Beta-glucosidase Production by *Aspergillus niger* using Breadfruit Seed Hull as Substrate under a Solid State Fermentation

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Abstract: Cost effective production of β-glucosidase which is of high value in most biotechnological processes requires an efficient microbial producer, technologies and biomass resources that are economical. This study aimed at evaluating the biochemical characteristics of β-glucosidase produced by *Aspergillus niger* under solid state fermentation using agro-industrial residues. Fungal isolates from spoilt yam were isolated and screened for the production of β-glucosidase. Study on the optimized conditions and industrial suitability of the produced β-glucosidase was carried out using the most potent fungal producer and the substrate that supported the highest yield of the enzyme. The β-glucosidase producer was identified culturally and morphologically as *Aspergillus niger*. The highest yield of β-glucosidase (161.4±3.2 U/g) was supported by bread fruit seed hull out of the four screened agro-waste. Optimal cultural conditions for the enhanced production of β-glucosidase ((210.1 U/g) were at pH 6.0, 35 ^{o}C, moisture level 75 %, inoculum size 2 % and incubation period of 72 hrs. The optimum activity of the crude enzyme was recorded at pH 6.0 and 60 ^{o}C. The pH stability of the enzyme was over a broad range of 4.0 – 8.0 with relative residual activity above 70 % recorded after incubation for 120 min. The β-glucosidase was thermostable as its half life was 4 hrs at 65 ^{o}C. This study showed that *Aspergillus niger* can effectively utilize a low cost substrate (breadfruit seed hull) for the production of β-glucosidase which might be suitable for diverse industrial processes as depicted by its biochemical characteristics.
Keywords: β-glucosidase; *Aspergillus niger*; solid state fermentation; breadfruit seed hull.

INTRODUCTION

Agricultural wastes are major cellulosic biomass which offers significant opportunities in renewable energy sector (Ahmed *et al*., 2017). Valorization of agro-industrial waste is one of the strategies to drive the actualization of zero waste concepts. The zero waste resolution adopted at the United Nations general assembly of March 2022 is a way to advance the UN 2030 agenda sustainable development goal number 11 and 12 which promotes friendly environment and reuse of waste for more resource efficient economy (United Nations, 2015; Geneva Environment Network, 2022). Utilization of agricultural wastes such as yam peels, cassava peels and potato peels as cellulose biomass feedstock will lessen the burden of waste disposal as well as reduce industrial production cost (Anwar *et al*., 2014).

Cellulose being a great source of renewable energy is faced with a hurdle of hydrolysis (Agbor *et al*., 2011). Any hydrolysis approach to be adopted in the conversion of cellulose must be environmentally friendly and cost effective. Overcoming these challenges has attracted industrial and research interest to the subject of cellulose breakdown.

Enzymatic hydrolysis of cellulose requires synergetic action of exoglucanase, endoglucanase and β-glucosidase (Lambertz *et al*., 2014; Noor El-Deen *et al*., 2014). However, the importance of β-glucosidase in the breakdown of cellulose cannot be overemphasized, as it enhances the function of cellulolytic enzymes by mitigating cellobiose inhibition and producing glucose from cellobiose (Lambetz *et al*., 2014).

Beta-glucosidase has displayed diverse industrial application such as: oligosaccharide and amino-glycoside synthesis, cassava detoxification, breakdown of cyanogenic glucoside in substrate for beer production, production of nonionic biodegradable surfactant, production of renewable energy and many others (El-Naggar *et al*., 2015, Ahmed *et al*., 2017). Beta-glucosidases are ubiquitous and have been previously isolated from fungi example *Aspergillus*, *Trichoderma* and *Fusarium,* as reported by some investigators Abdullah *et al*. (2020), Sun *et al*. (2021) and Olajuyigbe *et al*. (2016) respectively.

The rise in industrial demand of β-glucosidase due to its ability to utilize diverse cellulolytic substrates has attracted enormous research attention to it. In order to obtain an industrially efficient enzyme in an approach that is economically viable and environmentally sustainable, it is necessary that β- glucosiadases are deeply studied. Hence this work is aimed at studying the biochemical characteristics of β-glucosidase produced by fungi under solid state fermentation using agro-industrial residues**.**

MATERIALS AND METHODS

Sample collection, isolation of fungi and primary screening for beta-glucosidase production

Spoilt yam (*Dioscorea rotundata*), bitter yam (*Dioscorea dumetorum*) peels, breadfruit (*Treculia africana*) seed hulls, udara (*Chysophyllum albidum)* peels, and yam (*Dioscorea rotundata*) peels, were obtained from waste of food vendors in Orie Ugba market Umuahia, Abia state. Fungal strains were isolated from diseased parts of yam on a Potato dextrose agar (PDA) and identified using standard microscopic and macroscopic techniques described by Sangeetha & Thangadurai (2013), results were compared with the identification key in Wanatabe (2010). Isolated fungal strains were screened for beta-glucosidase production in a cellobiose containing broth (composition in g/L: cellobiose 20.0; $(NH_4)_2SO_4$ 0.5; yeast extract 0.5; KCl 0.5; $MgSO_4.7H_2O$ 0.2; $FeSO_4.7H_2O$ 0.01; $CaCl_2$ 0.1 and K_2HPO_4 1.0) adjusted at pH 5.0 (El-Naggar *et al*., 2015; Vaithanomsat *et al*., 2011). Incubation was carried out for 5 days at 28^{o}C and 160 rpm, using standard method as described by Wood and Bhat (1998) with β-glucosidase activity was determined in the fermentation broth supernatant. Positive isolates were maintained at 4^{o}C on a PDA slant.

Preparation of substrates for beta-glucosidase production

The agricultural wastes: Bitter yam peels (*Dioscorea dumentorum*), Udara peels (*Chrysophullum albidum*), Yam peels (*Dioscorea alata*) and Bread fruit seed hulls (*Treculia africana*), were washed with distilled water, and chopped into smaller pieces and then oven dried at 60^{o}C for 48 hours. Thereafter the dried peels were milled to about 0.3 mm mesh particle size.

Inoculum preparation

The *Aspergillus niger* was reconstituted at 28^{o}C for 48 h in an inclined Erlenmeyer flask (100 ml) containing 20 ml of a Potato Dextrose Agar. Spore suspension was obtained by adding 25 ml of the nutrient solution and gently dislodging the spore clusters using a sterile wire loop (Merheb-Dini *et al*., 2009). The spores were counted with Neubauer's counting chamber and diluted to a concentration of 10^5 spores/mL.

Production of beta-glucosidase in a solid-state fermentation

Five (5) grams of the various prepared substrates were weighed into different Erlenmeyer flasks of 250 ml capacity, and moistened with 5 ml of nutrient solution (Ammonium sulfate 1 g/L, Magnesium sulfate heptahydrate 1 g/L, Ammonium nitrate 1 g/L). The flask contents were autoclaved at 121 ℃ for 15 min, afterward cooled to room temperature, before inoculation with 5 ml of the spore suspension and then incubated at 28 ℃ for 96 h. The substrate that showed the best β-glucosidase production by the *Aspergillus niger* was selected for further studies. All the assays were performed in three replicates. The results were expressed as U/g referred to enzymes units per gram of dry substrate (El-naggar *et al*., 2015).

Enzyme extraction and beta-glucosidase assay

Into each fermented substrate was added 50 mL of 0.1 M phosphate buffer pH 6.0, and then agitated for 1 h at 150 rpm on a rotary shaker. This mixture was filtered and centrifuged at 3600 × g for 5 min.

The crude extract (supernatant) was used for the β-glucosidase assays. The method of Wood and Bhat (1998) was adopted with slight modification for the determination of β-glucosidase activity. Crude enzyme 0.1 mL was added into an Eppendorf tube containing 1.4 mL of 0.1 M sodium acetate buffer (pH 4.5), and 0.5 mL of 0.02 mM p-nitrophenyl β-D-glucopyranoside (pNPβG, Sigma), and incubated at 40°C for 15 min thereafter 2 mL of 0.2 M sodium carbonate was added to stop the enzyme reaction. The absorbance was measured at 410 nm to determine the activity of the enzyme. One unit of β-glucosidase activity was defined as the amount of enzyme that releases 1 µmol of nitrophenol per minute under standard assay condition.

Beta-glucosidase Production Studies

Impact of incubation period on beta-glucosidase production

Five milliliters of the nutrient medium and 1 ml of spore suspension (1x 10^5) was added into Erlenmeyer Flasks containing 5 g of the selected substrate (breadfruit seed hull), and incubated in a rotary shaker at 120 rpm and 28^{o}C ±2^{o}C. Beta-glucosidase was extracted and assayed at 24 h interval up to 120 h (5th day) (Abdullah *et al.*, 2019; El-Ghonemy, 2021).

Impact of moisture on beta-glucosidase production

To select the optimal moisture level for the production of β-glucosidase, initial moisture level were varied at 50 %, 66.7 %, 75 % and 70 % that is the substrate to nutrient mineral ratio (w/v) were 1:1, 1:2, 1:3 and 1:4 respectively. One milliliter of the spore suspension was inoculated into the substrate mineral mixture in the flask and incubated for 72 h (pre-optimized time) at 28 ^{o}C, thereafter enzyme was extracted and assayed as previously described (Abdullah *et al.*, 2019; El-Ghonemy, 2021).

Impact of inoculum concentration on beta-glucosidase production

The impact of inoculum size on the beta-glucosidase production was studied by employing different inoculum levels (10^4, 10^5, 10^6, 10^7, 10^8 and10^9 spores/mL), fermentation was carried out using 75 % moisture, and incubated at 28 ^{o}C for 72 h (Abdullah *et al.*, 2019; El-Ghonemy, 2021).

Impact of initial pH on beta-glucosidase production

The initial pH of the nutrient medium was adjusted to various levels (3.0 -9.0), so as to investigated the influence of initial pH on beta-glucosidase production. Various pH adjusted nutrient medium (75 %) was added to the substrate and fermented at previously optimized conditions at 28 ^{o}C (Abdullah *et al.*, 2019; El-Ghonemy, 2021).

Impact of temperature on beta-glucosidase production

The influence of temperature on the beta-glucosidase production was evaluated from 20 ^{o}C to 50 ^{o}C at 5 ^{o}C interval. Fermentation was carried out under previously optimized conditions (pH 6.0, 75% moisture, 10^6 spores/ml inoculum size, for 72 h) (Abdullah *et al.*, 2019; El-Ghonemy, 2021).

Studies on the characteristics of the beta-glucosidase

Effect of pH on the beta-glucosidase activity and stability

For the pH study buffers (50 mM) used were glycine HCl (pH 3.0 – 4.0), sodium citrate (pH 5.0 – 6.0), Tris-HCl (pH 7.0 – 8.0) and glycine –NaOH (pH 9.0 -11.0). Standard procedure for enzyme activity assay as previously described was adopted to determine the optimum pH for the β-glucosidase activity. Determination of the β-glucosidase pH stability was carried out by incubating the crude enzyme in the appropriate buffer of varying pH (3.0 -11.0) without substrate for 240 min at 40^{o}C. Thereafter, residual β-glucosidase activity was determined using standard procedure as described earlier (Ire *et al.*, 2017; El-Ghonemy, 2021).

Effect of temperature on the beta-glucosidase activity and stability

Reaction mixture was incubated at various temperatures ranging from 40 ^{o}C to 70 ^{o}C for 30 minutes, afterward the beta-glucosidase activity was determines as previously described.

The studied β-glucosidase thermostability was determined by incubating the crude enzyme at temperature ranging from 40 ^{o}C to 70 ^{o}C for 240 min. At 60 min interval enzyme aliquots (100 µL) were withdrawn and the residual activity determined Ire *et al*., 2017; El-Ghonemy, 2021).

Statistical analysis of analysis

All assays were performed in three replicates and the descriptive statistics (mean and standard deviation) of data obtained are presented in Figures.

RESULTS

The morphological characteristics of the isolated fungi species revealed their identity to be *Aspergillus niger*, *Botrydiplodia* sp. and *Rhizopus* sp. Screening these fungi for β-glucosidase production showed *Aspergillus niger* to be the only producer, and it was further studied. The substrate screening result as depicted in Figure 1, showed that of the four agricultural waste used as substrate for the production of β-glucosidase, breadfruit seed hull supported the highest production of the β-glucosidase (161.4±3.2 U/g), and the least β-glucosidase was produced from yam peels with 27.2±0.7 U/g.

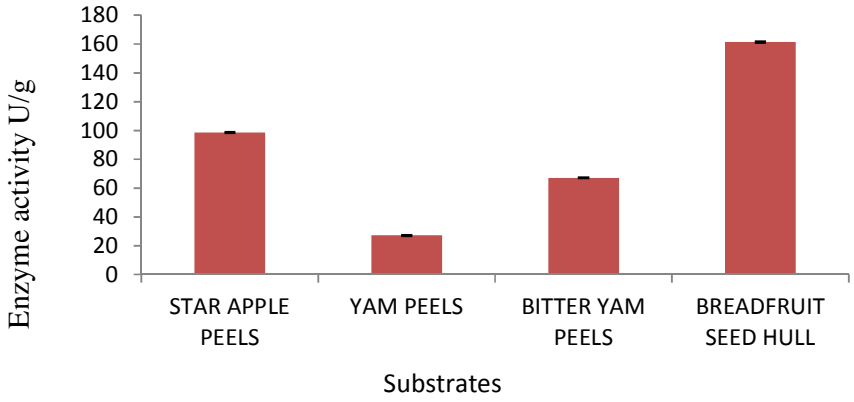


Figure 1: screening of substrates for β-glucosidase production

The influence of fermentation parameters on the studied *Aspergillus niger* ability to produce β-glucosidase using breadfruit seed hull as substrate was illustrated in Fig.2 (a-e), The impact of various fermentation times 0 h to 120 h (Figure 2a) showed that fermentation for 72 h was optimal for β-glucosidase production (179.25 U/g) by *Aspergillus niger*.

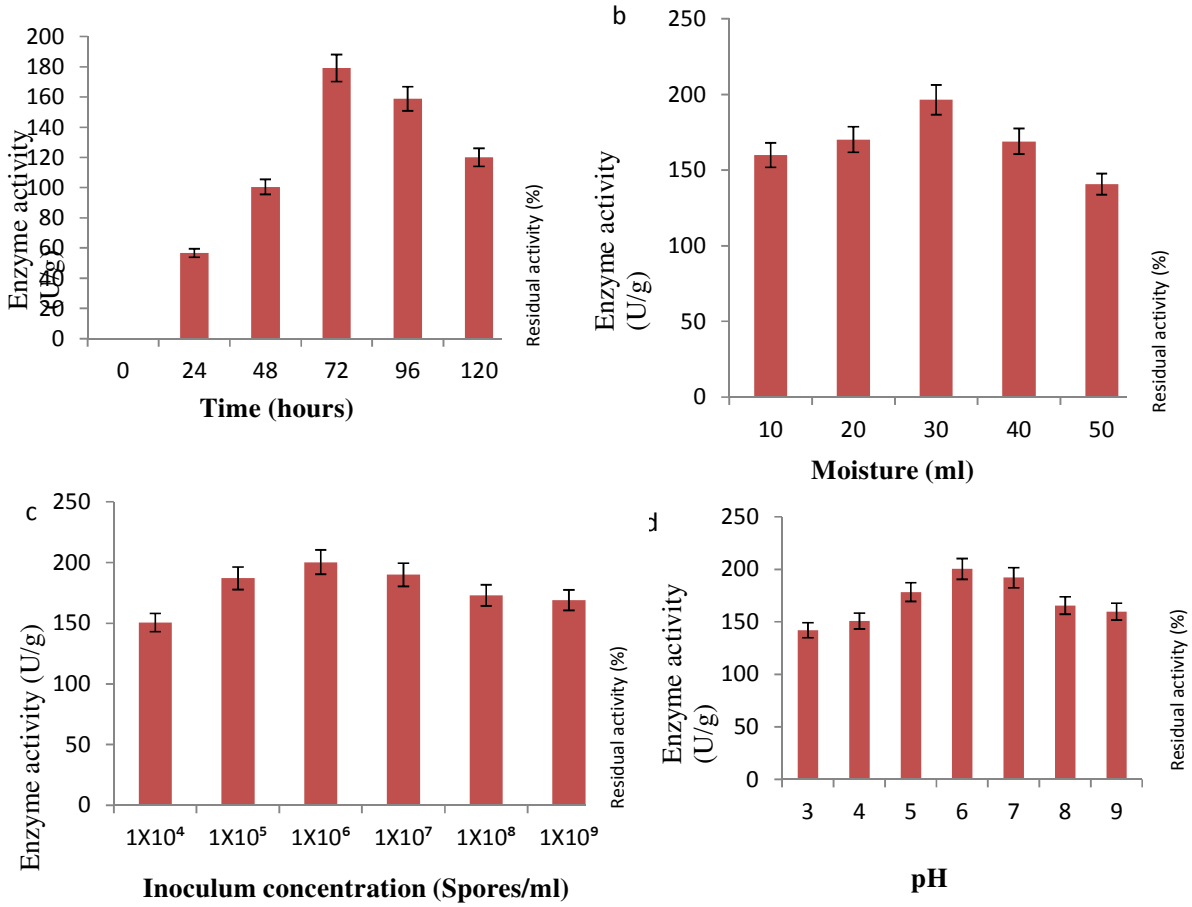
The moisture content effect on β-glucosidase production as shown in Figure 2b, which revealed that maximum β-glucosidase production of 196.5 U/g was obtained at 75 % moisture, that is ratio 1:3 hence 15 milliliters of medium to 5 grams of substrate was the optimum moisture content.

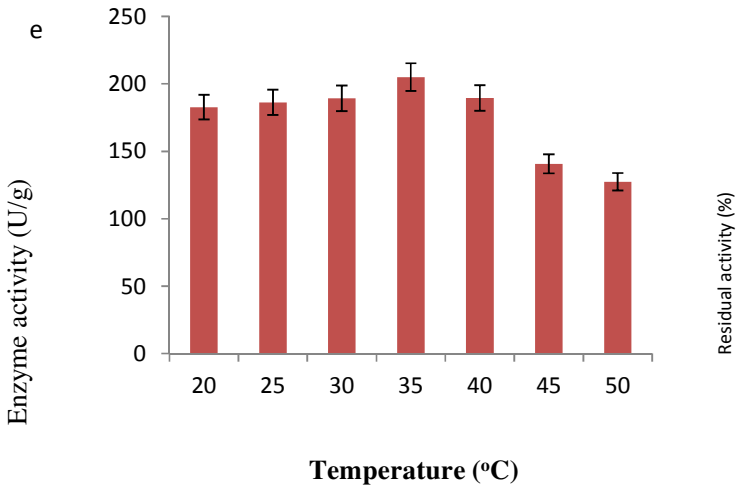
Different inoculum concentration of the *Aspergillus niger* had impact on the production of β-glucosidase as presented in (Figure 2c). The result obtained indicated that production of the enzyme increased with increase in inoculum size from $1x10^4$ – $1 x10^6$ spores/ml where the maximum production (200.3 U/g) was recorded. Further increase in inoculum size resulted to decline in the enzyme production.

As illustrated in Figure 2d, the effect of the fermentation medium initial pH on the enzyme production showed that initial pH 6 was optimum for the production of β-glucosidase by the *Aspergillus niger* Maximum production of 203.37 U/g was obtained at pH 6 while the least 142 U/g was obtained at initial pH 3.

Temperature of 35 °C (Figure 2e) supported the highest production of β-glucosidase (210.1 U/g) and the least quantity of β-glucosidase was produced at 50 °C (127.4 U/g).

The impact of the pH on the activity of β-glucosidase as reported in Figure 3 showed that as the pH increased there was an increase in the activity of the enzyme to pH 6.0 where optimum enzyme activity was recorded, afterward there was a decrease in enzyme activity. After 120 min incubation over different range of pH (3.0 – 11) the enzyme retained its original activity above 70 % over pH of 4.0 – 8.0. At 60 °C the *Aspergillus niger* β-glucosidase performed optimally and at 70 °C up to 65 % of its maximum activity was retained (Figure 4a). The β-glucosidase maintained about 52.5 % of its initial activity for 1 h at 70 °C, and at temperature 40 °C – 65 °C the enzyme was stable for up to 4 hours as presented in Figure 4b.





Initial activity at above 55% after incubation at 50 °C to 60 °C for up to 4 h, and at 65 °C the half life was 4 h (Fig. 4b).

Figure 2: Effects of fermentation parameters on β-glucosidase production by *Aspergillus* sp. in bread fruit husk (a) incubation time, (b) moisture content (c) inoculums concentration, (d) pH, (e) temperature.

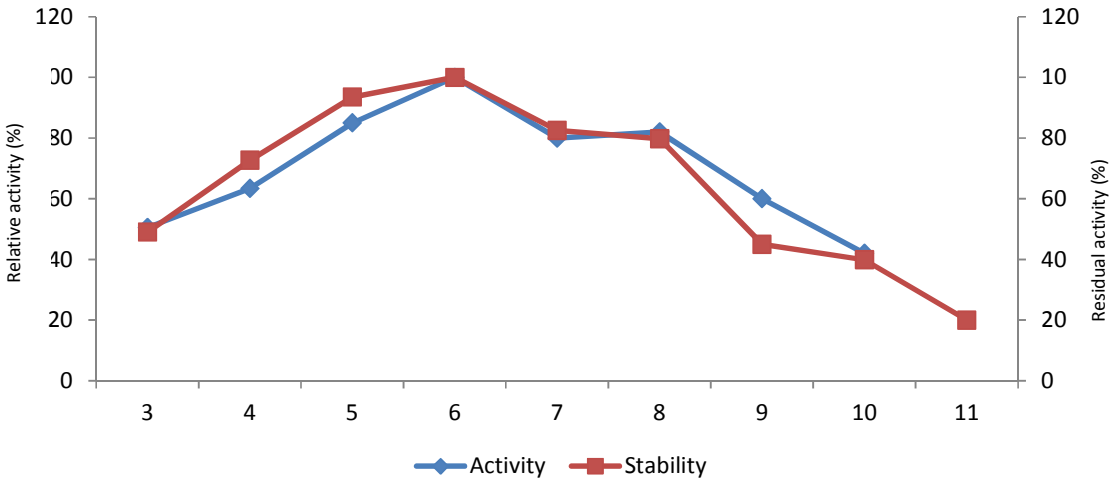


Figure 3: Effect of pH on the activity and stability of β-glucosidase from *Aspergillus niger*.

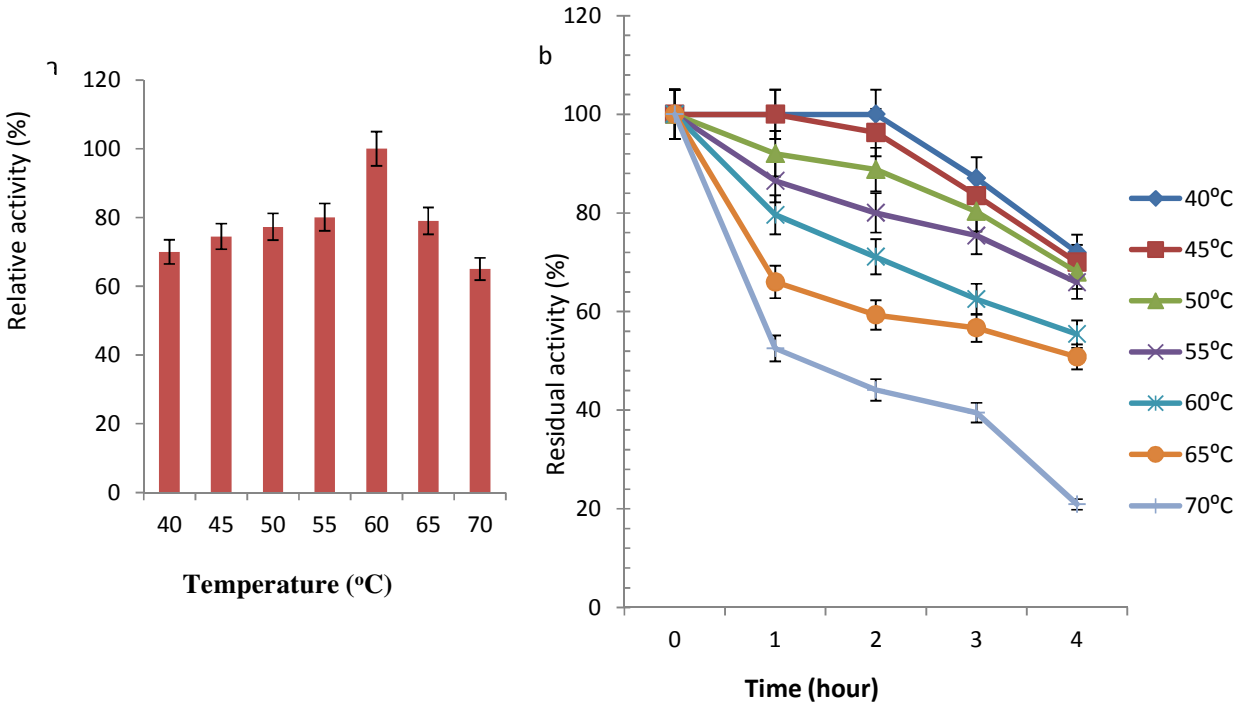


Figure 4: Effect of Temperature on the (a) activity (b) stability of β-glucosidase from *Aspergillus* niger.

DISCUSSION

The preliminary screening of the three fungal isolates showed that *Aspergillus niger* was a good producer of β-glucosidase, this could be attributed to the excellent system of protein secretion by *Aspergillus* as stated by Li *et al.* (2020). Members of the genus *Aspergillus* are known decomposers of complex molecules, especially lignocellulose biomass. Its ability to produce a glycoside hydrolase known as β-glucosidase has been reported by several researchers (Vaithanomsat *et al.*, 2011; El-naggar *et al.*, 2015; Abdullah *et al.*, 2020).

Screening of the four agro-wastes (Star apple peels, bitter yam peels, yam peels and breadfruit seed hulls) for optimum production of β-glucosidase using solid state fermentation technique showed that breadfruit seed hulls as substrate supported maximum production of the enzyme, with a high enzyme activity of 161.4 U/g. This might be due to the high nutritional content of the breadfruit seed (Ejidike & Ajileye, 2007; Nwajiobi *et al.*, 2019). Nwajiobi *et al.* (2019) reported a high cellulose content of 39% in breadfruit seed hull. Cellulose being an easily utilizable carbon source might enhance fungal growth, although as implied by Cuiyi *et al.* (2017) high cellulose content does not induce β-glucosidase production. Also breadfruit seed hull is high in mineral content (Ejidike & Ajileye 2007) particularly magnesium, calcium and potassium which are key inducers of β-glucosidase as presumed by Cuiyi *et al.* (2015).

Due to the uniqueness of each fungal strain, it is necessary to identify the most suitable fermentation conditions for enhanced production of metabolites. The influence of incubation time on the production of β-glucosidase by the *Aspergillus niger* showed that maximum production of the enzyme (172.29 U/g) was achieved at 72 h incubation period.

With extension of the incubation period beyond 72 h decrease in enzyme production was obtained. This reduction in enzyme production might be as a result of nutrient depletion, accumulation of products which might be inhibitors to the enzyme and the fungi and also enzyme proteolysis as reported by Cuiyi *et al.* (2017). Similar results were obtained by Abdullah *et al.* (2019) and Abdullah *et al.* (2020).

Varying basal medium volume in the range of 5ml to 25ml impacted on the β-glucosidase production by the *Aspergillus niger.* Beta-glucosidase production was maximum (196.5 U/g) at 15 ml (75 % moisture) of basal medium to 5 g of substrate. Moisture content being a critical requirement for microbial growth may still hamper its growth in excess, owing to the fact that the supply of oxygen and circulation of nutrient reduces due to substrate particles sticking together (Wang *et al.*, 2008; Sharanappa *et al.*, 2011). Generally the substrate absorption capacity determines the level of substrate moistening. Maximum yield of β-glucosidase has been obtained from different *Aspergillus* strain at different initial moisture level on different substrate. Highest activity value was recorded by El-Ghonemy (2021) at 70 % initial moisture content on jojoba meal also Noor El-Deen *et al.* (2014) reported maximum yield at 90 % on soybean flour.

Inoculum size is one of the factors that significantly affect β-glucosidase production (Sun *et al.*, 2021), inoculum concentration of 1.0 x 10^6 spores/mL on 5 g of breadfruit seed hull was optimum for maximum secretion of β-glucosidase at 200.3 U/g by *Aspergillus niger.* Higher inoculum size might result to decrease in available nutrient and also reduced available substrate surface area for the growth of microbes and enzyme production (Tsegaye and Gessesse, 2014). Reports by some researchers have shown that the size of the inoculum have impact on enzyme production by fungi (Puri *et al.*, 2013; Ire *et al.*, 2017; Sun *et al*, 2021).

Optimum initial pH for the production of enzyme by microorganisms varies from species to species (Ahmed *et al.*, 2017). The *Aspergillus niger* optimum pH for the production of β-glucosidase was at pH 6.0. Lower β-glucosidase activity was recorded at high acidity (pH 3.0) and high alkalinity (9.0), as opined by Sun and Xu (2008) slightly acidic environment is favorable for fungal growth. This result on pH 6.0 being optimum for *Aspergillus* production of β-glucosidase corroborates with reports of Noor El-Deen *et al.* (2014), Abdullah *et al.* (2019) and El-Ghonemy, 2021.

Fermentation temperature is important in the production of value-added metabolites. At too low temperature the metabolic reaction rate of the microorganism might be too slow and at too high temperature denaturation of enzyme might occur hence low value of enzyme activity. *Aspergillus niger* strain of this study produced maximal β-glucosidase at 210.1 U/g of breadfruit seed hull. Similar result was recorded on *Aspergillus* sp. by El-Ghonemy (2021), optimum temperature within the range 28 ^oC to 40 ^oC has been recorded by other researchers for the production of β-glucosidase by different species of *Asergillus* (Ahmed *et al.*, 2017).

The β-glucosidase showed that the best pH for its catalytic activity was pH 6.0, catalytic activity over 50 % was recorded over a wide range of pH (4.0-9.0), although decrease in activity was recorded at pH value above or below pH 6.0 which was the optimum. The enzyme was highly stable at pH 6.0, and retained its original activity above 70 % over pH 4.0 – 8.0, this characteristics might be of benefit in dairy industry. Enzymes are protein therefore change in pH might alter the ionic characteristics of its carboxylic or amino groups at the active site. Change in active site conformation affects the substrate-enzyme affinity (Li *et al.*, 2013). El-Ghonemy, (2021) recorded that pH 6.0 was optimum for the activity of *Aspergillus* sp.DHE7.

Temperature above or below optimum affects the activity and stability of enzymes, the *Aspergillus niger* β-glucosidase catalytic activity was optimum at 60 ^{o}C after incubation for 120 min. The thermostability studies showed that at temperature ranging from 40 ^{o}C – 65 ^{o}C the enzyme was stable for up to 4 hours, at 65 ^{o}C the half life of the enzyme was 4 hours, and at 70 ^{o}C the enzyme retained about 52.5 % of its initial activity for 1 h. Extreme temperatures result to loss of activity and stability due to loss of active site specificity as a result of denaturation (Olajuyigbe *et al.*, 2016). Despite the fact that most mesophilic microorganisms produce enzymes that are not thermally stable, just like this study researchers such as Olajuyigbe *et al.* (2016) and El-Ghonemy (2021) have extracted thermostable β-glucosidase from mesophiles.

CONCLUSION

The result obtained in this study revealed that an underutilized agro waste (breadfruit seed hull) is suitable for β-glucosidase production by *Aspergillus niger* under solid state fermentation. The biochemical characteristics of the produced β-glucosidase showed a thermostable and a pH versatile enzyme. The β-glucosidase retained above 50 % of its original activity at 70 ^{o}C and above 70 % of its original activity at pH 4.0 to 8.0. These properties suggest the enzyme suitability in various biotechnological processes.

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