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Screening and characterization of amylase producing bacteria from soil contaminated with cassava effluents from Abraka, Delta State

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Key words: Amylase producing bacteria, Soil, Characterization

Abstract

Amylases are starch hydrolyzing enzymes important in normal daily life and industry. There are different sources of amylase production however, enzymes derived from microorganisms are cheaper and thermos table among other features. The aim of this work was to isolate amylase producing bacteria from soil contaminated with cassava water effluents and to find out environmental/ optimization of amylase production. Soil samples were collected from four different sites. A total of 50 bacterial were isolated form soil samples, 14 showed area of clear zones on starch medium. Isolates that gave maximum yield of amylase were identified molecularly. The isolates were optimized for carbon sources, pH, nitrogen sources, incubation time, agro waste and chlorides. Biochemical and molecular analysis showed that *Citrobacter* sp and *Enterobacter cloaca* showed higher amylase activity. Glucose, nitrate, pH 7.0, magnesium chloride and temperature of 25°C produced maximum amylase. Plantain peel was the agro water that produced more amylase enzyme.

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Introduction

Amylases are important in the hydrolysis of starch into sugars. In humans, amylases are produced in pancreas and saliva glands and play a great role in digestion of foods containing large amount of starch into disaccharides and trisaccharides that are in turn converted by other enzymes to glucose (Oluwadamilare *et al*., 2019).

Amylases are found in animal, plant, bacteria and fungi (Ekka and Namdea, 2018). There are different forms of amylases; alpha, beta and gamma which act on α-1,4-glycosidic bonds. Amylases have many useful applications. Alpha (α)-is majorly a digestive enzyme that need calcium for functioning as such called calcium metalloenzyme which differentiates it from beta and gamma amylases (Geesha, 2016).

Bacterial and fungal amylases are widely used in industries due to shorter time of production, cheaper, consistency and thermostability (Fossi *et al*., 2005; Swetha *et al*., 2006). Amylases derived from fungal and barley are used in preparing oriental foods and in brewing industry (Veerapagau *et al*., 2016). Amylase is used in pharmaceutical industry in digestive tonics and to hydrolyse starch to produce different sugars (Saini *et al*., 2017). Due to the usefulness of this enzyme, this study was carried out to screen organisms for different sources for the production of amylase.

Materials and methods

Sample collection and Microbial Identification

Soil samples were obtained from Abraka area. Soil contaminated with cassava effluents were collected into sterile cellophane and transported into the Microbiology laboratory of the University.All culture media used for the laboratory work were prepared according to manufacturers instructions and 10g of samples was dissolved in 90mls of sterile distilled water and serially diluted. Pour plate method was used for analysis. One millilitre of appropriate dilutions were introduced into sterile petri dishes and nutrient agar already prepared and cooled to 55°C was poured then mixed and incubated at 37°C for 24hours

Screening for amylase producing bacteria and amylase enzyme screening

The method of Fasiku *et al.* (2020) was adopted with little modification. Nutrient agar was supplement with 1% starch. And the organism streaked at the centre of the plate and incubated for 24hours-48hour at 37°C at the end of incubation. Culture plate was flood with lugols iodine. Clear zone around culture indicated amylolytic activity.

Production of amylase and enzyme assay

Activated cultures (F15 and S14) showing clear zones around them were inoculated into fermentation broth. Fermentation medium contained soluble starch (10g/L), peptone (5g/L), (NH4)2 SO4 (2g/L), KH2PO4(1g/L), K2HPO4, (2g/L),mgCl2, 0.01) at pH 7.0. and incubate in shaker incubator at 150rpm 37°C for 24 hrs. At the end of incubation, the culture was centrifuged at 10.000 rpm for 10 minutes (Validya and Rathore, 2015; Ekka and Nemdo. 2018). The supernatant was used as source of crude enzyme. One milliliter of crude enzyme and phosphate buffer (pH 7.0) was introduced in a test tube and incubated for 10 minutes at 35°C, 2mls of DNS reagent was introduced to the test tube to stop the experiment and then put in boiling water for 10 minutes. The test tube was removed and allowed cool to room temperature and the final volume was made up to 10ml by adding distilled water. The readings were taken at absorbance 540nm against maltose the standard (Ekka and Nemdo. 2018).

Bacterial Identification

Pure cultures were obtained by further subcultures on nutrient agar. Cultures were purified by inoculating into fresh media. Microorganisms were identified using conventional and molecular characteristics.

Molecular Identification of bacterial Isolates

The DNA of pure bacterial isolates were identified using PCR method. Universal primers: Forward 27F: 5'AGAGTTTGATCMTGGCTCAG3' and reverse 1492R: TACGGTACCTTGTTACGACTT were used. The PCR amplification mixture was carried out using 10µL volume of the cocktail mix containing the following

constituents; 0.5µL each of forward and reverse primer, with 2.0µL DNA template, 1.0µL each of PCR buffer,mgCl_2, and DMSO, 0.8µL deoxynucleoside triphosphates (Soils Biodyn), 0.1 µL Taq polymerase (Soils Biodyne) and 3.1µL water. PCR condition was 94°C for 5 minutes for initial denaturation, 94°C at 30s for denaturation, annealing temperature was 56°C for 30s and extension (72°C for 45 seconds). PCR was carried out in 36 cycles. The final extension was 72°C for 7 minutes and hold temperature 10°C. The amplicons from the 16s rRNA PCR reaction above was loaded on 1.5% agarose gel and electrophoresis was carried out at 80V for 1hr.

Sanger sequencing was done using 3500 ABL genetic analyser at Inqaba, South Africa. The sequencer was used to generate sequences and visualized for base pairing and then edited using Bio edit from national Center for Biotech Information database. Similar sequences above 95% were downloaded and aligned using Clusta IW and phylogenetic tree was drawn with MEGA 6 software.

Optimization for amylase production

The optimization of amylase production by amylase enzyme were carried out. Different carbon sources (Lactose, glucose, mannose and sucrose), nitrogen source (potassium nitrate, urea, peptone and casein) different pH (3, 5, 7, 8, 9) at different incubation times (0,24,48,72,96) different chloride (ammonium chloride, ferric chloride, magnesium chloride, calcium chloride and sodium chloride) and temperature (15°C, 25°C, 35°C and 45°C) were used to induce amylase production in the medium. The enzyme activity was measured after 24hours of incubation (Alariya *et al.*, 2013)

Using Agrowaste as substrate

Yam peel, Plantain peel, egg shell and rubber seed scale were used. The experiment was carried out using Erlenmeyer flask containing 10g of the substrate and 20mls of sterile liquid nutrient medium containing (%): {KH2 PO4-0.1, NaCl-0.025, mgSO4.7H20-0.01, $CACl_2$-0.01} with agro waste by solid state fermentation process and inoculated with 1ml of the prepared inoculum and thoroughly mixed and incubated at 37°C for 5 days and enzyme assay was done (Sexane and Singh, 2011)

Results and discussion

Sample Collection and Microbial Identification

Samples were collected from four different sites from Abraka and Environ. Fifty organisms were isolated picked and screened for amylase activity, however only 14 showed clear zones from which two bacteria that produced appreciable zones of inhibition were picked for identification.

Table 1. Biochemical test of isolates and molecular identification.

Organism	Gram reaction	catalase	Oxidase	Urease	Citrate	Indole	coagulase	TSI	sucrose	lactose	Glucose	Acid/gas	H_2S	VR	MR	
F15	-rod	+	-	+	+	-	-	+	+	+	+	+	+	-	+	Citrobacter sp.
S14	-rod	+	-	-	+	-	-	+	+	-	+	+	-	+	+	Enterobacter cloacae

These two isolates were identified using biochemical tests and molecular identification and labelled F15 and S14 respectively. Biochemical tests conducted indicated *Citrobacter* sp and *Enterobacter* cloacoe.

Citrobacter sp and *Enterobacter* sp were identified as the isolates that produced amylase above (25.00mm). These organisms were isolated from soil contaminated with cassava effluent. Previous works reported amylase producing isolates for various soil.: sugarcane field (Ekka and Namdeo, 2018), forest soil (Oseni and Ekperigin, 2013); sewage enriched soil (Pokhrel *et al.*, 2013), potato waste dumpsite; (Vermaa *et al.*, 2011); and rotten potato (Orji *et al.*, 2009). Molecular identification confirmed the two isolates (Figs. 1-3).

Optimization of carbon source for amylase production

Fig. 4 shows amylase activity by the two isolates. The bacterium F15 compared to S14 produced more amylase activity.

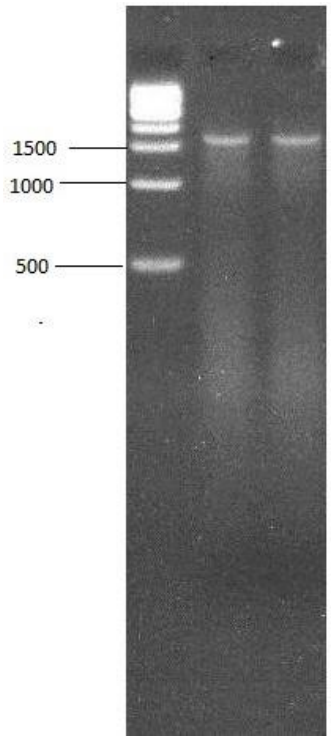

Fig. 1. Amplified PCR product generated from bacterial isolates.

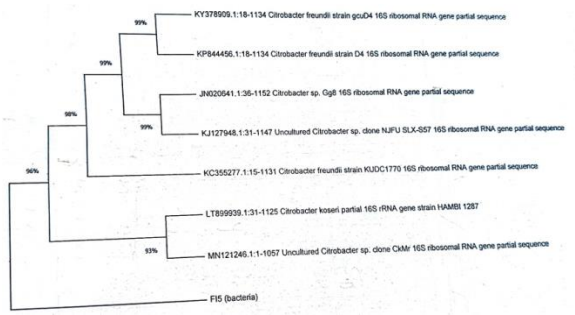

Fig. 2. Phylogenetic tree for F15.

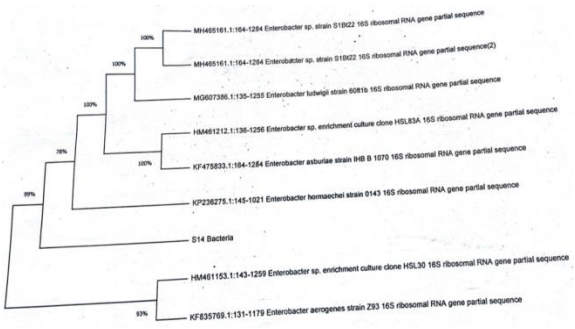

Fig. 3. Phylogenetic tree for S14.

Fig. 5 showed the carbon sources used to replace starch in amylase production at the end of 24hrs. results showed that glucose was the carbon source that produced highest amount of amylase for both bacteria, then lactose, before mannose while sucrose produced the least amount. This findings is consistent with (Ekka and Namdeo, 2018) in which sucrose produced the least amount of amylase while fructose produced highest in their study.

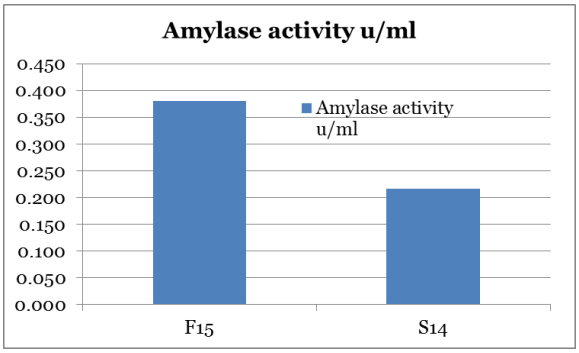

Fig. 4. Amylase activity.

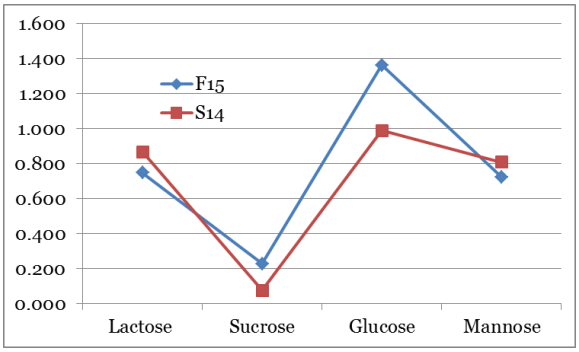

Fig. 5. Various sources of carbon such as Lactose, Sucrose glucose and mannose were used to replace Starch which was the original carbon source in growth media.

Optimization of Nitrogen source for amylase production

Results obtained for Fig. 6 showed contrasting results for the two organisms. For F15, potassium nitrate gave the highest production and peptone the least. For S14, peptone yielded the highest amylase production just as reported by Gupta *et al*. (2008) for amylase production by *Aspergillus niger*.

Optimization of amylase production by changing pH

The optimium pH for the amylase production was pH 7.0 as shown in Fig. 7 though all the organisms were able to grow within pH 3.0-8.0. Those result is consistent with Validya and Ruthore, (2015), whose study showed maximum amylase production by bacteria was pH 8.0 (Ekka and Namdeo, 2018).

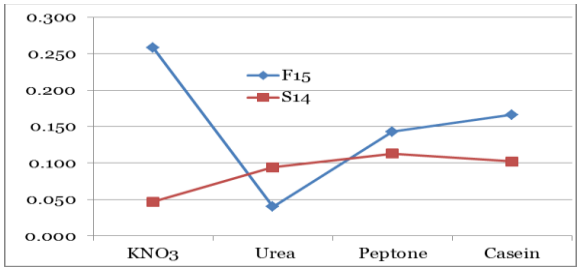

Fig. 6. Various sources of Nitrogen such as Potassium nirate, urea, peptone and casein.

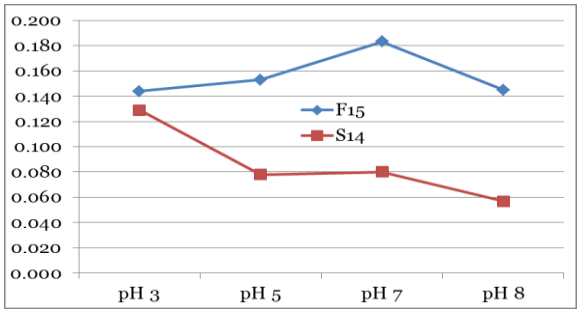

Fig. 7. All the two colonies were allowed to grow in media of different pH ranging from 3.0 to 8.0.

Optimization of chloride on amylase production.

Magnesium chloride was the most suitable chloride that yielded more quantity of amylase for both organisms.

Effect of temperature change

Results showed that the optimum temperature for amylase production was 25°C and the least temperature was 35°C. There is no definite temperature for enzyme production as results obtained depend on the organism involved and the source of such organisms Other reports showed varied temperature for enzyme production by various bacterial isolates from different sites 35°C (Bole *et al.*, 2013) 40°C (Validya and Rathore, 2018).

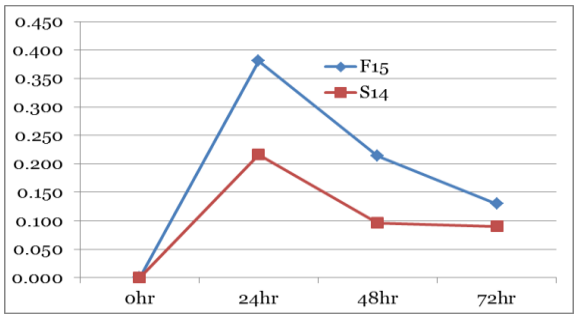

Fig. 8. Effect of incubation period on amylase production.

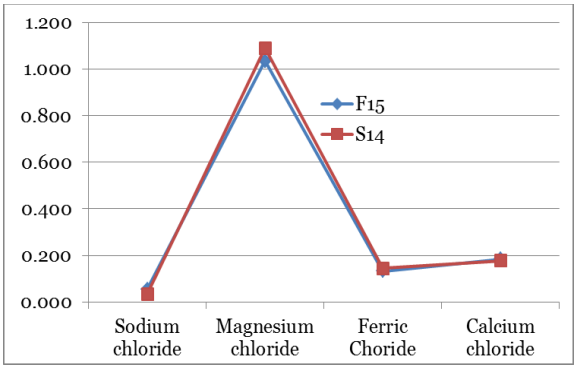

Fig. 9. Effect of chlorides on amylase production.

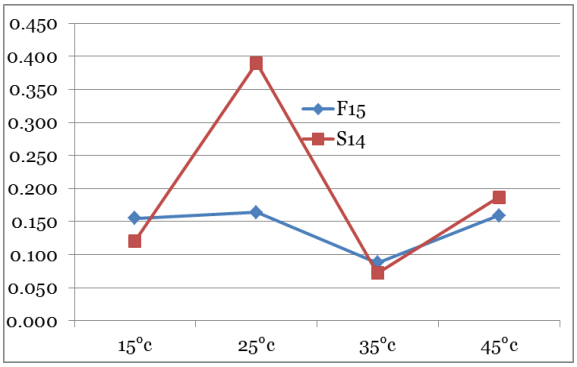

Fig. 10. Effect of temperature on amylase production.

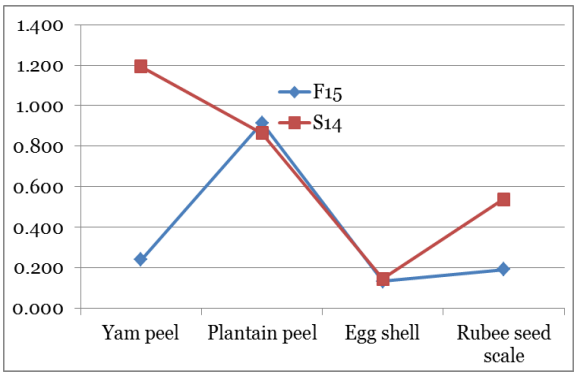

Fig. 11. Effect of agro waste on amylase production.

Effect of agrowaste

Plantain peel and produced the highest amount of amylase compared with egg shell and other agrowaste used in this study.

Conclusion

Citrobacter sp and *Enterobacter cloacae* isolated from soil contaminated with cassava effluent produced amylase. Plantain peel produced the highest amount of amylase compared with other agrowaste used in this study. These isolates may be useful in amylase production.

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