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Production of Alpha Amylase by *Bacillus subtilis* Using Maize Husk as Substrate

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AM, JBA and DAM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AM, JBA and DAM managed the analyses of the study. Author AM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To produce alpha amylase by *Bacillus subtilis* using maize husk as substrate.
Place and Duration of Study: Department of Microbiology, Ahmadu Bello University, Zaria between August 2016 and December 2016.
Methodology: The proximate composition of the maize husk substrate was determined following standard procedures described by Association of Official Analytical Chemists (AOAC). The effects of incubation temperature, initial pH, incubation period and moisture content on the production of alpha amylase were investigated. Alpha amylase was produced by the *B. subtlis* isolate using maize husk as substrate by solid state fermentation under predetermined optimum fermentation conditions. The type of amylase produced was identified by sugar analysis using glucose and maltose as standards on thin layer chromatography (TLC).
Results: The proximate analysis of the maize husk revealed percentages of ash (4.83%), crude protein (7.00%) and carbohydrates (60.37%) contents. All parameters optimized were found to significantly ($P < 0.05$) influence α-amylase production using maize husk. An overall yield of 51.65±0.13 U/g of alpha amylase was obtained under predetermined optimum fermentation conditions (35°C, pH 7.0, 72hrs and moisture content of 1:2). The product of starch hydrolysis on

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the TLC plate was found to appear as a spot with a retention factor (Rf) of 0.48 which is the same as that of standard maltose. Thus, indicating that the amylase produced was alpha amylase.
Conclusion: Maize husk contains sufficient organic nutrients for growth and amylase production by *B. subtilis*. The study also revealed the potential of maize husk as substrate in the production of alpha amylase by *B. subtilis*.

Keywords: Production; Bacillus subtilis; maize husk; fermentation.

1. INTRODUCTION

Amylase is one of the most widely used enzymes in the industry such as textile, paper, detergent, food, fermentation and pharmaceutical. It hydrolyzes starch which is used commercially for the production of sugar syrups which consists of glucose, maltose, and higher oligosaccharides [1].

Two major classes of amylases have been identified in microorganisms, namely α-amylase and glucoamylase. α-Amylases (endo-1, 4-α-D-glucan glucohydrolase, E.C. 3.2.1.1) are extracellular enzymes that randomly cleave the α-1,4-D glucosidic linkages between adjacent glucose units in the linear amylose chain. Glucoamylase (exo-1, 4-α-D-glucan glucanohydrolase, E.C. 3.2.1.3) hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin in a step-wise manner [2].

Amylase is among the most important enzymes with wider industrial applications in food, fermentation, textile, paper, detergent and pharmaceutical industries [3].

Agro wastes have been found to be good substrates for the cost effective production of several hydrolytic enzymes including alpha amylase and are thus attracting the attention of researchers for exploring different agro wastes as substrates for alpha amylase production [4]. In this regard, brans and flours of different grains and tubers, such as corn, rice, sorghum and wheat and peels of cassava and potato had been used in the development of fermentation medium to increase the productivity of amylases from bacteria and fungi [5-7].

Production of α-amylase had been carried out through submerged fermentation (SmF) and solid state fermentation (SSF) [8]. In recent years, the technique of solid state fermentation (SSF) process had been developed and used more extensively. It offers several advantages over SmF and had been reported to be the most appropriate process for developing countries [9].

There are so many baking, pharmaceutical, brewing and food industries in Nigeria that utilizes amylase for their production processes. However, amylase enzymes are yet to be produced commercially in Nigeria, making the cost of procurement high as a result of importation [10].

Synthetic medium tend to be very expensive and uneconomical, as such there is need to come up with more economically available agricultural and industrial by products, that can serve as good substrates for enzyme production through SSF.

This study was aimed at producing alpha amylase by *B. subtilis* using maize husk as substrate.

2. MATERIALS AND METHODS

2.1 Collection of Sample

One (1) kg of maize husk sample was procured from a maize milling station in Samaru, Sabon Gari Local Government Area of Kaduna State. The maize husk sample was packaged in a clean polythene bag, labelled appropriately and brought to the Industrial/Food Research Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for analyses.

2.2 Proximate Analyses of the Sample

Ten grams of the dried unfermented maize husk sample was used to carry out proximate analyses at the Product Development Research Programme, Institute of Agricultural Research, Ahmadu Bello University, Zaria in order to determine the moisture, ash, crude fibre, fat, crude protein and total carbohydrate contents as the percentage composition of the substrate according to standard methods described by Association of Official Analytical Chemists [11].

2.3 Alpha Amylase Production by Solid State Fermentation (SSF)

2.3.1 Inoculum preparation

The *Bacillus subtilis* (RD4) isolate was inoculated into nutrient broth with the following composition in (g/l): peptone, 5; Beef extract, 3; NaCl, 5, followed by incubation at 37°C for 24hours to obtained a standardized inoculum of 3.5 x 10^5 CFU/ml with 0.5 optical density (OD) at 600 nm [12].

2.3.2 Preparation of medium for SSF

For α-amylase production under solid state fermentation, mineral salt solution was used as the fermentation medium and comprised the following per litre: 0.1 g, KH_2PO_4; 0.1 g $(NH_4)_2SO_4$; 0.25 g, NaCl; 0.01 g, $MgSO_4$ and 0.01 g, $CaCl_2$

2.3.3 Solid state fermentation

Ten millilitres of the mineral salt solution was mixed with 5g of maize husk substrate in a 250 ml cotton-plugged Erlenmeyer flask in duplicate and labeled appropriately. The contents were homogenized and the initial pH adjusted to 7.0, then sterilized at 121°C for 15 min. After which, the flasks were inoculated with 1.0 ml of freshly prepared inoculum (3.5 x 10^5 CFU/ml) and incubated aerobically at 35°C for 72hrs [13].

2.4 Parameters Optimized for α-Amylase Production

The following parameters were optimized; effects of incubation temperature (20, 25, 30, 35 and 40°C), initial pH (5, 6, 7, 8 and 9), incubation period (24, 48, 72, 96 and 120 hours) and moisture content (1:1, 1:2, 1:3, 1:4 and 1:5) on alpha amylase production.

2.5 Amylase Assay

2.5.1 Enzyme extraction

After fermentation, 50 ml of 0.1 M phosphate buffer (pH = 7) was added and the flasks were placed in a shaker at 150 rpm for 1h. They were then filtered through a muslin cloth followed by centrifugation at 10,000 x g for 20 min. The clear supernatant was subjected to amylase assay [13].

2.5.2 Amylase activity

Amylase activity was determined by spectrophotometric method as described by [14]. According to the procedure, 1.0 ml of enzyme solution was taken into test tube and 1.0 ml of substrate (1% w/v starch) in 0.1 M phosphate buffer (pH 7.0) was added and incubated at 50°C for 30 min in water-bath. Then, 2.0 ml dinitrosalicylic acid (DNS) reagent was added to each tube to stop the reaction and kept in boiling water-bath for 5 min. The content was then cooled to room temperature and the volume made up to 10 ml with distilled water and absorbance was read at 540 nm. One unit (U) of α-amylase activity is defined as the amount of enzyme that releases 1µmol of reducing sugar as glucose per minute under 50°C for 30 min and expressed as U/g of dry substrate [13].

2.6 Identification of the Type of Amylase by Sugar Analysis on Thin Layer Chromatography (TLC)

To identify the type of amylase produced based on starch hydrolysate, thin layer chromatography was carried out. An aliquot of 0.9 ml 1% soluble starch was mixed with 0.3 ml crude enzyme solution and incubated for 30 minutes at 80^{o}C in a water-bath. The hydrolysate was then spotted on TLC plate along with standard known sugar (glucose and maltose) solutions. A one dimensional ascend was done using a solvent system (v/v) of butanol: ethanol: water (5:3:2). After ascend, air-dried TLC plate was sprayed with 50% (v/v) Methanol-H_2SO_4 mixture and heated for 10 min at 100^{o}C. The dark brown spot that appeared was identified by comparing with the standards [15].

2.7 Statistical Analysis

Data obtained from the optimization parameters were subjected to statistical analysis by comparison of means through one way analysis of variance (ANOVA).

3. RESULTS

3.1 Proximate Composition of Maize Husk

The proximate composition of the dried unfermented maize husk sample presented in (Table 1) revealed 4.83% of ash, crude protein of 7.00% and total carbohydrate contents of 60.37%.

Table 1. Proximate composition of maize husk

Parameter	Percentage (%)
Moisture	10.60
Ash	4.83
Fat	17.20
Crude Protein	7.00
Crude Fibre	3.45
Carbohydrate	60.37

3.2 Optimum Conditions Affecting Alpha Amylase Production by *Bacillus subtilis*

3.2.1 Effect of incubation temperature on alpha amylase production by *Bacillus subtilis*

Fig. 1 shows effect of different incubation temperatures (20, 25, 30, 35 and 40^{o}C) on α-amylase production by *Bacillus subtilis* at initial pH 7.0, moisture content of 1:2 for 72hrs. Highest α-amylase (38.83±2.17 U/g) was produced at 35^{o}C. There was an increasing trend in α-amylase production as the temperature increases up to 35^{o}C after which further increase in temperature resulted in decrease α-amylase production.

3.2.2 Effect of initial pH on alpha amylase production by *Bacillus subtilis*

Effect of various levels of initial pH (5, 6, 7, 8 and 9) on α-amylase production by *Bacillus subtilis* at 35^{o}C, moisture content of 1:2 for 72hrs is presented in (Fig. 2). Alpha amylase production of 39.97±0.72 U/g was observed at pH 7.0. Further decrease in α-amylase production was observed with increasing pH.

3.2.3 Effect of incubation period on alpha amylase production by *Bacillus subtilis*

Fig. 3 reveals effect of various incubation periods (24, 48, 72, 96 and 120 hours) on α-amylase production by *Bacillus subtilis* at 35^{o}C and initial pH 7.0 and moisture content of 1:2. Highest α-amylase production was observed after 72 hours of incubation (47.98±2.51 U/g). With increasing incubation period, a decrease in α-amylase production was observed.

3.2.4 Effect of moisture content on alpha amylase production by Bacillus subtilis

Effect of different moisture contents (1:1, 1:2, 1:3, 1:4 and 1:5) on α-amylase production by *Bacillus subtilis* at 35^{o}C, initial pH 7.0 and incubation period of 72 hours is presented in (Fig. 4). Highest α-amylase production was observed at moisture content of 1:2 (51.02±1.62 U/g) after which decrease in α-amylase production was observed with increasing moisture content.

3.4 Yield of Alpha Amylase Produced by *Bacillus subtilis* Using Maize Husk under Optimum Fermentation Conditions

Optimum α-amylase yield of 51.65±0.13 U/g as presented in (Table 2) was obtained from maize husk substrate with the selected *B. subtilis* isolate under predetermined optimum fermentation conditions of incubation temperature (35^{o}C), initial pH (7.0), incubation period (72hrs) and moisture content of 1:2 (substrate : water).

Table 2. Yield of alpha amylase produced by *Bacillus subtilis* using maize husk under optimum fermentation conditions

Parameter (Optimum value)	Yield of alpha amylase (U/g)
Incubation temperature (35^{o}C)	38.83 ± 2.17
Initial pH (7.0)	39.97 ± 0.72
Incubation period (72hrs)	47.98 ± 2.51
Moisture content (1:2)	51.02 ± 1.62
Overall optimum production	51.65 ± 0.13

3.5 Identification of the Type of Amylase by Sugar Analysis on Thin Layer Chromatography (TLC)

The spots developed on TLC plate for identification of the type of amylase produced are shown on Plate 1. The product of starch hydrolysis labelled S corresponds to the spot developed by maltose labelled M with a retention factor of 0.48 indicating the amylase produced was α-amylase.

4. DISCUSSION

Proximate composition of the maize husk revealed percentages of crude protein (7.00%), carbohydrates (60.37%) and ash (4.83%) contents. The crude protein showed the potential of the maize husk as a nitrogen source required for growth and efficient enzyme expression, whereas the carbohydrate content indicates high content of fermentable sugars required for

growth and enzyme production by the organism. The ash content indicates the richness of the substrate in minerals required for growth of the organism. These might have accounted for the α-amylase production (51.65±0.13 U/g) obtained in this study. The result is similar to the findings of [16] on the chemical and nutritional value of maize and maize products.

In this study, the effect of incubation temperature on α-amylase production by *Bacillus subtilis* under SSF revealed that 35°C was the optimum (38.83±2.17 U/g) among the tested incubation temperatures. Decrease α-amylase production as observed beyond the optimum temperature (35^{o}C) might be due to growth reduction and enzyme inactivation or suppression of cell viability. The yield of α-amylase produced by *Bacillus subtilis* at the different incubation temperatures was found to be statistically significant ($P < 0.05$). The result of this study is similar to the work of [17] who observed maximum α-amylase production by *Bacillus amyloliquefaciens* MTCC-610 using agro-byproducts under solid state fermentation at 35^{o}C. The finding also agrees with that of [18] who reported 35^{o}C as the optimum incubation temperature for α-amylase production by *Bacillus subtilis* using potato peel.

Maximum production of α-amylase by *B. subtilis* occurred at pH of 7.0 (39.97±0.72 U/g). This might be due to the physiological nature of pH 7.0 at which best metabolic functions are carried out. The enzyme yield sharply increased from pH 5 to pH 7 while a decrease in the enzyme yield

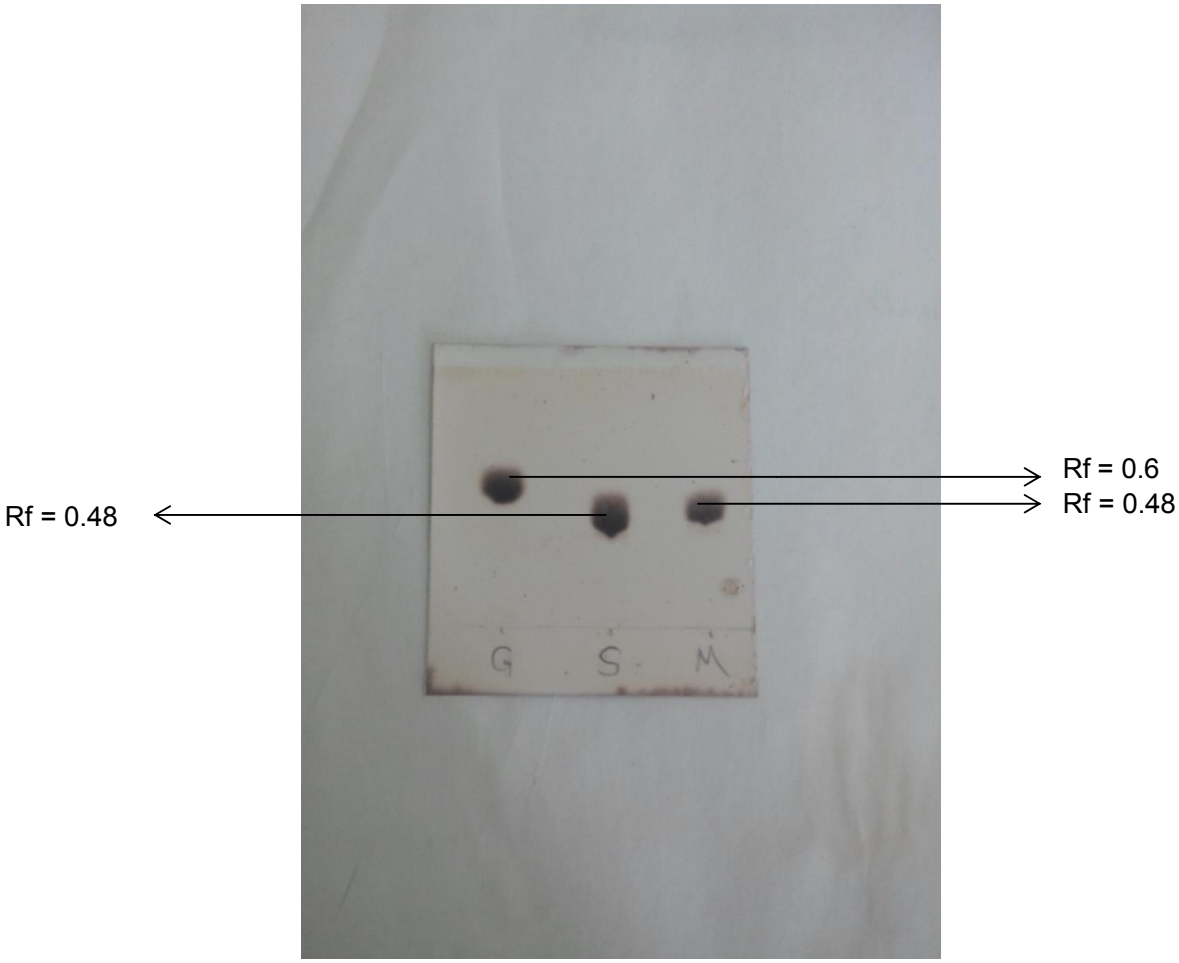


Plate 1. Spots of sugars on TLC plate for identification of type of amylase produced
Key: G = Glucose, S = Starch Hydrolysis Product, M = Maltose, Rf = Retention factor

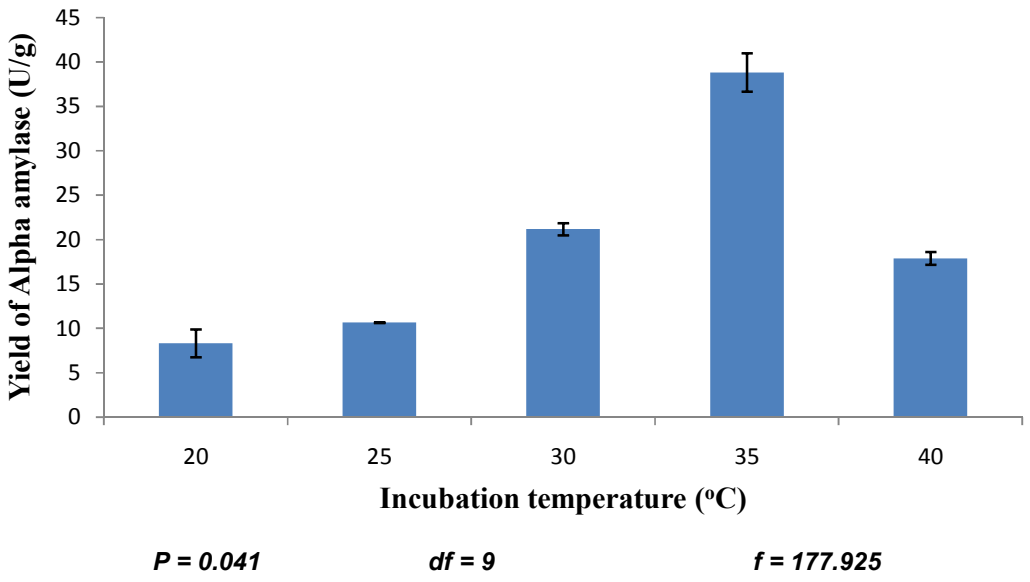


Fig. 1. Effect of incubation temperature on alpha amylase production by *Bacillus subtilis*

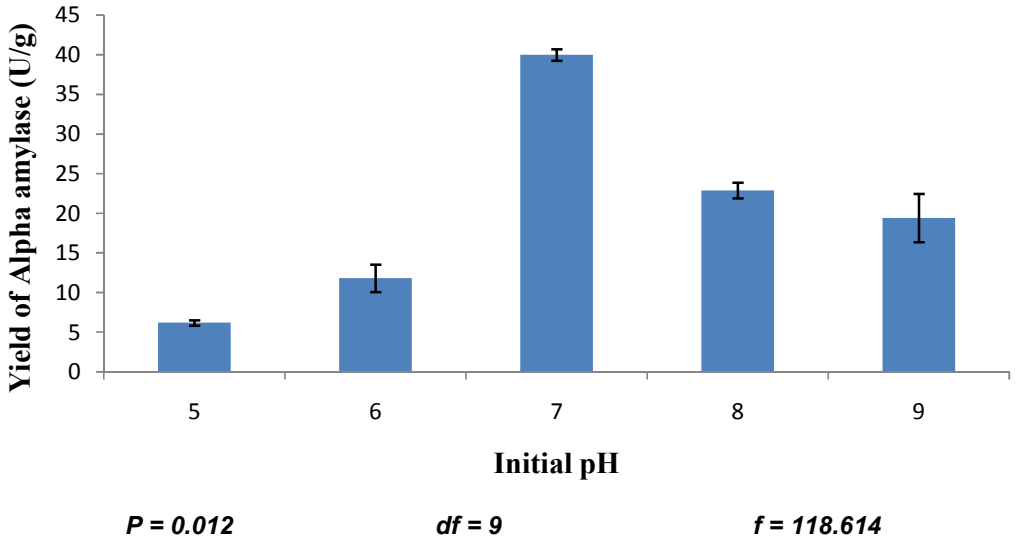


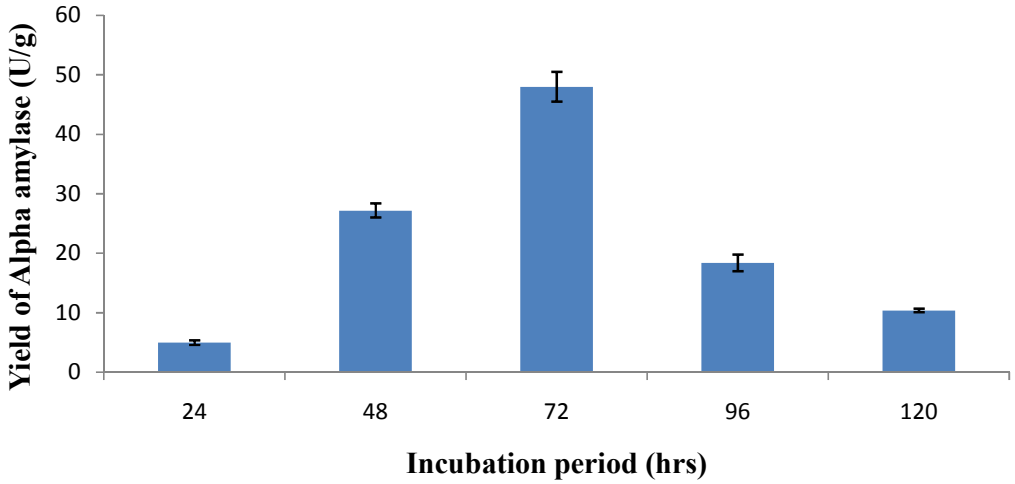
Fig. 2. Effect of initial pH on alpha amylase production by *Bacillus subtilis*

was observed from pH 8 to 9. This might be because the metabolic activities of microorganisms are very sensitive to rising pH. The yield of α-amylase produced by *B. subtilis* at the different initial pH of the fermentation medium was found to be statistically significant ($P < 0.05$). The results of this finding are in line with that of [13] who observed a maximum α-amylase production of 232.95 and 180.8 U/g by *Bacillus subtilis* NCTC-10400 and *Bacillus cereus* ATCC 14579 respectively using wheat bran at initial pH of 7.0.

The incubation time is governed by culture characteristics and also based on growth rate [17]. In this study, the effect of incubation period on α-amylase production by *Bacillus subtilis* under SSF revealed that 72h was the optimum (47.98±2.51 U/g) among the tested incubation periods. Beyond this period, the α-amylase production started to decrease. This might be due to the deficiency of nutrients present in the medium. It might also be due to proteolysis of α-amylase [19]. The yield of α-amylase produced by *Bacillus subtilis* at the different incubation

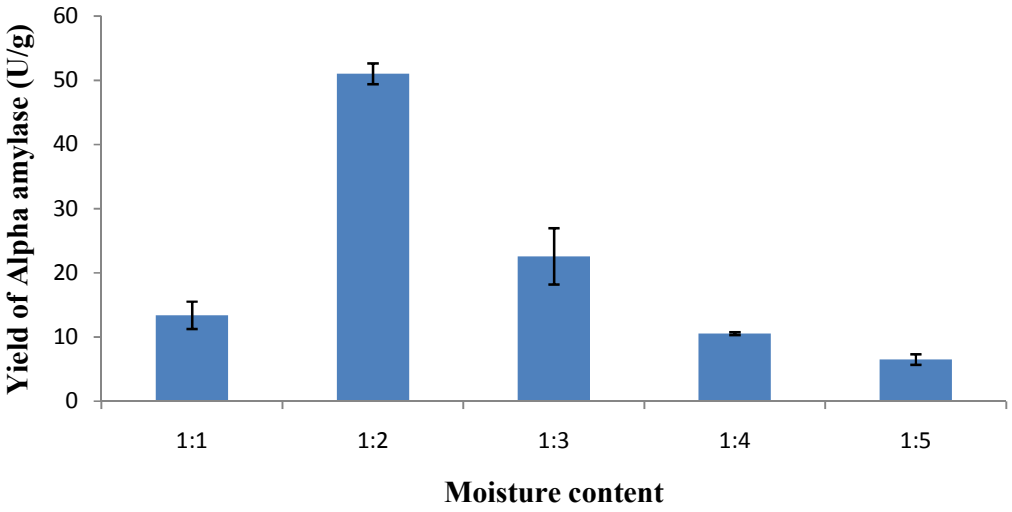
periods was found to be statistically significant ($P < 0.05$). Similar results were reported by [20] who found 72 h as the optimum fermentation period for alpha amylase production by *Bacillus amyloliquefaciens* by SSF using wheat bran and ground nut oil cake.

Moisture content is a critical factor in SSF processes and has been found to have great influence on microbial growth and biosynthesis and secretion of different metabolites [12]. Maximum production of α-amylase by *B. subtilis* was attained when the substrate: moisture ratio was 1:2 (51.02±1.62 U/g). The observed variations in the α-amylase produced with varying moisture contents could be explained by the fact that higher moisture levels can cause a reduction in enzyme yield due to steric hindrance



P = 0.034 ***df = 9*** ***f = 287.178***

Fig. 3. Effect of incubation period on alpha amylase production by *Bacillus subtilis*



P = 0.013 ***df = 9*** ***f = 118.305***

Fig. 4. Effect of moisture content on alpha amylase production by *Bacillus subtilis*

of the growth of strain due to reduction in porosity (interparticle spaces) of the solid substrate, thus interfering with oxygen transfer [8]. The yield of α-amylase produced by *Bacillus subtilis* at the different moisture contents was found to be statistically significant ($P < 0.05$). The results of present study are in line with the work of [21] who reported substrate: moisture ratio of 1:2 as the optimum for α-amylase production by *B. cereus* MTCC 1305 by solid state fermentation using wheat bran as substrate.

In this study, the total amount of α-amylase produced at optimum conditions by *B. subtilis* using maize husk was 51.65±0.13 U/g. This shows that agricultural residues such as maize husk could be a good substrate for the production of α-amylase.

The product of starch hydrolysis was found to appear as a spot with a retention factor (Rf) of 0.48 which is the same as that of standard maltose. Thus, indicating that the amylase produced was alpha amylase. This is in agreement with the report of [15] who stated that *Bacillus* spp. produce mainly α-amylase.

5. CONCLUSION

Maize husk contains organic nutrients in quantities sufficient to support the growth of *B. subtilis* and amylase production. Amylase production by *B. subtilis* using maize husk as substrate occurs when the solid state fermentation process was conducted at 35°C, initial pH 7.0, and moisture content of 1:2 72hrs. An overall yield of 51.65±0.13 U/g of α-amylase using maize husk as substrate was obtained.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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