IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 1, Issue 6 (Sep. – Oct. 2015), PP 01-09
www.iosrjournals.org

Detection of Alpha-Amylase Activity from Soil Bacteria

[1]Salihu, Y., [1]Saidu, A. Y., [2]Rabiu, G. A., [1]Umar A.A., [3]Zeynep, A., [3]Unzile, G.

[1]Department of Biological Sciences, Faculty of Science, Federal University Dutse, Jigawa State-Nigeria.
[2]School of Sciences, Department of Biology, Saadatu Rimi College of Education, Kunbotso, Kano State-Nigeria. [3]Department of Genetics and Bioengineering, School of Sciences and Engineering, Fatih University Istanbul, Turkey.

Abstract:

Background: *Alpha-amylase is one of the industrial enzymes that hydrolyze starch molecules into polymers composed of glucose units. The enzyme has potential application in a wide number of industrial processes such as food, textile, paper, detergent, fermentation and pharmaceutical industries. Alpha-amylase can be produced by microorganisms, plants or animals.*

Aim: *The aim of this study is to detect the activity of alpha-amylase from bacteria isolated from soil environment.*

Method: *Soil samples were inoculated onto the media that are rich in nutrient that favour the growth of the bacteria and incubated for 24 hours at 37^{o}C after which the bacterial growth was detected in form of colonies. In this study, bacterial species belonging to the genus Bacillus were identified through phylogenetic analysis using 16s-ribosomal RNA sequencing for detection of the enzymatic activity. Effects of pH and temperature on the enzymatic activity were observed using DNS activity assay method.*

Results: *Positive response to alpha-amylase activity by the soil bacteria was observed by the formation of clear zone of inhibition shown by the colonies on the petri plates.*

Conclusions: *The optimal pH and temperature activities showed that the bacteria exhibit enzymatic activity at mesophilic temperature and acidophilic or alkalophilic pH.*

Keywords: *Alpha-amylase, Bacillus, phylogenetic analysis, DNS activity assay.*

I. Introduction

Microorganisms have become increasingly important producers of industrial enzymes. In view of their biochemical diversity and the ease with which enzymes concentrations may be increased by environmental and genetic manipulation, several attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Amylolytic enzymes capable of degrading starch are most important in biotechnology industries with large application in food, fermentation, textile and paper industries [1].

Amylases can be obtained from several sources such as plants, animals and microorganisms [2]. The microbial source of amylase is preferred to other sources of its plasticity and vast abundance. Amylase obtained from microorganisms has almost surpassed the synthetic sources in different industries [1]. Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized into exo-acting, endo-acting and debranching enzymes. Among the amylases, beta-amylase is exo-acting, where alpha-amylase is endo-acting enzyme.

A vast number of organisms live in the soil [3], so great are microfloral number that dominate the biomass in spite of the minute size of each organism [4]. Together with the earthworms, the microflora monopolizes the metabolic activity in the soils. It is estimated that 60-80% of the soil metabolism is due to microflora. Not only do they destroy plant residues but they function in the digestive tracts of animals and eventually decompose the dead bodies of all organisms.

Alpha-amylases are hydrolytic enzymes that are widespread in nature being found in microorganisms, plants and animals [5]. They are among the most important in present day biotechnology. The enzyme has found numerous applications in commercial processes including thinning and liquefaction of starch alcohol, brewing and sugar industries [6].

The genus Bacillus produces a large variety of extracellular enzymes of which amylases and proteases are of significant industrial importance. Najafi et al [7] showed that the optimum pH and temperature of the alpha-amylase from Bacillus subtilus were 6.0 and 55^{o}C respectively. The end products of soluble starch were glucose (70-75%) and maltose (20-25%).

Demands for novel amylases worldwide are increasing day to day as these enzymes application spectra are spreading in various industrial sectors [8]. This class of industrial enzyme constitutes approximately 25% of the enzyme market covering many industrial processes [9].

This study is aimed at detecting alpha-amylase activity produced by some soil bacteria. The activity of the bacterial enzyme was detected at different pH and temperatures after which the optimal activities were obtained. The objectives of this study include: (1) Isolation and identification of the soil bacteria. (2) Gram's

staining characteristics of the bacteria. (3) 16s ribosomal sequencing of the alpha-amylase producing bacteria. (4) Enzymatic activity assay of the bacterial enzyme.

II. Material and Method

2.1 Study Site, Sample Collection and Media Preparation

This study was conducted in Fatih University Istanbul, Turkey. The soil samples were collected in clean dry sterile containers from different soil environments as follows: Behind E-block and car-pack of Fatih university, Istanbul; Buyucekmece Lake, Istanbul and Agricultural land of Bursa all in Turkey.

Upon soil samples collection, the culture media were prepared and the soil was inoculated onto the plates containing the media that support the growth of the bacteria. The plates were put in an incubator at 37^oC for 24 hours after which the bacterial growths were detected in form of colonies. Similarly, the bacterial samples that showed positive response to alpha-amylase were grown in LB-medium and placed in an incubator at 37^oC for 24 hours for the bacterial cell cultivation.

2.2 Preparation of Media and Regents 2.2.1 Alpha-amylase media

Two alpha-amylase media were prepared for the growth of soil bacteria. The media were enriched with the starch as the substrate for alpha-amylase enzyme (table 2.1). Following the mixture of the chemicals, the media were autoclaved at 121^oC for 15 minutes using autoclave (Nuve OT4060 steam sterilizer (Turkey)). They were poured on to the clean and dry Petri plates and allowed to cool after which they solidified. The plate containing the media were sealed with stretch film and stored at 4^oC before use. The preparation of the media was performed under aseptic conditions.

2.2.2 LB-Medium

For the preparation of 500Ml LB-broth medium, 10g powder was suspended in 500ml of distilled water. The media were autoclaved at 121^oC for 15 minutes, then cooled to 50^oC and stored at 4^oC before use.

2.3 Preparation of Buffers and Solutions 2.3.1 Phosphate buffer

Reagents used

Monobasic sodium phosphate: 2.78g of sodium dihydrogen phosphate was dissolved in 100ml distilled water (Merck, Germany).

Dibasic sodium phosphate (0.2M): 5.3g of disodium hydrogen phosphate (Sigma-Aldrich, Germany) was dissolved in 100ml distilled water.

Procedure

About 39ml of dihydrogen sodium phosphate (Merck, Germany) was mixed with 61ml of disodium hydrogen phosphate. This made up to 200ml with distilled water which gives phosphate buffer $(PO_4)_2$ of 0.2M.

The pH was measured with pH meter which was standardized with standard buffer and its electrode was washed with distilled water.

DNS Reagent

About 1g of dinitrosalicylic acid (DNS) (Merck, Germany) was dissolved in 50ml of distilled water. To the solution, 30g of sodium tartarate tetrahydrate (Merck, Germany) was added in small lots, the solution turned yellow in colour. 20ml of 2N NaOH (Merck, Germany) was then added, which turned solution to transparent orange –yellow colour. The final volume was made to 100ml with the distilled water. The solution was stored in an amber coloured bottle.

1% Starch solution

About $80cm^3$ of distilled water was heated in a beaker. 1 gram of starch was weighed, mixed with a splash of distilled water. When the water was a near boiling, the starch was added to make up to $100cm^3$ and brought to boil.

Biochemical activation of the colonies

The colonies that showed positive response to alpha-amylase were detected by the formation of clear zone of inhibition. This shows that the bacteria were able to break down alpha-amylase. The colonies were sub-cultured, stocked and stored at 4^0C.

Isolation and characterization of the colonies

In order to isolate and identify the group of bacteria that were responsible for alpha-activity, the colonies that showed positive response to alpha-amylase were subjected to microbial characterization via Gram's staining and microscopy.

Gram's staining

Gram's staining technique was employed for the characterization of the colonies so as to know the nature of the bacterial cell-wall based on Gram's reactions. Clean dried slides were used for fixing the smears. After fixing the smears on the slides using fire flame, the fixed smears on the slides were subjected to Gram's staining steps as follows:

Staining Techniques

The slides were flooded with the crystal violet solution (primary stain) for 1 minute and then washed with water not five seconds and drained. The slides were flooded with Gram's iodine solutions (modant) for about 1 minute, washed with water and drained. The slides were flooded with 95% alcohol (decolourizer) for 10 seconds, washed with water and drained. The slides were flooded with safranin (counterstain) for 30 seconds, washed with water and drained. All the slides were examined under the microscope using Nikon Labophot-2 Electric Microscope (Germany) with oil immersion lens (times 100 magnifications).

Microscopy

Following Gram's staining, the stained slides were observed microscopically under oil immersion lens. The colonies Gram's stain characteristics which include their morphologies and nature of Gram's stain reaction were observed and recorded.

Phylogenetic analysis

In order to identify the species of bacteria isolated from the soil environment that showed positive response to alpha-amylase activity, the samples were sent to MACROGEN (Korea) and REFGEN (Ankara) for phylogentic analysis using 16s ribosomal RNA sequencing technique.

16S ribosomal RNA is a component of the 30s small prokaryotic ribosomes subunit. It is 1.542kb (or 1542 nucleotide) in length. The genes coding for it are referred to as 16s rDNA and are used in constructing phylogenies. It is highly conserved between different species of bacteria and archea.

The result of a phylogentic analysis is expressed in a phylogenetic tree where closely related organisms have a high degree of agreement in the molecular structure of DNA, RNA and proteins.

Alpha –amylase activity assay procedure

Following identification of bacterial species that showed positive response to alpha-amylase activity, the samples were subjected to activity assay via DNS method. The bacterial samples were grown in LB-medium overnight at 37^{o}C.

Upon the overnight growth of the bacterial samples in LB-medium, the samples were centrifuged for 10 minutes using a centrifuge (Beckman counter (Germany)) and the pellets were collected after discarding the supernatants. The cells were suspended in 1000ul phosphate buffer (pH 7). The cells were subjected to sonication for lyses using a sonicator (Bendelin electronic (Germany)). Following cell lyses, the samples were further centrifuged for 10 minutes after which the supernatants were collected.

Having collected the supernatant after centrifugation, DNS method was employed for the bacterial enzymatic assay as described below:

DNS Activity Assay Method for Alpha-amylase:

- 250ul of the enzyme solutions (supernatants) were mixed with 250ul of starch solutions (at pH 6, 7 and 8) and incubated at different temperature (25^{o}C, 37^{o}C and 80^{o}C) for 10 minutes.
- 500ul of DNS reagents were added to the mixture.
- The solutions were boiled in a boiling water bath for 5 minutes.
- The solutions were cooled in a running tap water.
- 1ml of deionised distilled water was added to each solution.
- A blank was prepared without enzyme.
- The absorbance was read at 540nm using UV-Vis spectrophotometer (Nano-Drop, Thermo (U.S.A).

III. Result

Biochemical Activation of the Colonies

Following the overnight incubation of the culture media, the bacteria were able to degrade the media there by producing a clear zone of inhibition. The larger the zone of inhibition, the more active is the bacteria! The diameter of the zone of inhibition and that of the colonies were measured.

Table: 1 Biochemical Activation of the colonies.

Sample no.	Colony diameter (mm)	Zone diameter (mm)	Enzymatic index
A1.3.S2.1	7	17	2.42
A1.3.S2.2	6	16	2.66
A1.3.S2.3	5	16	3.2
A1.5.S3.2	7	14	2
A1.5.S3.2	7	14	2

Gram's stain Result

Following Gram's stain reaction, microscopic examination showed that the bacteria are Gram-positive (appear purple in colour). Also, the morphological examination showed that the bacteria are rod in shape (bacilli).

The result shows that the bacteria are Gram-positive and appear rod in shape (Bacilli). It was observed under electrical microscope at 100 times magnification (under oil immersion lens).

Phylogenetic Analysis Result

Based on the nature of 16s ribosomal RNA, the colonies that were identified from Gram's stain reaction and microscopic examination were sequenced and the sequence analysis showed that the bacteria belonged to the family Bacillaceae as can be seen from the phylogenetic tree. The analysis was carried out using computer programme as follows:

First, a blast search for the contig sequence was made. In the blast search, about 10 species were picked from the top. Then, their fasta formats were saved. After saving, they were copied and pasted on ClustalW2 programme for the alignment between the species. In the ClustalW2 programme, a tree construction link was used for the automatic construction of the phylogenetic tree after the alignment between the species.
The phylogenetic trees for the three bacterial species are as follows:

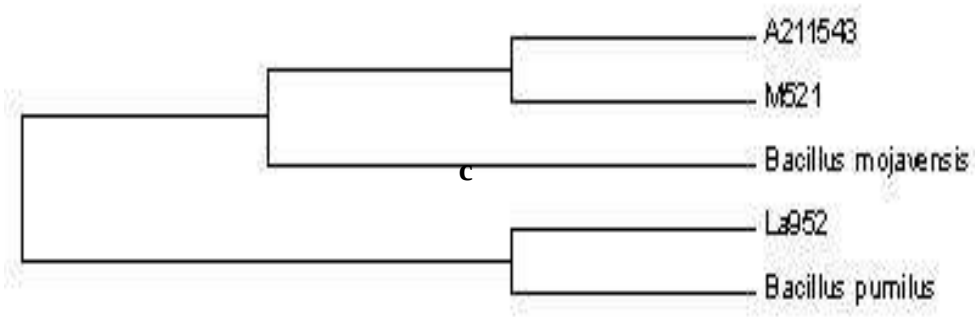


Figure: 5 Phylogenetic tree of Bacillus mojavensus.

The figure: 5 shows the phylogeny of the bacteria after the blast search using ClustalW2 programme. It shows a Bacillus mojavensus and related member of the family (Bacillus pumilus).

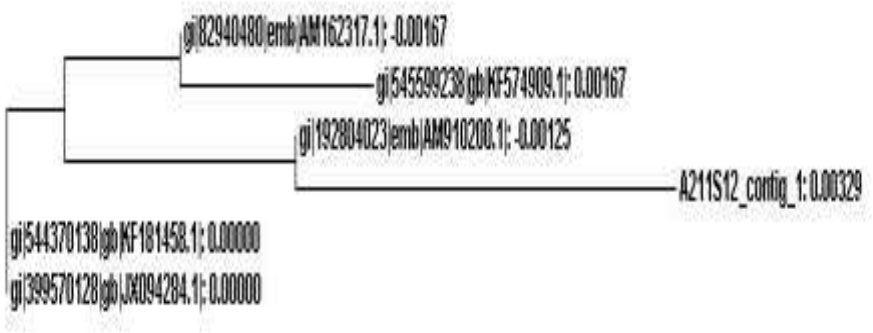


Figure: 6 Phylogenetic Tree of Bacillus subtilus.

The figure: 6 above shows the phylogeny of Bacillus subtilus after the blast search using ClustalW2 programme. A2.11.S1.2 is the sample number of the bacterial specie.

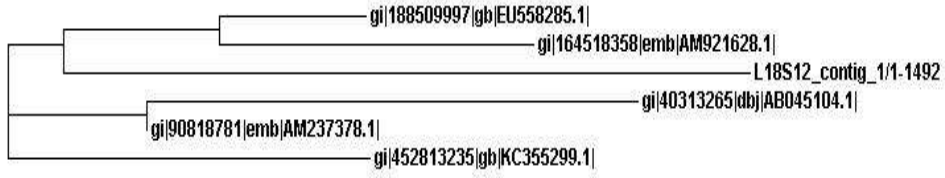


Figure: 7 Phylogenetic tree of Paenibacillus sp B3a.

The figure above shows the phylogeny of Paenibacillus sp B3a after the blast search using ClustalW2 programme. L.18.S1.2 is the sample number of the bacterial specie.

Alpha-amylase Activity Assay Results

Dinitrosalicylic (DNS) method was employed for the alpha-amylase activity assay. Absorbance of the enzyme was measured sphectrophotometrically and the effects of pH and temperature were determined. The enzymatic activities of the enzyme differ with respect to the bacterial species in relation to pH and temperature dependent as can be seen in the tables below:

Table: 2 Alpha-amylase Activity Result for Bacillus mojavensus.

pH	TEMPERATURE (^{0}C)	ABSORBANCE (nm)
6	80	0.272
6	37	0.250
6	25	0.249
7	80	0.291
7	37	0.289
7	25	0.251
8	80	0.239
8	37	0.636
8	25	0.262

Table: 3Alpha-amylase Activity Result for Bacillus subtilus.

pH	TEMPERATURE (^{0}C)	ABSORBANCE (nm)
6	80	0.765
6	37	0.769
6	25	0.778
7	80	0.447
7	37	0.452
7	25	0.483
8	80	0.325
8	37	0.336
8	25	0.369

Table : 4 Alpha-amylase Activity Result for Paenibacillus sp B3a.

pH	TEMPERATURE (^{o}C)	ABSORBANCE (nm)
6	80	0.351
6	37	0.409
6	25	0.483
7	80	0.369
7	37	0.819
7	25	0.803
8	80	0.325
8	37	0.323
8	25	0.793

Bacillus mojavensus

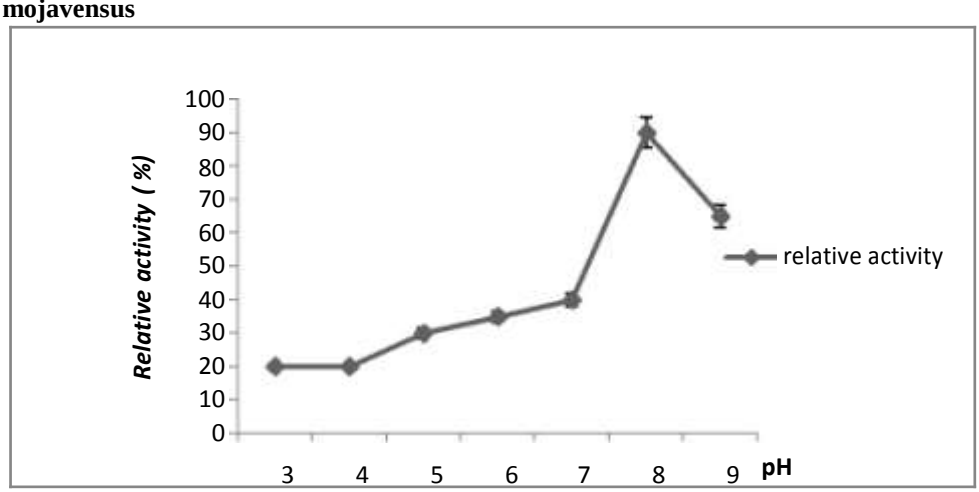


Figure: 8 Graph of pH gradient with respect to relative enzymatic activity for Bacillus mojavensus.

The figure: 8 above shows the bacterial amylase activity when studied at different pH gradients. The maximum activity of the enzyme was obtained at pH 8. The enzymatic activity was studied under alkaline condition not acidic because the activity was low at acidic medium.

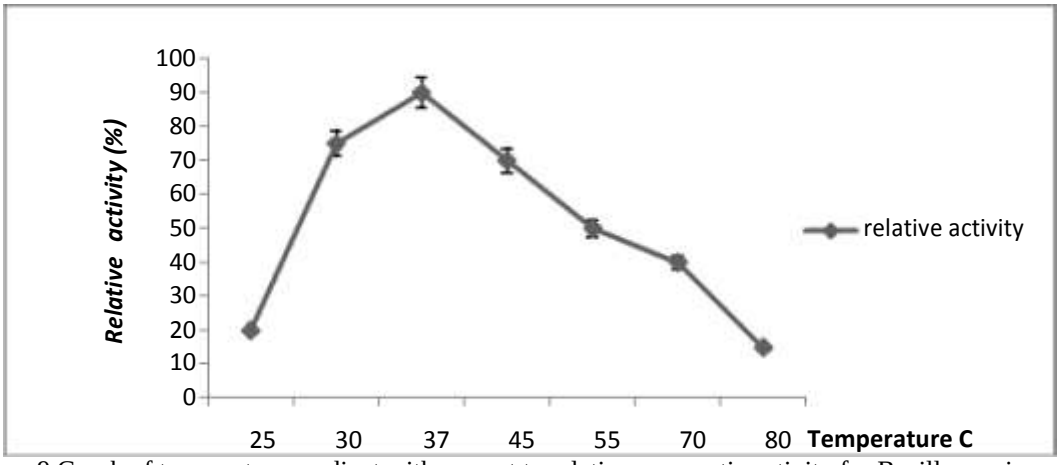


Figure: 9 Graph of temperature gradient with respect to relative enzymatic activity for Bacillus mojavensus.

The figure above shows the maximum activity of bacterial alpha-amylase when studied at temperature gradients. 37^{o}C was found to be the maximum temperature. Other temperatures show little activity. In the conclusion, the maximum enzymatic activity was found to be at 37^{o}C and pH 8.**2 Bacillus subtilus**

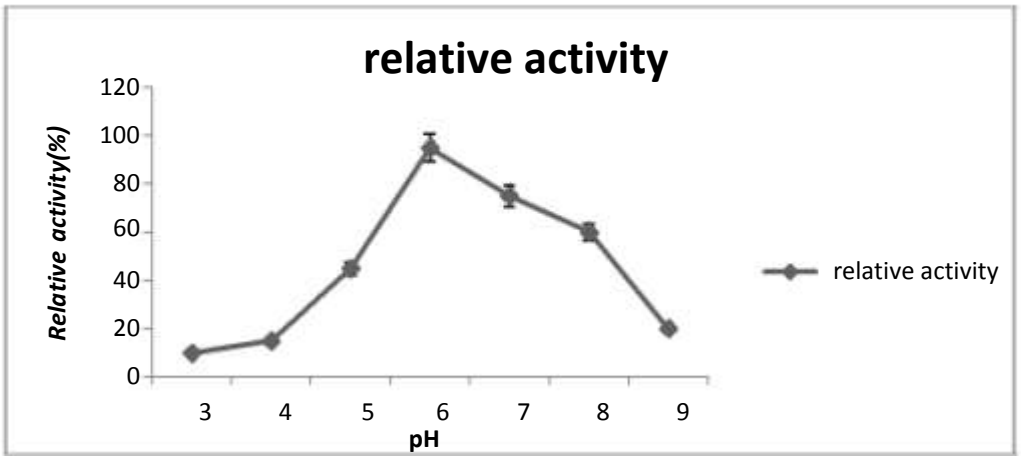


Figure: 10 Graph of pH gradient with respect to relative enzymatic activity for Bacillus subtilus.

The figure above shows the bacterial alpha-amylase activity assay at different pH gradients using DNS alpha-amylase activity assay. Our enzyme shows maximum activity at pH 6.

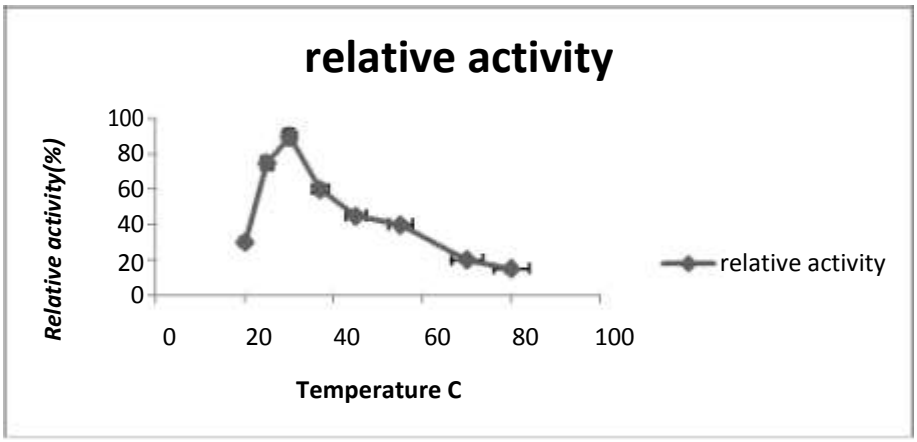
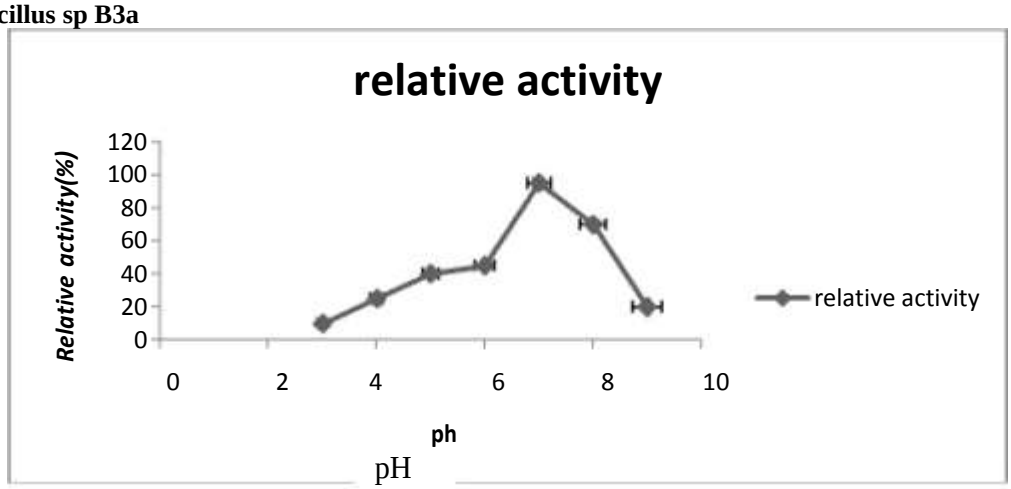


Figure: 11 Graph of temperature gradient with respect to relative enzymatic activity for Bacillus subtilus.

The figure: 11 above shows the bacterial alpha-amylase activity at different temperature using DNS alpha-amylase activity assay method. The enzyme shows maximum activity at 30^{o}C. In the conclusion, our enzyme shows maximum activity at pH 6 and 30^{o}C temperature.

Paenibacillus sp B3a



pH

Figure:12 Graph of pH gradient with to relative enzymatic activity for Paenibacillus sp B3a

The figure above shows the bacterial alpha-amylase activity at different ph gradients using DNS alpha-amylase activity assay method. The enzyme shows maximum activity at pH 7.

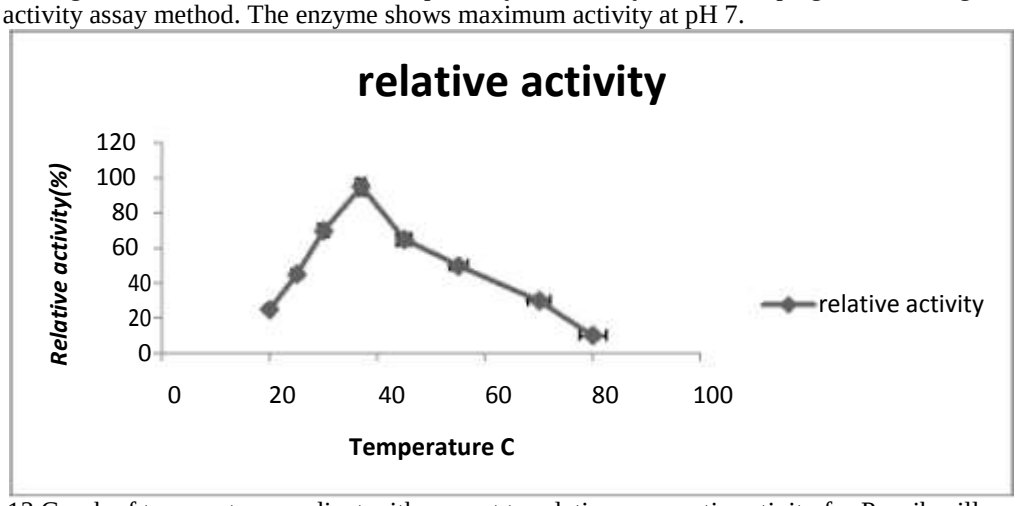


Figure:13 Graph of temperature gradient with respect to relative enzymatic activity for Paenibacillus sp B3a.

The figure: 13 above shows alpha-amylase activity at different temperature gradients using DNS alpha-amylase activity assay method. The maximum enzymatic activity is at 37^{o} C. In the conclusion, our enzyme shows maximum activity at pH 7 and 37^{o} C.

IV. Discussion

Different types of soil favour the growth of alpha-amylase producing microorganisms such as fungi and bacteria. In this study, we were able to isolate and identify three bacterial species that are capable of producing alpha-amylase activity from different soil environments. Our investigation shows that, the alpha-amylase producing bacteria grow best in a soil that is rich in nutrients that favour the growth and survival of these bacteria. Growth and survival of soil bacteria is attributed to the possession of spores that help them to resist adverse environmental conditions in the soil such as severe heat, cold and drying. To cope with the adverse environmental conditions mentioned above, these bacteria have the ability to produce endospore as the cell envelope. The nutrients that favour the growth of these bacteria in the soil originated from decayed leaves, kitchen- wastes or dead organic matters. Sasnita Mishra and Niranjar Behera were able to isolate and identify bacteria that show amylase activity from soil receiving kitchen wastes [10]. Similarly, Oseini, O.A. and Ekperigin M.M., isolated and identified bacterial strains from soil in the forest that show alpha-amylase activity [11].

As it is obtained from the result, majority of the soil bacteria that show alpha-amylase activity belong to the genus Bacillus. They belong to the family Bacillaceae that are Gram-positive, spore-forming, aerobic or facultative anaerobe and rod in shape. Following Gram's staining technique; the colonies that show alpha-amylase activities are Gram-positive and appear rod in shape morphologically.

Furthermore, phylogenetic analysis based on 16s ribosomal RNA sequence, shows that Bacillus mojavensus, Bacillus subtilus and Paenibacillus sp B3a were the identified bacterial species that showed positive response to alpha-amylase activity. Many researchers have reported the isolation and identification of these bacteria from different soil environments as the major producers of alpha-amylase. For example, Noomen et al isolated and identified Bacillus mojavensus from the soil of marine water as an organism that produces alpha-amylase [12].

On the basis of their temperature requirement, bacteria are classified into psychrophilic, mesophilic and thermophilic type. Similarly, bacteria can also be classified into acidophilic, alkalophilic and neutrophilic on the basis of their pH requirement. Therefore, pH and temperature play a vital role in the growth and survival of bacteria in different environment. Also ezymatic activity can be altered by these parameters. For this reason, the effects of pH and temperature on the alpha-amylase activity shown by these bacteria were studied spectrophotometrically using DNS activity assay method. As it is obtained from the result, the enzymatic activity varies with respect to the bacterial specie. Bacillus mojavensus shows maximum activity at 37^{o}C and pH 8. Bacillus subtilus shows maximum amylase activity at 30^{o}C and pH 6. Finally, Paenibacillus sp B3a shows the maximum enzymatic activity at pH 7 and 37^{o}C. Therefore, the results show that the bacterial species identified show alpha-amylase activity at the pH range of 6 to 8 and temperature range of 30^{o}C to 37^{o}C. The variation in the pH and temperature can be attributed to the type of bacteria and the soil type. In contrary to this research, some researchers were able to isolate and identify bacteria that produce thermostable alpha-amylase. It means the enzyme produced by the bacteria is at higher temperature (40^{o}C to above). Example, Vasakaran, S. et al

reported the isolation and identification of bacterial strain that are capable of producing thermostable alpha-amylase [13]. Similarly, some researchers have reported the isolation and identification of halophilic alpha-amylase producing bacteria. Prakash et al reported the isolation, purification and characterization of two extremely halotolerant, thermostable and alkali-stable alpha-amylase from chromohalobacter sp TVSP 101. [14].

Generally, all the bacterial species identified, show optimal alpha-amylase activity at mesophilic temperature (30^{o}C to 37^{o}C) out of which two species (Bacillus mojavensus and Paenibacillus sp B3a) show maximum alpha-amylase activity at 37^{o}C while, the other specie (Bacillus subtilus) shows 30^{o}C as maximum enzymatic activity. In terms of pH, both Bacillus mojavensus and Paenibacillus sp B3a show the maximum alpha-amylase activity at alkalophilic pH (7 and 8) while Bacillus subtilus show the maximum enzymatic activity at acidophilic pH.

V. Conclusion

Phylogenetic analysis based on 16s ribosomal RNA sequence showed that species of bacteria belonging to the family Bacillaceae were responsible for showing alpha-amylase activity, also it can be concluded that, alpha-amylase activity can be detected from different species of soil dwelling microorganisms particularly bacteria.

Competing Interests

Authors have declared that no Competing interest exist.

Reference

[1] Pandey, A., Nigam P., Soccol VT., Singh D. and Mohan R. Advances in microbial amylases. Biotechnol. Appl. Biochem, Vol. 31, pp. 135-152, 2000.

[2] Kathiresan, K. and Manivannan S. Alpha-amylase production by Penicillium fellutanum isolated from mangrove rhizospheric soil. Afr. J. Biotechnol, Vol. 5, pp. 829-832, 2006.

[3] Schmidt, S., Martin A., Schadt, C., Lipson, D., Meyer, A., Costello, E., Nemergut, D., West, A., and Metchalf, J. Profound seasonal changes in microbial diversity and function Alpine Environment. Mc-Graw Hill, New York, pp. 776-795, 1991.

[4] Rusell, E.W. Soil conditions and plant Growth. 10th eds. William Clowes and Sons Ltd., London, pp. 150, 1977.

[5] Octavio, L.F., Daniel, J.R., Francislete, R.M., Carlos Bloch Jr., Carlos, P.S. and Maria F.G. Activity of wheat alpha-amylase inhibitors towards burchid alpha-amylase and structural explanations of observed specificities. Eur. J. Biochem, Vol. 267, pp. 2166-2173, 2000.

[6] Richele, D.W., M.B. and Lubbert, D. Engineering of factors determining alpha-amylase and cyclodextrin glycosyltransferase specificity in the cyclodextrin glycosyltransferase from Themoanaerobacterium thermosulfurigenes EMI. European Journal of Biochemistry, Vol. 253 (3), pp. 598-605, 1998.

[7] Najafi, M.F. and Deobagkar, D. Protein Expression and Purification, Vol. 41, pp. 349-354,2005.

[8] Kiran, O.I., Comlekcioglu, U.I. and Arikan, B. Effects of carbon sources and various chemicals on the production of a novel amylase from a thermophilic Bacillus sp. K-12. Turk. J. Biol, Vol. 29, pp. 99-103, 2005.

[9] Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K.M., Soccol, C.R. and Pandey, A. Alpha-amylase from microbial sources – an overview on recent development. Food Technol. Biotechnol, Vol. 44, pp. 173-174, 2006.

[10] Sasmita Mishra and Niranjan Behera. Amylase activity of starch degrading bacteria isolated from soil receiving kitchen wastes. African Journal of Biotechnology, Vol. 7 (18), pp. 3326-3331, 2008.

[11] Oseni, O.A. and Ekperigin, M.M. Isolation and activity of alpha-amylase from selected bacteria strains in the forest soils. Global Journal of Bioscience and Biotechnology, Vol. 1, pp. 17-20, 2013.

[12] Noomen Hmidet, Hana Maalej, Anisa Haddar and Moncef Nasri. A novel alpha-amylase from Bacillus mojavensus A-21: Purification and Biochemical Characterization. Appl. Biochem. Biotechnol., Vol. 162, pp. 1018-1030, 2010.

[13] Vaseekaran, S., Balakumar, S. and Arasaratnam, V. Isolation and identification of bacterial strain producing thermostable alpha-amylase. Tropical Agricultural Research, Vol. 22(1), pp. 1-17, 2010.

[14] Prakash, B., Vidyasagar, M., Madhukumar, M.S., Muralikrishna, G.J and Sreeramulu, K. Production, purification and characterization of two extremely halotolerant, thermostable and alkali stable alpha-amylases from Chromohalobacter sp. TVSP-101. Process Biochemistry, Vol. 44, pp. 210-215, 20