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Isolation and Screening of *Aspergillus niger* and *Bacillus coagulans* as Potential Candidates for Amylase and Glucose Isomerase Production

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Abstract

There is an increasing demand for high fructose corn syrup as an alternative to glucose especially for use by people with diabetes due to its low glycemic index. The high cost of this product coupled with its high demand has attracted the attention of many researchers to search for an alternative and sustainable production route. Therefore, the focus of this work was isolating and screening Aspergillus niger and Bacillus coagulans as possible producers of amylase and glucose isomerase respectively, which are needed to produce high fructose corn syrup. Nine (9) samples of loamy soil were taken from three (3) separate locations (BG = Botanical Garden, RD = Refuse Dumpsite, FB = Flower Bed) to isolate the bacterium and fungus. Based on the lacto-phenol cotton blue stain preparation under a microscope, colonies that were thought to be Aspergillus niger colonies were observed and further identified. Cultural, microscopic, and biochemical characteristics were used to confirm the identification of colonies that were thought to be Bacillus coagulans. The probable isolates of Aspergillus niger and Bacillus coagulans were then screened for amylase and glucose isomerase production respectively. Out of the total of nine (9) soil samples analysed, 5 (55.6 %) were positive for Aspergillus niger. A higher isolation rate of Aspergillus niger, 2 (66.7 %) was recorded in soil samples from botanical garden (BG) and flower bed (FB). The lowest isolation rate, 1 (33.3 %) was observed in soil obtained from the refuse dump. Out of all the isolates screened for their potential to produce amylase, the isolate from the botanical garden showed the highest zone of starch hydrolysis (28 mm), and the isolate from the flower bed showed the least zone of hydrolysis of starch (13 mm). On the other hand, out of the nine (9) soil samples analysed, 6 (66.7 %) were positive for Bacillus coagulans and the highest occurrence of Bacillus coagulans, 3 (100 %) was recorded with soil from botanical garden (BG1), while the least occurrence, 1 (33.3 %) was observed in soil from flower bed (FB2). The probable isolates of Bacillus coagulans screened for glucose isomerase production revealed that all the six (6) isolates produced glucose isomerase, with isolate from refuse dump (RD2) producing the highest concentration of glucose isomerase (4.7014 g/L).

Keywords: Isolation, Screening, Aspergillus niger, Bacillus coagulans, Amylase, Glucose isomerase, High fructose corn syrup (HFCS)

INTRODUCTION

Soft drinks and other food items are sweetened with high fructose corn syrup (HFCS). The superior solubility of glucose and fructose in comparison to sucrose and the reduced tendency of HFCS to crystallize in a variety of food products are its technical advantages (Sathya and Ushadevi, 2014). The production of high-fructose corn syrup (HFCS) begins with the enzymatic hydrolysis of starch to glucose, which is followed by the isomerization of glucose to fructose (Souzanchi *et al.*, 2019). According to Fernandes (2018) and Neifar *et al.* (2019), HFCS is more fructose-rich than corn syrup (100 % glucose), which is pure glucose.

Due to its economic and technological importance, amylase has drawn considerable interest worldwide (Aullybux and Puchooa, 2013).

The strain type, growing techniques, cell development, nutritional requirements, length and temperature of incubation, metal ions present, pH, and thermostability all affect the amylase yield during production.

A crucial role in microbial sugar metabolisms is played by the commercially significant enzyme

glucose isomerase/xylose isomerase, which is especially important in the food industry (Seyhan and Alagoz, 2008). The synthesis of high-fructose corn syrup depends heavily on the conversion of glucose to fructose, which is catalysed by the enzyme glucose isomerase (HFCS). Because of the increasing demand for HFCS as sweetener in food industries and for other applications, there is need to search for alternative, cheap and sustainable production route, hence the objective of this research was to identify local strains of *Bacillus coagulans* and *Aspergillus niger* from soil and evaluate their potentials to produce amylase and glucose isomerase enzymes which could be used in the production of High Fructose Corn Syrup (HFCS).

MATERIALS AND METHODS

Collection of Soil Samples

A total of Nine (9) loamy soil samples were collected from different locations namely: Botanical Garden of the Department of Biological Sciences, Ahmadu Bello University, Zaria; Refuse dump site as well as flower bed around the Department of Microbiology, Ahmadu Bello University, Zaria. Three (3) samples were collected from each location at a distance 100 m equidistant from each otherand at a depth of 5 cm. Each sample was packed into a clean polythene bagand labelled appropriately before they were brought to the main research laboratory, Department of Microbiology, Ahmadu Bello University Zaria, for analyses.

Isolation of *Aspergillus niger* from Soil Samples

Ten (10) grams each of the soil samples were separately suspended in 250 mL Erlenmeyer flask containing 90 mL of sterile distilled water and mixed thoroughly. A ten-fold serial dilution was carried out (10^{-1} - 10^{-5}) and aliquot of 0.1mL of 10^{-3} and 10^{-5} dilutions were inoculated onto plates of Potatoes Dextrose Agar (PDA) by spread plating using sterilized bent glass rod. The inoculated plates were incubated at room temperature for five days (Sakshi, 2016).

Identification of the *Aspergillus niger* Isolates

The identification of *Aspergillus niger* was primarily based on the macroscopic observation using coloured mycological atlas and microscopic identification using microscope. The characteristic cultural and microscopic morphologieswere observed and recorded as also described by Toshi and Kumar (2017). Morphological characteristics such as the shape, size and colour of the mycelia including colour of the reverse side were observed and recorded. Wet mount preparation and fungal staining were carried out to observe the isolates' microscopic characteristics as also described by Toshi and Kumar (2017).

Screening of the *Aspergillu niger* Isolates for Amylase Production

The starch agar plate method was used to check the test isolates for their ability to produce amylase. On the starch agar medium, the *A. niger* isolates were spot-inoculated centrally. However, standardization of the inoculum was presumed to be unnecessary since the isolates were only being screened for their potential to produce amylase. The inoculated plates were incubated for 72 h at room temperature (25 - 28 °C). The plates were then flooded with Logul's iodine solution to observe clear starch hydrolysis zones surrounding the amylase-producing fungal colonies. Using a transparent ruler, the diameter of each zone of hydrolysis was measured in millimeters (Soares *et al.*, 2012).

Isolation of *Bacillus coagulans* from the Soil

The isolation of *Bacillus coagulans* was carried out by separately suspending twenty-five (25) grams of each of the nine (9) loamy soil samples (used for the isolation of *A. niger*) into 150 mL of sterile distilled water and mixed thoroughly. The soil suspensions were heat-shocked at 80 °C for 15 min. Serial dilution (10^{-1} to 10^{-5}) for each sample were carried out and 0.1 mL from each of the last three dilutions (10^{-3} to 10^{-5}) were inoculated separately onto nutrient agar plates by spread plate method. All the plates were then incubated at 37 °C for 24 h and examined for the appearance of colonies. The colonies that appeared round or irregular with dull surface; thick, opaque, and cream coloured were sub-cultured onto nutrient agar slants for further identification as described by Kumar *et al.* (2012) and Amin *et al.* (2015).

Identification of the *Bacillus coagulans* Isolates

The identification of the suspected *Bacillus coagulans* isolates was carried out based on theircharacteristic morphological, cultural, and biochemical properties (Sneath *et al.*, 1986). Cultural characteristics including colonial pigmentation and morphology such as size and elevation were observed. Motility test, Gram staining, endospore staining and various biochemical tests (catalase, methyl red (mixed acid fermentation), Voges-Proskauer (Acetoin fermentation), citrate utilization, nitrate reduction and indole tests) were also carried out according to standard procedures described by Cowan and Steel (1993).

Screening of the *Bacillus coagulans* for Glucose Isomerase Production

The screening of the *Bacillus coagulans* isolates for glucose isomerase production was carried out using Seliwanoff's test as described by Sathya and Ushadevi (2014). The screening was carried out by culturing *Bacillus coagulans* on nutrient broth fortified with 4 % glucose. The tubes were incubated at 37 °C for 48 h. One millilitre from each of the incubated test tubes was transferred each into different test tubes, 2 mL of resorcinol (Seliwanoff"s reagent) was then added. Thereafter the solutions were heated for one minute and examined for colour change. The development of cherry-red colour indicates the presence of fructose formed from glucose by the action of the glucose isomerase produced by the *B. coagulans*. The absorbance was recorded at a wavelength of 540 nm using UV spectrophotometer along with a standard curve for fructose as a reference (Sapunova, 2014).

RESULTS

Occurrence of *Aspergillus niger* in the Soil Samples

Five isolates were tentatively found to be *Aspergillus niger* based on cultural and morphological characteristics. All the five isolates were tentatively confirmed to be *Aspergillus niger* after microscopic examination (Table 1). The percentage occurrence of *Aspergillus niger* based on the sampling location revealed 66.7 % each for the soil samples from botanical garden and flower bed, whereas 33.3 % occurrence was observed in the samples from refuse dump site (Table 2).

Amylase Production Potential of the *Aspergillus niger* Isolates

Out of all the nine soil samples analysed, five were found to be positive for *Aspergillus niger*, giving an overall occurrence of 55.6 %.

On the starch agar media, all the five isolates that were tested for their ability to produce amylase displayed distinct zones of starch hydrolysis (Table 3). Therefore, all the isolates were found to be capable of producing amylase enzyme with isolate BG1produced the largest zone of hydrolysis (28 mm) and FB3 produced the least (13 mm).

Percentage Occurrence of *Bacillus coagulans* in the Soil Samples

Six (6) isolates (BG1, BG2, BG3, FB1, FB3, and RD2) were tentatively confirmed to be *Bacillus coagulans* based on cultural, microscopic as well as biochemical characteristics as shown on Table 4. All the isolates were Gram positive, spore-forming, mixed acid fermenting (MR +ve) rods and formed creamy opaque colonies on nutrient agar medium

Table 5 shows the percentage occurrence of *Bacillus coagulans* per sampling area with soil from botanical garden having the highest percentage of occurrence of 100 %, and soil from flower bed had the least percentage occurrence of 33.3 %. The organism was however isolated from all the sampling locations, where six of the nine soil samples were positive for *Bacillus coagulans*, giving an overall occurrence of 66.7 %.

Glucose Isomerase Production Potential of the *Bacillus coagulans* Isolates

While the isolate RD2 from the refuse dumpsite showed the highest potential of glucose isomerase production, thus highest fructose concentration (4.70 g/L) during screening, isolate BG3 from flower bed showed the least glucose isomerase production potential observed from the least fructose concentration (0.73 g/L) produced from the glucose in the medium as shown on Table 6.

Table 1: Cultural Characteristics and Conidiophore Structure of the fungal isolates

Isolate's Code	Colour change of colony	Colour of reverse side	Conidiophore	Probable Organisms
BG1	White to Black	Yellowish	Smooth & Long	*A. niger*
BG2	White to Black	Yellowish	Smooth & Long	*A. niger*
FB1	White to Black	Yellowish	Smooth & Long	*A. niger*
FB3	White to Black	Yellowish	Smooth & Long	*A. niger*
RD1	White to Black	Yellowish	Smooth & Long	*A. niger*

Key: RD= Refuse dump, **BG** = Botanical Garden, **FB**= Flower bed.

Table 2: Occurrence of *Aspergillus niger* in Soil Samples from Various Locations

n= 9

Sampling Location	Number of Samples Collected	Number Positive (%)
Botanical garden	3	2 (66.7)
Flower bed	3	2 (66.7)
Refuse dump site	3	1 (33.3)
Total	9	5 (55.6)

Key: n =Total number of samples collected

Table 3: Amylase Production Potential of the *A. niger* isolates based on Starch hydrolysis

Isolate's Code	Zone of Starch Hydrolysis (mm)
BG1	28
BG2	19
FB1	15
FB3	13
RD1	17

Key: BG= Botanical Garden, **RD**= Refuse dump, **FB**= Flower bed, **mm**= millimetre

Table 4: Cultural, Microscopic and Biochemical Characteristics of the Bacterial Isolates

Isolate's code	Colonial Morphology	GRM	ES	Biochemical Characteristic							Tentative Identity
				M	C	CAT	I	MR	VP	NR	
BG1	COC	G+ve rods	+(oval)	+	+	+	-	-	+	-	*B. coagulans*
BG2	COC	G+ve rods	+(oval)	+	+	+	-	-	+	-	*B. coagulans*
BG3	COC	G+ve rods	+(oval)	+	+	+	-	-	+	-	*B. coagulans*
FB1	COC	G+ve rods	+(oval)	+	+	+	-	-	+	-	*B. coagulans*
FB3	COC	G+ve rods	+(oval)	+	+	+	-	-	+	-	*B. coagulans*
RD2	COC	G+ve rods	+(oval)	+	+	+	-	-	+	-	*B. coagulans*

Key: BG = Botanical Garden, **RD**= Refuse dump, **FB**= Flower bed, **NA**= Nutrient agar, **GRM** = Gram reaction and Morphology, **ES**= Endospore Staining, **M**= Motility, **C**= Citrate Utilization, **I**= Indole, **MR**= Methyl red, **VP**= Voges-Proskauer, **NR**= Nitrate Reduction, **+** = Positive, **-** =Negative; COC= Creamy, opaque colonies

Table 5: Occurrence of *B. coagulans* in Various Soil Samples Collected

Sample location	Number of Sample Collected	Number Positives (%)
Botanical garden	3	3(100)
Refuse dump site	3	2 (66.7)
Flower bed	3	1 (33.3)
Total	9	6 (66.7)

Table 6: Glucose Isomerase Production by *B. coagulans* Isolates

Isolate's code	Absorbance at 540nm	Concentration of fructose (g/L)
BG 1	3.06	4.41
BG2	2.98	4.29
BG3	0.49	0.69
FB1	0.52	0.73
FB2	2.08	2.98
RD2	3.26	4.70

Key: BG =Botanical Garden, **RD**= Refuse dump; **FB**= Flower bed.

DISCUSSION

From the results of the frequency of occurrence of *A. niger* in soil samples from the botanical garden and flower bed (each 66.7%) and the trash dumpsite (33.3%), the difference in the amount of organic matter in the different soils at the sampling sites might be the cause of the observed variations in the isolation. This agrees with the findings of Wissam *et al*. (2019) who showed that soil is the best source of *Aspergillus niger* with 35.1 % percentage occurrence, whereas percentage occurrence of 34.3 % and 31.3 % were from dough and rice sources respectively. The present study however disagrees with the work of Madika *et al*. (2020) who reported highest percentage occurrence of *Aspergillus niger* (60 %) in soil samples from refused dung site.

Analysis of the clear zones of starch hydrolysis developed around the fungal colonies on starch agar medium revealed that all five of the isolated *Aspergillus niger* strains produced amylase. The botanical garden's isolate, BG1 had the largest hydrolysis zone, measuring 28 mm, whereas the fungal isolate FB3 from flower bed had the smallest hydrolysis zone (13 mm). The observed difference was likely due the variations in the isolates' potential for metabolism. This might possibly be a result of the type of soil they were isolated from. Previous research by Oguche *et al*. (2021) reported zone of starch hydrolysis of 21 mm (0.21 cm) produced by *Aspergillus niger*. According to previous findings of Onyia *et al*. (2020), soil is known to be a reservoir of amylase-producing organisms. *Bacillus coagulans* was identified based on culture, microscopic, and biochemical characterizations. Soil from the botanical garden soil had a 100 % incidence of *Bacillus coagulans*, whereas flower bed soil had only a 33.3 % occurrence. The observed variations in the isolation rate might be caused by the variations in the nutritional make-up of the sampling sites.

All the six (6) isolates of *Bacillus coagulans* obtained produced glucose isomerase with isolate from refused dump (RD2) producing the highest concentration of glucose isomerase (4.70 g/L) produced. The study falls in line with the work of Mukesh *et al*. 2012 who described the ability of *Bacillus* species to produce glucose isomerase. Research conducted by Ogbonnaya, (2015) showed a range between 0.11 and 3.62 mg/mL fructose, with isolate designated AO9 as a potent GI producer as determined quantitatively based on the release of 3.62 mg/mL of fructose from glucose contained in the assay medium.

CONCLUSION

Five fungi and six bacteria were isolated from soil samples collected from the three different sites namely, flower bed, refuse dump and botanical garden. From the results obtained in this research, it was concluded that *Aspergillus niger* and *Bacillus coagulans* were isolated from all the soil samples analysed with respective overall occurrences of 55.6 % and 66.7 %. It was also concluded that all the isolates of *Aspergillus niger* and *Bacillus coagulans* obtained from the three soil sources demonstrated the ability for amylase and glucose isomerase production respectively. *Aspergillus niger* BG1 from botanical garden demonstrated the highest zone of hydrolysis (28 mm), whereas *B. coagulans* RD2 from refuse dump showed the highest glucose isomerase concentration (4.70 g/L fructose equivalent).

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