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Extraction and Purification of Amylase from *Bacillus Cereus* Isolated from Soil

Ashraf Abd Alhussien[*1a] and Rahman Laibi Chelab[1b]

[1]*Biology Department, College of Education for pure science / University of Thi -Qar , Thi -Qar,Iraq.*

[b]E-mail: Rahmanlaeby2007@yahoo.com
[a*]Corresponding author: Ashraf.Bio2022@utq.edu.iq

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***Abstract*—The goal of this study was to isolate bacteria that produce amylase from soil samples that were taken from Al-Chibayish Marsh, which is located south of the Thi-Qar Governorate in Iraq. Using plating and repeated dilution techniques, isolation was accomplished. From the gathered soil samples, eighteen bacteria were identified. Using the starch agar plate method, all isolates were tested for starch activity. Of the 18 bacterial isolates, only 6 were able to make amylase; the two isolates with the highest production efficiency were chosen. Bacillus cereus was determined to be the source of the two isolates (the putative strain) by molecular, biochemical, and microscopic tests. The 48-hour incubation time, pH of 5, temperature of 37°C, starch concentration of 2%, and the use of dextrose as a carbon source and meat extract as a nitrogen source produced the highest levels of enzyme activity. The results of the present study showed that B. cereus isolates were capable of producing amylase enzyme, and the results indicated that nutrients and medium characteristics played a pivotal role in amylase enzyme production.**

***Key words*— Bacillus cereus, starch, amylase, Lugol's solution.**

I. INTRODUCTION

Alpha-D-glucopyranose polymers, which make up starch molecules, are broken down at alpha-1,4 and alpha-l,6-glycoside linkages by the enzyme amylase [1]. Enzymes function as biological processes' catalysts. They have exceptional catalytic qualities that are significantly superior to those of synthetic agents [6]. Enzymes known as amylases catalyze the breakdown of starch into the simple sugars glucose and maltose [7]. Amylase is one of the most useful enzymes used by industries. This enzyme can hydrolyze starch molecules into polymers that contain glucose units. The food, fermentation, and pharmaceutical sectors are among those that use amylase in various manufacturing processes. Organisms, including microbes, plants, and mammals, can produce amylases [2]. Microbial amylases have the following benefits: they are inexpensive, consistent, require little space, require less time to produce, and are very simple to modify and optimize [6]. Enzyme synthesis and activity are influenced by a number of physical and chemical factors, including pH, temperature, incubation duration, carbon and nitrogen sources, e.tc [5]. The aim of the present study was to use soil samples taken from the soil of Al-Chibayish Marshes in Thi-Qar Governorate, Iraq, to isolate and identify the amylase-producing bacteria, *B. cereus*. To achieve high amylase enzyme production and high enzyme activity, the production settings (temperature, pH, carbon supply, nitrogen source, and starch matrix concentration) were adjusted.

II. MATERIALS AND METHOD

A. Soil Collection

The sample used in the study was isolated from soil samples collected from Al-Chibayish marshes in Thi-Qar Governorate. The soil was collected at a depth of 10 cm below the soil surface. Sterile plastic bags were used to collect these samples and during their transportation to the laboratory, they were stored in ice boxes. Until their isolation, all samples were kept in the refrigerator. The samples were dried at room temperature for 7 to 10 days and packed in hermetically sealed bags [9].

B. Bacterial Isolation

One gram of materials was serially diluted in the range of 10^{-1} to 10^{-6} using nine milliliters of sterile distilled water. The serially diluted samples were cultured using the spread plate method with nutrient agar (NA) medium. The culture media was sterilized for 15 minutes at 15 pressure at 121°C. The plates were incubated at 37°C for 24 to 48 hours. Before being needed for analysis, the isolates were kept on slops (NA with 2% starch) at 4°C in the refrigerator after being filtered using the drip method [4].

C. Screening of bacteria for amylase production

Using a starch hydrolysis experiment on a starch agar plate (starch 2%, yeast extract 1%, MgSO4.7H2O 0.1%, KH2PO4 0.2%, Agar 2%), bacterial isolates were examined for hydrolytic activity. On starch agar plates, bacteria were streaked and then incubated for 48 hours at 37°C. The starch agar plate was submerged in Lugol's solution following

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incubation. The colonies' surrounding clear zones showed signs of amylase production. The bacteria with the greatest clearing zone were chosen for additional research after the diameter of the clearing zones was measured [11].

D. Identification of bacteria

The approach that was followed for identifying the isolated bacteria was standard and included the use of Bergey's Manual of Critical Bacteriology (1957) in addition to a number of biochemical tests (Fawole and Osho, 2004; Willey et al., 2008). Molecular analysis was done on the bacteria that had the highest level of amylolytic activity. For the purpose of verifying the identity of the bacterial strains, and the small subunit *16srRNA* genes were amplified from the genomic DNA using R27: 5-AGA GTT TGA TCC TGG CTC AG-3 and F1492 : 5-GGT TAC CTT GTT ACG ACT T-3 primer [2].

E. Amylase Extraction

About 100 µl of the bacterial suspension was put into a 250 ml glass flask with sterile starch broth medium in order to produce enzymes. 48 hours were spent incubating the production medium at 37°C. The culture broth was centrifuged for 10 minutes at 10,000 rpm after incubation. The top liquid was utilized as crude enzyme for enzyme analysis following centrifugation. To prepare the reaction mixture for additional analysis, one milliliter of crude enzyme and one milliliter of 0.1M phosphate buffer solution pH 7 were added. It was kept at 50°C for thirty minutes. After that, the reaction was stopped by adding around 2 milliliters of DNS and heating the mixture in a water bath for 10 minutes at 90°C.The release of reducing sugar was recorded at 540 nm using a spectrophotometer [8].

F. Optimal Conditions of Amylase Production

effect of incubation period on amylase activity:

The chosen bacterial isolate was cultivated in starch broth and incubated at 37°C for 24, 48, 72, 96, and 120 hours in order to ascertain the impact of incubation length on extracellular enzyme production. The supernatants from which the extracellular xylanase activity would be determined were then obtained by centrifuging the culture broth for 10 minutes at 10,000 rpm.

G. Effect of temperature on amylase activity:

This particular bacterial isolate was cultured in starch broth and incubated at 32, 37, 45, 50, and 55°C for 48 hours to see how temperature affected the production of extracellular enzymes. To assess the extracellular amylase activity, the culture broth was centrifuged for 10 minutes at 10,000 rpm to extract the supernatants.

H. Effect of pH value on amylase activity:

The effect of initial media pH on amylase production was performed by adjusting starch broth to pH 5.0, 6,7, 8, 9,10 and 11 before bacterial inoculation. After 48 h of incubation at 37°C, the culture starch broth was then centrifuged at 10,000 rpm for 10 min to obtain the supernatants from which extracellular amylase activity was measured.

I. Effect of starch concentration on amylase activity:

Under optimal temperature, pH, and incubation period. Five concentrations of the main substrate represented by starch (0.5%, 1%, 1.5%, 2%, 2,5%) were tested. The production of the enzyme amylase was measured.

J. Effect of carbon sources on amylase activity:

Under optimum temperature, pH, and incubation period. Five different carbon sources (Lactose, Maltose, Dextrose, Fructose, Glucose) with the same concentration of 2% were tested for amylase production.

K. Effect of nitrogen sources on amylase activity:

The effect of different nitrogen sources was selected by adding 1% of the nitrogen sources (peptone, Beef extract, Meat extract, Urea, KNO3). All of these media were sterilized separately at 121 ℃ for 1h. The flasks were incubated at 37℃ for 48h. The amount of enzyme produced was estimated [12].

III. RESULTS

A. Isolation and screening of amylase-producing bacteria

Amylase-producing bacteria were isolated from soil samples of Al-Chibayish marshes by serial dilution and spread plating on starch agar. Starch agar is a selective medium for the growth of amylase-producing bacteria and can only use starch as a carbon source. Based on the purification zone surrounding the colony on starch agar medium, amylase-producing bacterial isolates were screened. The appearance of the clear zone around the colony after adding Lugol's solution was strong evidence that the bacteria produced amylase for starch hydrolysis. The amylase-producing bacterial strains in the clear zone around the colony were identified as efficient amylase-producing bacteria and the B. cereus isolate showed the maximum clear zone in diameter (Figure 1) and was tentatively identified by Gram staining, colony morphology and biochemical tests as shown in Table 1.

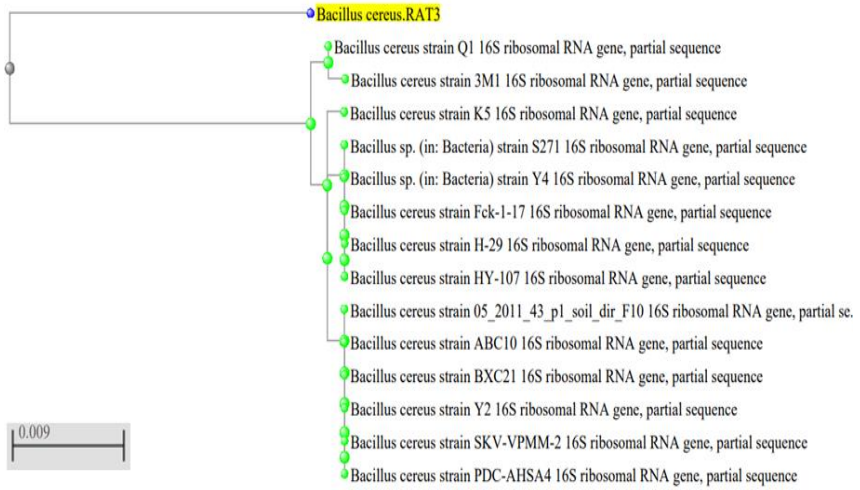
Fig. 1: Clear area indicates starch hydrolysis by B. cereus grown on starch agar medium after incubation at 37°C for 48 h.

Table 1: morphological and biochemical characteristics for the identification of isolates

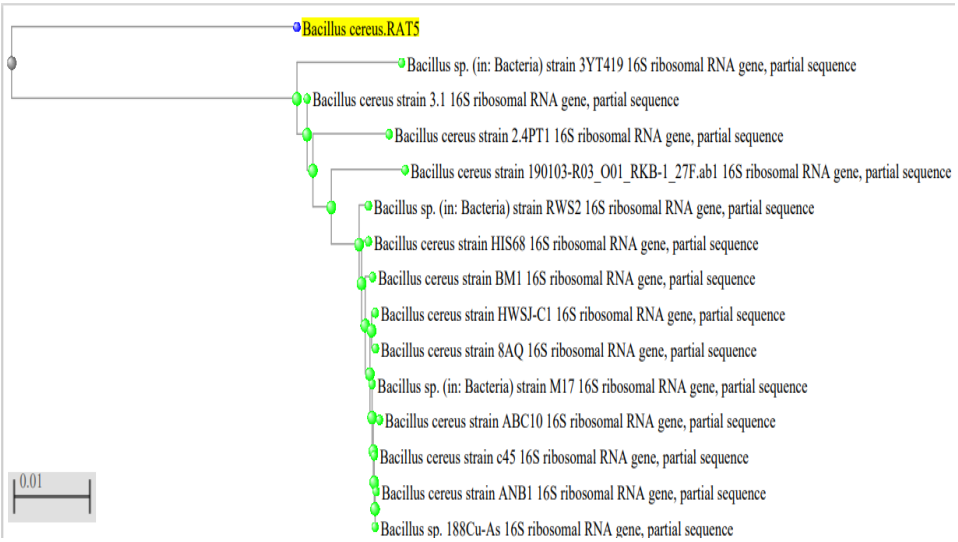
Morphological and biochemical	*B. cereus* RAT3	*B. cereus* RAT5
Gram stain	+	+
Endospore Stain	+	+
Shape	**Rod**	**Rod**
Starch test	+	+
Blood hemolysis	***β***	***β***
Catalase test	+	+
Oxidase	+	+
Gelatin	-	-
Mannitol	-	-
Klikler Iron test	**K/A**	**K/A**
Indole test	-	-
Methyl Red test	+	+
Voges proskar test	+	+
Motility	+	+

B. Molecular characterization and identification of starch-degrading bacteria

The *16S rRNA* gene was amplified for molecular identification of the isolate. Using BLAST analysis, a 95% similarity was found between the sequence and various *B. cereus* reports from around the world stored in the NCBI database. *B. cereus* was identified as RAT3 and RAT5 based on molecular analysis. Figure 2 (A / B) shows the close relationship identified by the phylogenetic tree between the detected bacterial species and other Bacillus species in Gen Bank.



A



B

Fig. 2: A: Identification of *B. cereus* RAT3 by using Phylogenetic tree method indicated that strain was closely related with *B. cereus* strain. B | Identification of *B. cereus* RAT5 by using Phylogenetic tree method indicated that strain was closely related with *B. cereus* strain.

C. *Effect of incubation time on amylase production*

The results showed that studying the time taken to produce the maximum amount of enzyme, it was found that the maximum production was at 48h of incubation for the enzyme produced by *B. cereus* RAT5 0.355, *B. cereus* RAT3 0.251 Figure 3.

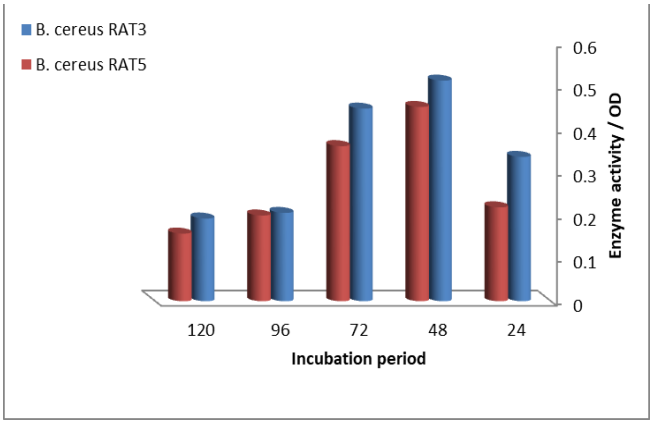


Fig. 3: Effect of incubation period on enzymes production from *B. cereus* RAT3, *B. cereus*RAT5.

D. *Effect of temperature on amylase production*

With a particular *B. cereus* isolate, 37°C was determined to be the ideal temperature for enzyme synthesis. The highest amylase production was recorded at 0.793 by*B. cereus* RAT3, 0.435 by *B. cereus* RAT5 as shown in the Figure 4.

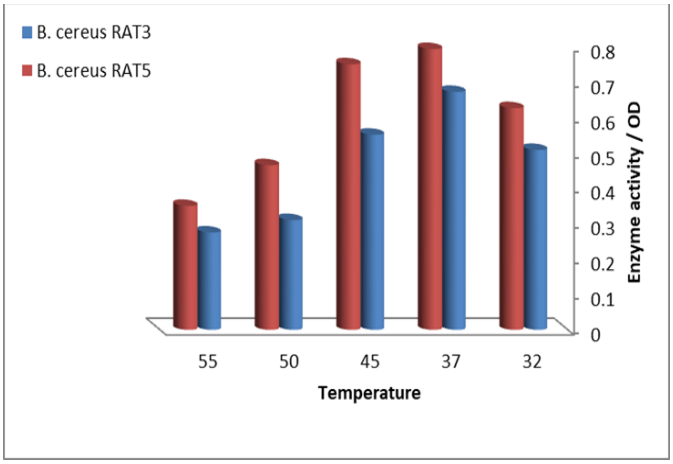


Fig. 4: Effect of temperature on amylase production from *B. cereus* RAT3, *B. cereus* RAT5.

E. *Effect of pH on amylase production*

After 48 hours of incubation at 37°C, Figure 5 illustrates the impact of varying pH on the synthesis of amylase. pH 5 was shown to be the maximum amylase production level. (0.678, 0.560) by *B. cereus* RAT3and *B. cereus* RAT5 respectively and minimum amylase production was recorded at pH 11 (0.678, 0.560) by *B. cereus* RAT3 and *B. cereus* RAT5 respectively.

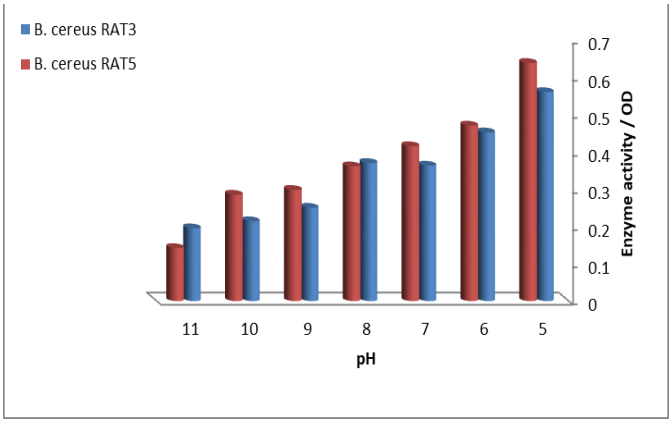


Fig. 5: Effect of pH on the activity of*B. cereus* RAT3, *B. cereus* RAT5 conditions: substrate 2% and temperature of 37°C using 0.1M phosphate buffer.

F. *Effect of starch concentration on amylase production*

The synthesis of amylase from *B. cereus* RAT3 and *B. cereus* RAT5 was examined in relation to varying quantities of starch (0.5% to 2.5% w/v) in the production medium. Maximum amylase production was observed at a starch concentration of 2% w/v with activity of (0.621, 0.598) respectively. However, enzyme production decreased as starch concentration increased beyond 2% w/v Figure 6.

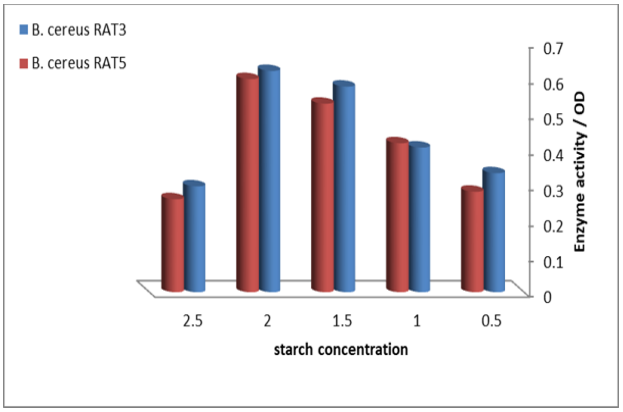


Fig. 6: Effect of starch concentration on amylase production from *B. cereus* RAT3, *B. cereus* RAT5.

G. *Effect of carbon sources on amylase production*

After 48 hours of incubation at 37°C, Figure 7 illustrates how different carbon sources affect the generation of amylase. The highest amount of amylase was produced in a medium supplemented with Dextrose (0.623, 0.550) by *B. cereus* RAT3, *B. cereus* RAT5, and the lowest amount was produced in a medium containing glucose (0.331, 0.194) by *B. cereus* RAT3, *B. cereus* RAT5, respectively.

H. *Effect of organic nitrogen sources on amylase production*

After 48 hours of incubation at 37°C, Figure 8 illustrates the impact of various nitrogen sources on the production of amylase. The meat extract produced the most amylase, as was seen. (0.655, 0.939) added to the medium by *B. cereus* RAT3, *B. cereus* RAT5 respectively, and the lowest enzyme

activity was observed in the KNO3 (0.191, 0.319) by *B. cereus* RAT3, *B. cereus* RAT5 respectively.

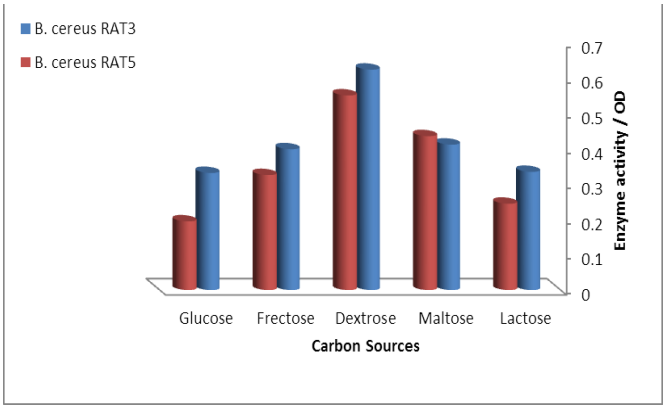


Fig. 7: Effect of different carbon sources on the production of amylase by *B. cereus* RAT3, *B. cereus* RAT5.

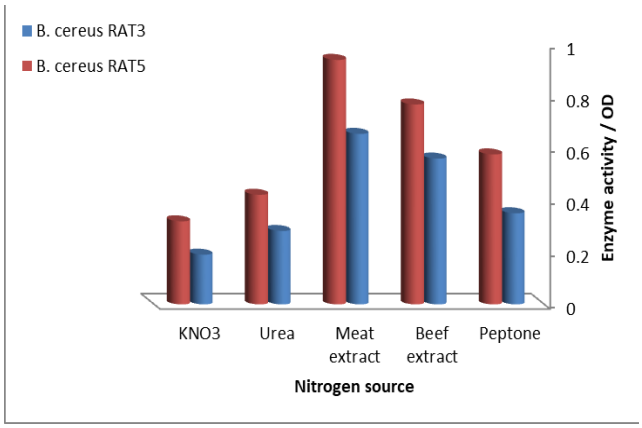


Fig. 8: Effect of nitrogen sources on enzyme production by *B. cereus* RAT3, *B. cereus* RAT5.

IV. DISCUSSION

Initial screening of *B. cereus* strain revealed hydrolytic zones with a diameter of 21 mm on starch agar plates. This distinct zone indicated that the amylase enzyme produced by Bacillus cereus is capable of hydrolyzing starch. The results of this study are in agreement with [4].The genus Bacillus produces a wide range of extracellular enzymes, of which amylase is particularly important for industry. The chosen bacterial isolates were recognized as Bacillus cereus based on morphological and biochemical features. The findings of this investigation concur with [11]. In accordance with the findings of [2], the strain's phylogenetic tree of various bacilli species revealed that it was closely linked to the *Bacillus cereus* strain. The fermentation period of 48h was found to be optimal for fermentation according to the results of the incubation period for amylase production. After that, increasing the fermentation period led to a decrease in enzyme production because of increased bacterial growth produces toxic compounds that prevent enzyme synthesis. These results are consistent with the results of [1]. It has been proven that 35°C is ideal for amylase synthesis, and amylase is produced by bacilli species when grown between 35°C and 37°C. The results of all isolates showed that the temperature at which enzyme production was highest at 37 °C was much lower than 50 ℃, 55 ℃ these results are consistent with the results of [4]. The pH values of the medium greatly affect many enzymatic processes and the transport of compounds across the cell membrane. Maximum amylase production was achieved at pH 5 by *B. cereus* and enzyme production decreased at pH 10, 11. this indicates that the bacteria are able to tolerate acidic conditions of the medium according to these results, as these results are consistent with the results of [10].The concentration of starch has an impact on the amount of amylase that B. cereus produces; the maximum amount of enzyme was produced at a concentration of 2%, and the amount of amylase produced dropped as the concentration rose. The insertion of mono- or polysaccharides, which are carbon sources, can influence the synthesis of amylase. Among carbon sources, Dextrose was found to support amylase production. The results of all isolates showed that enzyme productivity was high when lactose was used as a carbon source. Enzyme productivity decreased when glucose was used as a carbon source. Also, nitrogen sources can affect amylase production. Among nitrogen sources, meat extracts produced the maximum amount of amylase enzyme, and enzyme production decreased when urea and KNO3 were added as nitrogen sources.

V. CONCLUSIONS

According to the results of the present study, *B. cereus* is an amylase-producing strain. *B. cereus* can be used for amylase production by submerged fermentation method using soluble starch as a carbon source because it has low nutrient requirements and can produce high amounts of enzyme.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

REFERENCES

[1] R. A. Arfah, A. Ahmad, M. Natser, M. Anis and M. Zakir "Production Optimization and Characterization of Amylase Enzyme Isolated from Termofil Bacteria Bacillus sp RSAII-1b from Lejja Hot Spring South Sulawesi" *American Journal of Biomedical and Life Sciences*, Vol., 3 No., 6 PP., 115-119 2015.

[2] M. Arekemase, O. Alfa, T. Agbabiaka, N. Ajide-Bamigboye, O. Aderoboye, J. Orogu and T. Ahmed "Optimization of Amylase Produced from Bacteria Isolated from Cassava Peel Dumpsite using Submerged Fermentation" *Science World Journal*, Vol., 15 No., 1 PP., 64-75 2020.

[3] M. Aladejana, O. Oyedeji, O. Omoboye and K. Bakare "Production, purification and characterization of thermostable alpha amylase from Bacillus subtilis Y25 isolated from decaying yam (Dioscorea rotundata) tuber" *Notulae Scientia Biologicae*, Vol., 12 No., 1 PP., 154-171 2020.

[4] D. P. Elechi, O. C. Jason, A. Onyewuchi and O. Akuma "Optimization of B. cereus PW4 alpha amylase production by OVAT technique" *GSC Biological and Pharmaceutical Sciences*, Vol., 20 No., 1 PP., 083-090 2022.

[5] S. E. Ibrahim, H. B. El Amin, E. N. Hassan and A. M. E. Sulieman "Amylase Production on Solid State Fermentation by Bacillus Spp" *Food Public Health*, Vol., 2 No., 1 PP., 30-35 2012.
[6] H. H. Raplong, P. O. Odeleye, C. Hammuel, M. O. Idoko, J. I. Asanato and E. H. Odeke "Production of Alpha Amylase by Bacillus cereus in Submerged Fermentation" *Aceh International Journal of Science and Technology*, Vol., 3 No., 3 PP., 124-130 2014.
[7] A. Madika, J. B. Ameh and D. A. Machido "Isolation and Screening of Bacillus subtilis from Soil for Amylase Production" *UMYU Journal of Microbiology Research (UJMR)*, Vol., 2 No., 2 PP., 82-86 2017.
[8] A. Samanta and S. C. Jana "Optimization of cold active amylase production by mesophilic Bacillus cereus RGUJS2023 under submerged fermentation" *Journal of Environmental Biology*, Vol., 45 No., 1 PP 16-24 2024.
[9] S. Sharif, A. H. Shah, A. Fariq, S. Jannat, S. Rasheed and A. Yasmin "Optimization of amylase production using response surface methodology from newly isolated thermophilic bacteria" *Heliyon*, Vol., 9 No., 1 PP., 12901 2023.
[10] T. Sivakumar, T. Shankar, P. Vijayabaskar, J. Muthukumar and E. Nagendrakannan "Amylase Production Using Bacillus cereus Isolated from a Vermicompost Site" *International Journal of Microbiology Research*, Vol., 3 No., 2 PP., 117-123 2012.
[11] G. G. Srikant and V. Beniwal " Isolation, Screening and biochemical characterization of xylanase producing Bacillus cereus" *Annals of Agri Bio Research*, Vol., 25 No., 1 PP., 1-19 2020.
[12] C. H. Yang, K. C. Cheng, W. H. Liu "Optimization of Medium Composition for the Production of Extrac-Ellular Amylase by Thermobifida fusca Using a Reponse Surface Methodology" *Food Science and Agricultural Chemistry*, Vol., 5 No., 1 PP., 35-40 2003.