

SUSTAINABLE BIOPROCESS DEVELOPMENT FOR ENHANCED PRODUCTION OF NOVEL β -AMYLASE OF GH-14 FAMILY

Ayisha Aman Ullah^{1,2}, Ayesha Siddiqui¹, Azkah Qayyum², Rashida Rahmat Zohra², Mahnaz Ahmad¹ and Raheela Rahmat Zohra^{1*}

¹Department of Biotechnology, Faculty of Science, University of Karachi, Karachi-75270, Pakistan;

²Department of Biotechnology, Faculty of Science, Jinnah University for Women, Karachi, Pakistan.

*Email: rrzohra@uok.edu.pk

ABSTRACT

Bacterial cells are extensively utilized for the production of various enzymes, particularly those involved in starch degradation. Among these, amylolytic enzymes are widely recognized for being secreted extracellularly in substantial amounts. β -amylases are exo-acting enzymes that possess broad applications in food industry. The current study was based on taxonomy and molecular phylogeny of bacteria from genus *Bacillus* that has potential of synthesizing β -amylase. Optimization of various physicochemical parameters was investigated using OFAT method for improved production of β -amylase from isolated strain. Among various purified strains, *Bacillus subtilis* C5W was selected on the basis of efficient β -amylase production. Parametric optimization resulted in the production of maximum β -amylase in a modified starch production medium. Enhanced enzyme yield was attained at 50 °C after 24 hours of incubation period in modified culture medium (pH 7.0). Improved production of β -amylase was noticed when starch (1.5%) as a carbon source, peptone (0.05%) as an organic nitrogen source and NaCl (1%) was incorporated in medium. The potential of isolated strain to thrive and produce β -amylase under wide pH ranges with least fermentation time, makes *B. subtilis* as potential candidate for the production of β -amylase on large scale for starch saccharification.

Keywords: β -Amylase, *Bacillus subtilis*, Starch, Physiochemical parameters, OFAT

INTRODUCTION

Industrial biotechnology offers significant advantages over traditional methods in various industrial and analytical sectors. A key strategy involves utilizing biological systems to optimize media components, minimize by-product formation, and enhance the yield of desired bio-products in an eco-friendly manner (Tiwari *et al.*, 2022; Saini *et al.*, 2017). Bioprocess technology utilizes living cells or their components (enzymes, metabolites) for chemical transformations. It enables large-scale production of various products through biocatalysis and is widely applied in food, textiles, fine chemicals, pharmaceuticals, and therapeutics. The use of biocatalysts (enzymes) instead of chemical catalysts in industrial processes is a growing global trend. These biological macromolecules, produced by living organisms, regulate cellular metabolism and are synthesized by plants, animals, and microorganisms (Mesbah, 2022). Microbial enzymes hold significant commercial value due to their advantages over other sources, including easy isolation, cost-effective and rapid production, high yield, stability in harsh conditions, environmental friendliness, adaptability, and abundant availability (Gaur *et al.*, 2024; Mukherjee *et al.*, 2023). Amylolytic enzymes are highly valued in industrial biotechnology due to their diverse and widespread applications (Kumar *et al.*, 2024). Amylolytic enzymes, a class of glycoside hydrolase (GH) contribute up to 30% of global enzyme's market that have introduced new edges of several industrial biotechnology setups (Paul *et al.*, 2021).

β -Amylases (EC 3.2.1.2), also called α -1,4-maltoglucan hydrolases, are exo-enzymes that break α -1,4 glycosidic bonds at the non-reducing ends of starch, releasing maltose. While many plant species produce β -amylases abundantly, microbial β -amylases is preferred due to its superior stability, cost-effectiveness, and efficient production process. They are widely utilized in industries such as saccharification, starch brewing, and distilling, where their activity is vital for fermentable sugar production (Nag *et al.*, 2021). They are specially used for the production of maltose syrup. Maltose is widely used as a sweetener in candies, confectionery, ice cream, and various foods due to its rich flavor, lower Maillard reaction rate compared to glucose, and resistance to crystallization (Kojima, 2010).

In the era of industrialization, advancements in biotechnology have enabled the bioprocessing of agro-industrial waste for the production of value-added enzymes (Bala *et al.*, 2023). However, enzyme production remains expensive due to fermentation media and processing costs. To reduce production expenses and meet industrial demands, diverse microbial strains and cost-effective agro-industrial substrates are now utilized (Bibi *et al.*, 2023). Globally, food and agriculture industries generate vast amounts of agro-industrial waste, contributing to waste

*Corresponding Author: Raheela Rahmat Zohra: Associate Professor, Department of Biotechnology, University of Karachi, Pakistan; Cell# 0333-3992390; Email: rrzohra@uok.edu.pk; ORCID: <https://orcid.org/0000-0002-8057-296X>

disposal and environmental pollution challenges (Dey *et al.*, 2021). Rich in essential nutrients, this waste serves as a valuable raw material, providing sufficient micro and macronutrients to support microbial growth for the cultivation of industrially significant enzymes. Lignocellulosic substrates, the primary components, contain fibers, sugars, cellulose, hemicellulose, lignin, proteins, and minerals (Jahangeer *et al.*, 2024; Mujtaba *et al.*, 2023).

Microbial enzyme production is influenced by various physiochemical parameters such as incubation time, pH, temperature, agitation, and nutrient sources. Optimizing these parameters is essential for maximizing yield. Typically, each factor is optimized sequentially while keeping others constant. This systematic approach enhances efficiency and ensures cost-effective fermentation (Farooq *et al.*, 2021; Niyonzima *et al.*, 2020).

A large number of reports on alpha and gamma amylases regarding optimization and characterization are present. But β -amylase specifically from bacterial source was constantly passed over though it has also several industrial applications. Limited researches are available on β -amylase optimization and production. So, the aim of current work is to isolate extremophilic bacteria and investigate the influence of various physical and chemical cultural conditions on the biosynthesis of β -amylase by *Bacillus subtilis* C5W under static flask fermentation.

MATERIALS AND METHODS

Isolation of β -amylase producing bacteria

To isolate β -amylase-producing bacteria, soil and water samples were aseptically collected from extreme environments in Pakistan, including hot springs, the sea, and salt mines. Bacterial species were isolated using the spread plate method on Luria Bertani (LB) medium through serial dilution. The isolates were then screened using a qualitative starch hydrolysis assay, and the strain with the highest enzyme index (EI) was selected for optimization studies.

Phenotypic and molecular characterization of a pure bacterial culture

The selected bacterial strain was identified based on both taxonomic and molecular characteristics. For taxonomic characterization, macroscopic and microscopic observations, along with biochemical assays, were conducted following *Bergey's Manual of Determinative Bacteriology*. Molecular identification of the isolated strain was performed through 16S rDNA analysis using the Sanger sequencing method (chain termination technique) (Sanger and Coulson, 1975). The obtained 16S rDNA sequence was compared with available nucleotide sequences in the NCBI database using the BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the strain's identity. Subsequently, multiple sequence alignment (MSA) was carried out, and a phylogenetic tree was constructed using MAFFT MSA software version 7.0.

Preparation of original inoculum

To prepare the inoculum, Luria broth (composed of 1% NaCl, 1% Tryptone, and 0.5% Yeast extract) was utilized. A loopful of freshly grown bacterial culture was aseptically introduced into the sterilized broth. The culture was then incubated at 45°C for 24 hours under continuous shaking and subsequently used as an inoculum for enzyme production.

Selection of appropriate fermentation medium for β -amylase production

To identify the most suitable fermentation medium for maximum β -amylase production, six different reported culture media were initially screened (Table 1). The pH of each medium was adjusted to 7.0 using 0.1M HCl and 0.1N NaOH before sterilization. Following autoclaving, six 100 mL Erlenmeyer flasks, each containing 49 mL of fermentation medium, were inoculated with 1 mL of fresh starter culture. The cultures were then incubated at 50°C for 24 hours. After incubation, the cell-free broth was obtained by centrifuging the culture at 5000 rpm for 15 minutes at 4°C to separate the cell pellet. The resulting supernatant was used as the crude enzyme source for enzymatic assays. Once the optimal medium for amylase production was identified, its composition and concentrations were further modified to enhance β -amylase yield.

Table 1. Media selection for β -amylase production from *Bacillus subtilis* C5W.

Media	Composition	Reference
M1	Peptone 10g/L; Yeast extract 0.2 g/L; Soluble starch 10.0 g/L; NaCl 2.0 g/L; MgSO ₄ .7H ₂ O 0.5 g/L; KH ₂ PO ₄ 3.0 g/L; CaCl ₂ 0.5 g/L	(Al-Johani <i>et al.</i> , 2017)
M2	Soluble starch 10.0 g/L; CaCl ₂ 0.01 g/L; Maltose 10.0 g/L; (NH ₄) ₂ SO ₄ 2.0 g/L; K ₂ HPO ₄ 17.41 g/L; MgCl ₂ .6H ₂ O 0.2 g/L	(Rasooli <i>et al.</i> , 2008)
M3	Soluble starch 2.5 g/L; NaCl 1.25 g/L; MgSO ₄ .7H ₂ O 1.25 g/L; KH ₂ PO ₄ 0.75 g/L; K ₂ HPO ₄ 0.75 g/L	(Indriati <i>et al.</i> , 2018)
M4	Soluble starch 10.0 g/L; MgSO ₄ .7H ₂ O 0.2 g/L; CaCl ₂ 0.1 g/L; K ₂ HPO ₄ 1.0 g/L; KNO ₃ 0.5 g/L; FeCl ₃ 0.01 g/L	(Al-Awsy <i>et al.</i> , 2017)
M5	Yeast extract 3.0 g/L; Soluble starch 10.0 g/L; MgSO ₄ .7H ₂ O 0.02 g/L; KH ₂ PO ₄ 0.2 g/L; CaCl ₂ 0.01 g/L; K ₂ HPO ₄ 1.2 g/L; Tryptone 3.0 g/L	(Rekadwad, 2015)
M6	Peptone 10g/L; Yeast extract 4.0 g/L; Soluble starch 20.0 g/L; NaCl 0.5 g/L; MgSO ₄ .7H ₂ O 0.5 g/L; CaCl ₂ 0.2 g/L	(Bano <i>et al.</i> , 2011)

Analysis of β -Amylase Activity

The β -amylase activity in the crude extract was assessed using the Bernfeld method (Berfed, 1955). A maltose calibration curve was established using a 4 mg/mL maltose stock solution to quantify the concentration of maltose released during the DNS amylase assay and to calculate the total enzyme units produced.

"One unit of β -amylase is defined as the amount of enzyme required to liberate one micromole of reducing sugar (maltose) per milliliter per minute under standard assay conditions."

Systematic Optimization of Culture Conditions for Maximum β -Amylase Yield

To achieve efficient β -amylase production from the isolated bacterial strain, various physicochemical parameters were optimized using the One Factor at a Time (OFAT) approach. The optimized physical parameters included incubation temperature (40°C, 50°C, 60°C, and 70°C), incubation time (18, 24, and 72 hours), and medium pH (ranging from 5.0 to 9.0).

Nutritional requirements and their influence on β -amylase production were also evaluated. Eight different agro-industrial residues (1%), including corn husk, corn cob, banana peel, sugarcane bagasse, potato peel, wheat bran, wheat straw, and rice husk, were tested as carbon sources, with starch (1%) serving as the control. Additionally, varying starch concentrations (5–30 g/L) were assessed for their impact on enzyme production.

Various organic and inorganic nitrogen sources (0.5 g/L), such as tryptone, peptone, yeast extract, ammonium sulfate and potassium nitrate, were also examined. Furthermore, different peptone concentrations (0.25–1.25 g/L) were tested to enhance β -amylase production. The effect of varying NaCl concentrations (0–30 g/L) on enzyme production was also investigated.

Data analysis and statistical evaluation

All experiments were conducted in triplicate, and the collected data were statistically analyzed using Microsoft Office Excel 2013. The results were expressed as mean \pm S.E.M.

RESULTS AND DISCUSSION

Isolation and Characterization of the Bacterial Strain

Several bacterial strains were isolated and purified from soil and water samples. β -amylase production in all strains was evaluated using the qualitative starch hydrolysis plate assay. Among them, strain C5W exhibited the highest amylolytic activity on starch agar medium. The selected strain was subsequently characterized based on its morphological, biochemical (Table 2), and molecular properties. Further analysis confirmed its halothermophilic nature, as previously reported (Aman *et al.*, 2019)

Table 2. Morphological and Biochemical Profile of *Bacillus subtilis* C5W.

Cellular morphology	
Shape	Rod
Size	Small
Arrangement	Scattered
Gram reaction	Gram positive
Shape	Rod
Size	Small
Colonial morphology	
Shape	Irregular
Margin	Undulate
Elevation	Slightly raised
Size	Large
Texture	Rough
Surface	Wrinkled
Appearance	Dull
Opacity	Opaque
Pigmentation	Off white
Physiological tests	
Growth at 37°C to 45°C	+
Growth in NaCl (up to 10%)	+
Mode of respiration	Aerobic
Biochemical assays	
Indol production	-
Methyl red test	-
VogesProkaurer test	-
Citrate utilization test	-
Simple carbohydrate fermentation	-
Catalase	+
Motility test	Non motile

For molecular characterization, the bacterial genomic DNA was extracted, and the 16S rDNA sequence was amplified using PCR. The resulting PCR product was purified and subsequently sequenced. BLAST analysis was then performed to determine the bacterial species. The isolated strain was identified as belonging to the *Bacillus* genus and characterized as *Bacillus subtilis* C5W. The obtained 16S rDNA sequence was submitted to GenBank (NCBI) and assigned with accession number MK758086.1.

For multiple sequence alignment, the 16S rDNA query sequence was compared with other 16S rDNA sequences available in the GenBank repository of NCBI. A phylogenetic tree (Fig.1) was constructed using MAFFT MSA software version 7.0. The resulting phylogenetic analysis indicated that the query sequence (*B. subtilis* C5W) shares 98% 16S rDNA similarity with other *B. subtilis* strains, confirming its close evolutionary relationship.

Selection of Medium for Optimization Studies

Although different species within the *Bacillus* genus exhibit similar growth patterns and enzyme production profiles, their optimal growth conditions vary depending on the specific strain. Optimizing physicochemical parameters is essential to maximize enzyme production and ensure cost-effectiveness in the production process (Elmansy *et al.*, 2018).

To determine the optimal conditions for amylase production, the isolated strain *Bacillus subtilis* C5W was subjected to various process parameters. The enhancement of β -amylase production was assessed using six different reported media, revealing variations in enzyme production across different media formulations (Fig. 2). This variation in amylase production indicates the distinct nutritional requirements of *Bacillus subtilis* C5W. Among the six tested media, medium #4 exhibited the highest enzyme production (11,111.1 U/mL), whereas medium #3 resulted in the lowest extracellular β -amylase synthesis.

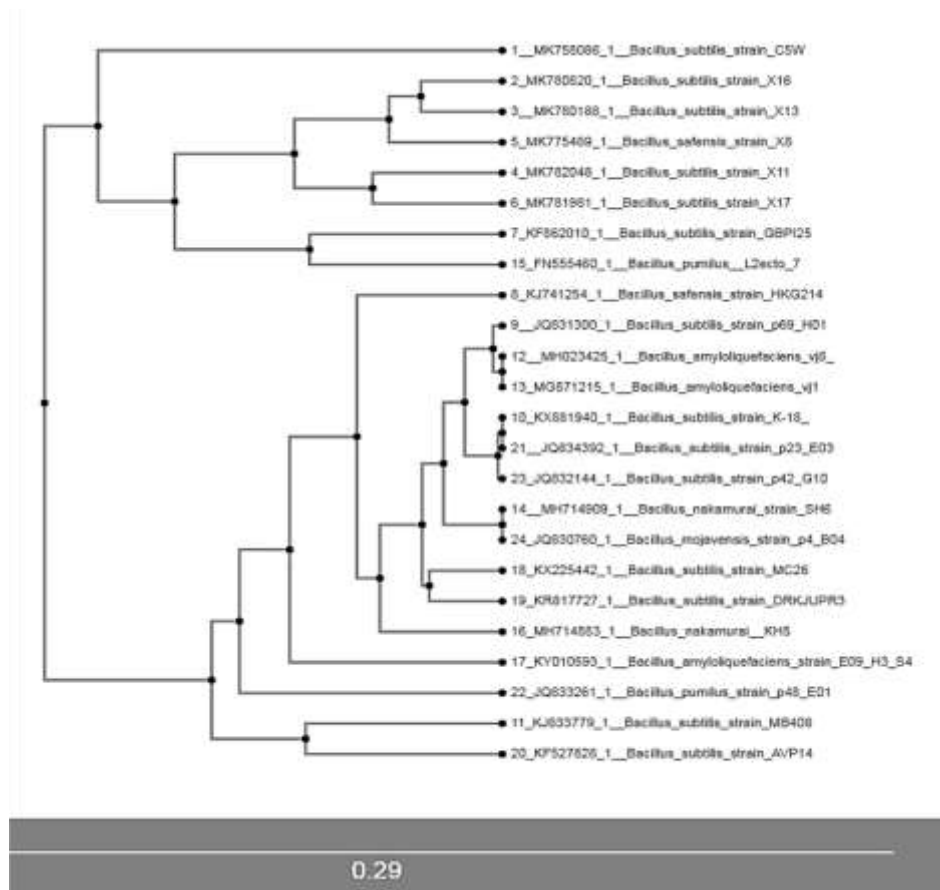


Fig. 1. Phylogenetic Analysis of *Bacillus subtilis* C5W Using MAFFT MSA Software Version 7.0 version 7.0.

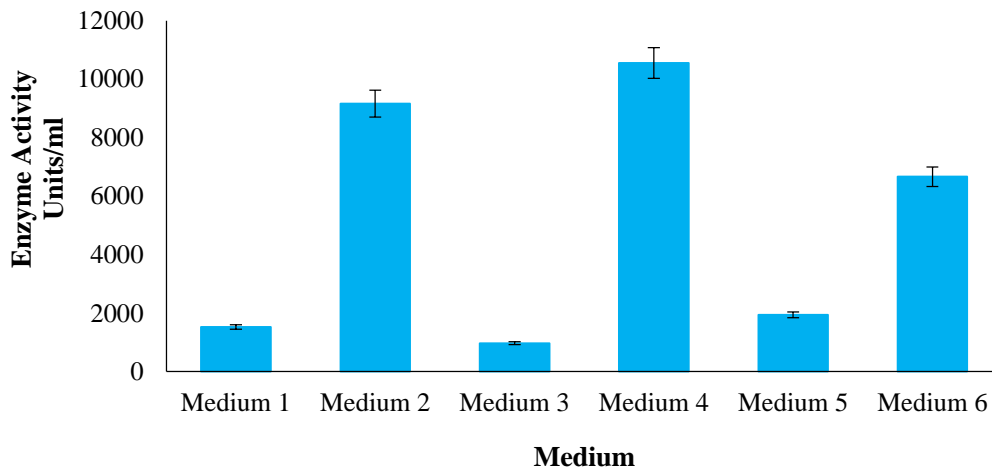


Fig. 2. Optimizing Culture Medium for Maximum β -Amylase Yield in *Bacillus subtilis* C5W (Mean \pm S.E.M., n=3).

Influence of physical parameters on β -amylase production by *Bacillus subtilis* C5W

Incubation temperature

Incubation temperature is a critical physical factor affecting enzyme production and stability. In microbial cells, temperature directly influences biochemical pathways, regulating the synthesis and secretion of extracellular enzymes (Patil and Dayanand, 2006). The optimal temperature for microbial growth and enzyme production varies

among different species (Kumar and Takagi, 1999). In this study, the culture medium was incubated at temperatures ranging from 40°C to 70°C for 24 hours. The highest β -amylase production was observed at 50°C, while a sharp decline in enzyme synthesis was noted as the temperature increased to 70°C (Fig. 3). Several mechanisms have been proposed to explain enzyme denaturation at elevated temperatures. One explanation suggests that excessive metabolic heat disrupts enzymatic catalytic activity, leading to the shutdown of biochemical pathways, which ultimately halts bacterial growth and enzyme production (Demirkan *et al.*, 2017). Conversely, at lower temperatures (40°C and below), enzyme synthesis remained at a basal level due to insufficient activation energy required for optimal metabolic activity. The findings of this study align with previous reports. *Bacillus cereus* var. *mycoides* exhibited maximum amylase production at 50°C (Takasaki, 1976). Other *Bacillus* species have been reported to produce amylase efficiently within a temperature ranges of 30–70°C (Farooq *et al.*, 2023; Sharif *et al.*, 2023; Saha and Mazumdar, 2019; Dash *et al.*, 2015).

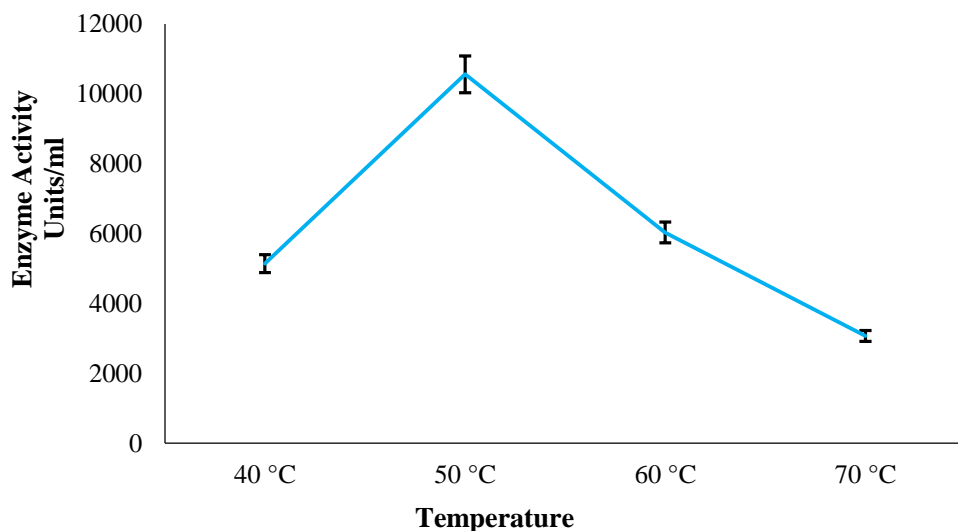


Fig. 3. Effect of temperature on β -amylase production by *Bacillus subtilis* C5W (Mean \pm S.E.M., n=3).

pH

Another key physical factor influencing enzyme production is pH. Changes in pH can alter the morphological characteristics of microorganisms, subsequently affecting their biochemical pathways and enzyme synthesis capabilities (Wiley, 2008). In culture media, microbial cells are highly sensitive to H⁺ ions, making pH a critical parameter that determines the chemical nature of metabolic end products, whether acidic or alkaline, which is essential for commercial applications.

Several studies have reported the optimal pH conditions for enzyme production in *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis* (Gaur *et al.*, 2012). In this study, the effect of medium pH on β -amylase production by *Bacillus subtilis* C5W was examined across a pH range of 5.0 to 9.0. Notably, enzyme production was observed across this broad pH range, with the highest yield recorded at pH 7.0 (Fig. 4). Similar findings have been reported, where *Bacillus specie* optimally synthesized amylase at pH 7.0 (Farooq *et al.*, 2023; Sharif *et al.*, 2023; Saha & Mazumdar, 2019). The ability of *Bacillus subtilis* C5W to produce amylase across a wide pH range (5.0–9.0) enhances its potential for industrial applications, as it can withstand both acidic and alkaline conditions in various industrial processes.

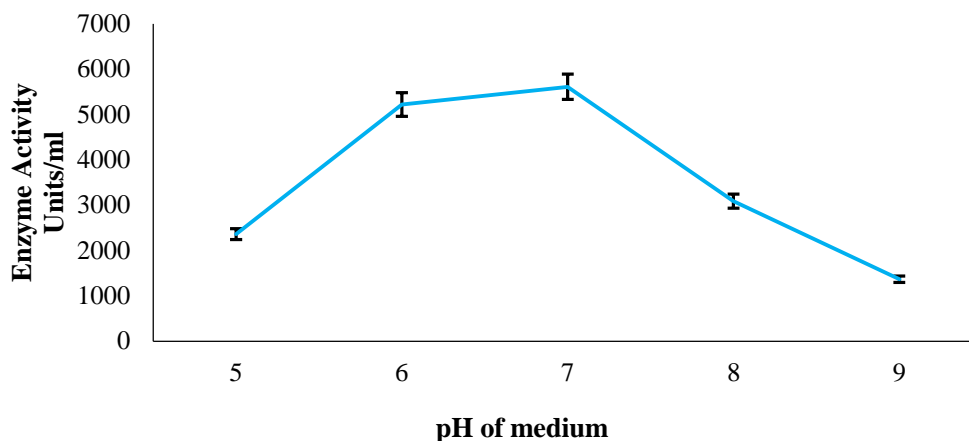


Fig. 4. Effect of pH on β -amylase production by *Bacillus subtilis* C5W (Mean \pm S.E.M., n=3)

Incubation Period

Incubation time is a crucial factor influencing bacterial growth and metabolism. To determine its effect on amylase production, *Bacillus subtilis* C5W was incubated for different time intervals. The highest amylase production (6027.77 U/ml) was recorded after 24 hours of incubation (Fig. 5). Lower enzyme production during the initial 18 hours could be attributed to the lag and log phases of bacterial growth, during which the microorganisms adapt to the new medium before initiating active division.

Since amylase is an inducible enzyme, its synthesis predominantly occurs during the stationary phase. In this study, maximum enzyme yield was achieved at 24 hours. However, extending the incubation period beyond 24 hours resulted in decreased amylase activity, likely due to the depletion of essential nutrients in the medium, leading to physiological stress in bacterial cells (Flores *et al.*, 1997).

In industrial biotechnology, rapid enzyme production is desirable, making the extracellular synthesis of β -amylase within 24 hours a commercially significant outcome. Dash *et al* (2015) also reported extracellular amylase production from *Bacillus subtilis* BI19 strain with in 24 hours of incubation. On contrary, optimum amylase production by *Bacillus* specie was achieved after 48 hours of incubation period (Sharif *et al.*, 2023; Saha & Mazumdar, 2019). Variations in the optimal incubation period for enzyme production depend on factors such as the strain's origin, morphological and physiological characteristics, and genetic adaptability to growth conditions. Prolonged incubation often results in reduced bacterial growth and enzyme synthesis, which may be attributed to catabolic repression caused by the readily available starch substrate (Lin *et al.*, 1998).

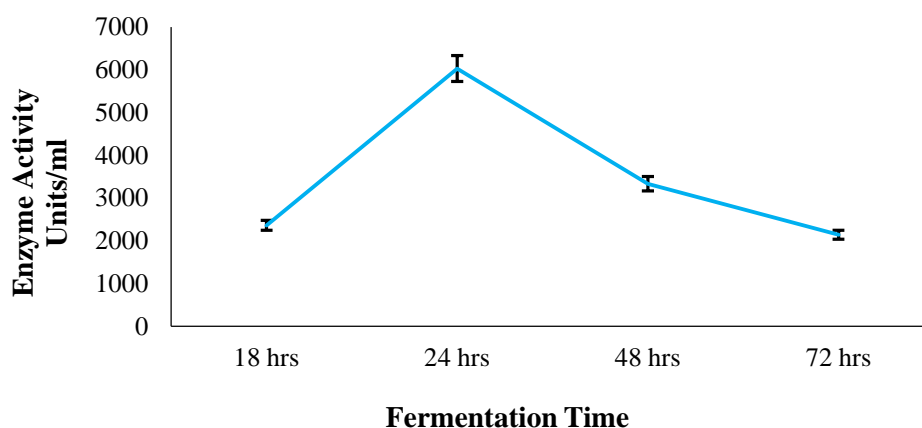


Fig. 5. Effect of incubation time on β -amylase production by *Bacillus subtilis* C5W (Mean \pm S.E.M., n=3)

Influence of nutritional components on β -Amylase production by *Bacillus subtilis* C5W

Carbon Source

The use of agricultural waste as a raw material for enzyme production offers an economical and sustainable approach by reducing the reliance on synthetic media while simultaneously addressing environmental pollution caused by these residues (Anto *et al.*, 2005). Agro-industrial byproducts serve as rich carbon sources that support microbial growth, and their type and concentration in culture media play a crucial role in the extracellular amylase production process (Akcan, 2011).

In this study, β -amylase biosynthesis was investigated using various carbon-based agro-industrial wastes by halothermophilic *Bacillus* strain. A total of eight agro-industrial substrates were evaluated alongside starch as a control to enhance enzyme production. Among all tested substrates, soluble starch resulted in the highest β -amylase yield (4444.44 U/ml), although significant enzyme production was also observed with other agro-wastes (Fig. 6). Similar findings were reported by Bello *et al.* (2021), who documented increased amylase biosynthesis in the presence of starch as a carbon source.

Among the agro-industrial residues, rice husk exhibited the highest β -amylase activity, contributing 57.5% relative enzyme production, followed by corn husk (50%). The utilization of corn cob and sugarcane bagasse resulted in 43.74% relative enzyme activity, while banana peel induced 40.62% enzyme production. Conversely, wheat straw, wheat husk, and potato peel yielded lower amylase levels (<40%) in *Bacillus subtilis* C5W. Several researches have been documented for the production of amylases using agro-industrial wastes. Rice bran has been proved to be cheapest agro-industrial substrate for amylase production by *Bacillus tequilensis* on commercial scale (Paul *et al.*, 2020). Mojumdar *et al.* (2019) reported the use of rice bran, potato peel and wheat bran for the production of amylase by *Bacillus amyloliquefaciens* via solid-state fermentation. Similarly, Singh *et al.* (2022) recorded the production of amylase using apple peels as carbon substrate by *Bacillus subtilis* at 50 °C, pH 5 after 24 hours of incubation (1394.145 IU/ml).

Based on these results, it is evident that while soluble starch remains the most effective substrate for amylase production, agro-industrial residues also offer considerable potential. All tested residues supported substantial enzyme synthesis, making them viable alternatives for cost-effective and sustainable β -amylase production.

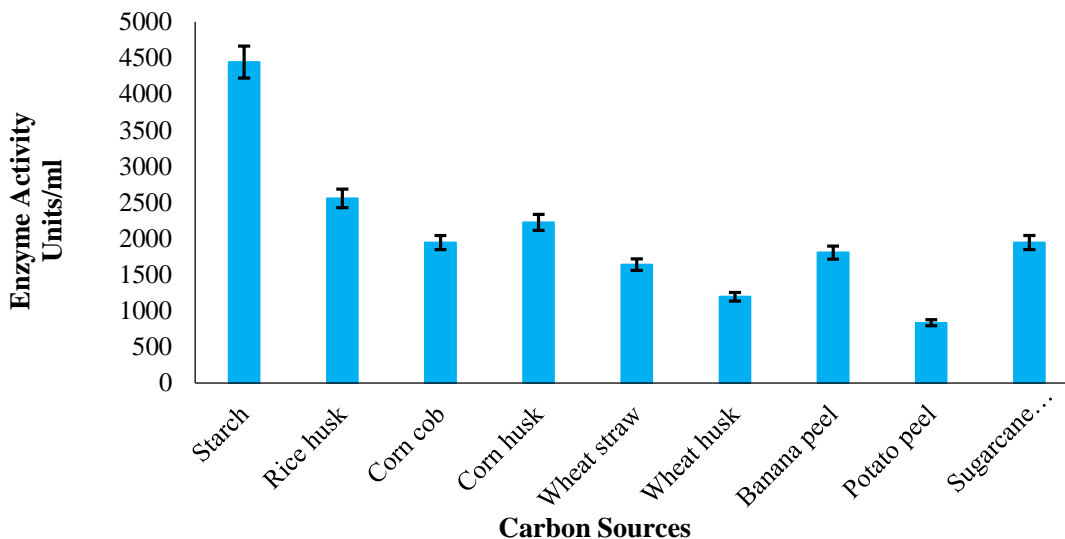


Fig. 6. Effect of carbon substrate on β -amylase production by *Bacillus subtilis* C5W (Mean \pm S.E.M., n=3)

Starch Concentration

The appropriate concentration of carbon substrate is crucial for maximizing enzyme production. *Bacillus subtilis* C5W exhibited a strong preference for starch as a carbon source; therefore, varying concentrations of starch were incorporated into the culture medium to determine the optimal level for β -amylase synthesis. The results indicated that enzyme production increased with raising starch concentrations, reaching its peak at 1.5% starch (Fig. 7). However, further increases beyond this threshold led to a decline in bacterial growth, which directly impacted β -amylase production. This reduction in enzyme yield at higher starch concentrations may be attributed to the rapid

depletion of starch by bacteria, triggering the accumulation of toxic metabolic by-products that inhibit microbial growth and enzymatic activity (Mishra and Behera, 2008). Previous studies have recommended starch concentrations ranging from 1% to 2% for efficient enzyme production in submerged fermentation systems (Zohra and Ahmad, 2012). While El-Kady *et al* (2017) reported that *Bacillus* species could produce amylase effectively at a starch concentration of 2.5%.

Nitrogen Source

During the fermentation process, microorganisms require both organic and inorganic nitrogen sources for extracellular amylase synthesis. To evaluate their impact, different nitrogen sources were tested. The results revealed that peptone significantly enhanced β -amylase production by *Bacillus subtilis* C5W (Fig. 8). Among the tested nitrogen sources, organic ones, particularly peptone and tryptone, supported higher enzyme yields compared to inorganic alternatives. These findings align with those of Al-Johani *et al.* (2017), who also identified peptone as the most effective nitrogen source for amylase production.

Conversely, inorganic nitrogen sources such as urea, ammonium sulfate, and potassium nitrate resulted in lower β -amylase production. Additionally, other studies have indicated that potassium nitrate enhances amylase yields in *Bacillus subtilis* KPA and *Bacillus subtilis* AK (Khusro and Aarti, 2015). Some reports also suggest that ammonium salts exert a stimulatory effect on enzyme synthesis in *Bacillus* species (Mahmood and Rahman, 2008). However, the findings of the present study contrast with these reports, as ammonium sulfate was observed to suppress β -amylase production in *Bacillus subtilis* C5W.

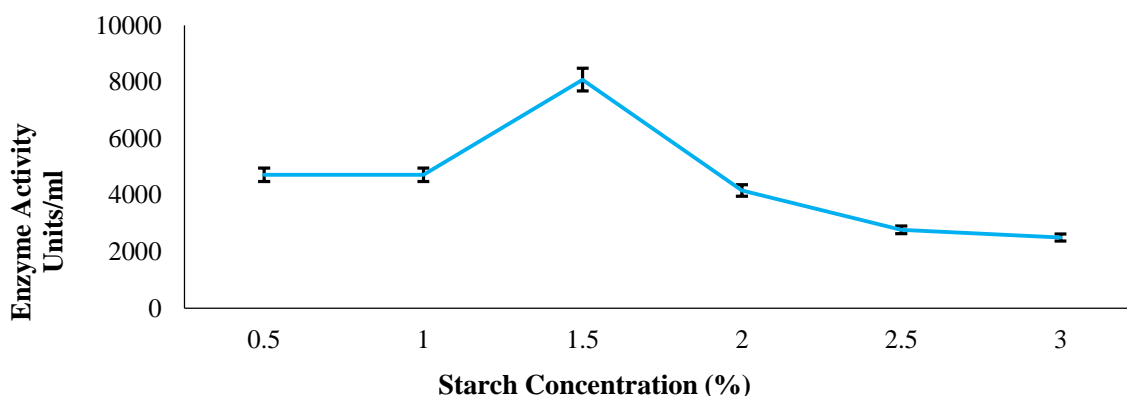


Fig. 7. Effect of substrate concentration on β -amylase production by *Bacillus subtilis* C5W (Mean \pm S.E.M., n=3)

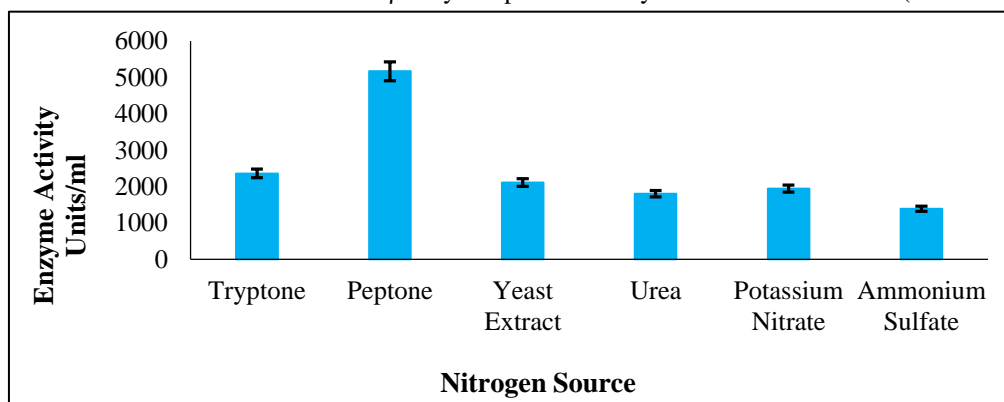


Fig. 8. Effect of nitrogen sources on β -amylase production by *Bacillus subtilis* C5W (Mean \pm S.E.M., n=3)

Peptone Concentration

An adequate supply of nutrients in the culture medium is essential for microbial growth and enzyme synthesis. After identifying peptone as an effective inducer for amylase production, different concentrations (0.025–0.125%) were tested to determine the optimal level for maximum β -amylase yield. The results revealed that a minimal

amount of peptone was sufficient to stimulate enzyme production in *Bacillus subtilis* C5W, with the highest enzyme activity (4831.11 U/ml) observed at 0.05% peptone concentration (Fig. 9).

Interestingly, increasing the concentration of peptone beyond this level had an inhibitory effect on amylase production. These findings contrast with those of Al-Johani *et al.* (2017), who reported that 1% peptone enhanced bacterial growth and amylase synthesis. The reduction in enzyme yield at higher peptone concentrations could be attributed to alterations in the surface charge (hydrophobicity) of the bacterial cell wall. Previous studies suggest that nitrogen source concentration significantly influences cell surface hydrophobicity, which in turn affects microbial metabolism and enzyme secretion (Prisca *et al.*, 2005).

Sodium chloride Concentration

The presence of an optimal concentration of NaCl in the microbial environment is a crucial chemical factor that can either promote or inhibit bacterial growth and enzyme synthesis. Sodium chloride plays a vital role in regulating cellular metabolic activities, while metal ions act as cofactors for many enzymes, facilitating various biochemical processes within bacterial cells. Most amylases are categorized as metalloenzymes, requiring specific ions for their activity.

To determine the impact of NaCl on β -amylase production, different concentrations (0–3%) were tested. The results indicated that a 1% NaCl concentration facilitated the highest enzyme yield (Fig. 10), highlighting the importance of monovalent ions in enhancing the production of starch-hydrolyzing enzymes. Retnaningrum and Purwestri (2016) similarly reported that *Bacillus* species exhibited maximum amylase production at 1M NaCl. However, as the concentration of cations increased beyond the optimal level, a decline in enzyme production was observed. Although *Bacillus subtilis* C5W demonstrated the ability to survive and grow in up to 10% NaCl at elevated temperatures—classifying it as a halothermophilic strain (Aman *et al.*, 2019)—it did not efficiently produce amylase at higher NaCl concentrations in this study. In contrast, Gaur *et al.* (2012) reported that amylase production was significantly enhanced at 5% NaCl concentration.

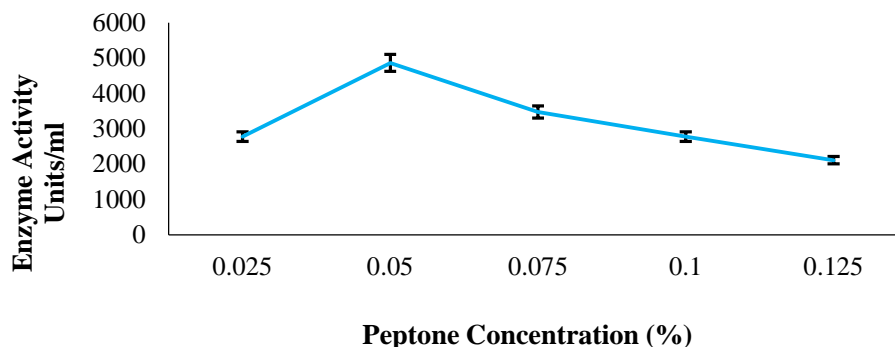


Fig. 9. Effect of peptone concentration on β -amylase production by *Bacillus subtilis* C5W (Mean \pm S.E.M., n=3)

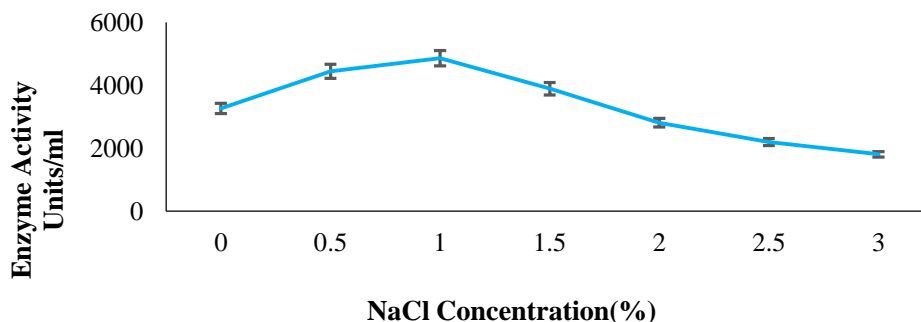


Fig. 10. Effect of NaCl concentration on amylase production by *Bacillus subtilis* C5W (Mean \pm S.E.M., n=3)

Micronutrients

Micronutrients in the fermentation medium play a crucial role in regulating the osmotic pressure of the culture environment, thereby influencing bacterial growth and enzyme synthesis. The incorporation of various

micronutrients can enhance microbial substrate utilization, ultimately improving enzyme production. The presence of trace elements in the medium supports optimal enzyme synthesis.

Potassium and organic phosphates are essential for the biosynthesis of both primary and secondary metabolites (Yoon *et al.*, 1989). A deficiency or complete absence of phosphorus ions in the medium can hinder bacterial growth and enzyme production, whereas excessive phosphorus levels may inhibit cell division and growth.

Additionally, magnesium and ferric ions were found to have a regulatory effect on β -amylase production by *Bacillus subtilis* C5W. Amylases are typically metal ion-dependent enzymes, requiring divalent cations such as manganese, magnesium, zinc, and ferric ions for their activity. Calcium ions, in particular, contribute to the thermostability of amylases. This effect is likely due to the exposure of hydrophobic amino acid residues on the protein surface, leading to a more compact protein structure through salting-out mechanisms facilitated by calcium ions. Furthermore, metal ions are believed to function as molecular bridges, linking the enzyme's active site to the substrate and enhancing amylase activity (Sirohi and Prakash, 2015).

CONCLUSION

In this study, the impact of different fermentation parameters, including temperature, incubation duration, pH, carbon and nitrogen sources, as well as NaCl concentration, was assessed to optimize β -amylase production by *Bacillus subtilis* C5W. The findings indicate that *Bacillus subtilis* C5W holds significant potential as a β -amylase producer, making it a promising candidate for industrial applications. However, ongoing research is being carried out to further explore the utilization of this strain for the development of biotechnological processes.

PREPRINT:

Ayisha Aman ullah, Azkah Qayyum ,Ayesha Siddiqui, Rashida Rahmat Zohra, Mahnaz Ahmad and Raheela Rahmat Zohra. Valorization of lignocellulosic waste using Halothermophilic *Bacillus* sp.: A sustainable approach for the production of glycoside hydrolase. Research Square. posted 24 Aug, 2023. 28 pages. URL: <https://doi.org/10.21203/rs.3.rs-3204586/v1>. This work is licensed under a CC BY 4.0 License

REFERENCES

- Akcan, N. (2011). High-level production of extracellular α -amylase from *Bacillus licheniformis* ATCC 12759 in submerged fermentation. *Romanian Biotechnology Letters*, 16(6): 6833–6840.
- AL-Awsy, M., S. Al Obiady and A. Al Obaidi (2017). Production of amylase enzyme from thermophilic bacteria using agricultural wastes as a substrate. *Australian Journal of Basic and Applied Sciences*, 11: 158-164.
- Al-Johani, N.B., M.N. Al-Seeni and Y.M. Ahmed (2017). Optimization of alkaline α -amylase production by thermophilic *Bacillus subtilis*. *African Journal of Traditional, Complementary and Alternative Medicines*, 14: 288-301. <https://doi.org/10.21010/ajtcam.v14i1.31>.
- Aman, A., R.R. Zohra, M. Ahmad and R.R. Zohra (2019). Screening of halothermophilic bacterial strain for starch saccharification enzyme. *International Journal of Biology and Biotechnology*, 16: 277-281.
- Anto, H., U. Trivedi and K. Patel (2005). Alpha amylase production by *Bacillus cereus* MTCC 1305 using solid-state fermentation. *Food Technology and Biotechnology*, 44: 241-245.
- Bala, S., D. Garg, K. Sridhar, B.S. Inbaraj, R. Singh, S. Kamma, M. Tripathi and M. Sharma (2023). Transformation of agro-waste into value-added bioproducts and bioactive compounds: micro/nano formulations and application in the agri-food-pharma sector. *Bioengineering (Basel)*, 10: 152. <https://doi.org/10.3390/bioengineering10020152>.
- Bano, S., S.A. Qader, A. Aman, M.N. Syed and A. Azhar (2011). Purification and characterization of novel α -amylase from *Bacillus subtilis*. *AAPS PharmSciTech*, 12: 255-261. <https://doi.org/10.1208/s12249-011-9586-1>.
- Bello, A. Y., N. Abdulkadir, S. Abubakar and A. Lawal (2021). Studies on screening and optimization of amylase enzyme production using bacteria isolated from soil. *Journal of Microbiology & Experimentation*, 9(6), 196–200. <https://doi.org/10.15406/jmen.2021.09.00343>
- Bernfeld, P. (1955). Amylases α and β , In: Colowick, S.P., Kaplan, N.O., (Eds.), *Methods in Enzymology*. Academic Press, New York pp. 149-158.
- Bibi., N. Ilyas, M. Saeed *et al.* (2023). Innovative production of value-added products using agro-industrial wastes via solid-state fermentation. *Environmental Science and Pollution Research*, 30: 125197-125213. <https://doi.org/10.1007/s11356-023-28765-6>.
- Dash, B.K., M.M. Rahman and P.K. Sarker (2015). Molecular identification of a newly isolated *Bacillus subtilis* BI19 and optimization of production conditions for enhanced production of extracellular amylase. *BioMed Research International*, 2015: 1–9. <https://doi.org/10.1155/2015/859805>.

- Demirkan, E., T. Sevgi and M. Başkurt (2017). Optimization of physical factors affecting the production of the α -amylase from a newly isolated *Bacillus* sp. M10 strain. *Karaelmas Fen ve Mühendislik Dergisi*, 7: 23-30.
- Dey, T., T. Bhattacharjee, P. Nag, N. Ritika, A. Ghata and A. Kuila (2021). Valorization of agro-waste into value-added products for sustainable development. *Bioresource Technology Reports*, 16: 100834. <https://doi.org/10.1016/j.biteb.2021.100834>.
- El-Kady, E.M., M.S. Asker, M.S. Hassanein, E.A. Elmansy and F.M. El-Beih (2017). Optimization, production, and partial purification of thermostable α -amylase produced by marine bacterium *Bacillus* sp. NRC12017. *International Journal of Pharmaceutical and Clinical Research*, 9(8): 558–570. <https://doi.org/10.25258/ijpcr.v9i08.9581>
- Elmansy, E.A., M.S. Asker, E.M. El-Kady, S.M. Hassanein and M. Fawkia (2018). Production and optimization of α -amylase from thermo-halophilic bacteria isolated from different local marine environments. *Bulletin of the National Research Centre*, 42: 1-9. <https://doi.org/10.1186/s42269-018-0033-2>.
- Farooq, M.A., S. Ali, A. Hassan, H.M. Tahir, S. Mumtaz and S. Mumtaz (2021). Biosynthesis and industrial applications of α -amylase: a review. *Archives of Microbiology*, 203: 1-12. <https://doi.org/10.1007/s00203-020-02128-y>.
- Farooq, M.A., S. Ali, A. Hassan, R. Sulayman, M.A. Kaleem, H. Shahzad, M. Summer, A. Latif and T. Tanveer (2023). Enhanced bacterial α -amylase production using mutant strains through submerged fermentation. *Punjab University Journal of Zoology*, 38: 99-107. <https://doi.org/10.17582/journal.pujz/38.1.99.107>.
- Flores, M.E., R. Perez and C. Huitron (1997). β -Xylosidase and xylanase characterization and production by *Streptomyces* species CH-M-1035. *Letters in Applied Microbiology*, 24: 410-416. <https://doi.org/10.1046/j.1472-765X.1997.00149.x>.
- Gaur, D., P.K. Jain and V. Bajpai (2012). Production of extracellular α -amylase by thermophilic *Bacillus* sp. isolated from arid and semi-arid region of Rajasthan, India. *Journal of Microbiology and Biotechnology Research*, 2: 675-684.
- Gaur, S., M. Kaur, R. Kalra, E.R. Rene and M. Goel (2024). Application of microbial resources in biorefineries: Current trend and future prospects. *Heliyon*, 10: e28615. <https://doi.org/10.1016/j.heliyon.2024.e28615>.
- Indriati, G. and R.R.P. Megahati (2018). Isolation of thermophilic bacteria and optimizing the medium growth conditions. *International Journal of Current Microbiology and Applied Sciences*, 7: 1457-1464. <https://doi.org/10.20546/ijcmas.2018.701.177>.
- Jahangeer, M., M.U. Rehman, R. Nelofer, M. Nadeem, B. Munir, W. Smulek, T. Jesionowski and S.A. Qamar (2024). Biotransformation of lignocellulosic biomass to value-added bioproducts: insights into bio-saccharification strategies and potential concerns. *Topics in Catalysis*. <https://doi.org/10.1007/s11244-024-01941-9>.
- Khusro, A. and C. Aarti (2015). Molecular identification of newly isolated *Bacillus* strains from poultry farm and optimization of process parameters for enhanced production of extracellular amylase using OFAT method. *Research Journal of Microbiology*, 10(9): 393-420. <https://doi.org/10.3923/jm.2015.393.420>
- Kojima (2010). A report on new products introduction—thermostable microbial β -amylase: the first successful industrial-scale production in the world. *Enzyme Wave*, 13.
- Kumar, A., S. Dhiman, B. Krishan et al. (2024). Microbial enzymes and major applications in the food industry: a concise review. *Food Prod. Processing and Nutrition*, 6: 85. <https://doi.org/10.1186/s43014-024-00261-5>.
- Kumar, C.G. and H. Takagi (1999). Microbial alkaline protease: from a bioindustrial viewpoint. *Biotechnology Advances*, 17: 561-594. [https://doi.org/10.1016/S0734-9750\(99\)00027-0](https://doi.org/10.1016/S0734-9750(99)00027-0).
- Lin, L.L., C.C. Chyau and W.H. Hsu (1998). Production and properties of a raw-starch-degrading amylase from thermophilic and alkaliphilic *Bacillus* sp. TS-23. *Biotechnology and Applied Biochemistry*, 28: 61-68. <https://doi.org/10.1111/j.1470-8744.1998.tb00513.x>.
- Mahmood, S. and S.R. Rahman (2008). Production and partial characterization of extracellular α -amylase by *Trichoderma viride*. *Bangladesh J. of Microbiology*, 25(2): 99-103. <https://doi.org/10.3329/bjm.v25i2.4870>.
- Mesbah, N.M. (2022). Industrial biotechnology based on enzymes from extreme environments. *Frontiers in Bioengineering and Biotechnology*, 10: 870083. <https://doi.org/10.3389/fbioe.2022.870083>.
- Mishra, S., N. Behera (2008). Amylase activity of starch-degrading bacteria isolated from soil receiving kitchen wastes. *African Journal of Biotechnology*, 7: 3326–3331.
- Mojumdar, A. and J. Deka (2019). Recycling agro-industrial waste to produce amylase and characterizing amylase–gold nanoparticle composite. *International Journal of Recycling of Organic Waste in Agriculture*, 8: 263–269. <https://doi.org/10.1007/s40093-019-00298-4>
- Mujtaba, M., L.F. Fraceto, M. Fazeli, S. Mukherjee, S.M. Savassa, G.A. De Medeiros, A.D.E.S. Pereira, S.D. Mancini, J. Lipponen and F. Vilaplana (2023). Lignocellulosic biomass from agricultural waste to the circular

- economy: a review with focus on biofuels, biocomposites and bioplastics. *Journal of Cleaner Production*, 402: 136815. <https://doi.org/10.1016/j.jclepro.2023.136815>.
- Mukherjee, P., I. Mondal, D. Dey, E. Dan, F. Khatun and S. Tewari (2023). An overview on microbial enzymes and their industrial applications. *Journal of Survey in Fisheries Sciences*. <https://doi.org/10.53555/sfs.v10i1s.2120>.
- Nag, M., D. Lahiri, S. Garai, D. Mukherjee and R.R. Ray (2021). Regulation of β -amylase synthesis: a brief overview. *Molecular Biology Reports*, 48(9): 6503-6511. <https://doi.org/10.1007/s11033-021-06613-5>.
- Niyonzima (2020). Microbial enzymes: roles and applications in industries. In: *Microorganisms for Sustainability*. <https://doi.org/10.1007/978-981-15-1710-5>.
- Patil, S.R. and A. Dayanand (2006). Optimization of process for the production of fungal pectinases from deseeded sunflower head in submerged and solid-state conditions. *Bioresource Technology*, 97: 2340-2344. <https://doi.org/10.1016/j.biortech.2005.10.025>.
- Paul, J.S., E. Beliya, S. Tiwari, K. Patel, N. Gupta and S.K. Jadhav (2020). Production of biocatalyst α -amylase from agro-waste 'rice bran' by using *Bacillus tequilensis* TB5 and standardizing its production process. *Biocatalysis and Agricultural Biotechnology*, 26: 101648. <https://doi.org/10.1016/j.bcab.2020.101648>
- Paul, J.S., N. Gupta, S. Beliya Tiwari and S.K. Jadhav (2021). Aspects and recent trends in microbial α -amylase: a review. *Applied Biochemistry and Biotechnology*, 193: 2649-2679. <https://doi.org/10.1007/s12010-021-03546-4>.
- Prisca, S.Z., D. Marie-Lise, D. Nicola, A. Michael and U. Job (2005). Influence of fermentation medium composition on physicochemical surface properties of *Lactobacillus acidophilus*. *Applied and Environmental Microbiology*, 71: 8165-8173.
- Rasooli, I., S.D.A. Astaneh, H. Borne and K.A. Barchini (2008). A thermostable α -amylase producing natural variant of *Bacillus* spp. isolated from soil in Iran. *American Journal of Agricultural and Biological Sciences*, 3: 591-596. <https://doi.org/10.3844/ajabssp.2008.591.596>.
- Rekadwad, B.N. (2015). Characterization of amylase from industrially important thermophilic microorganism: *Geobacillus thermoleovorans* strain Rekadwadsis. *International Journal of Life Sciences Biotechnology and Pharmaceutical Research*, 4: 26-30.
- Retnaningrum, E. and Y.A. Purwestri (2016). Molecular identification and optimization of culture conditions of amylase-producing bacteria isolated from green algae in the coast side of Southern Sea, Yogyakarta, Indonesia. *AIP Conference Proceedings*, 1755: 030002. <https://doi.org/10.1063/1.4958473>.
- Saha, S.P. and D. Mazumdar (2019). Optimization of process parameter for alpha-amylase produced by *Bacillus cereus* amy3 using one factor at a time (OFAT) and central composite rotatable (CCRD) design-based response surface methodology (RSM). *Biocatalysis and Agricultural Biotech.*, 19: 101168. <https://doi.org/10.1016/j.bcab.2019.101168>.
- Saini, R., H.S. Saini and A. Dahiya (2017). Amylases: Characteristics and industrial applications. *Journal of Pharmacognosy and Phytochemistry*, 6: 1865-1871.
- Sanger, F. and A. R. Coulson (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.*, 94 (3): 441-8.
- Sharif, S., A.H. Shah, A. Fariq, S. Jannat, S. Rasheed and A. Yasmin (2023). Optimization of amylase production using response surface methodology from newly isolated thermophilic bacteria. *Heliyon*, 9: e12901. <https://doi.org/10.1016/j.heliyon.2023.e12901>.
- Singh, R., S. Langyan, S. Sangwan, P. Gaur, F. N. Khan, P. Yadava, and P. K. Sahu (2022). Optimization and production of alpha-amylase using *Bacillus subtilis* from apple peel: comparison with alternate feedstock. *Food Bioscience*, 49: 101978. <https://doi.org/10.1016/j.fbio.2022.101978>
- Sirohi, R. and V. Prakash (2015). Effect of metal ions on amylase production using *Bacillus subtilis* isolated from soil of Almora District, Uttarakhand, India. *International Journal of Pure and Applied Bioscience*, 3: 37-41.
- Takasaki, Y. (1976). Production and utilization of amylase and pullulanase from *Bacillus cereus* ver *mycoides*. *Agricultural and Biological Chemistry*, 40: 1515.
- Tiwari, K., G. Singh, G. Singh, S.K. Sharma and S.K. Singh (2022). Industrial biotechnology: An Indian perspective. *Journal of Applied Biology & Biotechnology*, 10: 22-33. <https://doi.org/10.7324/jabb.2022.100503>.
- Willey, J.M., L.M. Sherwood, C.J. Woolverton, Prescott, Harley and Kleins (2008). *Microbiology*, 7th Ed. McGraw Hill Co. Inc Boston.
- Zohra, R.R. and M. Ahmad (2012). Optimization of cultural conditions for production of amylase from thermophilic *Bacillus* sp. *Pakistan Journal of Biochemistry and Molecular Biology*, 45(2): 99-103.

(Accepted for publication April 2025)