Industrially Important Amylase Enzyme Producing Bacteria Isolated and Characterized From Mangrove Soil of Bhitarkanika

Eliza Priyadarshini
MITS School of Biotechnology, Bhubaneswar, Odisha, India

Abstract— Amylases are catalysts that catalyze the hydrolysis of starch into oligosaccharides, maltose and glucose. They are generally disseminated in microbial, plant and creature realms. α-amylases are one of generally significant and utilized compounds whose scope of use has augmented in numerous parts, which incorporate assembling of High Fructose Corn Syrup (HFCS).This study is planned for auditing related writing on secluding and creation of amylase from microscopic organisms. Writing checked on in this article incorporate those on confinement of microscopic organisms delivering amylase, creation and streamlining forms included and other firmly related and late writing. This paper depicts the disconnection of microscopic organisms from Bhitarkanika mangrove biological system of Orissa and screening for their anti bacterial properties. This examination additionally tries to find research hole in this field of study that should be filled by scientists and proper suggestions made. The compounds from microbial sources are steadier and acquired inexpensively. Amylases are among the most significant chemicals and are of incredible hugeness in present day industry. Starch debasing microorganisms are generally significant for enterprises, for example, food, maturation, material and paper.

Keywords: Preparation of Amylase, Dextrinising Activity, Assay Method, Enzyme Characterization, Total Protein Estimation, Enzyme Assay, Reducing Sugar Activity

I. INTRODUCTION

The microbial metabolism at intracellular and extracellular levelincreasing a lot of significance to give different valuable results of modern significance viz. compounds, sugars, anti-toxins and natural acids and so forth. Such enhanced microbial frameworks are likewise announced from mangrove biological system .Mangroves, more than a large number of years, have advanced both morphologically and physiologically to adjust to marshy and saline conditions. Comparable versatile attributes in the structure and capacity may happen with the related micro flora in such situations. Amylase was the primary chemical to be found and detached (by AnselmePayen in 1833). (Richard, 2002). Starches are a significant class of particles utilized for food by all creatures. Proteins digest these unpredictable sugars and convert them into less complex atoms, acting in a fixation subordinate style without being changed themselves. One normal catalyst is amylase, which separates starch into glucose. The protein is found in numerous spots in the body and has been a staple of investigations intended to read enzymatic reactions for a long time. α-Amylase separate the α(1–4) glycosidic securities in starch into the basic sugar glucose that is expended during glycolysis. Starch debasing amylase compounds are generally significant in the biotechnology enterprises with enormous application in food, aging, material and paper. Since microorganisms are managable to hereditary building, strains can be improved for acquiring higher amylase yields.

II. TYPES OF AMYLASE

1) α-amylase (1,4-α-D-glucanglucanohydrolase; glycogenase) Amylase is calcium metallo compounds and act aimlessly areas along the starch bind lead to breakdown of long chain sugars into maltotriose and maltose from amylose, or maltose, glucose and cutoff dextrin" from amylopectin, α-Amylase will in general be quicker acting than β-amylase and in creatures, it is a significant stomach related chemical having ideal pH is 6.7 - 7.0. In human physiology, both the salivary and pancreatic amylases are α-Amylases and furthermore found in plants (grain), beats, growths (ascomycetes and basidiomycetes) and microscopic organisms (Bacillus).
2) β-amylase (interchange names: 1,4-α-D-glucanmaltohydrolase; glycogenase; saccharogen amylase). β-amylase is additionally integrated by microbes, growths, beats seeds and plants catalyzes the hydrolysis of the second α-1,4 glycosidic security, severing off two glucose units (maltose) at a time. During the maturing of organic product, β-amylase separates starch into sugar that brings about improving of ready natural product. β-amylase is available before germination, while α-amylase and proteases show up toward the beginning of germination. Creature tissues don't contain β-amylase, in spite of the fact that it might be available in microorganisms contained inside the stomach related parcel.
3) γ-amylase (elective names: Glucan 1,4-α-glucosidase; amyloglucosidase; Exo-1,4-α-glucosidase; glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucanglucohydrolase) γ-amylase severs last α(1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose alongside α(1-6) glycosidic linkages. In contrast to different types of amylase, γ-amylase is generally effective in acidic situations and has an ideal pH 3.

A. Uses:

Amylases are the one which are broadly utilized in enterprises where the use is stretched out from different food preparing and material manufacturing plants to clinical fields. Among different wellsprings of amylase creation, their creations from microorganisms are supported in wide-arriving at divisions. Amylase is yielded from numerous microbes and other miniaturized scale living beings in littler sums even without the presence of starch, yet its creation is high when adequate starch is given. A few sorts of sugars are accessible, for example, galactose, fructose, sucrose, lipase, glucose and maltose of which fructose is reasonable for most extreme amylase creation.

III. MATERIALS AND METHODS:

A. Sample collection site:

The sediment samples were composed of three sections which were selected at Bhitarkanika area. All the samples are collected named as Bhitarkanika sample (BKS) 1,2 and 3.The surface sediment were collected near mangrove trees viz. Khola, Dangmal and Rangani distributed throughout the mangrove forest and are 5-10 km apart from each other.

B. Soil sample collection:

The soil samples were collected from three different locations of the National Park. Top layer of soil (about 1 cm) was removed. Three soil samples were collected from three spots from three different locations. Samples were mixed thoroughly and put in sterile polythene bags with proper labelling, stored in ice box and brought to the laboratory for further analysis. In the laboratory, the samples were stored at 4 ± 0.1° C in a refrigerator.

C. Isolation of Bacteria:

1 gm of each soil sample was serially diluted in 9 gm of sterile (121oC for 15mins) distilled water up to 10-6 NA (2.8gm in 100ml of distilled water) plates were prepared and they were left to solidify. After solidification 0.1µl of each serially diluted sample were spread over the solidified NA plates with a sterile L spreader. The plates were labelled correctly and were kept in the BOD incubator at 30 ± 0.1°C for 24 hrs for further incubation. Incubation time was 24hrs for the bacteria. After 24hrs the resulted colonies were randomly isolated from the plate based on the colony morphology. The isolated colonies were then striped on NA plates to obtain single colonies. After obtaining single colonies the bacteria were transferred to NA slants for further uses.

1) Composition of nutrient agar plate:

28gms of NA powder was suspended in 1000ml of distilled water.

For 100ml of distilled water 2.8gms of NA powder.

As these bacteria are salt tolerant 0.5% extra NaCl was added to the media while preparing.

D. Screening of Amylase Activity:

All bacterial strains are isolated from different sample were screened for the invention of amylase on plates of starch agar containing (w/v) 0.2% soluble starch,1.3% nutrient broth , and 1.5% agar. Marine bacteria were cultured on ZoBell's 2216E marine agar plates and a small amount of cell paste was scraped and deposited onto the surface of starch agar. The plates were then incubated for 48hr at 28 ° C. Afterwards the plates were flooded with iodine solution [(0.2% (w/v) iodine and 2 % (w/v) potassium iodide]. A colourless zone around cells of marine bacteria indicated amylase activity.

E. Colony and cell Morphology of Bacterial Identification

Bacteria were isolated from BKS-1, BKS-2 and BKS-3 marine samples. Bacteria were identified after different serial dilution like 10^{-1} to 10^{-6} dilutions. All thirty one bacterial isolates were selected for further studies on the basis of the colony morphology and the colour keeping in mind that there may be different representative for each sample. Colonial pigmentation of the bacterial isolates include pale yellow , pale white , yellow , creame, and off white colour. These colonies were irregular and regular in form while few were rounded projection, tiny, zigzag and spreader in form. Most of the colonies surface was smooth (shiny, glistering) and (dull, granular or mate). Colony elevations were flat and convex. few colonies were small in size and few were large. Most of the colonies were opaque and few were translucent. Different bacterial isolates displayed different cell sizes like 0.5, 0.7, 1.0, 1.2, and 1.6 and different morphologies. When viewed under microscope, they were rod shaped, Baciilus and Coccobacil were very rare in individual or chain from.

F. Screening of all bacterial sample for amylase

After isolation of 31 bacterial strains from three sampling sites, colony morphology was studied and noted. Further all 31 bacterial strains were screened for amylase activity on agar plates, out of 31 strains 9 no of bacteria showed positive result. Out of 9 no of amylase positive strains three strains showed best result .The biochemical characterization for those three (BKS 03/02, 03/08, 03/12) were carried out.

G. Amylase producing bacteria and zone size on starch agar plates

All 31 isolated bacterial strains were screened on agar plate using starch as a substrate, out of 31 strains were positive on starch plates. On repeated screening the best three (BKS - 03/08, 03/02 & 03/12) bacterial strain were selected for identification by biochemical characterization.

On measurement of zone size on starch agar plate, out of the best three BKS 03/08, 03/02 & 03/12, BKS 8 showed the highest zone size. Hence BKS 8 was selected for further studies like enzyme assay and effect of environmental parameters.

(Fig: Starch plates showing zone for Amylase activity)

Sl no.	Bacterial code	Gram staining	Cell Morphology	Indole test	MR Test	VP Test	Citrate Test	TSI Test	Oxidase Test	Catalase Test	Tributyin Test	Tween 20 Test	Casin test
1	BKS-2	+ve	Bacilus	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve
2	BKS-8	+ve	Cocco	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve

			bacili										
3	BKS-12	+ve	Cocco bacili	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve

Table 1: Biochemical analysis of best three amylase producers

– The biochemical tests were carried out only for the best amylase producers.

Bacteria isolated from starch rich materials may have better potential to produce enzyme under adverse condition (Mishra and Behera 2008). Microorganisms that produce amylases could be isolated from places such as mills, cassava farms and processing factories as well as flour markets (Fossi et.al 2005). In the present study the amylase producing bacteria were isolated from mangrove area which is rich in mangrove plant litter falls.

H. Dextrinising Activity

In the standard assay the reaction mixture containing 250µl of enzyme solution, 250µl of 0.2 mol/l buffer (optimum pH) and 500µl of 2gm/l soluble starch in same buffer incubated at optimum temperature for 30 min. The reaction was stopped by 1ml of 1.5N acetic and 1ml iodine reagent [0.2%(w/v) iodine and 2.0%(w/v) potassium iodide].The absorbance of the mixture was read at 690nm (Saito et al 1975).One unit of amylase activity was defined as the amount of enzyme necessary to degrade 1µgram of starch per min under the assay condition.

I. Assay Method

1) Reducing Sugar Activity:

In the standard assay, the reaction mixture containing 250µl of enzyme solution,250µl of 0.2mol/l phosphate buffer(pH 7.0) and 500µ of 2 gm/l soluble starch in the same buffer incubated at 37ºC for 30 min. The reaction was stopped by placing the tube in a boiling water bath and the liberated glucose was estimated by dinitro salicylic acid method (Miller 1959). One unit of activity was defined as the amount of enzyme necessary to liberated glucose was estimated by dintro salicylic acid method (Miller 1959). One unit of activity was defined as the amount of enzyme necessary to liberate 1µmol of glucose per min under the experimental conditions.

2) Preparation of Crude Enzyme

The Bacterial strains were cultured in liquid broth medium (pH 7.0) containing-

Peptone	0.5%
Yeast Extract	0.3%
Starch	1 gm
Nacl	0.5gm
50% Distilled water	100 ml

And incubated for 48 hours at 37º C in a rotatory shaker (200 rpm) cultured cells were harvested by centrifugation at 10,000 rpm for 30 minutes at 4º C. Supernatent were collected in sterilized conical flask and pallet containing cells were suspended in 10mM Tris-HCI buffer (pH 7.2) and disrupted by freezing and thawing followed by grinding with glass power in an ice bath. After centrifugation the supernatant fluid was used as crude enzyme.

3) Standard Curve of Glucose:

For Glucose Standard Curve, aliquots of the glucose solution in different concentration (0.25mM, 0.50mM, 0.75mM, 1.0mM, 1.0mM, 1.25mM, 1.50mM, 1.75mM, 2 mM, 2.25mM and 2.5mM) were taken in test tubes and incubated at 37º C for 30 minutes followed by adding 3ml of DNS (Di-nitro salicylic acid) reagent. After 5 minutes in boiling water bath and quick cooling to room temperature, the degree of enzymatic hydrolysis of the cellulose were determined spectrophotometry (UV-Vis Spectrophotometer, Syntronics, Model-117, India) by measuring the absorbance at 575 nm. The unit of enzyme required to release 1 µmol of reducing sugars as amylase equivalent min g^1 of the enzyme sample.

IV. ENZYME CHARACTERIZATION

A. Amylase activity:

Amylase activity was measured with soluble starch as the substrate. Here aliquots of the enzyme solution in different concentration (50µl, 100µl, 150µl, 200µl, 250µl, 300µl, 350µl, 400µl, 450µl and 500 µl) were taken and buffer solution of pH (7.0) was added to make volume 500ul. Then 500ul of starch solution was added to each sample followed by incubation at optimum 37 º C for 30 minutes.

B. Effect of pH:

For effect of pH, aliquots of the enzyme solution and 2 ml of respective buffer were incubated with 4 ml of 02% starch solution and 1 mi of crude enzyme at 37 º C at different pH values (pH 3 to 5.5 using 0.1 M sodium citrate; pH 5.5 to 8 using 0.2 M sodium phosphate.) These reactions were stopped by adding 1 ml of 1.5 N Acetic acid and 1 ml of iodine reagent.

C. Effect of Time:

The degree of enzymatic hydrolysis of the amylase was determined by spectrophotometriacally by measuring the absorbance at 690 nm (UV-Vis Spectrophotometer, Syntronics, Model-117, India). The unit of enzyme required to release 1umol of reducing sugars as Amylase equivalent min/g of the enzyme sample.

D. Effect of Temperature:

For the effect of Temperature, aliquots of the enzyme solution and 2 ml of respective buffer were incubated with 4 ml of 0.2% starch solution at different temperature (4 º C, 10 º C, 20 º C, 37 º C, 60ºC, 100 º C) .These reactions were stopped by adding 1 ml of Acetic acid and 1 ml of Iodine reagent. The degree of enzymatic hydrolysis of the amylase was determined by spectrophotometry (UV-vis Spectrophotometer, Syntronics, Model-117 India) at 690 nm. The unit of enzyme required to release 1µmol of reducing sugars as amylase equivalent min/g of the enzyme sample.

E. Effect of Salinity:

Effect of salinity was examined by using Nacl solution ranges from 1%-12%. For this, 0.5 ml of the enzyme solution and 0.5 ml of the enzyme solution and 0.5ml of respective buffer were incubated with 2 ml of 0.2 % starch

solution and 0.5 ml of respective buffer were incubated with 2 ml of 0.2% starch solution at different Salinity (1,2,3,4,5,6,7,8,9,10,11,12) at optimum temperature. After incubation reaction was stopped by adding 1 ml acetic acid and 1 ml Iodine reagent. The absorbance of the mixture was read at 690 nm.

1) Total Protein Estimation:

The protein concentration of the best amylase procedure (BKS 03/08) was estimated by Barford method and the protein estimated was 0.15mg/ml protein concentration (Mx+c) formula, showing in fig: 1.1.

2) Enzyme Assay:

Amylase enzyme played an important role in biotechnological industries and has a number of potential applications in food, fermentation, textile and paper industries. The spectrum of applications of - amylases has widened in many sectors such as clinical, medicinal and analytical chemistry.

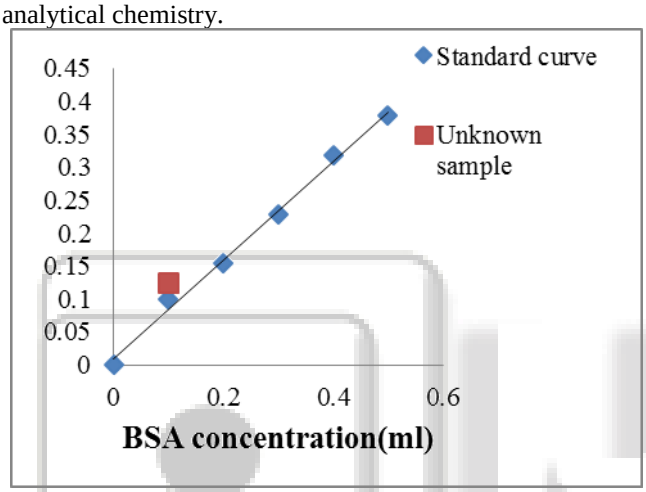

Fig. 1.1: Standard Curve of Total Protein Estimation

Amylases can be obtained from several sources such as plants, animals and microbes. Many microorganisms especially several species belonging Bacillus sp. are known to produce a variety of extracellular enzymes with a wide range of industrial application. The microbial source of amylase is favoured to other sources because of its plasticity and vast availability. Micro-organisms have become increasingly important as producers of industrial enzymes due to their biochemical diversity and the ease of improving the enzyme productivity through environmental optimization and genetic manipulation. There are various reports on starch degrading micro-organisms from different sources and respective amylase activity. Among bacteria, Bacillus sp. is widely used for amylase production to meet the industrial needs. Amylase production in different Bacillus sp has been reported by several workers. In the currenamylase producing ability of three Bacillus sp. were checked and all were found to be positive, but B. megateriumwas found to be the best amylase producer. However Vipulvermaet al. found the maximum amount of amylase production in B. subtilisfollowed by B. megaterium, among nine strains tested which included B. cereus, B. megateriumandB. subtilis. These enzymes have traditionally been obtained from submerged cultures because of ease of handling and greater control of environmental factors such as temperature and PH. The maximum amylase producing - B. megateriumwas taken for optimization studies through submerged fermentation by and nitrogen source, since the production of amylase enzymes are influenced by diverse physico-chemical and biological factors.

3) Enzymatic Activity of Amylase:

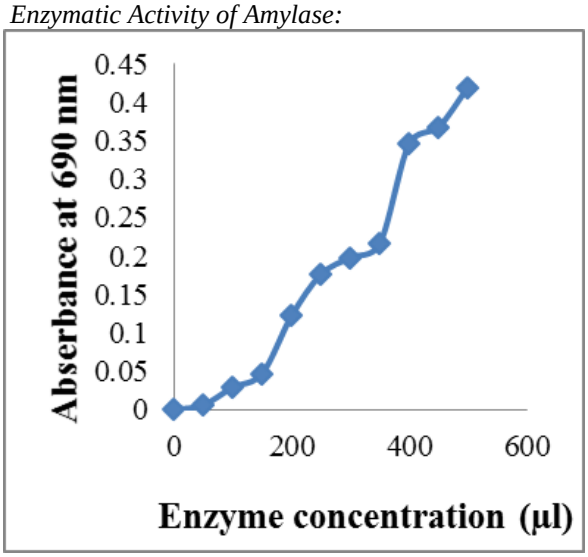

Fig. 2.1: Amylase activity with increased concentration

4) Reducing Sugar Activity:

Reducing sugar, expressed as glucose was measured by the dinitrosalisylic acid reagent method of Miller (1959). In both the method keeping enzyme amount constant, we increase the substrate concentration, which showed increase in enzyme activity.

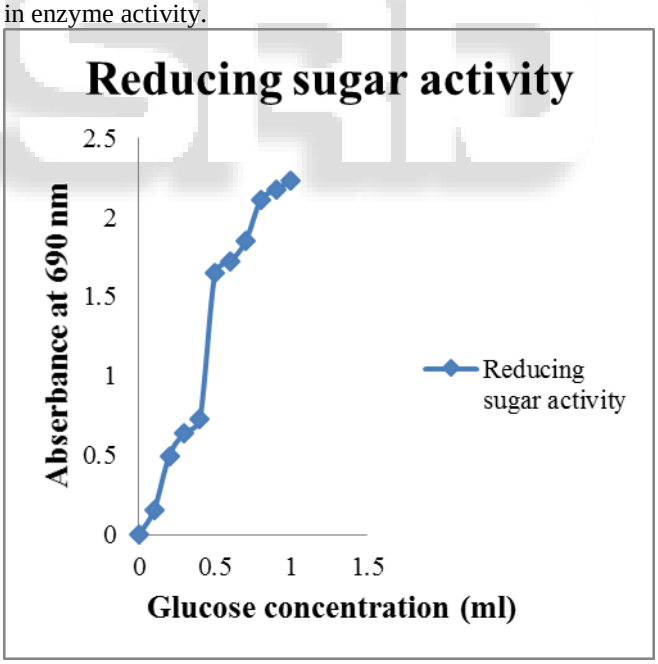

Fig. 2.2: Reducing Sugar Activity with increased concentration

V. Optimization of Incubation Time and Temperature on Amylase Production

A. Effect of Time Period:

The effect of different incubation time was taken on amylase production .The maximum amount of amylase activity was found at 30 min incubation time at room temperature. The stability of crude enzyme at different temperature was

studied ranging from 4º C to 100º C. above 40º C the enzyme activity was drastically reduced. There was almost no enzyme activity above 60º C.

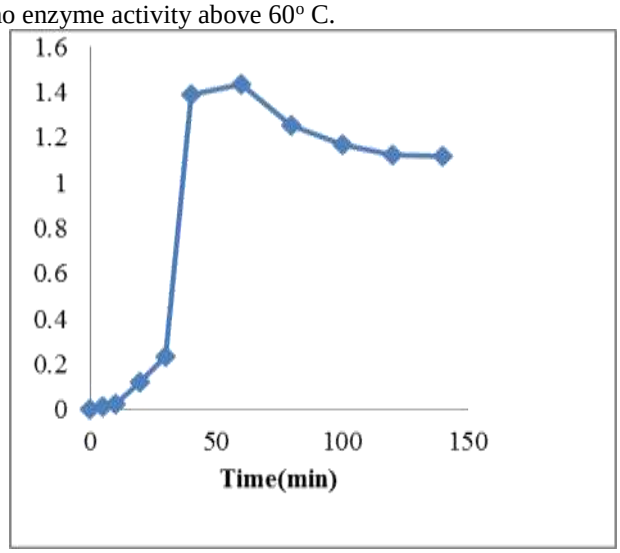

Fig. 3.1: Activity of enzyme with different time period

B. Effect of Different Temperature:

Fig no 3.2showed the optimum temperature for the maximum amylase activity of our test bacteria. The enzyme activity of these bacteria was found to be within the range of the value reported earlier for this enzyme from Acinetobactersp (Onishi et al 1978), Halobacterium (Onishi 1972).

C. Effect of Different pH:

Amylase yield by the test bacteria depends on pH value of the media. Result illustrated clearly show the amylase production, expressed as amylase activity. The enzyme activity gradually increased as the pH value increase from 3 to 10 and showed the maximum activity at pH 7.5. The pH optima is almost similar with the value reported for this enzyme from other halophilic bacteria like halo bacterium halobium (Onish1972). The amylase activity was less in other tested pH levels where enzyme activity was minimum at pH 3 and it indicates a sudden increase at pH 5 and 7.5 further this activity started decreasing to reach the lowest at pH 10.

D. Effect of Different Salinity:

The maximum enzyme activity was recorded at 3% sodium chloride concentration as in the sea water. Reaction mixture showed least activity within Nacl. Enzyme activity reduced drastically. After 6% Nacl. In general amylase required 1-6% Nacl for exhibiting maximum activity. Even at higher activity concentration of Nacl 10% considerable activity was record

E. Effect of Different Heavy Metals:

The effect of metal ions on amylase activity shows that the activity is not much affected by major cations of backwater cu^{+2}, mn^{+2} and fe^{+2} at 4 PPM but inhibited by more than 4 PPM. The enzyme activity also not much at lower metal concentration 2 PPM mg^{+2} almost does not support the enzyme activity in comparision to other test metals. Presence of certain metal ions provided good enzyme activity like cu^{+2} (as most amylase is known to be metalloenzymes). Manganese sulphate also was found to be another suitable sulphate source for enzyme activity (Sodhi et al 2005), but fecl3 and MgSO4 exhibited negative influence by suppressing enzyme activity.

VI. Conclusion

The paramount importance of mangrove vegetation especially in the Bhitarkanika area of Odisha keeping in view the topographic diversities, Zonal pattern, vegetational characteristics, faunal composition, and aquaculture feasibility though last on the conservation sites has been extensively researched. The research in this study on different aspects indicates the productivity of that environment. The heterotrophic bacteria at the brackish water environments play an important role in the decomposition of organic matter and in the regeneration of minerals. It has been investigated a total 31 different morphotype bacteria were meticulously isolated from three different stations of the study area and have been identified by biochemical characterization.

Growth curve of some important bacteria also have been studied to assess the viability of those strains. Also a total protein estimation has been carried out which shows the bacteria are producing different kinds of proteins and enzymes in good amount , which indicates the study area is quite productive and the nutrient cycle is active which indirectly boost the fauna and flora composition. To understand some of the important microbiological parameters like enzyme activity and to assess the degrees of degradation by microbes which help in the flows of food chain a work was carried out to study enzymatic activity.

References

[1] Amira El-Fallal, M. A.-S. (2012). Starch and Microbial α-Amylases: From Concepts to Biotechnological Applications. In C.-F. Chang, Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology (pp. 459-476). CC BY 3.0 license
[2] Amira El-Fallal, M. A.-S. (2012). Starch and Microbial α-Amylases: From Concepts to Biotechnological Applications. In C.-F. Chang, Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology (pp. 459-476). CC BY 3.0 licence.
[3] Anselme, Payen and Jean-François Persoz, 1833.Memoir on diastase, the principal products of its reactions and their applications to the industrial arts)".Annales de
[4] Chimieet de Physique 2 series, 53: 73-92
[5] Kathiresan R, (2006). α-Amylase production by Penicilliumfellutanum isolated from mangrove rhizolspheric soil. Afr. J. Biotechnol., 5: 829-832.
[6] Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for the determination of reducing sugar.Anal. Chem. 31, 426 – 428.
[7] Mishra S, Behera N (2008) Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen waste. African J of Biotechnology 7:3326–3331.
[8] Pandey, A.; Nigam, P.; Soccol, C.R.; Soccol, V.T.; Singh, D.; Mohan, R. (2000). Advances in microbial amylases.BiotechnolApplBiochem 31 (Pt 2), 135-152

[9] Rani Gupta, P. G. (2003). Microbial α-amylases: a biotechnological perspective. Process Biochemistry, 1-18.
[10] Richard, B. (2002). The Organic Chemistry of Enzyme-catalyzed Reactions, 2nd ed. London, England: Acadamic Press
[11] Sodhi HK, Sharma K, Gupta JK, Soni SK (2005) Production of a thermostable α-amylase from Bacillus sp. PS-7 by solid-state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. ProcBiochem 40:525–534. doi:10.1016/j.procbio.2003.10.008.
[12] ZoBell CE (1996) Marine microbiology.A monograph on hydrobacteriology. ChronicaBotanicaCo., Waltham.

