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Review IJBBR

Production and Optimization of Amylase Enzyme Using *Bacillus subtilis* Under Solid State Fermentation and Its Characterization

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Abstract

This study employed solid state fermentation to maximize amylase production by Bacillus subtilis (OR578403). The research began by discovering and analyzing amylase-producing bacteria in soil samples followed with biochemical, molecular characterization. By using commercially available substrates, like wheat bran, rice bran, and wheat Rava, were obtained from the local market and employed as solid substrates, which may influence amylase production and to be assessed. The identified organism was Bacillus subtilis, confirmed through BLAST analysis and phylogenetic tree construction. Further the Bacillus subtilis sample was used in this study to produce amylase, which was then optimized in the fermentation media. Changes in fermentation conditions, such as fermentation hours, temperature, inoculum size, carbon sources, metal salts, effect of moisture content, and all have a significant effect on enhancing amylase output. Thermal, pH, and effects of metal stability of partly purified amylase were studied and characterized using the SDS PAGE. Finally, From the present study it was considered that this Bacillus subtilis was a potential bacterium producing maximum amylase enzyme.

Keywords: *Bacillus subtilis*, isolation, phylogenetic tree, amylase enzyme, enhanced production with effect of pH, temperature, metal salts, SDS-PAGE characterization

INTRODUCTION

Amylases, which hydrolyze starch molecules to produce finely divided products, such as glucose, oligosaccharides [1–34], and dextrins molecules, are among the most important hydrolytic enzymes with significant commercial applications [17, 37]. Alpha-amylases, which hydrolyze starch molecules at the –1, 4 bonds site, can be obtained from plants, microbes, and animals; however, it is the enzymes generated from the latter sources that commonly fulfill industrial demand [19, 20]. According to a review of the literature, microbial–amylases have prospective uses in the fine, food, pharmaceutical, chemicals, bakery, and textile laundry detergent sectors, in addition to starch hydrolysis. Amylases are responsible for the glucosidal bonding of complex polysaccharides [17]. These enzymes are extremely significant in a variety of industrial operations. Fungi that generate amylase include *Rhizopus* sp., *Penicillium* sp., *Aspergillus niger* and *Cephalosporium* sp. [14, 15, 16]. Furthermore, the synthetic medium is too costly for commercial synthesis of industrial enzymes. Traditional optimization methods are time demanding and

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typically neglect factor interactions. Since decades, the ubiquitous starch breaking enzyme amylase [EC 3.2.1.1] has been a focus of research. It breaks starch to release simple sugars such as glucose, maltose, and maltotriose [11]. Because of its widespread usage in industry, amylase has been produced on a massive scale utilizing submerged and solid-state fermentation with the assistance of microorganisms such as bacteria and fungus. However, solid state fermentation outperforms submerged fermentation due to a variety of additional benefits such as simplicity, cost effectiveness, ease of availability, higher productivity, and lower water output [12].

As the manufacturing use of alkaline amylases is expected to increase dramatically in the coming era, efforts have been directed in recent years to investigate ways to reduce amylase production costs through improved yield and the use of either cost-free or low-cost agricultural byproducts as substrate(s) for amylase production [31]. Solid-state fermentation (SSF), which involves the growth of microbes on moist solid substrate(s) in the absence of free-flowing water, has gained tremendous momentum in recent years due to certain advantages over conventional submerged fermentation (SmF), such as low production cost, water, and energy savings, less waste effluent problem, and product stability, due to less dilution in the medium [3, 10]. Furthermore, SSF technology offers enormous potential for job creation, particularly in developing countries [4, 5]. Furthermore, it has been observed that wild-type strains of microorganisms (fungi or bacteria) function better in SSF systems than genetically engineered microorganisms, decreasing energy and cost requirements even further [6,]. Many agro-industrial wastes, including barley bran, wheat bran, potato peel, maize meal, rice bran, molasses bran, coconut oil cake, soybean meal, and others, have recently been investigated as low-cost solid substrates for microbial production of amylase in SSF [8, 9]. A review of the literature reveals that some solid substrates have better potential than others for supporting/inducing the synthesis of amylase (or even other enzymes) by microorganisms. Wheat bran and potato peel, for example, have been found to be better than the other solid substrates investigated for optimal-amylase synthesis by several microbes under similar SSF conditions [21, 24]. However, the actual reason(s) for such a larger enzyme synthesis by microorganisms when growing on some precise substrates has never been investigated, and no logical explanation for the above observed effect has been presented to yet. The synthesis of thermostable-amylases in solid culture is primarily confined to Bacillus, and numerous Bacillus species including *B. polymyxia*, *B. vulgarus*, *B. megaterium*, *B. licheniformis*, *B. coagulans*, and *B. subtilis*, have been reported to generate amylase [22].

As a carbon source, starch is a cost-effective raw material in the industrial sector. The combination of starch and amylase producing bacteria improves the fermentation process and generates amylase enzyme in a single step, lowering the overall fermentation cost. The primary goal of this study was to isolate and screen potential amylase producers and enzymes producing *Bacillus subtilis*, as well as to detect amylase production ability for industrial applications in bioprocessing and fermented foods; thus, these *Bacillus subtilis* are generally regarded as potential producers.

SCREENING OF AMYLASE PRODUCING ORGANISMS

The bacterial strain was isolated from a flour mill soil sample using the serial dilution method, and they were tested for amylase production using the plate assay method. For screening amylase generating organisms, sterile nutrient agar with 1% soluble starch was utilized. Plates were examined for the zone of clearing using iodine after the incubation time. The isolated colonies were further sub-cultured and characterized for morphological and biochemical identification based on Bergey's Manual of Determinative Bacteriology.

Gram Staining

After 24 hours, the amylase producing culture was gram stained and examined under a light microscope.

Biochemical Test

12 different tests, like Indole test, Methyl red test, Voges Proskauer's test, citrate utilization test, and eight different carbohydrates utilization test, Sorbitol, Adonitol, glucose, lactose, Mannitol, Arabinose,

Rhamnose, and sucrose, were tested using the pure culture from the above isolation. Based on the biochemical characteristics the organism was identified by using Bergey's manual.

DNA Extraction

By extracting genomic DNA from bacteria using the conventional phenol chloroform procedure, two dominating colonies were found using the 16s rRNA sequencing method. In the thermal cycler, an 800 bp DNA fragment was amplified using high-fidelity PCR polymerase. Using forward and reverse primer sequences, the PCR products were amplified bidirectionally. The forward primer sequence is 5'-GGTCGTGCGAGAGCGATGCGGT-3', while the reverse primer sequence is 5'-GTCCCGCTTTCTTCCC-3'. The following PCR mixture was used to amplify the product: DNA 3µl, Forward Primer (10 pM)-1 µl, Reverse Primer (10 pM)-1 µl, 2x ampliqon master mix-12.5 µl, and Nuclease free water made up to 25 µl. The aforesaid combination was amplified for 5 minutes at 95°C, followed by 35 amplification cycles at 95°C for 45 seconds, 56°C for 45 seconds, and 72°C for 1.30 minutes, with a final extension at 72° C for 10 minutes. DNA was detected using 1% Agarose gel electrophoresis. The UV absorbance at 260/280 nm was used to calculate the concentration of DNA.

Phylogenetic Tree Construction

The sequence similarity was deep-rooted by Basic Local Alignment Search Tool in the sequence database. Phylogenetic tree was created with different subspecies of Bacillus and partial sequence was used to compare using neighbor joining method.

PREPARATION OF INOCULUM

A 25-ml volume of nutritional broth in a 100-ml Erlenmeyer flask. The flask was sterilized in an autoclave at 15 pounds of pressure (121°C) for 20 minutes. A loop full of culture was injected from a 24-hour-old slant and stored at 30°C in a rotary shaker after the medium was cooled. 1 ml of this nutrient broth culture was utilized as the inoculum after 24 hours of incubation.

FERMENTATION MEDIUM

Commercially available wheat bran, rice bran, and wheat Rava were obtained from the local market and utilized as solid substrates to test their influence on amylase production. The most suitable substrate was chosen and utilized in following studies.

AMYLASE PRODUCTION BY SOLID STATE FERMENTATION

10g of wheat bran, wheat Rava and rice bran were taken in 250 ml conical flask and moistened with 15ml of distilled water. The flasks were sterilized at 121ºC for 20 min. After sterilization, 4 ml of inoculum were added to each flask and mixed well. Then, the flask was incubated at 30ºC for 5 days. At every 24 h interval, samples were collected and analyzed for amylase activity.

ENZYME EXTRACTION

1 g of fermented bran was taken, to which 10ml of 100mM citrate phosphate buffer (pH 5.0) was added and grinded using mortar and pestle. The slurry was squeezed through muslin cloth. Extracts were collected and centrifuged at 4°C for 15 min at 10000 rpm. The clear supernatant was used for enzyme assays by DNS (3, 5-dinitroslicylic acid) method. For DNS enzyme extraction method, 0.9 ml of 1% starch and 0.1 ml of enzyme was incubated at 50ºC for 10 minutes. After incubation 3 ml DNS reagent was added to stop the reaction. Later the mixture was boiled for 10 min at 80ºC to develop red brown color.1ml of 40% potassium sodium tartarated solution was added to stabilize the color. Zero the spectrometry with blank and measure the absorbance at 540 nm. The absorbance was plotted against glucose concentration to get a standard graph.

AMYLASE ACTIVITY ON DIFFERENT SUBSTRATES

The different substrates supplement on amylase production were evaluated by adding rice bran, wheat bran and wheat Rava. The enzyme from the fermented bran was extracted and same as above method. The crude enzyme extract was analyzed for amylase activity.

EFFECT OF INCUBATION PERIOD

The effect of incubation period was determined by incubating production medium for Different incubation periods (24, 48, 72, 96, and 120 h) at 37ºC.

AMYLASE ACTIVITY ON DIFFERENT CARBON SOURCES

To enhance the amylase activity the different carbon sources on amylase production were used and evaluated by adding carbon sources (1% w/w) such as soluble starch, maltodextrin, lactose, sucrose, glucose, and fructose.

AMYLASE ACTIVITY ON DIFFERENT STARCH CONCENTRATIONS

To enhance the amylase activity the Wheat bran was supplemented with soluble starch at different concentrations, such as 0.5, 1.0, 1.5, 2.0, and 2.5% w/w, were used individually.

AMYLASE ACTIVITY ON DIFFERENT METAL SALTS

To enhance the activity and production the different metal salts are supplement on amylase production were added and evaluated by adding NaCl, $MgCl_2$, $CaCl_2$, NH_3Cl_2, and $MnSO_4$ at a concentration of 0.05%.

EFFECT OF MOISTURE CONTENT ON AMYLASE PRODUCTION

To check the effect of moisture content in amylase enzyme production, Wheat bran was supplemented with different ratios of water like 10, 15, 20, and 25 ml individually and evaluated.

EFFECT OF INOCULUM ON AMYLASE PRODUCTION

The effect of inoculum size on amylase production was examined by incubating the flasks containing 10 g of substrate with 15 ml of moisture content at different range of inoculum size from 2 ml, 4 ml, 6 ml, and 8 ml of preinoculum broth.

PARTIAL PURIFICATION OF ENZYME

The enzyme isolated from the fermented bran using the preceding methods was ground with a mortar and pestle. Muslin fabric was used to squeeze the sludge. To separate the enzyme and extract, extracts were collected and centrifuged at 4°C for 15 minutes at 10, 000 rpm for 15 minutes. The clear supernatant was utilized to purify enzymes. The enzyme was purified at a temperature of 4°C. The supernatant was utilized to purify enzymes. The supernatant was saturated to 80% with solid ammonium sulfate and permitted to stand overnight at 4°C. The precipitate was recovered by centrifugation at 12,000 g (4°C) for 20 minutes and redissolved in a citrate phosphate buffer containing 100 mM pH 5. The resultant elute was tested for total protein and enzyme specific activity using Lowry's technique.

Characterization of Protein Using SDS-PAGE

The protein from the above protocol was further processed for the SDS-PAGE analysis for identifying the size in KDa. The eluted protein was resuspended in 6X SDS PAGE loading buffer in equal volume and boiled for 5 to 10 minutes. The combinations were subjected to 12% sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoresed in running buffer at 70 v. The electrophoresed gels were then stained with staining solution containing Coomasie brilliant blue and destained with methanol.

CHARACTERIZATION OF ENZYME

The characterization of amylase enzyme production was analyzed by optimizing the temperature, pH, and Metal Ions are follows.

EFFECT OF TEMPERATURE

The effect of temperature was characterized from the purified amylase enzyme was determined by incubating the reaction mixtures at different temperatures which are ranging from 30ºC–80°C temperature at 5°C intervals and followed with enzyme assay using the standard method.

EFFECT OF pH

Amylase activity was determined using the usual assay technique using starch as the substrate at various pH levels. The pH of the reaction mixtures was adjusted using buffers containing (100 mM) citrate phosphate (pH 3.0–6.0) and phosphate buffers (pH 7.0–9.0), with final buffer concentrations of 100 mM. The pH stability of the enzyme was determined by incubating the enzyme solution for 1 hour at various pH values ranging from 3.0 to 9.0 at 50 degrees Celsius. The residual amylase enzyme activity was measured using starch as the substrate under normal assay conditions.

EFFECTS OF METAL IONS

The effects of various metal ions on amylase activity were determined by adding the relevant ion to the reaction mixture at concentrations of 1 mM and 10 mM, and the test was done under standard conditions. The following ions were tested: $NaCl_2$ (Na+), $CaCl_2$ (Ca^{2+}), KCl (K^+), $MgCl_2$ (Mg^{2+}), $ZnCl_2$ (Zn^{2+}), $CuSO_4$ (Cu^{2+}), NH_3Cl_2, $MnSO_4$, and $AgNO_3$. The relative activity was estimated in relation to the control, in which the reaction was performed in the absence of metal ions, and the residual activity was determined after one hour of incubation.

RESULTS AND DISCUSSION

Screening of Amylase Producing Organisms

Twenty bacterial colonies were isolated by culturing the soil sample by serial dilution. Out of 20 colonies screened by iodine staining, 10 colonies showing clear hydrolysis zone on soluble starch nutrient agar plate due to having the capability of hydrolyzing the starch by the presence of enzyme using iodine test (Figure 1). Out of 10 colonies, selectively one colony processed further for the Amylase production by inoculating in broth 24hrs of incubation at 37ºC.

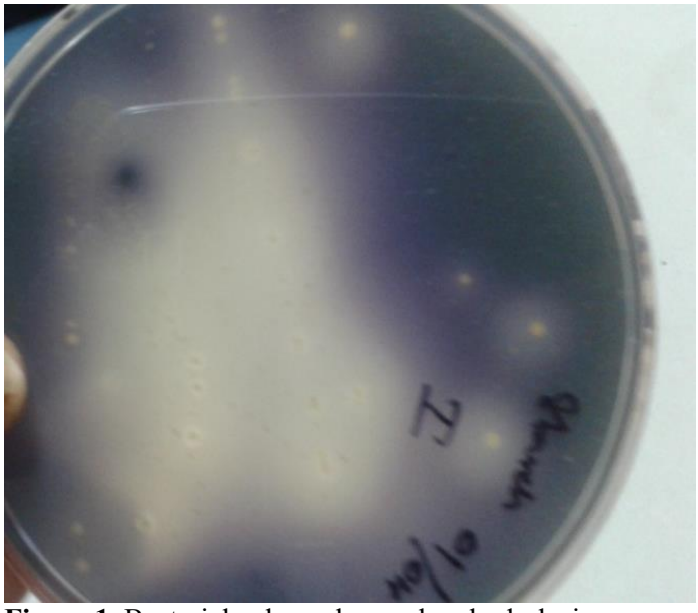


Figure 1. Bacterial colony shows clear hydrolysis zone.

Gram Staining

After 24 hours, the culture was gram stained and examined under a light microscope which resulted in Gram negative motile rods (Figure 2).

Biochemical Test

The Amylase producing bacteria isolated from soil sample was found to be Negative for Indole, Methyl red, Adonitol, Arabinose, Lactose, and Positive for Voges Proskauer, citrate utilization, Glucose, Sorbitol, Mannitol, Sucrose test. The amylase producing organism was identified as *Bacillus subtilis* as per the Bergey's manual of determinative bacteriology.

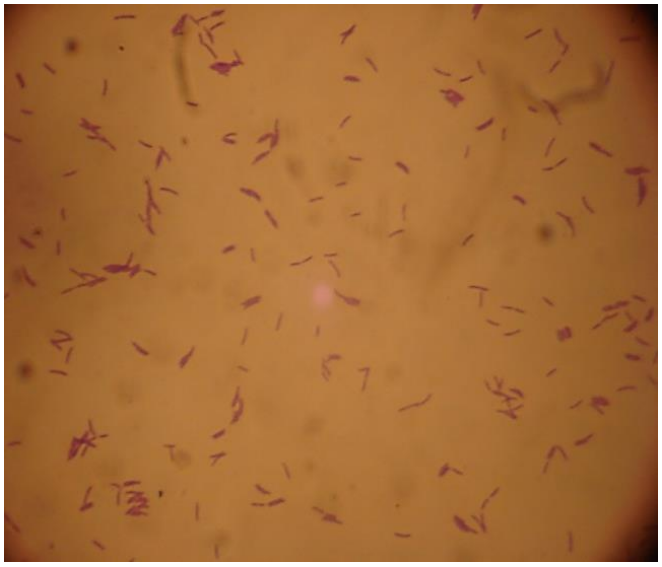


Figure 2. Gram staining results in Gram-negative organisms.

DNA Extraction

The DNA was extracted from the culture using conventional Phenol Chloroform method. The concentration of genomic DNA isolated from *Bacillus subtilis* culture was found to be 102.2 ng/µl. Amplified PCR product was found to be 760bp in size and showed in Figure 3. Further the amplified product was processed for sequencing, and the resultant sequence was submitted to GenBank and the resultant accession number was OR578403.

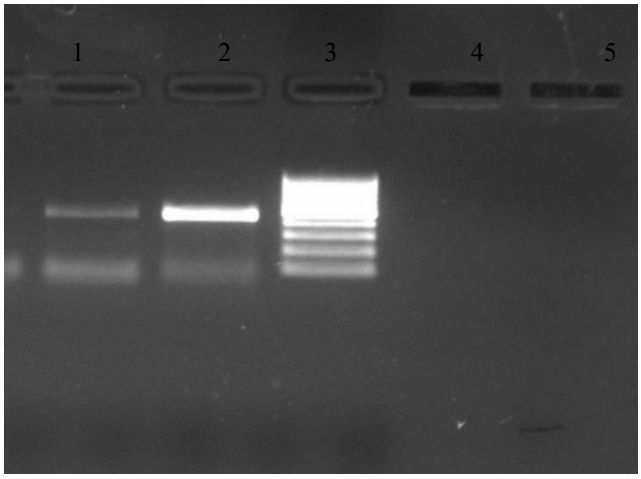


Figure 3. Amplified PCR product.

- *Lane 1:* Amplified *Bacillus subtilis* resulting 760 bp.
- *Lane 2:* Amplified *Bacillus subtilis* resulting 760 bp.
- *Lane 3:* DNA Ladder 1 kbp.
- *Lane 4: 5:* Negative Control.

Phylogenetic Tree Construction

The isolated bacterial strain was identified molecularly using 16S rDNA sequence analysis. The results revealed that the partial sequence of the 16S rDNA gene is approximately 760 bp long and contains both variable and conserved sections. Using the NCBI website, the sequence was matched to bacterial species documented in the GenBank database and identified as *Bacillus subtilis* with 99% similarity. OR578403 is the accession number assigned by the NCBI.

The organism was shown to have 99% sequence homology with *Bacillus subtilis* OR578403 based on multiple sequence alignment using the CLUSTALW tool and phylogenetic tree mapping using the neighbor joining method. A phylogenetic tree was generated and shown in Figure 4, which was compared to the already available *Bacillus subtilis* sequence (Gene bank accession number of the screened isolate is OR578403).

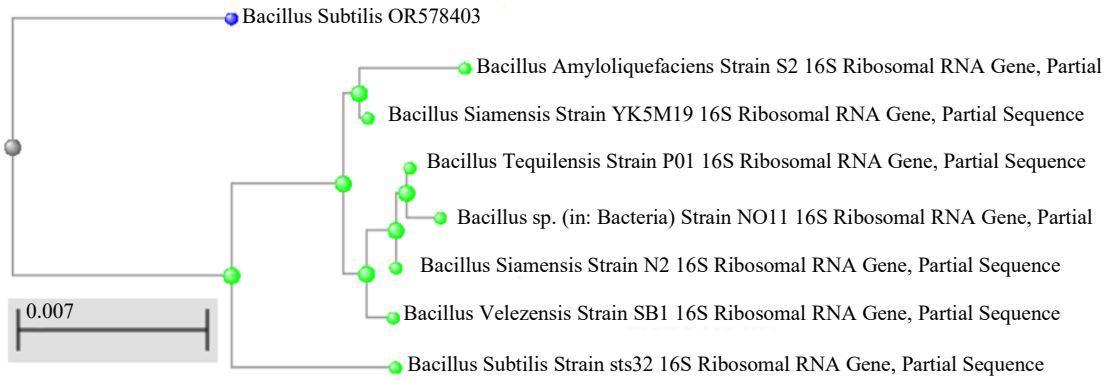


Figure 4. Constructed phylogenetic tree using NCBI website.

AMYLASE ACTIVITY ON DIFFERENT SUBSTRATES

Wheat bran has the highest enzyme activity of any substrate supplement on amylase production. The enzyme was isolated and ground using a mortar and pestle from fermented wheat bran, wheat Rava, rice bran, etc. Muslin fabric was used to squeeze the sludge.

To separate the enzyme and extract, extracts were collected and centrifuged at 4°C for 15 minutes at 10, 000 rpm for 15 minutes. The amylase activity of crude enzyme extract was determined using the 3, 5-dinitrosalicylic acid technique. Finally, it was discovered that all the substrates promoted growth and enzyme synthesis. Wheat bran contained a high concentration of enzymes. When compared to other agricultural leftovers, wheat bran was determined to be the best substrate for amylase production. This might be owing to the existence of enough nutrients and the capacity to remain lost under damp circumstances. Wheat bran is employed in later trials to produce amylases.

AMYLASE PRODUCTION BY SOLID STATE FERMENTATION

Choosing a good substrate for production is a crucial factor for amylase enzyme in solid-state fermentation (SSF). In the Figure, wheat bran was the most effective substrate for amylase production by the *Bacillus subtilis* OR578403 among the other solid substrates (such as wheat bran, rice bran, and wheat Rava). *Bacillus subtilis* OR578403 produced the most with wheat bran (48 U/gds), followed by rice bran (34 U/gds).

The lowest amylase production occurred when wheat Rava was used as the substrate (17 U/gds). The image depicts the influence of varied inoculum concentrations on amylase production by *Bacillus subtilis* OR578403. The production of amylase increased with increasing inoculum size, as seen in the graph with the glucose standard (Figures 5 and 6, Table 1).

Table 1. Production of amylases (U/g) on different substrates by solid-state fermentation.

	Substrates		
Hours	**Wheat bran**	**Rice bran**	**Wheat Rava**
Enzyme production (U/gds)			
24	11	03	0.05
48	25	09	04
72	35	25	15
96	48	34	19
120	41	16	7

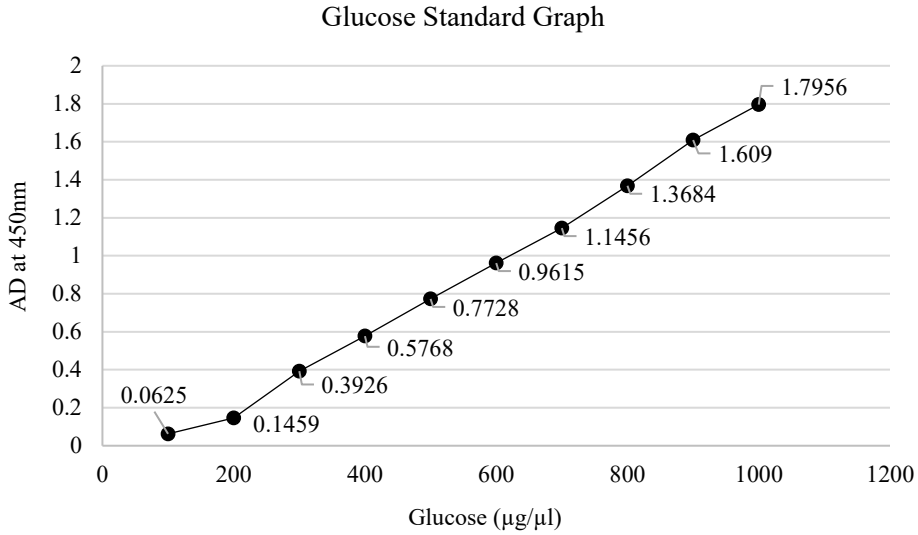


Figure 5. Glucose standard Graph.

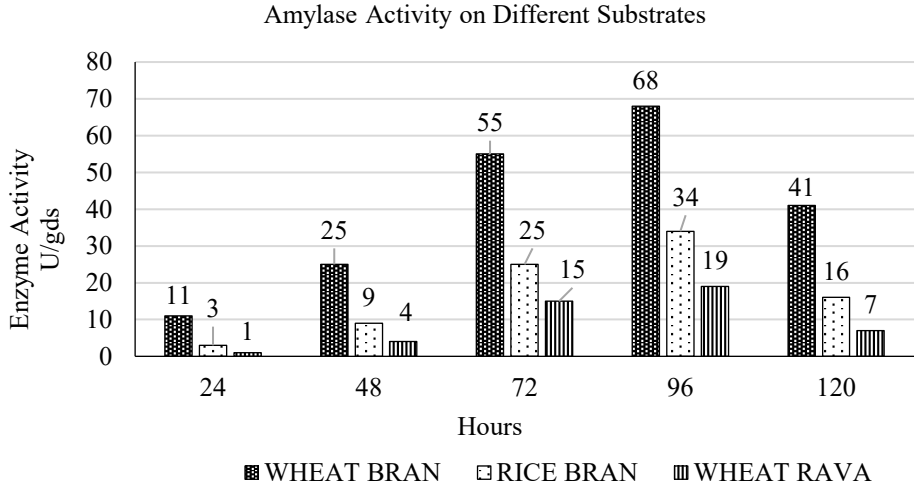


Figure 6. Amylase activity on different substrates.

EFFECT OF INCUBATION PERIOD

The influence of incubation duration was investigated by incubating production medium at 30ºC for various incubation periods (24, 48, 72, 96, and 120 h). The ideal incubation period for maximal enzyme synthesis is determined by the culture's properties and is dependent on the microorganisms' growth rate and enzyme production pattern. Amylase synthesis peaked after 96 hours of incubation (Figure 7). The enzyme output gradually decreased as the fermentation duration was extended.

This might be due to moisture loss, enzyme denaturation or breakdown, resulting in pH variations during fermentation, or interaction with other components in the culture medium. In most cases, the optimal incubation duration for amylase synthesis in SSF employing bacteria ranged from 48 to 120 hours, depending on the ambient circumstances; for subsequent studies, an incubation period of 96 hours was used. Amylase production by KR1 was examined at various incubation times up to 120 hours in the Table 2. *Bacillus subtilis* OR578403 produced the most amylases after 48 hours (58.88 U/gds). With increasing incubation time, there was a decrease in amylase output. *Bacillus subtilis* OR578403 produced the least amylase after 24 hours (32.27 U/gds) (Table 2).

Table 2. Amylase activity on substrate wheat bran for 24, 48, 72, 96, and 120 hours.

Trials	Enzyme activity u/gds 24 hours	Enzyme activity u/gds for 48 hours	Enzyme activity u/gds for 72 hours	Enzyme activity U /gds 96 hours	Enzyme activity U /gds for 120 hours
1	1.6851	4.33	29.62	46.77	23.31
2	0.3703	1.85	34.98	56.81	32.29
3	0.3888	2.0925	29.85	58.88	32.27
4	0.4814	2.75	17.49	42.98	22.25

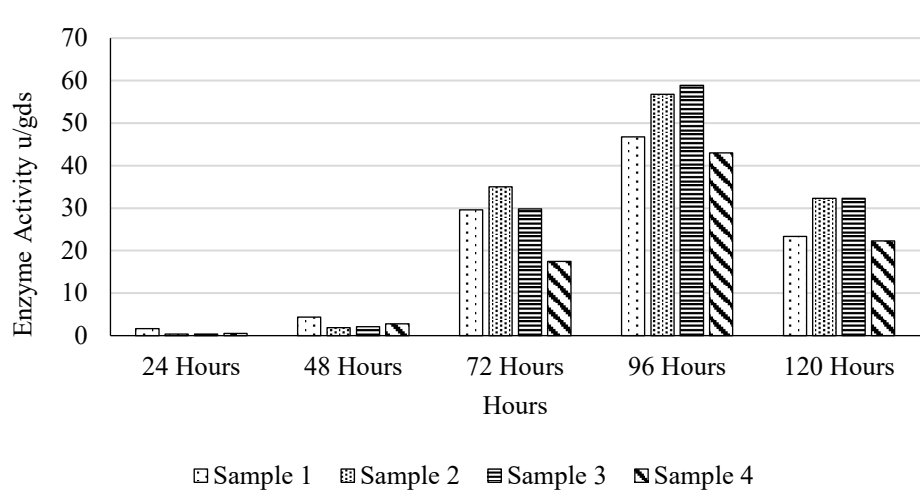


Figure 7. Effect of incubation period.

AMYLASE ACTIVITY ON DIFFERENT CARBON SOURCES

The nutrients available in the growth medium have a significant impact on the growth and enzyme production of any organism. The carbon sources contained in the media have a significant impact on the enzyme production behavior. Some carbon sources promoted good development while limiting enzyme output, whereas others promoted both good growth and enzyme secretion. Among the carbon supplements tested, starch encouraged the most enzyme synthesis, followed by lactose, maltodextrin, glucose, fructose, and sucrose (Table 3 and Figure 8).

Table 3. Amylase activity on different carbon sources.

Sample	Enzyme activity U/gds
1. Maltodextrin	55.12
2. Lactose	30.4
3. Soluble Starch	63.87
4. Sucrose	39.33
5. Glucose	22.07
6. Fructose	16.96

AMYLASE ACTIVITY ON DIFFERENT SOLUBLE STARCH CONCENTRATION

Since carbon compounds provide an energy source for the growth of *Bacillus subtilis* OR578403 and associated enzyme production it is likely that the presence of starch in the medium stimulated increased production of amylase. In our study, 1.5% of starch was most suitable for enzyme production by *Bacillus subtilis* OR578403 under Solid state fermentation. In the subsequent experiments, starch with 1.5% is used to produce amylase (Table 4).

AMYLASE ACTIVITY ON DIFFERENT METAL SALTS

Among the different metal salts are supplement on amylase production were evaluated by adding $NaCl_2$, $MgCl_2$, $CaCl_2$, NH_3Cl_2, and $MnSO_4$. The addition of salts of specific metal ions has been noticed

to impact microorganism growth, and thereby promote enzyme synthesis. From these different metals, manganese sulphate shows the highest enzyme activity from these nine different salts with the enzyme activity of 42.37 u/gds, next to manganese sulphate, sodium chloride shows high activity with the enzyme units of 26.11 u/gds. In the subsequent experiments manganese sulphate with 0.05% is used to produce amylase (Table 5).

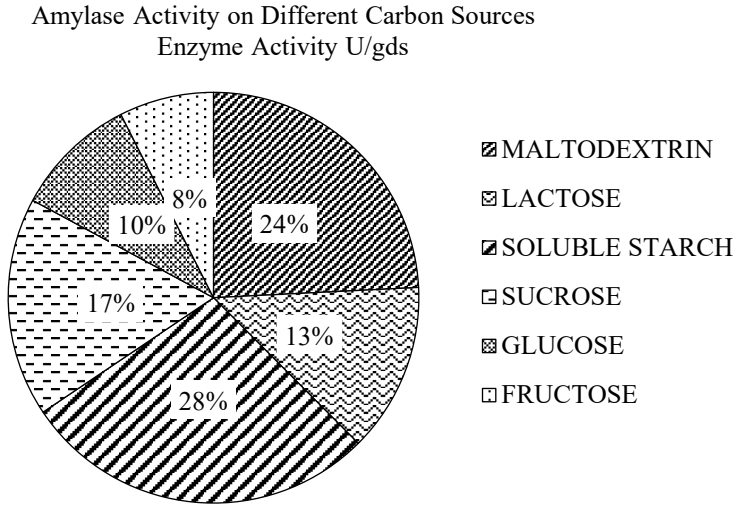


Figure 8. Amylase activity on different carbon sources.

Table 4. Amylase activity on substrate wheat bran with soluble starch.

Sample	Enzyme activity U /gds on 72 hours	Enzyme activity U /gds on 96 hours	Enzyme activity U /gds on 96 hours
0.25 g soluble starch + WB	29.98	50.37	59.20
0.5 g soluble starch + WB	35.09	58.14	69.12
0.75 g soluble starch + WB	46.16	65.55	81.46
1 g soluble starch + WB	54.48	73.87	55.98

Table 5. Amylase activity on metal salts.

Metal ions	72-hour enzyme activity U/gds	96-hour enzyme activity U/gds
Sodium chloride	59.11	66.6
Magnesium chloride	53.96	65.06
Ammonium chloride	42.02	55.52
Calcium chloride	51.4	63.26
Manganese sulphate	66.94	72.37

EFFECT OF MOISTURE CONTENT ON AMYLASE PRODUCTION

Wheat bran was supplemented with water different concentrations (10, 15, 20, 25) individually. The importance of the moisture level in SSF and its influence on the biosynthesis of enzymes has been attributed to the interference of moisture in the physical properties of solid particles. Enzyme production was found to be decreased with lower moisture content. Amylase enzyme activity on substrates with different moisture level from incubation of 72, 96, and 120 hrs, the log of substrate with 15ml of moisture content results in highest enzyme activity by *Bacillus subtilis* OR578403, the remaining moisture content also results in good activity but not with the highest enzyme production. In the subsequent experiments wheat bran with 15ml is used to produce amylase (Table 6).

Table 6. Amylase activity on substrate wheat bran with different moisture levels.

Sample	Enzyme activity U/gds on 72 hours	Enzyme activity U/gds on 96 hours	Enzyme activity U/gds on 120 hours
10 ml + WB	41.31	62.51	42.51
15 ml + WB	52.29	82.90	62.90
20 ml + WB	42.62	54.38	44.38
25 ml + WB	34.87	43.77	33.77

EFFECT OF INOCULUM ON AMYLASE PRODUCTION

The effect of inoculum level on amylase production was examined by incubating the inoculated flasks containing 10 g substrate with 15 ml of moisture content at different range of inoculums level from 2 ml, 4 ml, 6 ml, and 8 ml of culture broth. The 4 ml of the inoculums level in the10 g substrate with moisture content shows the highest amylase enzyme activity by the *Bacillus subtilis* OR578403 (Table 7 and Figure 9).

Table 7. Amylase activity on substrate wheat bran with different inoculum level.

Inoculum + wheat bran	Enzyme activity U/gds on 72 hours	Enzyme activity U/gds on 96 hours	Enzyme activity U/gds 120 hours
2 ml + WB	12	16.6	11.11
4 ml + WB	37	40.7	27.7
6 ml + WB	18.5	27.7	13.8
8 ml + WB	5.5	9.25	1.85

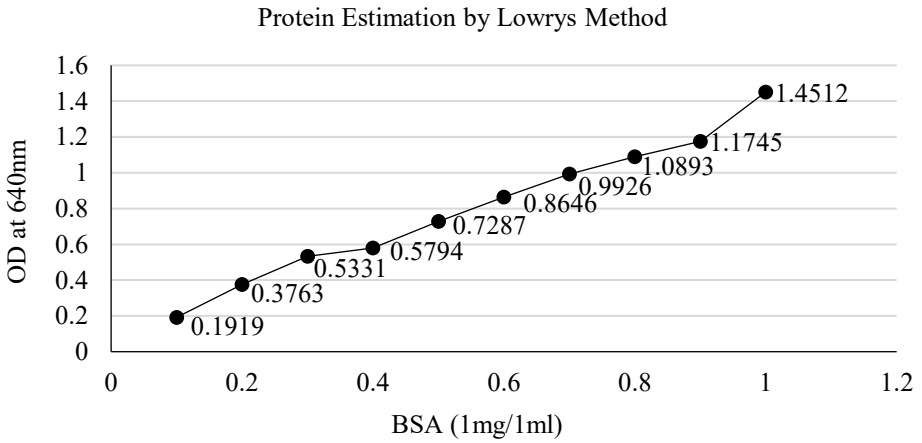


Figure 9. Protein estimation by Lowrys method.

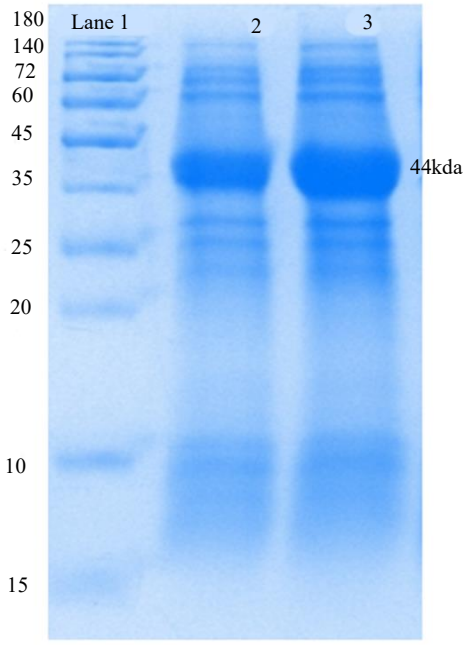
Characterization of Protein Using SDS-PAGE

In 8% SDS-PAGE gel, amylase was isolated from a SSF extract after 48 hours. Figure 10 depicts the observation; the gel profile revealed that, in comparison to the Protein Ladder. SDS-PAGE was used to determine the purity of the protein and the molecular mass of amylase. The purified amylase was found to be a thick band with an estimated molecular mass of 44 kDa from the sample 4ml with substrate in previous experiment in duplicates.

CHARACTERIZATION OF ENZYME: EFFECT OF TEMPERATURE

The temperature of incubation affects not only the development of microorganisms but also their biological activity. The impact of temperature on *Bacillus subtilis* OR578403 amylase activity was investigated by altering the temperature from 30°C to 60°C, and the findings are shown in Table 8. The results show that a temperature of 50°C is most suited for amylases and activity, with a maximum

activity of 68.5 for amylases. It was found that enzyme activity was low at 30°C and gradually increased when the temperature was raised to 35°C. A rise in temperature resulted in a reduction in enzyme synthesis. Amylase enzyme temperature stability ranges from 30°C to 55°C, with enzyme activity decreasing above 60°C.



Lane 1: Protein Ladder, 2 & 3 Protein Sample

Figure 10. Characterized amylase protein in SDS page.

Table 8. Temperature activity.

Temperature	Enzyme activity (u/gds)	Enzyme stability (u/gds)
30	22.5	62.92
35	42.8	67.30
40	55.5	64.27
45	59.38	66.5
50	68.5	67.5
55	60.21	61.21
60	42.85	36.85

Effect of pH

Amylase activity of purified amylase enzyme was measured at different pH values by the standard assay method using starch as the substrate. The pH of the reaction mixtures was varied using the following buffers with (100 mM) citrate phosphate (pH 3.0–9.0) final concentrations of the buffers were 100 mM. The pH stability of the enzyme was evaluated by incubating the enzyme solution for 1 h at different pH values ranges from 3.0 to 9 at 50°c. The residual amylase enzyme activity was determined under the standard assay conditions using soluble starch as the substrate. It was clearly found that the amylase enzyme activity on pH 5 with activity of 64.24 and the stability of the enzyme at pH 5 with 68.15 of enzyme units (Table 9).

Effects of Metal Ions

Supplementation of salts of certain metal ions is found to influence the growth of *Bacillus subtilis* OR578403, and thereby stimulate or inhibit enzyme production. Among the metal ions supplemented potassium chloride supplements the growth of microorganism and stimulates enzyme production.

Whereas the other metal ions also supplement the growth by not inhibiting the growth of the organism. The effect of metal ions shown in relative activity and residual activity on the concentration of 1 mM and 10 mM (Table 10).

Table 9. pH activity.

pH	Enzyme activity (u/gds)	Enzyme stability (u/gds)
3	31.73	21.70
4	42.8	39.99
5	64.24	68.15
6	53.3	51.3
7	22.5	49.85
8	16.5	34.63
9	13.1	27.98

Table 10. Effects of metal ions relative and residual activity.

Metal ions relative activity	1 mM	10 Mm
Sodium chloride	104	101
Silver nitrate	106	101
Zinc chloride	104	102
Potassium chloride	106	118
Magnesium chloride	101	105
Ammonium chloride	105	104
Calcium chloride	103	103
Cupric sulphate	96	116
Manganese sulphate	105	109
Metal ions residual activity		
Sodium chloride	76	56
Silver nitrate	90	42
Zinc chloride	98	62
Potassium chloride	103	83
Magnesium chloride	99	65
Ammonium chloride	101	69
Calcium chloride	98	65

DISCUSSION

Wheat bran was the most effective substrate for amylase synthesis by *Bacillus subtilis* OR578403 among all agro-residual substrates employed for amylase production. *Bacillus amyloliquefaciens* MTCC [25], *Bacillus subtilis* [26], *Bacillus subtilis* MTCC 121 [27], *Bacillus licheniformis* RT7PE1 [28], Bacillus sp., *B. amyloliquefaciens* and Bacillus sp., on the other hand, have been found to produce excellent amylase utilizing corn gluten meal and mustard oil seed cake as substrates [29].

Rice bran was reported to be the greatest source of amylase production from Streptomyces MSC702 under submerged fermentation conditions [30]. The amount of the inoculum has a significant impact on amylase production in solid-state fermentation (SSF). *Bacillus subtilis* OR578403 generated the most amylase at 10% v/w inoculum level in the current study, and a similar finding has been reported for *Bacillus subtilis* ATCC6633 (Maity et al., 2015). *B. subtilis*, on the other hand, produces the most amylase at 20% v/w. Cells grow slowly at lower inoculum levels, therefore, it takes longer to achieve effective substrate use, resulting in reduced enzyme production [32].

Culture conditions, as well as growth rate and enzyme synthesis, determine the incubation duration. Maximum amylase production was reported in *Bacillus subtilis* OR578403 after 48 hours of incubation.

Previously, optimal amylase production was found for *B. amyloliquefaciens* [33], B. Subtilis DM-03, and Bacillus sp. After 48 hours of incubation, *B. amyloliquefaciens* MTCC1270, B. Subtilis ATCC 6633, and Bacillus sp. MRS6 produced the most amylase. On the contrary, optimal amylase production under SSF after 24 hours for *B. amyloliquefaciens* has been observed [34, 35].

Mineral salt solutions have been utilized to boost Bacillus coagulans amylase production [23]. Salts, such as $MgSO_4$, NaH_2PO_4, and K_2HPO_4, and amylase synthesis in the presence of these salts may reduce total industrial production costs. Tap water (pH 8.5–9.0) was shown to be the most efficient moistening agent for B. Subtilis DM-03 [8], Bacillus PS-07 [1], and B. Subtilis DM-03 [24] amylase production [7].

For *Bacillus subtilis* OR578403, the optimal beginning pH for amylase synthesis was 5. *Bacillus subtilis* OR578403 amylase titer decreased significantly in the pH range of 6.5–8.0. Optimal starting pH 7.0 has already been described for *B. amyloliquefaciens*, while Bacillus sp. MRS6 has shown optimal beginning pH 8.0 [34]. On the other hand, an initial pH of 8.5 has been described as optimal for *B. licheniformis* SPT 27 [39]. The optimal incubation temperature for *Bacillus subtilis* OR578403 amylase production was 35 °C. B. Subtilis MTCC 121 previously produced optimal amylase at 40°C under optimized circumstances [27].

Hence the current study sought to determine the potency of *Bacillus subtilis* OR578403 in terms of its capacity to degrade diverse agro-industrial substrates, as well as to optimize various production settings. The isolate demonstrated substantial amylase production capacity by degrading a variety of environmentally favorable natural substrates. Furthermore, it has shown potential in decomposing pretreated wheat bran, making the organism a prospective contender.

CONCLUSION

Amylase derived from *Bacillus subtilis* OR578403 has a huge demand in industrial work due to their promising characterization of fermentation hour, temperature, inoculum size, carbon sources, metal salts, effect of moisture content resilience using the solid-state fermentation (SSF). Further research will be conducted with the objective of producing it on a trial scale. This study's findings will aid in the development of experimental setups for large-scale amylase production. Other biochemical and biophysical properties, such as sensitivity Thermal, pH, and effects of metal stability, will be evaluated to confirm its application in industries. Because divalent cations, such as NaCl, $MgCl_2$, $CaCl_2$, NH_3Cl_2, and $MnSO_4$ ions, improve amylase thermal stability, it will be important to investigate the impact of these ions in fermentation media to improve amylase thermal stability. This May pave the way for its commercial utility to be justified.

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