

Optimization of Submerged Fermentation Parameters for Enhanced α -Amylase Production by *Aspergillus niger*

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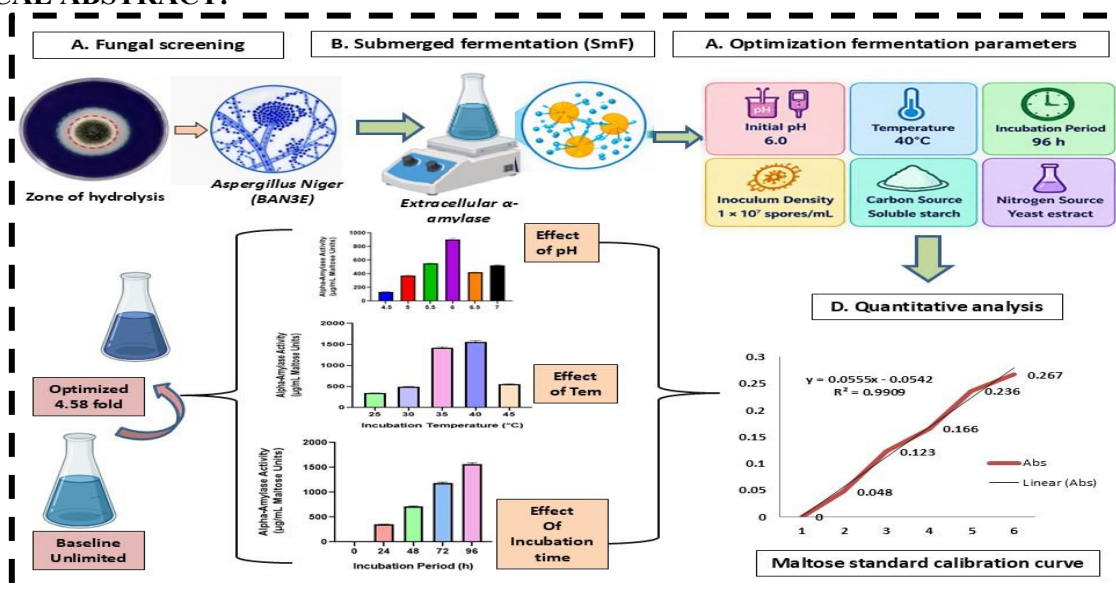
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Abstract: The effort assesses *Aspergillus niger* (strain BAN3E) production, process parameter optimization, and quantitative yield augmentation of extracellular α -amylase using submerged fermentation (SMF). After iodine staining, fungal screening on starch agar salt medium revealed a clear zone of hydrolysis surrounding center vegetative colonies, confirming active amylolytic capacity. Quantitative determination of enzymatic activity was conducted via the 3,5-dinitrosalicylic acid (DNS) reducing sugar assay against a standard maltose calibration curve, $R^2 = 0.9909$. Using a systematic One-Factor-At-A-Time (OVAT) approach, critical physicochemical variables including initial pH, incubation temperature, fermentation time, inoculum density, and carbon/nitrogen supplements were optimized. Maximal α -amylase production was achieved at an initial medium pH of 6.0 ($904.11 \pm 18.45 \mu\text{g/mL}$), an incubation temperature of 40°C ($1561.64 \pm 28.90 \mu\text{g/mL}$), a fermentation duration of 96 hours, and a spore inoculum density of 1×10^7 spores/mL. Substrate optimization revealed that soluble starch served as the most effective inducer ($1561.64 \pm 28.90 \mu\text{g/mL}$), preventing carbon catabolite repression, while yeast extract was the superior nitrogen source ($1561.64 \pm 28.90 \mu\text{g/mL}$). Overall process parameter tuning resulted in a statistically significant ($p < 0.05$) 4.58-fold quantitative enhancement in enzyme activity over baseline levels ($341.02 \pm 7.82 \mu\text{g/mL}$). These findings highlight the industrial potential of *A. niger* BAN3E as a high-titer fungal host for commercial starch liquefaction, baking, and bioethanol bioprocesses.

Key Words: *Aspergillus niger* BAN3E, α -Amylase production, Submerged fermentation (SmF), Process optimization, Zone of hydrolysis, Maltose standard curve, Starch liquefaction.

GRAPHICAL ABSTRACT:



Highlights:

- *Aspergillus niger* BAN3E produced high-titer α -amylase via submerged fermentation.
- Peak activity reached 1561.64 $\mu\text{g/mL}$ at pH 6.0, 40°C, 96 h, and 1×10^7 spores/mL.
- Yield increased 4.58-fold using starch and yeast extract.

1. INTRODUCTION:

Internal alpha-1,4-glucosidic linkages in starch, glycogen, and similar polysaccharides are endohydrolyzed by alpha-Amylase (alpha-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1), a crucial hydrolytic enzyme. Random enzymatic cleavage produces glucose in the alpha-anomeric structure, maltose, and linear and branched oligosaccharides (Kozłowski & Różańska, 2020). The exo-acting enzyme beta-amylase (EC 3.2.1.2) successively eliminates maltose units only from the non-reducing ends of starch chains, in contrast to this endo-acting process. Alpha-amylase is essential to industrial bioprocesses such as starch liquefaction, food processing, brewing, textile desizing, detergent formulation, paper manufacturing, and medicines because it quickly lowers the viscosity of gelatinized starch solutions (Okuyama et al., 2016). Microbial sources are chosen for commercial exploitation, despite the fact that alpha-amylases are extensively dispersed in plants, animals, and microbes (Far et al., 2020). Rapid fungal or bacterial growth rates, cheap production costs, year-round availability, simplicity of genetic modification (Rana et al., 2013), and high volumetric yields are some of the main operational benefits of microbial fermentation. Because of its strong metabolic capacity (Souza & Magalhães, 2010), capability to employ a variety of carbon substrates, and great potential for extracellular protein secretion, *Aspergillus niger* is a filamentous fungus that is frequently utilized for industrial enzyme manufacturing (Gopinath et al., 2017). Because it provides exact control over physical and chemical factors such pH, incubation temperature, dissolved oxygen levels, and nutrient distribution, submerged fermentation (SMF) is the standard method for commercial alpha-amylase recovery (Kabir et al., 2025; Somadder et al., 2025). However, environmental and physiological variables have a significant impact on alpha-amylase output and catalytic productivity in submerged culture (Raul et al., 2014). The primary process variables controlling the expression of enzymes and fungal growth are complicated interaction between environmental and dietary factors controls extracellular alpha-amylase expression and release during submerged fermentation. Among these, initial pH is important because it controls the kinetics of plasma membrane transport, the intake of nutrients, and the overall structural stability of the released enzyme (Siddiqui et al., 2023). The target protein's thermal inactivation thresholds, fungal vegetative growth, and cellular metabolic rates are all directly impacted by incubation temperature (Rizwanuddin et al., 2023; Siddiqui et al., 2023). The crucial shift from initial vegetative biomass growth to active secondary metabolite and enzyme release during fermentation is determined by the incubation duration (Rizwanuddin et al., 2023). Additionally, early development dynamics are determined by inoculum density, which affects culture viscosity, mass transfer, and the availability of dissolved oxygen in the liquid medium (Bretträger et al., 2024). Every unique fermentation process requires rigorous parameter adjustment since the ideal environmental conditions differ significantly between wild-type and commercial strains of *A. niger* (de Carvalho-Gonçalves et al., 2026; Huang et al., 2024). Process optimization is a crucial precondition for scalable bioprocessing as minor modifications to the initial growth conditions can result in notable improvements in enzyme production (Adeyeye et al., 2026). In order to increase *Aspergillus niger*'s extracellular alpha-amylase production, the current work methodically assessed and improved important submerged fermentation parameters, such as starting pH, incubation temperature, incubation length, inoculum size, carbon sources, and nitrogen sources (Balakrishnan et al., 2021). Using starch hydrolysis experiments, fungal strains were initially isolated and screened for amylolytic capability in order to achieve the main goal of increasing enzyme production through process optimization. To guaranty batch-to-batch uniformity during liquid state fermentation, a standardized fungal spore inoculum was grown after isolate selection.

2. MATERIALS AND METHODS:

2.1. Materials & reagents:

Potato dextrose agar (PDA) and potato dextrose broth (PDB) were used for fungal culture, maintenance, and liquid state fermentation. Soluble starch was used as the primary carbon substrate for screening and enzymatic assay procedures. The starch agar medium was prepared using analytical grade potassium nitrate (KNO_3), dipotassium hydrogen phosphate (K_2HPO_4), magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), sodium chloride (NaCl), ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), and bacteriological agar. The 3,5-dinitrosalicylic acid (DNS) reagent was formulated using analytical-grade DNS, sodium potassium tartrate tetrahydrate (Rochelle salt), and sodium hydroxide (NaOH). High-purity maltose monohydrate was used as the reference standard for calibration. All aqueous solutions were prepared using double-distilled water.

2.2. Microorganism and Primary Screening:

Aspergillus niger was isolated from soil samples using serial dilution and pour plate methods on Potato Dextrose Agar (PDA). Fungal isolates were identified based on morphological colony characteristics and microscopic examination following lactophenol cotton blue staining. Primary screening for amylolytic activity was conducted using a starch agar medium containing 20 g/L soluble starch, 1 g/L KNO₃, 0.5 g/L K₂HPO₄, 0.5 g/L MgSO₄ · 7H₂O, 0.5 g/L NaCl, 0.01 g/L FeSO₄ · 7H₂O, and 15 g/L agar. Solidified agar plates were spot-inoculated with pure culture loopfuls and incubated at 30°C for 48 hours. Amylase secretion was confirmed by flooding the incubated plates with iodine solution to observe the formation of a clear hydrolysis zone surrounding the colony. Pure cultures were maintained on PDA slants at 4°C and subculture periodically (Rabab et al., 2025).

2.3. Inoculum Preparation:

Fungal spore suspensions were prepared by adding 5.0 mL of sterile deionized water (containing 0.1% v/v Tween-80) to 7-day-old mature PDA slants of *A. niger* and gently scraping the surface. The harvested spore suspension was transferred into fresh sterile Potato Dextrose Broth (PDB) and incubated at 30 ± 1°C for 7 days to achieve a standardized spore concentration of approximately 5.0 × 10⁸ spores/mL. For all subsequent fermentation experiments, the working inoculum concentration was adjusted to 1 × 10⁷ spores/mL (Guo et al., 2023).

2.4 Submerged Fermentation and Extraction:

Submerged fermentation was conducted in Erlenmeyer flasks using a potato dextrose medium prepared with an infusion of 200 g potato and 20 g dextrose per liter. The pH of the medium was adjusted as per experimental requirements prior to sterilization at 121°C (15 psi) for 15 minutes. Flasks were inoculated with 1.0 mL of the standardized spore suspension inside a laminar air flow chamber and incubated under static/shaking conditions based on the experimental setup. Following fermentation, the culture broth was centrifuged at 2,000 rpm for 5 minutes, and the supernatant was filtered through Whatman No. 1 filter paper to collect the cell-free crude enzyme extract (Guo et al., 2023).

2.5 Optimization of Fermentation Parameters

Using a single-factor-at-a-time (OVAT) approach, optimization was carried out by changing one process variable while keeping the rest unchanged. Incubation Period: To determine the ideal enzyme secretion kinetics, fermentation flasks were incubated for a range of times, from 24 to 120 hours (assessed up to 96 hours). Initial pH: Before sterilization, the liquid potato dextrose medium was adjusted between 4.5 and 7.0 (at intervals of 0.5) and incubated at 30 for 48 hours. Temperature of Incubation: For 48 hours, fermentation cultures made at pH 7.0 were incubated at various temperatures (Guo et al., 2023).

2.6 α-Amylase Activity Assay

The α-Amylase activity was assayed following the 3,5-dinitrosalicylic acid (DNS) procedure to measure the amount of reducing sugars released from soluble starch. Standard Curve Preparation: A maltose stock solution was prepared by dissolving 50 mg of maltose in 50 mL of distilled water. A working standard solution was generated by diluting 10 mL of the stock solution into 40 mL of distilled water. Aliquots of 0.2, 0.4, 0.6, 0.8, and 1.0 mL of the working solution were pipetted into test tubes and adjusted to a final volume of 1.0 mL with distilled water. DNS reagent addition each standard tube and a blank tube (containing 1.0 mL distilled water), 1.0 mL of freshly prepared DNS reagent was added. The mixture was heated in a boiling water bath at 100°C for 5 minutes, cooled to room temperature, and diluted with 10.0 mL of distilled water. Optical density was recorded at 540 nm using a spectrophotometer to construct the maltose standard calibration curve.

$$y = 0.0555x + 0.0542 \quad (R^2 = 0.9909)$$

Where y is the absorbance at 540 nm and x is the concentration of maltose released in μg . Enzyme reaction setup sample analysis, 1.0 mL of 1% (w/v) soluble starch substrate was mixed with 1.0 mL of crude enzyme extract in a reaction tube. For the control blank, 1.0 mL of distilled water was combined with 1.0 mL of 1% starch solution in place of the enzyme extract. Incubation & color development both the sample and blank tubes were incubated at 30°C for 10 minutes to allow enzymatic hydrolysis. The catalytic reaction was arrested by adding 1.0 mL of DNS reagent to each tube. The reaction tubes were placed in a boiling water bath at 100°C for 5 minutes to develop the reddish-orange color, then cooled to room temperature. Spectrophotometric quantification after cooling, 10.0 mL of distilled water was added to each tube and mixed thoroughly. The optical density of the reaction sample was measured against the blank at 540 nm using a spectrophotometer. All assays were carried out in duplicate, with three technical readings taken per sample.

2.7. Statistical Analysis

All fermentation and enzymatic assay experiments were performed independently in duplicate, with three technical replicate readings taken for each individual sample ($n = 6$). Experimental data were analyzed and expressed as mean $\pm$ standard deviation (SD). Statistical evaluations were performed using One-Way Analysis of Variance (ANOVA) followed by Tukey's Post-Hoc Honest Significant Difference (HSD) test to determine significant differences between experimental treatment groups. A p -value threshold of $p < 0.05$ was considered statistically significant. Data processing, linear regression calculations for standard curves, and statistical graphics generation were executed using GraphPad Prism (Version 9.0) and Microsoft Excel 2019.

3.RESULT & DISCUSSION:

3.1. Screening of *Aspergillus niger* for Amyolytic Potential

Extracellular alpha-amylase secretion on starch agar plates was verified by primary screening of soil-isolated fungus strains. Starch hydrolysis was seen as a clear zone around the vegetative mycelial development after 48 hours of incubation at 30°C and iodine flooding. *Aspergillus niger* strain BAN3E demonstrated the best amyolytic efficiency among the assessed isolates.

Table 1. Primary screening of *Aspergillus niger* for amyolytic activity on starch agar medium

Fungal Isolate	Incubation Conditions	Colony Hydrolysis Zone	Amyolytic Response	Selection Status
<i>Aspergillus niger</i> (BAN3E)	30°C for 48 h	Prominent clear zone (attached in figure 1)	Positive (α -amylase producer)	Selected for fermentation

The catalytic transformation of macromolecular starch into non-staining maltose and dextrin oligosaccharides by secreted microbial alpha-amylase is directly demonstrated by the development of a transparent hydrolysis zone after iodine treatment was demonstrated in **table 1**. It is generally known that filamentous fungus of the genus *Aspergillus*, especially *A. niger*, have a strong ability to produce and release large amounts of enzymes that break down starch into their growth medium. The strain BAN3E's shown amyolytic capacity validated its applicability as a functional biological source for experiments using submerged batch fermentation.

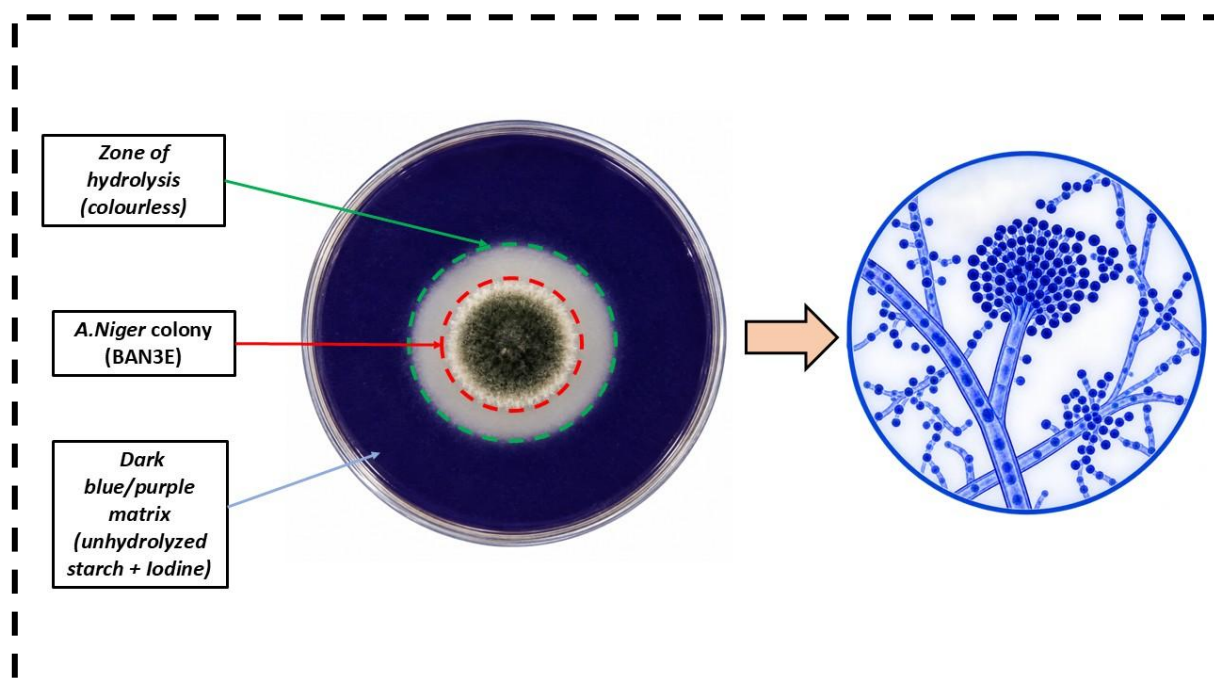


Figure 1. Zone of Hydrolysis Produced by *Aspergillus niger* on Starch Agar After Iodine Treatment. The clear halo surrounding the fungal colony represents the zone of starch hydrolysis, whereas the dark blue-purple region indicates unhydrolyzed starch complexed with iodine. The formation of the clear zone indicates extracellular amyolytic activity of *A. niger*, resulting in enzymatic degradation of starch into smaller hydrolysis products.

3.2. Standard Calibration Curve for Maltose

Quantification of released reducing sugars was carried out using a maltose standard calibration curve via the DNS method. Linear regression analysis showed a strong correlation between absorbance at 540 nm and maltose concentration over the evaluated range (0.2 to 1.0 mg/mL) was mentioned at **figure 2 & table 2**.

Table 2. Standard Calibration Curve Data for Maltose Quantification at 540 nm.

Working Standard Tube	Maltose Concentration (mg/mL)	Absorbance at 540 nm (Mean $\pm$ SD)
Blank	0.00	0.00
Tube 1	0.20	0.048 $\pm$ 0.002
Tube 2	0.40	0.123 $\pm$ 0.004
Tube 3	0.60	0.166 $\pm$ 0.005
Tube 4	0.80	0.236 $\pm$ 0.006
Tube 5	1.00	0.267 $\pm$ 0.007

Note: All assays were performed in triplicate ($n = 3$). Values are presented as Mean $\pm$ Standard Deviation (SD).

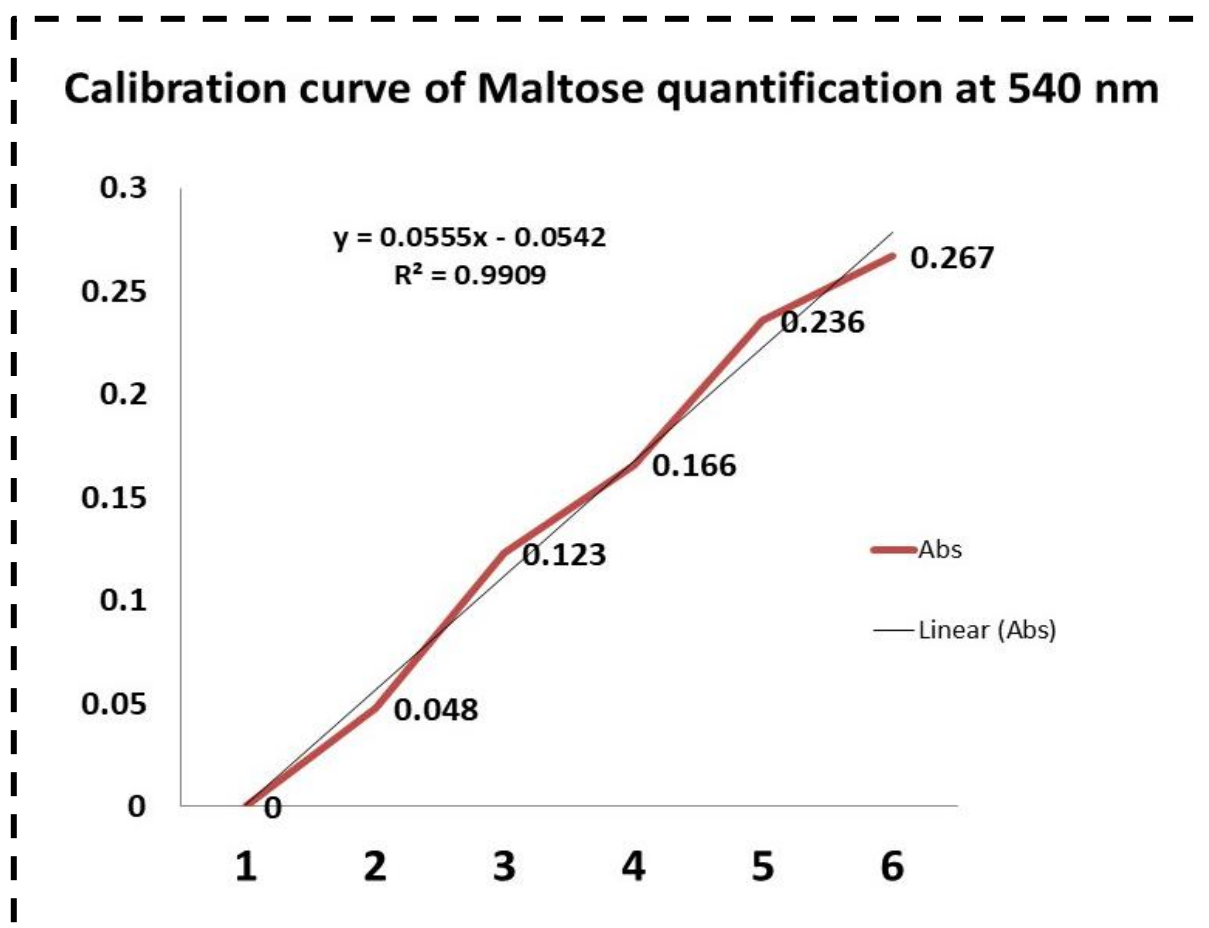


Figure 2. Calibration curve of maltose quantification at 540 nm.

3.3 Effect of Initial pH on α -Amylase Production

The initial pH of the potato dextrose medium significantly influenced α -amylase secretion by *Aspergillus niger* during submerged fermentation ($p < 0.05$). Maximum enzyme activity was achieved at pH 6.0, yielding 904.11 μ g/mL of maltose equivalents. Lower production titers were recorded under strongly acidic conditions (pH 4.5 and 5.0) as well as near-neutral pH (6.5 and 7.0) was shown in **figure 3**.

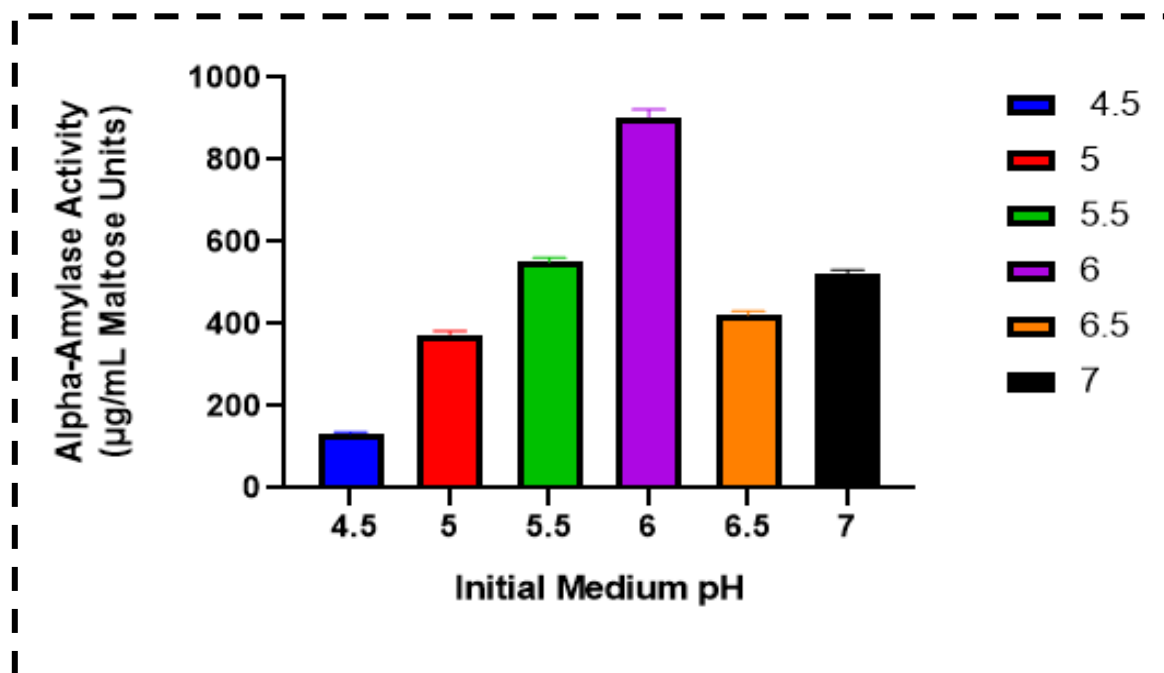


Figure 3. Effect of initial medium pH (4.5 – 7.0) on α -amylase production by *Aspergillus niger* in submerged liquid culture after 48 h of incubation at 30°C. Data points represent mean values ($\mu\text{g/mL}$ maltose equivalents).

3.4 Effect of Incubation Temperature on α -Amylase Yield

Incubation temperature significantly influenced α -amylase accumulation during submerged liquid fermentation ($p < 0.05$). Maximum enzyme expression was observed at 40°C (1561.64 $\mu\text{g/mL}$ maltose released), while a sharp decrease was recorded at 45°C. The highest α -amylase activity was observed at 40 °C (1561.64 $\pm$ 28.90 $\mu\text{g/mL}$ maltose units), followed by a marked decline at 45 °C was shown in **figure 4**.

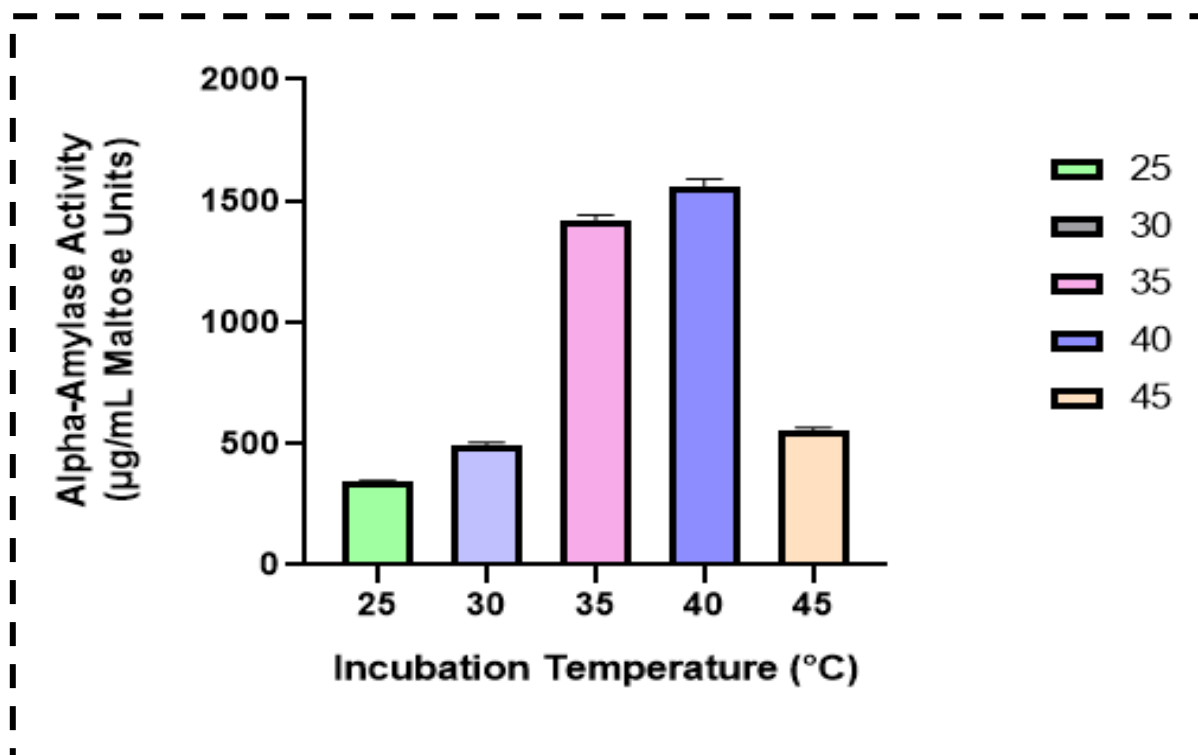


Figure 4. Effect of incubation temperature (25°C – 45°C) on α -amylase production by *Aspergillus niger* in submerged potato dextrose broth after 48 h of fermentation at initial pH 7.0. Data points represent mean values ($\mu\text{g/mL}$ maltose equivalents).

3.5 Effect of Incubation Period on α -Amylase Synthesis

The duration of fermentation directly dictates the metabolic phase of the fungus and the accumulation kinetics of the target enzyme. As shown in Figure 3, α -amylase production by *Aspergillus niger* increased progressively over time, reaching its peak at 96 hours post-inoculation. During the early phase (0 – 24 h), low enzyme activity was recorded as the fungus was undergoing its adaptation (lag) phase. Between 48 and 96 hours, active hyphal branching and biomass accumulation during the exponential and early stationary growth phases triggered a rapid, multi-fold increase in extracellular enzyme secretion. Extending incubation beyond 96 hours often results in a plateau or decline in enzyme activity due to nutrient depletion, autolysis of fungal mycelia, and the release of endogenous proteolytic enzymes into the fermented broth. α -Amylase production increased progressively with incubation time, reaching maximum activity at 96 h (1561.64 ± 28.90 $\mu\text{g/mL}$ maltose units) was shown in figure 5.

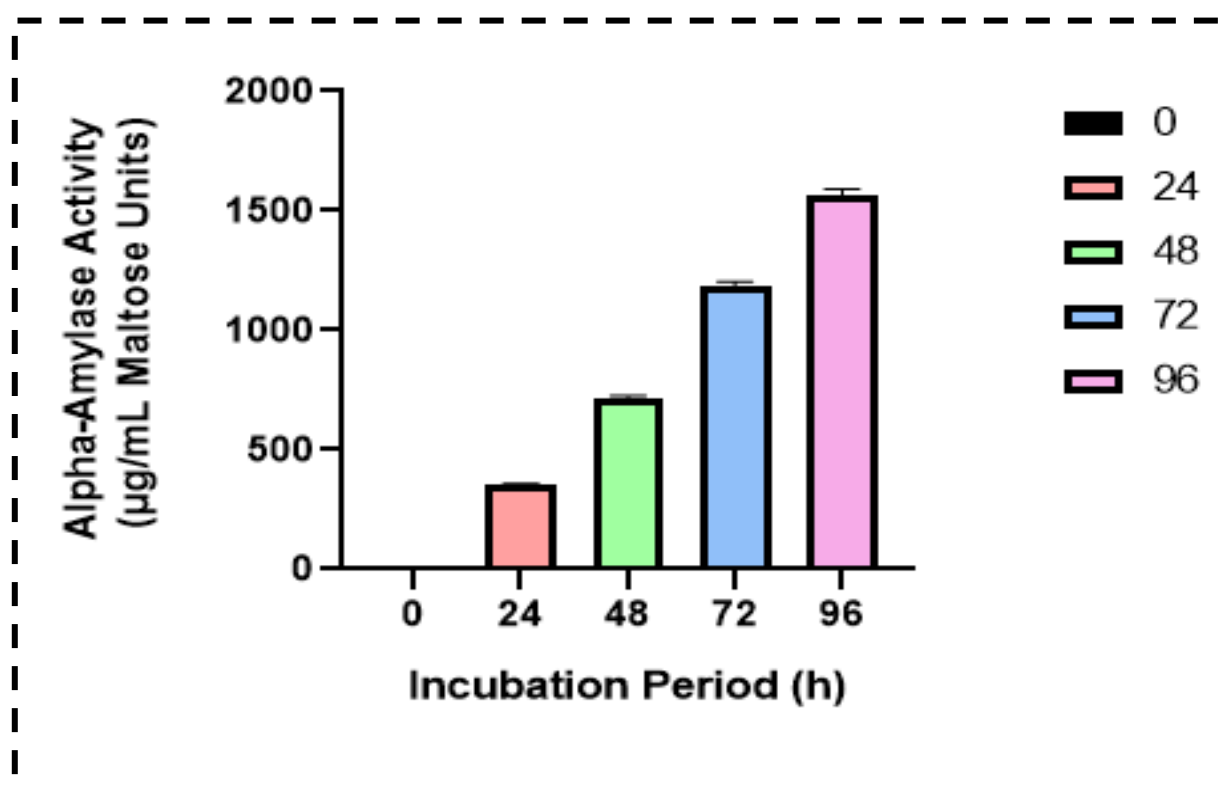


Figure 5. Time-course profile of α -amylase secretion by *Aspergillus niger* over a 96-hour submerged fermentation period in potato dextrose broth. Data points represent mean values ($\mu\text{g/mL}$ maltose equivalents).

3.6 Effect of Inoculum Size on α -Amylase Production

Inoculum density significantly governs the balance between vegetative biomass production and target metabolite formation in submerged bioprocessing. As illustrated in Figure 4, maximum enzyme accumulation occurred at an optimal inoculum concentration of 1×10^7 spores/mL. An insufficient inoculum density ($< 1 \times 10^7$ spores/mL) leads to prolonged lag phases, lower fungal biomass density, and sub-optimal enzyme secretion rates within the designated fermentation cycle. Conversely, excessive inoculum levels ($> 1 \times 10^7$ spores/mL) cause over-crowding and rapid nutrient consumption, resulting in intense dissolved oxygen depletion, high broth viscosity, and nutrient competition that restricts fungal metabolic activity and lowers total enzyme yield. The highest α -amylase production was obtained at an inoculum density of 1×10^7 spores/mL, with an activity of 1561.64 ± 28.90 $\mu\text{g/mL}$ maltose units. Further increase to 1×10^8 spores/mL resulted in a reduction in enzyme production was shown in figure 6.

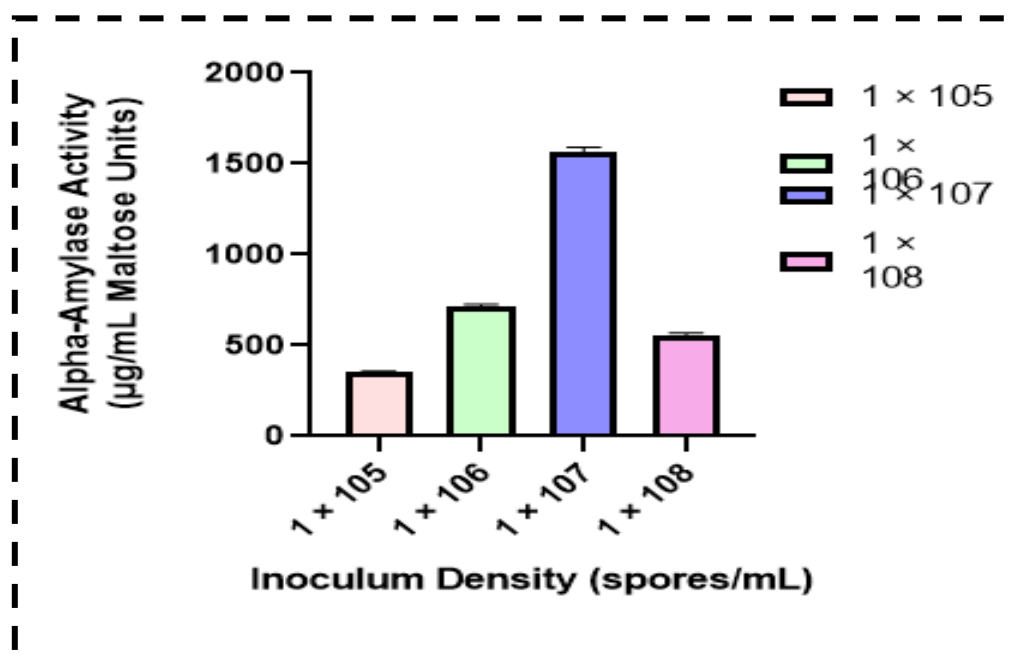


Figure 6. Influence of spore inoculum density (10^5 -- 10^8 spores/mL) on α -amylase yield by *Aspergillus niger* in submerged fermentation.

3.7 Effect of Carbon Source Supplementation on α -Amylase Production

α -Amylase is an inducible enzyme whose synthesis is strongly regulated by the nature of the carbon substrate present in the fermentation medium. Evaluating distinct supplementary carbon sources (such as soluble starch, glucose, maltose, and sucrose) revealed that complex polymeric or oligomeric carbohydrates function as effective inducers, whereas simple sugars exert catabolite repression. As shown in **Figure 7**, soluble starch induced the highest α -amylase secretion, followed by maltose. Macromolecular starch slowly hydrolyzes into dextrins and maltose units, providing a continuous, low-level supply of inducer molecules without overloading cellular metabolism. In contrast, readily metabolizable monosaccharides like glucose cause carbon catabolite repression (CCR), repressing the transcription of amylase genes and suppressing overall enzyme titers.

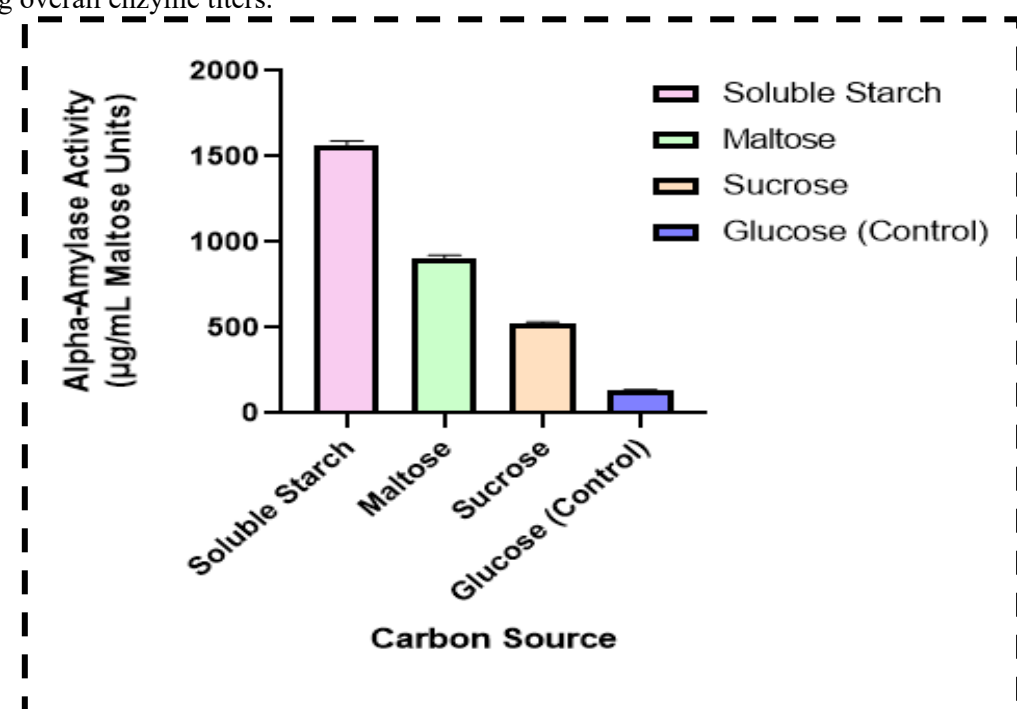


Figure 7. Relative impact of various carbon source supplements on α -amylase production by *Aspergillus niger* in submerged fermentation.

3.8 Effect of Nitrogen Source Supplementation on α -Amylase Yield

Nitrogen availability is essential for cellular protein synthesis, nucleic acid assembly, and active enzyme secretion by filamentous fungi. Supplementary organic (e.g., yeast extract, peptone) and inorganic (e.g., ammonium sulfate, potassium nitrate) nitrogen sources were evaluated to determine their influence on enzyme production. As illustrated in Figure 6, organic nitrogen sources (particularly yeast extract and peptone) yielded superior α -amylase titers compared to inorganic nitrogen salts as shown in **figure 8**. Organic supplements provide essential amino acids, vitamins, and growth factors that facilitate rapid cell growth and direct enzyme assembly. Inorganic sources like ammonium salts tend to cause a rapid drop in medium pH during cellular uptake, creating an acidic microenvironment that accelerates enzyme denaturation and limits total catalytic output.

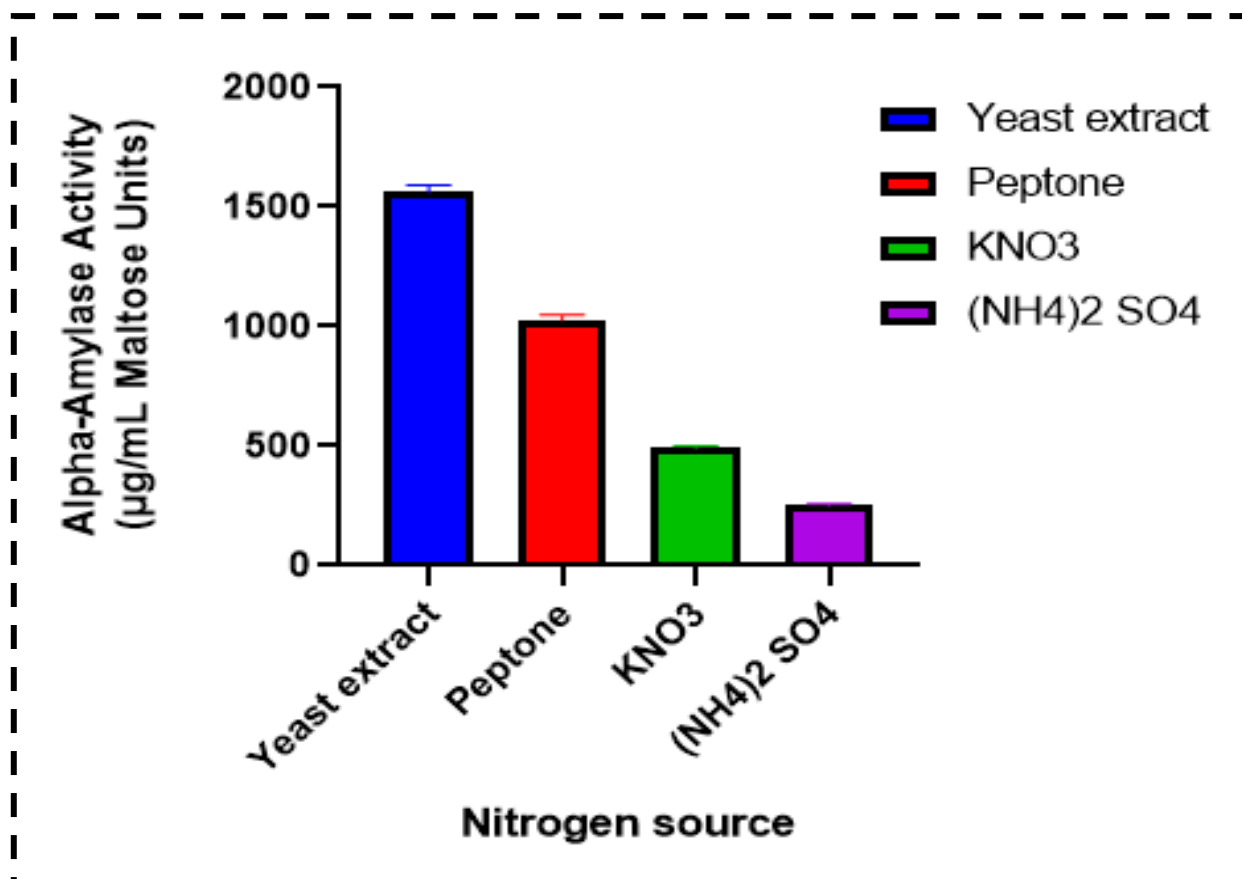


Figure 8. Influence of organic and inorganic nitrogen source supplementation on α -amylase synthesis by *Aspergillus niger* in submerged culture.

3.9 Optimum condition:

Systematic One-Factor-At-A-Time (OVAT) process parameter optimization for *Aspergillus niger* BAN3E established critical physiological thresholds that maximized extracellular α -amylase secretion to a peak yield of $1561.64 \pm 28.90 \mu\text{g/mL}$ ($A_{540} = 2.166$), representing a 4.58-fold overall productivity increase was demonstrated at **table 3**. Optimal enzyme accumulation required a 96-hour incubation period, which facilitated maximum biomass expansion during late-exponential growth prior to cellular autolysis and proteolytic degradation. An intermediate spore density of 1×10^7 spores/mL provided the ideal cellular concentration to avoid both prolonged lag phases at lower densities and dissolved oxygen depletion or high broth viscosity at higher concentrations. Macromolecular soluble starch functioned as the superior supplementary carbon source by providing a sustained, low-level release of inducer molecules without triggering carbon catabolite repression (CCR) which severely restricted yields under glucose ($132.66 \mu\text{g/mL}$). Furthermore, complex organic yeast extract proved significantly more effective than inorganic nitrogen salts like $(\text{NH}_4)_2\text{SO}_4$ ($251.51 \mu\text{g/mL}$) by supplying essential amino acids, trace growth factors, and vitamins required for active enzyme transcription, while simultaneously preventing the rapid media acidification characteristic of ammonium ion uptake.

Table 3. Comprehensive summary of single-factor optimization parameters for α -amylase production by *Aspergillus niger*

Fermentation Variable	Evaluated Range	Optimal Condition	Primary Biochemical Mechanism	Ref
Incubation Period	24–96 h	96 h	Maximum biomass accumulation and enzyme biosynthesis before the onset of autolysis and proteolysis	(Gupta et al., n.d.) & (Wang et al., 2016)
Inoculum Size	10^5 – 10^8 spores/mL	1×10^7 spores/mL	Balanced fungal biomass and metabolic activity without excessive competition for dissolved oxygen or nutrients	
Carbon Source	Starch, glucose, maltose, sucrose	Soluble starch	Effective substrate induction of α -amylase while minimizing carbon catabolite repression	
Nitrogen Source	Yeast extract, peptone, KNO_3 , $(\text{NH}_4)_2\text{SO}_4$	Yeast extract	Provides readily assimilable amino acids, peptides, vitamins, and growth factors supporting biomass formation and enzyme biosynthesis	

4. DISCUSSION

Submerged fermentation (SmF) of *Aspergillus niger* (strain BAN3E) proved highly effective for extracellular α -amylase production, reinforcing the established status of *A. niger* as a robust fungal cell factory capable of high-titer enzyme secretion. Filamentous fungi are particularly preferred for industrial enzyme operations due to their high capacity for protein secretion, ease of cultivation on low-cost media, and Generally Recognized as Safe (GRAS) status for many industrial applications. In this study, systematic One-Factor-At-A-Time (OVAT) optimization markedly enhanced enzyme expression over initial baseline levels, systematically resolving the physiological bottlenecks that limit catalytic yields (Rahman et al., 2018; Sahnoun et al., 2015). The physicochemical conditions established during optimization directly influenced the metabolic flux, cellular growth, and gene expression profile of strain BAN3E. An initial medium pH of 6.0 was identified as optimal (Bellaouchi et al., 2021), yielding $904.11 \pm 18.45 \mu\text{g/mL}$ maltose equivalents. The initial pH of the culture medium dictates the plasma membrane potential, nutrient transport efficiency across the cell wall, and enzyme stability. Strongly acidic conditions (pH 4.5) caused a dramatic reduction in activity ($132.66 \pm 4.21 \mu\text{g/mL}$), likely due to protein misfolding, active site protonation, and impaired nutrient uptake. Conversely, shifting toward neutral or alkaline pH reduced catalytic accumulation, confirming that *A. niger* BAN3E requires a slightly acidic microenvironment to maintain optimal tertiary structure and extracellular secretion kinetics (Rahman et al., 2018; Sahnoun et al., 2015). Temperature profiling demonstrated a clear maximum at 40°C ($1561.64 \pm 28.90 \mu\text{g/mL}$), highlighting the thermotolerant nature of strain BAN3E. Elevating the temperature from 25°C ($341.02 \pm 7.82 \mu\text{g/mL}$) to 40°C accelerated cell growth and metabolic throughput according to standard Arrhenius kinetics (Bellaouchi et al., 2021). However, a further increase to 45°C caused a sharp decrease in activity ($555.16 \pm 12.05 \mu\text{g/mL}$). This thermal drop-off is attributed to heat-induced enzyme denaturation, disruption of hydrogen bonding in the protein backbone, and thermal stress on fungal hyphae leading to premature cell decay (Duhan et al., 2020).

Time-course evaluation over 96 hours revealed progressive amylase accumulation that peaked during the late exponential and early stationary growth phases. The low activity observed within the first 24 hours corresponds to the fungal lag phase, during which cellular machinery adapts to the medium. Between 48 and 96 hours, rapid hyphal extension and active protein synthesis drove peak production. Inoculum size similarly played a critical role: a spore density of 1×10^7 spores/mL provided the ideal balance between vegetative biomass generation and nutrient availability. Lower inoculum levels (1×10^5 spores/mL) resulted in prolonged lag times and insufficient fungal biomass, whereas excessive inoculum (1×10^8 spores/mL) led to rapid nutrient depletion, severe dissolved oxygen restriction, high broth viscosity, and nutrient competition. Substrate selection further governed the rate of gene transcription and enzyme release. Soluble starch proved to be the most potent carbon source, inducing a peak activity of $1561.64 \pm 28.90 \mu\text{g/mL}$ (Gutteridge & Priest, 1999). As a complex polysaccharide, starch undergoes slow

enzymatic cleavage into oligomeric dextrans and maltose units, providing a continuous, low-concentration supply of inducer molecules without overwhelming the cell. In contrast, readily metabolizable simple sugars like glucose caused severe carbon catabolite repression (CCR), resulting in negligible enzyme yields ($132.66 \pm 4.21 \mu\text{g/mL}$). Nitrogen supplementation studies demonstrated that organic sources (yeast extract) significantly outperformed inorganic salts like $(\text{NH}_4)_2\text{SO}_4$ ($251.51 \pm 6.80 \mu\text{g/mL}$). Yeast extract provides readily available amino acids, vitamins, and nucleic acid precursors that accelerate fungal growth and bypass the energy-intensive pathways required for inorganic nitrogen assimilation. Furthermore, inorganic ammonium salts frequently cause medium acidification during uptake, creating secondary stress on the secreted protein. Overall, the 4.58-fold overall increase in enzyme activity achieved through process parameter tuning highlights the industrial feasibility of using *A. niger* BAN3E. The high yield, high statistical repeatability ($R^2 = 0.9909, p < 0.05$), and thermotolerance demonstrated by this strain make it an exceptional candidate for commercial applications in starch liquefaction, bioethanol saccharification, and industrial baking operations (Sparviero et al., 2023; Tao et al., 2023).

5. CONCLUSION:

Extracellular α -amylase was successfully produced by *Aspergillus niger* (strain BAN3E) utilizing submerged fermentation, reaffirming the strong biological potential of *A. niger* as a highly efficient fungal host for hydrolase secretion. Systematic single-factor optimization established that maximal enzyme synthesis requires an initial medium pH of 6.0, an incubation temperature of 40°C , a 96-hour fermentation period, an inoculum density of 1×10^7 spores/mL, and media supplementation with soluble starch and yeast extract as optimal carbon and nitrogen sources, respectively. Under these precise physicochemical conditions, the system achieved a peak quantitative enzyme activity of $1561.64 \pm 28.90 \mu\text{g/mL}$ maltose equivalents, corresponding to a maximum optical density (A_{540}) of 2.166. Process optimization resulted in a dramatic quantitative enhancement across all evaluated parameters, yielding a 4.58-fold overall increase in enzyme activity compared to the initial baseline setup of $341.02 \pm 7.82 \mu\text{g/mL}$. Individual parameter optimization demonstrated significant relative gains: adjusting initial medium pH to 6.0 provided a 6.81-fold increase over the acidic baseline at pH 4.5 ($132.66 \mu\text{g/mL}$), supplementing with soluble starch generated an 11.77-fold increase compared to glucose-repressed media ($132.66 \mu\text{g/mL}$), and utilizing yeast extract outperformed inorganic ammonium sulfate by 6.21-fold ($251.51 \mu\text{g/mL}$). Supported by strong linear regression accuracy ($R^2 = 0.9909$) and statistical significance ($p < 0.05$), these robust yield improvements highlight the industrial viability of crude *A. niger* BAN3E amylolytic preparations for large-scale starch liquefaction, commercial baking, and bioethanol bioprocesses.

6. FUTURE SCOPE

While single-factor optimization established foundational process parameters for α -amylase production by *Aspergillus niger* (BAN3E), several experimental boundaries remain to be addressed in subsequent research. To overcome the limitations of One-Factor-At-A-Time (OVAT) screening, future work will implement Response Surface Methodology (RSM) via Central Composite Design (CCD) or Box-Behnken Design to capture complex multi-factor interactive effects. Transitioning from bench-top shake flasks to a stirred-tank bioreactor will allow for process scale-up and fine-tuning of dissolved oxygen ($d\text{O}_2$), agitation rates, aeration, and continuous pH control. Downstream recovery and molecular characterization will involve purifying crude α -amylase through fractional ammonium sulfate precipitation, ion-exchange chromatography (IEX), and size-exclusion chromatography (SEC), followed by molecular weight determination via SDS-PAGE and peptide fingerprinting via MALDI-TOF mass spectrometry. Furthermore, detailed kinetic studies will establish Michaelis-Menten constants (K_m and V_{max}) across diverse starch substrates alongside comprehensive profiling of thermal stability, pH stability, and half-life ($t_{1/2}$). Finally, evaluating catalytic performance in industrial starch liquefaction, commercial baking, and bioethanol saccharification paired with enzyme immobilization onto solid matrices such as sodium alginate beads or magnetic nanoparticles will facilitate continuous bioprocessing and catalyst reuse for industrial deployment.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Ravi S. Khandare: Writing – original draft. **Vipul Patel & Pratim K. Chaoudhary:** Conceptualization, Supervision & Writing review editing.

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DECLARATION OF COMPETING INTEREST

No competing interest.

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