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BIOUTILIZATION OF *ADANSONIA DIGITATA* FRUIT PULP BY *BACILLUS* SPECIES FOR AMYLASE PRODUCTION

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ABSTRACT: In recent years the cost of carbon source for amylase production has necessitated a drive towards cheaper and sustainable sources. In this study, the utilization of *Adansonia digitata* fruit pulp as substrate for bacterial amylase production and the effects of varying incubation period, temperature, pH of medium, inoculum size and substrate concentration on amylase production were investigated. The amylolytic activity of the isolates was screened base on halo of clearance on starch medium, while the amylase activity of the selected *Bacillus* species was monitored using 3, 5 - dinitrosalicylic acid (DNS) method. Out of 24 *Bacillus* species screened, *B. licheniformis* shows the highest amylolytic activity of 5.04 mm while *B. subtilis* has the least activity of 0.86 mm. Therefore *B. licheniformis* was selected for studies on the effect of fermentation conditions on amylase activity. The highest amylase activity (2.20 IU/ml/min) was observed after 3 days of fermentation at initial substrate concentration of 10g/L, pH 5.8, inoculum size of 8% and temperature of 75^{o}C. The results of this study suggest that *Adansonia digitata* fruit pulp can be harnessed at low concentration for large scale bacterial amylase production, and the alpha-amylase produced by *B. licheniformis* strain could be a thermostable enzyme with novel characteristics suitable for application in starch and other food processing industries.

Keywords: *Adansonia digitata,* Amylase, *Bacillus licheniformis,* fermentation, dawadawan botso

INTRODUCTION

In recent years the high cost of carbon sources for industrial production of microbial amylase has necessitated a shift to other cheaper sources of carbon. The potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms [1; 2; 3] Amylase is an enzyme that breaks down starch to reducing sugar. Amylase is present in human saliva, where it begins chemical process of digestion. The pancreas also makes amylase (alpha amylase) to hydrolyse dietary starch into di and trisaccharides which are converted by other enzymes into glucose to supply the body with energy [4]. All amylases are glycoside hydrolyser and act on alpha- 1,4 glycosidic bonds [4]. Industrially, alpha amylase (*EC 3.2.1.1*) is used particularly in starch liquefaction, brewing, textile, pharmaceuticals, paper, detergents, drugs, toxic wastes removal and oil drilling [5]. The enzyme production is largely dependent on the type of strain, medium composition, cell growth, initial pH and thermostability [6; 7; 8].

Amylases are among the most important enzymes and are of great significance in present day biotechnology taking approximately 25% of the enzyme market [9]. New amylases could be potentially useful in pharmaceutical and fine chemical industries if enzymes with suitable properties could be identified [10]. With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many other fields, such as clinical, mechanical and analytical chemistry [3].

Amylases can be derived from several sources, such as plants, animals and microorganisms. Because of their short growth period, the enzymes from microbial source generally meet industrial demand [3]. At present, *Bacillus*, *Aspergillus* and *Rhizopus* species are considered to be the most important sources of industrial amylases [11]. Growth conditions and nutrients promote high yield of microbial amylases. However, carbon sources such as dextrin, fructose, lactose, maltose, glucose and starch are very expensive for commercial production of these enzymes [12]. Agricultural wastes are being used for both liquid and solid state fermentation to reduce the cost of fermentation medium. In Nigeria, most of these products are used as source of food for humans and livestock. It is necessary to search for other substrates which when exploited will not have any negative economic impact on humans or livestock, create wealth for the people. *Adansonia digitata* fruits pulp is among the most common but underutilized fruits in Africa. This project is aimed at utilizing *A. digitata* fruit pulp as substrate for bacterial amylase production and to access the effects of varying incubation period, temperature, pH of the medium, inoculum size and substrate concentration on the production of α-amylase by selected *Bacillus* species.

MATERIALS AND METHODS

One thousand grams of *Adansonia digitata* fruit was purchased from a local market in Sokoto city, Northern Nigeria. A clean mortar and pestle were used to separate the seed from the pulp. The pulp was sieved through 0.1 millimeter pore size sieve to obtain a fine powder .

The fermentation medium was prepared by dissolving 6g of $MgSO_4.7H_2O$, 0.5g KCl, in to two litre distilled water after which 100 ml of the medium was dispensed into 250-ml conical flasks. Different weight (10, 15, 20, and 25g) of *Adansonia digitata* fruit pulp was then added to the flasks containing the medium and autoclaved at 121^{o}C for 15min. The medium was allowed to cool at room temperature before inoculation.

The *Bacillus* species were previously isolated from dawadawan botso (a fermented soup condiment in Northern Nigeria) using standard procedures as described by [13], and identified following series of biochemical test as described by [14].

Screening for Amylolytic Activity of the Isolates

The amylolytic activity of the isolated organism was determined according to the method of [15]. Briefly, a loop full of each isolate was streaked aseptically on starch medium. The plate was then incubated at 37^{o}C for 24 hours. After incubation period, lugol's iodine solution was flooded over the plate, and allowed to stand for 20 minutes. A zone of clearance formed around the bacterial colonies. The zone of clearance was measured using a meter rule. This represents the amylolytic activity of the bacterial species.

Effect of fermentation conditions

The effects of fermentation conditions such as pH, temperature, inoculums size, incubation period and substrate concentration were studied as described by [15]. Initial pH of the medium was set at 3.6, 4.2, 5.0, and 5.8 using 1 N HCl or 1 N NaOH (at constant inoculum size of 4%, temperature 55^{o}C, incubation period of 3 days and 1%). Based on the results of this experiment a pH of 5.8 was adopted in subsequent experiments. The inoculum size of 2, 4, 6, and 8%; temperatures 45^{o}C, 55^{o}C, 65^{o}C, and 75^{0}C, incubation periods of 1, 2, 3, and 4 days and substrate concentration of 1, 2, 3, 4 and 5% were studied using the same procedures. At certain intervals the fermentation medium was agitated. Each assay was carried out in duplicate and the mean of the duplicate analysis was reported in each figure.

Amylase assay

Amylase activity was measured by the 3, 5-dinitrosalicylic acid (DNS) method [16; 17] by monitoring the amount of reducing sugar liberated from starch.Amylase was assayed by adding 1ml of enzyme (fermented broth supernatant) to 0.5 ml of 1% soluble starch and incubated for 30 min at 37^{o}C. The reaction was stopped by adding 1 ml of 3, 5 dinitrosalicylic acid, followed by boiling for 10 min. The final volume was made to 5 ml with distilled water and the absorbance due to the produced 3-amino, 5-nitrosalicyclic acid measured at 540 nm with a spectrophotometer (Jenway 6100).

One amylase unit (U) was defined as the amount of enzyme per millilitre culture filtrate that released 1 microgram glucose per minute.

RESULTS AND DISCUSSION

The amylolytic activity of *Bacillus* isolates following the zone of clearance on starch medium is presented in Table 1, Starch reacts with iodine to give blue black colouration. The test would, however, be negative in the presence of amylase which hydrolysis starch to released glucose. Twenty four species of *Bacillus* species were isolated from dawadawan botso. All the *Bacillus* isolates were gram positive, rod shape, spore formers, and hydrolyser's of starch. As seen in Table 1, *B. licheniformis* has the highest amylolytic activity of 5.04 mm followed by *B. amyloliquefaciens* 3.74 mm, while *B. subtilis* showed the least zone of clearance of 0.86 mm. Therefore, *B. licheniformis* was selected for further studies. Variation in amylolytic activity produced by these isolates may probably be as a result of differences in their genetic makeup. The amylolytic activity of *B. licheniformis* in this study is somewhat greater than *Bacillus* species isolated from cassava dump site [17] and that yeast strains isolated from starchy soil [15]. These workers also attributed the observed variation in amylolytic activity to genetic makeup of their isolates. Results of this study show that the *B. licheniformis* with high amylolytic activity could be a potential candidate for large scale of amylase production.

Table 1. Result of amylolytic activity of the identified *Bacillus* species

Isolates	Identity	Zones of clearing on starch agar (mm).
A_1	*B.licheniformis*	5.04
A_2	*B.licheniformis*	2.10
A_3	*B.subtilis*	0.86
A_4	*B. megaterium*	2.18
A_5	*B. brevis*	-
B_1	*B.licheniformis*	2.18
B_2	*B.licheniformis*	2.32
B_3	*B.subtilis*	2.32
B_4	*B.licheniformis*	2.22
B_5	*B. amyloliquefaciens*	2.34
C_1	*B. megaterium*	2.00
C_2	*B. pumilis*	2.08
C_3	*B. pumilis*	2.56
C_4	*B.subtilis*	-
C_5	*B.licheniformis*	2.22
C_6	*B.licheniformis*	2.10
C_7	*B. laterosporus*	2.10
D_1	*B. amyloliquefaciens*	3.74
D_2	*B. pumilis*	2.38
D_3	*B. laterosporus*	2.94
D_4	*B.licheniformis*	2.72
E_1	*B. amyloliquefaciens*	2.28
E_2	*B. brevis*	2.16
E_3	*B. subtilis*	2.44

Figure 1, shows the effect of temperature on amylase production by *Bacillus licheniformis*. The highest amylase activity (2.20 IU/ml/min) was observed at 75^{0}C. The fact that amylase activity drastically increased after 55^{o}C may suggest that the alpha- amylase produced by *B. licheniformis* is a thermophilic variant which could be suitable for industrial application. Our result was, however, in contrast with the findings of [18] who reported 35^{0}C as the maximum temperature for amylase production by *Bacillus subtilis*. Also contrary to this result, was reported by [19] who found that optimum amylase production was at 45^{0}C for free and immobilized cells of *Bacillus* species. Unlike the thermolabile *Bacillus* species which are known to be inhibited at high temperature [3; 20; 21], we supposed that at high temperature, the growth of our thermophilic *B. licheniformis* was greatly influenced and enzyme formation stimulated. More so once *Bacillus* spores are activated, germination set in, which will result to increase metabolic activity. A wide range of temperature (35-80°C) has been reported for optimum growth and alpha-amylase production in bacteria [22]. Konsula and Liakopoulou-Kyriakides [23] reported that a thermophilic *B. Subtilis* strain, isolated from fresh sheep's milk, produced maximum extracellular thermostable alpha-amylase at 40°C in a medium containing low starch concentration.

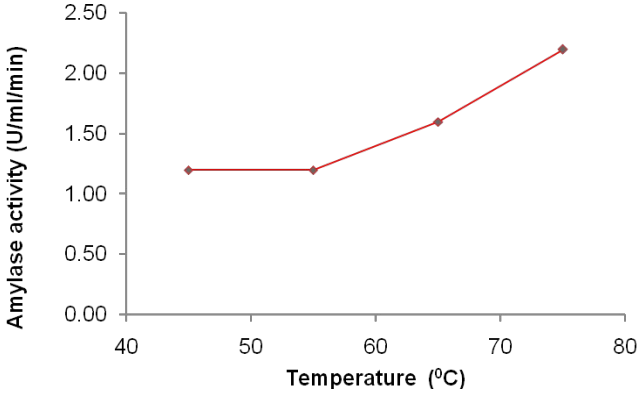

Fig. 1 Effect of varying incubation temperatures on amylase production by *Bacillus licheniformis* cultured on *Adansonia digitata* fruit pulp at 10g/L, initial pH of 5.8 and inoculum size of 8%.

Figure 2, shows the effect of pH on amylase production by *B. licheniformis*. The highest amylase activity (1.80 IU/ml/min) was observed at pH 5.8. At low pH, the amylase activity was low probably due to accumulation acidic end products. As the pH increased to 5.8 the enzyme production increased. Terui *et al.* [24] reported pH 6.8 as the optimum pH for the production of amylase enzyme by *B. subtilis.* Dhanya *et al*. [25] who worked on solid culturing of *Bacillus amyloliquefaciens* for α - amylase production reported maximum amylase production at pH 4.0. Haq *et al.* [26] reported a pH of 7.5 - 8.0 to be the best for α -amylase production by *Bacillus subtilis*.

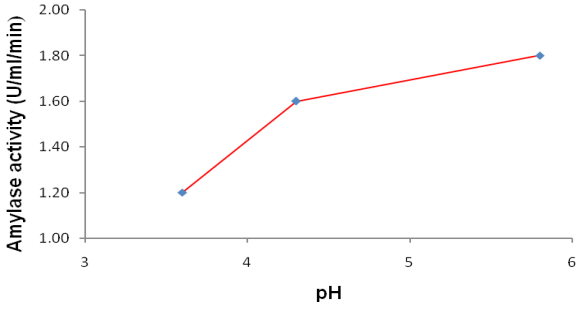

Fig. 2 Effect of varying pH of medium on amylase production by *B. licheniformis* cultured on *Adansonia digitata* fruit pulp at 10g/L, initial temperature of 75^{o}C, incubation period of 3 days and inoculum size of 4%.

The effect of inoculums size on *Bacillus licheniformis* amylase production is presented in Figure 3. The highest amylase activity (2.20 IU/ml/min) was attained at 8% inoculums size. The result shows increase in amylase activity with increasing inoculum size. Omojosola *et al.* [27] reported highest cellulase production at 10% inoculum size of the fungi. This may be explained that as the inoculum size increases, the amount of metabolically active cells may increases, which in turn increases the production of the enzyme.

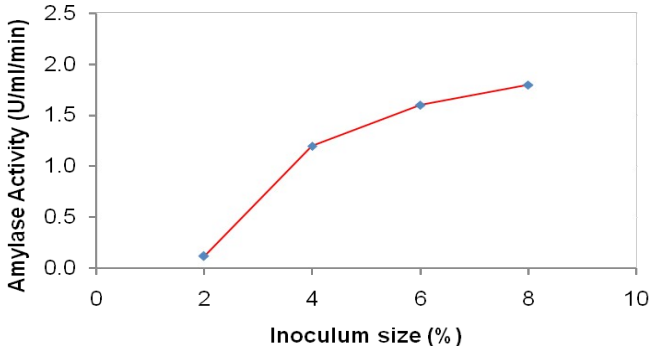

Fig. 3 Effect of varying inoculum size on amylase production by *B.licheniformis* cultured on *Adansonia digitata* fruit pulp at 10g/L, initial pH 5.8, temperature of 75°C and incubation period of 3 days.

The effect of *Adansonia digitata* fruit pulp concentration on amylase production by *Bacillus licheniformis* was evaluated and result presented in Figure 4. The highest amylase enzyme activity was observed at 10g/L (1%) of substrate at 2.00 IU/ml/min. Omojasola *et al.* [27] reported high cellulase production at 5% substrate concentration by fungi cultured on pineapple waste. This shows that *A. digitata* better support the growth of *Bacillus* species and biosynthesis of amylase at low concentration. It has been reported by [28] that synthesis of carbohydrate degrading enzyme in most species of genus *Bacillus* leads to catabolic repression by readily metabolizable substrates such as glucose and fructose. Glucose was observed to represses the production of amylase in hyperthermophilic archeon *Sulfolobus solfataricus* [29]. Whether the decreased amylase production at high *Adansonia digitata* fruit pulp concentration (Figure 4) was due to catabolic repression metabolizable substrates such as glucose and fructose awaits further research.

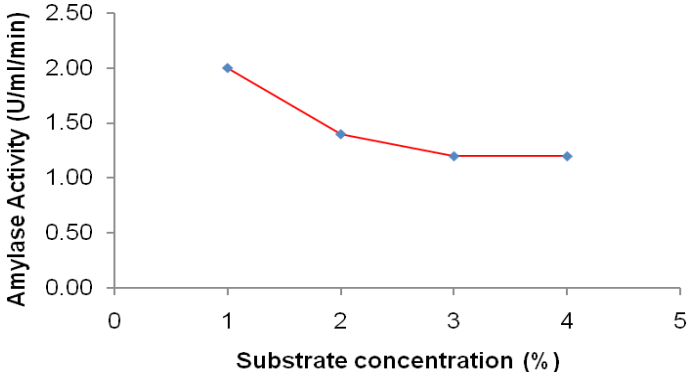

Fig. 4 Effect of varying substrate concentration on amylase production by *B.licheniformis* cultured on *Adansonia digitata* fruit pulp at initial pH 5.8, temperature of 75°C, inoculum size of 8% and incubation period of 3 days

Figure 5 shows the effect of fermentation period on amylase production by *Bacillus licheniformis* cultured on *Adansonia digitata* fruit pulp. Amylase activity increased progressively and attained the peak (2.20 IU/ml/min) at the third day of incubation and declined on the 4th day. The current result is in accordance with that of [25] who work on solid culturing of *Bacillus amyloliquefaciens* for alpha - amylase production and obtain an increase in amylase production from the first three days of fermentation and a decrease at the 4th and 5th day. They explained the decline in amylase activity on 4th and 5th day by the fact that at this stage the isolates have entered their late stationary phase. In other words, the isolates have a short lag and initial stationary phase. Sudharhsan *et al.* [30], suggested that their isolates exhibited a short lag phase and moderate log phase. Production of amylase is usually initiated during the log phase of the growth and reaches maximum levels during the initial stationary phase [30. Even though the extra cellular enzymes are produced from log phase to initial stationary phase, within the phase the production may vary.

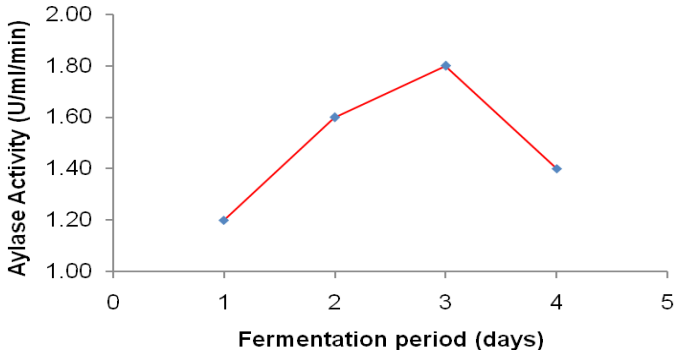

Fig. 5 Effect of varying fermentation period on amylase production by *B.licheniformis* cultured on *Adansonia digitata* fruit pulp at 10g/L, initial pH 5.8, temperature of 75ºC and inoculum size of 8%.

CONCLUSION

The present study shows that *A. digitata* fruit pulp, at low concentration, is a good substrate for amylase production by *Bacillus licheniformis.* The characteristics high temperature, slightly acidic pH of 5.8 as well as short lag and initial stationary phase observed is this study, are novel qualities for application of the *Bacillus licheniformis* in industrial amylase production, which could be exploited in starch and other food industries.

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