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Optimization of *α*-amylase Activity Enzyme Produced By *Bacillus subtilis* and *Aspergillus niger*

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Abstract

Many microorganisms are known to be good sources for *α*-amylase enzyme production, with its recognized industrial importance. *Bacillus subtilis* and *Aspergillus niger* are appropriate isolates for production of *α*-amylase using solid state fermentation (SSF). Efforts were done to increase *α*-amylase production targeting to decrease the SSF cost. The aim of this study was evaluating certain significant factors as carbon source, temperature, pH, volume of container and minerals for enhancing *α*-amylase production from *B. subtilis* and *A. niger* in lab scale SSF. Results showed that the maximum *α*-amylase productivity was obtained in units and one unit of enzyme activity defined as (the quantity of enzyme that release 1Mmole of sugar / minute at 37°C for 48 hours incubation or 45°C for 24 hours incubation by used *B. subtilis*. Inoculum size of 0.5 % v/v was used, and the maximum *α*-amylase productivity was obtained unit at temperature of 37°C for 7 days by *A. niger*. The maximum activity was found to be at 60°C using *B. subtilis*. Inoculum volume of 1000 mL showed the maximum amylase production from B. subtilis, while inoculum volume of 500 mL showed the maximum amylase production from *A. niger*. $ZnSO_4$ showed the best enzyme production from *B. subtilis* and $FeSO_4.7H_2O$ showed the best enzyme production from *A. niger*. The optimum pH for the best enzyme activity in this study was 6.5 from *B. subtilis*.

Key of words: Amylase activity, SSF Technique, pH, Temperature.

1. Introduction

Amylases are enzymes that cleave α-1, 4 glycosidic bonds in an endo-acting manner, freeing the low molecular weight subunits, primarily maltose and glucose [1]. At the same time, α-amylase hydrolyzed starch can be used as a sizing and coating agent in the paper sector instead of expensive chemically modified starch. [2]. Despite these benefits, enzymes are only used in a few industrial applications. Several variables are soluble aqueous medium and it is difficult to recover them from reactor effluents, including the high cost of enzymes, their instability, and availability in small doses. Different kinds of microbes, particularly bacteria and fungi, have been identified as substantial suppliers of α-amylase, leading to industrial requests. It's generally being studied as a result of an increase in large-scale application. *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are known to produce thermostable α -amylase, and these bacteria have been widely employed for commercial production of the enzyme for a variety of purposes. Thermostable α-amylases have been reported from a variety of bacterial strains using both SmF (submerged fermentation) and SSF. SSF has been found to be more favourable than SmF in regards of enzyme production costs [2, 3]. *Bacillus Sp*. has become pervasive in nature, necessitating adequate dietary requirements for amplification and producing the highest amount of *α*-amylase. Food, feed, detergents, textiles, medicines, and paper are just a few of the industries that use amylase [3, 4]. Microbiological *α*-amylase is the most common source of industrial-amylase. Ethanol, amino acids, citric acids, nitrates, nitrites, fine compounds, and significant molecules might all be manufactured via biological methods. Enzyme-catalyzed reactions provide technology a big boost in terms of cost and environmental friendliness [3]. The attachment of free or soluble enzymes to various types of supports, resulting in a reduction or loss of enzyme mobility, is known as enzyme immobilisation [5]. Amylases account for around 25% of the global enzyme trade business, according to annual estimates. The scope of α-amylases encompasses not just industrial

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applications, clinical and medicinal procedures, but also their global ubiquity. Its living domains make it an ideal molecule for studying evolutionary events [3]. Simultaneously, instead of more expensive chemically modified starch, *α*-amylase hydrolyzed starch which can be utilised as a sizing and coating agent in the paper sector. Despite these benefits, enzymes are only used in a few industrial applications: Several factors, most notably the high cost of enzymes, their instability and availability in small amounts in soluble aqueous media, make recovering them from reactor effluents at the end of catalytic procedures challenging. The current study was designed with the primary purpose of effectively isolating, screening, manufacturing, and purifying α-amylase, based on the previous background information. Solid state fermentation (SSF) is a popular method for producing amylases. In general, various agro-residues can be used in solid state fermentation [7].

Our study aims to enhance the production and the activity of *α*-amylase produced by *Bacillus subtilis* and *Aspergillus niger*, via optimization of certain parameters during SSF.

MATERIALS AND METHODS:

Materials

Microorganisms

B. subtilis bacteria was donated by Dr. Ahmed Badawi, El Azhar University faculty of science, microbiology department. *A. niger* fungus was bought from Mycological Centre, Assiut University, Egypt. *A. niger* is well-known production organism used in the food and food/feed additives industry. Fermented foods produced by *A. niger* was shown to be aflatoxin-free. *A. niger* was tested and demonstrated no detectable amount of aflatoxin.

The chosen bacterial strains were inoculated in nutrient broth containing (g L-1): peptone, 5; Beef extract, 3; NaCl [7]. *B. subtilis* can grow in the gastrointestinal (GI) tract of animals, it is not considered a human pathogen. In fact, *B. subtilis* along with other species of *Bacillus* is considered a GRAS (Generally Regarded as Safe) organism by the Food and Drug Administration (FDA).

Effect of various sources on α-amylase production

Different carbon sources were used as substrate such as wheat straw, maize straw, rice straw, potatoes, and mustard, were procured from local market and powdered to obtain a particle size of 1.0 mm to 2.0 mm.

Methods

SSF Technique

Experiments were conducted in 100ml Erlenmeyer flasks holding 3g of substrate and 10ml of sterile liquid nutrition mixture containing 0.3g KH_2PO_4 and 0.5g $MgSO_4.7H_2O$. The flasks were autoclaved and inoculated with 1ml of the prepared inoculum, thoroughly mixed and followed by incubation at 37ºC, pH 7 for 3 days. Periodically, aseptically extracted samples were tested for α-amylase activity [2]. Size of inoculum of 0.5 % v/v was used for further experiments in this study.

α-amylase assay

The crude enzyme sample was incubated for 10 minutes at 45oC in pH 5 with 1% soluble starch in 0.1M Citrate buffer. 3 mL 3,5-dinitrosalicyclic acid (DNS) was heated for 15 minutes in a boiling water bath to estimate the released reducing sugars. A reference blank of two ml of 0.1 M Citrate buffer was used. A spectrophotometer set to 575 nm was used to measure the optical density of the colour developed. The results were compared to a standard curve using glucose concentrations varying from 0.10 to 1.0 mg/ml. Under conventional test conditions, one unit of enzymatic activity (IU) was defined as the quantity of enzyme that released 1 µmol of sugar in 1 minute [8].

Effect of various culture media on *α*-amylase production

The components of the culture medium and the fermentation process must be optimised in the synthesis of industrial enzymes. Wheat straw, maize straw, and rice straw were soaked separately for 7 days, washed with water 3 times, and left to dry in oven at 60ºC overnight, then pre-treated with H_2SO_4 (1%) and washed. Potatoes peal, mixture of potatoes with mustard cake, mustard cake was used as carbon substrates. The mustard cake was prepared by boiling the mustard seeds with water for 15 minutes, filtered, and the plant matrix remained on filter paper was used as a substrate.

The mixture of potatoes and mustard produced the most α-amylase; thus, it was utilised as the substrate for the rest of the study's tests to optimise *α*-amylase production. The enzyme was extracted after the candidate strain was inoculated into the culture medium [9]. All substrates were exposed to SSF, and their enzyme production was measured using an assay [4].

Effect of pH on α-amylase production

It was discovered how pH affects *α*-amylase production. Individually, several pH buffers (90%, v/w) were mixed with the solid medium. The buffer solution's pH ranged from 6.0 to 11.0. After fermentation, the enzyme was extracted and the α-amylase assay was performed [10].

Effect of different minerals on *α*-amylase production

Seven different minerals were used separately in SSF; $MgCl_2.6H_2O$, $ZnSO_4$, $K_2S_2O_7$, KCl, $MgSO_4.7H2O$, $FeSO_4.7H_2O$, and $MnSO_4.H_2O$. Some of them enhance growth of microorganisms and others act as cofactor for enzyme [11,12]

Effect of different container volumes on α-amylase production.

Different volumes were used; 50, 100, 250, 500, or 1000 ml separately.

Effect of different temperatures on α-amylase production

Various temperatures were used to achieve the best α-amylase production. To check which temperature can enhance the production [13]. *B. subtilis* was incubated separately for three days in different temperatures; 37ºC, 45ºC, and 37ºC for the first 48h then 45ºC for 24h, also, to check stability of the enzyme at different temperatures especially at 45 [2, 10]. Results are the average of triplicate experiments.

A. niger. was incubated separately in 27ºC for 7 days, in 37ºC for 7 days, and finally in 45oC for 3 days then incubated in 27ºC for 4 days [14]. Results are the average of triplicate experiments.

Temperature and pH effect on α-amylase activity

The optimal temperature for the enzyme assay was determined by varying the assay's incubation temperature from 30ºC to 70ºC [15, 16]. Results are the average of triplicate experiments.

Different pH such as 2.5, 4.5, 6.5, 8.5, 11.5 were controlled by NaOH and H_2SO_4 separately [15, 16]. Results are the average of triplicate experiments.

RESULTS AND DISCUSSION

B. subtilis and *A. niger* were utilised in this study to produce α-amylase in SSF, and the optimal conditions for their production were studied.

All the experiments were performed in triplicates and data presented is the average of three parallel experiments. Error bars are shown for standard deviation.

In terms of carbon sources, Table 1 and Figure 1 show that utilizing a mixture of potatoes and mustard cake as a substrate resulted in the most α-amylase production, while rice straw resulted in the lowest. The combination of potato and mustard cake appears to be an effective fermentation substrate for producing large *α*-amylase yields from *Bacillus sp*. This could be because the potato-mustard cake mixture has a lower proteinaceous matter content and a larger carbohydrate content than the other substrates [17, 18].

B. subtilis was reported to produce the most α-amylase when incubated at 37°C for 48 hours and then 45°C for 24 hours (Table 2 and Figure 2). This revealed a unique feature of this *B. subtilis* strain, which grows at 37°C as a mesophile yet produces active and stable enzyme at high temperatures (45-70ºC). Temperatures of 35–45°C were reported to induce maximal α-amylase production in *B.* strains [6, 8].

A. niger produced the most α-amylase units after being exposed to 37°C for 7 days, and the least after being exposed to 45°C for 3 days and subsequently 27°C for 4 days (Table 3 and Figure 3) [19, 20]. The low temperature is not ideal for *A. niger* growth, whereas the high temperature depletes the medium water content and reduces oxygen concentration due to evaporation [4]. Thus, both extremes limit the enzyme production [10].

Table (1): Effect of different substrates on production of *α*-amylase produced by *B. subtilis*.

Optimization Substrate	Mean
Potatoes + mustard	0.8746667 **IU**
Mustard cake	0.735 **IU**
Treated Wheat straw	0.4616667 **IU**
Maize straw	0.4696667 **IU**
Rice straw	0.4426667 **IU**
Potatoes	0.5456667 **IU**

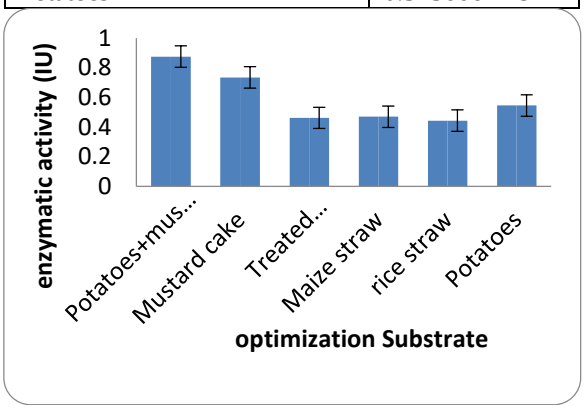

Figure (1): Effect of different substrates on production of *α*-amylase produced by *B. subtilis*.

Table (2): Effect of different temperatures on production of *α*-amylase produced by *B. subtilis*.

Temperature ºC	Mean
37ºC for 72h	0.7796667 **IU**
37ºC for 48h, 45ºc for24h	0.7753333 **IU**
45ºC for 72h	0.3743333 **IU**

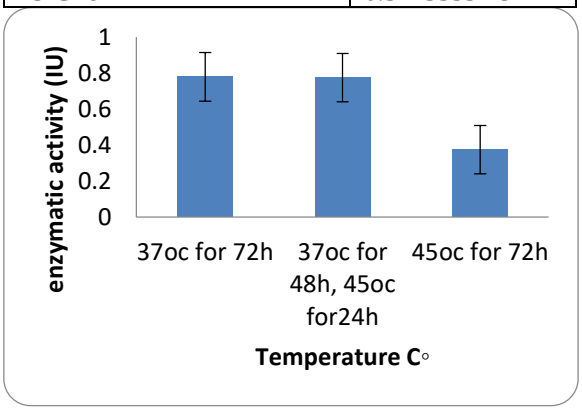

Figure (2): Effect of different temperatures on production of *α*-amylase produced by *B. subtilis*.

Table (3): Effect of different temperature on production of *α*-amylase produced by *A. niger*.

Temperature ºc	Mean
27ºc for 7days	0.25 **IU**
37ºc for 7days	0.5566667 **IU**
45ºc for 3days then 27ºc for 4days	0.044 **IU**

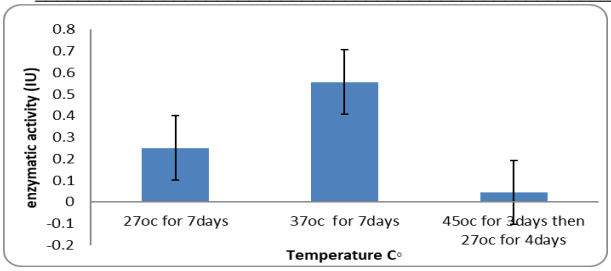

Figure (3): Effect of different temperature on production of *α*-amylase produced by *A. niger*.

In considerations of container volume, the inoculum volume of 50ml produced the lowest amount of *α*-amylase from both *B. subtilis* (Table 4 and Figure 4) and *A. niger* (Figure 5). The capacity of containers is one of the most important biological parameters, as it affects biomass production in the fermentation process, which is lowest when the inoculum volume is the smallest (50ml). In the case of *B. subtilis*, the inoculum volume of 1000 mL resulted in the highest α-amylase production (Figure 4), but in the case of *A. niger*, the inoculum volume of 500 mL resulted in the highest *α*-amylase production (Figure 5). The maximum enzyme production is thought to be the consequence of a balance between the nutrient supply and the growing biomass [15, 21, 22], which can be the reason for the 1000 ml inoculum volume is not optimum for *A. niger*.

Volume ml	Mean
50 ml	0.2533333 **IU**
100 ml	0.45 **IU**
250 ml	0.7533333 **IU**
500 ml	0.9433333 **IU**
1000 ml	1.1866667 **IU**

Table (4): Effect of different volume on production of *α*-amylase produced by *B. subtilis*.

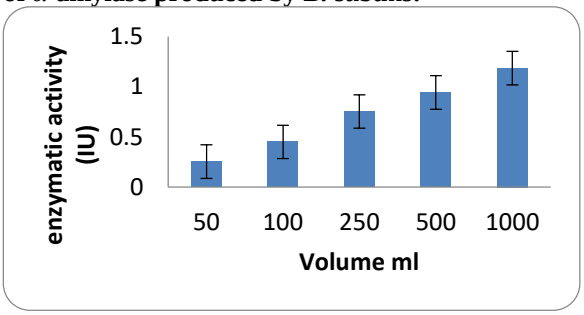

Figure (4): Effect of different volume on production of *α*-amylase produced by *B. subtilis*.

Volume ml	Mean
50 ml	0.2533333 **IU**
100 ml	0.34 **IU**
250 ml	0.55 **IU**
500 ml	0.6433333 **IU**
1000 ml	0.4666667 **IU**

Table (5): Effect of different volume on production of *α*-amylase produced by *A. niger*.

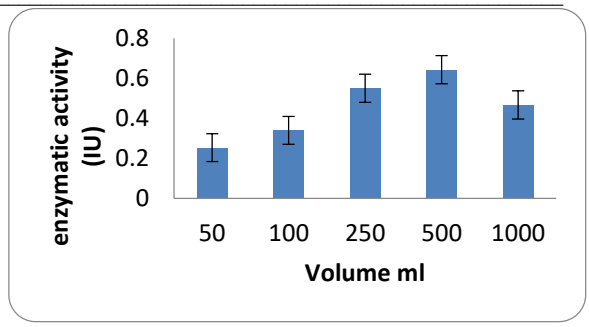

Figure (5): Effect of different volume on production of *α*-amylase produced by *A. niger*.

Observations of mineral variation during SSF revealed that the use of $ZnSO_4$ showed the best enzyme production from *B. subtilis* (Table 6 and Figure 6), and $FeSO_4.7H_2O$ showed the best enzyme production from *A. niger* (Table 7 and Figure 7). The *α*-amylase production of *A. niger* was suppressed using $MgCl_2.H_2O$ and K_2SO_7. In their investigation, Babar and Khadse [14] looked into the influence of changing metal composition in the media. [21, 22].

Mineral	Mean
$Mgcl_2.6H_2O$	0.683 **IU**
$ZnSO_4$	0.885 **IU**
K_2SO_7	1.738 **IU**
KCl	0.464 **IU**
$MgSO_4.7H_2O$	0.648 **IU**
$FeSO_4.7H_2O$	1.214 **IU**
$MnSO_4.H_2O$	0.715 **IU**

Table (6): Effect of different minerals on production of *α*-amylase produced by *B. subtilis*.

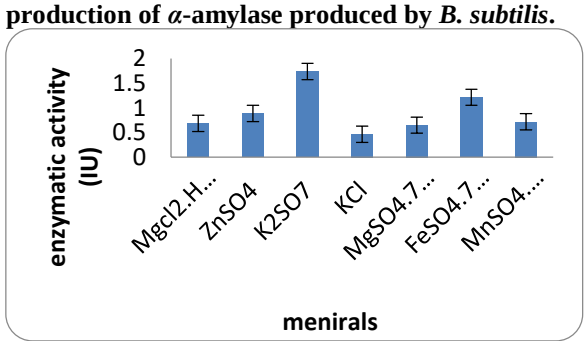

Figure (6): Effect of different minerals on production of *α*-amylase produced by *B. subtilis*.

Mineral	Mean
$Mgcl_2.6H_2O$	0.002 **IU**
$ZnSO_4$	0.028 **IU**
K_2SO_7	0.104 **IU**
KCl	0.001 **IU**
$MgSO_4.7H_2O$	0.155 **IU**
$FeSO_4.7H_2O$	0.189 **IU**
$MnSO_4.H_2O$	0.564 **IU**

Table (7): Effect of different minerals on production of *α*-amylase produced by *A. niger*.

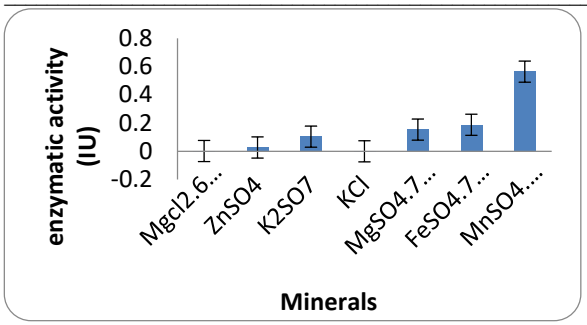

Figure (7): Effect of different minerals on production of *α*-amylase produced by *A. niger*

The α-amylase activity produced by *B. subtilis* cultured at various temperatures and pH levels was studied. Table 8 and Figure 8 show that *α*-amylase activity was highest at 60ºC and lowest at 70ºC. Table 9 and Figure 9 show that at 6.5 pH, *α*-amylase activity was highest, whereas at 2.5 pH, it was lowest. The effects of temperature and pH on enzyme activity have been studied extensively. This variation could be explained by the influence of nutritional and physical factors [4, 23].

Temperature ºC	Mean
30ºC	0.8966667 **IU**
40ºC	0.9646667 **IU**
50ºC	1.11 **IU**
60ºC	1.2933333 **IU**
70ºC	0.7266667 **IU**

Table (8): Effect of different temperature on activity of α-amylase produced by *B. subtilis*.

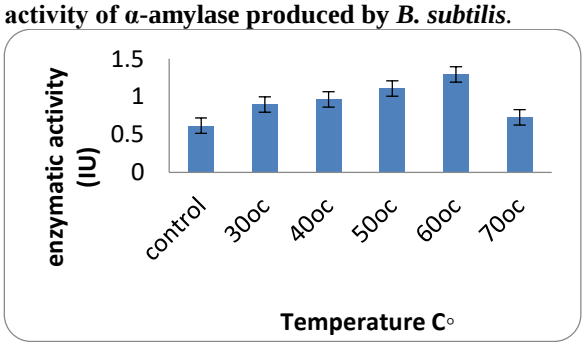

Figure (8): Effect of different temperature on activity of *α*-amylase produced by *B. subtilis.*

pH value	Mean
2.5	0.35 **IU**
4.5	0.66 **IU**
6.5	2.37 **IU**
8.5	2.23 **IU**
11.5	1.1333333 **IU**

Table (9): Effect of different pH values on activity of *α*-amylase produced by *B. subtilis*.

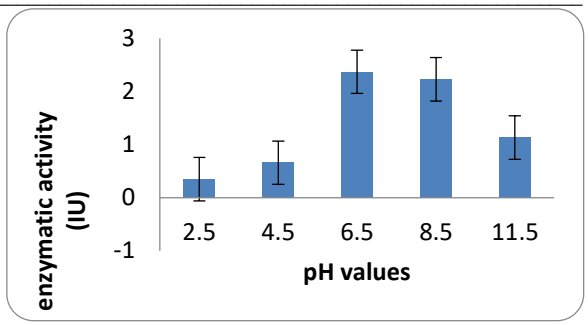

Figure (9): Effect of different pH values on activity of *α*-amylase produced by *B. subtilis*.

CONCLUSION

SSF was used to optimise several parameters for maximal *α*-amylase production from *B. subtilis* and *A. niger*. *B. subtilis* produced the highest yield of *α*-amylase when incubated at 37°C for 48 hours or 45°C for 24 hours, while *Aspergillus niger* produced the highest yield of *α*-amylase unit when incubated at 37°C for 7 days. At 60oC, the greatest activity was reported. *Bacillus subtilis* produced the highest yield of *α*-amylase when the incubation volume was 1000 mL, while *A. niger* produced the highest yield of *α*-amylase when the incubation volume was 500 mL. *Bacillus subtilis* produced the highest yield enzymes with $ZnSO_4$ and *Aspergillus niger* produced the highest yield enzymes with $FeSO_4.7H_2O$. In this study, the optimum pH for the best enzyme activity was reported to be 6.5. Further studies should be performed in pilot-scale for scaling up before commercialization and testing larger samples in production scale.

AUTHOR CONTRIBUTIONS

All authors made significant contributions to the experimental design, analysis and data interpretation; took part in writing the manuscript or revising it critically; agreed to submit to the current journal.

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