Isolation and Characterization of Alpha-amylase Producing Bacillus Stearothermophilus from Banana

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Abstract

Starch as a natural polymer has attracted significant interest and is currently used in numerous industrial applications. This is because of its renewability, biodegradability, abundance, and cohesive film-forming properties. Moreover, the hydroxyl (OH) groups associated with the hydro glucose units provide it with several modification possibilities. These features have resulted in substantial interest for its use in several advanced functional material applications in addition to the typical consumer plastic applications. The

goal of this review is to shed light on the recent advances achieved in the utilization of starch for advanced functional material applications and its derivatives. The review specifically focuses on applications ranging from electronics, drug delivery, pharmaceuticals, and antimicrobial materials to structural materials.

Keywords: *Enzymatic Activity, Starch Degradation, Alpha-amylase, Banana, Temperature Optimization, Characterization, Amylase Assay, Partial Purification, Protein Annotation.*

1. Introduction

Plants, microbes, and higher creatures are all capable of producing Alpha-amylase (1,4 glucanohydrolase). This enzyme belongs to the endo-amylases family, which is responsible for the first breakdown of starch into glucose and maltose by breaking the linkages in the starch (Magaji, 2018). Several bacteria may make alpha-amylase, but commercial use requires a specific kind of microbe. Bacillus is the primary source of alpha-amylase. In the food, textile, and paper sectors, alpha-amylases from Bacillus licheniformis, Bacillus stearothermophilus, and Bacillusamyloliquefaciens have potential use. Alpha-amylases and their derivatives are economically significant enzymes produced by several Bacillus species(Tangphatsornruang et al., 2005). This enzyme is used in a variety of production processes, including industrial, environmental, and food biotechnology.

Detergent quality can be improved by modifying bleaching using alpha-amylase, a common enzyme. The addition of enzymes to laundry detergents and soap bars improves the durability and efficacy of bleach. (Haq et al., 2010). Alpha-amylase has broad applications to hydrolyze and solubilize starch in the majority of the textile industry. The coated paper uses alpha-amylase for starch production and modification. In addition to enhancing paper quality, the coating also protects it from mechanical damage and enhances its rigidity and strength. Alpha-amylase is required for the sizing and coating of paper because it converts raw starch into glucose and fructose. As a result, alpha-amylase is frequently employed in the paper-sizing process (Bozic et al., 2011).

2. Material and Method

2.1. Sample collection

The organisms were isolated from banana samples taken in a sterile polythene bag and precautions were taken to minimize the contamination.

2.2. Media preparation

Petri plates were taken and autoclaved in a heating oven for 120 minutes at 180˚ C. The starch agar media was used and each nutrient was measured by using an electric weighing balance. After pouring, the plates were placed in incubation for 24 hours at 370 C(Hajra et al., 2023).

2.2.1. Streaking of bacterial sample

The agar plate was streaked by the quadrant streak method.

2.3 Screening and isolation

After 48 hours, bacterial colonies were observed on the colony counter. The colorless and rod-shaped colonies were observed and counted. Then these new colonies were picked and the streak-plate method was repeated under the sterilized condition of Laminar Air Flow to obtain pure colony. After re-streaking, the Petri plates were placed in incubation at 370 C for 24 to 48 hours(Usman et al., 2023).

2.3.1. Hydrolysis test

This test was used to screen the alpha-amylase-producing bacteria by the zone formation.(Rehman et al., 2015).

2.4. Identification of microbe

The structural and functional attributes of bacteria were examined under a microscope morphologically, staining, and biochemical testing.

2.5 Characterization of a bacterial enzyme

Alpha amylase-producing bacterial colonies expressed as the largest clear zones on Petri plates were used for characterization. The isolated bacterial colony was used by changing different physical parameters at different carbon sources, nitrogen sources, incubation periods, temperatures, pH, and metal ions and the maximum enzyme activity was calculated. The bacteria were grown in LB media to carry out the characterization of bacterial enzymes (Yassin et al., 2021).

2.6 Amylase assay

Dinitro salicylic acid method was applied to perform the amylolytic assay. Extracellular and Intracellular assays were done in U/ml.

2.7. Bioinformatics analysis

Prediction of the alpha-amylase sequence of Bacillus stearothermophilus was done using different bioinformatics tools. Sequence alignment, determination of the composition of protein through preparation, protein secondary structure prediction using PSIPRED, JPRED, Protein Prediction, and PROTEUS. Tertiary structure prediction using PS2 and Swiss model (Velteri et al., 2004).

3. Results and Discussion

3.1. Isolation and screening of amylase-producing bacteria

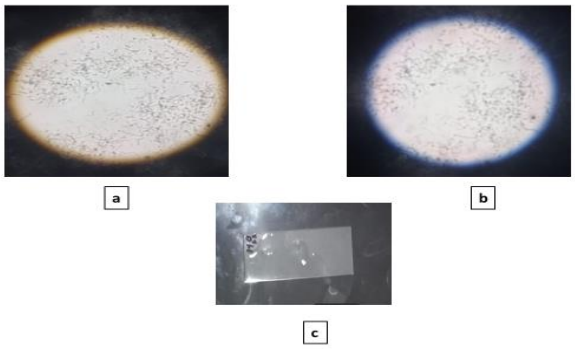
For screening, a hydrolysis test was performed. When the iodine solution was added to the streaking plates having bacterial colonies, clear zones of hydrolysis of bluish-purple color were observed as shown in Fig. 3.1. In this way, amylase-producing bacteria were screened with clear zones around their colonies.

Figure 3.1: Visualization of clear zones of hydrolysis of bluish-purple color.

3.2. Biochemical and stainingtesting

Biochemical tests were performed to identify alpha amylase-producing bacteria. Biochemical and staining tests that were conducted were endospore staining, gram staining, and catalase test.

Figure 3.2(a) Endospore staining of alpha-amylase-producing bacteria which appeared to be negative (b) Gram staining of alpha-amylase-producing bacteria which appeared to be gram-negative (c) Catalase test of alpha-amylase producing bacteria which appeared to be negative (Ayesha and Usman. 2023)

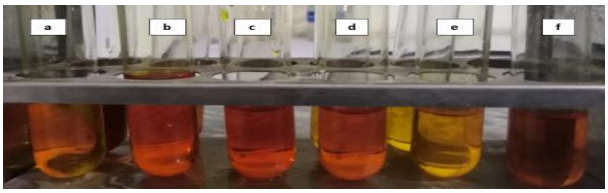


3.3. Characterization of alpha-amylase enzyme

The crude alpha-amylase was subjected to characterization at a different temperature, time of incubation, pH, carbon sources, and nitrogen sources and their effects on enzyme activity were calculated (Demirkan, 2011).

3.3.1. Effect of carbon sources

Figure 3.3: Enzymatic activity of alpha-amylase at different carbon sources.

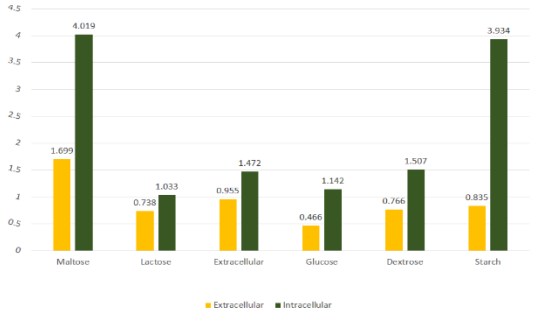


(a) Maltose, (b) Lactose, (c) Sucrose, (d) Glucose, (e) Dextrose, (f) Starch

Table 3.1: Extracellular and Intracellular enzymatic activity of alpha-amylase at different carbon sources.

Sr.no	Carbon Sources	Extracellular O.D at 540nm	Extracellular Enzyme activity U/ml	Intracellular O.D at 540nm	Intracellular Enzyme activity U/ml
1	Maltose	1.185	1.699	2.84	4.091
2	Lactose	0.52	0.738	0.724	1.033
3	Sucrose	0.67	0.955	1.028	1.472
4	Glucose	0.332	0.466	0.8	1.142
5	Dextrose	0.54	0.766	1.052	1.507
6	Starch	0.6	0.835	2.732	3.934

Figure 3.4: Graphical representation of alpha-amylase at different carbon sources. Carbon sources are taken as the x-axis and O.D is taken as the y-axis



3.3.2. Effect of nitrogen sources

Figure 3.5: Enzymatic activity of alpha amylase at different nitrogen sources.

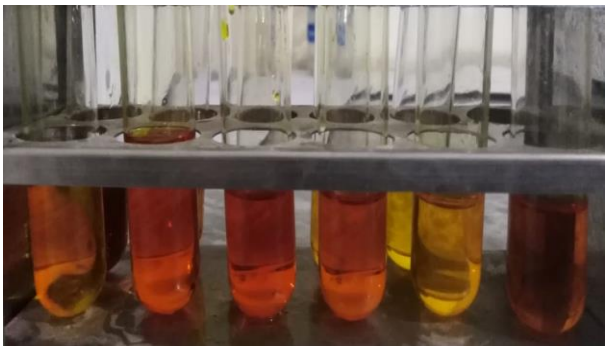
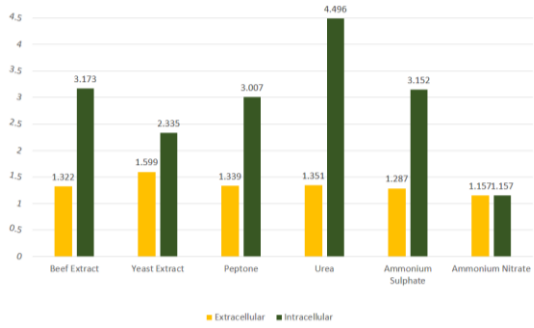


Table 3.2: Extracellular and Intracellular enzymatic activity of alpha-amylase at different nitrogen sources.

Sr. No	Nitrogen Sources	Extracellular O.D at 540nm	Extracellular Enzyme activity U/ml	Intracellular O.D at 540nm	Intracellular Enzyme activity U/ml
1	Beef Extract	0.924	1.322	2.205	3.173
2	Yeast Extract	1.116	1.599	1.625	2.335
3	Peptone	0.936	1.339	2.09	3.007
4	Urea	0.944	1.351	3.12	4.496
5	Ammonium Sulphate	0.9	1.287	2.19	3.152
6	Ammonium Nitrate	0.81	1.157	2.37	1.157

Figure 3.6: Graphical representation of alpha-amylase at different nitrogen sources.



3.3.3. Effect of metal ions

Figure 3.7: Enzymatic activity of alpha amylase at different metal ion sources.

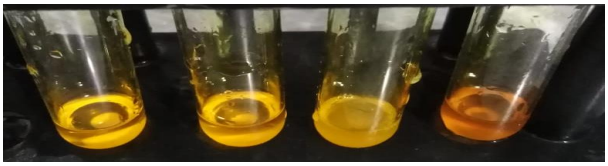
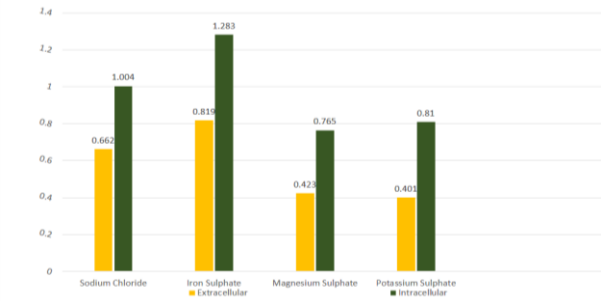


Table 3.3: Extracellular and Intracellular enzymatic activity of alpha-amylase at different medallion sources.

Sr.no	Metal Ions	Extracellular O.D at 540nm	Extracellular Enzyme activity U/ml	Intracellular O.D at 540nm	Intracellular Enzyme activity U/ml
1	Sodium Chloride	0.468	0.662	0.704	1.004
2	Iron Sulphate	0.576	0.819	0.897	1.283
3	Magnesium Sulphate	0.302	0.423	0.539	0.765
4	Potassium Sulphate	0.287	0.401	0.570	0.810

Figure 3.8: Graphical representation of alpha amylase at different metal ions.



3.3.4. Effect of incubation times

Figure 3.9: Enzymatic activity of alpha amylase at different pH

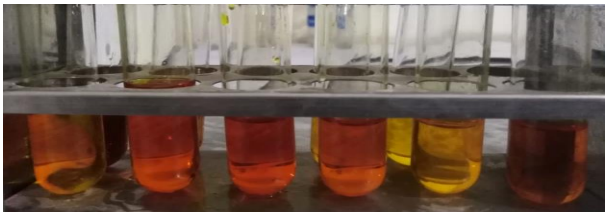
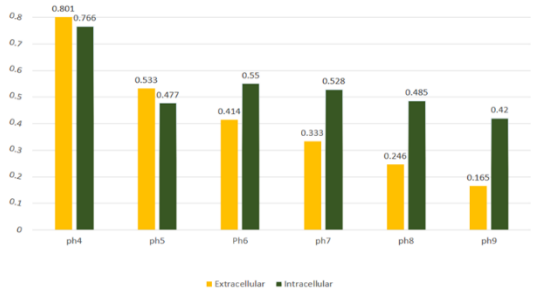


Table 3.4: Extracellular and Intracellular enzymatic activity of alpha amylase at different pH.

Sr. no	Ph	Extracellular O.D at 540nm	Extracellular Enzyme activity U/ml	Intracellular O.D at 540nm	Intracellular Enzyme activity U/ml
1	4	0.564	0.801	0.54	0.766
2	5	0.392	0.553	0.34	0.477
3	6	0.296	0.414	0.39	0.550
4	7	0.24	0.333	0.375	0.528
5	8	0.18	0.246	0.345	0.485
6	9	0.124	0.165	0.3	0.420

Figure 3.10: Graphical representation of alpha-amylase at different pH.



3.3.5. Effect of temperatures

Figure 3.11: Enzymatic activity of alpha amylase at different temperatures.

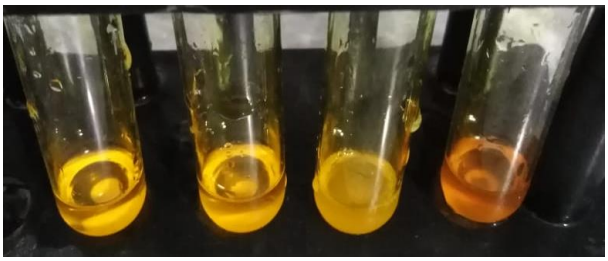
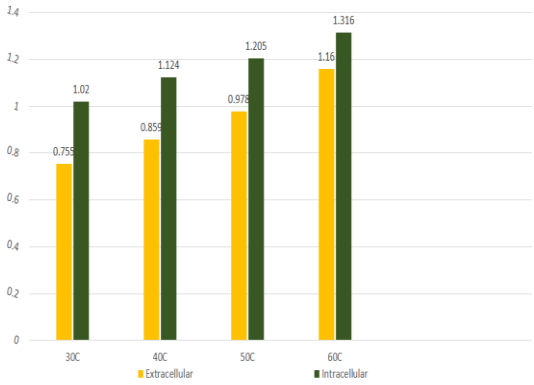


Table 3.5: Extracellular and Intracellular enzymatic activity of alpha amylase at different temperatures.

Sr. no	Temperatures	Extracellular O.D at 540nm	Extracellular Enzyme activity U/ml	Intracellular O.D at 540nm	Intracellular Enzyme activity U/ml
1	30ºC	0.532	0.755	0.715	1.020
2	40ºC	0.604	0.859	0.787	1.124
3	50ºC	0.686	0.978	0.843	1.205
4	60ºC	0.812	1.160	0.92	1.316

Figure 3.12: Enzymatic activity of alpha amylase at different temperatures.



3.4. Partial purification of alpha-amylase

At each step of purification, the assay was performed to check the activity of the enzyme. As the process of purification increases it was observed that the specific activity increases. Partially purified amylase exhibited specific activity of 0.038 U/ml/mgwhich corresponds to 5.7 purification fold and 68 % Yield (Pandey et al., 2009).

Table 3.6: Steps for partial purification of amylase performed by ammonium sulphate precipitation followed by dialysis displaying the volume, enzyme activity, protein activity, specific activity, purification fold and yield

Steps for Purification	Volume	Enzyme activity (mg)	Protein activity (U/ml/mg)	Specific activity (U/ml/mg)	Purification Fold	Yield %
Crude extract	100	0.640	68	0.006	1	100

Ammonium precipitation	9.5	0.533	38	0.016	2.6	83
Dialysis	7.2	0.362	9	0.038	5.7	68

3.5. Bioinformatics analysis

3.5.1. Prediction of secondary structure of enzyme

Secondary structures of protein were identified by using different bioinformatics tools including Predict Protein, proteus2, PSIPRED, and JPRED. These tools predict the presence of Alpha helix by symbol H, Beta strand by E and used letter C for Coils. As Alpha-Helix are made of at least 6 amino. Each tool described presence of secondary structure in different form either by using symbols or by using colors and some by visual display.

3.5.1.1.JPred:

Figure 3.13: Secondary structure prediction of alpha-amylase of Bacillusstearothermophilus, accession number M57457.1 by JPRED.

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3.5.1.2 Predict Protein

Figure3.14: Secondary structure prediction of alpha-amylase of Bacillusstearothermophilus, accession number M57457.1 by Predict Protein

3.5.1.3. PROTEUS 2

Figure 3.15: Secondary structure prediction of alpha-amylase of Bacillusstearothermophilus, accession number M57457.1 by PROTEUS2

3.5.4.PSIPRED

Figure 3.16: Secondary structure prediction of alpha-amylase of Bacillusstearothermophilus, accession number M57457.1 by PSIPRED

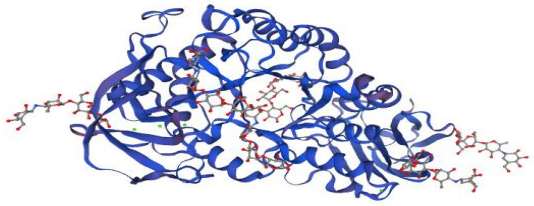
3.5.2.Prediction of the tertiary structure of the enzyme

Prediction of the tertiary structure of the enzyme from online software gave the 3-Dimensional structure of alpha-amylase of Bacillusstearothermophilus, accession number M57457.1. These online tools used different modeling strategies to predict tertiary structure.

3.5.2.1. SWISS-MODEL

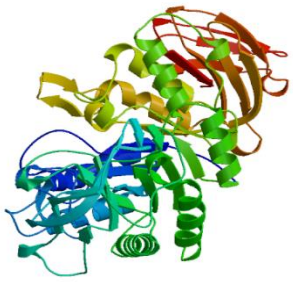
SWISS-MODEL predicted the 3-Dimensional structure of alpha-amylase of Bacillus stearothermophilus, accession numberM57457.1on basis of its comparative homolog modeling (Arnold et al., 2009)

Figure 3.17: 3D structure of Alpha-Amylase of Bacillus stearothermophilus, accession number M57457.1 obtained from the Swiss model



3.5.2.2. (PS)2

Figure 3.18:3D structure of Alpha-Amylase of Bacillus stearothermophilus, Accession number M57457.1. Obtained from (PS) 2



4. Conclusion

In this research, bacteria were extracted from banana fruit and were screened for alpha-amylase activity on starch agar plates and subjected to morphological and biochemical

testing for identification purposes. Distinct zones producing colonies were used for the characterization. It was characterized at different times of incubation, temperatures, pH, carbon sources, nitrogen sources, and metal ions. The maximum activity was determined at 60˚C with 72 hours of incubation. Maltose was identified as the best carbon source, urea was recognized as the best nitrogen source and FeSO4 was the best metal ion source. The maximum activity was at neutral pH 4 and distinct colors were produced in assay results. Partially purified amylase exhibited specific activity of 0.038 U/ml/mg which corresponds to 5.7 purification fold and 68 % yield.

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