RESEARCH ARTICLE

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Studying the solid-liquid extraction of enzyme amylase: influence of type of solvent, temperature, contact time and their interrelationship

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ABSTRACT

Amylases are one of the most essential and widely used enzymes in a number of industries, including food, fermentation, paper, textile and pharmaceutical. The industrial production of enzyme amylase is carried out using two methods which are submerged fermentation and solid-state fermentation. The important factors that affect the fermentation process are pH, temperature, carbon and nitrogen sources, and metal ions. This study aims at investigating the solid-liquid extraction of amylase from *Aspergillus niger*, focusing on the influence of solvent type, temperature, contact time, and their interrelationship. The effects of different solvents, temperatures, and contact times on the extraction efficiency and activity of amylase were examined using solvents of different polarity (water, methanol, ethanol, glycerol and acetone), varying temperatures (15 °C, 30 °C and 45 °C) and varying contact times (15, 30 and 90 minutes). The results provided insights into optimizing the extraction process parameters for enhanced amylase yield and activity. Polar solvents like glycerol extracted amylase with the highest enzyme activity levels compared to less polar solvents. A temperature of 30 °C was identified as the optimum for amylase extraction. The results showed that the majority of the amylase was recovered within the first 90 minutes of the process, with diminishing returns for longer contact times. Thus, this research concluded that the interplay between solvent type, temperature, and extraction time is complex, and further optimization through a systematic experimental design would be necessary to identify the ideal combination of these factors for maximizing amylase extraction efficiency and yield.

Key words: amylase, *Aspergillus niger*, solid state fermentation, extraction efficiency

Introduction

Enzymes are biological catalysts, which initiate and accelerate the biochemical reaction in living cells. Their structure is much specialised, and they have an active site that a substrate molecule may attach to and transform into a product (Vitolo, 2020). Nowadays, enzymes are utilised in a wide range of industries, including food, pharmaceuticals, paper, textiles, detergents, and many more.

According to Drauz et al. (2012), amylases are highly sought-after among the many commonly utilised enzymes due to their function in the hydrolysis of starch and their employment in hydrolytic action. They have been reported to represent around 25 % of the total enzyme sales and are one of the most significant groups of industrial enzymes (de Souza and Magalhães, 2010). Enzyme amylases have been employed in the food, textile, detergents, animal feed, pharmaceutical, paper and pulp, biofuels and waste treatment industries (Nunes & Kumar, 2018; Ahmad et al., 2019).

Amylases are produced by use of submerged fermentation (SmF) and solid-state fermentation (SSF) processes (Gupta et al., 2003). However, a number of nutritional and physicochemical parameters, including pH, inoculum age, inoculum concentration, agitation, incubation temperature, incubation duration, sources of carbon and nitrogen, as well as metal ions, have a great impact on the synthesis of amylase by fermentation (Sales et al., 2012).

The particular enzyme activity of the extracted amylase has also been reported to be greatly influenced by the solvent used, suggesting that solvent selection is an important extraction process parameter. The different solvents' propensities to interact with and solubilize the amylase molecules, as well as their effects on enzyme stability, are probably the cause of the variations in enzyme activity

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amongst them (Vermuë et al., 2014). To optimise the yield of amylase extraction and guarantee the effective recovery of this important enzyme for a range of industrial uses, the type of solvent used needs to be carefully chosen.

This study focuses on investigating the influence of solvent type, temperature, and contact time on the solid-liquid extraction of amylase from *Aspergillus niger*, aiming to optimize the extraction process for enhanced amylase yield and activity. Understanding how these parameters interact and influence each other is vital for developing an optimized extraction process.

Materials and Methods

Overview of the study

The study explores the effects of different solvents, temperatures, and contact times on the extraction efficiency and activity of amylase. The research was carried out at the University of Zimbabwe, department of biotechnology and biochemistry.

Culturing of the fungi

Aspergillus niger used in this study was provided by the department of Biotechnology and Biochemistry. *Aspergillus niger* was inoculated in a broth medium containing minimal amount of moisture and incubated at 37 °C under optimal conditions for amylase production, including pH, and aeration. To achieve maximum amylase production, the culture was allowed to grow for a period of 2 days.

Confirmation of amylase producing strain

The two days cultured *Aspergillus niger* was centrifuged and the supernatant produced was investigated for presence of amylase enzyme using the starch agar test. Starch agar media was prepared and the supernatant was added into the wells made in the media. The media was incubated for 48 hours. After the incubation, the plates were flooded with gram's iodine and observed for the zone of hydrolysis. Iodine and starch combine to make a blue tint, but the bacteria that produce amylase break down the starch by the action of amylase around them and use it as their only source of carbon, creating a translucent zone. Clear zone development on iodine-flooded starch agar plates served as the major criterion for selecting amylase-producing microorganisms.

Biomass preparation

The fungi were cultivated in an optimised growth medium containing 10 g of wheat bran, 0.05 % (w/w) urea, 0.25 % (w/w) $MgCl_2$, 0.25 % (w/w) KCl, ribose 1 % (w/w), 25 ml deionized water which was autoclaved for 20 minutes at 121 °C, under controlled conditions to maximize amylase production. After the incubation period, the fungal biomass was soaked in selected solvents for different contact times at varying temperatures for amylase extraction.

Extraction process

Solid-Liquid Extraction Setup

A set-up with three different sets of extraction experiments with variations in solvent type, temperature, and contact time was prepared. A range of solvents with varying polarity were used to examine the effect of solvent type. Multiple extraction conditions were set, including different temperatures (15 °C, 30 °C and 45 °C) and contact times (15 minutes, 30 minutes and 90 minutes).

Extraction procedure

Extraction was conducted in three sets of 100 ml conical flasks using 2 g of biomass in 50 ml of six solvents (10 % (v/v) methanol, 10 % (v/v) ethanol, 10 % (v/v) acetone, 10 % (v/v) glycerol, distilled water and 50 mM sodium chloride). A control was also included containing 2.5 % (v/v) glycerol. Extraction was done by soaking the biomass with several solvents while shaking the sample in a shaking water bath. The parameters selected for this study were type of solvent, soaking time and soaking temperature. The experimental procedure was repeated thrice in order to collaborate on the validity of results.

Separation of solid and liquid phases

After the designated contact times, the solid biomass was separated from the liquid extracts using filtration and centrifugation techniques to remove insolubles. The clear extracts obtained, which is the crude enzyme, were assayed for amylase activity.

Enzyme activity determination

The enzyme activity was assayed following the method of Bernfeld which is known as the DNS method (Bernfeld, 1955) using 3,5-dinitrosalicylic acid. In a test tube, the reaction mixture (containing 1 ml of soluble starch solution mixed with 1 ml of potassium phosphate buffer, pH 6.9) was mixed with 0.1 ml of the crude enzyme and incubated for 15 minutes at room temperature. After the incubation, 2 ml of the DNS reagent was added and the reaction mixture was terminated by immersing the tube in boiling water (100 °C) for 10 min. The absorbance was measured at 540 nm against blank prepared as above without incubation. A glucose standard curve was constructed using absorbance values measured at varying concentrations of glucose. The curve was used to estimate the glucose concentrations produced by amylase extracted with different solvents, on starch hydrolysis. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmole of reducing sugar as glucose per minute under the assay conditions specified.

Results

Confirmation of amylase producing fungi

The cultured *Aspergillus niger* colonies showed a clear zone during starch agar test confirming the presence of amylase. Thus, the colonies were then further used in solid state fermentation to produce the enzyme amylase.

Determination of the Amylase activity

The clear extracts obtained, which is the crude enzyme, from three different sets of extraction experiments with variations in solvent type, temperature, and contact time were investigated for their amylase activity and results are shown in Figures 1, 2 and 3. All the Figures show that the solvent glycerol is the most efficient in amylase extraction as explained by highest enzyme activity as compared to all the other solvents, with the control 2.5 % glycerol proving to be more efficient than 10 % glycerol. The solvent sodium chloride was found to be the least effective in amylase extraction as shown by the lowest enzyme activity obtained.

Figure 1 shows the interrelation of amylase activity at 30 minutes' contact time and varying temperature for all the solvents used in extraction. The highest activity was observed at 45 ºC recorded as 0.193 U/mg and the least at 15 ºC recorded as 0.11 U/mg for all the experiments. The activity of amylase was found to increase with increase in temperature for all the solvent types. Results from Figure 1 shows that the optimum temperature for amylase extraction at 30 minutes' contact time is 45 ºC for all the solvents used.

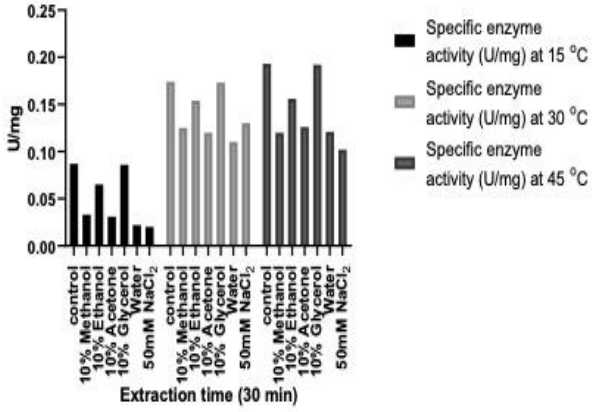

Figure 1. *Specific enzyme activity of amylase measured at varying temperatures (15 ºC, 30 ºC and 45 ºC) for the selected solvents types when the contact time was kept constant at 30 minutes.*

Figure 2 shows the interrelation of amylase activity at 60 minutes' contact time and varying temperature for all the solvents used in extraction. The highest activity was observed at 30 ˚C recorded as 0.615 U/mg and the least at 15 ˚C recorded as 0.286 U/mg. These results show that the optimum temperature for amylase extraction at 60 minutes' contact time is 30 ˚C for all the solvents used.

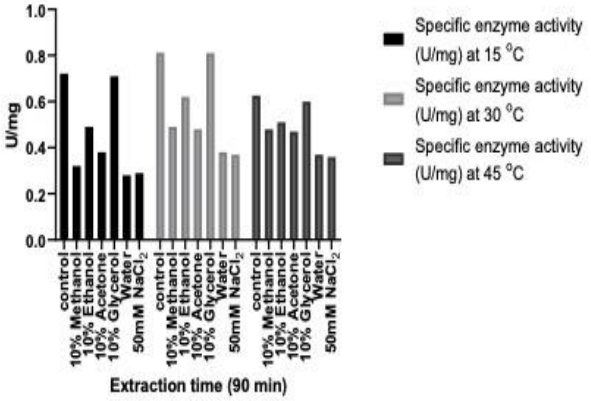

Figure 2. *Specific enzyme activity of amylase measured at varying temperatures (15 ºC, 30 ºC and 45 ºC) for the selected solvents types when the contact time was kept constant at 60 minutes.*

Figure 3 shows the interrelation of amylase activity at 90 minutes' contact time and varying temperature for all the solvents used in extraction. The highest activity was observed at 30 ˚C recorded as 0.812 U/mg and the least at 15 ˚C recorded as 0.28 U/mg. These results show that the optimum temperature for amylase extraction at 90 minutes' contact time was 30 ˚C for all the solvents used.

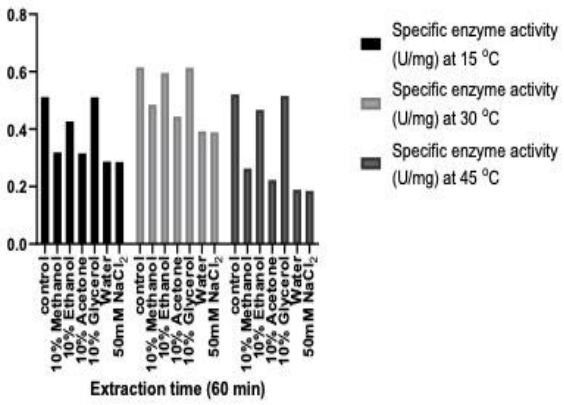

Figure 3. *Specific enzyme activity of amylase measured at varying temperatures (15 ºC, 30 ºC and 45 ºC) for the selected solvents types when the contact time was kept constant at 90 minutes.*

Discussion

In this study, the effects of different solvents, temperatures, and contact times on the extraction efficiency and activity of amylase were examined using solvents of different polarity (water, methanol, ethanol, glycerol and

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acetone), varying temperatures (15 °C, 30 °C and 45 °C) and varying contact times (15, 30 and 90 minutes).

The amylase enzyme extracted using glycerol showed the highest levels of enzyme activity when compared to amylase extracted using the other solvents. However, amylase extracted from 10 % glycerol showed specific enzyme activity of 0.9 U/mg and the control which was 2.5 % glycerol yielded amylase with a specific activity of 1.0 U/mg. As the activity is almost the same, the 10 % glycerol may have better advantages like greater viscosity or improved enzyme protection hence may be a solvent of choice instead of the 2.5 % control. Generally, glycerol works well as an extraction solvent for amylase because of its high viscosity and capacity to create strong hydrogen bonds (Palit & Banerjee, 2001). It is less polar and less effective in rupturing the hydrophobic sections of enzymes. According to Hmidet et al. (2008), this permits the proteins to retain their natural stability and structure.

Additional insights into the kinetic parameters controlling the extraction process were obtained by further analysing the time-temperature effects. Enzymes work best in certain temperature ranges; over these limits, their structural integrity may be compromised, making the enzymes inert (Aehle, 2007). The best temperature for amylase extraction was found to be 30 °C from 60 to 90 minutes of the procedure regardless of the solvent utilised. Lower temperatures, such 15 °C, may cause inadequate levels of enzyme activity and solubility, which would reduce extraction yields. On the other hand, the structural stability and integrity of the enzyme may be jeopardised at higher temperatures, such as 45 °C, which might lead to a drop in the specific activity due to partial denaturation or conformational changes (Rao et al., 1998). This may explain why the specific enzyme activity of amylase extracted at 45 °C was lower than that of amylase extracted at lower temperature for extraction time 90 minutes, which was a longer exposure time.

Throughout all three temperature conditions, the 2.5 % glycerol solvent continuously extracted amylase with the greatest specific enzyme activity, demonstrating its superior performance. According to Michałowska-Kaczmarczyk et al. (2015), this discovery implies that glycerol is a useful solvent for extended extraction times since it can preserve the structural integrity of the enzyme and avoid denaturation over time. Glycerol's ability to form hydrogen bonds and its viscosity may be responsible for its protective qualities, preventing harmful alterations to the enzyme's structure and maintaining its catalytic activity throughout the prolonged extraction procedure. Furthermore, even over extended periods of time, the polarity of glycerol makes it easier to solubilize and extract the amylase enzyme, resulting in a more effective and reliable recovery (Vaidya et al., 2020).

The effectiveness of the extraction process is greatly influenced by the contact time. In this study, 90 minutes proved to be the best contact time for amylase extraction. Extended periods of contact provide a more comprehensive interaction between the solvent and the solid substrate, hence raising the probability of extracting the intended constituents. Incomplete extraction may occur from shorter contact durations (Kaufmann & Christen, 2002). One important component of the contact time needed for extraction is the rate of diffusion. The solute's diffusion coefficient in the solvent determines how long it takes for the solvent to permeate the solid substrate and dissolve the required components. The diffusion rate can be influenced by elements such as the solid substrate's porosity, particle size, and composition (Vaidya et al., 2020).

Conclusions

The best conditions for amylase extraction are 30 °C, 2.5 % glycerol solvent, and 90 minutes of extraction time. These specifications enable the greatest recovery of the particular activity of the enzyme, which is essential for figuring out the process's total yield and efficiency.

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Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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