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Screening on the Amylase Producing Activity of some Bacterial Strains Isolated from Hot Spring

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Abstract: ***Twelve bacterial strains were isolated from soil samples of hot springs. Among 12 bacterial isolates, 3 isolated bacterial strains showed the amylolytic activity. Cultural and morphological characteristics of the selected bacterial were studied. The quantitative determination of reducing sugar formation was carried out by using dinitrosalicylic acid (DNS) method in which starch was used as an inducer substrate for amylase enzyme. According to reducing sugar formation, T1, T2 and T3 showed the reducing sugar formation with the values of 2.986 mg/ml, 4.77 mg/ml and 2.805 mg/ml, respectively. The optimal incubation period for reducing sugar formation was found at 48 hours for T1 and at 36 hrs for T2 and T3. The optimum incubation temperature for T2 was at 50 ºC whereas at 60 ºC was observed for T1 and T3. The optimum condition of pH 6.5 and PH 7 was observed in T1and T2, T3. The growth curve of effective bacteria T3 strain was studied. The growth rate of these bacteria reached maximum value (7.0x108cfu/ml) at 60ºC and 36hrs of incubation period.***

Keywords: thermostable, dinitrosalicylic acid, amylolytic activity, quantitative, thermophilic bacteria

1. Introduction

Starch is the substrate of the amylase and the most abundant sort of polysaccharides which are stored in plants. It constitutes a cheap source to produce syrups which contain glucose, fructose or maltose which are widely utilized in food industries [1]. In starch processing industry, chemical hydrolysis of starch has almost been completely replaced by amylases which have approximately 25% of the enzyme market [2].

Thermostable α-amylases possess mass commercial applications in sugar production, starch processing, and brewing [3]. Thermophilic processes, which are more stable, rapid and less expensive can, facilitate the reactant activity and product recovery. Thermostable α-amylases are commercially produced by using the genus Bacillus.

Nowadays, amylases (alpha- amylases, beta- amylases and glucoamylases) represent one of the most important enzyme groups within the field of biotechnology. alpha- amylases (EC 3.2.1.1, 1,4-alpha-D-glucan glucano-hydrolase, endo-amylase) is a classical calcium containing enzyme catalyse hydrolysis of starch and related carbohydrates by randomly cleaving internal alpha-D-(1-4) glycosidic linkage, yielding glucose, maltose, maltotriose, and other oligosaccharides. [4].

Among the most important enzymes amylases are used in biotechnology, particularly in process involving starch hydrolysis. Though amylases originate from different sources (plants, animals and microorganisms), the microbial amylases are the most produced and used in industry, due to their productivity and thermostability [5]. Thermostable enzymes from micro-organisms have found a number of commercial applications because of their overall inherent stability [6]. The most widely used thermostable enzymes are the amylases in the starch industry [7, 8, and 9]. Scientists have thoroughly investigated the amylase production by fermentation. It was found that the process can be affected by a variety of physiochemical factors. The most common factors are pH, temperature, carbon and nitrogen sources and the media composition. In this research, amylase production was optimized by growing isolated bacteria from hot spring soil sample on nutrient media supplemented with different carbon and nitrogen sources and growth conditions.

2. Material and Methods

2.1 Samples Collection

Soil samples were collected from two different sites of hot springs located in Taung Lay Lone and Yae Nwaye village, southern Shan state, Myanmar. All of the samples were taken from (4-5cm) depth of soil. The samples were transferred to the laboratory for isolation of amylase producing bacteria..

2.2 Isolation

Isolation of the microorganisms was performed by soil dilution plate technique. The nutrient medium supplemented with soluble starch (1%) was used for bacteria isolation. The bacterial isolates were further sub-cultured to obtain pure culture. Pure isolates on starch agar slants were maintained at 4 ºC (for further study).

2.3 Screening of Amylase Producing Activities

Bacterial isolates were screened for amylase production by starch hydrolysis test on starch agar plates with starch as the only carbon sources. The microbial isolates were streaked on the starch agar plate and incubated at 37 ºC for 48 hours. After incubation, 1% iodine solution (freshly prepared) was flooded on the starch agar plate. Presence of blue color around the bacterial growth on starch agar plate indicated negative result and clear zones around the growth indicated hydrolysis of starch (positive result) and were considered as amylase producers and were used for further analysis.[10, 11]

2.4 Characterization of Amylase Producing Bacteria

The cultural characteristics, microscopic morphology and biochemical characteristics of selected strains were studied in this research.

2.5 Production of Amylase

Each selected bacterial strain was inoculated in each flask containing 100ml of complex medium broth (1.0 % starch, 0.04 % yeast extract, 0.4% (NH4)2 HPO4, 0.1% KCl and 0.05% MgSO4.7H2O). Then, the flasks were incubated at 40ºC, 120 rpm for 108hr. The samples were harvested at 12 hours interval in duplicate and then the cells were separated by centrifugation at 4ºC, 5000 rpm for 20 minutes. The supernatant containing amylase was collected.

2.6 Determination of Amylase

Amylase was determined by reducing sugar assay with UV-VIS spectrophotometer. For reaction, the substrate (sodium phosphate buffer pH 7 containing 1% soluble starch) was mixed with the enzyme (supernatant) in 1:1 ratio. Then, the reaction was taken at 50°C for 10 minutes. After that, the reaction mixture and the indicator (DNS reagent) were mixed in 1:1 ratio and kept in boiling water bath for 10 minutes. The samples were cooled at room temperature. Finally, the samples were diluted with distilled water at 1:1.5 ratios and determined the reducing sugar concentration by using UV-VIS spectrophotometer at 540 nm.

2.7 Determination of Cultural Conditions

Cultural conditions of the selected bacterial strains were determined at various carbons (bacteriological starch, corn starch, rice starch, potato starch and maltose) and nitrogen source (yeast extract, casein, sodium nitrate, urea and peptone), various temperatures (30, 40, 50, 60 and 70 ºC) and various pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0). All the experiments were carried out in duplicate.

2.8 Growth Determination

The growth of the selected microbial strain (T3) was measured by plate counting method and turbidity method. The selected bacterial strain (T3) was inoculated in a flask containing nutrient broth supplement with 1% starch and then incubated at 42°C, 150 rpm for 108 hr. The samples were taken out at various times and measured the growth.

3. Result and Discussions

In this study, eight bacterial strains (S1, S2, S3, S4, S5, S6, S7 and S8) were isolated from hot spring located at Yae Nwaye village. On the other hand, four bacterial strains (T1, T2, T3 and T4) were isolated from hot spring located at Taung Lay Lone village.

Amylolytic activities of the isolates were screened by using nutrient medium supplemented with 1% concentration of four types of starch (bacteriological starch, corn starch, rice starch and potato starch). Among them, three bacterial strains (T1, T2, and T3) were selected based on their amylolytic activities. And then, reducing sugar assay was used for quantitative analysis of amylase with 12 hrs incubation time interval. According to the data, T1 produced its maximal reducing sugar formation (2.986 mg/ml) after 48 hours incubation. However, T2, and T3 strains produced (4.771 mg/ml) and (2.805, mg/ml) after 36 hrs incubation, respectively. The optimum cultural conditions for the selected strains were also studied.

The optimum temperatures for T1 and T3 were 60°C with the reducing sugar concentration of 1.1314 mg/ml and 3.0347 mg/ml, respectively. However, the optimum temperature for T2 strains was 50 °C with the maximum reducing sugar concentration of 3.3141 mg/ml. The optimum pH of the selected bacterial strains was determined by culturing at various pH values 5, 5.5, 6, 6.5, 7, 7.5 and 8. For two bacterial strains T2 and T3, the optimum reducing sugar formation was found at pH 7 with the reducing sugar concentration 3.2311 mg/ml and 3.4803 mg/ml, respectively. T1 strain showed the optimum reducing sugar formation at pH 6.5 with the concentration of 2.9380 mg/ml.

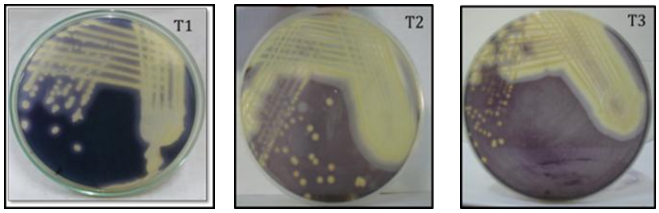

Figure 1: Screening of amylase activity on Nutrient media supplemented with 1% bacteriological starch

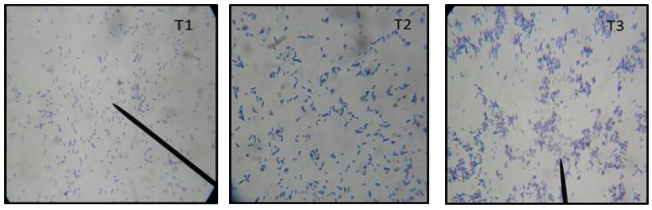

Figure 2: Microscopic Morphology of the Isolated Bacteria

Table 1: Colonial and microscopic Morphology and Some Biological Characteristics of Three Isolated Strains

Strains / *Tests*	*T1*	*T2*	*T3*
Form	Regular	Regular	Regular
Colour	Creamy	Creamy	Creamy
Gram Staining	+	+	+
Shape	Rod	Rod	Rod
Catalase	+	+	+
Motility	+	+	+
Indole	-	-	-
TSI	Yellow	Yellow	Yellow
MR	-	-	-
VP	-	-	-

+ = positive, - = negative, Yellow = Glucose fermented also lactose and or sucrose

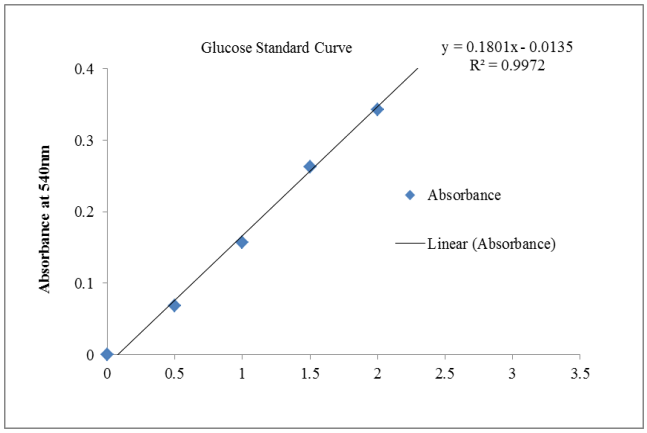

Figure 3: Standard curve for Glucose at 540 nm

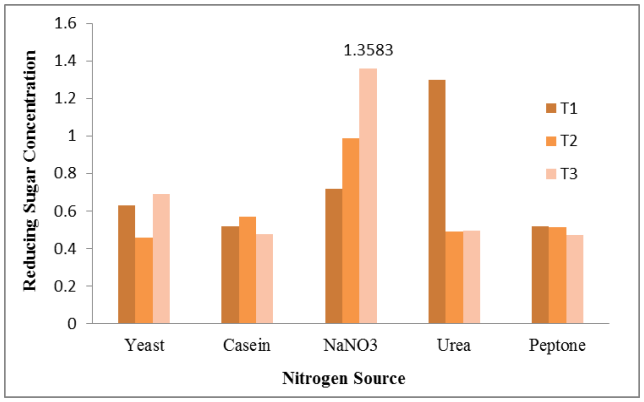

Figure 7: Effects of nitrogen source on Amylase production of isolated strains (T1, T2 and T3)

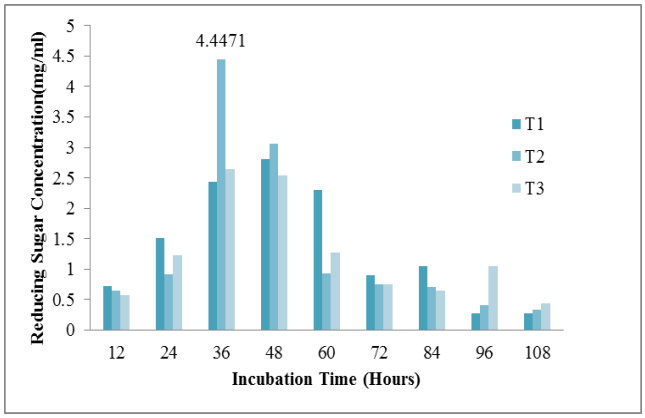

Figure 4: Amylase production of Isolated strains (T1, T2 and T3) at 37 ºC during the course of fermentation

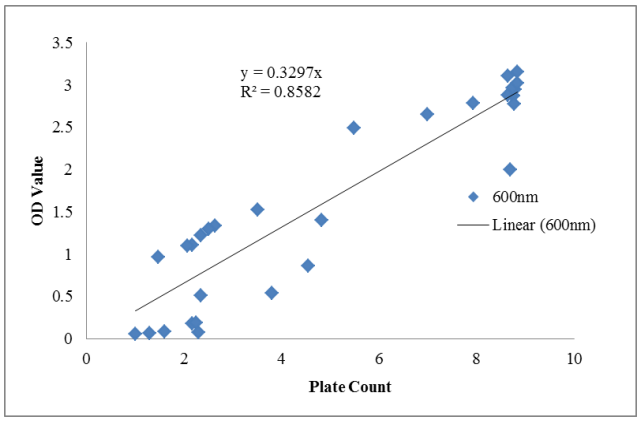

Figure 8: Plate Count (log no. of cells/ml) Vs. OD Values (660nm) Standard Curve of Strain No.T3

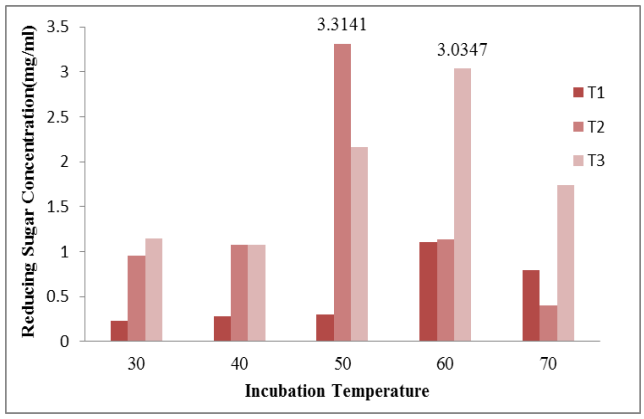

Figure 5: Effects of temperature on amylase production of isolated strains (T1, T2 and T3)

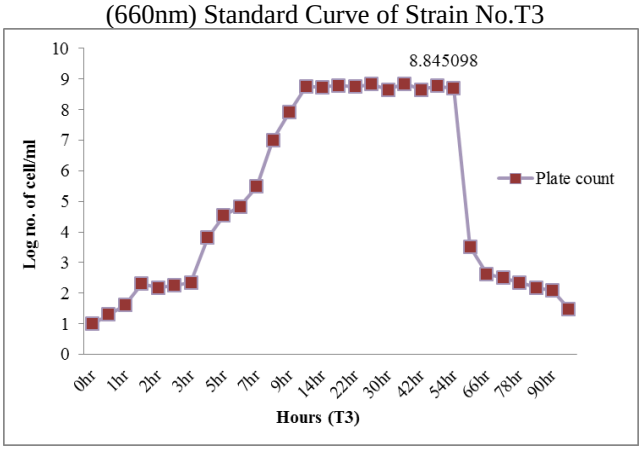

Figure 9: Plate Count (log no.of cell/ml) for T3

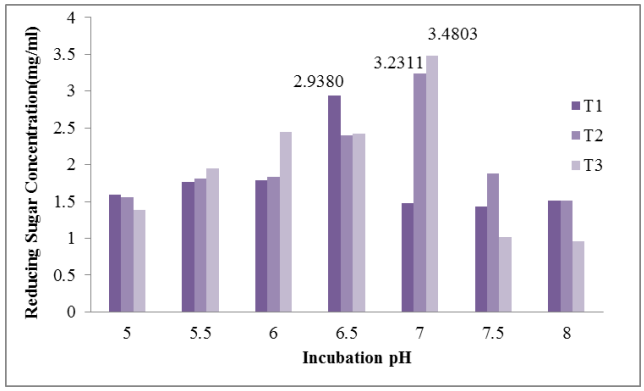

Figure 6: Effects of pH on amylase production of isolated strains (T1, T2 and T3)

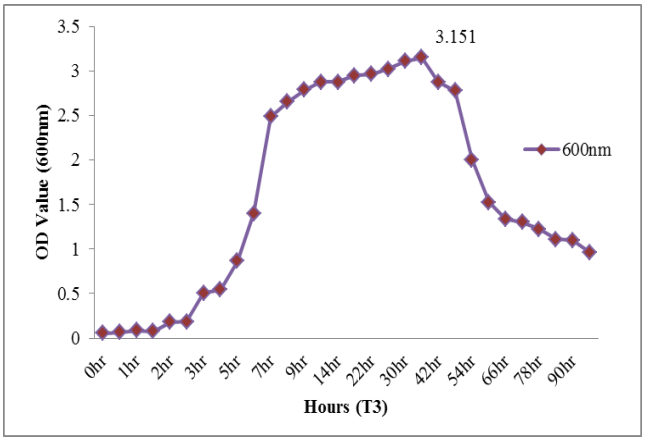

Figure 10: OD value (600nm) for T3

The best nitrogen source was selected from various nitrogen sources (urea, casein, NaNo3, peptone and yeast). Among them NaNO3 was the best, nitrogen source for T2 and T3 isolated strains with 0.9892 mg/ml and 1.3583 mg/ml whereas urea was for T1 1.2963 mg/ml respectively.

The growth conditions and product formation of T3 was studied during the course of fermentation. In growth determination, plate count (cfu/ml) and OD- values were measured with serial dilution method and UV- VIS spectro-photometric analysis at 600nm, respectively.

In plate count measurements, the maximum population ($7.0x10^8$cfu/ml) of the T3 strain was reached at 36hr incubation period. The maximum OD value of the T3 strain was 3.15 at 36hr incubation period. Based on the plate count measurements and OD values, the calibration curve of T3 strain was plotted (Fig 9 and 10).

3. Conclusions

Twelve bacterial strains were isolated from the soil sample of hot spring and screened the production of amylolytic activity. The quantitative analysis was studied on various culture conditions, (temperature and pH, carbon and nitrogen source) for the optimum production of amylase from the selected bacterial strains. From this observation, T2 strain was found the maximum reducing sugar formation 3.2311 mg/ml at 50 ºC and pH 7 after 36 hours incubation period. T3 strains showed the optimum reducing sugar production 3.4803 mg/ml at 60 ºC and pH 7 for 36 hours incubation. T1 strain produced the optimum reducing sugar 2.9380 mg/ml at 60ºC and pH 6.5 for 48 hours incubation. Among the three strains, T3 strains was found the best in production of reducing sugar at 60 ºC .Therefore, T3 was selected for further study because of thermo-tolerance strains.

4. Acknowledgements

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