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CHARACTERIZATION OF AMYLASE-PRODUCING *BACILLUS* SPECIES AND INSIGHT INTO ITS AMYLASE ACTIVITY

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ABSTRACT

Microbial amylase enzymes and their screening are crucial for accelerating the production of various products, from food and beverages to biofuels, through efficient starch hydrolysis, reducing costs and environmental impact. Amylase-producing bacterial isolates were collected from garden soils and the isolate with the highest starch degrading index (SDI) was subjected to identification and characterization. The isolate was identified as *Bacillus subtilis* through analysis of its 16S rRNA gene sequence. As the concentration of starch increased, the isolate exhibited enhanced growth and amylase activity up to a starch concentration of 8%. However, amylase activity sharply declined when the starch concentration reached 16%. The most significant increase in bacterial population occurred at 37ºC, although there was notable growth even at 50ºC. A slight reduction in amylase activity was noted as the temperature ranged from 25ºC to 55ºC. At pH 7, the isolate showed its highest growth, however, the amylase activity didn't show any significant changes at pH ranges from 6 to 8. Characterization aids in fine-tuning production procedures, cutting down expenses and boosting output. It enables the identification of valuable enzyme sources, optimization of production processes, and the development of sustainable and efficient solutions in various fields.

Keywords: Amylase, starch, characterization, optimization, *Bacillus*, Bangladesh

INTRODUCTION

Amylase is a remarkable enzyme that holds a pivotal role in the world of biology and industry. It is classified as a hydrolase enzyme, primarily responsible for breaking down starch and glycogen into simpler sugars, namely maltose and glucose, through hydrolysis. This enzymatic action is of fundamental importance in various biological processes, including digestion in humans and other animals, as well as in the germination of seeds. Beyond its biological significance, amylase has found extensive utility in a wide array of industrial applications, ranging from the production of alcoholic beverages and food processing to textile and detergent manufacturing. This versatile enzyme, derived from various sources, such as fungi, bacteria, and plants, continues to captivate researchers and industries alike, as its diverse applications and enzymatic properties unlock new possibilities in the fields of biotechnology and beyond (**de Souza & de Oliveira Magalhães, 2010; Jujjavarapu and Dhagat, 2019**). However, amylase produced by microorganisms is widely known for cost-effectiveness, quick production for rapid growth of microorganisms, requires small space for production, environment- and user-friendly technique, and lower manpower. The role of amylase in industries is huge as amylase contributes 25% in various industries including food, detergent, paper, textile, and fermentation. The need for amylase enzymes is increasing day by day in different sectors like clinical, medicine, analytical chemistry, brewing, and distillation plants (**Luang-In *et al*., 2019**).

Enzymes are used in the detergent industry to enhance stain removal effectiveness and to make detergents more environmentally friendly. Amylases are regarded as the second most crucial enzyme in detergent formulations and are present in approximately 90% of all liquid detergent products. These enzymes have many beneficial roles in the laundry and cleanup of automatic dishwashers for mostly starchy residues that are stuck in the machine, for example, custard, potato, chocolates, and so on (**Gurung *et al*., 2013**).

Amylase production from bacteria is getting more attention day by day because its stability is higher than that of plant and animal sources. Thermostable amylase has been prioritized in the industrial sector for commercially important starch-degrading products such as crystalline dextrose, glucose, maltose, dextrose syrup, and maltodextrins. *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis,* and *Bacillus amyloliquefaciens* are well-established and widely utilized for the commercial production of thermostable *α*-amylase, with *Bacillus stearothermophilus* and *Bacillus licheniformis* amylases being commonly employed in the starch industry. Soil is a perfect habitat for various microorganisms including bacteria, yeast, and fungi. They play a vital role in the breakdown of complex organic materials to simply accessible forms by enzyme secretion and improve the condition of the soil to uptake the nutrients for plants. *Bacillus* spp. is also easily found in soils and amylase-producing *Bacillus* strains can be isolated from the soil (**Gurung *et al*., 2013; Islam and Zerin, 2019**). For our research, we selected garden soil due to its high nutrient content. Following preliminary screening, we isolated and examined amylase-producing bacteria, evaluating their amylolytic activity by optimizing factors such as temperature, pH, and substrate concentration. Local screening for amylase-producing bacteria is vital for economic, environmental, and scientific reasons, as it facilitates local enzyme production, reduces environmental impact, and supports regional growth and innovation.

MATERIALS AND METHODS

Sample collection and processing

Soil samples were collected in a sterile beaker using a sterile spatula at a depth of 2-3 cm from gardens of Bangladesh including Ramna Park in Dhaka, Muktagacha in Mymensingh, and Siddeshwari campus of Stamford University Bangladesh (SUB), Dhaka. Using the sterile sampling tool, a sufficient amount of soil from different spots within the chosen area was collected. The soil samples were placed into sterile containers (plastic bags or containers with lids) and sealed tightly to avoid any contamination during transport. The soil samples were transported cool to the lab to preserve the microbial activity. A 1-gram sample of soil was combined with 99 milliliters of sterile normal saline and mixed thoroughly, as described by Cappuccino and Sherman (**Cappuccino and Sherman, 1996**). From this mixture, one milliliter was taken and introduced into 9 milliliters of sterile normal saline, and serial dilutions were subsequently performed up to a dilution factor of 10^{-8}.

Isolation and screening of amylase-producing bacteria

Bacterial isolates were obtained and evaluated for amylase activity using the starch hydrolysis method on starch agar plates, following the procedure outlined by Islam and Zerin (**Islam and Zerin, 2019**). In brief, 100 µl of serially diluted bacterial suspensions were spread onto nutrient agar plates and incubated at 37°C for 24

hours. Individual colonies that developed were then streaked onto starch agar plates, where starch served as the sole carbon source, to assess their ability to hydrolyze starch. The plates were incubated at 37°C for a period of 24 to 48 hours. Following incubation, the plates were treated with Gram's iodine solution (prepared by dissolving 250 mg of iodine crystals in a solution of 2.5 g potassium iodide and 125 ml of water, stored at room temperature) to visualize starch hydrolysis. Colonies producing a clear zone around their growth were considered positive for amylase activity, while a deep blue coloration indicated no starch degradation. Pure isolates exhibiting clear halos were periodically subcultured and preserved on nutrient agar slants at 4°C.

Determination of Starch Degrading Index (SDI)

SDI refers to the capacity of the isolates to break down starch. It is calculated by the ratio of the total diameter of clear zone and colony diameter. According to the degrading index, potential colonies with the best efficiency were selected as the best starch-degrading colonies (**Nusrat and Rahman, 2007**).

Morphological, cultural, and biochemical characterizations

The amylase-producing bacterial isolates were tentatively characterized through Gram staining, spore staining, motility examination, and an analysis of their cultural and biochemical traits, following the classification guidelines provided in Bergey's Manual of Determinative Bacteriology (**Bergey and Holt, 2000**). The biochemical evaluations included a series of tests such as methyl red, Voges-Proskauer, citrate utilization, indole production, hydrogen sulfide (H_2S) production, motility, gelatin liquefaction, sugar breakdown, oxidase activity, catalase reaction, and various carbohydrate fermentation assays.

Molecular characterization

DNA extraction

Genomic DNA was extracted from a pure bacterial culture, 24 h grown in Nutrient Broth (Oxoid, UK) at 37°C, and centrifuged (SCILOGEX, Germany) for 15 min at 15000 g. The remaining pellet was resuspended in 100 µl of molecular biology-grade nuclease-free water using a vortex mixer (SCILOGEX, USA) after the supernatant was removed. The mixture underwent another centrifugation step for 10 minutes at 15,000 g. The residual pellet was collected and resuspended with nuclease-free water and heated for 10-15 min at 100°C in a heat block (SCILOGEX, digital dry bath) as a conventional boiled DNA method. The heating step is followed by cooling on ice. In the final step, centrifugation was performed to obtain the supernatant, which was then stored at -20°C for subsequent genomic analysis.

PCR conditions and the reaction mixture

The extracted bacterial DNA served as the template for PCR amplification, which was carried out using a thermal cycler (Model AERIS, 96 wells, ESCO Micro Pte. Ltd, Singapore). To target the 16S rRNA gene and enable species-level identification, specific oligonucleotide primers were used: forward (F) 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse (R) 5'-GGTTACCTTGTTACGACTT-3'.

A 15 µl PCR reaction mixture was prepared using GoTaq Green master mix. Each reaction included 1 µl of the forward primer, 1 µl of the reverse primer, and 1 µl of DNA template. The thermal cycling conditions were set as follows: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute and 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute and 30 seconds. The expected PCR product was approximately 1465 base pairs in size, which was confirmed by running the samples on a 1% agarose gel stained with ethidium bromide and visualized using a gel electrophoresis system (Biometra, Germany).

The amplified products were purified using a PCR cleanup kit (FAVORGEN, Taiwan), following the manufacturer's protocol. Between 0.50 and 1.00 µg of the purified DNA was then sequenced using a 3500 Genetic Analyzer (Applied Biosystems).

The resulting partial sequences obtained from both forward and reverse primers were analyzed with MEGA 6 software. To identify the bacterial species, the sequences were compared against the GenBank database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) available at http://www.ncbi.nlm.nih.gov/GenBank.

Characterization of Growth Under Varying Conditions:

The selected bacterial isolate was cultivated in a starch medium without agar, supplemented with varying starch concentrations (0.25%, 0.5%, 1%, 2%, and 4%) to assess its growth. To determine the ideal temperature for growth, cultures were incubated at 25°C, 37°C, and 50°C. Similarly, the influence of pH was examined using media adjusted to pH levels of 3, 5, 7, and 9. Bacterial growth under these conditions was monitored by measuring optical density at 600 nm at different time intervals.

Preparation of Cell-Free Enzyme

Amylase production was initiated by inoculating a loopful of the pure bacterial culture into a production medium comprising starch (10 g/L), peptone (5 g/L), ammonium sulfate ($(NH_4)_2SO_4$, 2 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (2 g/L), and $MgCl_2$ (0.01 g/L) at pH 7. The culture was incubated at 37°C in a shaking water bath at 120 rpm for 24 hours, following the method previously described (**Vaidya and Rathore, 2015**). After incubation, 10 ml of the 24-hour-old culture was centrifuged at 5000 g for 15 minutes to remove the cells. The supernatant, containing the crude amylase enzyme, was collected and used for further optimization of enzymatic assay conditions.

Characterization of Amylolytic Activity

Amylase activity was evaluated using the 3,5-dinitrosalicylic acid (DNS) method, as outlined by Bernfeld (**Bernfeld, 1955**) with slight modifications. A 1% starch solution was freshly prepared by dissolving 1 g of soluble starch in 100 ml of 0.02 M sodium phosphate buffer (pH 7). For the enzyme assay, 1 ml of starch solution was mixed with 0.5 ml of crude enzyme and incubated at 50°C for 30 minutes. The reaction was terminated by adding 3 ml of DNS reagent, followed by boiling the mixture in a water bath for 10 minutes. The solution was then cooled under running tap water, diluted to 10 ml with distilled water, and the absorbance was recorded using a spectrophotometer. Control samples were prepared without enzyme addition. Enzyme activity was quantified by constructing a standard curve using known glucose concentrations (see Supplement 1). One unit (U/ml) of enzyme activity is defined as the amount of amylase needed to produce 1 µmol of reducing sugar (glucose) per minute under the given assay conditions.

Effect of Different Parameters on Amylase Activity

To examine how various factors influence enzyme activity, assays were conducted using different starch concentrations (0.25%, 0.5%, 1%, 2%, 4%, and 8%), temperatures (25°C, 35°C, 45°C, and 55°C), and pH levels (6, 6.5, 7, 7.5, and 8).

Statistical Analysis

All experiments were conducted in triplicate, and the results were presented as the mean ± standard deviation. The data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Isolation and Screening of Isolates

Among the 27 isolates (11 from Ramna Park soil, 8 from Mymensingh soil, and 8 from Stamford University Bangladesh garden soil) that showed prominent growth on starch agar media, 6 isolates (RP5, RP9, RP10, MC2, MC5, and MC6) exhibited good starch hydrolyzing capability according to clear zones they produced during the starch hydrolysis test (Table 1).

Table 1 Screening for Starch hydrolysis potential of the isolates isolated from three individual places.

Ramna Park		**Mymensingh**		**SUB**	
Isolates	Starch hydrolysis	Isolates	Starch hydrolysis	Isolates	Starch hydrolysis
RP1	++	MC1	-	SUB1	+
RP2	+	MC2	+++	SUB2	-
RP3	++	MC3	+	SUB3	-
RP4	+	MC4	+	SUB4	-
RP5	+++	MC5	+++	SUB5	+
RP6	+	MC6	+++	SUB6	+
RP7	-	MC7	+	SUB7	-
RP8	+	MC8	+	SUB8	-
RP9	+++				
RP10	+++				
RP11	-				

Isolate MC2 exhibited the highest starch degrading index as indicated by its clear zone-to-colony diameter ratio. Figure 1 illustrates the SDI of MC2. Perceived as having the highest starch degrading capability, MC2 was chosen for further experiments.

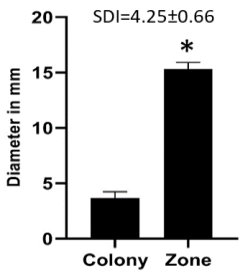

Figure 1 The starch degrading index (SDI) for isolate MC2 represents the ratio between the zone and colony. The error bars represent the standard deviation of the mean from three independent experiments, each performed in triplicate. An asterisk (*) denotes a statistically significant difference with a p-value less than 0.05.

Presumptive Identification of MC2

The result in Table 2 reveals that based on the microscopic, morphological, and biochemical properties, the isolate MC2 presumptively belonged to the genus *Bacillus*. Representative pictures of bacterial physiology, colony morphology, and starch hydrolysis are presented in Figure 2.

Table 2 Microscopy, Colony morphology and biochemical characterization of MC2 isolate

Isolate	Microscopy			Colony Morphology				Biochemical Tests											Presumptively identified bacteria
												TSI							
	Shape	Arrangement	Gram Reaction	Size	Pigmentation	Margin	Elevation	Oxidase	Catalase	MR	VP	Slant	Butt	H_2S	Gas	Indole	Citrate	Casein	
MC2	Rod	Chain	+	Medium	Off White	Entire	Raised	+	+	–	+	K	A	–	–	–	+	+	*Bacillus* sp.

Legend: MR, Methyl Red test; VP, Voges–Proskauer test; TSI, Triple Sugar Iron test; K, Alkaline; A, Acidic.

Figure 2 Microscopy (A), colony morphology (B), and starch hydrolysis assay (C) of the isolate

Molecular Identification of MC2 isolate

Genome sequencing of MC2 isolate was carried out followed by bioinformatics analysis to confirm its identity. Using reference sequences from BLAST search, a phylogenetic tree (Figure 3) was constructed which clearly identified MC2 isolate as *Bacillus subtilis.*

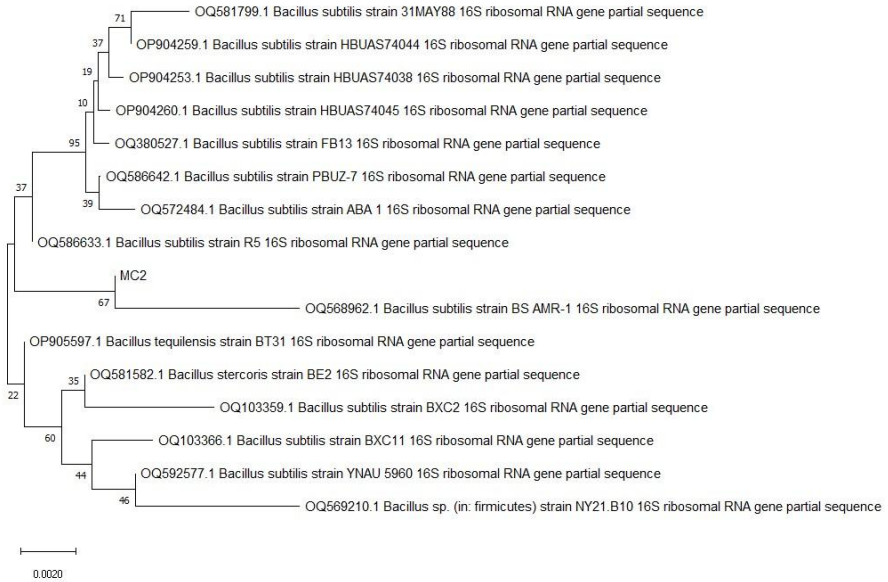

Figure 3 The evolutionary relationships were analyzed using the Neighbor-Joining method implemented in MEGA11. The most optimal phylogenetic tree is displayed, with bootstrap values (based on 1000 replicates) shown beside the corresponding branches, indicating the reliability of the groupings. The tree is scaled according to branch lengths, which reflect the evolutionary distances used in the analysis. These distances were calculated using the Jukes-Cantor model and are expressed as the number of nucleotide substitutions per site.

Determination of Optimal Growth Condition:

Having been presumptively identified as *Bacillus* sp., the optimal growth conditions of isolate MC2 were assessed by growing it in a range of starch concentrations (Figure 4A), temperatures (Figure 4B), and pH (Figure 4C). With increasing starch concentration, the isolate demonstrated improved growth, peaking at 8% starch concentration. Nevertheless, there was a rapid decline in growth once the starch concentration reached 16%. This isolate demonstrated the highest growth at 37ºC temperature and a pH value of 7.0.

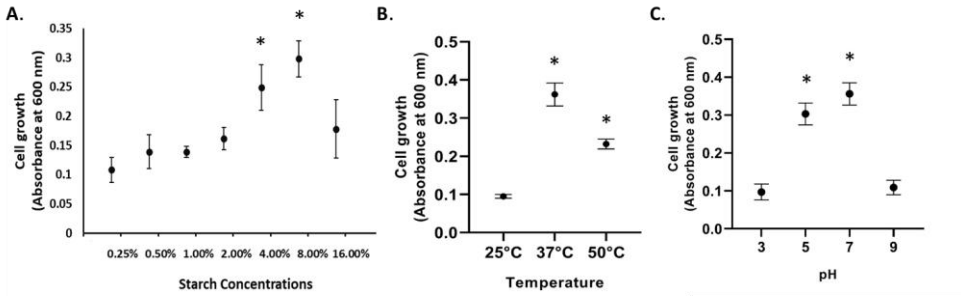

Figure 4 Characterization of cell growth of MC2 isolate in terms of starch concentrations (A), temperatures (B), and pH (C). The error bars indicate the mean ± standard deviation derived from three separate experiments, each conducted in triplicate. An asterisk (*) signifies a statistically significant difference with a p-value less than 0.05.

Determination of Amylase Activity under Varying Conditions:

Similar to the growth of the bacterial isolate, the activity of the amylase enzyme was recorded at different starch concentrations (Figure 5A), temperatures (Figure 5B), and pH (Figure 5C). Amylase activity reached its maximum level at a starch concentration of 8%, a temperature of 25ºC, and a pH of 8. However, no significant differences in amylase activity were observed with increasing temperature and pH. Nonetheless, increasing starch concentrations increased amylase activity up to 8% starch concentration which significantly drops to 16%. Error bars presented mean values of the standard deviation of triplicates of three independent experiments. ANOVA was performed with a p-value ≤ 0.05 considered significant.

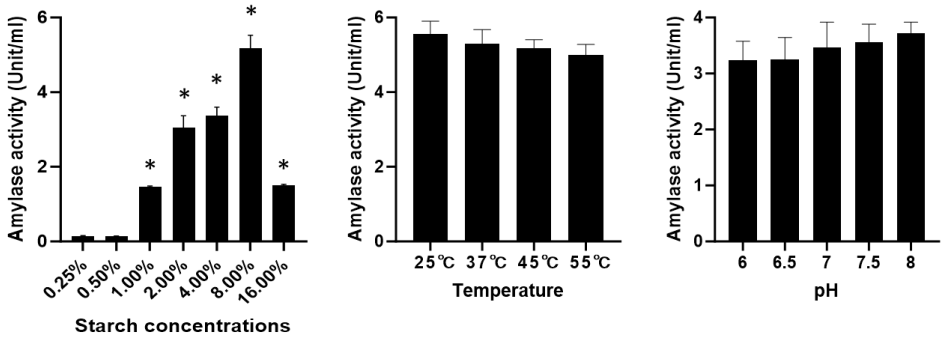

Figure 5 Characterization of amylase activity of MC2 isolate in terms of starch concentrations (A), temperatures (B), and pH (C). The error bars represent the average ± standard deviation from three independent experiments, each performed in triplicate. An asterisk (*) denotes a statistically significant difference with a p-value below 0.05.

DISCUSSION

According to a previous study, bacteria capable of producing amylase enzymes have a higher probability of being isolated from sources rich in starchy materials (**Mishra and Behera, 2008**). As indicated, we selected garden soil as our choice of sample. Because gardens usually harbor different types of plants, it could be easier to obtain amylase-producing bacteria from them. Collecting soil samples from different sources allows for a comprehensive assessment of amylase activity across diverse ecological settings within Bangladesh, facilitating comparative analysis and providing insights relevant to the local context.

After screening 27 isolates initially, only one, known as MC2, was chosen for further study because it consistently showed the highest Starch Degradation Index (SDI) in several experiments. The aforementioned isolate was subjected to grow under varying conditions to identify its optimum growth parameters. According to the studies of (**Hong *et al*., 2019; Nur Indah *et al*., 2017**), the optimum temperature for growth of different strains of *Bacillus subtilis* is 37ºC. Our isolate also gave a similar temperature pattern. In addition, its optimum pH for growth was observed to be at pH 7.0, consistent with the previous findings (**Nur Indah *et al*., 2017; Satapute *et al*., 2012**). In addition to temperature and pH, MC2 was grown in the plates supplemented with increasing starch concentrations as media components have shown to influence the growth of bacteria as well as the production of amylase enzyme **(Srivastava and Baruah, 1986**). Similar to the studies of Mishra and Behera, our isolate also exhibited higher growth as the starch concentration was increased and grew the highest at 8% starch concentration (**Mishra and Behera, 2008**).

Afterwards, the effect of varying conditions including starch concentration, temperature, and pH on the amylase enzyme activity was studied. According to several past studies, amylase enzyme activity usually increases with the increase in soluble starch concentration up to a certain amount which correlates with our study (**Bharathiraja, 2016; Santos and Martins, 2003**). A significant rise in amylase activity was observed as the starch concentration increased from 1% to 8%, followed by a sharp decrease at 16% starch concentration. However, it's essential to note that there is a limit to how much starch can be utilized by bacteria. Beyond a certain concentration, the rate of bacterial growth and amylase activity may plateau or even decline because other factors, such as nutrient competition, waste product accumulation, or toxic effects of high starch concentrations, can come into play.

A number of studies have been conducted for determination of optimum temperature for the activity of bacterial amylase. Results of all such experiments demonstrated that the optimal temperature can range anywhere from 30 to 100ºC (**Divakaran *et al*., 2011**). In the case of our study, enzyme activity at four different temperature points (25ºC, 37ºC, 45ºC & 55ºC) was almost similar with the highest activity being recorded at 25ºC.

pH is another important factor that can heavily influence the activity of enzymes. As such, a plethora of studies have been made for determination of pH for optimal enzyme activity. The amylase we extracted from the bacterial isolate exhibited a gradual rise in activity along a range of pH from 6.0 to 8.0 where each interval had a difference of 0.5. The highest activity was measured at pH 8.0 which is similar to the earlier study **(de Carvalho et al., 2008**). However, characterizing MC2 isolate with high amylase activity might provide valuable insights into potential applications in biotechnology industries.

CONCLUSION

Local screening of amylase-producing bacteria is crucial for economic, ecological, and scientific rationales since it enables localized enzyme manufacturing, mitigates environmental effects, and fosters regional progress and creativity. Through rigorous screening and evaluation, we can unearth bacterial strains that exhibit exceptional amylase production capabilities. However, the journey to finding these strains is often a complex and multifaceted process. It involves not only isolating high-yield amylase producers but also considering their suitability for specific industrial applications, their genetic diversity, stability, and environmental impact. Moreover, the optimization of culture conditions, substrate concentrations, and other environmental factors may be necessary to fully harness their potential.

Conflict of Interest: The authors declare that there are no conflicts of interest associated with this study.

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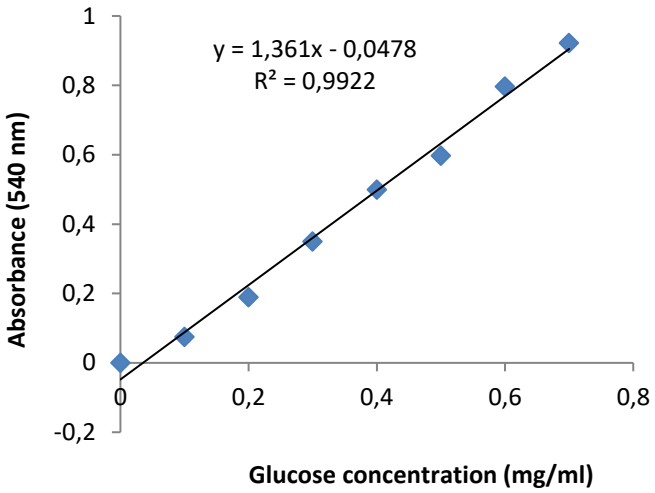
Supplement 1:



Supplement 1: Standard curve of glucose.