Study of amylase producing bacteria isolated from vegetable market dumping ground

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***Abstract*-Amylases are one of the most important industrial enzymes that hydrolyze starch molecules into fine products such as dextrin and maltose. Microbes were isolated and identified from vegetable waste dumping area. In recent years, interest in microbial production of the amylase has increased dramatically due to its wide use in the bakery, food, textile and the detergent industry. The purpose of this research study is to isolate indigenous amylase producing bacteria from vegetable waste dumping area. Samples collected from in an around Bhiwandi, district - Thane, Maharashtra. Randomly 19 isolates were selected based on variation in appearance for further studies. All isolates were tested for Quantitatively amylase production using DNSA. The potent isolate showing higher amylase activity of 144 µg/ml/min was identified as belonging to *Bacillus thuringiensis* by using 16s rRNA gene sequencing. The maximum enzyme production found at 37 °C at pH 7 with 1% starch. The enzyme activity was enhanced in the presence of NH_4SO_4 , $MnSO_4$ and sago using colorimetric assay. Immobilization yield is found 50% by the entrapment method with 3% (w/v) Na-alginate.**

***Index Terms*- Amylase, *Bacillus thuringiensis*.,16s rRNA, DNSA, Entrapment.**

I. INTRODUCTION

About 1.3 billion tons of food is wasted worldwide each year, which is equal to one third of the food produced [1]. During the year, millions of tons of municipal solid waste (MSW) are generated, 70% of which are organic substances contributing to the carbohydrate content. Food waste, which is generated in hotels, restaurants, canteens and household kitchen waste, is the main contributor of organic matter [2].To minimize the problem of waste disposal, organic waste can be used in the production of fermentable sugars, which can be further used to produce other value-added products. Starch, a white granule polymer created by plants as a reserves food source, has a significant impact on the dietary needs of plants, animals, and microorganisms [3].

It is a hetero polymer made up of amylose and amylopectin, and when it is hydrolyzed, other oligosaccharides are produced that have reducing industrial applications [4]. Starch hydrolysis produces branching oligosaccharides, which are useful as antitumoral oligoisaccharides, and dextrin, which has Starch is a crucial component of the human diet and is digested chemically and hydrolyzed to create a wide range of products, including starch lipase, sucrose syrups, carbohydrate, and equivalents of dextrose or cyclodextrins. However, its usage in various commercial food applications is constrained due to factors such as low shear tolerance, low good thermal stability, thermal disintegration, and a high potential for depolymerization [5][6][7]. Starch is one of the carbohydrates that is presently receiving more focus due to its applications in various culinary items [8]. A glucose polymer called starch is joined to another via a glycosidic bond, and there are two different kinds of glucose molecules: amylose and amylopectin. Amylose is a straight chain polymer made up of 1,4-glycosidic linkages connecting up to 6000 glucose units, while amylopectin is a straight chain polymer made up of 1,4-glycosidic linkages connecting up to 6000 glucose units.

Microorganisms are being used for many purposes, such as absorption of heavy metals, genetic engineering, digestion, production of new antimicrobial substances and especially for the production of industrial enzymes. Bacteria, actinomycetes and fungi are among the most widely used microorganisms for the production of alpha-amylase. Some bacteria can produce alpha-amylase under harsh conditions, such as thermophilic bacteria producing alpha-amylase at high temperatures [9]. Enzymes are natural substances that function as catalysts and have very specialised structures [10]. Microbial enzymes stand out due to their stability and much more affordable production. Amylase is one of the most often utilised enzyme production [11], and glycoside hydrolase is another name for it. Amylases break down the 1,4 and 1,6 glycosidic connections in starch and glycogen, a polysaccharide linked to starch, and facilitate the breakdown of starch molecule into smaller monomers consisting of glycosidic bonds and sugars like maltose and dextrins [12].

In 1811, Kirchhoff made the first discovery Ohlsson suggested both alpha and beta amylase classes of starch digestion enzyme in malt in 1930. Amylase enzymes are found in a wide variety of microorganisms, including fungus and bacteria. Bacillus is the most widely used bacterium for producing amylases. Exoenzymes and endoenzymes are categories for amylases made by organisms. Microbes, fungi, plants, and creatures all make amylase, but microorganisms have a significant capability to improve to Industrial applications due to effective manufacturing techniques. Solid-state fermentation (SSF) technology has been developed and employed increasingly often in recent years due to its straightforward process, minimal capital expenditure, affordable synthesis of an enzyme with higher physiochemical characteristics, lower levels of catabolite repression, and improved product recovery [13]. The choice of an appropriate substrate and microbe, substrate particle density, Inoculum concentration, and substrates moisture level are the main variables that influence the bacterial activity of enzymes in an SSF system.

Amylases are two classes of enzymes, endoamylase and exoamylase. Endoamylases cleave -1,4 glycosidic bonds between adjacent glucose units present in the starch chain, while exoamylases either catalyze the cleavage of the -1,4 glycosidic bond or both -1,4 and -1,6 glycosidic bonds [14]**.** Amylases from plant and microbial sources have been used as food additives for centuries. Microbial sources, especially fungal and bacterial amylases, are used for industrial production due to advantages such as cost-effectiveness, consistency, less time and space required for production, and ease of process modification and optimization [15]. Fungi of the genus Aspergillus are most often used for the production of amylase. Enzyme production by solid-phase fermentation (SSF) has become a cost-effective production technique [16]. Amylases have a 3D structure, have the ability to bind to the substrate and promote the cleavage of glycosidic bonds through the action of catalytic groups. The protein contains three domains: A, B and C. The active site of alpha amylase is located in a long cleft located between the carboxy terminus of the A and B domains [17]. The enzyme has potential uses in virtually every sector, including the paper, detergents, fabric, food, baking, pharmaceuticals, ethanol production, starch conversion, and treatment of agricultural wastes industries. The traditional approach of using acids to hydrolyze starch into glucose has a number of drawbacks, but enzymes are used to produce high pure fructose, which is more environmentally benign due to its reduced processing requirements and biodegradable end products [18].

II. METHODOLOGY

1. Collection of sample

Using a sterilized spatula, a soil sample was obtained from a vegetable disposal site in and near Bhiwandi. In aseptic environment, collected samples were moved to non-contaminated plastic bags.

2. Enrichment and Isolation

1g of soil sample was added in St. Nutrient broth with 1% starch of 100 ml. It was further incubated for 48 to 72 hrs at RT °C After incubation 1ml of enrichment broth solution is taken and followed the 10 fold dilution with saline contain NaCl and plating technique. 1% of starch nutrient agar media is used for growth of colonies by streak plate method for 24 h at 37°C. Selected colonies were again streak on same media for Better isolation

3. Screening of amylase activity (starch iodine test)

By performing a starch hydrolysis experiment on a starch agar plate, bacterial cultures were examined for amylolytic activity [19]. Pure isolated colonies was plated on 1% starch agar plate (Peptone -1.0g; Sodium chloride 0.5g; Beef extract 0.3 g; Starch 1%; Distilled water 100 ml; Agar 2.5g at pH 7.4)and Incubated at RT for 24 to 48 hours After incubation the gram`s iodine is flooded on plate. If there is a clear zone in the medium around the colonies, it means the isolate is producing the amylase enzyme, which has hydrolyzed the starch. Observed of clear zone around the colonies were selected and maintain on St. Nutrient agar slant with 1% starch at 4°C.

4. Crude enzyme extraction

A full loop bacterial culture was transferred from starch nutrient agar slants to 1% starch medium at pH 7. Inoculated broth is kept on shaker at At the end of fermentation , The fermented medium was centrifuged at 10,000 rpm for 15 min to obtain the crude enzyme source. The clear supernatant is used as the crude enzyme source.

5. Enzyme activity test

Estimation of amylase producing activity was carried out by using the DNSA (3,5 – dinitrosalicylic acid) method [20]. Used of different concentration of standard glucose from 200mcg/ml to 1000mcg/ml and unknown concentration were used which is from enzyme production. The reaction between enzyme and 1% of starch kept for 5 minutes. To stop the reaction 2 ml of DNSA was added in all the test tube including unknown. Further tubes were kept in boiling water bath for 10 minutes and 6 ml of distilled water was added in all the tubes after cooling. The absorbance was taken at 540 nm and recorded.
The highest reading showed were selected for potent amylase production.

6. Identification of potent amylase producing bacteria

a. Morphology identification

A single isolated colonies were picked and prepared a smear on grease free slide and fixed the side by heat. Few drops of crystal Violet is added on smear for 1 minute and rinsed with running tap water. Then the slide were flooded with Grams's iodine for 1 minute. This was followed by the 70% of Ethanol and rainsed with running tap water. The slide was stained with counter stain saffranin and rinsed with running tap water. Allow to air dry and observed under oil immersion lens 100X [21].

b. 16S rRNA gene sequencing and phylogenetic analysis

DNA isolation from isolate was done using the Biobee Spin EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd., Forward primer 27F(5'AGAGTTTGATCTGGCTCAG3') and reversed primer 1492R 5' TACGGTACCTTGTTACGACTT 3' are used by adding 5 µL of isolated DNA in 25 µL of PCR reaction solution. PCR performed under conditions such as initial denaturation 95˚C 2 min, denaturation 95˚C 30 sec 25 cycles, annealing 50˚C 30 sec, extension 72˚C 2 min and final extension 72˚C 10 min. Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).Single-pass sequencing was performed on each template using below 16s rRNA universal primers. An ethanol precipitation process was used to separate the fluorescently labelled fragments from the unregulated terminators. The samples were electrophoresed in an Applied Biosystems ABI 3730xl sequencer after being reconstituted in distilled water.. The 16S rRNA sequence was blast using NCBI blast similarity search tool for the phylogeny analysis

of query sequence with the closely related sequence. The program MUSCLE 3.7 was used for multiple alignments of sequences and resulting aligned sequences were cured using the program Gblocks 0.91b. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

7. Optimization of amylase production

a. Determination of the optimum pH with time

Optimization of pH is carried out at 5 min time interval unto 30 minutes. 100 ml of phosphate buffer was prepared and transfer into 5 different flask i.e 20 ml each. With the help of NaCl and NaOH the different pH was set as 5,6,7,8 as well as 9 and cross checked with pH paper. Transferred the 10 ml from each flask to 5 different tubes respectively. Addition of equal volume of substrate (starch) to the 5 different tube. 1 ml of mixture was taken and addition of 2 ml of DNSA reagent was added with respect to different pH at 5 minutes further it was kept in boiling water bath for 10 minutes and addition of 6ml of distilled water. It was followed for all the pH at different time interval as same as above. Absorbance was taken at 540nm.

b. Determining the optimum temperature

Enzyme activity was optimized for temperature using range of temperature viz. 0°C ,10°C, RT°C, 37°C, and 55 °C. 5 tubes was taken for different temperature, 0.5 ml of enzyme and 0.5 ml of substrate was added in each tube and kept for different respective temperatures for 5 minute. The Addition of 2 ml DNSA to stop the reaction and kept in boiling water bath for 10 minutes. Then all the tubes were added of 6ml distilled water. The activity was checked at 540nm using calorimeter.

c. Determining the effect of heavy metal ion on enzyme activity

Effect of heavy metal ion was checked on amylase production, the metal ions include $MnSO_4$, Zn , $CdCl_2$, $CoCl_2$ and $HgCl_2$ of 1mM concentration. 0.5 ml of 1mM concentration of metal ion in Tris-HCL buffer taken out in newly tube and addition 0.5 ml of enzyme in all tubes of different metal ions kept at RT for 5 minutes for reaction. 2ml of DNSA in each tube was added and 6ml of distilled water. The effects of different metal ions at 1mM concentration was check at OD 540nm.

d. Utilization of carbon and Nitrogen source

Carbon : Different carbon sources such as sago, carrot, banana, beetroot, and potato was checked where 1% Starch Nutrient agar broth was made of 100 ml and distributed in 10 different tubes including 5 control tubes. Each tube is mark as a different carbon source. pH were adjusted at 7.0 and the tubes were autoclave at 121°C for sterilization of media. The test culture was inoculate in test tube and incubated at 37°C for 48 hours. After the incubated period, the tubes were centrifuged and supernatant was collected. 1ml of DNSA is added in all the tubes and kept for boiling water bath for next 10 minutes. 6ml of distilled water was added in each the test tube and OD was taken at 540nm.

Nitrogen : Different nitrogen sources such as NH_4SO_4, Casein , NaNo3, Peptone and NaNo2 was checked where 1% Starch Nutrient agar broth was made of 100 ml and distributed in 10 different tubes including 5 control tubes. Each tube is mark as a different nitrogen source. pH were adjusted at 7.0 and the tubes were autoclave at 121°C for sterilization of media. The test culture was inoculate in test tube and incubated at 37°C for 48 hours. After the incubated period, the tubes were centrifuged and supernatant was collected. 1ml of DNSA is added in all the tubes and kept for boiling water bath for next 10 minutes. 6ml of distilled water was added in each the test tube and OD was taken at 540nm.

8. Determining the enzyme Immobilization yield

3% sodium alginate in 50mM of sodium phosphate buffer was prepared by warming and allowed to cool. 1 ml of stock crude enzyme solution was mixed with 9 ml of sodium alginate solution and total volume become 10 ml. The mixture was taken in 10 ml pipette and beads were formed by dropping down the solution in 1M NaCl chilled solution. The beads were collected by filtration and wash with distilled water and the filtrated NaCl solution were used to determine the enzyme activity. The immobilization yield in percentage was carried out by using formula.

Immobilization yield (%) = (Activity of immobilized enzyme / A – B) × (100)

A - is activity of free enzyme added

B - is the activity of remaining enzyme in washed water and filtrated calcium chloride solution.

III. RESULT AND DISCUSSION

1. Isolation of amylase – producing bacteria

Collected enriched broth was streaked on sterile 1% starch agar plates. Well-isolated colonies were selected from plates after 24 hours of incubation at 37 °C. 19 different colonies were selected based on their appearance on starch agar plates streaked with enriched broth from a soil sample. The 19 different isolates were named A1, A2, A3, A4...A19 (Table 1).

Table 1 Colony characteristics of 19 different isolates

Isolate	Size (mm)	Shape	Colour	Margin	Opacity	Consistency	Elevation	Gram's nature and Morphology
A1	Medium	Circular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A2	Medium	Circular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A3	Medium	Circular	Off	Entire	Opaque	Raised	Mucoid	Gram-positive

			White					rods
A4	Small	Circular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A5	Medium	Circular	White	Irregular	Translucent	Raised	Mucoid	Gram-positive rods
A6	Medium	Irregular	White	Entire	Translucent	Flat	Dry	Gram-positive rods
A7	Medium	Circular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A8	Small	Irregular	White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A9	Medium	Circular	Off White	Irregular	Opaque	Raised	Mucoid	Gram-positive rods
A10	Medium	Circular	Off White	Entire	Opaque	Flat	Smooth	Gram-positive rods
A11	Medium	Circular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A12	Small	Circular	Off White	Entire	Opaque	Flat	Smooth	Gram-positive rods
A13	Medium	Irregular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A14	Medium	Circular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A15	Medium	Circular	White	Entire	Translucent	Raised	Mucoid	Gram-positive rods
A16	Small	Circular	Off White	Irregular	Opaque	Flat	Smooth	Gram-positive rods
A17	Small	Circular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A18	Medium	Irregular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods
A19	Medium	Circular	Off White	Entire	Opaque	Raised	Mucoid	Gram-positive rods

2. Screening of amylase activity (starch iodine test)

After 24 hours of incubation at 37°C, the bacterial cultures were tested for amylolytic activity by a starch hydrolysis assay on a starch agar plate. Pure, isolated colonies were plated on starch agar plates with starch as the sole carbon source. Individual petriplates were covered with Gram's iodine, and a clear zone (halo zone) quickly appeared around the growth of the isolates. As these isolates hydrolyzed starch, the clear zone suggests that they were amylase producers. It forms a blue-brown complex. Hydrolyzed starch does not produce a color change. The halo zone is an indication of amylase production, and all 19 different isolates showed starch hydrolyzing activity. It was found that the thermotolerant bacterial strain's capacity to produce amylase was examined by allowing the bacteria to grow on LB agar plates that contained 1% (w/v) soluble starch [22]. Plates were filled with iodine solution after one day at 70 °C of incubation to observe for the appearance of a visible halos zone around bacterial colonies. The screening of bacteria that produce amylase by submerging them in a 1% iodine solution for 5 minutes and then washing the remaining color away with water [23].

3. Enzyme activity test

Amylase-producing activity was carried out by using the DNSA (3,5-dinitrosalicylic acid) method [24] By using different concentrations of standard glucose from 200 mcg/ml to 1000 mcg/ml and an unknown concentration for enzyme production. The highest OD for potent amylase production is 0.56 (Table 2 and figure 1), and its found to be 144 µg/ml/min. The different concentrations of standard glucose from 200 mcg/ml to 1000 mcg/ml are found to be 0.07, 0.23, 0.65, 0.72, and 0.82, respectively. 30 minutes of incubation with 1% starch in a 0.05 M phosphate buffer, *Bacillus sp.* 3.5AL2 showed the greatest specific amylase enzyme activity of 1.97 0.41 U/mg protein under the optimal circumstances of 60 °C and pH 7.0 [25]. The highest amylase assay activity was found to be 3179.62 IU/ml/min from a soil sample of *Bacillus species* [26].

Table 2 DNSA Enzyme assay

Isolate	**Substrate (1% starch) (ml)**	**Crude Enzyme of specific isolate (ml)**	**Total volume (ml)**	**Enzyme and substrate Reaction (min)**	**DNS A (ml)**	**Boiling water bath (min) And Cool it.**	**Distilled water (ml)**	**Absorbance at 540nm**
A1	0.5	0.5	1	5	2	10	6	0.26

A2	0.5	0.5	1	5	2	10	6	0.33
A3	0.5	0.5	1	5	2	10	6	0.30
A4	0.5	0.5	1	5	2	10	6	0.52
A5	0.5	0.5	1	5	2	10	6	0.30
A6	0.5	0.5	1	5	2	10	6	0.27
A7	**0.5**	**0.5**	**1**	**5**	**2**	**10**	**6**	**O.56**
A8	0.5	0.5	1	5	2	10	6	0.41
A9	0.5	0.5	1	5	2	10	6	0.22
A10	0.5	0.5	1	5	2	10	6	0.15
A11	0.5	0.5	1	5	2	10	6	0.14
A12	0.5	0.5	1	5	2	10	6	0.26
A13	0.5	0.5	1	5	2	10	6	0.38
A14	0.5	0.5	1	5	2	10	6	0.12
A15	0.5	0.5	1	5	2	10	6	0.42
A16	0.5	0.5	1	5	2	10	6	0.20
A17	0.5	0.5	1	5	2	10	6	0.13
A18	0.5	0.5	1	5	2	10	6	0.21
A19	0.5	0.5	1	5	2	10	6	0.28

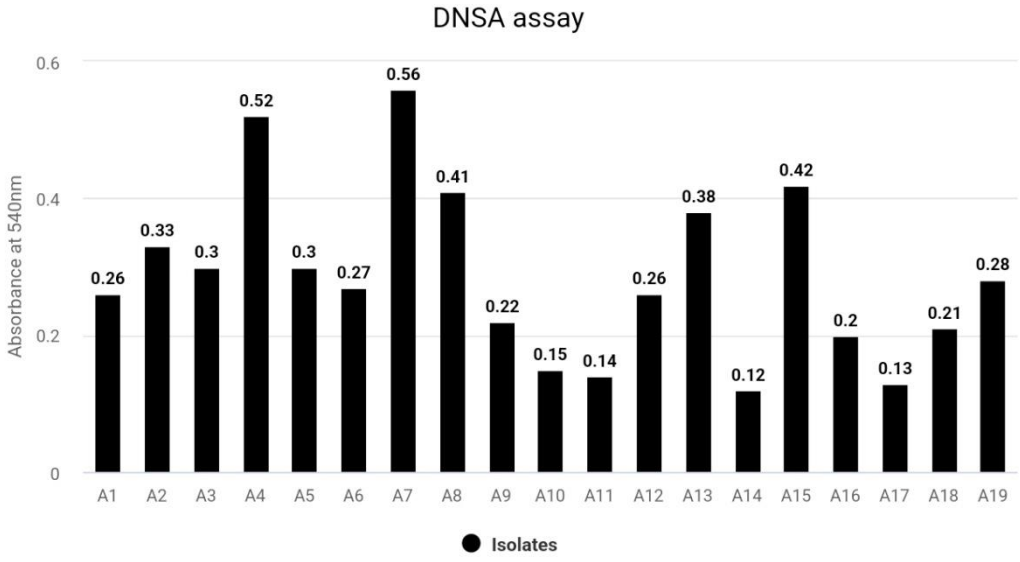


Figure 1 DNSA Enzyme assay - OD at 540nm of 19 different isolate

4. Identification of potent amylase producing bacteria

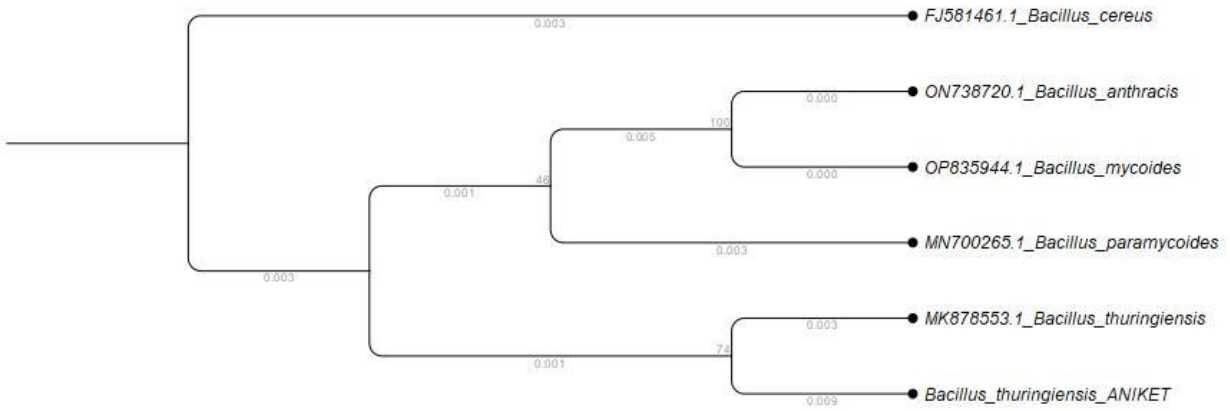
a. **Morphological identification**:

The isolate A7 showing maximum activity was observed under a microscope by performing the gram staining and was found to be Gram's positive rods.

b. **16S rRNA gene sequencing and phylogenetic analysis**

With the help of the Biobee Spin EXpure Microbial DNA isolation kit manufactured by Bogar Bio Bee stores Pvt Ltd., forward primer 27F (5'AGAGTTTGATCTGGCTCAG3') and reversed primer 1492R (5' TACGGTACCTTGTTACGACTT 3'), the chosen isolate A7 is subjected to 16S rRNA gene sequencing to identify the strain. *Bacillus thuringiensis* was identified as the query sequence in a phylogenetic study of the 16S rRNA sequence using the NCBI blast similarity search tool (Figure 2). Multiple sequence alignments were performed using the programme MUSCLE 3.7, and the aligned sequences were then corrected using the programme Gblocks 0.91b. Finally, phylogenetic analysis was performed using PhyML 3.0 aLRT and the HKY85 Substitution model..

Figure 2 phylogenetic tree of presenting the relationship between ***Bacillus thuringiensis*** with other closely related *Bacillus sp.*



5. Optimization amylase production

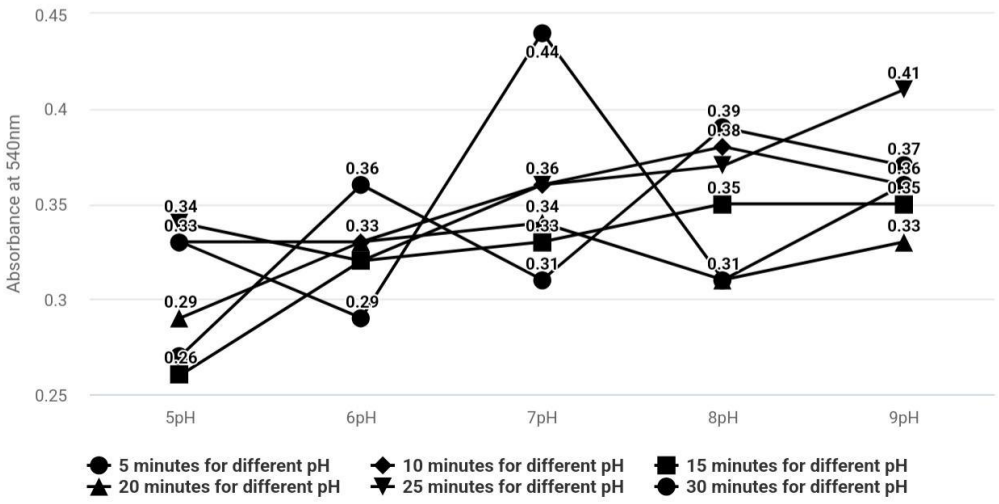
a. Determination of the optimum pH with time

format The optimum temperature for enzyme production was studied by incubating the fermentation medium at different pH values with respect to time, which is one of the important physicochemical factors that promote changes in cell morphology and growth. pH 7 is the optimum pH for amylase production and showed maximum amylase activity in 5 minutes at 540 nm when compared to different pHs that are 5, 6, 7, 8, and 9. The maximum activity was found at pH 7 (Table 3). This may be due to the nature of bacteria, which are stable above an acidic pH. A further increase in hydrogen concentration results in decreased amylase production and minimal enzyme production at an acidic pH. The organism liked a neutral pH in our investigation, and starch is best for the production of amylase. Dietary and physical variables had an impact [27]. According to research **,** Bacillus amyloliquefaciens produces amylase best at a neutral pH of 7.0 [28]. The majority of Bacillus bacteria use a pH range of 7.0–10.0 to produce amylase [29][30]**.**

Table 3 Effect on enzyme production at different incubation time with pH

pH → ---------- Time (min) ↓	5	6	7	8	9
5	0.33	0.29	**0.44**	0.31	0.36
10	0.33	0.33	0.36	0.38	0.36
15	0.26	0.32	0.33	0.35	0.35
20	0.29	0.33	0.34	0.31	0.33
25	0.34	0.32	0.36	0.37	0.41
30	0.27	0.36	0.31	0.39	0.37

Figure 3 Determination of optimum pH with time



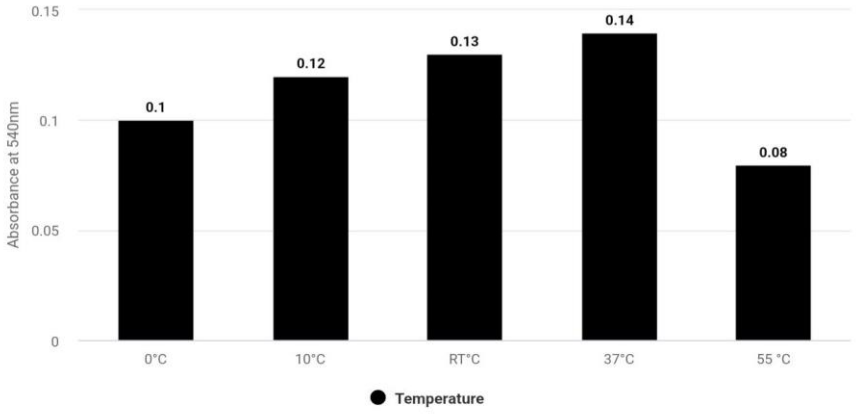
b. Determining the optimum temperature

The effect of temperature on enzyme production is shown in table 4. The results show that 37 °C is the optimum temperature for amylase production, with the highest amylase activity at pH 7 after an incubation time of 5 min. From the results in Figure 4, an increase in temperature causes a decrease in amylase production because the enzyme is easily denatured at high temperatures. The most widely employed It has been found that Bacillus sp. produces amylase between 37 and 60 °C [31]. In the current investigation, the production of amylases was determined to be at its peak at 37 °C. Due to the organism's extreme temperature sensitivity, further temperature increases resulted in negligible output [32]. According to studies on amylase production in Bacillus subtilis strains LZ-10 and LZ-11 the ideal temperature was 55 °C, which is higher than the 37 °C [33]

Table 4 Effect of temperature on amylase production

Temperature	O.D at 540nm
0°C	O.10
10°C	0.12
RT°C	0.13
37°C	0.14
55 °C	0.08

Figure 4 Effect of temperature on amylase production at 540nm



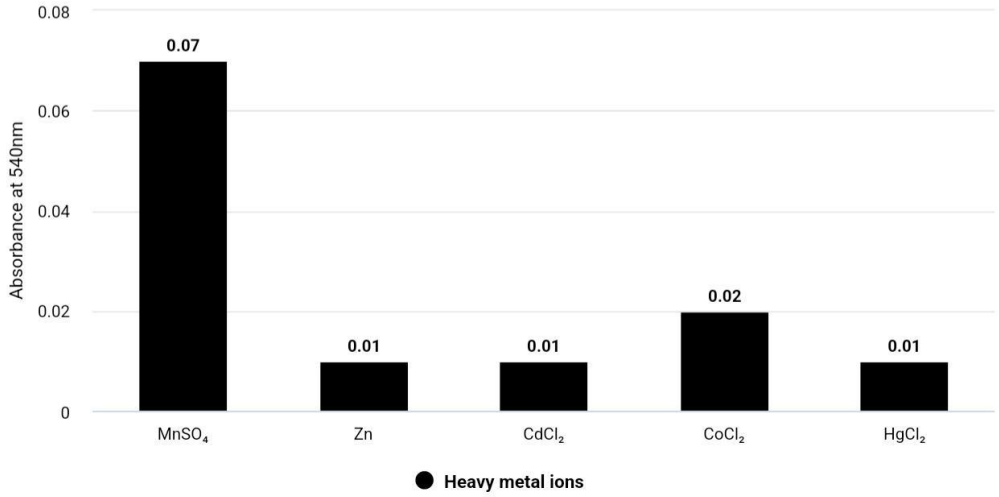
c. **Determining the effect of heavy metal ion on enzyme activity**

Extracted crude enzyme was tested with different heavy metal ions at 1 mM concentration in buffer solution. It's resultant that heavy metal ions help increase amylase production, as amylases are metalloenzymes. Table 5 shows the effect of metal ions on enzyme activity. It was observed that $MnSO_4$ enhanced the enzyme activity, showing maximum activity at 540 nm at 0.07 compared to Zn , $CdCl_2$, $CoCl_2$ and $HgCl_2$. The Zn , $CdCl_2$ and $HgCl_2$ show inhibitory effects on activity. Alpha amylase has its highest activity in the presence of $MnSO_4$ and $CaCl_2$ in the order of 165.2 (IU/ml) and 154.8 (IU/ml), respectively [34]. Several metals, including Mg^{2+}, K^+, and Ca^{2+}, as well as very little activity with Cu^{2+}, were observed to make crude enzymes extremely active [35]. Similar to this, the presence of Mg^{2+} and Ca^{2+} increased the thermostable amylase from Anoxybacillus AH1's activity, but Cu2+ had an inhibiting impact [36].

Table 5 Effect of heavy metal ions on enzyme production

Metal ions	O.D at 540nm
$MnSO_4$	0.07
Zn	0.01
$CdCl_2$	0.01
$CoCl_2$	0.02
$HgCl_2$	0.01

Figure 5 Effect of heavy metal ions on enzyme activity



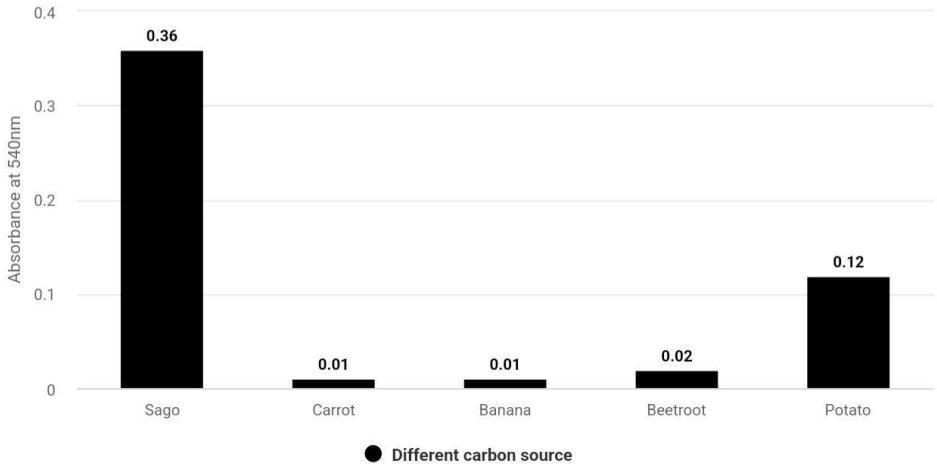
d. Utilization of carbon and Nitrogen source

For carbon: Five different carbon sources, i.e., sago, carrot, banana, beetroot, and potato, are used to check its utilization on amylase activity. It is found that Sago is showing the highest amylase activity of 0.36 at 540 nm from table no. 6. Sago waste and wheat bran, respectively, generated the highest amylase yields of 9.83 and 8.05 mg/ml/min at a pH of 7, Sago waste and wheat bran were the two starch wastes investigated, and after 24 hours of development of Bacillus subtilis, sago waste was determined to be the most desirable inexpensive substrate, followed by wheat bran, for enzyme (amylase) synthesis [37]. Amylase synthesis on pure starch medium did not increase bacterial amylase production, but in the current investigation, low-grade, inexpensive impure substrates such as sago waste and wheat brain produced significantly more amylase than pure starch [38].

Table 6 Effect of carbon source on amylase activity

Carbon source	Absorbance at 540nm
Sago	0.36
Carrot	O.01
Banana	0.01
Beetroot	0.02
Potato	0.12

Figure 6 Utilization of Carbon source

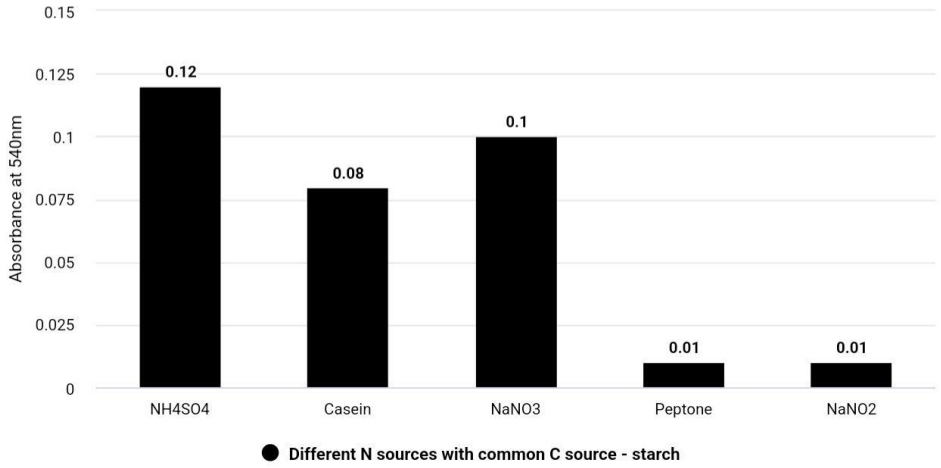


For Nitrogen : Five different nitrogen sources i.e NH_4SO_4, Casein , $NaNo_3$, Peptone and NaNo2 are used to check its Utilization on amylase activity It is found to be NH4SO4 is showing highest amylase activity 0.12 at 540nm from table no 7. Maximum enzyme production was discovered [39] using ammonium chloride (NH4Cl) as the nitrogen source at a concentration of 6g/l. Additionally, it appears that peptone and yeast extract can help Bacillus amyloliquefaciens produce amylase. The addition of yeast extract as a nitrogen source (594 U/g) and the lowest amount of ammonium sulphate increased the production of amylase [40].

Table 7 Effect of Nitrogen source on amylase activity

Nitrogen source	Absorbance at 540nm
NH_4SO_4	0.12
Casein	O.08
$NaNO_3$	0.10
Peptone	0.01
$NaNO_2$	0.01

Figure 7 Utilization of Nitrogen source



e. **Determining the enzyme Immobilization yield**

Percentage of immobilization yield is carried out by the entrapment method with 3% (w/v) Na-alginate solution in 50 mM sodium phosphate buffer at pH 7.0 with 1 mM concentration of calcium chloride using the formula, and it is found to be 50%. Immobilization yield was determined to be most prevalent (90%) for a final sodium alginate amount of 3% w/v, while our study noted a 50% immobilization yield [41] and immobilization of amylase with a 3% concentration of sodium alginate gave a maximum activity of 87.78 IU/g with the highest yield of 72.18 % [42].

Immobilization yield (%) = Activity of immobilized enzyme / (A – B) × (100)
Was found – Activity(0.03) , A (0.11) and A (0.05)

= 0.03 / (0.11 – 0.05) × 100
Immobilization yield (%) = 50 Percentage

A - is activity of free enzyme added
B - is the activity of remaining enzyme in washed water and filtrated calcium chloride solution.

IV. Conclusion

Vegetable market dumping grounds are areas where fruits and vegetables are sold and often discarded, leading to the accumulation of food waste. This waste can become a breeding ground for bacteria, and some of these bacteria produce amylase, an enzyme that breaks down starch into simple sugars. Amylase has various applications, such as in the production of industrial enzymes, food processing, and biotechnology. For example, the amylase produced by these bacteria can be used to improve the quality and texture of food products, reduce the time and cost of starch hydrolysis, and even produce biofuels. In the present study, an amylase-producing bacterial isolate from a vegetable market produced ***Bacillus thuringiensis*** by using a soil sample was identified by 16S rRNA gene sequencing and blast using NCBI blast similarity search tool for the phylogeny analysis of query sequence with the closely related sequence with program MUSCLE 3.7. The ***Bacillus thuringiensis*** showed maximum amylase activity of **144 µg/ml/min** by using the DNSA Method. The maximum activity of amylase given by ***Bacillus thuringiensis*** after 5 minutes of reaction between extracted crude enzyme and 1% of starch as substrate at pH 7 is 0.44 absorbance at 540 nm by using the colorimetric method. The maximum amylase produced by ***Bacillus thuringiensis*** follows the following conditions, 37 °C, 1mM concentration of metal ions in $MnSO_4$, carbon source as sago, nitrogen source as NH_4SO_4, and 50% immobilization yield in percentage.

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