

Research Article

Process Optimization for the Production of Alpha Amylase by *Aspergillus niger* Kbt-3 Using Submerged Fermentation

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Abstract | The use of alpha amylase as a substitute for chemical processes has gained significant momentum in the field of biotechnology. As a result, there is a growing need to explore untapped reserves of fungi with high potential for efficient alpha amylase production. This has led to the isolation of novel fungal strains capable of producing the targeted product. The aim of this study was to isolate a novel fungal strain and optimization of process parameter in order to enhance the alpha amylase productivity. The production of enzyme indigenously lessen reliance on external sources and promoting self-sufficiency. In this study, 15 amylolytic fungal strains were isolated and screened via submerged fermentation. The strain exhibiting the highest alpha amylase activity was identified using conventional methods, including morphology and microscopic features, and confirmed by 18S rDNA gene sequencing using specific ITS primers. The selected strain was then subjected to sequencing and phylogenetic analysis to further characterize its properties. The result indicates the selected strain was found to be *A. niger* and this strain was given code *A. niger* KBT-3. The Five fermentation media were also screened. The Medium2 gave higher titer of alpha amylase activity 1q and found to be the best medium. Different parameters including time and temperature of incubation, pH, Inoculum size, volume, carbon and nitrogen sources were also tested. Optimal enzyme production was obtained at 72 h, 30C, pH5.5, 1ml inoculum, 50ml volume and 1.5% lactose and 1% yeast extract.

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Introduction

Alpha amylase, which is also referred to as α -1,4-glucan-glucanohydrolase (EC 3.2.1.1), is a widely distributed enzyme that breaks down starch in a variety of natural sources. The random hydrolysis of α -1,4 glucosidic links inside the starch molecule is

catalyzed by this extracellular enzyme, which yields both monosaccharides and oligosaccharides, such as alpha limit dextrin, glucose, and maltose (Saha *et al.*, 2014; Soy *et al.*, 2021). Alpha amylases are among the most important and frequently used enzymes, having a wide range of uses in pharmaceutical clinical and analytical chemistry.

They are widely used in many different industries, including culinary, baking, brewing, detergent, textile, and paper manufacture, in addition to their function in starch saccharification. The need to develop quick and efficient ways in order to produce alpha amylase is growing due to upsurge in its consumption; the production of enzyme indigenously lessen reliance on external sources and promoting self-sufficiency (Gupta *et al.*, 2008; Balakrishnan *et al.*, 2021; Singh *et al.*, 2022).

Numerous creatures, including plants, animals, and microbes, are sources of alpha amylase. However, production from plants and animals is constrained due to several factors. Due to the low concentration of alpha amylase obtained from plants processing significant amounts of plant material is necessary. However, the availability of animal-derived enzyme is limited because it is a byproduct of the meat industry. Microbial sources, on the other hand, such as bacteria and fungi, provide a good substitute for large-scale production meeting market demands numerous bacterial and fungal strains have been widely used to produce alpha amylase, providing a more scalable and effective alternative.

Filamentous fungi are renowned for their natural ability to secrete enzymes that break down starch and cellulose. The ability to produce substantial amounts of extracellular proteins renders them a perfect option for industrial enzyme synthesis. Several species of fungi have been commonly utilized for this purpose, including *Trichoderma*, *Thermomyces lanuginosus*, *Penicillium griseoroseum*, *Fusarium moniliformis*, *Actinomyces*, and *Alternaria*. These fungi have been exploited for their ability to produce a broad range of enzymes (Elyasi *et al.*, 2020; Balakrishnan *et al.*, 2021).

One of the most important things in developing a biotechnological process is choosing an appropriate strain. The ability of the strain to continuously produce high yields, its physiological stability, and incubation time needed for ideal production was consider very crucial for choosing an appropriate strain. The strain's resistance to several environmental stresses, like temperature, aeration, and shear stress, is also an important factor (Laluce *et al.*, 1991).

The culture process has a major influence on the amount of enzymes produced. Both submerged and

solid-state fermentation methods can be used to produce alpha amylase. The submerged fermentation offers more agitation, greater aeration, and easier enzyme separation, it is frequently chosen over solid-state fermentation, even if it has its own benefits. Submerged fermentation can result in increased enzyme yields and activity because the liquid culture facilitates more effective oxygen transfer and mixing. It is also easier to separate the enzyme, which makes it a more feasible option for large-scale enzyme production (Lokeswari, 2010; Fatima and Ali, 2012)

Optimizing cultural conditions is crucial for maximizing enzyme production. This involves carefully selecting the carbon and nitrogen sources, pH, and incubation temperature of the medium, as well as the inoculum size. Finding the optimal temperature and duration is especially crucial for getting the maximum amount of alpha amylase. Enzyme output can be greatly increased by adjusting these variables, which will increase productivity and efficiency across a range of industrial applications. The aim of this study was to isolate a novel fungal strain and optimization of process parameter in order to enhance the alpha amylase productivity.

Materials and Methods

Isolation of amylolytic fungi

Soil samples from various habitats were collected and stored in separate polythene bags for the isolation of fungal strains. In order to isolate amylolytic fungi from soil samples, the serial dilution approach was used. The starch agar plates were used for isolation of desired fungi. After spreading a 0.2 ml aliquot of the diluted suspension over culture medium plates, the plates were incubated at 30°C for three to four days to allow the culture to develop. The colonies exhibited starch hydrolysis zone was chosen (Chandrakara and Pandey, 2018). Submerged fermentation was used to screen the chosen cultures for the synthesis of alpha amylase. The strain showing highest amylolytic potential was identified on the basis of morphological and Molecular characteristic. The genomic DNA was extracted using a method adapted from Nisar *et al.* (2020). The PCR product was then amplified, purified, and sent for commercial sequencing to determine the genetic sequence of the isolated fungal strain.

Inoculum preparation

Conidia from slant cultures that were 3–4 days old

were utilized for inoculation. In order to form a conidial suspension in each slant exhibiting abundant conidial growth, 10 ml of saline water was added and vortex (Haq *et al.*, 2002).

Fermentation media

Submerged fermentation: After adding 1 ml of inoculum to 25 ml of fermentation medium, the mixture was put in an orbital shaking incubator and incubated at 30 degrees Celsius with a 200 g shaking speed. Following 72-hour the contents of the flasks were filtered and the filtrate and residue was used for enzyme and cell mass estimation, respectively.

Enzyme assay and dry cell mass (DCM) determination

The estimation of alpha amylase and DCM was performed according to Haq *et al.* (2010) and (Irfan *et al.*, 2011), respectively. For estimation of alpha amylase in a test tube, 1 ml of diluted filtrate was taken in order to measure the enzyme activity. Additionally, 1ml of 1% starch solution was added to it. One milliliter of distilled water was used in place of the filtrate in a parallel run of the blank. The reducing sugar released during a 10 minute incubation period at 40°C was detected at 546 nm using the DNS method (Miller, 1993). While by filtering the fermented broth through pre weighed Whatman filter paper No. 1 the dry cell mass was ascertained. The mycelia were washed with distilled water dried for two hours at 105°C in the oven. Weighing the dry cell mass, the final weight was subtracted from the initial weight to determine the dry cell mass.

Results and Discussion

Isolation identification and screening of amylase producing fungi

An important phase in the fermentation process is the isolation of a novel fungal strain that has the potential to yield a desired product. It is crucial to comprehend the features of the several fungal strains that were isolated and screened during the enzymatic production and optimization process. This knowledge is essential for a number of reasons, such as being able to choose the best strain for maximum enzyme production, seeing potential limitations and opportunities for improvement, and to ensure the consistency and efficiency of the fermentation process. Usually, morphological and biochemical characteristics are used to identify fungi, but a more comprehensive approach is required to differentiate

between closely related species. To identify the subtle differences between these species, molecular investigations must be conducted in addition to phenotypic study. Fungal species may be identified with more accuracy and precision by this integrated approach (Shahriarinnour *et al.*, 2011). In the current study 15 different amylolytic strains were isolated and screened via submerged fermentation in order to determine their ability to produce alpha amylase (Table 1).

Table 1: Screening of fungal isolates for alpha amylase production by using submerged fermentation.

S. No.	Fungal isolates	Enzyme activity (U/ml)	Dry cell mass (g/l)
1	Isolate 1	3.10±0.02	2.0±0.02
2	Isolate 2	4.50±0.03	2.6±0.03
3	Isolate 3	10±0.05	5±0.04
4	Isolate 4	2.11±0.02	2.0±0.01
5	Isolate 5	2.02±0.03	2.1±0.01
6	Isolate 6	4.11±0.02	3. ±0.03
7	Isolate 7	5.50±0.02	3.7±0.03
8	Isolate 8	4.23±0.03	3.2±0.04
9	Isolate 9	6.07±0.03	4.3±0.03
10	Isolate 10	1.43±0.01	1.8±0.01
11	Isolate 11	7.38±0.03	4.5±0.03
12	Isolate 12	5.70±0.02	3.8±0.03
13	Isolate 13	2.72±0.04	2.3±0.02
14	Isolate 14	2.66±0.02	1.9±0.02
15	Isolate 15	7.84±0.02	4.6±0.04

Every value is mean of triplicates ±indicates the standard deviation between replicates.

The isolate 3 gave optimal production of enzyme and identified both on the basis of morphological and molecular characters and designated the code KBT-3. The colonial characteristics of *A. niger* include a black colony color with white mycelia, and a thin, spreading shape. Microscopically, *A. niger* exhibits the following features: conidia are sub-globose to globose in shape, measuring between 4-6 µm in diameter. They are brown in color with a smooth to rough surface and are relatively small in size. The conidiophores, on the other hand, were hyaline, long, and globose in shape, with a smooth surface and brown in color (Figure 1a, b) (Atallah *et al.*, 2022).

The identity of *A. niger* was further confirmed through molecular analysis by sequencing the ITS region (5.8S rDNA).

Fermentation media

Fermentation media	Composition(g/l) pH=6
M1	Wheat bran 100, Zn SO ₄ .7H ₂ O 0.062, FeSO ₄ 0.068, Cu SO ₄ .7H ₂ O 0.0008.
M2	Starch 20, yeast extract 8.5, NH ₄ Cl 1.3, MgSO ₄ .7H ₂ O, 0.12, CaCl ₂ 0.06.
M3	Starch 10, MgSO ₄ .7H ₂ O 0.005, CaCl ₂ .2H ₂ O 0.2, FeSO ₄ 0.1, (NH ₄) ₂ SO ₄ 2, phosphate buffer 1000 ml.
M4	Glucose monohydrate 4.84, (NH ₄) ₂ SO ₄ 4.84, KH ₂ PO ₄ 3.87, MgSO ₄ .7H ₂ O 3.75, NaCl 1.80, CaCl ₂ .2H ₂ O 1.21, trace metal solution 0.12 ml.
M5	Glucose 50, NaNO ₃ 3, KH ₂ PO ₄ 1.0, KCl 0.5, MgSO ₄ .7H ₂ O 0.2, FeSO ₄ 0.01.

(Hayashida *et al.*, 1986; Spohr *et al.*, 1998; Nandakumar *et al.*, 1999; Haq *et al.*, 2002)

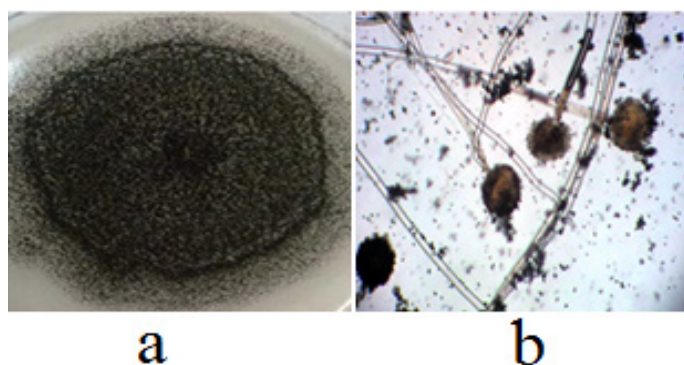


Figure 1: (a) Colony of *A. niger* KBT-3 (b) Microscopic structure of *A. niger* KBT-3.

The generated sequence was compared to existing sequences in GenBank using BLAST analysis, which revealed a high similarity of 100% with *A. niger*. Maximum Composite Likelihood method was used in order to construct phylogenetic tree using Mega 11 which provide a comprehensive view of the evolutionary relationships between *A. niger* and other related species (Figure 2).



Figure 2: Phylogenetic tree of *A. niger* showing close similarity to other fungal species.

Fermentation media plays a vital role in the production of enzyme as well as fungal growth by providing

the essential nutrients. The potential of five distinct fermentation media (M1, M2, M3, M4, and M5) to produce alpha amylase was examined in the current study. The most appropriate of them, medium M2, was found to produce the highest amounts of alpha amylase as well as dry cell mass (Figure 4a). Yeast extract and ammonium chloride most likely function as organic and inorganic nitrogen sources in the M2 medium, respectively. Because of its high nutritional value, yeast extract a complex nitrogen source that includes free amino acids and peptides is regarded as the perfect ingredient for the synthesis of enzymes. Additionally, as metal ions activate enzyme activity and are essential for maximizing enzyme performance, their addition to the fermentation medium has a substantial effect on both the production and stability of enzymes (Lin *et al.*, 1997; Malhotra *et al.*, 2000).

Essential ions including Ca⁺², Cl⁻, Mg⁺², and SO₄⁻² found in the M2 medium are vital to the fungus's growth and development as well as the synthesis of enzymes. In particular, calcium and chloride ions have a variety of functions that promote the best conditions for fungal growth and enzyme activity. These functions include stabilizing, binding, activating, and stimulating. These ions aid in maintaining cellular homeostasis, facilitate enzymatic reactions, and promoting protein folding, ultimately contributing to the successful production of alpha amylase (Haddaoui *et al.*, 1999). In comparison to the M2 medium, the other media (M1, M3, M4, and M5) produced less significant results.

This is probably because the other media lacked or contained insufficient amounts of critical components required for the optimal fungal growth and enzyme production. On the other hand, the repressor impact of some of the components in these media might have contributed to the decreased performance by impeding the organism's growth and, consequently, the production of enzymes.

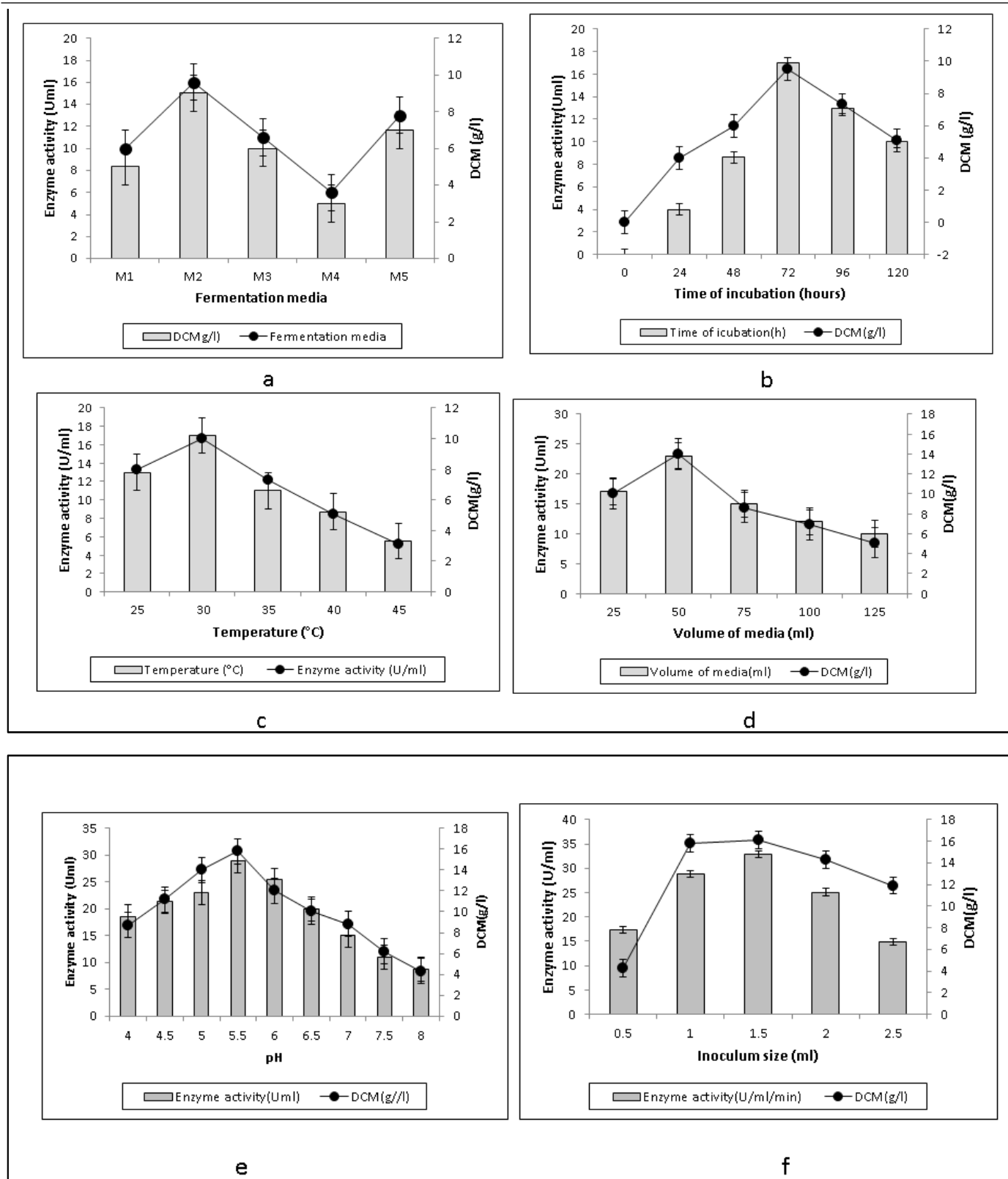


Figure 3: Impact of different parameter on alpha amylase production by *A. niger* KBT-2 (a) Media (b) Incubation time (c) Temperature (d) Volume of medium (e) pH (f) Inoculum size.

Incubation time, temperature and volume

The biosynthesis of alpha amylase was investigated at various incubation times (0-120 hours) to determine the optimal period. The findings demonstrated that after 72 hours of incubation maximum production of alpha amylase and dry cell mass was occurred

(Figure 3b). This is explained by the fact that the developing fungus had entered the stationary growth phase, which is characterized by optimal enzyme production, after reaching the end of its logarithmic phase. This result is in line with the findings of Prescott and Dunn (1987), who noted that fungal

strains produce their highest amount of alpha amylase during the stationary growth phase. Alpha amylase yield decreased with longer incubation times than 72 hours. After 72 hours of incubation, the output of alpha amylase decreased, most likely as a result of the enzyme becoming denatured from interactions with other substances in the fermentation medium. The decreased enzyme yield may also have been caused by nutrient depletion and the production of by products such as proteases in the fermentation medium (Ramesh and Lonsane, 1990; Kirshna and Chandrasekaran, 1996). Particularly proteases have the ability to degrade enzymes such as alpha amylase, leading to reduced activity and production.

In present research different temperature (25–45°C) was evaluated for optimal enzyme production. It was noted that 30°C found to be optimal for alpha amylase production. Above or below this optimal range decline in enzyme activity was recorded (Figure 3c). Our results are in line with Shafique *et al.* (2009). Optimization of the fermentation medium volume is crucial for adequate air supply, nutrient provision, microbial growth, and enzyme production. In this work, effect of different medium volumes (25–125 ml) was evaluated and found that maximum enzyme as well as DCM production was achieved with 50 ml of fermentation medium. Due to a decrease in agitation speed and air supply, enzyme production was shown to decrease with an increase in medium volume in contrast, the production of enzymes was similarly reduced at low medium volumes, presumably due to a lack of available nutrients to promote the growth of *A. niger* and subsequent enzyme production (Mimura and Shinichi, 1999; Ivanova *et al.*, 2001).

pH and inoculum size

The proliferation of microbial strains and, in turn, the production of metabolites is directly impacted by the pH of the medium. In addition, the medium pH has a major impact on several enzyme activities as well as the movement of various substances across the membrane.

The three-dimensional shape of the enzyme active site must be maintained at the optimal pH, and changes in the ionic bonding of the enzyme cause the loss of functional form (Mmango-Kaseke *et al.*, 2016).

Figure 3d shows the effect of initial pH (ranging from 4.0 to 8.0) on alpha amylase production. At pH 5.5,

the highest amount of enzyme production was noted. This is explained by the fact that fungal growth, which in turn promotes enzyme secretion, is supported by an acidic pH (Gangadharan *et al.*, 2008). At pH values either above or below the optimal range, a decrease in the synthesis of enzymes was observed. This is because enzymes have a narrow pH range for optimal activity and are extremely sensitive to even slight pH changes (Gupta *et al.*, 2008).

One important physical factor influencing the synthesis of enzymes is the size of the inoculum. In this study, the impact of various inoculum sizes (0.5–2.5 ml) on alpha-amylase production was investigated. According to our findings, 1 ml of inoculum produced the optimal enzyme. However, further increases in inoculum size led to decreased enzyme production, probably because of the overgrowth of *A. niger*, which consumed most of the substrate for growth and metabolic processes during fermentation and produced anaerobic conditions, which in turn reduced the production of enzyme. On the other hand, decreasing the inoculum size also resulted in reduced enzyme production, possibly because the smaller amount of conidia produced inadequate mycelia, leading to decreased enzyme production (Kashyap *et al.*, 2002).

Carbon and nitrogen sources

The impact of various carbon sources on enzyme production was assessed, and lactose was found to yield the highest enzyme production as well as biomass (Figure 4a). This can be attributed to lactose serving as a complex carbohydrate source, which is gradually metabolized by the microorganism, leading to enhanced accumulation of inducible alpha-amylase in the fermentation media (Nguyen *et al.*, 2000; Calik and Ozdamar, 2001). As the supplementary carbon source for enzyme production, lactose was chosen, and the ideal concentration was evaluated at different levels (0.5–2.5%). The findings indicated that the optimal lactose concentration for the production of enzyme was 1.5%. Reduced enzyme production resulted from additional lactose concentration increases or decreases. This is explained by the fact that excessive carbon levels (above 1.5%) led to catabolic suppression, which further reduce the production of enzyme, and insufficient carbon levels (below 1.5%) restricted microbial growth which also hindered enzyme production (Carlsen and Nielsen, 2001; Gupta *et al.*, 2008).

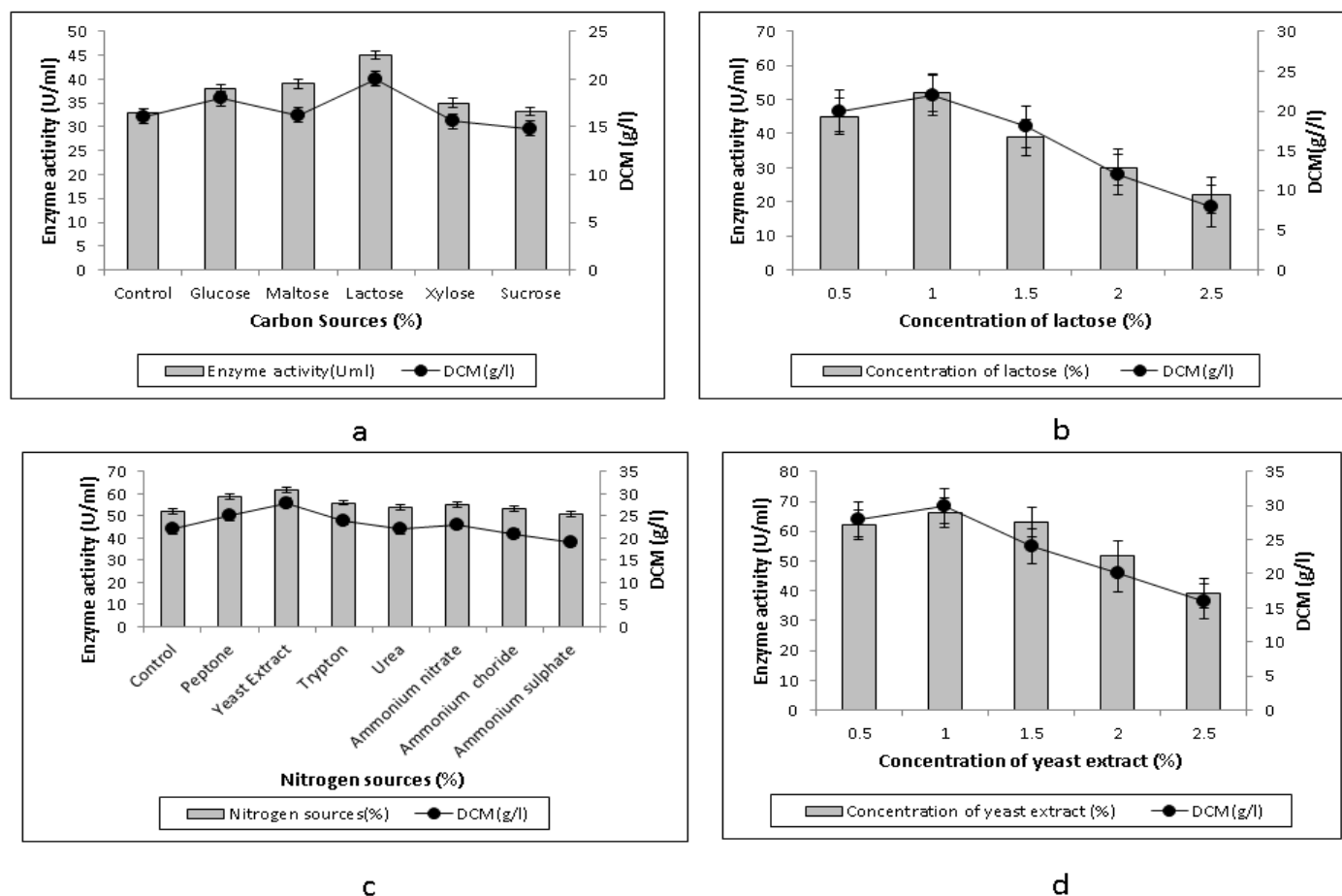


Figure 4: Impact of nutritional parameter on alpha amylase production by *A. niger* KBT-2 (a) carbon sources (b) Lactose concentration (c) Nitrogen sources (d) Concentration of yeast extract.

The impact of several different organic and inorganic nitrogen sources on α -amylase production by *A. niger* was investigated (Figure 4c). These additional nitrogen sources were added at a concentration of 0.5% and screened for their effect. Among all the nitrogen sources tested, yeast extract yielded the highest α -amylase production. To further optimize enzyme production, different concentrations of yeast extract (0.5-2.5%) were evaluated (Figure 4d). The optimal level of α -amylase production (66U/ml) and DCM (30g/l) was achieved at a yeast extract concentration of 1%. Our findings align with those of Irfan *et al.* (2012), who also identified yeast extract as an appropriate nitrogen source for optimal enzyme production.

Conclusions and Recommendations

In conclusion, this study successfully isolated and characterized a novel fungal strain, *A. niger* KBT-2, with high potential for efficient alpha amylase production. Through submerged fermentation the optimal conditions 72 h, 30°C, pH 5.5, 1 ml inoculum,

50 ml volume, 1.5% lactose, and 1% yeast extract were identified. These findings contribute to the growing momentum of using alpha amylase as a substitute for chemical processes in biotechnology, highlighting the importance of exploring untapped fungal reserves for efficient enzyme production. The results of this study provide valuable insights for further optimization and scaling up of alpha amylase production, paving the way for potential industrial applications. The alpha amylase potential of isolated can be further enhanced by mutation.

Author's Contribution

Roheena Abdullah: Supervision and manuscript writing.

Kinza Nisar: Experimental work.

Afshan Kaleem: Editing of manuscript.

Mehwish Iqtedar: Editing of manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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