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Optimization of the Breeding Conditions of *Aspergillus niger* for Enzyme Production Focusing on Temperature, Substrate Source, Nitrogen Source, Carbon Source, and pH

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ABSTRACT

Background. *Aspergillus niger* is extensively employed in the industry to produce organic acids, enzymes, and other metabolites. Optimizing the breeding conditions for *A. niger* to increase enzyme yields and boost the effectiveness of industrial fermentation processes is a crucial field of study.

Methods The effects of different temperature conditions (15°C, 30°C, and 45°C), carbon sources (oats, wheat, corn, and sugar cane), nitrogen sources (NH_4NO_3, NH_4CL, and $(NH_4)^2SO_4$), and pH (4, 7, and 10) on the production of enzyme amylase were determined. *A. niger* was fermented under controlled conditions in bioreactor shake flasks and enzyme activities were measured using standard colorimetric and fluorometric assays.

Results. The results showed an optimum temperature of 30°C for *A. niger* fermentation for the production of amylase, with the highest specific enzyme activity of 1.1 U/mg. NH_4SO_4 was found to be the best nitrogen source for the fermentation of *A. niger* for enzyme production. Whereas, wheat and corn proved to be the best carbon sources for the fermentation of *A. niger* for enzyme production. Wheat and corn produced amylase with the specific enzyme activity of 0.63 U/mg. The pH 4 was found to be ideal for the fermentation of *A. niger* for enzyme production The enzyme produced at pH 4 showed a specific enzyme activity of 0.5 U/mg.

Conclusion. The results suggest a positive correlation between optimising the breeding conditions of *A. niger* and the quality of amylase produced. It provides valuable insights to guide the scale-up and commercialization of *A. niger*-based bioprocesses for industrial enzyme manufacturing.

Keywords: *Aspergillus niger,* bioprocesses, enzyme production, fermentation

Highlights

- Optimising conditions for the fermentation of *A. niger.*
- Use of *A. niger* for the production of amylase enzyme.
- Optimum amylase activity for industrial usage.

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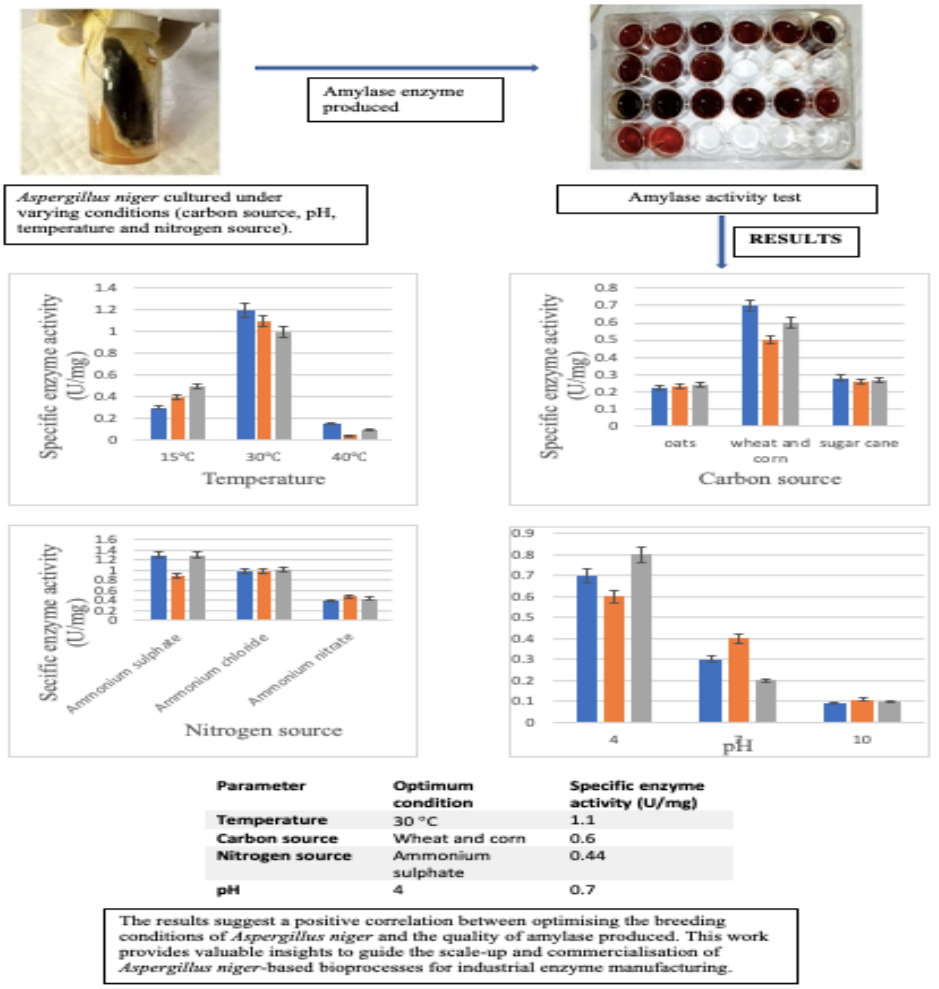
GRAPHICAL ABSTRACT



Parameter	Optimum condition	Specific enzyme activity (U/mg)
Temperature	30 °C	1.1
Carbon source	Wheat and corn	0.6
Nitrogen source	Ammonium sulphate	0.44
pH	4	0.7

The results suggest a positive correlation between optimising the breeding conditions of *Aspergillus niger* and the quality of amylase produced. This work provides valuable insights to guide the scale-up and commercialisation of *Aspergillus niger*-based bioprocesses for industrial enzyme manufacturing.

1. INTRODUCTION

Enzymes are biological catalysts that have many applications in various sectors [1]. Over the years, there has been a constant rise in the demand for enzymes in the manufacturing industry and in medicine [2]. Most enzymes used in industries are produced by fungi and bacteria, with a small share still being sourced from animals and plants [3, 4]. Recent advancements in molecular biology and genetic engineering have led to the development of various expression systems currently exploited for the commercial production of different types of analytical and industrial enzymes [5, 6].

Researchers have found that solid state fermentation (SSF) is one of the most effective methods of producing enzymes from

microorganisms, such as *Aspergillus niger*. A moist medium made by adding mineral salts and trace elements to sterilised wheat or rice bran acidified with HCl is commonly used. An inducer, such as 10% starch for the synthesis of amylase or gelatin and pectin for the creation of protein and pectinase, can also be added [7]. Fungi are frequently used to produce enzymes in SSF. In this method, the vaccinated bran is spread out in trays, damp air is circulated, and moisture is maintained by periodically spraying water. Cold air can be used to maintain the temperature at or below 30°C. Samples are taken at certain intervals and enzyme tests are used to identify the ideal output. The ideal output might take anywhere from 30-40 hours to up to 7 days [8].

The fermentation material is then crushed and dried at around 40°C. This dried material is a version of the enzyme that can either be extracted or stored. Compared to submerged culture, development in the semi-solid medium may promote the production of a variety of enzymes. *A. niger* is capable of producing enzymes, such as gluco-amylase, protease, and amylase [9, 10].

The material is rapidly cooled to around 20°C after drying in order to prevent contamination and enzyme degradation. Stabilizers, such as calcium salts and starch hydrolysates, can be added. Destabilizing agents can be removed using chelating agents, such as EDTA. It may also be beneficial to add antimicrobial preservatives approved for food use, such as sorbates and benzoates. Most of the enzymes produced industrially are extracellular, indicating that they are secreted into the fermentation medium. Occasionally, enzymes attach to cells and have to be removed mechanically. Vacuum filtration and centrifugation are then used to recover the enzymes [11].

The level of downstream purification depends on the intended use of the enzyme. At times, the enzyme is directly used after the clarification step. In other cases, purification is continued using chemicals, such as acetone, ammonium sulfate, methanol, and ethanol. Factors such as the type of substrate, nitrogen source, pH, carbon source, and temperature can be manipulated to create a fermentation environment that maximizes the growth of *A. niger* [12]. The purpose of this research is to find appropriate breeding conditions of *A. niger* that give a maximum yield of enzymes to meet their increasing demand. Amylase contributes 24% to the total cost of the starch liquefaction process which is essential in many industrial processes [13, 14]. There is a growing interest in reducing costs by increasing enzyme yields from microbial sources.

2. MATERIALS AND METHODS

All of the chemicals used in this study were provided by the Biotechnology and Biochemistry Laboratory Department, University of Zimbabwe.

2.1. *Aspergillus niger* Culture

A. niger strain (ATCC 1004) was used in this study. To obtain a pure culture of *A. niger*, stored cultures were sub-cultured on nutrient agar plates. These sub-cultures were stored in the refrigerator at 5°C. Solid State Fermentation (SSF) was the fermentation method used throughout the experiment.

2.2. Effect of Carbon Source on Enzyme Production

Oats, wheat, corn, and brown sugar were the carbon sources used. All of these sources were obtained from the store. A measure of 10 g of each carbon source was placed in separate 250 ml beakers. A diluent was made by adding 114.6 mg of ammonium chloride, 0.2 mg of copper sulfate,

1.7 mg of ferrous sulfate, and 1.5 mg of zinc sulfate into 250 ml distilled water. This was adjusted to pH 7 and 10 ml of this diluent was added into each beaker. The beaker containing oats was labeled as glucose, beaker containing wheat and corn was labelled maltose, and the beaker containing brown sugar was labelled as sucrose. The beakers were sterilized by autoclaving for 15 minutes at 121°C and 15 psi. Then, 1 ml of inoculum containing *A. niger* was added into each beaker. The beakers were placed into an incubator at 30°C for 72 hours.

2.2.1. Extraction of Enzyme Amylase. After 72 hours, the beakers were retrieved from the incubator. *A. niger* had fermented. A total of 100 ml of distilled water was added to each beaker and the beakers were placed into a shaking incubator for an hour at 160 rpm at 37°C. After an hour, the contents were filtered and the filtrate was used for the estimation of enzyme amylase. After filtration, the residue was treated again with 100 ml distilled water in the same way and filtered.

2.2.2. Enzyme Assay. To check for enzyme activity, starch agar plates were created and the filtrate was inoculated onto the plates at specific regions. A positive control starch agar plate was made onto which salivary amylase was inoculated. The plates were incubated for 2 days. Iodine solution was added to the plates. Iodine turns blue black in the presence of starch and remains brown in its absence. An aliquot of 2 ml of each filtrate was pipetted into a test tube. An aliquot of 1 ml of 1% starch solution was added into the test tube and incubated at 40°C for 30 minutes. To halt the reaction, 2 mm of DNS solution were added to each test tube and the tubes were submerged in a bath of boiling water (100°C) for 5 minutes. The tubes were then cooled to room temperature. Using distilled water, the final volume was reduced to 20 mm. A spectrophotometer set to 540 nm was used to determine the absorbance of each test solution and compared to a standard curve made with 0.10 to 1.0 mg of glucose per mm. International Units (IU) were used to express enzyme activity. Using the glucose standard curve, one IU is defined as one µmole of glucose equivalents released per milliliter under standard test conditions. The estimate of enzyme activity was made using suitable dilution factors. Absorbance was measured at 540 nm with a microprocessor visible spectrophotometer LI-722.

2.3. Effect of Temperature on Enzyme Production

A measure of 10 g of oats was placed in 3 separate 250 ml beakers. A diluent was made by adding 114.6 mg of ammonium chloride, 0.2 mg of copper sulfate, 1.7 mg of ferrous sulfate, and 1.5 mg of zinc sulfate into 250 ml distilled water. This was adjusted to pH 7 and 10 ml of the diluent was added into each beaker. The beakers were sterilized in an autoclave at 121°C at 15 psi for 15 minutes. A 1 ml of inoculum containing *A. niger* was added into each beaker. The beakers were incubated at 15°C, 30°C, and 45°C for 72 hours. After 72 hours, the beakers were taken out to conduct enzyme extraction and enzyme assay.

2.4. Effect of Nitrogen Source on Enzyme Production

A measure of 10 g of wheat and corn was placed in 250 ml separate beakers. An aliquot of 10 ml diluent was added into each beaker. Diluents were made in 3 separate beakers by adding 114.6 mg of ammonium chloride (NH_4Cl) into Beaker 1, 114.6 mg of ammonium nitrate (NH_4NO_3) into Beaker 2, 114.6 mg of ammonium sulfate $(NH_4)^2SO_4$ into Beaker 3, as well as the same amounts of 0.2 mg of copper sulfate, 1.7 mg of ferrous sulfate, and 1.5 mg of zinc

sulfate in 250 ml distilled water. Each diluent was adjusted to pH 7 and its 10 ml amount was added into each beaker. The beakers were labeled NH_4NO_3, NH_4Cl, and $(NH_4)^2SO_4$. They were sterilized in an autoclave for 15 minutes at 121℃ and 15 psi. An inoculum measuring 1 ml containing *A. niger* was added. The enzyme assay was prepared as described above.

2.5. Effect of pH

A digital pH meter was used to measure the culture broth's pH. Before usage, the pH meter was given 30 minutes to warm up. The electrode was taken out of the distilled water in the storage beaker and dried. It was then calibrated to the same value and put in a beaker with a pH 7 buffer solution. The electrode was taken out, rinsed in distilled water, and placed in the culture broth to be evaluated. Contact between the glass and the electrode was avoided. The pH was measured using a digital pH meter. The experiment was run separately at pH 4, 7, and 10. The enzyme amylase was determined during a 72-hour incubation period at 30℃.

3. RESULTS

As presented in Figure 1, the optimization of different temperature conditions yielded different results. Firstly, 30℃ yielded amylase with the highest specific activity, as compared to the temperatures of 15℃ and 40℃. Also, ammonium sulfate, as a source of nitrogen, yielded the amylase with the highest specific activity, as compared to ammonium chloride and ammonium nitrate, as shown in Figure 2. Whereas, wheat and corn yielded amylase with the highest specific enzyme activity as compared to oats and sugar cane, as shown in Figure 3. A pH of 4 produced amylase with the highest specific activity as compared to pH 7 and pH 10, as shown in Figure 4. The results from ANOVA (Table 2) show that there was a statistically significant difference in the specific enzyme activity from amylase produced by *A. niger* subjected to the different levels for each parameter. Implying that optimum conditions indeed contribute to production of amylase with a higher specific enzyme activity as summarized in Table 1.

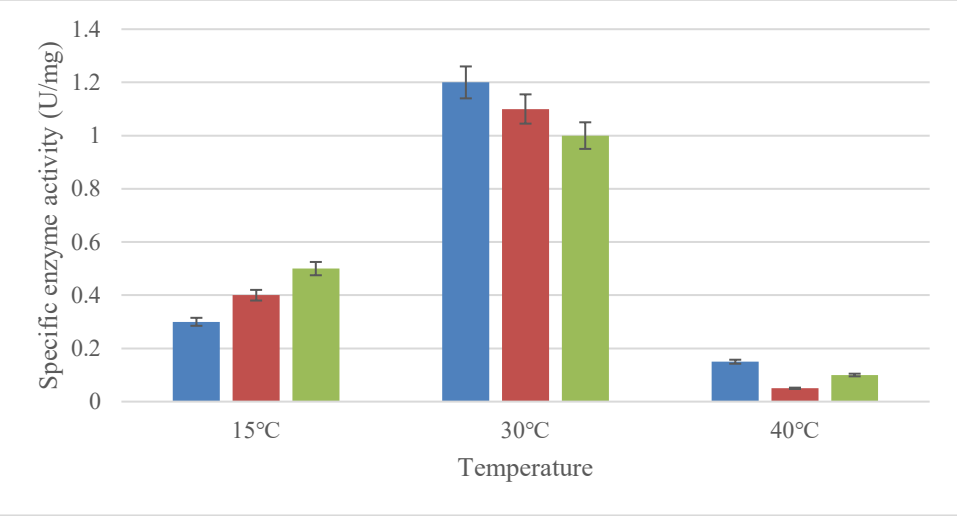


Figure 1. Effect of Temperature on Fungal Amylase Production

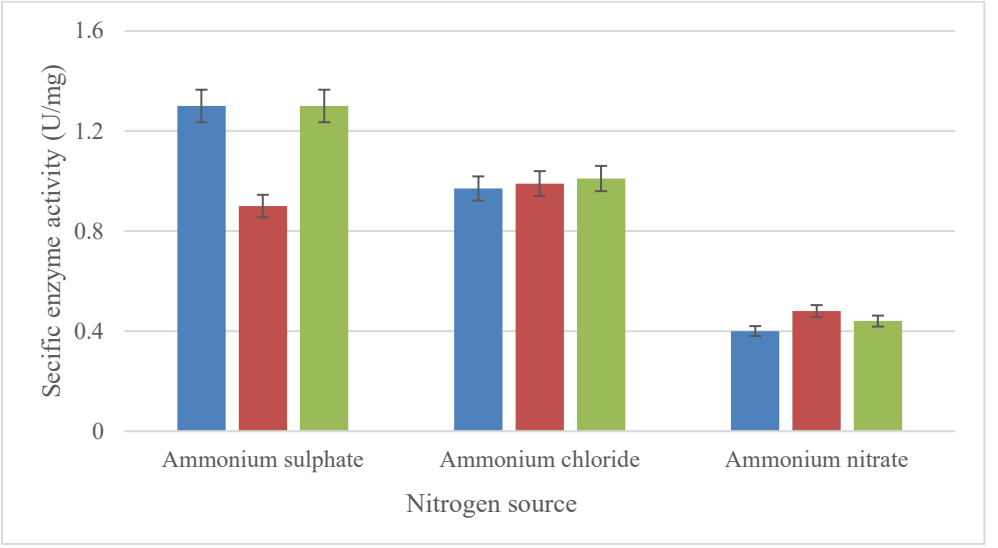


Figure 2. Effect of Nitrogen Source on Fungal Amylase Production

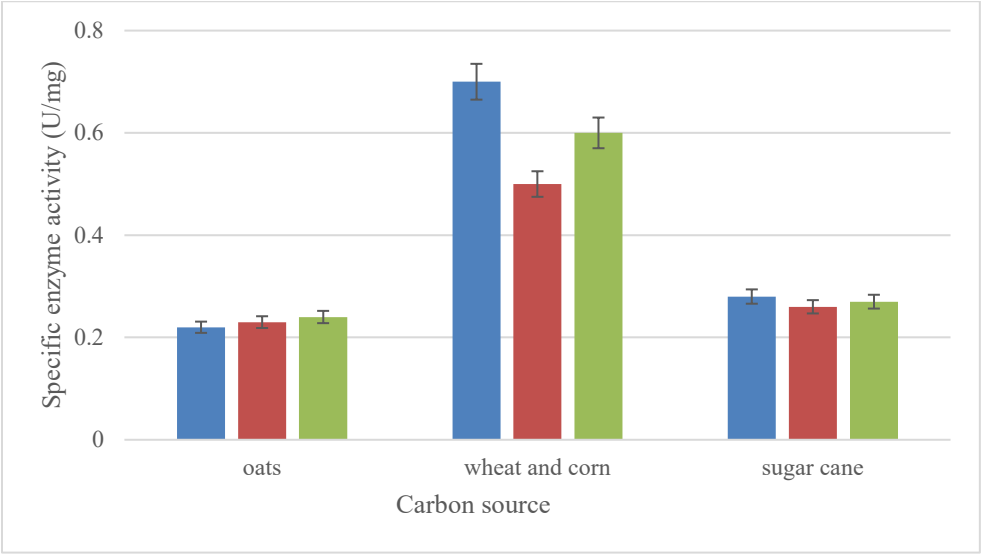


Figure 3. Effect of Carbon Source on Fungal Amylase Production

Table 1. Summary of the Optimum Conditions and Specific Enzyme Activity

Parameter	Optimum condition	Specific enzyme activity (U/mg)
Temperature	30 °C	1.1
Carbon source	Wheat and corn	0.6
Nitrogen source	Ammonium sulphate	0.44
pH	4	0.7

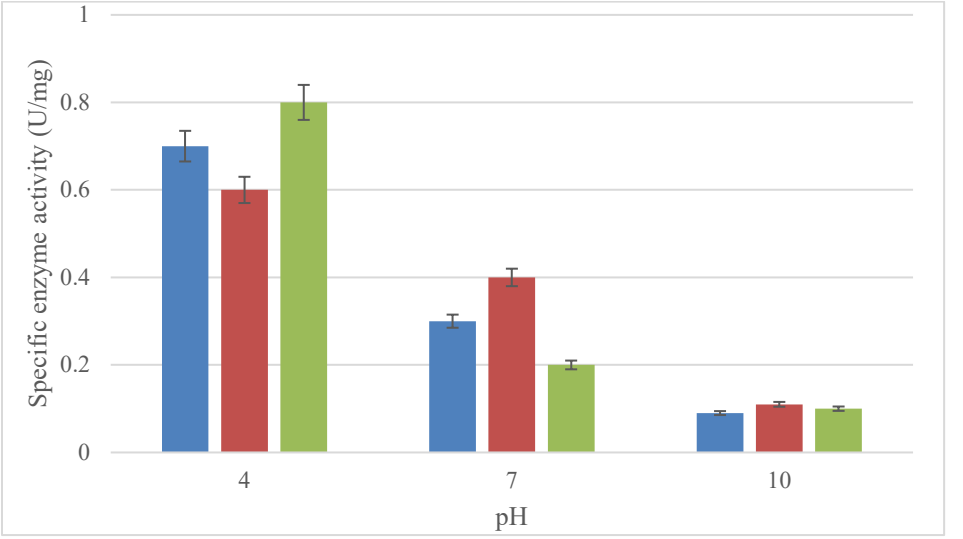


Figure 4. Effect of pH on Fungal Amylase Production

Table 2. ANOVA Summary

Parameter	SSB	SSW	MSB	MSW	*F* statistic	*P* value	Conclusion
Nitrogen source	0.750	0.084	0.375	0.014	26.8	0.001	Statistically significant difference
Carbon source	0.247	0.020	0.1237	0.003	36.4	0.004	Statistically significant difference
pH	0.560	0.402	0.280	0.007	41.81	0.000 3	Statistically significant difference
Temperature	1.608	0.045	0.8038	0.008	107.18	0.000 1	Statistically significant difference

4. DISCUSSION

The current study reports 30°C as the optimum temperature for the fermentation of *A. niger* for amylase enzyme production. This indicates that deviations from this temperature range could disrupt the equilibrium, resulting in reduced enzyme yield [15]. At a lower temperature (15°C), the enzyme activity may be reduced, resulting in a lower activity. Similarly, at a higher temperature (40°C), the enzyme might become denatured or lose its activity, leading to a decrease in its production.

Ammonium sulfate is the nitrogen source that produced amylase enzyme with the highest activity in this study. Nitrogen is essential for microbial growth and enzyme production. Nitrogen sources can vary in their availability and suitability for

the organism. Ammonium sulfate is a highly soluble and readily available nitrogen source [16]. It was used to provide nitrogen for *A. niger* in this study. The availability of nitrogen from ammonium sulfate was optimal to support the growth and metabolic processes of the fungus, resulting in increased fungal amylase production. The metabolic pathways and enzymes involved in assimilating and utilizing these nitrogen sources may vary, which may affect overall growth and enzyme production by the fungus [12, 17].

Wheat and corn were found to be the best sources of carbon for the production of amylase by the fermentation of *A. niger.* This could be due to the fact that wheat and corn are known to have a higher starch content, as compared to oats and sugar cane. Starch serves as a preferred substrate for fungal amylase production [18, 19]. *A. niger* produces fungal amylase to break down starch into more basic sugars such as maltose and glucose, which can be easily utilized by the fungus. The higher starch content in wheat and corn provides a more abundant and suitable carbon source for fungal amylase production, resulting in higher yields. Wheat and corn also contain a broader variety of nutrients, including the necessary vitamins, minerals, and amino acids, for microbial growth and enzyme synthesis. The availability of these nutrients in wheat and corn supports the overall metabolic activity of *A. niger*, leading to increased fungal amylase production [20].

In contrast, oats and sugar cane have a relatively lower nutrient composition, limiting the growth and enzyme production of the fungus. Also, the carbon-to-nitrogen ratio (C/N ratio) in the fermentation medium plays a crucial role in microbial growth and enzyme production [21]. *A. niger* prefers a moderate C/N ratio for optimal fungal amylase production. Whereas, wheat and corn provide a more balanced C/N ratio, facilitating the growth and metabolic activity of the fungus. On the contrary, oats and sugar cane have a C/N ratio that is less favorable for fungal amylase production by *A. niger,* which may have metabolic preferences for certain carbon sources.

A pH of 4 proved to be the optimum pH for the production of amylase by *A. niger.* This could be due to the fact that it is an acidophilic fungus [22], indicating that it thrives in acidic environments. It has adapted to be more efficient in nutrient uptake, growth, and enzyme production under acidic conditions. Consequently, providing an acidic pH of 4 would create a favorable environment for the growth and metabolic activity of *A. niger*, leading to increased fungal amylase production. Also, the fermentation medium's pH can influence the expression of genes involved in fungal amylase production, leading to the production of good quality amylase [23]. Lowering the pH to 4 may act as an inducer for the expression of fungal amylase genes in *A. niger*, resulting in higher levels of enzyme production. This induction mechanism at pH 4 may trigger the fungus to allocate more resources towards fungal *amylase* synthesis, ultimately leading to increased yields. Also, fungal *amylase*, like many enzymes, has an optimal pH range at which it exhibits the highest activity.

Fungal *amylase* may also be more stable at the pH of 4, which allows for prolonged enzyme activity. The availability of substrates, such as starch, can be influenced by pH, which leads to higher yields. Starch may be more soluble and accessible to *A. niger* at pH 4, as compared to pH 7 or pH 10. As a result, there is more efficient utilization of substrates by the fungus, leading to enhanced fungal *amylase* production [23].

The starch hydrolysis enzyme assay is used to determine the activity of an enzyme, such as amylase, that breaks down starch into smaller sugar molecules [24]. A specific volume of the fungal *amylase* enzyme extract was added to reaction tubes. The results of this assay provide information about the catalytic efficiency of the enzyme by measuring the rate at which starch is hydrolyzed. This information is important to understand the properties of the enzyme and its potential applications. It can also help to identify the optimal conditions for fungal *amylase* activity.

Performing the assay at varying conditions of temperature, pH, and substrate concentrations can determine the optimum parameters for maximum enzyme activity. Hence, the enzyme's performance can be optimized for various industrial laboratory applications. The assay data can be used to analyze the kinetic parameters of *amylase,* providing insights into the enzyme's affinity for starch. The calculated parameters, such as Michaelis-Menten constant and response rate (Vmax), can be used to aid in the optimization and designing of enzymatic processes [25].

The specific enzyme activity of the combined optimized parameters was 0.7 U/mg as summarized in Table 2 and these results are in accordance with previous studies [26, 27], which also concluded that the optimum conditions contribute significantly to the specific enzyme activity.

4.1. Conclusion

A. niger can be fermented under laboratory conditions to produce enzymes. Fungal amylase produced from *A. niger* is able to hydrolyze starch. The optimum conditions for the production of amylase included 30℃, pH 4, ammonium sulfate as the nitrogen source, and wheat and corn as carbon sources. At these conditions, amylase with the highest specific activity was produced.

Author Contribution

Marcia Matongorere: validation, writing-original document. **Tanaka Madingi:** investigation. **Rumbidzai Mangoyi:** validation, Supervision, Writing-Review & editing

Conflict of Interest

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

Data Availability Statement

Data availability is not applicable as no new data was used for the research article.

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Generative AI Disclosure Statement

The authors did not used any type of generative artificial intelligence software for this research.

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