for cellulase, Iodine Reagent for amylase, and Coomassie Brilliant Blue for protease are flooded onto the plates to verify the presence of the enzymes.

Lipase activity is assessed based on the development of clearance zones. For spectrophotometric enzyme activity quantification employing enzyme assays, selected bacteria are inoculated in suitable production conditions (Vijayalakshmi *et al.,* 2016). This is one example of the process of producing enzyme from endophyte.

Table 1 Endophytic bacterial strains producing extracellular enzymes (Khan *et al.,* 2017).

Species	Enzyme Produced	Detection Method
Actinomyces pyogenes *Bacillus circulans* *Bacillus megaterium* *Staphylococcus* sp. *Pseudomonas*	Amylase, esterase, lipase, protease	Agar medium
Bacillus sp. *Bacillus clausii* *Bacillus pumilus*	lipase, protease, cellulose, amylase	Agar medium
Pseudomonas *Micrococcus caseolyticu* *Enterobacter ludwigii* *Chryseobacterium* *Bacillus aerophilus*	cellulose, amylase, xylanase, pectinase	Agar diffusion method

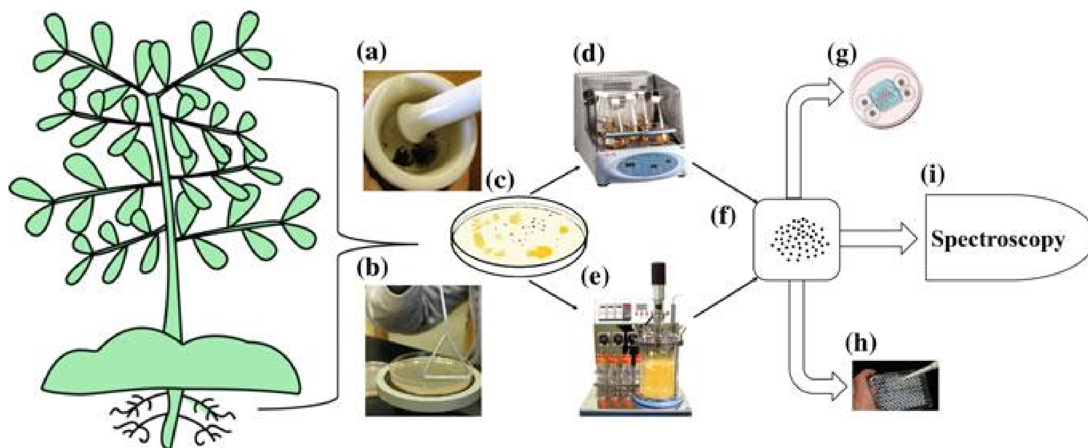

Figure 5. a) Plant crushing, b) Endophyte isolation, c) Growth in pure culture, d) The process of fermenting e, f, g, h) Enzyme extraction, and i) Assessment using refined chromatographic methods (Khan *et al.,* 2017)

2.4. Amylase enzyme

Amylases can be divided into exoamylases and endoamylases. These enzymes break down starch molecules, yielding substances like dextrins and smaller glucose polymers. Actinomycetes produce amylases to the cell surface for extracellular digestion. Starch-degrading amylolytic enzymes account for around 25% of the demand for enzymes worldwide and are used extensively in a variety of biotechnological applications, including food processing, fermentation, and the paper and textile industries (Rajagopalan, 2008). Amylases can come from microorganisms, animals, and plants. In most businesses, there is a high demand for enzymes of microbial origin. *Streptomyce*s (Reddy *et al.,* 2003), the genus regarded as an active supplier of amylases, frequently exhibits the feature of amylase presence in actinomycetes. Strains include *Streptomyces avermitilis, Streptomyces* sp. *SLBA-08, Streptomyces strain A3,* and *Streptomyces rochei BTSS 1001* are used for amylase production in industries like starch, detergent, food, and textiles. Additionally, these strains are effectively used in medical research. The breakdown of starch in industrial processes has been enhanced by the use of thermostable amylolytic enzymes. The strain of *Streptomyces gulbargensis DAS 131* that is alkali-thermotolerant has recently shown the highest extracellular amylase output (Hwang *et al.,* 2013). A marine *Saccharopolyspora sp. strain A9* that can create surfactants, oxidants, and detergent stable amylase was isolated from maritime sediments. Isolated from strain A3 is the surfactant-, detergent-, and calcium ion-independent amylase, which has several uses in the detergent and

pharmaceutical industries. Amylases possess enormous promise and find numerous uses beyond industrial processes, including therapeutic, pharmaceutical, and analytical chemistry domains. They have shown successful in starch saccharification procedures and are also applied in industries such as textiles, gastronomy, brewing, and distilling (Gupta *et al.,* 2003).

2.4.1 History of amylase enzyme

The initial observation of a starch-degrading enzyme in wheat was made by Kirchhoff in 1811. This discovery laid the foundation for the exploration and examination of amylase, marking the inception of the study of amylases in history. The reports of digestive and malt amylases that came after this were numerous. In 1833, Anselme Payen discovered the first amylase. It's worth mentioning that the first enzyme to be manufactured industrially was amylase, which came from a fungal source in 1894. This enzyme was used as a medication to treat illnesses of the digestive system (Gopinath *et al*., 2017).

Amylases are ubiquitous enzymes found in animals, fungi, plants, as well as unicellular eukaryotes, bacteria, and archaea. While both plants and animals produce amylases, those originating from microbial sources are largely exploited in industrial applications (Tiwari *et al.,* 2015). Amylases perform a critical role in breaking down starch into sugars, syrups, and dextrins. These hydrolysates are used in the synthesis of many processed food products and provide vital carbon and energy sources for fermentations (Lealem and Gashe, 1994). Amylases also occupy substantial importance in industries such as starch processing, detergent manufacturing, beverage production, and textiles. The commercial production of amylases from microbes constitutes a major share, around 25–33%, of the global enzyme market. Making commercial enzyme manufacturing economically viable frequently includes using low-cost substrates, such as agricultural byproducts, in the production medium. One of the most significant cellulosic agro-industrial byproducts, sugarcane bagasse, has seen increased focus in recent years on its effective use.

2.4.2 Sources of amylases

Microorganisms, animals, and plants can all be isolated for their α-amylase. According to Sundarram and Murthy (2014), the enzyme has been isolated from rice and barley plants. Although they can also be found in plants and animals, amylases have most frequently been observed to occur in microorganisms. In microorganisms, there are two main kinds of amylases that have been identified: GA and α-amylase. Furthermore, β-amylase, which is often derived from plants, has also been found in a few microbial sources (Aguilar *et al.,* 20000).

Microorganisms include bacteria, fungus, and certain types of algae have the ability to generate the enzyme α-amylase.The breakdown of starch into smaller, easier-to-digest carbohydrates like glucose and maltose depends on this enzyme. As part of their metabolic activities, microorganisms use α-amylase to extract energy from substrates in their environment that contain starch (Rana *et al.,* 2013).

Bacillus amyloliquefaciens, Bacillus licheniformis, and Bacillus stearothermophilus are among the bacteria that have been proven to be capable of producing large amounts of alpha-amylase, which makes them useful for industrial purposes. Some bacteria have the ability to manufacture alpha-amylase under extreme settings; for example, certain thermophilic bacteria may create alpha-amylase at increased temperatures. Due to the high temperature requirements of some starch processing phases, such as saccharification, gelatinization, and liquefaction, thermostatable alpha-amylase is useful for improving processes under severe circumstances (Far *et al.,* 2020).

2.4.3 Amylase production by endophytic bacteria

Endophytic bacteria, residing within plant tissues, are effective producers of amylase, an enzyme that hydrolyzes starch into simple sugars. This relationship supports plant growth by enhancing nutrient availability. Industrially, endophytic bacteria are exploited for amylase production due to their high enzyme yield and stability under various conditions. Optimized fermentation techniques further enhance amylase production, making it viable for applications in food, biofuel, and pharmaceutical industries (Qian *et al.,* 2022). Research indicates that endophytic bacteria can offer sustainable and efficient enzyme sources, contributing to advancements in biotechnology.

2.5. Classes of amylase enzymes

2.5.1 Alpha- amylase (α- Amylase (EC 3.2.1.1))

The α-amylase (EC 3.2.1.1, 1,4-alpha-D-glucan glucanohydrolyase) enzyme catalyzes the hydrolysis of starch, yielding diverse products such as glucose, maltose, and different forms of malto-oligosaccharides. α-amylases that primarily generate glucose and maltose find utility in sugar syrup manufacturing and alcohol fermentation processes. Conversely, those generating maltooligosaccharides are exploited in food processing applications (Rajagopalan *et al.,* 2008). Alpha-amylase, a widely distributed endoamylase, is present in different microorganisms, including both Archaea and bacteria. Its enzymatic action results in the formation of oligosaccharides with different lengths, characterized by α configuration, and α-limit dextrins, which create branching oligosaccharides (Tiwari *et al.,* 2015).

The starch processing industries heavily rely on alpha-amylases, which are essential extracellular enzymes. Alpha amylases are thermostable and work optimally at pH ranges between 2 and 12. In the liquefaction of starch, paper, food, sugar, and pharmaceutical industries, these enzymes have demonstrated value. Amylolytic enzymes find widespread usage in the food sector for manufacturing glucose syrups, high fructose corn syrups, maltose syrup, and lowering the viscosity of sugar syrups. They are also used to dissolve and saccharify starches, generate clarified fruit juice with a longer shelf life, and postpone the staling of baked goods.

Alpha-amylase can be generated by a vast array of organisms, spanning from microorganisms including aquatic bacteria, fungi, and actinomycetes to plants and animals. However, microbes serve as the principal source, creating considerable quantities of the enzyme due to their high reproduction and growth rates. Moreover, genetically altered microbes can be designed to create novel alpha-amylases with desired features such as thermostability. These bacteria can provide copious enzyme amounts, which can be further increased utilizing various strategies including response surface methodology. Among microorganisms, bacteria, actinomycetes, and fungi are often exploited for alpha-amylase synthesis. Notable bacteria such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis,* and *Bacillus stearothermophilus* are capable of producing enormous quantities of alpha-amylase, appropriate for industrial uses (Far *et al.,* 2020).

2.5.1.1 The Structure alpha amylase

Alpha-amylase features a three-dimensional structure enabling it to connect to the substrate while fostering the breakdown of glycoside bonds via specific catalytic groups. Typically, this enzyme is calcium-dependent, having a single oligosaccharide chain containing 512 amino acids, with a molecular weight of 57.6 kDa. It is present in humans as well (De Souza *et al.,* 2010).

Endo-amylases are enzymes responsible for commencing the breakdown of starch by catalyzing the hydrolysis of α-1,4-glycosidic bonds, resulting in the creation of shorter oligosaccharides. It's vital to note that alpha-amylase cannot cleave terminal glucose residues or α-1,6-linkages. The results of amylase activity include oligosaccharides of diverse lengths and configurations, along with limit dextrins, which further break down into smaller oligosaccharides such as maltose, maltotriose, and various 1,4 and 1,6 oligoglucans. While other enzymes also play a part in starch breakdown, alpha-amylase is often the enzyme that initiates the process (Hailemichael Fikadu, 2020).

Figure 6. In the shown structure of alpha-amylase, Domain A has been marked in red, Domain B in yellow, and Domain C in purple. The catalytic center includes a calcium ion represented by a blue sphere and a chloride ion shown by a yellow sphere. Furthermore, molecules bound to both the surface binding sites and the active site are indicated by green structures (De Souza *et al.,* 2010).

2.5.2. β- Amylase (E.C. 3.2.1.2)

The exo-enzyme β-amylase (EC 3.2.1.2) is recognized for its ability to specifically cleave the α-1,4 glucosidic bond of starch from its non-reducing end, resulting in the release of maltose as a byproduct. Belonging to family 14 of glycoside hydrolases, β-amylase is extensively utilized in starch processing, particularly for making maltose syrup and brewing (Duan *et al.,* 2021). It is present in fungi, bacteria, and plants, but not in humans. Unlike α-amylase, β-amylase only destroys starch from the non-reducing end of the polymer chain by hydrolyzing the second α-1,4 glycosidic bond, culminating in the generation of maltose, constituted of two glucose units. Similar to α-amylase, β-amylase is found in seeds and exploited in numerous applications such as malting. One of its principal applications is in the manufacturing of high maltose syrups. Additionally, β-amylase, together with glucoamylase, transforms the anomeric structure of released maltose from α to β. These enzymes are present in a wide spectrum of bacteria (Tiwari et al., 2015).

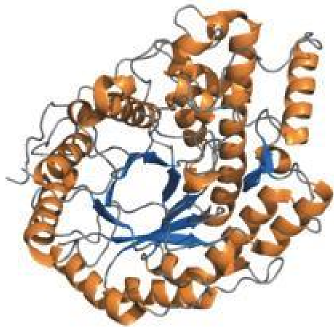

Figure 7. Structure of beta amylase

2.5.3 γ-Amylase (EC 3.2.1.3)

The enzyme γ-amylase is not as frequently used in industry. It hydrolyzes the final α-1, 4 glycosidic bond to break down starch from the non-reducing end, producing one glucose unit in the process. It also has the ability to hydrolyze α-1,6 glycosidic bonds. The pH optimum of the enzyme is lower than that of other amylases.

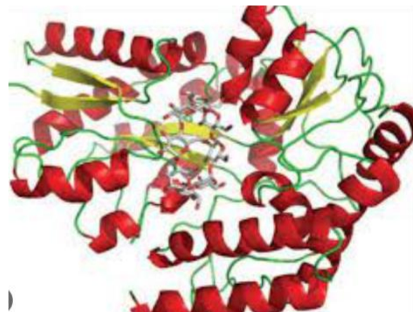

Figure 8. Structure of gamma amylase

2.6 Production of amylase by submerged fermentation

Submerged fermentation is the cultivation of microorganisms in a liquid nutrient broth. Many benefits come with submerged fermentation, such as uniform nutrient distribution throughout the culture medium, easy monitoring and control of environmental parameters like pH, temperature, and dissolved oxygen levels, and effective mass transfer of nutrients and oxygen to the growing microorganisms (Barragán *et al.,* 2016).

Since submerged fermentation (SmF) is more manageable and offers more control over environmental parameters like pH and temperature, it has long been a preferred method for producing industrially significant enzymes. For the most part, industrial enzymes have the desired property of thermostatability. Mesophilic bacteria such as *Bacillus licheniformis, Bacillus* sp. ANT-6*, Bacillus* sp. ASMIA-2*,* and *Bacillus subtilis* are known to produce thermostable α-amylases (Ozdemir *et al.,* 2011).

2.7 Purification of amylase enzyme

In the purification process of amylase, ammonium sulfate precipitation serves as an initial step for partial purification from crude extracts (Roe, 2001). Here, the steady addition of ammonium

sulfate to the protein solution leads to protein precipitation, which occurs based on the proteins' solubility at changing salt concentrations.

Through careful adjustment of salt concentration, specific proteins like amylase can be selectively precipitated while other contaminants remain in solution. Following precipitation, the protein pellet containing amylase is collected via centrifugation. Subsequently, dialysis is employed to further refine the purification (Singh *et al.,* 2014). Dialysis involves placing the partially purified amylase solution in a dialysis bag or tubing with a semi-permeable membrane immersed in a dialysis buffer. Through osmosis, small molecules, salts, and impurities diffuse out of the protein solution into the buffer, while the larger amylase molecules are retained inside the dialysis bag, resulting in a cleaner solution. Finally, SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) is utilized for molecular weight determination and protein characterization. In SDS-PAGE, denatured proteins are separated based on size by electrophoresis through a gel matrix. The resulting protein bands are visualized and compared to molecular weight markers, allowing estimation of the molecular weight of the purified amylase (Ivanova *et al.,*1993). By employing these sequential purification techniques, researchers can obtain a highly purified sample of amylase suitable for various applications or further analysis.

2.8. Industrial application of alpha amylase

Amylases have received considerable attention across many industries, especially in the food sector, due to their multiple applications. Approximately 25% of the global enzyme business is assigned to this enzyme group, showing its relevance in biotechnology (Msarah *et al.,* 2020). Alpha-amylase, the principal substrate of which is starch, comprised of amylose (25–30%) and amylopectin (70–75%), plays a vital role in different industrial processes.

In the starch business, alpha-amylases are largely utilized during starch liquefaction, where starch is transformed into fructose and glucose syrups. There are multiple steps in this enzymatic starch conversion process, including saccharification, liquefaction, and gelatinization. Liquidity is reduced and partial hydrolysis occurs during liquefaction, whereas starch granules dissolve and a thick suspension is formed during gelatinization. Saccharification further breaks down the starch into glucose and maltose (De Souza *et al.,* 2010).

Apart from the starch business, amylases find applications in various other industrial processes, including those in the food, fermentation, and pharmaceutical sectors. Their adaptability and efficacy make them useful in numerous applications, fuelling the demand for alpha-amylases in industrial settings.

2.8.1 In Starch conversion

The usual use for alpha-amylases is in the starch liquefaction process, which turns starch into syrups containing fructose and glucose. There are multiple steps in this process: saccharification (where glucose and maltose are produced by further hydrolysis), liquefaction (where partial hydrolysis and a decrease in viscosity occur), and gelatinization (where starch granules dissolve and a viscous suspension is formed) (Hwang *et al.,* 2013).

2.8.2 In Food industry

Amylases have significant application in different processed food industries, including the manufacturing of cakes, beer, fruit juices, digestive aids, and starch syrups. In the baking work, alpha-amylases are extensively exploited, particularly in bread dough, where they aid in breaking down the starch in the flour into smaller dextrins. This breakdown allows fermentation by yeast. The addition of alpha-amylase to dough accelerates fermentation rates and reduces viscosity, consequently boosting the volume and texture of the finished product. Furthermore, amylases are applied in clarifying beer, fruit juices, and animal feed before processing to promote fiber digestibility (John Jinu, 2017).

2.8.3 In fuel alcohol production

In fuel alcohol manufacturing, ethanol stands out as the most frequent liquid biofuel. Given its affordability and extensive availability as a raw material across most locations, starch emerges as the major substrate for ethanol synthesis. In this process, starch undergoes initial solubilization before going through two enzymatic steps to yield fermentable sugars. The first phases, liquidification, and saccharification include the conversion of starch into sugar, performed either through the activity of amylolytic bacteria or enzymes such as α-amylase. Subsequently, fermentation takes place, wherein the sugar is converted into ethanol by an ethanol-fermenting microbe (Pervez *et al.,* 2014).

2.8.4 In Detergent industry

The primary users of enzymes in terms of quantity and value are the detergent sector. Enzymes are introduced into detergent formulas to boost their ability to remove difficult stains and to render them environmentally friendly. Among the enzymes utilized in enzymatic detergents, amylases form a substantial class, appearing in nearly 90% of all liquid detergents (Mitidieri *et al.,* 2006). These enzymes efficiently convert leftovers from starchy foods like potatoes, gravies, custard, and chocolate into smaller oligosaccharides and dextrins, which are then used in automatic dishwashing machines and laundry detergents.

Since amylases exhibit activity at lower temperatures and an alkaline pH while maintaining essential stability under detergent conditions, their oxidative stability is a critical characteristic for their use in detergents, especially in environments where the washing conditions are highly oxidizing (Bhange *et al.,* 2016). Removing starch residues from surfaces is essential to achieving the intended whiteness effects since they can attract different types of particulate soils. Some of the amylases used in the detergent industry come from species of *Bacillus* or *Aspergillus* (Ruban et al., 2013).

2.8.5 In paper Industry

Alpha-amylases are used in the pulp and paper industry to change the starch used in paper with coatings, producing low-viscosity, high-molecular-weight starch. This change enhances the paper's capacity to write by providing a surface that is both resilient and smooth. For the purpose of paper sizing, the natural starch used in this application usually has a viscosity that is excessively high. However, this can be adjusted using batch or continual procedures that entail partial breakdown of the polymer employing alpha-amylases. Starch is not only a good covering for paper but also a great sizing agent for finishing paper, which improves the paper's quality and erasability. Sizing is the process of making paper stronger and more rigid, which enhances its overall durability and adaptability to a range of uses (De Souza *et al.,* 2010).

2.8.6 In textile industry

Amylases play a significant function in the desizing process within the textile industry. Sizing agents such as starch are often used to yarn before fabric manufacturing to promote a speedy and secure weaving process. Due of its cost, widespread availability, and ease of removal, starch is a highly favored sizing agent. Subsequently, in a wet process undertaken by the textile finishing

industry, the starch is removed from the woven cloth (Saha *et al.,* 2018). This removal of starch from the fabric, which serves as a reinforcing agent to prevent the breaking of warp threads during weaving, is referred to as desizing.Alpha-amylase produced from Bacillus strains has long been exploited in the textile industry for desizing reasons. Unlike other procedures, alpha-amylase selectively eliminates sizing chemicals without causing injury to the fibers (Rehman *et al.,* 2023).

Table 2 Industrial application of various microbial enzymes (Singh *et al.,* 2016).

Industry	Enzyme	Function	Microorganism
Dairy	Acid proteinase Neutral proteinase	Milk coagulation, faster cheese making, ripening, debittering	*Aspergillus* sp., *Bacillus subtilis*
Baking	Amylase Lipase	Flour adjustment, dough stability and conditioning, bread softness	*Aspergillus* sp., *Bacillus spp.*
Pulp and paper	Protease Amylase	Deinking, drainage conditioning, Biofilm removal	*Bacillus licheniformis* *Bacillus subtilis*
Detergent	Amylase Cellulase	Carbohydrate Stain removal	*Aspergillus* sp., *Bacillus subtilis,* *Aspergillus niger*
Cosmetics	Superoxide dismutase, Endoglycosidase	Free radical scavenging (skin care), Teeth and gum tissue care	*Corynebacterium* spp., *Glutamicum* *Lactobacillus* *Mucorhiemalis* *plantarum*
Waste management	Amylase, Amyloglucosidase	Bio-remediation of vegetable wastes, Starch hydrolysis for bio-remediatiom	*Bacillus licheniformis,* *Aspergillus niger* *Aspergillus* sp.

3. Materials and Methods

3.1 Study area

Plant samples were collected from Hawassa. Hawassa is located in Ethiopia's Great Rift Valley. It is situated on Lake Hawassa's eastern shore. Despite being right across the border in a subtropical highland environment, the area has a tropical savanna climate. There are two distinct seasons: a lengthy, albeit relatively dry, wet season that runs from March to October and a short, dry season that runs from November to February. Despite having a larger sun angle, the wet season is nevertheless considerably colder than the dry season since it contains more clouds than the later. Nonetheless, during the dry season, the mornings are normally the coldest. The city has an average temperature of 26 °C. It is 1,708 meters above sea level.

3.2 Study design

Cross–sectional and experimental study design was used for collection of the plants and screening of amylase producing endophytes respectively.

3.3 Sampling techniques and sample collection

Purposive sampling was used to select the plant samples from the study areas. Healthy leaves, roots, stems, and fruits from *Datrua*, *Solanum*, Enset, and Coffee plants were meticulously harvested using sterile scissors. These plant parts were then placed in sterile plastic bags and transported to the Microbiology laboratory of Hawassa University using an icebox to maintain their freshness and integrity during transit.

3.4 Isolation of endophytes

The plant parts were thoroughly cleaned with tap water for fifteen minutes and were cut into smaller pieces of approximately 2 – 3 cm using sterile scissors (Hung and Annapurna, 2004; Anjum & Chandra, 2015). The pieces were surface sterilized using 70% alcohol for one minute, 2.5% sodium hypochlorite for one minute, 96% ethanol for thirty seconds, and finally rinsed three times in sterile distilled water. The surface sterilized plant pieces were further crushed into smaller pieces and serially diluted from 10^{-1} to 10^{-5} in sterile saline solution by taking 1 mL from the test tubes containing homogenized plant material. Then, 0.1 mL volume was transferred into nutrient agar plates and spread plated. The plates were then incubated at 37 °C for 24 hours

(Hung and Annapurna, 2004). Distinct colonies were separately transferred into nutrient broth media and purified by repeated sub-culturing on nutrient agar. The pure colonies were stored under refrigeration conditions at 4 ^{O}C till further analysis.

3.5 Screening of isolates for amylase production on solid media

The pure colonies were cultured in two copies on starch agar plates at 37°C for 24 hours and , flooded with Lugol's iodine solution (1% iodine with 2% KI). The amylase-positive isolates showed a distinct hallo zone surrounded by a dark blue backdrop created by the starch-iodine complex and were maintained on nutrient agar plates and incubated in refrigerator till further analysis (Thippeswamy *et al.,* 2006).

3.6 Characterization of the endophytic isolates

3.6.1 Cultural characterization

The cultural characteristics of the pure colonies of the amylase-producing endophytic bacterial isolates were characterized. The isolates were grown on nutrient agar plates and incubated at 37 °C for 24-48 hours. Colony morphology, including shape, size, color, margin, elevation, and texture, was observed and recorded as described in (Boiu-Sicuia *et al.,* 2017). Additionally, Gram staining was performed to determine the Gram reaction and cell morphology of the isolates. The results were documented for further analysis.

3.6.2 Morphological and biochemical characterization

3.6.2.1. Gram staining

Smear of the pure colony of the amylase producing endophytic isolates were separately smeared onto clean microscope slide and heat fixed by passing through the flame of Bunsen burner. The slides were subsequently flooded with crystal violet , iodine solution , alcohol and finally counter stained with safranin (Beveridge TJ, 2001). The stained bacterial cells were examined under oil immersion microscope.

3.6.2.2. Catalase test

Production of catalase enzyme was detected using the method of (Reiner Karen, 2010). A few drops of 3% hydrogen peroxide on a clean microscope slide were gently mixed with a colony of 24 h old culture of the organism. Evolution of gas bubbles were considered as a positive test for catalase production.

3.6.2.3 Determination of carbohydrate utilization

Carbohydrate utilization was assessed according to Goa *et al.,* (2022). Briefly, a pure colony was picked by a sterile inoculation needle for even distribution. Then the inoculated media was incubated at 37 ℃ for 24 hours. Production of acid, gas and hydrogen sulfide was detected respectively by color change to yellow, bubble in the medium, and black precipitate at butt portion.

3.6.2.4 Determination of sulfide, indole and motility

Sulfide Indole Motility (SIM) a media was used to assess sulfide and indole production as well as the motility of the endophytic isolates. Briefly, the pure culture of each endophytic bacterium was separately inoculated into the SIM medium by stabbing with sterile inoculating needle. The tubes were then incubated at 37 ^{O}C for 24 hours (Ederer *et al.,* 1975). The change on the media such as black pricipitate and appearance of red color was indicative of sulfide and indole production. The growth of the isolates in diffuse growth pattern in the stabbed media was indicative of the motility of the organism.

3.7 Identification of the seven best amylase producing endophytic bacteria

The full-length 16S rRNA gene was amplified using the primer pair 27F: 5'-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5'-GGTTACCTTGTTACGACTT-3' under the following conditions: an initial denaturation at 94°C for 10 minutes, followed by 32 cycles of 94°C for 30 seconds, 55°C for 20 seconds, and 72°C for 55 seconds, and a final extension at 72°C for 5 minutes (Peijie *et al.,* 2024).

The total reaction volume for each tube was 25 µL. The amplicons were sequenced using the Sanger method as described by Bai *et al.,* (2023) with slight modifications. Specifically, cycle sequencing was conducted with the forward primer 27F and the reverse primer 1492R using the ABI PRISM® BigDye™ Terminator cycle sequencing kit. Electrophoresis was performed on an ABI PRISM 3730XL Sequencer platform at Beijing Sangon Biotechnology Company.

The 16S rRNA sequences of the bacterial isolates sequenced in this study were compared against the GenBank database using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) to

identify top matches. A threshold of 97% or higher sequence identity with the type strains of related bacterial species in the rRNA gene region was used for species identification.

3.8 Production of amylase enzyme by submerged fermentation

3.8.1 Growth conditions for amylase production

Two basal media were used in this study. The first medium (g/L) was composed of 10 g of starch soluble, 5 g of peptone, 0.5 g of magnesium sulfate, 0.5 g of sodium chloride, 0.15 g of calcium chloride, and 2 g of yeast extract in distilled water (Swain *et al.,* 2006). The second media (g/L) was composed of 20 g of soluble starch, 10 g of peptone, 4 g of yeast extract, 0.5 g of magnesium sulfate, and 0.2 g of calcium chloride (Elmansy *et al.,* 2018). 20 ml of medium was prepared on each 100ml conical flask and each medium inocullated with 1 mL 24 hours fresh culture then incubated on incubator shaker(Incubator Shaker, Programmable-Southern Labware) at the agitation of 150 rpm for 72 hours at 28 °C and the pH of the media was adjusted 6.8 before autoclaving. The crude product was obtained using an Eppendorf 5418 R Refrigerated Centrifuge. Following centrifugation at 5000 rpm for 15 minutes at 4 °C, the cell-free culture supernatant was used to assess amylase production capacity (Divakaran *et al.,* 2011).

3.8.2 Optimization of amylase production

3.8.2.1 Effect of carbon sources

Using a variety of carbon sources, including glucose, sucrose, lactose, and starch, the production and activity of amylase were measured (Fouda *et al.,* 2021). One percent (w/v) of each carbon source was added to the basal media in order to assess the effects of the various carbon sources. The technique described by Hasan *et al.,* (2017) was used to evaluate the production of amylase and the use of sugar. The optimal culture condition was maintained throughout the amylase production process. The negative control was defined as the amount of amylase enzyme produced in the basal medium without the addition of extra carbohydrates.

3.8.2.2 Effect of nitrogen sources

Various organic and inorganic nitrogen sources, such as tryptone, peptone, yeast extract (YE), beef extract, and $(NH_4)_2SO_4$, were added to the basal fermentation medium replacing the nitrogen sources(10 g of peptone + 4 g of yeast extract) as directed by Lal *et al.,* (2016). To

assess the impact of each nitrogen source on fermentation, 2% (w/v) of each source was added to the medium while maintaining all optimal cultural conditions. The control was the amount of amylase that was produced on the basal media without the addition of extra nitrogen sources.

3.8.2.3 Effect of temperature

An investigation was conducted to observe how variations in temperature impact the production of amylase and the activity of the enzyme. In this investigation, a temperature range of 20 °C to 50 °C was tested. The temperature range was determined using the research of Fincan and Enez, (2014) and Vyas and Sharma, (2015).The optimal cultural growth conditions needed to produce amylase remain constant throughout the experiment (Elmansy *et al.,* 2018) at the same level and to get the required temperature, the shaker incubator's (Incubator Shaker, Programmable-Southern Labware) temperature was adjusted then incubated at the revolution of 150 rpm for 72 hours.

3.8.2.4 Effect of pH

Bacterial isolates were grown in 20 mL of ideal conditions to test their tolerance to pH (Elmansy *et al.,* 2018). Using 1 M HCl or NaOH, the pH of the medium was adjusted (Hailemichael Fikadu, 2020) to 5.0–10.0 in a 100 mL conical flask. It was then incubated at 28 °C at 150 rpm for 72 hours.

3.8.2.5 Enzyme assay

Amylase activity in the cell-free supernatant (CFS) was measured using a series of processes. Initially, 0.25 mL of soluble starch (1%) and 0.5 mL of sodium phosphate buffer (0.05M, pH 7) were combined with 0.25 mL of the CFS and incubated at 37 °C for 25 minutes in a water bath. The reaction was subsequently stopped by adding 2 mL of 3,5-DNS reagent (containing of 3,5 dinitrosalicyclic acid, 1.0 g; Sodium hydroxide, 20 g; Sodium potassium tartarate, 30 g; and Distilled water, 100 mL), which promoted color development. This combination was subsequently cooked in a boiling water bath for 5 minutes. After cooling, the reaction mixture was diluted with 2 mL of water, and the absorbance was measured at 540 nm using a UV-Vis Spectrophotometer (JENWAY-6405 UV-Vis spectrophotometer) (Oliveira *et al.,* 2010; Kanimozhi *et al.,* 2014).

Subsequently, the amount of reducing sugar in the combination was measured by the dinitrosalicylic acid (DNS) technique. The activity of the enzyme was estimated using a standard

curve generated using varying concentration (mg/mL) of maltose (0.1, 0.2, 0.3, 0.4 and 0.5). The absorbance of the varied maltose concentrations and the reaction mixture was measured at 540 nm (Hailemichael Fikadu, 2020).

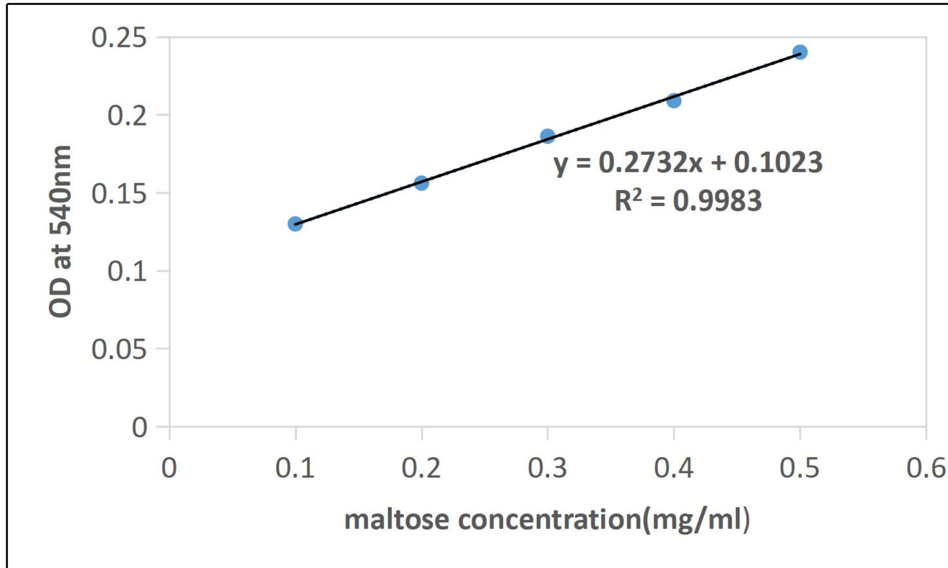

Figure 9.Standard curves to determine reducing sugars (maltose)

Activity (U/mL) = ([RS] released × RV / 342.3) × 10^3 × (1/t) × (1/EV) × F

Where[RS]released: The released reducing sugar concentration, expressed in mg / mL, is determined using the “a” of the standard curve equation (y=ax+b or y=ax => Abs=a[RS]stdcv+b or Abs=a[RS]stdcv), thus [RS]released (mg/mL)= Abs/a or [RS]released (mg/mL)=(Abs-b)/a Abs: Absorbance, RV is the reaction volume, which equals the volume of the substrate plus the volume of the diluted or undiluted enzyme solution (in mL). t is the reaction time (in minutes). EV is the volume of the enzyme solution (in mL). F is the dilution factor, if the enzyme has been diluted; otherwise, it is 1. The molar mass of glucose is 342.3 g/mol.

The activity is represented in U/mL, with 1 U (1 Unit) of amylase activity defined as the quantity of enzyme that releases 1 µmol of reducing sugar equivalent to maltose per minute under the assay conditions.

3.9. Partial purification of amylase enzyme

3.9.1. Precipitation by ammonium sulfate

Partial purification of the amylase enzyme was done using the method of Singh *et al.,* (2014) with slight modification. The raw enzyme was subjected to varying degrees of saturation (0%–30%, 30%–40%, 40%–50%, 50%–60%, and 60%–70%) using solid ammonium sulfate, which was continuously stirred overnight at room temperature. Precipitation was started by the process of salting in, which involves gradually adding ammonium sulfate ($(NH_3)_2SO_4$) to the crude enzyme solution in a conical flask while using a magnetic stirrer, continuing until the salt is fully dissolved.The precipitate was recovered using centrifugation (6000 rpm, 20 minutes) and then dissolved in 0.1M phosphate buffer (pH = 7) (Roe Simon, 2001).

3.9.2. Dialysis

Using dialysis tubing from Sigma, the pellet was reconstituted in 0.1 M sodium phosphate buffer (pH 7.0) and exposed to dialysis against the same buffer for an entire night (Matpan *et al.,* 2021). The enzyme solution was sealed within a selectively permeable membrane bag, which was then immersed in a large volume of buffer and agitated using a magnetic stirrer at room temperature. The semipermeable membrane's pores prohibit large-molecule proteins from flowing through, while enabling tiny molecules like sulfate ions and ammonium to equilibrate across the membrane until the internal and external volumes are balanced (Witazora *et al.,* 2021).

3.9.4 Partial characterization of amylase enzyme

3.9.4.1. Effect of pH on amylase activity

The effect of pH on amylase activity was assessed using the acidic or basic phosphate buffer solution method (Kiran *et al.,* 2018). The pH was lowered by adding acidic buffer solutions, which were usually made of sodium acetate, while the pH was raised by adding basic buffer solutions which was made of sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4), which were made of the same ingredients but in different ratios. With a pH meter that was calibrated, the addition of buffer solutions was done gradually while the pH was monitored and adjusted to ranges from 3 to 9.

This provided precise control over the pH changes procedure, which is essential for determining how pH affects amylase activity and production. The reaction mixture containing the substrate and enzyme source was incubated under carefully regulated conditions once the pH was adjusted. The incubation process was typically conducted at a specific temperature of 50 °C. The reaction mixture consisted of 0.1 ml of amylase, 0.25 ml of 1% starch solution, and 0.5 ml of 0.2 M sodium phosphate buffer (Kanimozhi *et al.,* 2014; Negi and Banerjee, 2009). After 25 minutes of incubation, 0.25 ml of 3, 5-dinitrosalicylic acid (DNS) reagent was added, boiling for 5 minutes, and the mixture was cooled. The absorbance was measured at 540 nm using a UV-Vis Spectrophotometer (JENWAY-6405 UV-Vis spectrophotometer) and the enzyme activity was calculated by the formula that was described previously at section 3.7.2.5.

3.9.4.2. Effect of temperature on amylase activity

The temperature effect for partially purified a-amylase activity was determined in 0.02M phosphate buffer, pH 7 by incubating at different temperatures ranging from 20°C to 60°C (Lodi *et al.*, 2023) for 25 minutes before the DNS and for 5 minutes after the DNS was added then the absorbance and enzyme activity was calculated as described by Al-Agamy *et al.,* (2021).

3.9.4.3. The Ca^{2+} in effect on amylase activity

The effect of calcium ions (Ca^{2+}) on amylase activity was assessed by adding $CaCl_2$, following the method of Shalini and Solanki, (2014). The reaction mixture included 0.1 ml of amylase, 0.25 ml of a 1% starch solution, and 0.5 ml of 0.2M sodium phosphate buffer, with varying concentrations of $CaCl_2$ ranging from 0.1 to 1.5 mM (Yuk *et al.,* 2008). The pH was adjusted to 7 using buffer solutions made of sodium dihydrogen phosphate (NaH_2PO_4) (Kiran *et al.,* 2018). The mixture was then boiled for 25 minutes at 50 °C, followed by the addition of 0.25 ml of DNS. After boiling for an additional 5 minutes, the reaction mixture was cooled, and the enzyme activity was measured.

3.9.5 Statistical analysis

The origin software and MS excel 2013 were used to analyze the experimental data.

4. Results

4.1 Isolation and screening of amylase producing endophytic bacteria

A total of 60 distinct endophytic bacteria were isolated from the four plants and screened for amylase production using starch agar media (Fig. 10). Thirty two isolates were found to produce amylase, 16 isolates with better amylase production capacity were used for further production of the enzyme under submerged fermentation conditions. Among the 16 isolates, 7 were selected based on the production capability under submerged fermentation. The seven isolates were designated as (C10R1, P10R1, P10R2, D10L1, C10L2, D10F2 and I10L2).

Table 3 Diameter of amylolytic clear zone (Mean ± SD) of the seven isolates.

Source of endophyte	***Endophyte Code***	***Clear Zone Diameter (mm)***
Coffee- leaf	C10L2	10.271 ± 0.14
Coffee- root	C10R1	5.034 ± 0.05
Datura- fruit	D10F2	11.531 ± 0.34
Datura- leaf	D10L1	9.089 ± 0.07
Potato- root	P10R2	6.999 ± 0.13
Potato- root	P10R1	7.047 ± 0.46
Enset- leaf	I10L2	4.481 ± 0.54

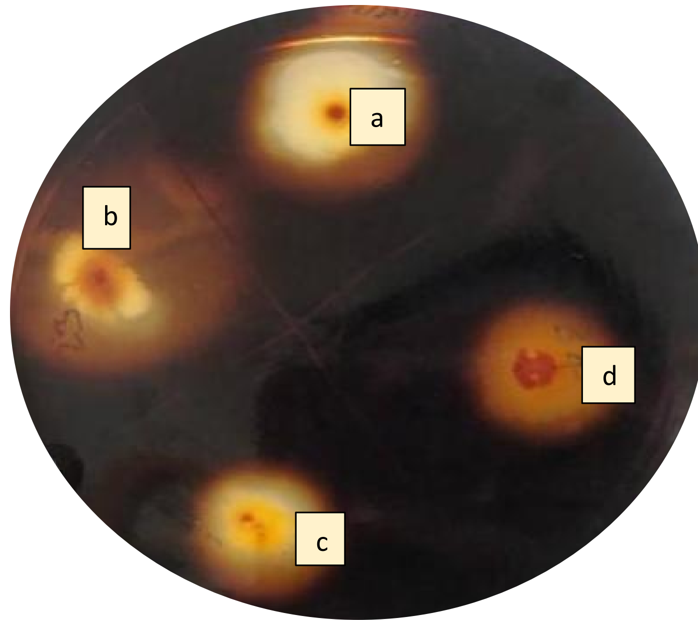

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Figure 10. Screened isolates for amylase production showed clear zone on starch agar plate
The letters a, b, c, and d represent the isolates C10L2, D10F2, P10R1 and P10R2 respectively.

Among the seven isolates, the largest clear zone (11.53 ± 0.34 mm) was recorded for isolate D10F2 and the smallest (4.481 ± 0.54 mm) was recorded for isolate I10L2 (Table 1). Endophytes from plants Datura and Coffee were better producers of the enzyme than the endophytes from Enset and Potato plant.

4.2 Characterization of amylase producing endophytic bacteria

The morphological and biochemical characteristics of the three endophytic bacteria that produced amylase enzymes better than the other isolates were characterized. All the three isolates were catalase positive, motile, with no sulfide production and used only dextrose. Isolates C10L2 and D10L1 had white colony color with entire margin, and were Gram positive endophytic bacteria. But, D10F2 is a Gram negative bacterium with yellowish flat colony that had wavy margin (Table 4).

Table 4 The morphological and biochemical characteristics of the three isolates.

Characteristics	D10F2	C10L2	D10L1
Colony size	small	large	small
Colony shape	round	circular	irregular
Color	yellow	white	white
Margin	wavy	entire	entire
Elevation	flat	raised	flat
Gram staining	+	+	+
Cell shape	rod	rod	rod
Sugar uilization	Only dextrose	Only dextrose	Only dextrose
Motility	motile	motile	motile
Sulphide production	_	_	_
Catalase	+	+	+
KOH	_	_	_

4.3 Identification of amylase producing endophytic bacteria

The seven top amylase-producing endophytic bacteria were identified, all belonging to the geneus *Bacillus* (Table 5). The bacterium isolated from the fruit of Datura (D10F2) was identified as *Bacillus subtilis* strain. The isolate from the leaf of Datura (D10L1) was identified as *Bacillus tropicus*, and the one from the leaf of coffee (C10L2) as *Bacillus tequilensis*. These endophytic bacteria produced the largest clear zones on solid starch agar media, indicating strong extracellular amylase production.

Table 5 Identification result for seven best amylase producing endophytic bacteria.

Sample ID	Species	Identity	Accession Number
C10L2	*Bacillus tequilensis strain 10b*	100	NR_104919.1
C10R1	*Bacillus proteolyticus strain MCCC 1A00365*	100	NR_157735.1
D10F2	*Bacillus subtilis strain soilG2B*	100	MT641205.1
D10L1	*Bacillus tropicus strain MCCC 1A01406*	100	NR_157736.1
I10L2	*Bacillus subtilis strain DSM 10*	100	NR_027552.1
P10R1	*Bacillus proteolyticus strain MCCC 1A00365*	100	NR_157735.1
P10R2	*Bacillus cereus strain IAM 12605*	100	NR_115526.1

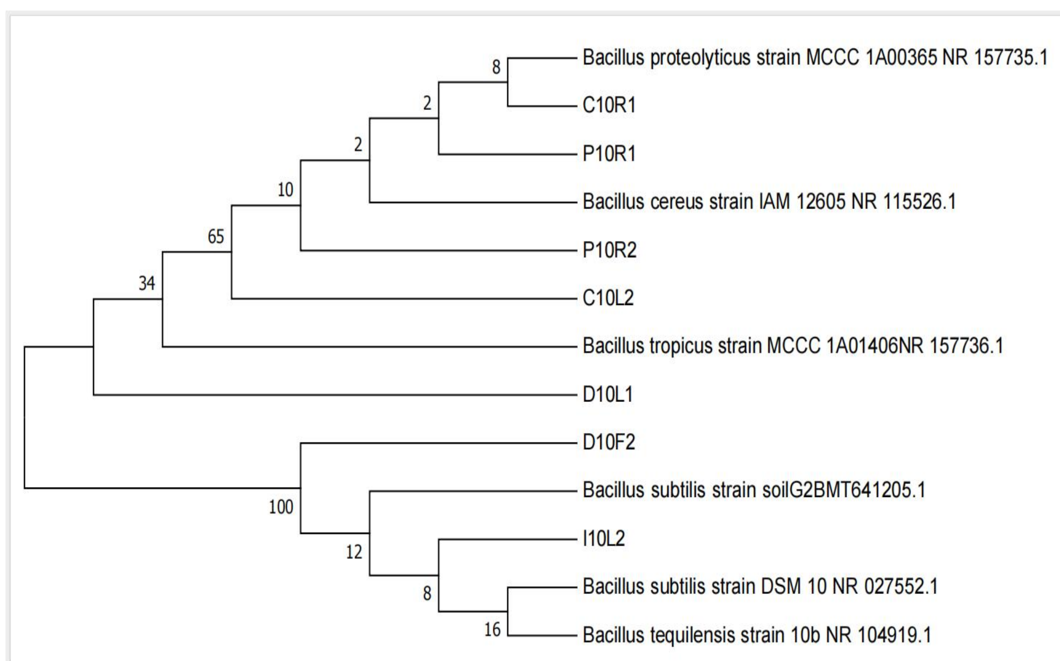

Figure 11. Evolutionary relationships of the seven isolates

4.4 Growth condition for amylase production by submerged fermentation

The best fermentation condition for amylase production was determined in the basal media consisting 1.6 grams of soluble starch, 0.8 grams of peptone, 0.32 grams of yeast extract, 0.04 grams of magnesium sulfate, and 0.016 grams of calcium chloride with pH 6.8 incubated at 28°C for 72 hours.

4.4.1 Optimization of amylase production

4.4.1.1 Effect of carbon sources on amylase production

The production of amylase varied amongst the endophytic bacterial isolates. For most of the endophytic bacteria the highest activity of amylase was recorded for the fermentation media supplemented with glucose followed by starch, and the lowest was recorded for the one supplemented with lactose. The activity of amylase enzyme from D10L1 was significantly higher ($p<0.05$) in the presence of each sugar type used in the fermentation compared to the other isolates. Statistically significant differences were not observed ($p >0.05$) in the amylase activity for most of the isolates when grown in liquid media containing starch or glucose (Figure 12). The enzyme activity between most of the isolates grown on starch and glucose containing liquid media didn't differ significantly ($p > 0.05$) (Figure 12).

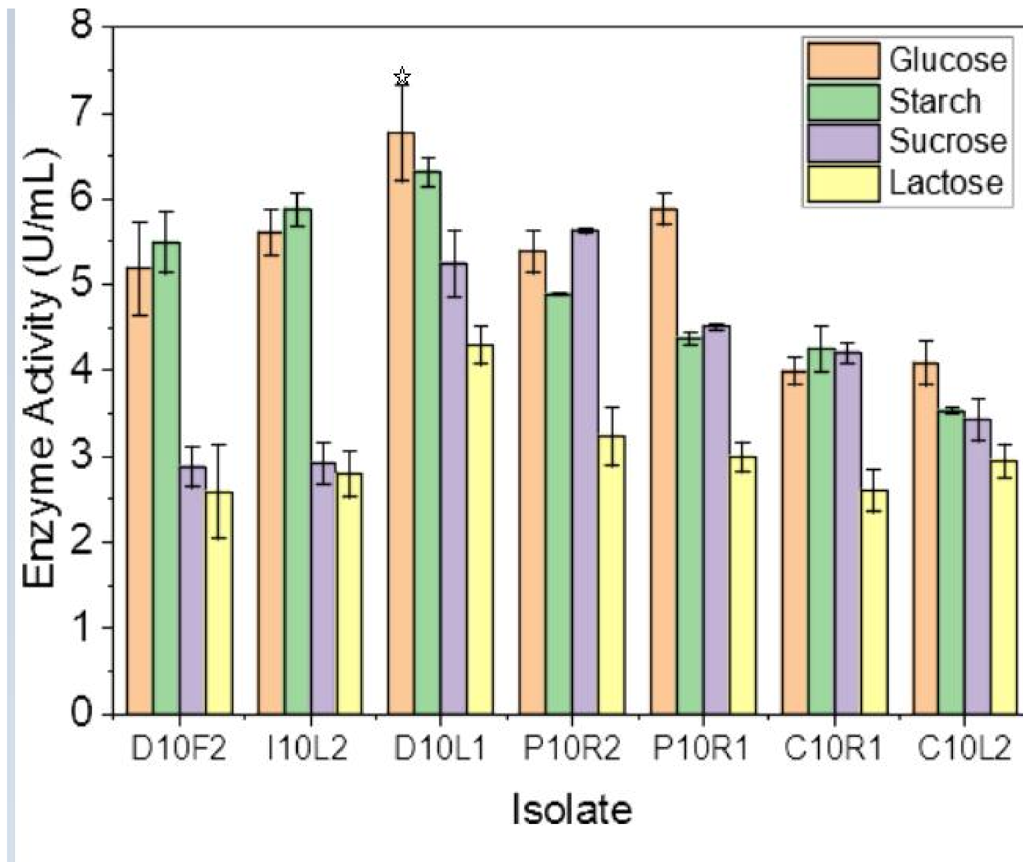

Figure 12. Effect of carbon source on amylase activity

4.4.1.2 Effect of nitrogen sources on amylase production

Production of amylase in the fermentation media differed for the nitrogen sources evaluated. In most of the cases, significantly higher ($p < 0.05$) amylase production was recorded in the presence of ammonium sulfate compared to the other nitrogen sources. Isolate D10F2, was able to produce higher concentrations of amylase compared to the other isolates for the respective nitrogen sources used in the fermentation media. The second best amylase producer was P10R1 (Figure 13).

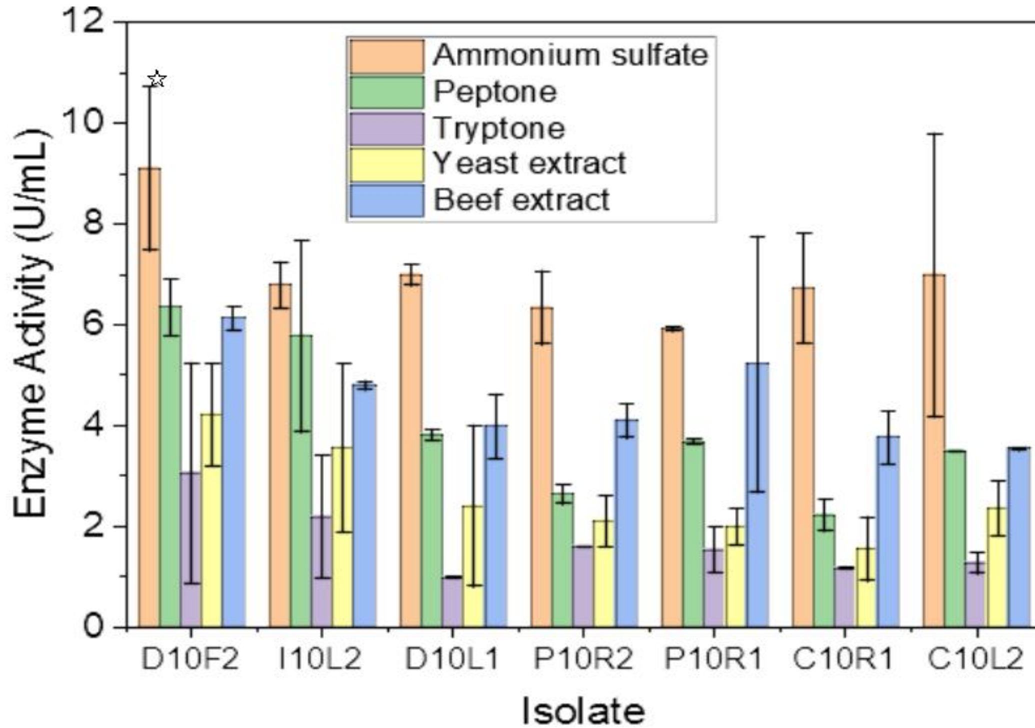

Figure 13. Effect of nitrogen sources on amylase activity

4.4.1.3 Effect of temperature on amylase production

Production of amylase enzyme was evaluated at temperatures ranging from 20 °C to 50 °C. The optimum temperature for the production of the amylase enzyme was 30 °C below and above which production of amylase decreased for all the endophytic bacteria tested. The highest concentration of enzyme was recorded for C10L2 (Figure 14).

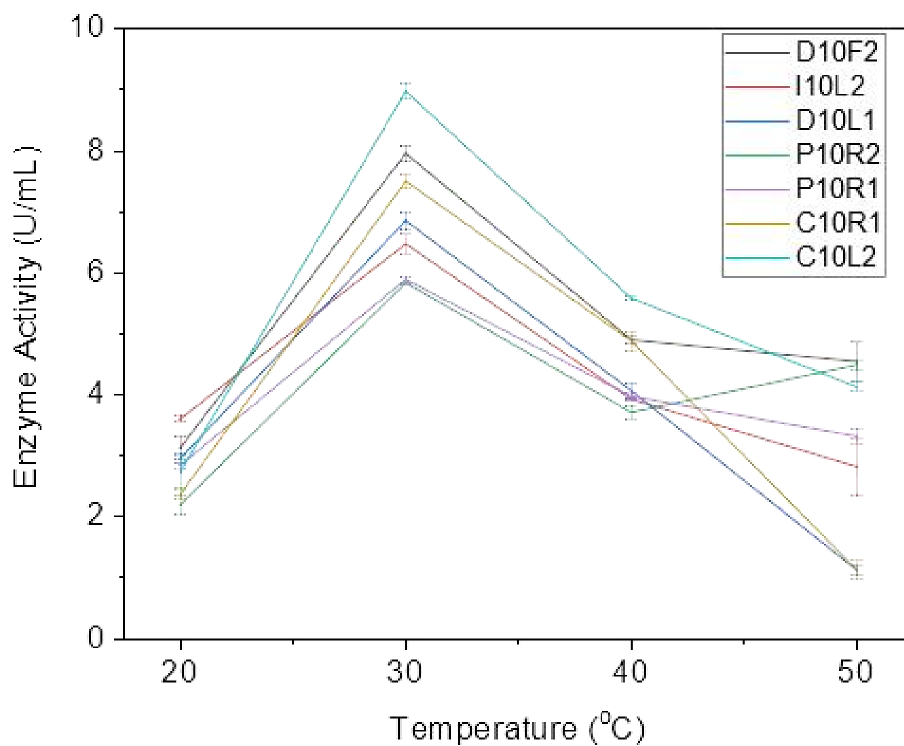

Figure 14. Effect of temperature on amylase activity

4.4.1.4 Effect of pH on amylase production

The largest level of enzyme production was reported for isolate D10L1 at pH 5.0, but a rapid reduction in the synthesis of the enzyme was detected when the pH climbed above 5. At neutral pH and pH 8 C10L2 produced the largest amount of the enzyme. For most of the isolates considerable ($p < 0.05$) reduction in the synthesis of amylase was detected above pH 8 (Figure 15).

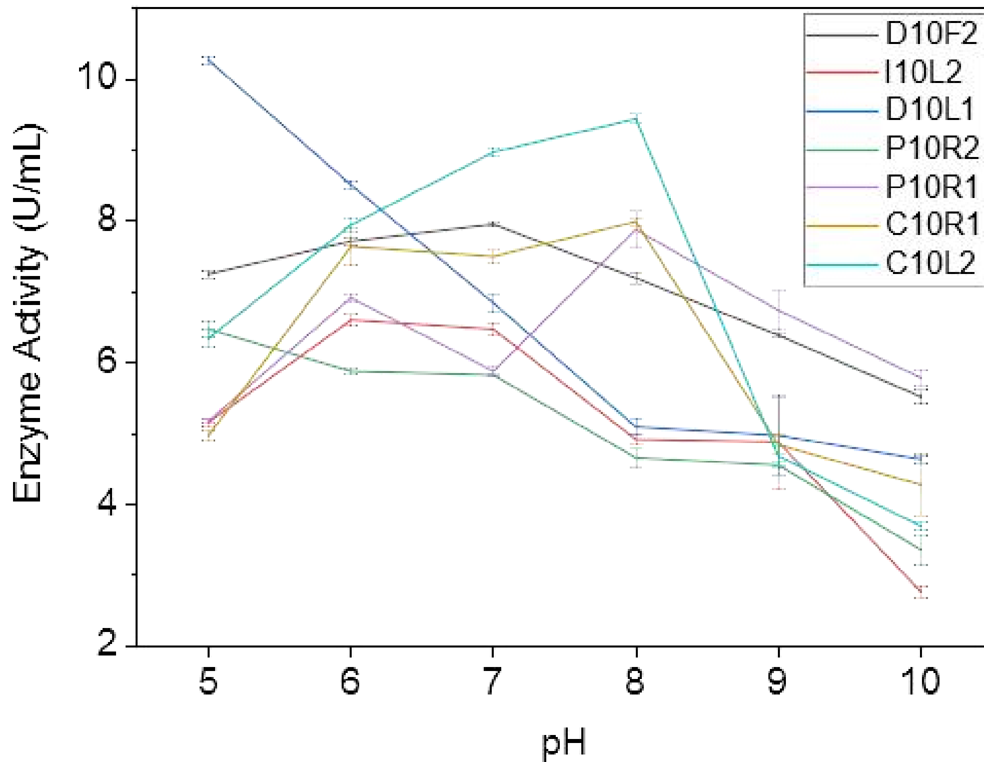

Figure 15. Effect of pH on amylase activity

4.5. Partial purification of amylase enzyme

4.5.1. Precipitation by ammonium sulfate

The three best isolates, D10F2, D10L1, and C10L2, were chosen for partial purification and characterization out of the seven. The ammonium precipitation was carried out using different degrees of precipitation; for D10F2, the precipitate was collected from the entire range (0-30% to 60%-70%); for D10L1, it was (0-30% to 50%-60%); and for C10L2, it was (0-30% to 40%-50%).

4.6. Characterization of partially purified enzyme

4.6.1. Effect of pH on the activity of partially purified enzyme

Three partially purified enzymes from D10F2, D10L1, and C10L2, were evaluated for activity at different pH. The highest activity of amylase enzyme was revealed at neutral pH (pH=7), followed by an alkaline pH (pH=9) (Figure 17).

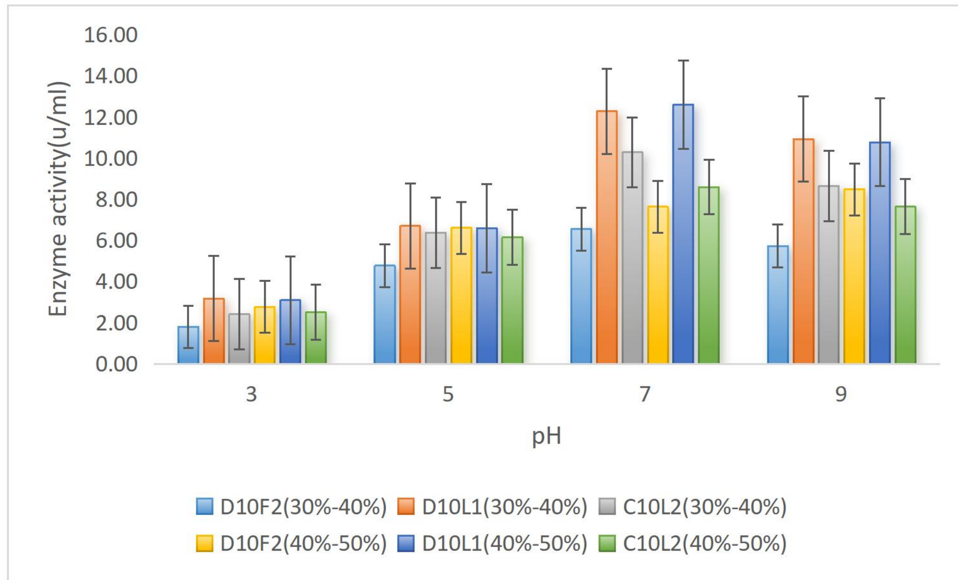

Figure 17. Enzymatic activity at varying pH after partial purification

4.6.2. Effect of temperature on the activity of partially purified enzyme

The highest activity of amylase enzyme for the three isolates (D10F2, D10L1, and C10L2) was observed at 50ºC, maintaining a pH of 7. Nonetheless, 30°C was the ideal incubation temperature for amylase production, as indicated by the highest amount of amyloid activity detected. During ammonium precipitation, D10L1 exhibited the highest activity with saturation levels of 30%–40% and 40%–50%, respectively, whereas C10L2 exhibited the next highest activity with saturation levels of 40%–50%.

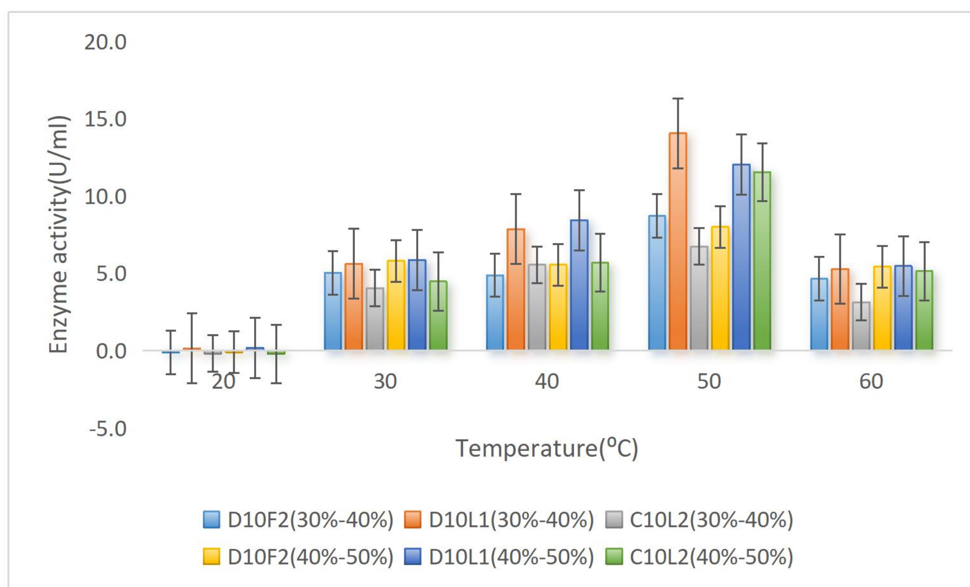

Figure 18. Enzymatic activity at varying temperature after partial purification

4.6.3. Effect of Calcium ion on the activity of partially purified enzyme

The effect of varying Ca^{2+} concentrations on amyloitic activity was investigated while maintaining a constant pH and temperature (pH=7 and 50°C). The maximum amylase activity was detected using the DNS method at 1 mM and 0.5 mM Ca^{2+} concentrations.

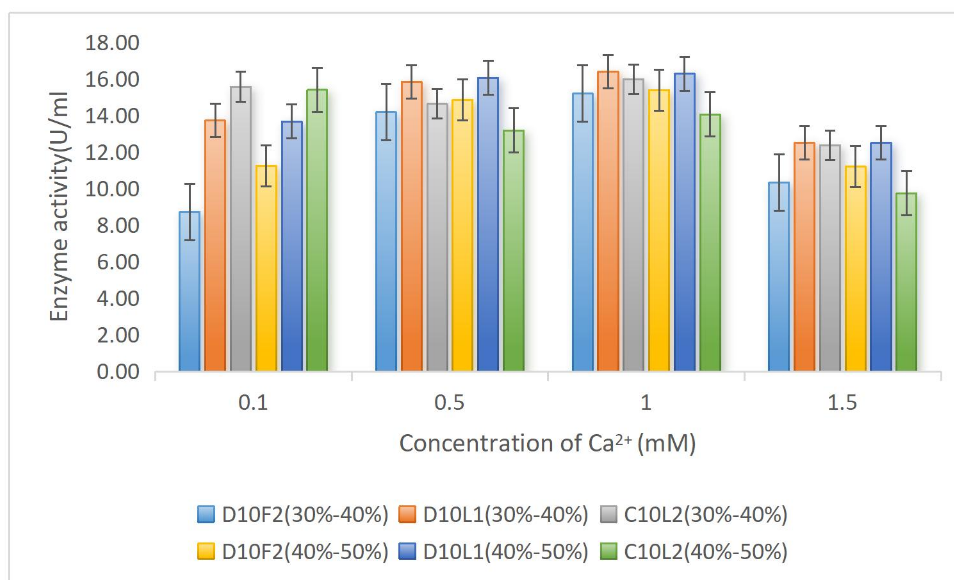

Figure 19. Enzymatic activity with Ca^{2+} after partial purification.

5. Discussion

In this study starch hydrolyzing endophytes were isolated from different plants; most of the isolates were Gram positive bacteria and rode shaped. Amylase producing bacteria were also reported form different plants (ALKahtani *et al.,* 2020; Duhan *et al.,* 2020;Hu *et al*., 2022). Starch is the substrate for amylase enzyme and is found in abundance in plants like potato and Enset (*Ensete ventriosum*) (Gebremariam and Schmidt, 1996; Grommers and Krogt, 2009). In order to grow and survive in different ecological areas bacteria produce broad range of enzymes (Wozniak *et al.,* 2023).

The evolutionary history was determined using the Neighbor-Joining method (Saitou & Nei, 1987). The resulting optimal tree has a total branch length of 0.05441258. Bootstrap analysis, conducted with 1000 replicates, shows the percentage of times each taxon clustered together, indicated next to the branches (Felsenstein, 1985). Evolutionary distances were estimated with the Kimura 2-parameter method (Kimura, 1980), measured as the number of base substitutions per site. The analysis included 13 nucleotide sequences, with all positions containing gaps or missing data removed, resulting in a dataset of 938 positions. Evolutionary analyses were performed using MEGA7 (Kumar et al., 2016).

Production of amylase in better quantities by microorganisms requires modification of growth conditions such as temperature, pH, carbon, nitrogen and mineral sources (Khusro *et al.,* 2017). In account of this, variability in the amylase production was noted for endophytes in the current study as different carbon sources were used (Figure 12). Amylase production in the current study didn't significantly differ when the media were supplemented with starch or glucose. In contrast, significantly higher amylase production was recorded in the presence of starch than glucose (Konsula and Liakopoulou-Kyriakides, 2004). The best and least production of amylase in the presence of glucose and lactose respectively was in agreement with reports from other studies (Krishnakumar *et al*., 2015; Abel-Nabey and Farag 2016). Production of amylase in the presence of maltose was also in agreement with another report (Abel-Nabey and Farag 2016). According toSreekanth *et al.,* (2013), the type of carbon source in the growth medium significantly affects amylase production. Different carbon sources can influence the metabolic pathways and enzyme synthesis rates of microorganisms. They found that certain carbon sources, such as glucose, were

particularly effective in promoting higher levels of amylase production. This is likely because glucose provides an easily accessible energy source, which can enhance microbial growth and enzyme synthesis.

Supplementing the medium with both organic and inorganic nitrogen sources had varied effects on amylase enzyme synthesis. When using $(NH_4)_2SO_4$ as an inorganic nitrogen source, amylase exhibited the highest activity, a finding consistent with other reports (Salman *et al.,* 2016; Abo-Kamer *et al*., 2023). Among the nitrogen sources, beef extract showed the second highest result overall and the highest among organic nitrogen sources, which aligns with the findings of (Simair *et al.,* 2017). Tryptone, however, resulted in the least amylase activity for all isolates. In contrast, Deb *et al.,* (2013) reported that, tryptone was an effective nitrogen source for amylase production. Bacteria often prefer inorganic nitrogen sources because they are readily assimilable, requiring less energy for conversion into amino acids and other cellular components compared to organic nitrogen sources. This preference can lead to more efficient growth and enzyme production, such as amylase. For instance, ammonium sulfate, a commonly preferred inorganic nitrogen source, provides a readily available form of nitrogen that can be directly incorporated into metabolic processes (Abo-Kamer *et al.,* 2023).

The highest amylase production for the endophytic bacteria under submerged fermentation conditions occurred at 30 ^{O}C (Figure 14). Similarly, 30 ^{O}C was reported as the optimum temperature for amylase production (Abd-Elhalem *et al.,* 2015). In the town where the samples were collected, the average temperature is 26.5°C. The adaptation of endophyte within the plant to the specific environmental temperature might be the reason enzyme production was the highest around 30°C.

The synthesis of enzymes, including amylase, is significantly influenced by the pH of the medium and differences in amylase production were observed between different isolates (Figure 15). The result is in agreement with previous studies (Ashwini *et al.,* 2011;Arekemase *et al.,* 2020;Abo-Kamer *et al*., 2023; Sobia *et al.,* 2023). The growth of microorganisms and the stability and activity of the enzyme can both be impacted by pH (Archana and Satyanarayana,

2012). The differences in amylase production at different pH values might be due to differences in the organisms, plants species and agro-ecological conditions.

The activity of the isolated enzyme was assessed based on three parameters: pH, temperature, and the effect of Ca^{2+} ions. The pH values of 3, 5, 7, and 9 were tested using six samples (D10F2, D10L1, and C10L2) from the 30%-40% and 40%-50% partially purified enzyme fractions. The highest enzyme activity was observed at pH 7, aligning with other findings (Ulya *et al.,* 2019), pH 6-7, Yasser *et al.,* (2013) for amylase from different species of bacteria.. Better activity of the enzyme at pH 8 was also reported by Rathour *et al.,* (2020) for another species of bacteria. Conversely, amylase activity decreased significantly in acidic conditions, indicating the enzyme's sensitivity to low pH, consistent with observations by Kuddus and Ahmad, (2012). This shows differences in pH sensitivity of enzymes from different microorganisms and the level of purity of the enzyme could also affect its optimum activity.

The effect of temperature on the activity of partially purified enzyme was investigated within the range of 20–60 °C. Enzyme activity increased steadily from 20°C, peaking at 50°C. This finding aligns with the findings of Tang-um and Niamsup, (2012), who reported optimal amylase activity between 50 °C and 60 °C from endophytic bacteria. On the other hands, Zakir *et al.,*(2005) noted an optimal temperature of 40 °C for amylase from another species of bacteria. These results indicate a need for further investigation into enzyme stability.

Maximum enzyme activity was observed at calcium concentrations of 0.5-1 mM, while the lowest activity occurred at 1.5 mM. Amylases require calcium ions for structural stability and catalytic function (Deb *et al.,* 2013; *Hu et al.,* 2022). Optimal concentrations of Ca^{2+} enhance amylase activity by stabilizing its structure, thereby improving its interaction with the substrate. This study has shown that amylase activity peaks at specific Ca^{2+} concentrations, typically around 1 mM, while higher concentrations can lead to a decline in activity due to structural disruption or competitive inhibition with other ions (Gupta *et al.,* 2003). Thus, understanding and optimizing ion concentrations are crucial for maximizing enzymatic efficiency in industrial and biotechnological applications.

6 . Conclusion and Recommendation

6.1. Conclusion

Amylase producing endophytes were isolated from different plants. It was found that isolate from Datura plant had shown the highest amylolytic activity compared to those from other plants. The best carbon and nitrogen sources were glucose and starch and ammonium sulphate and beef extract. The pH for amylase production varied from isolate to isolate. When measuring amylase activity using the DNS approach, partially purified amylase enzyme showed better results than crude enzyme. The maximum activity of the isolated enzyme was seen at 50ºC and pH 7 when 1 mM Ca^{2+} was present. These findings highlight the potential industrial uses of endophytic microbial enzymes in many biotechnological processes, especially alpha-amylase.

6.2. Recommendation

These isolates have encouraging potential for use as alpha-amylase production candidates. Further optimization, molecular identification of the isolates and scale up of production conditions for the promising isolates is important.

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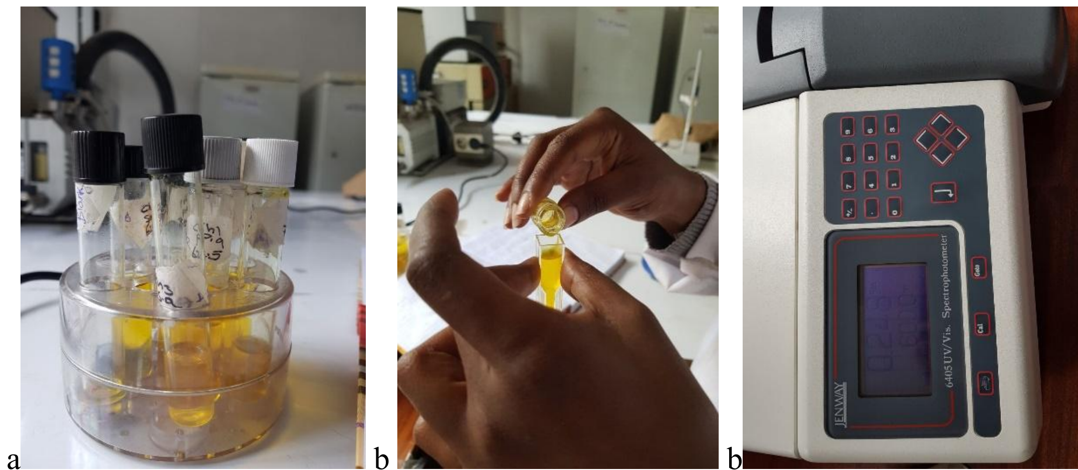
Appendices

Appendix 1.Pictures taken during plant sample collection from the study site

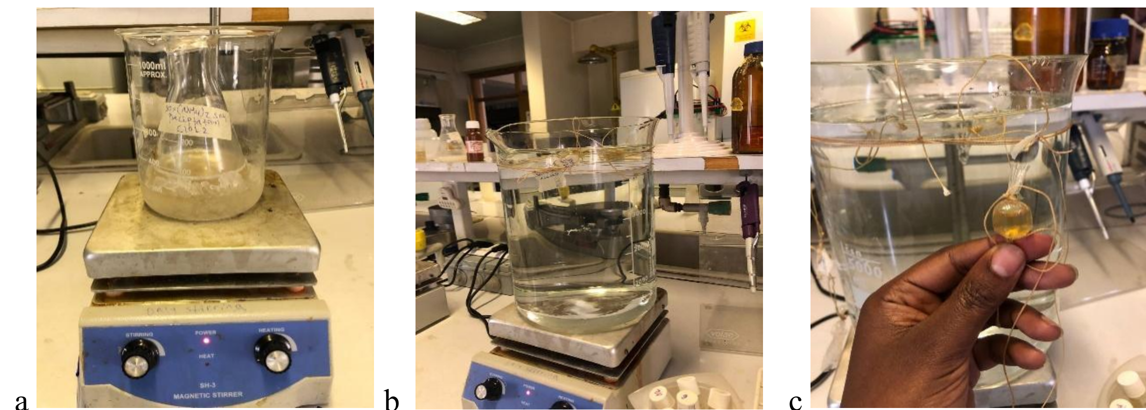
Appendix 2. Collection Details of Plant Samples Used for Endophyte Isolation

Local name	Scientific name	Temperature	Elevation(meters)	Date
Etsefaris	*Datura stramonium*	19	1693.34	01/02/23
Dinich	*Solanum tuberosum*	19	1703.52	01/02/23
Enset	*Ensete ventricosum*	21	1701.16	03/02/23
Buna	*Coffea Arabica*	21	1710.62	03/02/23



Appendix 3. Give this title

OD measurement using JENWAY- 6405 UV-Vis spectrophotometer. A. is the sample prepared in DNS method. B. is transferring the sample to cuvette and c. is the JENWAY- 6405 UV-Vis spectrophotometer.



Appendix 4. The partial purification process.

a. ammonium precipitation b. the dialysis against the given buffer and c the dialysis tube containing the enzyme sample.