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Efficient Extraction and Optimization of Amylase and Protease from *Bacillus* Species: A Comprehensive Study

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ABSTRACT

This review paper provides a comprehensive analysis of the crucial role of *Bacillus* species in the production of amylase and protease enzymes. *Bacillus* is one of the most important bacterial sources of enzymes, known for producing high yields of neutral and alkaline amylases and proteases. By breaking down complex starches and proteins into simpler, functional components, these enzymes play a critical role in enhancing efficiency and product quality across various industries, including food processing and textiles. These enzymes exhibit remarkable stability even under extreme conditions, such as high temperatures, fluctuating pH levels, and exposure to organic solvents, detergents, and oxidizing agents. Some *Bacillus* strains are highly efficient at secreting large quantities of extracellular enzymes, making them essential for industrial enzyme production. The primary objective of this review is to examine the processes involved in the production, purification, and characterization of amylases and proteases from *Bacillus* species, with a particular focus on those found in the rhizosphere. The first section of the paper discusses the properties and functions of these exoenzymes and highlights key factors that influence their optimization. The second section explores the use of cost-effective substrates for fermentation, aiming to enhance enzyme activity and stability. Given the extensive applications of amylases and proteases in various sectors, significant research has been dedicated to improving their production and efficiency. This review aims to advance our understanding of microbial enzyme systems and support the development of enhanced enzyme production strategies for emerging biotechnological applications.

KEYWORDS: *Bacillus*, amylase, protease, rhizosphere, fermentation substrates, enzymes

INTRODUCTION

Soil is the unconsolidated material on the earth's surface consisting of disintegrated rock and humus which is marked by different physical and chemical properties such as water holding capacity, pH, temperature, moisture, soil atmosphere, etc. which altogether create a heterogeneous environment for the growth and sustenance of life including microorganisms. Soil and the tiny organisms living in it are shaped by many connected factors, varying from place to place. That's why each environment has its unique mix of microbes. For instance, forest soil that's moist and full of dead leaves will have different microbes than farm soil that's often plowed and treated with chemicals (Balser *et al.*, 2010). The rhizosphere serves as an active hotspot for soil microbiota due to the stimulatory effects of root exudates and activities. Enzyme activity in both the rhizosphere and surrounding bulk soil is shaped by contributions from root cells, decaying root matter, microbial and macrofaunal remains, as well as free or soil-bound extracellular enzymes (Jones *et al.*, 2004; Nannipieri *et al.*, 2017). Enzymes are known for their specificity, high efficiency, and ability to work under mild conditions and provide a promising solution to combat challenges (Boominadhan *et al.*, 2009; Saeed *et al.*,

2025). Major sources of enzymes are living organisms, including plants, animals, and microorganisms. Microbial enzymes account for the major volume, however, less than 50 species are used to produce the microbial enzymes (Tejnecký *et al.*, 2025). Enzyme activity in the rhizosphere can be of intracellular origin, released after microbial cell disruption or root cell sloughing (Farrar *et al.*, 2003), and may be associated with soil colloids and cell debris, or they may be actively secreted by plant roots or root-associated microorganisms. Rhizosphere microorganisms release extracellular enzymes for the initial degradation of high molecular polymers such as cellulose, chitin, lignin, and proteins, leading to the mineralization of various minerals including nitrogen, phosphorus, and sulphur (Devi *et al.*, 2025). Amylase is a degrading enzyme that aids in the breakdown of carbohydrates by breaking the bonds between sugar molecules in polysaccharides through a hydrolysis reaction. It can be found in animals, plants, and bacteria. Amylases are classified into two classes, endoamylase (α -amylase) and exoamylases (glucoamylase) (Osunde *et al.*). Amylase can be classified into three types: α -amylase, β -amylase, and γ -amylase. Many microorganisms have the potential to produce amylases including *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*,

Lactobacillus spp, *Escherichia coli*, *Pseudomonas* spp, and *Streptomyces* spp (Syarifah et al., 2025). Protease (also called peptidase or proteinase) is an enzyme that performs the proteolysis, protein catabolism by the hydrolysis of the peptide bonds. It can be found in animals, plants, fungi, bacteria, archaea, and actinomycetes. Proteases can be classified into many types: serine proteases, cysteine proteases, aspartic proteases, threonine proteases, glutamic proteases, metalloproteases, and asparagine peptide lyases. Microbial sources have occupied an invincible domain in the production of all three—acidic, neutral, and alkaline—major types of proteases. Alkaline proteases, an important group of industrial enzymes, are produced by a wide range of organisms including animals, fungi, and bacteria. *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Halomonas*, *Pseudomonas*, and *Serratia* are the major bacterial genera that contribute to proteases (Jisha et al., 2013). Alkaline proteases produced by *Bacillus* remain stable under high temperatures and pH levels. As a result, there is a strong focus on developing high-performance alkaline proteases for commercial applications, particularly in detergents (Levine, 1975; Negi et al., 2025)

Bacillaceae Family: One of the earliest bacteria to be described was —*Vibrio subtilis* by Ehrenberg in 1835 (Petit et al., 2024; Xu & Kovács, 2024). In 1872, Cohn renamed the organism *Bacillus subtilis* (Wang et al., 2024). That organism was an early member of a diverse genus established by Cohn, belonging to the *Bacillaceae* family. A key characteristic of this family is its ability to produce endospores, highly refractile structures that can be round, oval, or cylindrical, forming within bacterial cells. *Bacillaceae* primarily consists of aerobic or facultatively anaerobic chemoorganotrophic rod-shaped bacteria with a typical Gram-positive cell wall (Sandler & Keynan, 1988). Major taxonomic changes have taken place, and consequently, the family now accommodates representatives of the genus *Bacillus* and other newly formed genera with related nomenclatures such as *Paenibacillus*, *Geobacillus*, and *Halobacillus*. The most effective approach to *Bacillus* taxonomy may be the analysis of 16S rRNA molecules by oligonucleotide sequencing (Acharya & Subedi). The genus *Bacillus* has long attracted scientific interest due to its unique characteristics, including the exceptional resistance of its spores to chemical and physical agents, its developmental cycle, widespread presence, and the pathogenicity of *B. anthracis*. Since the early days of bacterial research, and consistently since 1913, the presence of endospores has been a primary trait used in bacterial classification (Andersen & Doherty, 2005; Kochi, 1994).

The Genus Bacillus: The genus *Bacillus* is widely recognized as a group of aerobic, spore-forming bacteria. Ubiquitous in nature, these microorganisms are found in diverse environments, including soil, dust, air, and water. Of the approximately 70 known *Bacillus* species, most are predominantly present in soil and

water and are commonly encountered as airborne contaminants in medical laboratories. (Claus & Berkeley, 1986; Mathur & Mukherjee, 2025; Septian et al., 2025). Most *Bacillus* species are saprophytic and encompass a wide range of physiological adaptations, including thermophilic, psychrophilic, acidophilic, alkaliphilic, halotolerant, and halophilic representatives. These bacteria can thrive under extreme conditions of temperature, pH, and salinity, where few other organisms can survive. (Abd-Elgawad, 2024; Boyno et al., 2024). The physiological diversity of *Bacillus* is remarkable, encompassing species capable of degrading a wide array of plant and animal-derived substrates, including cellulose, starch, proteins, agar, and hydrocarbons. Additionally, members of this genus include antibiotic producers, heterotrophic nitrifiers, denitrifiers, nitrogen fixers, iron precipitators, selenium oxidizers, manganese oxidizers and reducers, facultative chemolithotrophs, acidophiles, alkaliphiles, psychrophiles, and thermophiles. As spore-forming members of the zymogenous soil flora, *Bacillus* species are considered opportunistic. When exposed to suitable germinant and substrates, they actively participate in and contribute to the diverse microhabitats within the soil's heterogeneous ecosystem. This diversity is evident even through classical phenotypic characterizations based on morphology particularly the size and position of endospores within the vegetative cell as well as nutritional requirements. (Sandler & Keynan, 1988; Schäfer et al., 2000; Webb et al., 1997).

Bacillus and Soil: Bacteria represent the most dominant and diverse group of microorganisms, thriving across a vast array of ecological niches, including extreme environments within the lithosphere and hydrosphere. Their metabolic versatility is essential for geochemical nutrient cycling and the synthesis of numerous industrially significant compounds. The gram-positive bacteria play a crucial role in soil microbiota. (Choudhary & Johri, 2009). The term *Bacillus* and related classifications are often used operationally to describe these organisms, with *Bacillus* being the most abundant genus in the rhizosphere. A study evaluated the impact of a *Bacillus* spp. rhizobacteria consortium on the early developmental stages of two micro-propagated banana cultivars. The findings suggest that this bacterial consortium holds promise as a viable strategy for enhancing plant health and improving survival rates in commercial nurseries (Jaizme-Vega et al., 2003; Kapoor et al., 2008). The *Bacillus* species are extensively found in various environmental habitats, likely due to the long-lasting survival of their endospores (Figure. 1). The case of the insect-pathogenic *B. thuringiensis* exemplifies the ubiquity of *Bacillus* spp. Additionally, bacilli like *B. subtilis*, *B. cereus*, and *B. mycoides* are recognized as beneficial rhizobacteria that enhance plant growth as biofertilizers or safeguard plants against pathogens,

with *B. subtilis* also serving as a biopesticide (Outtrup & Jørgensen, 2002).

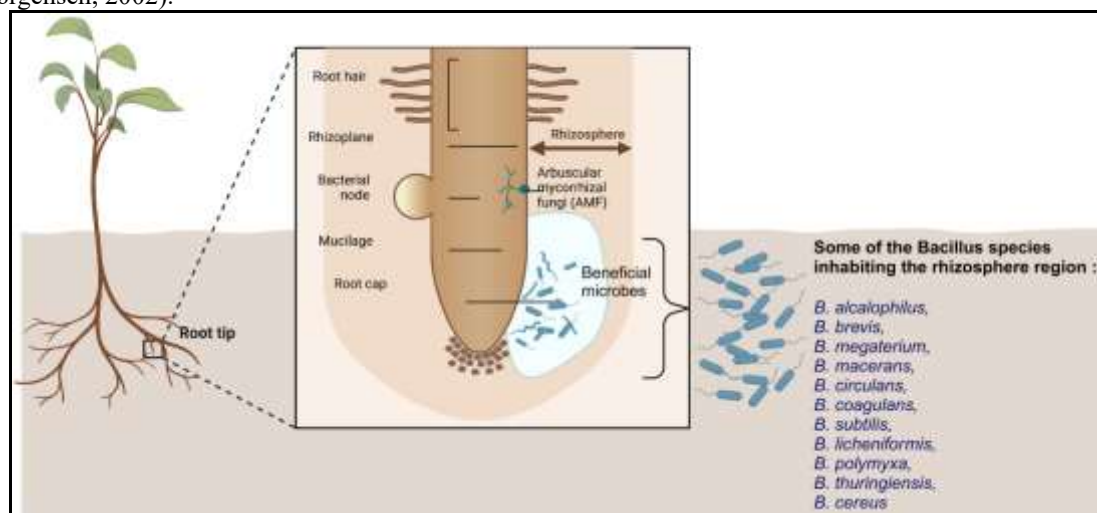


Figure 1. Illustration of the rhizosphere and beneficial microbes associated with plant roots. Beneficial microbes, such as *Bacillus* species and arbuscular mycorrhizal fungi (AMF), play a crucial role in plant health and growth. A list of *Bacillus* species commonly found in the rhizosphere are also provided.

The importance of *Bacillus* in Enzyme Production and Industrial Applications: The Gram-positive bacteria form an important part of the microbiota in many soils. The *Bacilli* plays a crucial role in the decomposition of plant-derived materials, humus, pesticides, and hydrocarbons in soil. Additionally, *Bacillus* and related genera are widely utilized for producing a diverse range of valuable products in medical, agricultural, pharmaceutical, and industrial fields (Su *et al.*, 2020). These include antibiotics, enzymes, amino acids, and sugars. Each strain can generate multiple enzymes involved in hydrolysis, oxidation-reduction, and metabolism (Figure. 2). Among these, amylases, lipase, protease, and cellulase are particularly significant, finding applications in industries such as food, pharmaceuticals, textiles,

paper, and leather. The world market for industrial enzymes is estimated to be US\$ 1.6 billion, split between food enzymes (29%), feed enzymes (15%), and general technical enzymes (56%) (Devi *et al.*, 2010). It is estimated that *Bacillus* spp., enzymes make up about 50% of the total enzyme market (Lyngwi & Joshi, 2014; Radhakrishnan *et al.*, 2017). Two *Bacillus* strains, *Bacillus subtilis* MA9 and *Bacillus cereus* MK8, were isolated from the eastern Himalayan region and have been identified as producers of thermostable α -amylase enzymes (Cercignani *et al.*, 1974; Rashid *et al.*, 2004). Most members of the genus *Bacillus* can produce low molecular weight antibiotics, which possess different biological activities, including antimicrobial, antiviral, and antitumor activities (Priest, 1977).

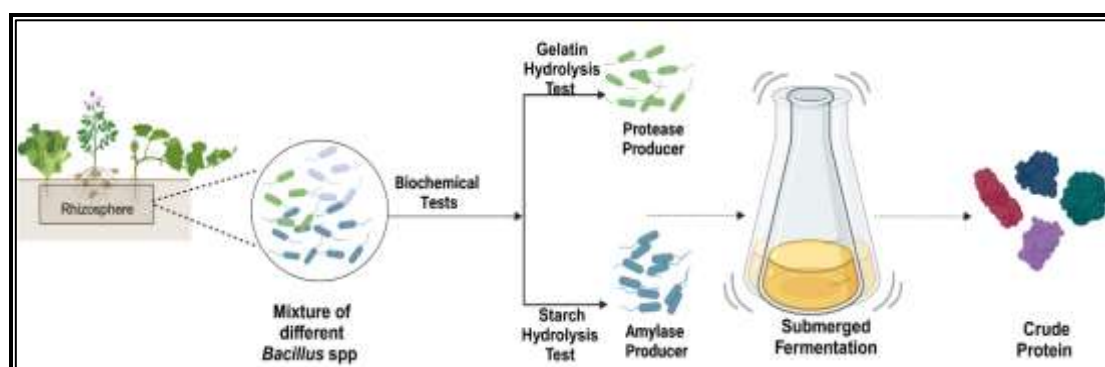


Figure 2: Schematic representation of the isolation and screening process of rhizospheric *Bacillus* spp. for enzyme production. Soil samples from the rhizosphere were used to isolate diverse *Bacillus* strains, which were then subjected to biochemical assays—gelatin hydrolysis for protease activity and starch hydrolysis for amylase activity. Enzyme-producing isolates are subsequently cultured under submerged fermentation conditions to obtain crude protein extracts (Created with BioRender.com).

Enzyme Cellular Location: Enzymes may be membrane-bound in young cells and released as exoenzymes as the culture enters the stationary phase (Levine, 1975) or solubilized by relatively mild

procedures including washing the cells with water or concentrated salt solution (Sargent & Lampen, 1970). Enzymes produced by bacilli are often referred to as "periplasmic" because their laboratory extraction

methods are like those used for isolating periplasmic enzymes from Gram-negative bacteria. Among these, amylases and proteases are the most widespread. Based on enzyme production, *Bergey's Manual of Determinative Microbiology* lists 48 *Bacillus* (Nagata et al., 1974). Exoenzymes such as amylase and protease are both membrane-bound and truly extracellular molecules, the remaining cell-bound enzymes, 50% are tightly associated with the cytoplasmic membrane, and 50% are associated with periplasmic vesicles (Coleman & Elliott, 1962). Similarly, cell-bound amylases were found in various strains, including *B. subtilis*, *B. natto*, *B. amyloliquefaciens*, and *B. subtilis* var. *amylosacchariticus* (Ogino et al., 2008). The protease is found intracellularly located in various species of *Bacillus*, more precisely in the *Bacillus amyloliquefaciens* (Reed Rodrigues Coelho & Drozdowicz, 1978). Proteases extracted from the *Bacillus* can be divided into alkalophilic protease, aminopeptidase, esterase, halophilic protease, metal protease, and serine protease.

Protease: Proteolytic enzymes play a crucial role in digestion by breaking down peptide bonds in dietary proteins, facilitating the release of essential amino acids required for various physiological functions (Gupta et al., 2002). The medical significance of these enzymes is underscored by clinical studies demonstrating their therapeutic potential in oncology, inflammatory disorders, blood rheology regulation, and immune modulation. Additionally, they contribute to targeting protein-based organisms, including parasites, fungi, and bacteria. Proteases are widely distributed in nature, with microbes serving as a preferred source due to their rapid growth, minimal spatial requirements for cultivation, and the ease of genetic manipulation to engineer novel enzymes with enhanced properties suited for various biomedical and industrial applications (Yousten & Nelson, 1976).

Microbial proteases have been divided into four groups based on the mechanism of action. Proteases are classified into four main types: (i) serine proteases, (ii) metal-dependent or metal chelator-sensitive proteases, (iii) thiol proteases, and (iv) acid proteases. In the *Bacillus* genus, nearly all extracellular proteases belong to either the serine or metal-dependent categories (Priest, 1977). The serine proteases can be divided into two groups based on their general properties. Serine proteases in *Bacillus* are categorized into two groups: Group A, represented by enzymes from *B. licheniformis* and *B. pumilus*, and Group B, which includes proteases related to *B. subtilis*, such as those from *B. amyloliquefaciens*. In contrast, species like *B. megaterium*, *B. polymyxa*, *B. thermoproteolyticus*, and *B. thuringiensis* exclusively secrete metal-dependent proteases. Several *Bacillus* species known for protease production include *B. cereus*, *B. stearothermophilus*, *B. mojavensis*, *B. megaterium*, and *B. subtilis* (Chahal, 1983; Vijayalakshmi et al., 2013).

Amylase: Amylases are essential starch-degrading enzymes that break down internal α -1,4-D-glycosidic bonds in polysaccharides while retaining the anomeric configuration of the products. Most amylases function as metalloenzymes, requiring calcium ions (Ca^{2+}) for their activity, structural integrity, and stability. As part of the hydrolase enzyme group, amylases play a crucial role in various industrial applications, including the conversion of starch into sugar syrups and the production of cyclodextrins for pharmaceutical use. These enzymes contribute to approximately 30% of global enzyme production (Boominadhan et al., 2009). Microorganisms produce different kinds of industrial enzymes. Due to their biochemical diversity and the ease of environmental and genetic modification, these enzymes have replaced those traditionally derived from complex eukaryotic organisms (Duckworth, 1996).

Amylase enzymes are categorized into four main groups: endoamylases, exoamylases, debranching enzymes, and transferases. Endoamylases break internal α -1,4 bonds, producing an anomeric product, while exoamylases cleave α -1,4 or α -1,6 bonds at the ends of glucose residues, also yielding anomeric products. Debranching enzymes specifically hydrolyze α -1,6 bonds, leaving behind long polysaccharide chains. Transferases, on the other hand, cut α -1,4 glycosidic bonds in donor molecules and transfer a portion to a glycosidic acceptor, creating a new glycosidic bond (Boominadhan et al., 2009).

The production of microbial amylases by bacteria is influenced by various factors, including the bacterial strain, medium composition, cultivation method, cell growth, nutrient availability, incubation time, pH, temperature, metal ions, and enzyme thermostability. Among Gram-positive isolates, *Bacillus* species are a dominant group found in soda lakes and soil environments (Bareither & Pollard, 2011; Nielsen et al., 1995; Taipa et al., 2019).

Industrially significant *Bacillus* species are commercially valuable due to their rapid growth, short fermentation cycles, efficient protein secretion, and safe handling. The first alkaline amylase from an alkaliphilic *Bacillus* strain was reported by (Kaissar et al., 2025; Priest, 1977), several *Bacillus* species involved in amylase production include *B. amyloliquefaciens*, *B. caldolyticus*, *B. coagulans*, *B. licheniformis*, *B. macerans*, *B. stearothermophilus*, *B. subtilis*, *B. cereus*, *B. megaterium*, *B. polymyxa*, and alkalophilic *Bacillus* species (Vasantha-Srinivasan et al., 2025).

Amylase and Protease Activity: Fermentation is the key method for enzyme production, utilizing both solid-state and submerged fermentation. However, submerged fermentation is typically preferred for bacterial enzyme production due to its higher water potential requirements (Mukendi et al., 2025). The use of submerged culture is advantageous because of the ease of sterilization and process control is easier to engineer in these systems.

Amylase Activity: In submerged fermentation, amylase production reached its highest level of 4 U/ml at 10 hours of incubation (Bernfeld, 1986). Further increase in the incubation period did not show any significant increase in enzyme production rather it was decreased. Thus, the optimum time of enzyme synthesis was to be 10 hours after inoculation. Enzyme production began approximately 6 hours after incubation in media containing 0.2% or 1.0% soluble starch (Mulimani & GN Patil, 2000). The fermentation medium used was [g/l] 6.0g Bacteriological peptone; 0.5 g MgSO₄.7H₂O; 0.5g KCl; 1.0 Starch, pH7. Following incubation, the fermentation medium was centrifuged at 5000 rpm for 20 minutes at 4°C, and the supernatant was collected for amylase activity assessment. The production of enzymes by the shake flask culture was further explained by (Rani et al., 2025) from *Bacillus amyloliquefaciens* (Acharya & Subedi 2025) The organism was cultured in a shake-flask using a basal medium containing essential nutrients and 1% soluble starch (pH 6.5) for extracellular amylase production. After 48 hours of incubation at 37°C, the enzyme activity reached 35.0 U/ml (Table 1).

Amylase activity can be measured using the method described by (Rawal et al., 2025). In this assay fixed enzyme aliquots are incubated with 0.5 ml of 1% starch in 0.01M phosphate buffer (pH 6.9) at 37°C for 6 minutes. The reaction is then halted by adding 1 ml of DNS reagent, followed by heating in boiling water for 5 minutes (Bernfeld 1955). Following cooling, the solution was made up to 10 ml with distilled water. The absorbance was read at 540 nm. In a control tube, enzyme activity was arrested at zero time by adding 1 ml DNS reagent. Enzyme activity was calculated from differences in absorbance of control and test assay mixtures using a maltose calibration curve. Aliquots of a standard solution of maltose (1 mg/ml) over the range of 0.1-1 ml, were pipetted into a series of test tubes and made up to 2 ml with distilled water. DNS reagent (1 ml) was added to each tube and the contents were covered with glass marble. The tubes were heated in boiling water for 5 minutes, then cooled, and 10 ml of double-distilled water was added. The absorbance of the resulting orange color was measured at 540 nm (Gupta et al., 2002).

Optimization for amylase production: The nature and amount of carbon sources in culture media are important factors in producing extracellular amylase (Yabuki & Fukui, 1970). It was found that amylase production was maximum when starch was used as the carbon source (Mishra & Behera, 2008). Enzyme biosynthesis occurred not only in the presence of starch but also with alternative carbon sources. Wheat bran was used as a substrate for enzyme production, while among the tested sugars, lactose at a 1% concentration was the most effective inducer of amylase production. Additionally, maltose was also found to be a strong inducer of amylase (Deb et al., 2013; Gupta et al., 2002).

Bacterial amylases are produced across a broad temperature range. *Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, and *B. stearothermophilus* are among the most commonly utilized *Bacillus* species, capable of amylase production at temperatures between 37°C and 60°C (Vijayalakshmi et al., 2012). Enzyme production was found to gradually increase with increasing temperature and maximum enzyme production was observed at 42°C (Aygan et al., 2008). Enzyme production by *Bacillus subtilis* was significantly inhibited at 40°C. While maximum activity was observed at 50°C, certain halotolerant species exhibited enzymatic activity across a wide temperature range, extending up to 80°C (Banerjee & Bhattacharyya, 1992; Terui, 1973).

The pH of the growth medium is a crucial factor influencing enzyme secretion and stability. Most studies have shown that bacterial growth and enzyme production are optimal within a pH range of 6.0 to 7.0. However, (Deb et al., 2013) reported maximum enzyme activity at a slightly alkaline pH of 9.0, while enzyme activity remained extremely low at neutral pH (Aygan et al., 2008; Joshi & Ball, 1993). Studies indicate that optimal pH for amylase production varies among organisms. *Bacillus amyloliquefaciens* showed maximum amylase production at pH 6.0 in a fermenter, while *B. subtilis* exhibited optimal production at pH 6.8, as reported by (Terui, 1973). Deviations from these optimal pH values negatively impact microbial growth and enzyme yield (Kanekar et al., 2002; Mulimani & GN Patil, 2000)

Table 1: Factors Affecting Amylase Activity

Factors	Details	References
Carbon Sources	Starch is the best carbon source for maximum amylase production. Other carbon sources such as wheat bran, lactose (1% concentration), and maltose are also good inducers.	Yabuki & Fukui, 1970 Mulimani et al.,2000 Akcan, 2011 Gupta et al.,2002
Temperature	Bacterial amylases are produced in a wide temperature range (37°C–60°C). Maximum enzyme production was observed at 42°C. Some halotolerant species show activity up to 80°C.	Mishra & Behera, 2008 Deb et al.,2013 Aygan et al.,2008
Bacterial Strains	<i>B. amyloliquefaciens</i> , <i>B. subtilis</i> , <i>B. licheniformis</i> , and <i>B. stearothermophilus</i> are commonly used for amylase production. <i>B. subtilis</i> enzyme production was inhibited at 40°C.	Mishra & Behera, 2008
pH Influence	The optimal pH for most bacterial strains is between 6.0 and 7.0. Some studies reported maximum enzyme activity at pH 6.0, while others found activity at pH 9.0.	Acharya & Subedi, 2025

pH Effects A deviation from the optimal pH leads to poor microbial growth and reduced enzyme production. *B. subtilis* has an optimum pH of 6.8 for amylase production. Teru, 1973

Protease Activity: The highest protease activity (99 U/mL) was achieved at 48 hours of cultivation, coinciding with the stationary phase of cell growth. Compared to previously reported shaken flask experiments, enzyme production was 1.8 times higher; however, peak activity was observed 24 hours later (Joshi & Ball, 1993; Vijayalakshmi *et al.*, 2013). Protease activity was reported in alkaliphilic species *B. sphaericus*, *B. bravis*, and *B. lactosporus* at 0.65, 1.55, and 1.85 U/mL, respectively, using a conventional medium consisting of nutrient broth supplemented with 4% gelatin at pH 10, following 24 hours of incubation at 37°C. However, in the study conducted by (Abo-Kamer *et al.*, 2025), the protease activity of *B. alcalophilus* was 12.3 units/ml and 18.3 units/ml at 24 hours and 48 hours of incubation, respectively, at ambient temperature 28±2°C under shake culture conditions using gelatin as the substrate (Switzer & Garrity, 1999) (Table 2).

For the assessment of protease activity, isolates exhibiting significant enzyme production indicated by a clearance zone exceeding 15 mm were selected for further quantification (Switzer & Garrity, 1999). Cell-free supernatants (CFS) were obtained by centrifugation at 12,000g for 15 minutes using a refrigerated centrifuge. These supernatants were then used to estimate protease activity with tyrosine as the substrate, following a modified method by (Horikoshi, 1971). The enzyme assay was conducted using 1 mL of cell-free supernatant (CFS) and 4 mL of 0.625% (w/v) casein as the substrate in Tris-HCl buffer (pH 9). The mixture was incubated at 37°C for 30 minutes. The reaction was then terminated by adding 5 mL of 5.0% (w/v) trichloroacetic acid, and tyrosine concentration was determined using the Folin-Ciocalteu reagent. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µg of tyrosine per milliliter per minute under the assay conditions. Research findings indicate that *Bacillus alcalophilus* isolates demonstrated the highest level of protease activity among the tested strains. This suggests that *B. alcalophilus* has a strong potential for protease production, making it a promising candidate for industrial and biotechnological applications where

high enzymatic activity is required (Switzer & Garrity, 1999).

Optimization for Protease Activity : The influence of different carbon and nitrogen sources on protease production was analyzed using simple and complex carbon sources such as sucrose, maltose, xylose, galactose, and starch instead of dextrose. Similarly, nitrogen sources like peptone, ammonium nitrate, ammonium sulfate, urea, and ammonium chloride were evaluated as alternatives to yeast extract (Pant *et al.*, 2015). Each source was used at concentrations of 1% w/v and 0.55% w/v. It was found that *B. subtilis* showed higher proteolytic activity on 0.03 (w/v) gelatin than on casein or skimmed milk in nutrient agar medium.

The optimal temperature for enzyme production varies depending on the species. It was determined by adjusting the incubation temperature with and without the presence of 5 mM CaCl₂, highlighting the influence of environmental conditions on enzymatic activity. The activity measured at 30°C was expressed as 100%. The activity at 60~65°C in the presence of Ca²⁺ was about 70% and that at 60°C in the absence of Ca²⁺ was about 40%. Among the different incubation temperatures tested for protease production for *B. licheniformis* was 45 °C and for *B. subtilis* 50°C. However, it is found that *B. thuringiensis* retained 90% activity after 30 min of incubation at 70 °C, these enzymes are the least stable of them, *Bacillus* proteases (Cherdyntseva *et al.*, 1982). The protease activity of the crude enzyme from *B. subtilis* was tested at various temperatures. The enzyme exhibited peak activity at 50°C, but its activity declined beyond this temperature (Vijayalakshmi *et al.*, 2013).

The pH range during microbial growth significantly impacts product stability. Studies have shown that *B. licheniformis* can grow and produce protease across a broad pH range of 2.0 to 10.0. However, most *Bacillus* species exhibit optimal protease production within a pH range of 7.0 to 11.0 (Das & Prasad, 2010). The highest protease activity was found at pH 7.4 for *B. subtilis* (Joo & Chang, 2005).

Table 2: Factors Affecting Protease Activity

Factor	Details	References
Carbon Sources	Sucrose, maltose, xylose, galactose, and starch were tested instead of dextrose. Used at 1% w/v and 0.55% w/v concentrations.	(Pant <i>et al.</i> , 2015)
Nitrogen Sources	Peptone, ammonium nitrate, ammonium sulfate, urea, and ammonium chloride were tested instead of yeast extract.	Das & Prasad, 2010 Taipa <i>et al.</i> , 2019
Substrate Preference	<i>B. subtilis</i> showed higher proteolytic activity on 0.03% (w/v) gelatin compared to casein or skimmed milk in a nutrient agar medium.	(Pant <i>et al.</i> , 2015)
Temperature Influence	<i>B. licheniformis</i> : 45°C, <i>B. subtilis</i> : 50°C, <i>B. thuringiensis</i> retained 90% activity after 30 min at 70°C. Enzyme stability varied with Ca ²⁺ presence.	Horikoshi, 1971 Naidu, 2011

pH Influence

B. licheniformis grows and produces protease at pH 2.0–10.0. Most *Bacillus* spp. have an optimum pH of 7.0–11.0. *B. subtilis*: pH 7.4. Other optima: *B. megaterium* (pH 8.0), *B. amyloliquefaciens* (pH 9), *B. thuringiensis* (pH 11.0).

Joo & Chang, 2005
Bareither & Pollard, 2011

Application of Amylase and Protease: The commercial use of enzyme preparations began in 1914 when Rohm in Germany developed trypsin from a microbial source. By the 1930s, enzyme applications had expanded beyond cheese production to the food industry, particularly in fruit juice manufacturing. Pectinases, a group of enzymes with various activities, were introduced to clarify fruit juice. The large-scale application of microbial enzymes in the food industry gained momentum in the 1960s, especially in the starch industry. During this time, traditional acid hydrolysis of starch was entirely replaced by α -amylases and glucoamylases, allowing nearly complete starch conversion into glucose. In 1959, the first commercial *Bacillus*-derived protease was introduced, and its demand significantly increased in 1965 when Novozymes in Denmark began large-scale production, prompting widespread adoption by major detergent manufacturers (Leisola et al., 2001). With advancements in enzyme applications, amylases have become essential across industries due to their ability

to break down starch into simpler sugars (Figure. 3). Its versatility has driven the transition from traditional chemical methods to more sustainable, enzyme-based solutions in food processing, textiles, biofuels, and pharmaceuticals. Some of the industrial applications of amylases are:

INDUSTRIAL APPLICATION OF AMYLASES

Bioethanol production: Starch, a key component of many agricultural feedstocks, serves as a convenient substrate for bioethanol production but requires enzymatic hydrolysis into simpler sugars like glucose and maltose for microbial fermentation. Amylases play a crucial role in this process. Despite the long-standing use of starch-based bioethanol production, ongoing research aims to enhance yields and reduce enzyme and production costs (Castro et al., 2011). *Bacillus licheniformis* with amylase expression is a promising candidate for bioethanol production from renewable feedstocks, including the conversion of mango peel into ethanol (Valorization, 2011).

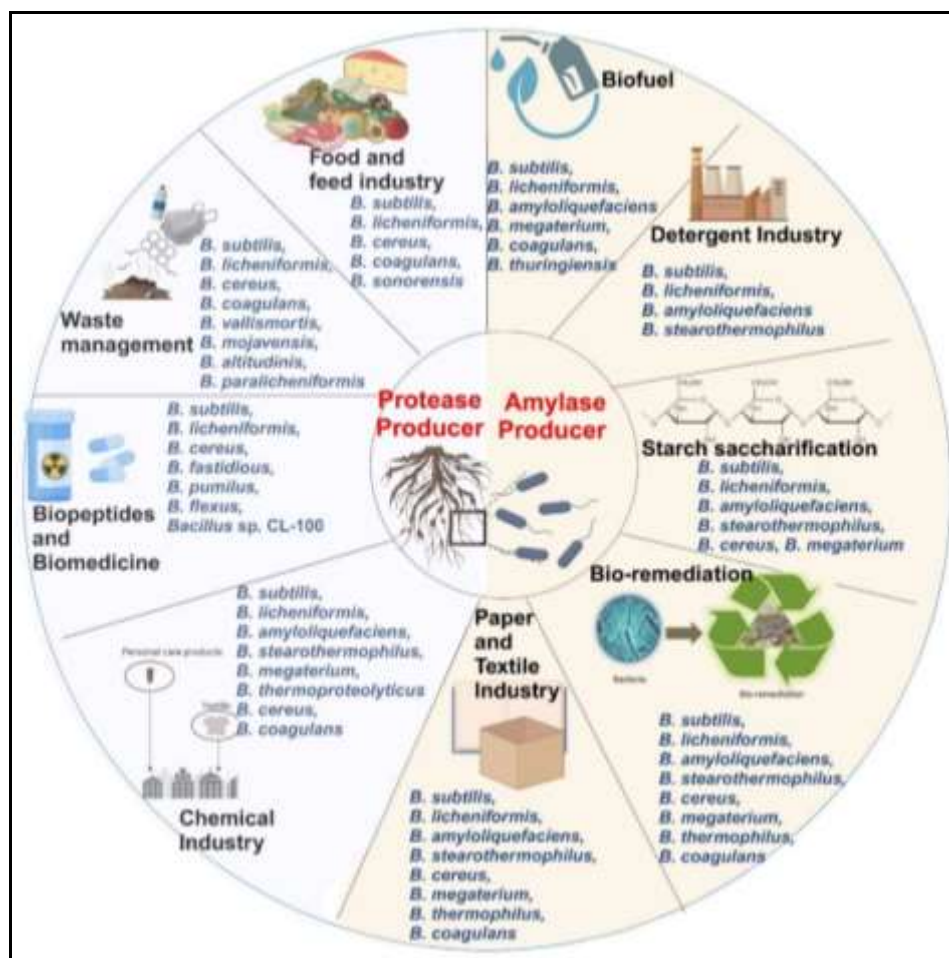


Figure. 3: *Bacillus* Species Producing Amylase and Protease in Various Industrial Applications. Amylase-producing *Bacillus* species (blue text) are utilized in industries such as biofuel production, detergent manufacturing, starch saccharification, and

bioremediation, where they help in breaking down starch into simpler sugars. Protease-producing *Bacillus* species (red text) play a crucial role in food and feed processing, waste management, bioremediation, biomedicine, chemical industry, and the paper & textile industry, where they aid in protein degradation and bioprocessing (Created with BioRender.com)

Starch saccharification: α -Amylases are widely used in the starch industry for hydrolysis during starch liquefaction, converting starch into fructose and glucose syrups. This enzymatic process involves gelatinization (starch granule dissolution), liquefaction (partial hydrolysis and viscosity reduction), and saccharification (glucose and maltose production). While *Bacillus amyloliquefaciens* was initially used, it has been replaced by *Bacillus stearothermophilus* and *Bacillus licheniformis* due to their superior thermostability and the availability of efficient expression systems for large-scale biotechnological applications (Blanco *et al.*, 2016).

Detergent industry: Amylases, found in 90% of liquid detergents, are the second most used enzymes, helping break down starchy food residues in laundry and dishwashing detergents. Their ability to function at lower temperatures and alkaline pH, along with oxidative stability, makes them ideal for detergent formulations. Additionally, starch removal improves fabric whiteness by preventing particulate soil accumulation. Amylases used in detergents are primarily derived from *Bacillus* and *Aspergillus* species (Blanco *et al.*, 2016).

Paper and Textile Industry: In the pulp and paper industry, α -amylases are used to modify starch in coated paper production, reducing its viscosity while maintaining a high molecular weight. This process improves paper smoothness, strength, and writing quality by enhancing its sizing and coating properties. Starch also contributes to paper stiffness, durability, and erasability, making it an essential finishing agent. Microbial amylases commonly used in this industry include Amizyme®, Termamyl®, Fungamyl®, BAN®, and α -amylase G9995® (Blanco *et al.*, 2016).

Bioremediation: Amylases play a vital role in bioremediation by breaking down starch-based pollutants into simpler sugars, facilitating microbial degradation. They help reduce organic wastewater from food, textiles, and paper industries, improve soil health by degrading starch pollutants, and aid in oil spill cleanup by breaking down biofilms and organic matter. Additionally, amylases contribute to the decomposition of starch-based biodegradable plastics and the removal of sizing agents from textile wastewater, making them valuable for eco-friendly waste management and pollution control (Sen *et al.*, 2009).

INDUSTRIAL APPLICATIONS OF PROTEASE

Food and feed industry: Proteases are essential in proteolysis, a critical process in food ripening, where they break down proteins into nitrogenous compounds that enhance flavor. *Bacillus* proteases are widely used in food processing, particularly in cheese ripening and fermented sausage production. Studies have shown that protease application accelerates cheese ripening, alters

texture, and enhances flavor, though excessive breakdown of β -casein may cause bitterness. In fermented sausage production, the addition of *B. subtilis* protease (Neutrase) enhanced nitrogen fractions, influencing protein solubility and amino acid composition.

Waste management: *Bacillus* proteases play a vital role in waste management, providing eco-friendly and efficient solutions. *Bacillus subtilis* UO-01 produces both amylases and proteases while significantly lowering the chemical oxygen demand (COD) of brewery wastewater, achieving reductions between 69.9% and 77.8% (Yadav *et al.*, 2024). Alkaline proteases help in sludge treatment by reducing pathogen counts, decreasing solids content, and enhancing deflocculation (Janiga-MacNelly & Lavado, 2024).

Biopeptides and biomedicine: Bioactive peptides generated using *Bacillus* proteases have shown significant antioxidant and antimicrobial properties. A study on ovine sodium caseinate hydrolyzed with *Bacillus* sp. P45 protease showed that peptide functionality changed over time, with antioxidant activity improving after 3–7 hours and antimicrobial effects against *Salmonella enteritidis*, *Escherichia coli*, *Corynebacterium fimi*, *Listeria monocytogenes*, and fungi appearing within one hour (Blanco *et al.*, 2016).

Chemical industry: Proteases with keratinase activity are particularly valuable in the textile industry due to their ability to degrade keratin, an insoluble fibrous protein found in hair, feathers, wool, and nails. Keratinases offer an eco-friendly alternative to traditional chemical treatments for wool processing, improving shrink resistance and handling properties. Research indicates that keratinase from *B. licheniformis*, combined with savinase from *B. lentus*, effectively minimizes wool shrinkage while maintaining fabric strength. Additionally, proteolytic bacteria such as *B. megaterium* and *B. thuringiensis* have demonstrated the ability to degrade wool fibers, leading to controlled weight loss while preserving fiber integrity, further highlighting the potential of microbial proteases in sustainable textile processing (Blanco *et al.*, 2016; Gorraeb *et al.*, 2024).

Enzyme Extraction: Traditional enzyme extraction methods involve organic solvents like ethanol and acetone, along with ammonium sulfate precipitation and ultrafiltration. However, these multi-step techniques require costly equipment, are labor-intensive, time-consuming, and often result in product loss. As an alternative, liquid-liquid extraction offers a more efficient purification approach by combining multiple early processing steps into a single operation. This method, which transfers specific components between immiscible or partially soluble liquid phases, is widely used in the chemical industry due to its

simplicity, cost-effectiveness, and scalability (Mrudula, 2024).

Purification of the Enzyme: Enzyme purification can be done by using the following techniques: Ammonium Sulphate Precipitation, Dialysis, Ultrafiltration, and Lyophilization (Vijayalakshmi *et al.*, 2013). For protease purification, a 500 ml Erlenmeyer flask containing 100 ml of basal medium was inoculated with 1% stationary-phase culture and incubated at 32°C with continuous shaking at 220 rpm for 40 hours. After incubation, the culture broth was centrifuged at 8000×g for 20 minutes at 4°C to obtain the supernatant. This supernatant was then dialyzed against a buffer solution containing 20mM Tris-HCl (pH 8.5), 50mM NaCl, and 0.5mM CaCl₂, serving as the crude enzyme extract (Erkiliç & Bayraktar, 2025). All the purification steps were performed at 4 °C. Pre-chilled acetone was added slowly to the solution up to 50% saturation in the first step. After removing the initial precipitate, additional acetone was added to the solution until reaching 80% saturation, followed by gentle stirring and a 1-hour incubation. The resulting precipitate was collected through centrifugation at 12,000×g for 20 minutes, dissolved in a minimal volume of buffer A, and dialyzed against the same buffer for 24 hours with two buffer changes. The purification process yielded 93.6% through ultrafiltration, 86.8% via dialysis, and 82% using ammonium sulfate precipitation (Silva *et al.*, 2025; Xin *et al.*, 2025).

The enzyme was purified through a multi-step process, starting with precipitation using 50% saturated ammonium sulfate to concentrate the protein. The resulting precipitate was then transferred into a dialysis bag and immersed in 100 ml of 0.025M phosphate buffer. To ensure the thorough removal of small molecules and salts, the mixture was continuously stirred for 24 hours using a magnetic stirrer. Following dialysis, further purification was performed using column chromatography with a Diethyl Amino Ethyl (DEAE) column, allowing for the separation of the enzyme based on its charge properties, resulting in a more refined and purified protein sample (Silva *et al.*, 2025).

From Lab to Large-Scale Production: A Fast and Efficient Path: After successfully purifying enzymes through methods such as ammonium sulfate precipitation, dialysis, ultrafiltration, and lyophilization, the next critical step is scaling up the process (Figure. 4). Transitioning from laboratory-scale purification to large-scale production is essential to maintaining enzyme activity and yield while meeting industrial demands (Liu *et al.*). This advancement is crucial in biotechnology, pharmaceuticals, and industrial enzyme production, where small-scale research helps refine techniques and

validate product effectiveness before large-scale manufacturing.

In the initial stages of process development, shaking flasks are frequently utilized for their high experimental capacity, simplicity, and cost-effectiveness (Al-Sharify *et al.*, 2025). They enable testing of cultivation media compositions and process parameters like pH and temperature while offering a larger liquid volume for more comprehensive analysis compared to microtiter plates. Additionally, their well-characterized physical properties, such as power input, make them valuable for scale-up studies. Although laboratory-scale systems offer flexibility and precise monitoring, scaling up is challenging due to variations in fluid dynamics, environmental inconsistencies, and the reliance on empirical rather than purely mathematical modeling. Consequently, iterative pilot experiments are necessary to optimize processes before full-scale industrial implementation (Acharya, 2019; Wang *et al.*, 2025).

Large bioreactors face challenges in heat and mass transfer, oxygen distribution, and mechanical stress. To ensure stable microbial growth and optimal yields, cultivation processes must tolerate temperature, pH, and nutrient variations, while increased back pressure is used to enhance oxygen solubility despite its impact on microbial strains (Esmeralda-Guzmán *et al.*, 2024; Ibrahim *et al.*, 2025).

Methods for enhancement of amylases and protease production: The increasing industrial demand for high-yield production of amylases and proteases has catalyzed significant advances in *Bacillus* species' genetic and metabolic engineering (Figure 5). As central workhorses in biotechnology, *Bacillus* strains are routinely engineered to maximize the output of these valuable enzymes, utilizing a combination of modern genetic tools and metabolic pathway optimization (Xiang *et al.*, 2020). Site-directed mutagenesis and gene editing have been proven to be powerful strategies for improving amylase and protease production in *Bacillus* species. For example, targeted mutations in the active site of *Bacillus subtilis* α -amylase have clarified the roles of key residues and enabled the creation of enzyme variants with altered catalytic efficiency and substrate binding. Recent work on *Bacillus velezensis* α -amylase used site-directed mutagenesis to improve both acid resistance and thermostability, resulting in mutants with higher activity and better performance in feed applications (Nie *et al.*, 2024).

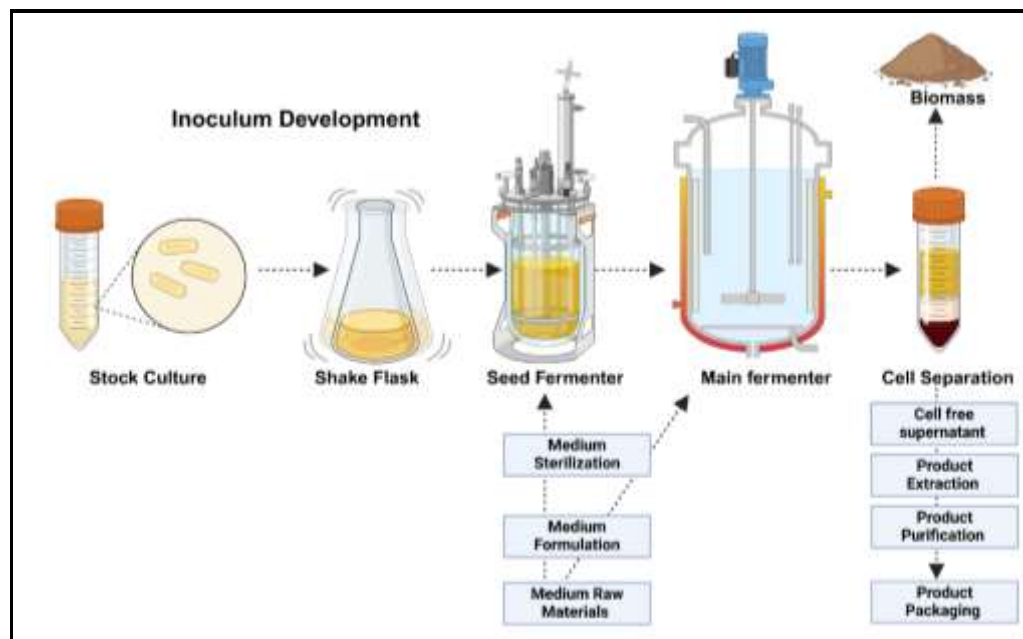


Figure. 4. Inoculum development for large-scale fermentation begins with a stock culture of microorganisms, which are cultivated in a shake flask for initial growth. The culture is then transferred to a seed fermenter, where medium formulation, sterilization, and raw material optimization enhance microbial proliferation. Once optimized, the inoculum is introduced into the main fermenter for large-scale production. After fermentation, cell separation yields biomass and a cell-free supernatant, which undergoes product extraction, purification, and packaging for industrial applications (Stanbury *et al.*, 2013). (Created with BioRender.com)

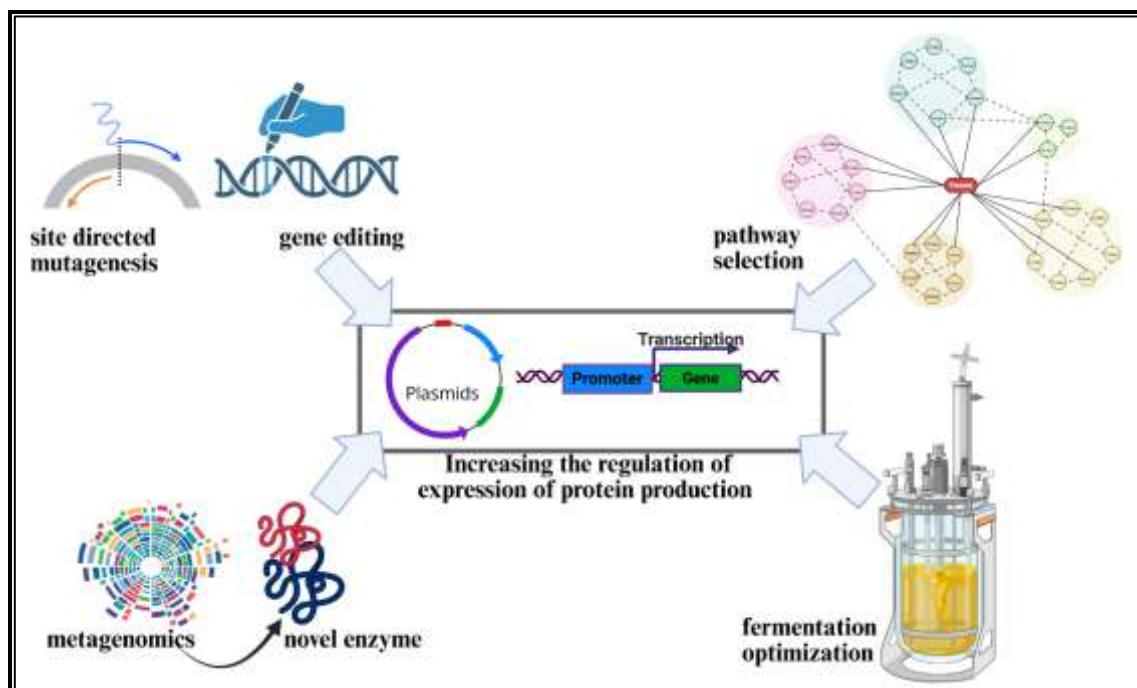


Figure. 5. strategies for boosting protein expression: site-directed mutagenesis, gene editing, pathway selection, metagenomics-driven enzyme discovery, and fermentation optimization enhance transcriptional regulation via engineered plasmids (Created with BioRender.com)

Advanced gene editing tools, especially CRISPR/Cas9, have revolutionized the engineering of *Bacillus* strains. CRISPR-based tools allow for multiplexed editing and iterative strain improvement, making it possible to optimize multiple traits simultaneously for industrial fermentation (García-

Moyano *et al.*, 2020). In *Bacillus*, pathway selection and modular engineering boost amylase and protease production by modifying genes to direct more resources to enzyme synthesis and reduce byproduct formation. For example, in *Bacillus amyloliquefaciens*, deleting genes involved in sporulation (such as *sigF*),

extracellular protease synthesis, and extracellular polysaccharide production significantly increased alkaline protease (AprE) output. Combining these modifications led to a 39.6% increase in protease production compared to the control strain (Zhang *et al.*, 2022). Environmental screening of soil samples is a key method for discovering *Bacillus* species with the natural ability to produce both amylase and protease. Studies have shown that soils from diverse environments, such as metropolitan areas and volcanic regions, harbor a wide variety of *Bacillus* including *B. cereus*, *B. aryabhattai*, *B. amyloliquefaciens*, and *B. velezensis*, that can synthesize these enzymes. For example, in a screening of soil samples from the Philippines, 33 putative *Bacillus* isolates were identified, with many producing both protease and amylase (Esmeralda-Guzmán *et al.*, 2024; Seidler *et al.*, 2024). By integrating fermentation optimization with advanced genetic expression systems, *Bacillus* strains have been engineered to produce industrially relevant quantities of amylase and protease. Adjusting factors like pH, temperature, dissolved oxygen, agitation, and nutrient composition can dramatically increase enzyme output. For example, optimizing these conditions in *Bacillus subtilis* using statistical models and response surface methodology led to a more than threefold increase in alkaline protease activity, reaching up to 1798.33 U/mL after optimization (Sun *et al.*, 2023). High-copy plasmids and strong, inducible promoters are used to drive robust expression of amylase and protease genes in *Bacillus*. These systems allow for fine-tuned control and high-level production of target enzymes. For instance, engineered strains with optimized regulatory elements and plasmid systems have shown substantial increases in enzyme yields, especially when combined with fermentation improvements (Sun *et al.*, 2023). Together, these strategies are gene editing, pathway engineering, the discovery of new genes, fermentation control, and advanced expression systems synergistically enhance the production of amylase and protease in *Bacillus* by maximizing transcription, translation, and secretion of the target enzymes (Yang *et al.*, 2021).

CONCLUSION

This comprehensive review underscores the pivotal role of *Bacillus* species in the production, optimization, and industrial application of amylase and protease enzymes. Recognized for their robust enzyme-secreting capabilities, *Bacillus* strains produce high yields of neutral and alkaline enzymes that remain stable under extreme pH, temperature, and chemical conditions. These characteristics make them ideal for diverse sectors such as food processing, textiles, and detergents (Danilova & Sharipova, 2020). A particular focus has been given to strains isolated from the rhizosphere, known for their high enzymatic potential (Tsotetsi *et al.*, 2022). The choice of carbon and nitrogen sources significantly influences enzyme

yields in *Bacillus*. Starch, lactose, and wheat bran boost amylase activity, while gelatin and peptone enhance protease production. Optimal production typically occurs at temperatures between 42°C–50°C and pH 7.0–11.0, though some strains can adapt to broader conditions ((Niyomukiza *et al.*, 2022).

To meet the growing industrial demand for robust enzymes, advanced genetic and metabolic engineering methods are now widely used. Techniques like site-directed mutagenesis and CRISPR-based editing have improved enzyme properties such as catalytic efficiency, heat stability, and pH tolerance (Schallmeyer *et al.*, 2004). Plasmid-based expression systems, combined with optimized fermentation and the use of cost-effective substrates, have further increased enzyme yields.

Historically, *Bacillus*-derived enzymes have evolved from traditional uses like cheese-making to become essential components in modern, sustainable biotechnological processes. Ongoing research-integrating genomics, synthetic biology, and process engineering-continues to expand the applications and efficiency of *Bacillus* enzymes in industry (Danilova & Sharipova, 2020).

CONFLICT OF INTEREST

We declare no competing interests affecting the work reported in this study.

AUTHORS' CONTRIBUTION

AA and SS led the literature review, and analysis and contributed to the synthesis and manuscript editing.

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