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Research Paper

Molecular identification and amylolytic potential of a thermophilic bacteria species from refuse dump in Ile-Ife, Nigeria

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Abstract

Molecular identification and amylolytic potential of a thermophilic bacterium species isolated from refuse dump was investigated. Bacterial isolates were identified by morphological and biochemical characterization while amylolytic bacterium of interest was identified by molecular analysis using 16S rRNA gene sequencing. The bacterium with the highest α-amylase activity was selected for enzyme production. The optimal conditions for α-amylase secretion were determined by varying the pH, temperature, percentage soluble starch, nitrogen sources and carbon sources. The isolated and identified bacteria were *Bacillus alvei* (40%) *Bacillus licheniformis* (40%) and *Bacillus brevis* (20%) while *Bacillus licheniformis* RD24 was identified by 16S rRNA gene sequencing. The peak of amylase production was at 20 h of incubation (925 µg/ml/min). The optimum pH and temperature for the enzyme production were 7 and 45^{o}C respectively. Enzyme production medium with 1% starch gave highest enzyme activity of 102 ± 5.3 µg/ml/min. Peptone gave an enzyme activity of 165 ± 8.97 µg/ml/min and yeast extract gave 52.26 ± 2.86 µg/ml/min. Of the raw starches, cassava flour gave the highest specific activity of 72 ± 0.07 Units/mg proteins, while sorghum starch gave the lowest specific activity of 5 ± 1.52 Units/mg proteins. The study concluded that starch-rich household waste can be employed for amylase production using *Bacillus licheniformis* RD24.

Keywords: *Alpha Amylase, Bacillus Species, Optimum, Soluble Starch, Thermophilic*.

1. Introduction

Starch production in the earth was estimated to be in the order of 2.0 x 10^{10} tonnes/year, which corresponds to about 80 % of total food production worldwide (Sarikaya *et al*., 2000). Enzymes are among the most important products acquired for human needs in the areas of industrial, environmental and food biotechnology through microbial sources. Alpha amylase is a hydrolytic enzyme and in recent years, interest in its microbial production has increased dramatically due to its wide spread use in food, textile, baking and detergent industries (Asgher *et al*., 2007). Amylases are hydrolases that function by the breakdown or hydrolysis of starch into reducing fermentable sugars, mainly maltose and reducing non-fermentable or slowly fermentable dextrins (Oyeleke *et al*., 2010). Among various extracellular enzymes, α-amylase ranks first in terms of commercial exploitation (Babu and Satyanarayana, 1993) and accounts for 12 % of the sales value of the world market (Baysal *et al*., 2003). Spectrum of applications of α-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in bakery, brewery, detergent, textile, paper and distilling industry (Ramachandran *et al*., 2004).

Alpha-amylase has been derived from several fungi, yeast, bacteria and actinomycetes; however, enzymes from fungi and bacteria sources have dominated applications in industrial sectors (Pandey *et al*., 2000). Evidences of amylase in yeast, moulds and bacteria have been reported and their properties documented (Buzzini and Martini, 2002; Oyeleke and Oduwole, 2009). For industrial applications, enzymes must be stable under process conditions. Therefore, thermophilic microorganisms are believed to be potentially good alternative sources of thermostable enzymes (Egas *et al*., 1998).

However, the cost of producing this enzyme is high and the cost of procurement by developing countries can be even higher as a result of importation. Cheap and readily available agricultural waste such as potato peels, which presently constitutes a menace to solid waste management, may be a rich source of amylolytic bacteria (Ali et al., 1998). Alpha-amylase can be produced by different species of microorganisms, but for commercial applications α-amylase is mainly derived from the genus Bacillus. Alpha amylases produced from *Bacillus licheniformis, Bacillus stearothermophilus,* and *Bacillus amyloliquefaciens* find potential application in a number of industrial processes such as in food, fermentation, textiles and paper industries (Konsoula and Liakopoulou-Kyriakides, 2007; Pandey *et al*., 2000). *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis,* and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications (Prakash and Jaiswal, 2009).

2. Methods

2.1. Collection of samples and isolation

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The samples were collected from four different refuse dumps along Ede road and on Obafemi Awolowo University Campus, Ile-Ife, Nigeria at a depth of 30 cm with temperature of 45°C. One gramme (1 g) of the decayed refuse material was serially diluted. One millilitre (1 ml) of the resulting dilution factors was pipetted into sterile Petri dish to which sterile nutrient agar was dispensed. The Petri dishes were incubated invertedly at 45ºC for 24 h and examined for colony growth. Discrete colonies were picked and purified on sterile nutrient agar plates using the streak method. The pure colonies were sub-cultured into sterile agar slants and kept in the refrigerator at 4°C at the Department of Microbiology laboratory, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

2.2. Identification of isolates

Bacteria isolates were identified using the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Bacterial isolates were characterized by colonial, morphological and physiological characteristics through biochemical tests. This was followed by 16S rRNA gene sequencing of the bacterium with the highest enzyme activity.

2.3. 16S rRNA gene sequencing

Extraction of DNA was done using CTAB method, the 16S rRNA gene was amplified by PCR using universal primer for bacteria: 16S forward, 5'-GAGTTTGATATGTACTGGCTCAG, reverse, 5'-GAAGGAGGTGACCTCCACTGCC. The amplified 16S rRNA gene PCR products (gene fragment of 1000 bp length) from this isolate, after purification by 2 M Sodium acetate wash techniques was directly sequenced on the Gene Sequencer (ABI machine) – Macrogen USA. The 16S rRNA gene fragment (1000 bp length) sequenced in both direction to obtain gene sequence in the form of A, C, T and G was then blasted on http://ncbi.nlm.nih.gov to assess the DNA similarities.

2.4. Methods for extraction of the raw starches

Sorghum, yam, cassava, corn starches were extracted by using the method of Singh *et al*. (2009); Walter *et al*. (2000); Gunorubon (2012); Moorthy (1991) respectively; and production of cassava flour was carried out by established protocol.

2.5. Alpha amylase production and extraction

The enzyme production was carried out in 250 ml Erlenmeyer flask containing 100 ml medium using 1 ml 0.5 McFarland standard inoculum. The modified medium of Femi-Ola and Olowe (2011) was made up of 1 g soluble starch, 0.1 g KH_2PO_4, 0.25 g Na_2HPO_4, 0.1 g NaCl, 0.005 g $MgSO_4.7H_2O$, 0.005 g, $CaCl_2$, 0.2 g $(NH4)_2SO_4$ and 0.2 g peptone; at pH 7.0. The medium was inoculated with the standard inoculum and incubated at 45°C for 48 h with a steady agitation at 150 rpm. This was centrifuged at 6000 rpm for 30 min to obtain the cell-free supernatant (CFS), the enzyme activity was determined by Nelson (1944) and Somogyi (1945) methods; and the protein concentration was quantified by Bradford method (1976) using 10 mM phosphate buffer at pH 7.

2.6. Optimization of alpha amylase production

The optimum pH (4.5 - 8.5), temperature (35°C – 60°C), percentage soluble starch composition (0.5 – 2.5%), different carbon and nitrogen sources for the production of α-amylase was determined with 0.5 ml inocula size in 50 ml of the basal medium and agitation at 150 rpm in an incubator shaker. The cell-free supernatant obtained was assayed for α-amylase activity.

2.7. Growth and enzyme production

The growth curve and enzyme production for *Bacillus* sp. RD24 was determined by inoculating a 250 ml enzyme production medium with 10 ml standard inoculum of 0.5 McFarland standards in an Erlenmeyer flask. This was incubated at 45°C for 48 h with agitation at 150 rpm. At 2 h interval, 5 ml samples were collected aseptically for a period of 48 h. The turbidity of the culture was checked at 680 nm using Spectrumlab 23A spectrophotometer and recorded as the cell optical density. The enzyme activity of each sample supernatant was assayed using the method stated above.

2.8. Effect of Some Raw Starchy Sources on Enzyme Production

Some raw starch sources namely yam, millet, cassava flour, corn starch and cassava starch were used to replace the soluble starch in the enzyme production media (50 ml) while other media components were kept constant. These were inoculated with 0.5 ml of the standardized inoculum of *Bacillus* sp. RD24 and incubated at 45°C for 48 h with 150 rpm agitation. The cell-free supernatant obtained was assayed for α-amylase activity and protein concentration as stated above.

3. Results

3.1. Thermophilic amylolytic bacteria

The isolated and identified bacteria were *Bacillus alvei* (40%) *Bacillus licheniformis* (40%) and *Bacillus brevis* (20%) as presented in Table 1. The result of the blasting of *Bacillus licheniformis* RD24 gene sequence revealed 91% maximum identity.

3.2. Growth and Alpha Amylase Production

Amylase production was evident in the culture supernatant at about 8 h of incubation with 17.43 Units/ml α-amylase activity, the lag phase for a period of 4 h was observed. The peak of amylase production was at 20 h of incubation which is 925.19 Units/ml (Fig. 1). Though α-amylase activity reduced after a period of 48 h, it did not result in total loss of activity.

3.3. Optimum pH and temperature for alpha amylase production by *Bacillus licheniformis* RD24

The optimum pH and temperature for the production of alpha amylase in the culture condition are 7.0 and 45°C with 150 ± 0.8 Units/ml and 58.1 ± 2.4 Units/ml respectively as presented in Fig. 2 and 3.

3.4. Effect of percentage starch composition, nitrogen, carbon sources and raw starches on the production of alpha amylase by *Bacillus licheniformis* RD24

The highest amylase activity was recorded with 1.5% starch as 102.3 ± 7.5 Units/ml (Fig. 4). The most suitable nitrogen source for the production of α-amylase was discovered to be peptone with enzyme activity of 165 ± 12.7 Units/ml followed by yeast extracts, $(NH_4)_2SO_4$ and calcium nitrate with 52.3 ± 4 Units/ml, 41.2 ± 0.01 Units/ml and 32.21 ± 4.99 Units/ml respectively (Fig. 5).

The production of α-amylase was discovered to be highest (32.9 ± 7 Units/ml) with starch as the sole carbon source in relation to maltose, glucose, melibiose, lactose and maltose (Fig. 6).

The use of raw starch such as cassava flour, cassava, yam, millet and corn starches respectively as a carbon source for the production of α-amylase gave an appreciable specific activity. Cassava flour gave the highest specific activity of 72.12 ± 0.09 Units/ mg protein followed by cassava starch 22.83 ± 1.30 Units/mg protein as shown in Fig. 7.

Table 1: Gram's Staining, Spore Staining and Biochemical Characteristics of the Isolates

Isolate Code	RD 13	RD 24	RD 18	RD 22	RD 14
Gram Reaction	+	+	+	+	+
Shape	Rod	Rod	Rod	Rod	Rod
Catalase	+	+	-	+	+
Starch hydrolysis	+	+	+	+	+
Citrate	-	-	+	+	-
Spore staining	+	+	+	+	+
Melibiose	A	-	-	-	A
Glucose	A	-	A	-	A
Mannitol	-	-	A	-	-
Rhamnose	-	-	-	-	-
Galactose	-	-	-	-	+
Xylose	-	-	-	-	A
Lactose	-	-	-	-	+
Arabinose	-	A	NT	NT	A
Methyl Red	+	-	-	-	-
VogesProskauer	+	+	-	+	+
6.5 % NaCl	+	+	+	+	+
NO_3 Reduction	+ gas	+ gas	+ gas	+ gas	+ gas
O/F	OX	F	F	F/OX	OX
H_2S Production	-	+	-	-	-
Indole test	-	-	-	-	-
Motility test	-	+	+	+	-
Growth @ 55 ºC	-	-	+	+	-
Urease test	-	+	+	+	-
Gelatin hydrolysis	+	+	+	+	+
Probable bacterium	*Bacillus alvei*	*Bacillus licheniformis*	*Bacillus brevis*	*Bacillus licheniformis*	*Bacillus alvei*

Key: + = Positive, - = Negative, OX = Oxidative, F = Fermentative, NT = Not tested, NA = Not applicable, H_2S = Hydrogen sulphide, NO_3 = Nitrate, NaCl = Sodium chloride.

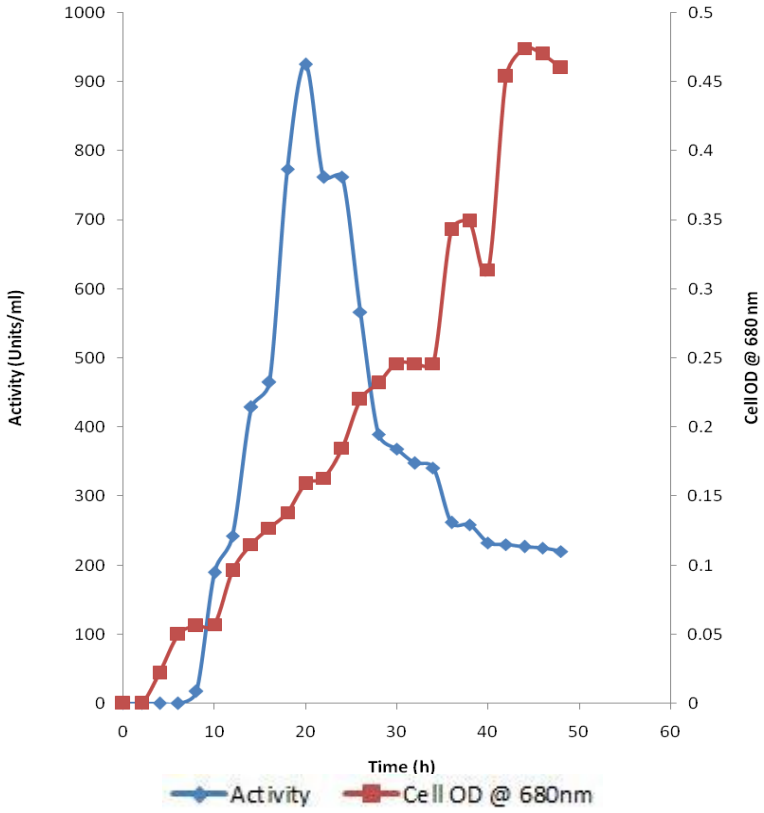


Fig. 1: Growth and Enzyme Production Curve at 45ºC and pH for 48 H.

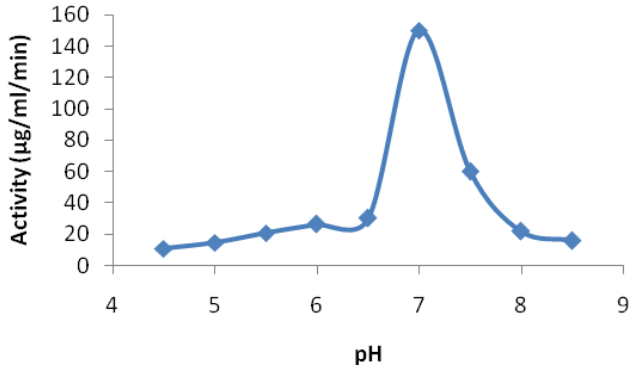


Fig. 2: Effect of PH on the Production of *Bacillus licheniformis* RD24 Crude a-Amylase

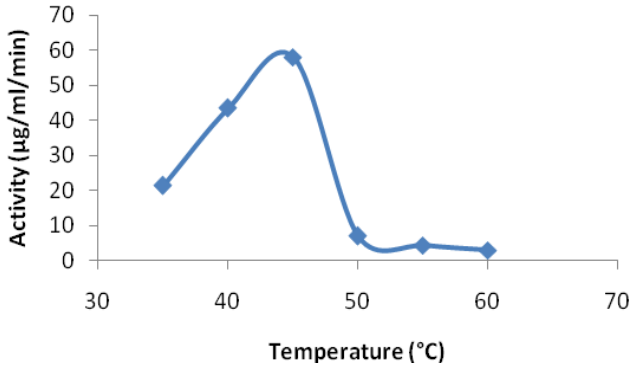


Fig. 3: Effect of Temperature on the Production of *Bacillus licheniformis* RD24 Crude a-Amylase.

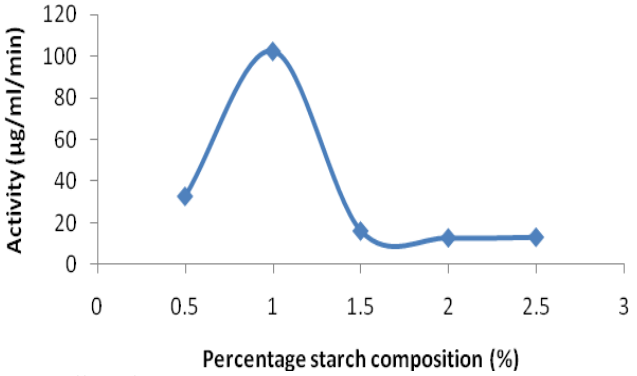


Fig. 4: Effect of Percentage (%) Starch Composition on the Production of *Bacillus licheniformis* RD24 Crude an Amylase.

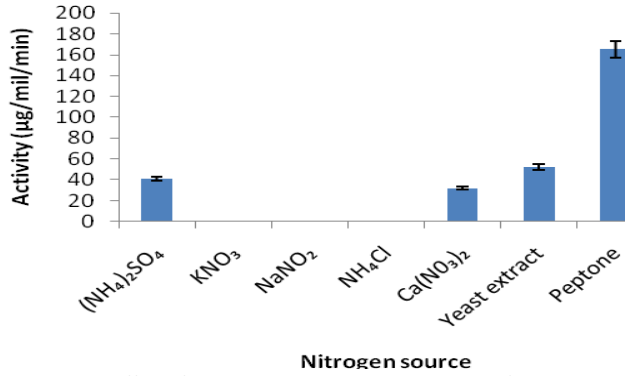


Fig. 5: Effect of Nitrogen Source on the Production of *Bacillus licheniformis* RD24 Crude a-Amylase.

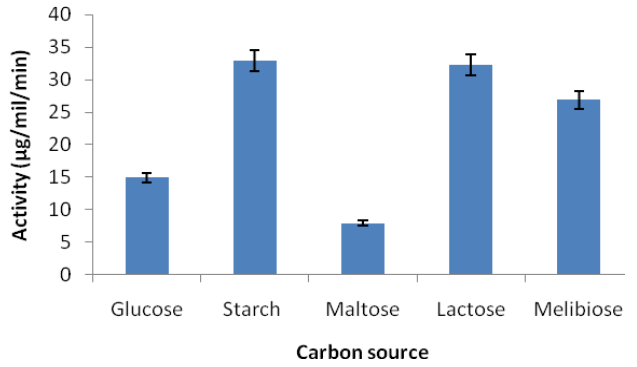


Fig. 6: Effect of Carbon Source on *Bacillus licheniformis* RD24 Crude a-Amylase Production

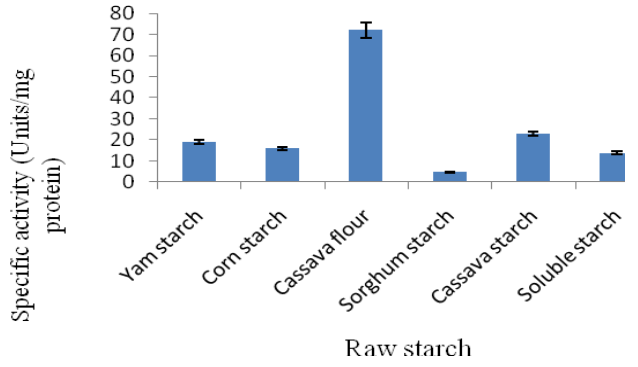


Fig. 7: Effect of Raw Starches on *Bacillus licheniformis* RD24 Crude a-Amylase Production

4. Discussion

The isolation of the thermophilic bacteria from the dumpsite in the study location with amylolytic properties is a prove of the fact that decay of food waste in such dumpsite is not just the function of chemical and physical changes at the site but also as a result of biological activities brought about by bacteria and other microorganisms. The growth and survival of the thermophilic bacteria can be said to be due to active decomposition of waste which involves release of energy, and therefore select for those that can adapt to high temperature. This is in accordance with the work of Ajayi and Fagade (2006); Adeniran and Abiose (2011) and Aynadis *et al*. (2013). The bacterium with the highest amylase activity subjected to molecular characterization by 16S rRNA gene sequencing and identified as *Bacillus licheniformis* RD24 with a maximum identity of 91 % to other *Bacillus licheniformis* is a clear indication that if biochemical characterization is improved upon, it will be very useful in identifying bacterial isolates; though still strongly dependent on molecular characterization for confirmation.

Production of α-amylase by *Bacillus* species is often dependent on growth of the bacterium in the appropriate media composition. It was observed in *Bacillus licheniformis* RD24 that peak of amylase production was at 20 h with gradual decline even when it seems the growth of the bacterium persisted (Fig. 1). The decline in amylase production may be due to exhaustion of the nutrients or accumulation of other products or metabolites which may be inhibitory to the growth of the bacterium and amylase production as noted by Prakash *et al*. (2009).

The optimum pH is in accordance with the results of some researchers who had reported pH 7 for alpha amylase production by species of Bacillus (Oyeleke *et al*., 2010; Mohammed *et al*., 2011). A wide range of temperature (35 - 80°C) has been reported for optimum growth and α-amylase production in bacteria (Burhan *et al*., 2003; Prakash *et al*., 2009). However, in this study, the optimum temperature for the production of α-amylase was observed to be 45^{o}C which confirms it as thermophilic in nature. This result agreed with the investigation of Mohammed *et al*. (2011); Aynadis *et al*. (2013) and Yasser *et al*. (2013) but is slightly better than 42 °C reported by Deb *et al*. (2013).

The findings of this study are a clear indication that though hydrolysis of starch may occur at different percentage starch composition of the medium, 1% is the most suitable. This is closely related to the findings of Mohammed *et al*. (2011) who observed maximum enzyme activity in the range of 1 to 1.5% of starch concentration and Yasser *et al*. (2013) who reported inhibition in the α-amylase activity beyond starch percentage concentration of

1.25%. The result obtained from the various nitrogen sources investigated may be due to the fact that peptone may be readily available to the bacterium for metabolism during growth and enzyme production as opposed to yeast extract. Other inorganic nitrogen sources such as KNO_3, $NaNO_3$ and NH_4Cl investigated in this study only supported the growth of the bacterium but not enzyme synthesis.

The outcome of the study on variation of carbon sources for the production of α-amylase is contrary to Dilli *et al*. (2006) who reported that *Bacillus subtilis* KCC103 secreted amylase with glucose as the major carbon source.

Of the raw starches tested as carbon sources, the high specific activity of cassava flour may be due to the presence of other metabolites in the material which can stimulate the synthesis of amylase, because cassava flour is a product of microbial transformation. Ajayi and Fagade (2006) reported that *Bacillus* species are better on corn starch than soluble starch. Similarly, Ruban *et al*. (2013) also reported that amylase production by low-grade cheap impure substrate sago waste and wheat bran produced very high amount of amylase than soluble starch by *Bacillus subtilis* and *Aspergillus niger*. These are in accordance with the report of this study as four out of the five carbon sources resulted in better specific activity than the soluble starch.

5. Conclusion

In conclusion, this study revealed that *Bacillus licheniformis* RD24 had a unique characteristic of thermostability and the ability to hydrolyze cheap raw starches for the production of α-amylase. Starch-rich household waste can therefore be converted for amylase production instead of constituting public nuisance.

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