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Amylase production potentials of bacterial isolates obtained from the gut of *Oryctes rhinoceros* larvae

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Abstract. Amylase is one of the main enzymes used in industry, such as food, detergent, textile, and pharmaceutical industry. Amylase can be produced by plants, animals, and microorganisms. However, bacterial and fungal amylases have dominated application in industries. This research was aimed to determine amylolytic activity of bacteria isolated from the gut of *Oryctes rhinoceros* larvae. Based on clear zone formation, 9 from 11 isolates showed amylolytic activity. Isolates with the widest clear zone, i.e *Bacillus subtilis* GOR1, *Bacillus cereus* GOR3, and *Bacillus pumilus* GOR2, were screened for amylolytic activity based on reduction sugar production. The result showed that *Bacillus subtilis* GOR1 was the most potential as amylase producer, showed by the widest clear zone 5.224 cm^2 and highest reduction sugar production 0.0235 mg/ml. Highest amylase specific activity (0.1447 U/mg protein) was obtained at 60^{o}C and pH 7. Amylase activity was stable for 3 hours at 60^{o}C with residual activity respectively was 59.7%.

1. Introduction

Enzyme is protein catalyst or biocatalisator that specific against chemical reactions and importantly used in industries [1]. Amylase is one of main enzyme in industry, such as food, detergent, textile, and pharmaceutical industry [2]. Amylase can be obtained by plants, animals, and also microorganisms. But, bacterial and fungal amylases have dominated application for industries. Microbial amylase is being economical, easy to manipulate, more controllable, and relatively more stable than the enzymes derived from plants and animals [3].

Amylase is enzyme which degrade starch into glucose through a hydrolysis reaction [4]. Amylase can be divided into several types: α-amylase, β-amylase, γ-amylase, pullulanase, α-glucosidase and cyclodextrin enzyme producer [5]. Endoamylase is a well-known α-amylase which able to cleave α,1-4 glycosidic bonds present in the inner part of the amylose or amylopectin chain. Exoamylase is either exclusively cleave α,1-4 glycosidic bonds such as β-amylase or cleave both α,1-4 and α,1-6 glycosidic bonds like amyloglucosidase or glucoamylase and α-glucosidase [6].

Microbial amylases are most preferred in industry. The gut of *O. rhinoceros* larvae is potential source of amylolytic bacteria producing amylase. The industrial application of enzymes often requires to be conducted at higher temperature for enzyme reaction, because under those conditions productivity improved with less contamination. Therefore, in the present study was mainly focused to determine amylolytic activity of bacteria isolated from the gut of *O. rhinoceros* larvae and to determine the optimum conditions, such as temperature, pH and stability for amylase activity.

2. Materials and Methods

Bacterial Strain
The isolates that were used in this study were obtained from the gut of *O. rhinoceros* larvae and had been identified based on 16S rRNA protein squence. Based on the 16S rRNA sequence, 10 isolates were classified into the *Bacillus* and 1 isolate was classified into *Citrobacter* [7].

Medium
Screening for the amylolytic activity used starch agar medium which composed of 0.2 g $NaNO_3$, 0.05 g $MgSO_4$, 0.005 g K_2HPO_4, 0.001 g $FeSO_4$, 0.002 g $CaCl_2$, 0.0002 g $MnSO_4$ and was added 0.05 g yeast extract, 2% agar and 1% *soluble starch.*
Amylase production used medium contained starch which as same as screening medium, without adding agar.

Screening for the amylolytic activity
Screening for the amylolytic activity was done based on clear zone formation on starch agar plate and reduction sugar production. Eleven isolates were grown on starch agar plates and were incubated at 37^{o}C for 48 hours. After incubation, the plates were flooded with Iodine (0.01 M I_2-KI solution) and observed for clear zone formation. 3 isolates with highest clear zone formation were screened based on reduction sugar production.

Amylase Production
Inoculum was produced by inoculating 10 ml *Luria Bertani* (LB) medium (containing 1% *soluble starch*) with 1 ml bacterial isolate and incubated at 30^{o}C, pH 7, 150 rpm for 24 hours. 10 ml inoculum inoculated in 100 ml production medium and incubated in the same condition.

Enzyme Extraction
To obtain crude enzyme, 24-hours old cultures were transferred to centrifuges tubes and centrifuged at 6000 rpm and 5^{o}C for 20 min. The resultant supernatant was used as crude enzyme extract.

Enzyme Assay
Amylase assay was carried out using a mixture reaction consisting of 1 ml substrate (1% *soluble starch* in 0.05 M phosphate buffer pH 7) and 1 ml crude enzyme extract. The mixture was determined with *dinitrosalicylic acid* (DNS) method. Total protein content was estimated by Bradford method using *Bovine Serum Albumin* (BSA) as the standard.

Characterization of Enzyme
2.1.1. Effect of Temperature.
To study the effect of temperature on amylase activity, 1 ml crude enzyme extract with 1 ml substrate (pH 7) was incubated at various temperature (30^o, 40^o, 50^o, 60^o, and 70^{o}C) for 30 min.

2.1.2. Effect of pH.
To study the effect of pH on amylase activity, 1 ml crude enzyme extract and 1 ml substrate (with different pH 4, 5, 6, 7, and 8) at optimum temperature for 30 min.

Stability Enzyme
Crude enzymes extract were incubated for 1, 2, 3, 4, and 5 hours at various temperature 30^o, 40^o, 50^o, and 60^{o}C. Then, analyzed for amylase activity at optimum conditions.

3. Results and Discussion

Amylase Producer

The bacteria isolated from the gut of *O. rhinoceros* larvae were screened based on clear zone formation. It was found that 9 strains showed amylase activity (table 1). Then, 3 strains with the widest clear zone formation were screened based on reduction sugar production (table 2).

Table 1. *Amylolytic activity based on clear zone formation.*

Bacteria	Clear Zone Formation (cm^2)
Bacillus subtilis GOR1	5.224*
Bacillus pumilus GOR2	4.229
Bacillus cereus GOR3	4.811
Bacillus megaterium GOR4	0.535
Bacillus thuringiensis GOR5	0.673
Bacillus aquimaris GOR6	0.239
Bacillus aryabhattai GOR7	0
Bacillus cereus GOR8	0.420
Citrobacter koseri GOR9	0
Bacillus clausii GOR10	0.946
Bacillus thuringiensis GOR11	0.808

*showed the highest amylolytic activity

Table 2. *Amylolytic activity based on reduction sugar production.*

Bacteria	Reduction Sugar Production (mg/ml)
Bacillus subtilis GOR1	0.0235
Bacillus cereus GOR3	0.0220
Bacillus pumilus GOR2	0.0213

According the tests, it was confirmed that *Bacillus subtilis* GOR1 was the most potential bacteria as amylase producer, showed by the widest clear zone 5.224 cm^2 and highest reduction sugar production 0.0235 mg/ml. Femi-Ola and Babalola [8] were reported that *Bacillus subtilis* isolated from the gut had ability to produce amylase.

Characteristics of Amylase

3.1.1. Effect of Temperature.

The highest specific activity of amylase was at 60ºC (0.1148 U/mg protein) (Figure 1). Specific activity increased from 30ºC and peaked at 60ºC. Above those temperature, the activity would

decrease. It proved that amylase had reached critical temperature, where enzymes were denaturated due to higher temperature. Sani *et al*. [9] proved that amylase from *Bacillus subtilis* had the highest specific activity at 60ºC.

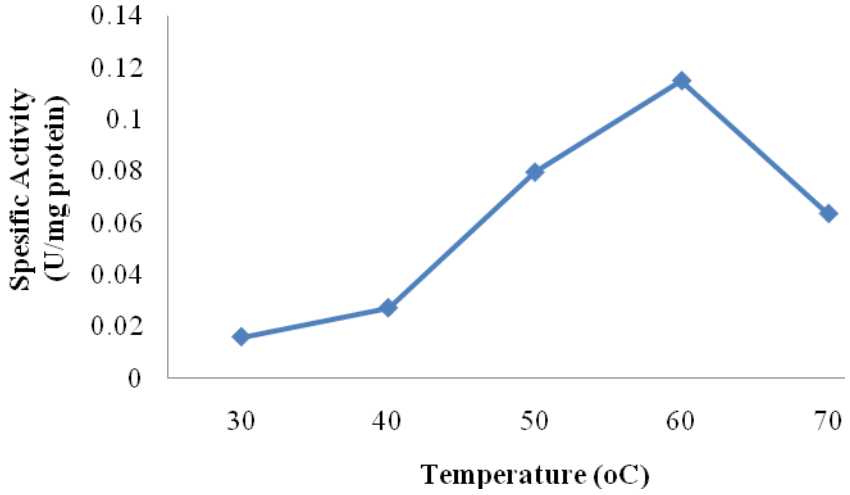

Figure 1. Effect of temperature on amylase activity *Bacillus subtilis* GOR1 at pH 7 and incubation for 30 min.

3.1.2. Effect of pH.

The effect of pH showed that the highest activity of amylase *Bacillus subtilis* GOR1 was at pH 7 (0.1447 U/mg protein) (Figure 2). The activity increased from pH 4 and peaked at pH 7. Enzymes are protein that has tertier structure and sensitive against pH condition [10]. Harris and Angal [11] reported that enzyme is very active at neutral pH. Similar results have been reported that amylase from *Bacillus subtilis* had the highest activity at pH 7 [12-13].

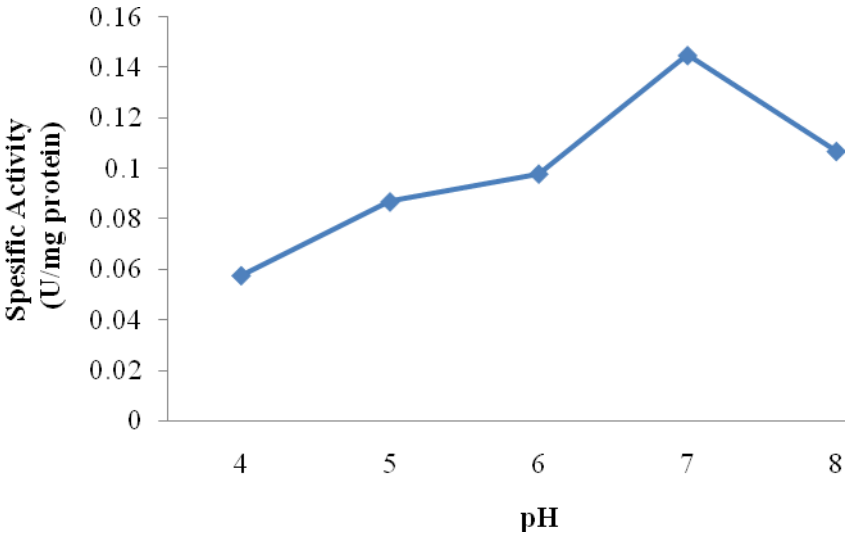

Figure 2. Effect of pH on amylase activity *Bacillus subtilis* GOR1 at optimum temperature (60ºC) and incubation for 30 min.

3.1.3. Stability of Amylase.

Stability of enzymes influence by several factors, including pH and temperature. The result showed that amylase was the most stable until 3 hours incubation at 60°C with residual activity respectively was 59.7% (Figure 3). Enzyme is stable if the residual activity was more than 50% [14].

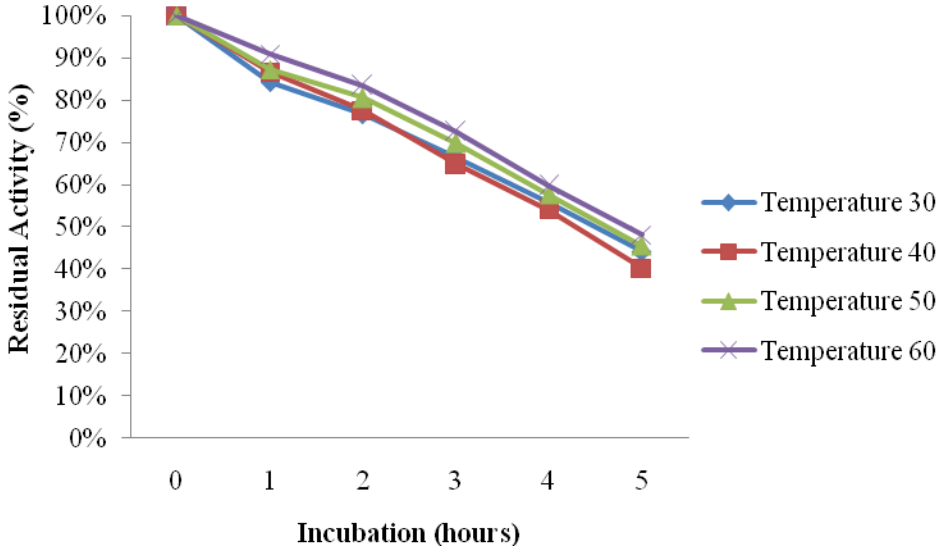

Figure 3. Effect of various temperature on stability of *Bacillus subtilis* GOR1 amylase. Activity was measured at optimum conditions (60°C, pH 7).

The results reported that the characteristics of amylase *Bacillus subtilis* GOR1 had optimum conditions at 60°C, pH 7 and most stable for 3 hours incubation at 60°C. The enzyme can be classified into thermostable enzyme that commonly used in industries.

4. Conclusion

The present study has revealed that *Bacillus subtilis* GOR1 was the most potential isolate as amylase producer, showed by the widest clear zone 5.224 cm^2 and highest reduction sugar production 0.0235 mg/ml. Highest amylase specific activity 0.1447 U/mg protein was obtained at 60°C and pH 7. Amylase activity was stable for 3 hours at 60°C with residual activity respectively was 59.7 %.

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