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Amylase and Biosurfactant Production from *Bacillus* species

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A B S T R A C T

Most *Bacillus* species have ability to produce secondary metabolites such as amylases and biosurfactants. This study was carried out to determine the amylase and boisurfactant production potentials of some *Bacillus* species obtained from agricultural soil. Soil samples were collected from agricultural research farm of Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria. All isolates were identified based on the morphological and biochemical characteristics using Bergey's manual of determinative bacteriology. Amylase-producing *Bacillus* species were selected and screened on soluble starch agar while biosurfactant activity was determined by three methods; blood haemolysis, oil spreading, and drop-collapse tests. The extent of amylase activity of each isolate was determined quantitatively using dinitrosalicylic acid (DNSA) assay method. A total of ten (10) *Bacillus* species were isolated and identified from the samples. These isolates include two *B. subtilis* (AMY5 and AMY13), *B. mycoides*, two *B. polymyxa* (AMY2 and AMY12), *B. azotoformans B. licheniformis, B. megaterium, B. pumilus* and *B. cereus*. The enzyme assay results indicated that *B. megaterium* (Isolate code: AMY17) had the best activity value of 1.12 *µ*mol/min/mL while *B. cereus* had the least value 0.27 *µ*mol/min/mL. All *Bacillus* species demonstrated great potentials for biosurfactant production except *B. subtilis* (AMY10). Overall, *B. licheniformis* (AMY17) and *B. subtilis* (AMY5) had the highest biosurfactant activity. Therefore, *Bacillus* species are promising sources of amylases and biosurfactants for the bioeconomy and their use should be encouraged.

Introduction

Amylases are biocatalytic proteins that act on starch molecules and hydrolyze starch to produce various products such as dextrin and smaller polymers composed of glucose units (Natasa *et al.*, 2011; Mohammed and Mastan, 2013). Enzymes are considered to be important in modern biotechnology with its applications ranging from food to detergent and paper making industries (Pandey *et al.*, 2000). Microbial amylases can be derived from microorganisms, a good number of microbial amylases are now available for commercial use and they have almost replaced chemical hydrolysis of starch in starch processing and related industries (Hussain *et al.*, 2013). Amylases can be

classified into three: (a) alpha amylase, (b) beta amylase and (c) gamma amylase. Alpha amylase: is an amylolytic enzyme that breaks down α, 1-4-glucosidic: linkages of starch into an endo form and produces oligosaccharides (Parka and Son, 2007). Beta amylase: acts on the non-reducing end by cleaving two glucose units (maltose) at a time (Bijttebier *et al.,* 2007). Gamma amylase: it cleaves the last α-(1-4)-glycosidic and α-(1-6)-glycosidic linkages at the non-reducing end of amylose and amylopectin to yield glucose (Adeoyo *et al.,* 2019). Gamma (γ)-amylase has been reported to be active in acidic environments (Kumar and Satyanarayana, 2009). Amylase is majorly produced by bacterial species of *Bacillus* (Muralikrishna and Nirmala, 2005), and *B. subtilis* and *B. licheniformis* are among the species that have been widely studied (Nidhi *et al*., 2005).

Amylase from microbial sources can be produced in large quantities to meet the high industrial and market demands, and the diversity of microbes as the source material for bio-based products (such as enzyme production) is gaining ground every day. Although, amylases can be derived from other sources, such as plants, and animals, but because of the short growth period, biochemical diversity and the ease with which enzyme concentrations can be increased by nutritional, environmental conditions and genetic manipulation, makes enzymes from microbial sources much better (Oliveira *et al.,* 2007; Mishra and Behera, 2008). Bacteria are a more dominant group of microorganisms in soil (Bodour *et al.,* 2003) with population of about a hundred thousand to several hundred millions for a gram of soil (Rangaswami and Bagyarag, 2004).

Biosurfactants are surface active compounds that accumulate at the boundary between two immiscible fluids or between a fluid and a solid. They reduce the repulsive forces (tension) between two different phases (liquid/air or liquid/liquid) which allow them to mix and thus enhance their solubility properties (Cheng *et al.,* 2008; Perfumo *et al.,* 2010; Fracchia *et al.,* 2012). They are amphiphilic compounds consisting of hydrophilic and hydrophobic domains. The hydrophilic domain can be carbohydrate, amino acid, phosphate group or some other compounds whereas the hydrophobic domain usually is a long chain fatty acid (Lang, 2002). Microorganisms have been reported to produce several classes of biosurfactants such as glycolipids, phospholipids, polymeric biosurfactants, neutral lipids or fatty acids (Banat *et al.,* 2000; Franzetti *et al.,* 2010).

Biosurfactants have some advantage over their commercially manufactured chemical surfactants because of their lower toxicity (pharmaceutical industry), biocompatibility, biodegradable nature and effectiveness at extreme temperature, pH, salinity, as well as the ease of production (Mulligan and Gibbs, 2004; Ilori *et al.,* 2005). Biosurfactants are more efficient critical micelle concentration (CMC) of biosurfactant is about 10–40 times lower than that of chemical surfactants, in other word, it requires lesser quantity than chemical surfactants to decrease surface tension (Desai *et al.,* 1997). Previously, biosurfactant-producing microbes had been produced on haemolytic media without hydrocarbons (Mulligan *et al.,* 1984) and the result indicated that it can serve as preliminary test for identification of related isolates. This test can be performed to determine the ability of bacterial colonies to induce haemolysis when grown on blood agar. A few biosurfactants are currently available in the market and efforts at discovering new ones are on top gear. Biosurfactants are useful in pharmaceutical, food, petroleum, detergent, agricultural, textile industries (Roy, 2017; Akbari *et al.,* 2018). This study aimed to determine the

amylase and biosurfactant activity of some *Bacillus* species obtained from agricultural soil.

Materials and Methods

Sample collection

Soil samples were collected from agricultural field of Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria from a depth of 10 cm – 15 cm with the aid of a soil auger. Samples were transferred into sterile plastic bags and brought to the Microbiology Laboratory of the Department of Microbiology, Adekunle Ajasin University under aseptic conditions and stored in a refrigerator at 4°C for further studies.

Isolation and identification of bacterial species

The organisms were isolated by growing on a nutrient agar medium after which they were observed carefully for colony morphology with respect to colour, shape, size, nature of colony and pigmentation (Mishra and Behera, 2008). The isolates were Gram stained and observed under the light microscope (Vipul, 2011). The isolates were characterized biochemically using catalase, coagulase, methyl red, sugar fermentation and starch hydrolysis tests (Kaur *et al.,* 2012). The organisms were identified using the Bergey's manual of determinative bacteriology, 9th edition (Olukunle, 2013; Adeoyo, 2019).

Screening for amylase producing bacterial species

Bacterial isolates were screened for amylolytic properties by starch hydrolysis test on starch agar plate with the following components (w/v): Starch (1.0%), yeast extract (0.05%), KCl (0.01%), $MgSO_4.7H_2O$ (0.05%), NaH_2PO_4 (0.01%), agar (1.5 g), water (100 mL). The microbial isolates were streaked along a line on the starch agar plate and plates were incubated at 37°C for 48 hours. After incubation 1% iodine solution (freshly prepared) was used to flood the starch agar plate (Ashwini *et al.,* 2011). Presence of blue-black colour around the growth indicated a negative result while a clear zone (halo) indicated a positive result. The isolates that produced clear zones (haloes) of hydrolysis were taken as amylase producers.

Assay for amylase activity

The enzyme assay was conducted using dinitrosalicylic acid (DNSA) assay method described by Miller (1959). A 100 mL nutrient starch broth was inoculated with 1 mL suspension of a bacterial isolate and incubated in a rotary incubator shaker at 37^oC for 48 hours. After incubation, the nutrient starch broth was centrifuge at 10000 x *g* for 15 min and the supernatant was used as cell free crude enzyme. A soluble starch (1%, w/v) was added to a 10 mL citrate-phosphate buffer (pH 6.8) in a Schott bottle. The mixture was stirred with a magnetic stirrer until completely dissolved.

A 1 mL of each crude enzyme was added to a separate 1 mL substrate (performed in triplicate). The blank contained 2 mL buffer only. All samples were incubated at 37°C for 60 min, followed by centrifugation at 10000 × *g* for 2 min. After centrifugation, a 1 mL supernatant was then withdrawn and transferred to a new test tube, and 1 mL DNSA reagent was added. This was followed by boiling for 5 min at 100°C and cooled on ice for 5 min. A 2 mL of each of either blank, controls or samples was placed in a cuvette and the absorbance read with the aid of a spectrophotometer at a 540 nm wavelength. A unit of enzyme activity was expressed as 1 μmol of maltose equivalent released per minute per millilitre of enzyme solution.

Screening for biosurfactant production from *Bacillus* species

Mineral salt medium (MSM) of minimal salt medium containing; K_2HPO_4 (1.0 g), $MgSO_4.7H_2O$ (0.2 g), $FeSO_4.7H_2O$ (0.05 g), $CaCl_2.2H_2O$ (0.1 g), $Na_2MoO_4.2H_2O$ (0.001 g), NaCl (0.03 g), used engine oil (1.0%, w/v), and water (1000 mL) (Nayarisseri *et al.*, 2018). A 100 mL of MSM was dispensed into each 250 mL Erlenmeyer flask, sterilized by autoclaving at 121^{o}C for 15 min and inoculated with a loopful of each bacterial culture.

The experiment was performed in an incubator shaker for 72 hours at 150 rpm (temperature 37^{o}C). After incubation, culture broth was centrifuged at 6000 rpm for 15 min. The supernatant was collected for various biosurfactant screening tests. Biosurfactant production was determined by three methods; blood haemolysis, oil spreading, and drop-collapse tests.

Blood haemolysis test

A 24-hour old culture of each isolate was streaked on blood agar plate. The plate was incubated for 72 hours at 37^{o}C. Each plate was observed for the presence of clear zone around each colony. A clear zone indicated the presence of biosurfactant.

Oil spreading test

A micropipette was used to take 10 µL of used engine oil, added to the surface of 40 mL of distilled water into a Petri dish to form a tin oil layer. A 10 µL of the crude biosurfactant was immediately dropped on the centre of the oil layer to form a clear zone. The result was read after 30 seconds, and a clear zone indicated the presence of biosurfactant (Morikawa *et al.*, 1993; Walter *et al.*, 2010).

Drop collapse test

A drop of the culture supernatant was placed carefully on an oil coated glass slide and was read after 1 min. If the drop of supernatant collapsed and spread on the oil coated surface, it signifies the presence of biosurfactant (positive). The diameter of clear zone on the oil surface indicated the extent of biosurfactant activity (oil spreading activity) (Walter *et al.*, 2010).

Results and Discussion

Isolation and identification of bacterial species

All isolates identified as *Bacillus* species were selected for further studies. The organisms were identified based on the characteristics observed with the use of Bergey's manual of determinative bacteriology. A total of ten (10) *Bacillus* species were identified from soil. These isolates include; two *B. subtilis* (AMY5 and AMY13), *B. mycoides*, two *B. polymyxa* (AMY2 and AMY12), *B. azotoformans B. licheniformis, B. megaterium, B. pumilus,* and *B. cereus*.

Amylase producing *Bacillus* species

Different *Bacillus* species showed varying enzyme activity on starch agar plates. The diameters of these microbes' hydrolytic zones range between 6 to 12 mm on agar plates. Plate a (*B. licheniformis*) shows more hydrolytic zone than the other tested *Bacillus* species (Figure 1) (Table 1).

Biosurfactant producing *Bacillus* species

Table 2 shows that *Bacillus licheniformis* (AMY17) had the highest biosurfactant production capacity, followed by *B. subtilis*

(AMY5), *B. azotoformans*, *B. polymyxa* (AMY24), *B. cereus*, and *B. megaterium*. Weak activity was observed with *B. subtilis* (AMY13), *B. pumilus* and *B. polymyxa* while *B. mycoides* did not show any activity.

Table.1 Amylase activities of *Bacillus* species

Isolate Code	*Bacillus* species	Diameter of hydrolytic zone (mm)	Amylase activity (μmol/min/mL)
AMY5	*B. subtilis*	10	0.89±0.021
AMY10	*B. mycoides*	9	0.76±0.030
AMY13	*B. subtilis*	7	0.33±0.016
AMY2	*B. polymyxa*	8	0.44±0.007
AMY24	*B. polymyxa*	9	0.62±0.029
AMY26	*B. azotoformans*	11	1.02±0.023
AMY17	*B. licheniformis*	12	1.12±0.035
AMY9	*B. megaterium*	8	0.33±0.010
AMY25	*B. pumilus*	7	0.30±0.016
AMY8	*B. cereus*	6	0.27±0.014

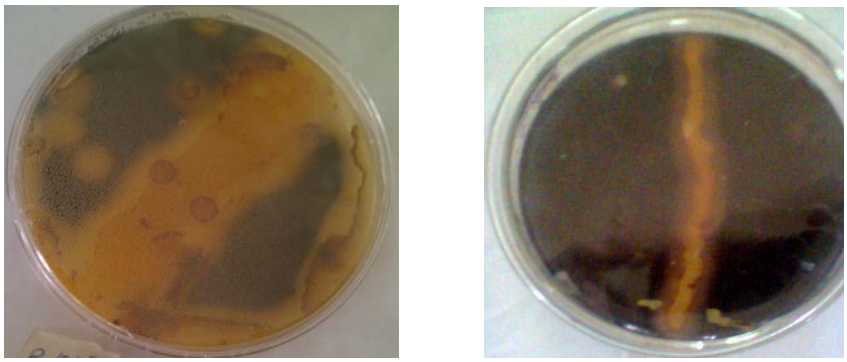
Table.2 Biosurfactant production potential of *Bacillus* species

Isolate Code	Organism	Haemolysis	Drop Collapse	Oil spreading
AMY5	*B. subtilis*	+	++	+++
AMY10	*B. mycoides*	-	-	-
AMY13	*B. subtilis*	+	+	+
AMY2	*B. polymyxa*	+	-	-
AMY24	*B. polymyxa*	+	++	++
AMY26	*B. azotoformans*	+	+	+++
AMY17	*B. licheniformis*	+	+++	+++
AMY9	*B. megaterium*	+	+	++
AMY25	*B. pumilus*	+	Nd	+
AMY8	*B. cereus*	+	++	++

Key: + = positive result, - = negative result, nd = not determined, ++ = efficient, +++ = very efficient

Figure.1 Screening for amylase enzyme production: (a) *Bacillus licheniformis* (AMY17); (b) *Bacillus megaterium* (AMY9)

(a) **(b)**



The study showed that ten (10) *Bacillus* species were isolated and identified from soil. They include; two *B. subtilis* (AMY5 and AMY13), *B. mycoides*, two *B. polymyxa* (AMY2 and AMY12), *B. azotoformans B. licheniformis, B. megaterium, B. pumilus* and *B. cereus*. These findings agree with the report of Pandey *et al.*, (2000); Mishra and Behera (2008) who reported that some *Bacillus* spp. isolated from soil and water samples were capable of hydrolysing starch. These results also corroborate the study of Mohammed and Mastan (2013) who reported amylolytic potential of some bacteria isolated from agriculture fields of Bhimavaram, India. *Bacillus licheniformis* (AMY17) had the highest activity value of 1.12 µmol/min/mL while *Bacillus cereus* (AMY8) had the least value of 0.27 µmol/min/mL.

Enzymes (for an example, amylase) from *Bacillus* species have been shown to contribute to improving nutrient digestibility and maintaining a beneficial gut microbiota in poultry birds (Latorre *et al.*, 2016). Furthermore, the study revealed that soil is a rich source of microorganisms (particularly bacterial species) which can be used for amylase production. The results of starch-agar screening method correlate with the enzyme activity assay across all isolates tested. Thus to determine enzyme activity of a large number of bacterial cultures, agar screening method provides means of identifying the best isolate(s) and reducing the number of positive isolates to a sizable number. The exploration of *Bacillus* species as microbial sources for amylases production will continue to increase productivity in detergent, pharmaceutical, food and confectionery industries.

Also, the study revealed that *Bacillus* species have varied biosurfactant activities. Haemolysis test is a major screening method for identifying biosurfactant-producing bacteria, and oil spreading, drop collapse are among other confirmatory tests (Shoeb *et al.*, 2015). From this study, *B. licheniformis B. subtilis* (AMY5), *B. azotoformans*, *B. polymyxa* (AMY24), *B. cereus*, and *B. megaterium* showed great potentials for biosurfactants production. These findings agree with the observation made on *B. azotoformans* by Adamu *et al.*, (2015). Similar report was made by Ndibe *et al.*, (2018) who reported the screening of biosurfactant-producing bacterial species obtained from River Rido, Kaduna, Nigeria. Moreover, Maneerat and Phetrong (2007) reported that *B. subtilis* and *B. pumilus* were among the bacterial species with biosurfactant activity. Consequently, the metabolic

products of these indigenous microbes have contributed to a major breakthrough for bioremediation purposes and other industrial applications (Boboye *et al.,* 2010; Olukunle, 2013; Akbari *et al.,* 2018).

In conclusion, amylases have wide range of applications and the demand for these enzymes is increasing daily, it is obvious that studies on amylases and their usefulness have progressed well over the last few decades. Biosurfactants on the other hand are key components in the clean-up strategy for bioremediation due to their biodegradability and low toxicity. They are also useful in detergent industry because they are biodegradable, pH stable; low CMC and environmentally friendly. *Bacillus* species are among bacteria that have shown attractive potentials for production of these useful biomolecules. Further studies are needed to characterize and optimize the production under certain growth conditions such as pH, temperature, carbon sources and nitrogen sources. Therefore, indigenous entrepreneurs and researchers are encouraged to invest in these important biomolecules that can benefit mankind, especially those from *Bacillus* species.

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