Studies on Crude Thermostable Amylase Produced by Bacillus Spp. Isolated from Starch Waste

Oluwafemi Adedire[1], Wuraola Ogundipe[2], Adekunle Farinu[3]

Federal College of Agriculture, Apata, Ibadan, Nigeria
fecky09@yahoo.com

Abstract: ***Thermostable amylase secreting bacterium was isolated through a high temperature screening procedure. The choice isolate, identified as Bacillus spp., appeared to be a facultative thermophile. Production of crude enzyme by the isolate was better when the medium was supplemented with organic nitrogen sources than the inorganic ones. The enzyme has an optimum activity at pH of 9.0, temperature of 65 0C and retains 82% of its activity at 70oC. The crude enzyme's activity was stimulated by 2mM Ca2+ , Mg2+, Zn2+ and Cu2+ but inhibited by EDTA, Acrylamide, dodecyl-sulfate, Fe2+ and Mn2+. It also showed resistance to both oxidizing and denaturating agents.***

Keywords: Thermostable, Bacillus spp., Facultative, Amylase.

1. Introduction

Amylases are enzymes that hydrolyse starch molecules into monomers or polymers composed of glucose units [1], they cleave the glycosidic linkage in starch with an endo mechanism that is in random fashion with the polysaccharide molecule [2]**.** Depending on the type of amylase, starch is degraded into simple sugar such as glucose, maltose or to oligosaccharides, maltooligosaccharides or dextrines [3]. They are of considerable practical utility in many industrial processes where starch must be hydrolysed such as textiles, laundry, medicine, food, brewing, automatic dish washing detergents, pulp and paper industries [4]. Temperature is one of the major criteria used in the selection of an industrial enzyme. Most enzymes used in industrial applications are preferred Thermostable [5]**.** For instance, the enzymes used in animal feeds industries must be Thermo-tolerant to survive the hot extrusion process used in animal feed manufacture [6]. With the availability of thermostable enzymes, a number of new possibilities for industrial processes have emerged [7]. The most widely used thermostable enzymes are the amylases in starch industries [8].

Amylases can be produced from several sources such as plants animals and microorganisms, the major advantage of using microorganisms for the production of amylase is its economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics [9]. To date, more than 70 genera and 140 species of thermophiles have been isolated from a variety of thermal environment [10]. Extracellular enzymes are synthesized by most microorganisms, these enzymes are synthesized into the cytoplasm, they cross the cytoplasmic membrane and are liberated into the environment [11].

This present study was as such tailored towards the production of heat-stable extracellular amylase from a facultative thermohpilic bacterium isolated from soil samples containing decayed starch wastes whose decomposition was allowed to complete at a controlled temperature.

2. Materials and Method

2.1 Screening and Isolation of Thermostable Amylase Producing Bacteria

The inner layer of compost heap containing decaying samples of starch wastes (mainly cassava peels) was taken from different dump sites of the post-harvest processing units of Federal College of Agriculture, Oyo State, Ibadan. Decomposition was allowed to continue within the soil sample for 6 days in a giant oven under controlled temperature of 55^{o}C; this was done to allow the selection of organisms with the potential of producing extracellular heat stable amylase because the temperature at which an enzyme retains full activity relates to the source organism's optimum temperature for growth [12].

Isolation of extracellular amylase producing bacteria was done by spreading 0.5 mL of each of the serially diluted soil samples from the decomposed preparation over a basal medium (6 g/L Bacteriological peptone, 0.5 g/L Mg SO_4. $7H_2O$, 0.5g/L KCl, 1.5g/L Soluble Starch and 15g/L Agar), this agar plate contained soluble starch as the only source of carbon for bacterial growth and the inoculated plates were incubated at 60 °C for 48 hours, the most abundant growing colony was subcultured, then transferred to declined agar slopes containing the same medium [13] and used as the experimental isolate.
Pure culture of the choice strain was grown at temperatures of 20, 30, 40, 50, 60 and 70 to determine its optimum growth temperature; and Amylase test was done on the isolate by pouring Lugol solution (0.15 g iodine and 1.5 g potassium iodide in 100 mL distilled H2O) over the agar plates; a distinct clear zone exceeding the colony diameter by 3-fold or greater was considered a good starch hydrolysis activity through the extracellular production of amylase.

2.2 Production and Recovery of Crude Enzyme

Amylase production was carried out in submerged fermentation. For maximum extracellular enzyme production, the broth culture of Bacillus isolate was grown

at 60°C on a rotary shaker at 150 rpm for 2days (48hrs) before harvest as illustrated by [2].

The bacterial culture was then centrifuged at 10,000 rpm for 15 minutes to remove the cells and the resulting supernatant was carefully decanted and used as crude enzyme for the evaluation of crude amylase activity.

2.3 Enzyme Assay

The reaction mixture contained 0.5mL of 1% (w/v) soluble starch in 100mM Na-phosphate buffer pH 7 as substrate and 0.5mL of the crude enzyme and the mixture was incubated at 50^0C for 10 minutes. Enzyme assay was performed according to the method described by [14]; where the enzyme activity was determined by analyzing the amount of reducing sugar released from starch by the crude amylase under each experimental condition through the addition of Dinitrosalicylic acid reagent to the reaction solution at the end of incubation time and the absorbance of the resulting solution was read using a spectronic 20 UV Spectrophotometer; one unit of amylase (U) was defined as the quantity of enzyme extract releasing 1µmole of reducing sugar from soluble starch in a minute. All experiments were done in replicates and the results presented as mean standard deviation were averages of duplicate determinations while controls contain solutions of heat killed enzyme.

2.4 Optimization of Enzyme Production

2.4.1 Effect of Organic and Inorganic Nitrogen Sources on the Production of Crude Amylase

The bacteria spp. was cultivated for 24 hours in a basal medium supplemented with 12 g/L each of both organic (Peptone, Tryptone, Beef extract, Yeast extract) and inorganic (Ammonium sulphate, Potassium nitrate, Sodium nitrate) nitrogen sources. [15]. To determine the enzyme production as influenced by each of these nitrogen sources, the crude enzyme was thereafter recovered and assayed as earlier described.

2.5 Characterization of Enzyme

2.5.1 Effect of Temperature on Amylase Activity and Stability

To determine the effect of temperature on the enzyme and as such identify the optimum temperature for its activity, crude amylase was assayed at different temperatures ranging from 40 to 90°C. The reaction mixture contained 0.5mL of 1% (w/v) soluble starch in 100mM Na-phosphate buffer pH 7 as substrate and 0.5mL of the crude enzyme and the mixture was incubated at different test temperatures of 40, 45, 50, 55, 60, 65, 70 and 75°C for 10minutes after which, the simple sugar released from soluble starch at each temperature was assayed. Also, the enzyme's stability was determined at temperatures of 50, 60 and 70°C respectively; to determine its thermostability, the crude enzyme was pre-incubated at the test temperatures for 30 minutes and the residual activity retained by the enzyme was analyzed at these temperatures as earlier described.

2.5.2 Effect of pH on Enzyme Activity

The amylase enzyme was incubated at different pH 4.0 to 11.0 for determination of optimum pH. The pH was maintained in the reaction mixture by preparing soluble starch in buffers; 100 mM Na-phosphate buffer (pH 4 to 8), 100 mM Gylcine - NaOH buffer (pH 9 to 10) and 100 mM Borax- NaOH buffer (pH 11) and the activity of the enzyme after incubation was assayed.

2.5.3 Effect of Substrate Concentration on Crude Amylase Activity

Soluble starch substrates in 100mM Na-phosphate buffer pH 7 was taken at concentrations 1, 2, 3, 4 and 5% (w/v) to evaluate the effect of substrate concentration on this crude enzyme. Starch substrate of desired concentration was used as enzyme's substrate and after the incubation period, amylase assay was performed.

2.5.4 Effect of some Cations and other Chemical Agents on Enzyme Activity

The effect of divalent cations (Ca^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+} and Cu^{2+}) on the crude amylase was analyzed by pre-incubating the enzyme with $CaCl.2H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $MgSO_4.6\ H_2O$, $ZnSO_4.7H_2O$ and $CuSO_4$ respectively before analyzing the enzyme's residual activity.

The chemical agents used were acrylamide, ethylene diamine tetraacetic acid (EDTA) and dodecyl sulfate (SDS). The crude amylase was pre-incubated at optimum conditions (Temperature of 65^0C and pH 8) with each of these chemicals and salts at 2mM (0.2% v/v) for 10minutes and the residual activity was thereafter assayed as earlier illustrated.

2.5.5 Effect of Oxidizing and Denaturing Agents on Crude Amylase Activity

Oxidizing and denaturing agents used were Hydrogen peroxide (H_2O_2) and Urea respectively. Urea, a well-known protein denaturant, is frequently used as a model to study enzyme stability [16], [17].the crude enzyme was pre-incubated with each of these agents at 2mM concentration each for 10minutes and the relative enzyme activity was thereafter determined and compared to its activity in absence of these agents.

3. Result and Discussion

Scanty growth was observed in most of the inoculated plates at the screening temperature of 60°C but the best growth at this incubation temperature was observed to be the inoculum traceable to the soil sample of cassava peel compost taken at the garri processing unit of animal science college and this was made the choice isolate. Morphological, Physiological and Biochemical tests performed on the isolate resulted in its identification on the basis of Bergey's manual of Systematic Bacteriology [18] as Bacillus spp. The organism showed significant growth at the temperature range of 50 to 60°C, though

optimum growth was observed at the temperature range of 40 to 60°, the isolate is most probably a facultative thermophile capable of growing at both the thermophilic and mesophilic temperature ranges [19].

Production of crude enzyme by the isolate was better when the medium was supplemented with organic nitrogen sources than the inorganic ones (Table 1). Maximum production was obtained when the medium was supplemented with 12g/L Peptone (117.86 U/mL), [20] reported that the addition of peptone shortens the lag period of growth and improves the synthesis of α-amylase.

Table 1. The effect of organic and inorganic nitrogen sources on the production of crude amylase

Nitrogen sources	*Amylase Activity (U/mL)*
Peptone	117.86 ± 3.60
Tryptone	110.79 ± 3.11
Beef Extract	104.68 ± 1.15
Yeast Extract	110.35 ± 3.28
Ammonium Sulphite	100.58 ± 2.17
Potassium Nitrate	107.42 ± 2.11
Sodium Nitrate	99.95 ± 1.21

Table 2 shows the effect of temperature on the production of reducing sugar from starch by the crude enzyme. Activity of the crude amylase was high at the temperature range of 50 to 70°C with optimum reducing sugar production at 65°C (95.3 U/mL). Thermostability is a critical characteristic for the industrial uses of α –amylases [7]. Most industrial processes involving amylases are operated at elevated temperatures exceeding 50 °C because of the higher reaction rates at these temperatures and a decreased risk of bacterial contamination of products [21], [7]. The enzyme retained 95, 88 and 82% of its activity after incubation at 50, 60 and 70°C for 30 minutes respectively.

Table 2. The effect of temperature on crude amylase activity

Temperature (°C)	*Amylase activity (U/mL)*
40	56.2 ± 1.41
45	80.0 ± 1.13
50	87.2 ± 0.42
55	92.4 ± 0.28
60	94.5 ± 0.13
65	95.3 ± 1.41
70	93.1 ± 2.16
75	83.2 ± 1.86

Table 3, illustrates that the enzyme was highly active at alkaline pH of 7.0 to 9.0, with optimum pH 9.0 (98.4 U/mL), indicating a broad pH range for enzyme activity; amylases with high activities at alkaline pH values are of potential use as components of detergents for automatic dishwashers and laundry machines [22]. [23] reported that the recombinant alkaline α- amylase from Bacillus alcalophilus and Bacillus subtilis were stable at pH range of 7.0 to 11.0. Several α-amylases have been reported to have optimum activities at pH values of 9.0 [24], 10.0 [25] and 11.0 [26].

Table 3. The effect of pH on crude enzyme activity

pH	*Amylase activity (U/mL)*
4	66.9 ± 1.67
5	66.3 ± 3.21
6	90.8 ± 3.11
7	96.1 ± 2.31
8	96.2 ± 1.89
9	98.4 ± 3.22
10	80.2 ± 1.19
11	78.5 ± 2.30

When the effect of various substrate concentrations on this crude amylase was studied, it was observed that enzyme activity increased with increasing concentration of substrate to 1.5% (97.9 U/mL, Table 4), after which there was a decline in its activity. Maximum activity at relatively low substrate concentration is an admired characteristic in industrial enzymes as specified by [6], that industrial enzymes should already be maximally active in the presence of low substrate concentration so that the desired reaction proceeds to completion in a realistic timeframe.

Table 4. The effect of substrate concentration on crude amylase activity

Substrate concentration (%)	*Amylase activity (U/mL)*
0.5	50.2 ± 1.38
1.0	90.3 ± 2.42
1.5	97.9 ± 1.87
2.0	86.2 ± 3.21
2.5	73.4 ± 2.33
3.0	73.1 ± 3.28
3.5	63.6 ± 1.18

When the effects of chemical agents viz EDTA, acrylamide and Sodium dodecyl sulfate (SDS) were studied on crude amylase, the enzyme activity showed a dramatic decrease to 62% with Sodium dodecyl sulfate (SDS), 70% with acrylamide and 67% with EDTA (Table 5) while Table 6 shows that the catalytic function of the crude enzyme was stimulated by Mg (120%), Ca^{2+}(119%), Zn^{2+}(118%) and Cu^{2+} (112%), Inhibition of the amylase enzyme by chemicals like EDTA and metal ions like Mn^{2+} (81%) and Fe^{2+} (85%) suggests that it is a metalloenzyme which could be inactivated by heavy metals causing irreversible inhibition of enzyme by binding strongly to their amino acid backbone [26]. Most known Ca^{2+}-sensitive α –amylases are very sensitive to EDTA [16], [27].

Table 5. Effect of chemical agents on crude amylase activity

Chemical Agent (0.2% v/v)	*% Relative activity*
EDTA	67 ± 5
Acrylamide	70 ± 1
SDS	62 ± 3
Urea	98 ± 2
H_2O_2	100 ± 5

Reference was made with Crude Amylase Activity in the absence of Chemical agent
100 % Crude Amylase Activity: 97.3 U/mL

Table 6. The effect of different metal ions on crude Amylase Activity

Metal Salts (2mM)	*% Relative Activity*
$MgSO_4.6\ H_2O$	120 ± 6
$ZnSO_4.7H_2O$	118 ± 3
$MnSO_4.H_2O$	81 ± 1
$CuSO_4$	112 ± 3
$CaCl.2H_2O$	119 ± 4
$FeSO_4.7H_2O$	85 ± 2

Reference was made with Crude Amylase Activity in the absence of Metal Salt
100% Crude Amylase Activity: 97.3 U/mL

The activity of the crude enzyme was tested in presence of an oxidant and a denaturant; it was observed that the amylolytic activity of this enzyme was unaffected by the presence of the oxidant (100%) and very slightly affected by Urea (98%) as represented in Table 5. The enzyme's resistance to these agents is also an admired characteristic in industrial enzymes as [28] reported that for applications in detergent industries, amylases must be stable in the presence of various aggressive detergent ingredients, such as chelators and oxidants.

From the derived results of this study, it could be concluded that Bacillus spp. with thermostable extracellular amylase was successfully isolated through a high temperature screening procedure. Production of Crude amylase from this organism was better influenced by organic than inorganic nitrogen sources. Moreover, the crude amylase was temperature stable, retaining most of its activity at 60 and 70^{o}C while it also showed resistance to both oxidizing (H_2O_2) and denaturizing (Urea) agents. Studies should though be made on how to further optimize heat-stable amylase production from Bacillus spp. that can be utilized at specific controlled conditions and as such making the enzyme and its catalytic products readily available for several high temperature industrial productions.

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Author Profile

Oluwafemi Michael Adedire received a B.Sc. degree in Microbiology from Obafemi Awolowo University, Ile-Ife in 2008; worked in the quality Control department at the baby care Section of Procter and Gamble, Ibadan between 2010 and 2011. He is currently a Lecturer at the Federal College of Agriculture, Ibadan with an M.Sc. in view from the University of Ibadan.