

Isolation, Molecular Characterization, Optimization and Purification of Amylase Enzyme from Locally Isolated *Bacillus* Species in Different Regions of Munshiganj, Bangladesh

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ABSTRACT: Amylase is a kind of enzyme that facilitates the breakdown of carbohydrates in the body. This enzyme has many applications in different industries. Generally, in pharmaceutical sector it is used for the treatment of pancreatic disorder, pancreatic enzyme replacement therapy (PERT) and as a digestive aid. In this study, the bacterial strain was isolated from soil samples collected from potato dumpsites in different areas of Munshiganj, Bangladesh. After subculturing on the nutrient agar plate, 31 colonies were obtained, of which 9 isolates were found as amylase producer on starch agar medium. Of these, 2 isolates (MC-04 and MC-15) were selected based on the starch hydrolysis clear zone ratio. The isolate MC-04 showed crude enzyme activity of 2.82 IU/ml and specific enzyme activity of 3.42 IU/mg, and isolate MC-15 showed crude enzyme activity of 3.16 IU/ml and specific enzyme activity of 3.77 IU/mg. The best isolate, MC-15, was then identified by morphology, biochemical and molecular characterization and confirmed by 16S-rRNA gene sequencing. After 16S-rRNA sequencing, the isolate (MC-15) was identified as *Bacillus subtilis*. This amylase production of this strain had also been optimized under certain conditions such as different incubation periods, pH, temperatures and different carbon sources. We found that the best incubation period was 48 h, the optimum pH-7.0, the optimum temperature at 40°C and 2% starch was considered as the best source of carbon. Finally, the crude amylase enzyme was purified by precipitation with ammonium sulfate, dialysis, and single-step gel filtration chromatography. The enzymatic activity of the purified amylase was found to be 8.91 IU/ml, that was 2.82-fold greater enzymatic activity than the raw enzyme. The experiments confirmed that *Bacillus subtilis* may be a good source of amylase enzyme for industrial application in Bangladesh.

Key words: Amylase, *Bacillus subtilis*, molecular characterization (16S-rRNA), optimization, purification.

INTRODUCTION

Enzymes are natural protein molecules that act as highly efficient catalysts in biochemical reactions, i.e., they help chemical reactions to take place quickly and efficiently.¹ They are also biodegradable.² Amylase is an enzyme that breaks down starch into glucose molecules and is known as a glycoside hydrolase enzyme.³ All alpha-amylases act on α -1,4-glycosidic bonds.⁴ Various origins of bacterial and fungal isolates are the choice for the

production of amylase enzyme. The predominant bacterial isolates are *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Bacillus megaterium*, *Escherichia coli*, *Pseudomonas* sp. *Lactobacillus* sp. et.⁵ Because of their profitability, short fermentation cycle and usability, these strains are used in the production of industrial enzymes.⁶

Various industries in our country use α -amylase to obtain high yields of products.^{7,8} It has a large scale of applications for the production of glucose syrups, high fructose corn syrups, maltose syrups, and saccharification of starch in the brewery and bio-ethanol industry.^{9,10} In the textile industry, it is used to remove starch from dirt cloth, short fiber from

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fabric, and also used to provide a washing effect on fabric.¹¹ Amylase is widely used in the bakery industry to improve the bread texture, accelerate the fermentation process, increase bread's volume, improve bread's color and flavor.¹² It is also used in the pharmaceutical industry for cyclodextrin production and medical application in pancreatic enzyme replacement therapy (PERT) and as a digestive aid.¹³ In the paper industry, it increases pulp fibrillation, water retention, and beating time in virgin pulp.¹⁴⁻¹⁶ That's why amylases are among the essential enzymes covering about 30% of the world's enzyme production.¹⁷

The recent industrial growth of Bangladesh stands on the backbone of the textile and pharmaceutical industries.¹⁸ Every year, we import a tremendous amount of amylase for food, textile, pharmaceutical & detergent usage and in the different industrial sectors.¹⁹⁻²¹ As a result, this sector is fully imported based and consumes huge amount of foreign currency every year. There is no enzyme industry in Bangladesh capable of responding to local demand. This is why this type of research will be wrathful to isolate bacterial strains that can lead to the production of amylase enzyme from the potato dump soil.

MATERIALS AND METHODS

Sample collection. Soil samples from potato dumpsites were collected in different areas of Munshiganj, Bangladesh. A sterile spatula, gloves and clear polyethylene bag were used for sampling. Soil samples were then kept in an insulated potable sample box, maintaining the temperature of 2-5°C.

Isolating amylase-producing bacteria. Freshly collected soil samples (10 g) was taken into a sterile conical flask and 90 ml of distilled water was added. It was then mixed properly and distributed in seven different test tubes for serial dilution.^{22,23} After serial dilution (10^{-1} to 10^{-7}), 100 μ l diluted samples were pipette out from each test tube, transferred into a nutrient agar media plate and was spread well by a glass spreader based on the spread plate technique.²⁴ For proper growth of bacteria, media plates were kept

in an incubator (Model: ICV404 ECO) at 37°C for 48 h. Bacterial colonies were isolated from each culture plate and the subculture was done in freshly prepared nutrient agar media. The subculture was continued until pure isolate was obtained.²⁵ Stock cultures were maintained on a nutrient agar plate at 4°C.

Identification of potent amylase-producing bacteria using starch hydrolysis test. Starch hydrolysis test was done to confirm amylase-producing bacteria.²⁶ With the help of a sterile inoculating loop, bacterial colonies were again subcultured in a starch agar plate by the dot method.²⁷ Starch agar plates were kept in an inverted position in an incubator for 48 h at a temperature of 37°C. Then the iodine solution was pipette out in the plates and bacterial isolate showed hydrolysis apparent zone ratio.²⁸ For the calculation of the hydrolysis clear zone ratio, the diameter of the clear zone and the diameter of the colony were considered. The test method was carried out three times for appropriate confirmation.

Amylase production using submerged fermentation process. Two bacterial isolates were inoculated in two separate 500 ml Erlenmeyer flasks. The working fermentation medium was made at pH 7.0 in a composition of soluble starch-1.0 g/100ml, yeast extract- 0.5 g/100 mL, MgSO₄-0.1g/100 ml, KH₂PO₄ - 0.2 g/100 ml, (NH₄)₂SO₄ -0.5 g/100ml, CaCl₂-0.05 g/100 ml. Medium (100 ml) was then put in each Erlenmeyer flask. The inoculation was carried out with fresh bacterial spores. Then the flasks were placed in a shaker incubator (Model: Stuart SI500) at 37°C with 130 rpm for 48 h. After incubation, the culture medium was centrifuged (Model:TT-15000R) at 9,000 rpm for 12 min. It was done in triplicate. After centrifugation, the obtained supernatant containing crude enzyme was stored in a screw cap test tube at 4°C.

Enzyme assay to determine amylase activity. To determine the amylase activity of bacterial isolate, the DNS method was used as described with some modifications.²⁹ So, two screw cap test tubes were taken in which 1% starch solution (1 ml), diluted crude enzyme (1 ml) and 0.1 M sodium phosphate buffer at pH 7.0 (1 ml) were put successively. The

solution was incubated for 30 min at 37°C. After 30 min of incubation, 1 ml DNS solution was added to each test tube. The samples were kept in the water bath and boiled for 10 min at 100°C then cooled down at room temperature using tap water, and 8 ml of distilled water was added to each test tube. The absorbance of the solution was taken against the blank solution at 540 nm using UV-visible spectroscopy (Model: Perkin Elmer, Lambda 365). The standard glucose curve for different concentrations was used to estimate the glucose concentration of each sample. One micromole of glucose is equivalent to one unit of amylase production under test conditions.²⁹ The process was conducted as triplicates.

Determination of specific enzyme activity. To determine the specific enzyme activity of bacterial isolate, Folin-Lowry method was used.³⁰⁻³² The absorbance of sample solution was taken against blank solution at 650 nm using UV-visible spectroscopy (Model: Perkin Elmer, Lambda 365). A standard bovine serum albumin curve with a different concentration was considered for each sample's specific enzymatic activity.

Morphological and biochemical identification. Bacterial isolate (MC-15) was morphologically characterized and examined under the microscope (Olympus, Model: BX53F2) after gram staining.²⁹ This isolate was characterized biochemically using the catalase test, simmon's citrate test, gelatin hydrolysis test, nitrate reduction test, V.P.(Voges Proskauer) test, casein hydrolysis test and indole test.

Molecular identification (16S-rRNA gene sequencing). The total genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA). Amplification of the 16S rRNA gene was performed by the primer sets of 27F (5'-AGA GTT TGA TCM TGG CTC AG- 3') and 1492 R (5'-GGT TAC CTT GTT ACG ACT T- 3'). PCR was carried out in a total volume of 25 µl with some modification that contained 12.5 µl GoTaq® G2 Hot Start Master Mix, 2 µl template DNA, 1µl forward primer 27 F, 1µl reverse primer 1492 R and 8.5 µl nuclease free water).³³ PCR steps included initial

denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 49°C for 30 sec, extension at 72°C for 90 sec and final extension at 72°C for 5 min. The PCR product was electrophoresed in 1% agarose gel and approximately 1.5 kb amplicons were generated. Afterwards, the DNA was purified from the PCR amplicons using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher, USA). The purified products were subjected to sequencing by the Sanger technique (ABI 3500 Genetic Analyzer, Applied Biosystems). The sequence data of bacteria and phylogenetic tree were determined with the help of reference bacterial sequence by comparing 16S rRNA using TYGS database (<http://tygs.dsmz.de>).³⁴

Purification of amylase enzyme. The purification of the enzyme is a series of processes such as ammonium sulfate precipitation, dialysis and gel filtration Sephadex G-100 column (20 mm diameter x 80 mm long) to isolate a single type of enzyme from a complex mixture.

Precipitation with ammonium sulfate. A 20 % ammonium sulfate solution was added to the crude enzyme solution, stirred for 30 min and chilled. The ice-cold suspension was centrifuged at 9000 rpm for 12 min at 4°C. And the precipitate was collected. The supernatant was then fractionated with an addition of ammonium sulfate at a concentration, 40%, 60% and 80% stirred for 30 min and chilled, then centrifuged at 9000 rpm, for 12 min at 4°C. This precipitate was collected at various concentrations and dissolved in sufficient amount of 0.1 M sodium phosphate buffer, pH 6.5. It was again centrifuged at 9000 rpm, for 12 min at 4°C and the supernatant was collected for further steps.³⁵

Dialysis against buffer. The supernatant is then placed in the dialysis bag (Cut-off 30,000 Daltons) in 1 liter of 0.1 M sodium phosphate buffer with a pH 6.5 and slowly stirred for 24 h. Dialyzed enzyme was stored at 4°C in the refrigerator.

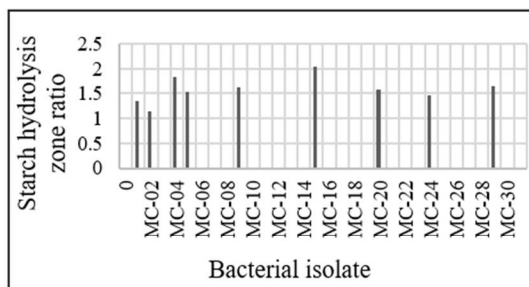
Gel filtration chromatography. The dialyzed enzyme solution was passed through the Sephadex G-100 column (20 mm diameter x 80 mm long) in gel filtration chromatography. For column equilibration,

0.1M sodium phosphate buffer with a pH 6.5 was used and the flow rate was 0.7 ml/min. After the baseline stability, the sample was mixed with 0.1M sodium phosphate buffer, pH 6.5, and passed through the column where the flow rate was 0.7 ml/min. The same buffer and flow rate were used in the washing and sample elution. The protein elution profiles were monitored at 280 nm.

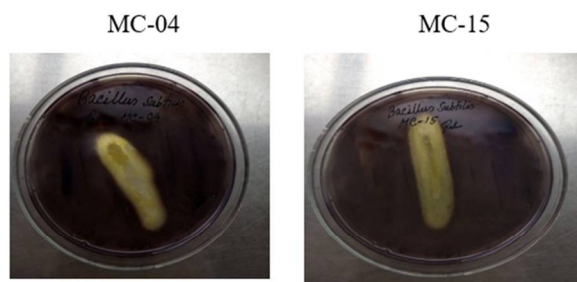
Optimization of incubation period, pH, temperature and carbon source for amylase production. For the optimization of amylase production different incubation periods (24, 48, 72 and 96 h), different pH (5.5, 6.0, 6.5, 7.0, 7.5 and 8.0), different temperature (30°C, 35°C, 40°C, 45°C, 50°C and 55°C) and different concentration of carbon sources (1% to 5% starch, 1% to 5% glucose, and 1% to 5% sucrose) were studied and the maximum yield of amylase was found by following above-mentioned DNS method.

RESULTS AND DISCUSSION

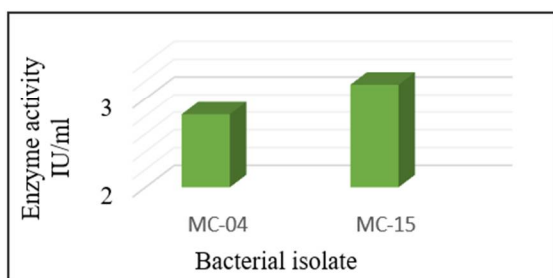
MC-15 isolate exhibited highest starch hydrolysis activity. Figure 1(a) represents the hydrolysis zone ratio of 9 isolates from 31 isolates on starch agar medium. The MC-04 and MC-15 isolates exhibited higher hydrolysis clear zone ratios. Figure 1(b) shows the representative snapshot of the hydrolysis zone of the MC-04 and MC-15 isolates. The graphical expression of figure 1(c) and figure 1(d) showed that the isolate MC-04 gave crude enzyme activity of 2.82 IU/ml and specific enzyme activity of 3.42 IU/mg; for isolate MC-15, it was 3.16 IU/ml and 3.77 IU/mg. In previous studies for *Bacillus* species showed that the crude enzyme activity result of 1.97 ± 0.41 U/mg and 2.243 $\mu\text{mol/ml/min}$ which was almost similar in our study.^{37,38}



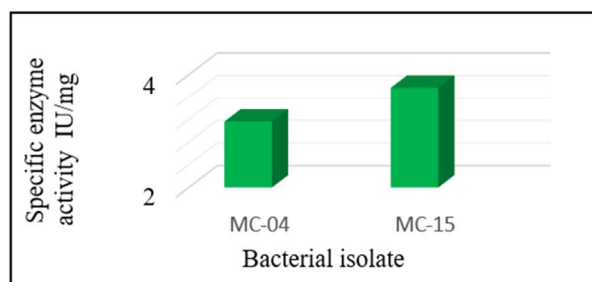
(a): Starch hydrolysis zone ratio of the amylase producing bacteria on starch agar plates.



(b): Isolate MC-04 and MC-15 showing positive starch hydrolysis test.



(c): The enzyme activity of isolate MC-04 and MC-15



(d): Specific enzyme activity of isolate MC-04 and MC-15

Figure 1. Graphical and pictorial overview of screening and enzymatic activity for isolate MC-04 and MC-15.

Microscopic and biochemical characterization of isolate MC-15. Morphological characteristics of the isolate MC-15 was determined. The results indicated that isolates MC-15 was rod shaped bacteria like bacilli. Moreover, the isolate was found to be Gram positive. Biochemical test was performed for this isolate and observed positive results by catalase test, simmon's citrate test, gelatin hydrolysis test, nitrate reduction test, V.P.(Voges Proskauer) test, casein hydrolysis test and found negative result by indole test.

Molecular identification of isolate MC-15. For the amplification of 16S rDNA, genomic DNA was extracted from Isolate MC-15 and PCR was performed. The gel electrophoresis showed an

appropriate band of 1500bp (Figure 2). After the purification of the PCR amplified 16S rDNA gene, the sequence was obtained and analyzed to identify the isolates.

On the basis of multiple sequence alignments, a phylogenetic tree was constructed for determining the relationship between isolates MC-15 and other reference strains using TYGS data base. The phylogenetic tree was constructed by the maximum likelihood method. It can be observed that the query sequence shares the same node with *Bacillus subtilis ATCC 6051* and *B. subtilis NCIB 3610* gene complete sequence and the bootstrap value is 78. This confirms that isolate MC-15 is as strain of *B. subtilis* in figure 3.

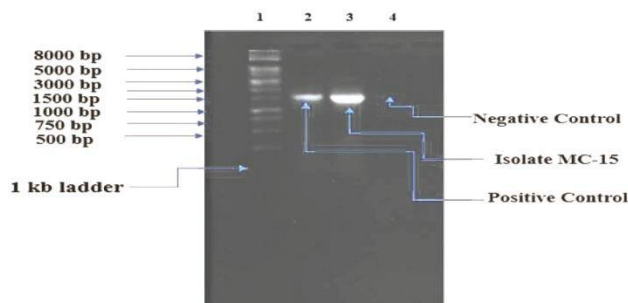


Figure 2. A purified PCR product of 16S rDNA (1500 bp) was visualized in gel electrophoresis. Lane 1-DNA ladder; lane 2-positive control; lane 3- 16S rDNA band of isolate MC-15; lane 4- negative control (mock-PCR), without any DNA template.

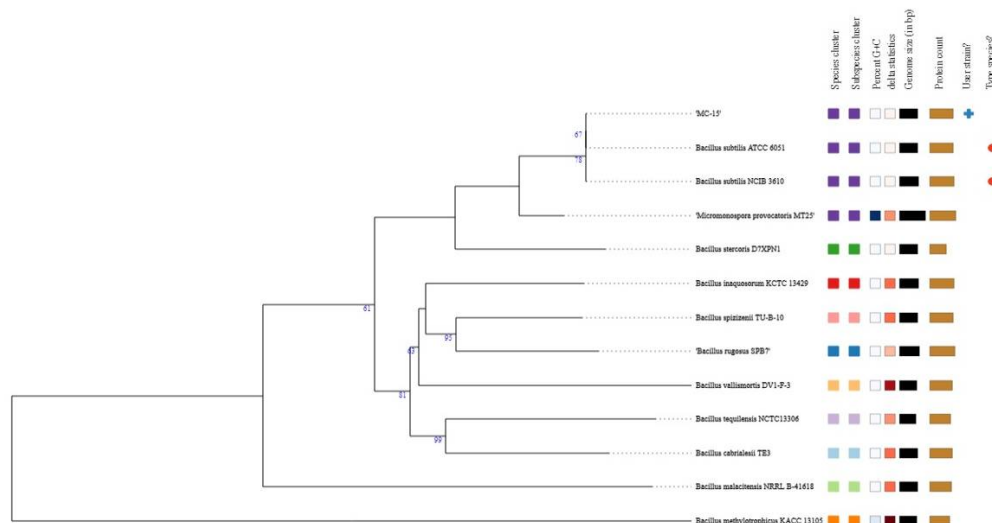


Figure 3. Phylogenetic tree based on homology sequence of 16S rRNA for isolate MC-15.

Purification of amylase enzyme for isolate MC-15. Collected crude enzyme was partially purified by the fractionation of various concentrations of ammonium sulfate solution (20% - 80%). Higher enzyme activity, 4.21 IU/ml, was found at 60% ammonium sulfate which was an approximately 1.33 times higher compared to the crude enzyme. For the removal of excess ammonium sulfate, dialysis was performed against 0.1 M sodium phosphate buffer, pH 6.5 for 24 h. Enzyme activity was found at 5.53 IU/ml, which was 1.75 times higher than the crude one. After dialysis, the enzyme solution was passed through the Sephadex G-100 column (20 mm diameter X 80 mm length) against 0.1M sodium phosphate buffer at pH 6.5. The protein elution profiles were monitored at 280 nm. Enzyme activity was found at 8.91 IU/ml, which was 2.82 times higher than the crude enzyme.

Optimization of amylase production for isolate MC-15 in different parameter. Optimization is very important for maximum yield of industrial enzyme production. Figure 4 represents the

amylase activity against different incubation periods, pH, temperature and concentration of carbon source. It reveals that MC-15 showed maximum amylase activity in an incubation period of 48 h. Previous studies also showed similar results of optimum activity of amylase at 48 h of incubation.^{31,39} When assay the enzyme activity at different pH, maximum amylase activity was observed at pH 7.0 (Figure 4b). Previous studies also showed similar results,^{38,42} which was almost similar in our study.

The temperature has an important effect on the denaturation of protein. Enzymatic reaction rate increases at a certain temperature, but when it exits, then reaction rate decreases with increasing the temperature. It was found that 40°C was the optimum temperature to achieve the highest yield. In previous studies, the optimal amylase activity was found at 37°C.^{38,42} In figure 4(d), the maximum activity of amylase was determined with starch (2%) solution which was used as the carbon source. In previous studies, best amylase activity found by using rice flour and starch as a carbon source.⁴²

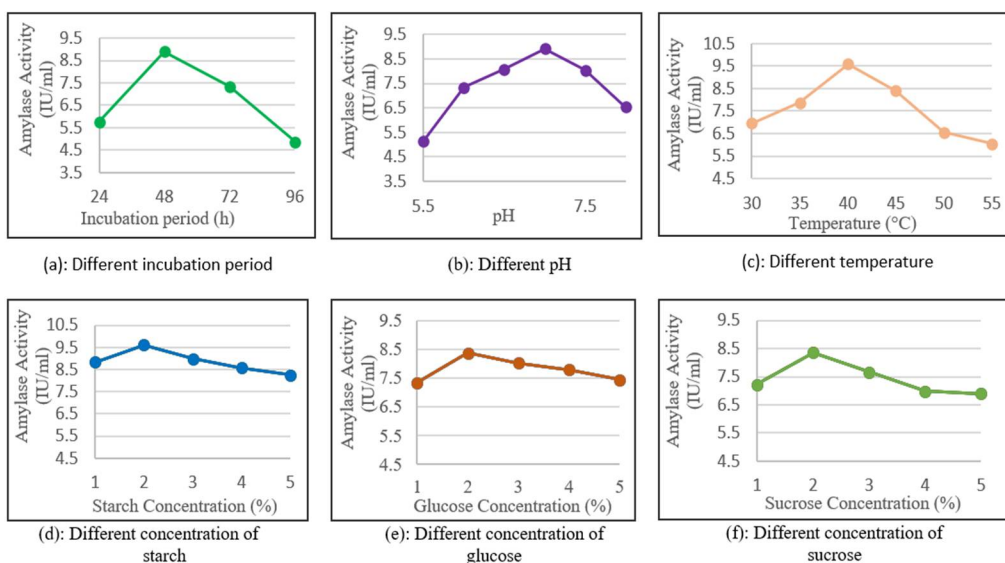


Figure 4. Optimization of incubation period, pH, temperature and carbon source on amylase production.

CONCLUSION

The research showed that the MC-15 isolate identified by morphology, biochemical and molecular

characterization using 16S-rRNA genetic sequencing of the bacterium and confirmed as *B. subtilis*. This isolate gave the best amylase activity after

optimization of various incubation periods, pH, temperatures and carbon sources. For amylase production, different industrial parameters were determined. The maximum yield of amylase obtained after 48 h of incubation, at pH-7.0, at 40°C with 2% starch as a carbon source. After purification, the activity of the enzyme was 8.91 IU/ml which was 2.82 times greater than that of crude enzyme. So, this isolated *B. subtilis* can be a good resource and considered as a commercial amylase producer in the industrial sector and it will be effectively applied especially in pharmaceutical textile and food industries of Bangladesh in the future.

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Conflicts of interest

The authors hereby declare no conflict of interest and no funding was received.

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