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BACILLUS SUBTILIS EMEA 22, POTENT MANGROVE RHIZOSPHERE AMYLASE PRODUCER: ISOLATION AND CHARACTERIZATION

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ABSTRACT

Amylases are one of the principal biocatalysts with significant applications in the fields of food, fermentation, textile, paper, detergent, and pharmaceutical industries. The present study focused on *Bacillus subtilis* EMEA 22, a halo-tolerant inhabitant of the soil of mangrove ecosystems capable of producing a greater amount of amylase. The whole isolates from different soil samples were further screened for amylase activity by introducing them into the starch agar. Furthermore, the amylase assay was also inspected. The bacteria with potential amylase activity were identified by biochemical and molecular characterization. Partial purification of the enzyme was carried out, which was further characterized by holding it at different temperatures, pH, and in the presence of metal ions. The outcome of which revealed that the isolated enzyme has the potential to maintain its maximal activity at pH ranges 7-9. The enzyme exhibited a high rise in activity in the presence of CaCl2 at 1 mM concentration, moreover, the study demonstrated the enzyme's textile starch removal capacity.

Keywords: *Bacillus subtilis*, Amylase, Mangrove, Rhizosphere

INTRODUCTION

Currently, amylase production accounts for up to 30% of the global enzyme market and is steadily rising. Because of their availability, quick fermentation cycles caused by their rapid growth rates, simplicity, and the ability to secrete proteins into an extracellular medium, maximal yield, and general handling safety, numerous microorganisms have been found and chosen as sources of amylase synthesis. However, amylase obtained from bacteria and fungi has predominated in several biotechnology applications, including those involving *Bacillus* sp., *Lactobacillus* sp., *Proteus* sp., *Escherichia coli*, *Pseudomonas* sp., *Streptomyces* sp., *Aspergillus* sp., *Talaromyces emersonii*, and *Thermomyces lanuginosus*, *Aspergillus niger*, *Penicillium*, *Rhizopus*, *Cephalosporium*, and *Neurospora* **(Ojha *et al*., 2020)**. Finding the bacterial strain that can produce amylase at a greater production rate and at a lower cost is still being researched. Thus, it is essential to have starch-degrading enzymes that are appreciably stable at high temperatures. The main advantage of using such a type of stable amylase in industrial processes comprises the decreased risk of contamination and the increased diffusion rate. Studying the microbial communities that can survive and reproduce in harsh settings is one of the key alternatives for reducing contamination in the industrial-scale production of biomolecules **(Rakaz *et al*., 2021; Yassin *et al*., 2021).**

There are three different types of amylases: α -α-α-amylase, β -β-β-amylase, and γ-amylase. The internal α, 1-4 glycosidic bonds of starches and other polysaccharides are broken down by an industrial enzyme known as alpha-amylase to produce a variety of products like glucose and maltose **(Agrawal *et al*., 2005)**. It belongs to the family glycoside hydrolase(GH) of GH13 (most of them), GH57, GH119, and GH126 and is one of the most widely used commercial enzymes **(Elyasi Far *et al*., 2020; Srivathsan *et al*., 2022**). Alpha-amylase is the extracellular enzyme with the most commercial range of uses, with particular growth in the fields of clinical, pharmaceutical, and analytical chemistry. Bacteria that break down starch are mostly used in the food, textile, fermentation, and paper sectors. The biotechnology industry places a high value on the isolation and manipulation of pure cultures of starch-degrading microorganisms from soil. Therefore, purifying and modifying pure culture from a variety of waste materials is crucial for many biotechnology enterprises **(Rakaz *et al*., 2021).** Amylases from animal and plant sources have low resilience to acidic, basic, and high-temperature environments when their origins are compared. In contrast, bacterial and fungal amylases have better stability in these circumstances and more cost-effective production processes **(Souza & Magalhães, 2010)**. Consequently, microbial enzymes are used in a wide range of applications **(Elmansy *et al*., 2018).**

Mangrove ecosystems are complex, diverse and dynamic ecosystems varying in salinity, water level, and nutrient availability. The ecosystem of mangroves is characterized by high rates of organic matter recycling and nutrient turnover between terrestrial habitats and the ocean. These are the most productive ecosystems for the isolation of microbial communities with great ecological, economic and social importance. Mangrove sediments are rich in diverse groups of the microbial community, which play a key role in the productivity and maintenance of the carbon budget. The enzymes produced by the mangrove-associated bacteria have reached industrial attention due to their elevated tolerance to withstand their native form at the extreme environment **(Allard *et al*., 2020; Gomes *et al*., 2010)**. In recent decades, the mangrove ecosystem has been consistently in jeopardy and stands dangerously close to extinction due to careless human interference and another natural disaster. Loss of these unique ecosystems triggers the loss of other dependent species, enriched soil and other unexplored assets such as microbial communities of this ecosystem **(Sandilyan & Kathiresan, 2012)**.

Utilizing microorganisms is being considered for many purposes, including heavy metal absorption, gene engineering, digestion, the production of novel anti-microbes, and particularly for the production of industrial enzymes, as opposed to chemical methods that require harsh conditions such as high pressure and temperature. In contrast to chemical processes that call for harsh conditions like high pressure and temperature, using microorganisms for a variety of purposes, such as heavy metal absorption, gene editing, digestion, the production of novel anti-microbes, and especially for the production of industrial enzymes, is being considered **(Elyasi Far *et al*., 2020)**. Due to its wide range of uses and practical advantages, alpha-amylase has attracted a lot of attention. Currently, and with continued growth, amylase manufacturing accounts for up to 65% of the global enzyme market. Starch liquefaction, paper, desizing of textile materials, making starch coatings for paints, removing wallpaper, the brewing sector, sugar induction through the creation of sugar syrups, and pharmaceuticals are just a few of the many industries where it is used. Microorganisms and larger organisms can both manufacture alpha-amylase **(Simair *et al*., 2017)**. The current work aimed to (a) isolate and identify the potent amylase-producing bacteria from the mangrove rhizosphere of Calicut, and (b) to isolate and characterize the partially purified amylase from the identified isolate.

MATERIALS AND METHODS

Isolation of amylase-producing bacteria

The mangrove rhizosphere soil samples from 6 different locations of the Kadalundi mangrove forest were collected and screened for bacterial flora through serial dilution and spread-plate technique as per standard procedures. For that, one gram of each soil sample was serially diluted with sterilized distilled water in the range of 10^{-1} to 10^{-6}. Thereafter, from dilutions of 10^{-5} and 10^{-6}, 0.1 ml suspension was transferred and equally spread on nutrient agar plates in aseptic conditions. These plates were incubated at 37 °C for 24 h. Individual bacterial isolates with different morphological appearances were picked up and subcultured further to obtain pure cultures.

Screening for amylase-producing bacteria

For screening of amylase producers, each isolate was streaked over the starch agar (Soluble starch -2g, Agar-2 g, Peptone- 0.5 g, Distilled water-100 ml) plates and incubated for 24 hrs at 37 °C. After incubation, the production of amylase was noticed by adding 2% of Lugol's Iodine solution. Bacteria exhibited amylase activity when further grown in starch broth (1 % starch) and incubated for 48 hours. The cell-free supernatant was collected after centrifuging at 8000 rpm for 15 minutes and added to the well bored on starch agar. A clear zone of starch hydrolysis around the well was observed, and the diameter was measured. The isolate that exhibited the largest clear zones around the well was selected for further studies.

Identification of a potential isolate

Morphological and Biochemical Characterization

The bacterial isolate was subjected to different microscopic and macroscopic examinations, such as colony characterization **(Silaban *et al*., 2020)**, Grams staining and endospore staining procedure. Furthermore, the bacterial isolate was biochemically characterized by Indole, Methyl red, Voges-Proskauer test, and Citrate utilization test. After that, the fermentation capacity of different sugars such as glucose, lactose, arabinose, sorbitol, mannitol, rhamnose, sucrose, and adonitol was also inspected **(Lechevalier, 1989)**.

Sequencing of the 16S rRNA region using universal primers.

The DNA was isolated from overnight cultures of isolate EMEA 22 by **Unal (1992)** method described. The specific primer for 16S-rDNA used was Forward-CAGGCCTAACACATGCAAGTC, Reverse-GGGCGGWGTGTACAAGGC. The PCR reaction was performed using standard reagents and a BIORAD T100TM thermal cycler (T100TM 200 Thermal cycler, BIORAD, Singapore). Initial denaturation was performed at 94 °C for 60 seconds, followed by denaturation at 94 °C for 60 seconds, annealing at 60 °C and elongation at 72 °C for 90 seconds (40 cycles). The final elongation was carried out for 30 seconds at 72 °C. Table 1 also shows the annealing temperatures for each primer. The PCR products were separated by electrophoresis on 1.5% agarose gels in 0.5X Tris-acetate-EDTA buffer for 60 min at 80 V using a gel documentation system (Mega Bio-Print 1000/26MX0, Vilber Lourmat, France).

ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five microlitres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37ºC for 15 minutes, followed by enzyme inactivation at 80 °C for 15 minutes. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following the manufacturer's protocol. The PCR mix consisted of the following components: PCR Product (ExoSAP treated)- 20 ng, Primer-3.2 pM (either Forward or Reverse), Sequencing Mix- 0.28 µl, Reaction buffer-1.86 µl, Sterile distilled water made up to 10µl. The sequencing PCR temperature profile consisted of a 1st cycle at 96 °C for 2 minutes, followed by 30 cycles at 96ºC for 30 sec, 50 °C for 40 sec and 60 °C for 4 minutes.

Sequence analysis and phylogenetic tree construction

The forward and reverse sequences obtained were further combined and subjected to using, BioEdit, and subjected to BLAST analysis for finding the regions of local similarity between the sequences in the NCBI database. Furthermore, some sequences were selected for phylogenetic tree construction based on the maximum identity score. For that, the sequences were aligned using multiple sequence alignment using Clustal W.

The quality of the sequence obtained was inspected using Sequence Scanner Software v1 (Applied Biosystems). The forward and reverse sequences of the gene were aligned and edited (if required) using BioEdit. The sequences obtained were then subjected to nBLAST analysis for finding the regions of local similarity between the sequences in the NCBI database and some sequences were selected for phylogenetic tree construction based on the maximum identity score. The selected sequences were further subjected to multiple sequence alignment using Clustal W and MEGA XI software, a phylogenetic tree was created, and their phylogenetic relationship was inferred by the maximum likelihood technique with a bootstrap value of 1000. The strain sequence was named and then submitted to Genbank using the BANKIT to get an accession number **(K.t & Sebastian, 2021).**

Enzyme production and partial purification

In the starch broth, the enzymes were produced in 250 ml Erlenmeyer flasks with 100 ml of pH 7.0 media, and 1 ml of inoculum that had been grown for 24 hours was cultured. A minimum of 24 to 48 hours was spent shaking the cultures at 37 °C. Following incubation, the fermented broth was centrifuged at 8000 rpm for 15 minutes at 4 °C. The supernatant was then harvested and filtered using a 0.22 µm membrane filter.

Amylase assay

The amylase activity was assayed following the method of **Bernfield (1955)** using 3,5-dinitrosalicylic acid(DNS reagent). An enzyme blank with 3,5-dinitrosalicylic acid added before the addition of the enzyme was used as a control. Amylase activity was assayed in the reaction mixture containing 0.5 ml of cell-free supernatant, 1 ml of 1% starch as substrate, 1 ml phosphate buffer (pH 6.9), and incubated at room temperature for 15 minutes. The reaction was arrested by adding 1 mL of DNS reagent. The inactivated reaction mixture was incubated in a water bath for 10 minutes and, made up to 10 ml and the absorbance was measured at 540 nm. Blank was prepared by immediate addition of 1ml DNS reagent to 0.5 ml enzyme, followed by the addition of 1 ml starch and 1 ml phosphate buffer. One unit of amylase activity is defined as the amount of enzyme required to release 1µg of reducing sugar (maltose) per ml per minute under the above assay conditions. The amount of reducing sugar released was quantified using 3,5-dinitro salicylic acid with maltose as a standard. A calibration curve established with maltose (0.2 to 1 mg in 1 ml. of H20) is used to convert the colorimeter readings into milligrams of maltose **(Baltas *et al*., 2016; Rakaz *et al*., 2021). The activity of the enzyme was calculated using the formula;**

$$\text{Enzyme Activity} = \frac{\text{Mannose (µmol/ml)x Total solution volume(ml)}}{\text{Enzyme addede(ml)x Reaction time(minutes)}}$$

Effect of Various factors on amylase activity

Effect of temperature and pH

For studying the effect of temperature on amylase activity 100 µl of cell-free supernatant (CFS) containing amylase was added to 5 ml of 1% starch solution was kept at a different temperature such as 27 °C, 37 °C, 47 °C and 57 °C and the same solution were prepared at various pH such as 1, 3, 5, 7, 9, and 11 and kept for 15 minutes. Moreover, the activity of the enzyme in respective solutions was measured by the DNS method **(Al-Dhabi *et al*., 2020).**

Effect of Metal Ions

Effect of different metal ions on the activity of amylase was observed by pre-incubated (10 min) the cell-free supernatant (CFS) containing 1mM metal ions such as MnSo4, ZnSo4, FeSo4, CuSo4, CaCl2, and MgSo4 respectively, after that, 0.5ml of which was incubated with 1 ml of 1% starch solution for 15 minutes and the corresponding activities were quantified by DNS method **(Al-Dhabi *et al*., 2020).**

Demonstration of amylase in the removal of textile starch

For displaying the activity of isolated amylase on the removal of starch, the starched cotton cloth was cut in to equal pieces of weight of 0.25 g and kept for 30 min in the sterile 5ml distilled water with 1mM concentration of CaCl2 and 1500 µL isolated amylase (CFS) and the amylase activity were observed by adding iodine solution to the respective tubes. The respective weight of the dried clothes was also evaluated.

RESULTS

Isolation of amylase-producing bacteria

Out of 52 isolates obtained from different locality of the Kadaludi mangrove ecosystem (named EMEA- 1 to EMEA- 52), of which 12 morphologically distinct bacteria showed amylase activity in starch agar, from which the isolate named

EMEA-22 showed 20 mm zone (Table 1 & Figure 1) for the starch hydrolysis was found as a good amylase producer among the other isolates on amylase plate assay.

Table 1 The zone of starch hydrolysis produced by isolates

SL.No	Isolate name	Amylase hydrolysis zone(mm)
1	EMEA-1	14 ±2
2	EMEA-3	14±2
3	EMEA-7	12±2
4	EMEA-12	12±2
5	EMEA-13	14±2
6	EMEA-16	16±2
7	EMEA-22	20±2
8	EMEA-34	18±2
9	EMEA-41	14±2
10	EMEA-43	16±2
11	EMEA-45	14±2
12	EMEA-49	12±2

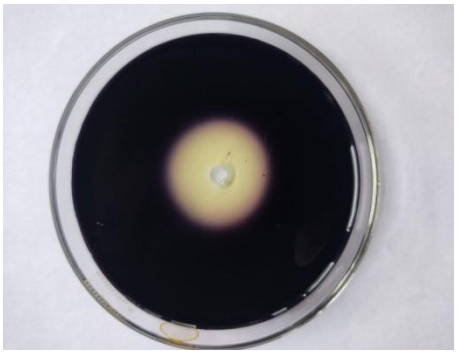

Figure 1 Cell-free supernatant of isolate showing the amylase activity.

Identification of a potential isolate

Morphological and Biochemical Characterization

Initially, the strain was identified phenotypically as Gram-positive, rod-shaped, endospore-forming bacteria with a positive reaction for catalase activity and negative for oxidase activity. It can grow at 50 ◦C temperature and shows a positive result for the methyl red test, and nitrate reduction, whereas it shows negative results for the indole test, Voges-Proskauer`s test, Citrate utilization, and urea hydrolysis. The bacteria didn't ferment glucose, lactose, arabinose, sorbitol, mannitol, rhamnose, sucrose, and adonitol (Table 2). These results reveal that the isolate belongs to *Bacillus* sps.

Table 2 Morphological and biochemical characterization of the potential isolate

TEST	Results
Colony	Rough, white, opaque colony with jagged edges
Gram's staining	Gram-positive
Endospore staining	Endospore forming
Indole	Negative
Methyl red	Positive
Vogesproskauer`s test	Negative
Citrate utilization	Negative
Glucose	Negative
Lactose	Negative
Arabinose	Negative
Sorbitol	Negative
Mannitol	Negative
Rhamnose	Negative
Sucrose	Negative
Adonitol	Negative
Nitrate reduction test	Positive
Catalase	Positive
Oxidase	Negative
Urease	Negative

Sequencing of the 16S rRNA region using universal primers

The forward and reverse sequences obtained after sequencing were combined (BioEdit Software) and the resulting sequence was compared with the sequence obtained by BLAST-n and was further aligned and compared with the homologous gene sequences. The evolutionary relationship of isolated bacterial 16S-rDNA sequences (EMEA 22) was inferred by a phylogenetic tree constructed using MEGA-XI software (Figure 2a). The constructed tree explored its similarity with *Bacillus subtilis* strain LL-16, which revealed that EMEA 22 belongs to the species *Bacillus subtilis.*

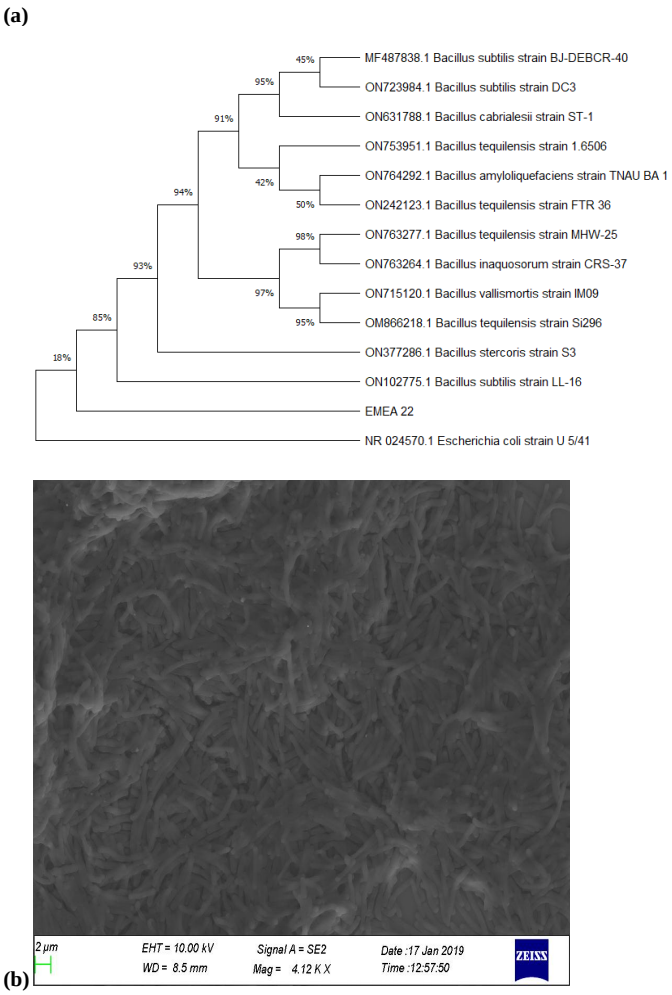

Figure 2 The constructed phylogenetic tree for *Bacillus subtilis* EMEA 22 strain(a) and its scanning electron microscopic view(b).

After analyzing the phylogenetic relationship, the sequence was submitted to Genebank through the BANKIT submission portal as *Bacillus subtilis* EMEA 22 with an accession NO: ON908837.

Enzyme production and partial purification

The amylase activity around the well filled with cell-free supernatant (CFS) of the potential isolate on starch agar reveals that the produced enzymes are extracellular (Figure 2). The amylase activity of CFS was also determined by DNS as 190 ±5U/m.

Effect of Various factors on amylase activity

Effect of Temperature and pH

The results of the effect of pH and temperature (Figure 3 (a) and (b)) showed that the optimal temperature among the tested is 37 °C (188±3 U/ml) and that of pH (189±7.5 U/ml) is at 9. The activity was reduced drastically when the pH was raised beyond 10. In the case of temperature, a drop in activity was observed at 47 °C.

Effect of Metal ions on amylase activity

Among the evaluated metal ions (Figure 3 (c)), the presence of CaCl2 (266± 7.5 U/ml) elevated the amylase activity than the control (195 U/ml). Whereas, all the other metals exhibited a negative effect at 1 mM concentration.

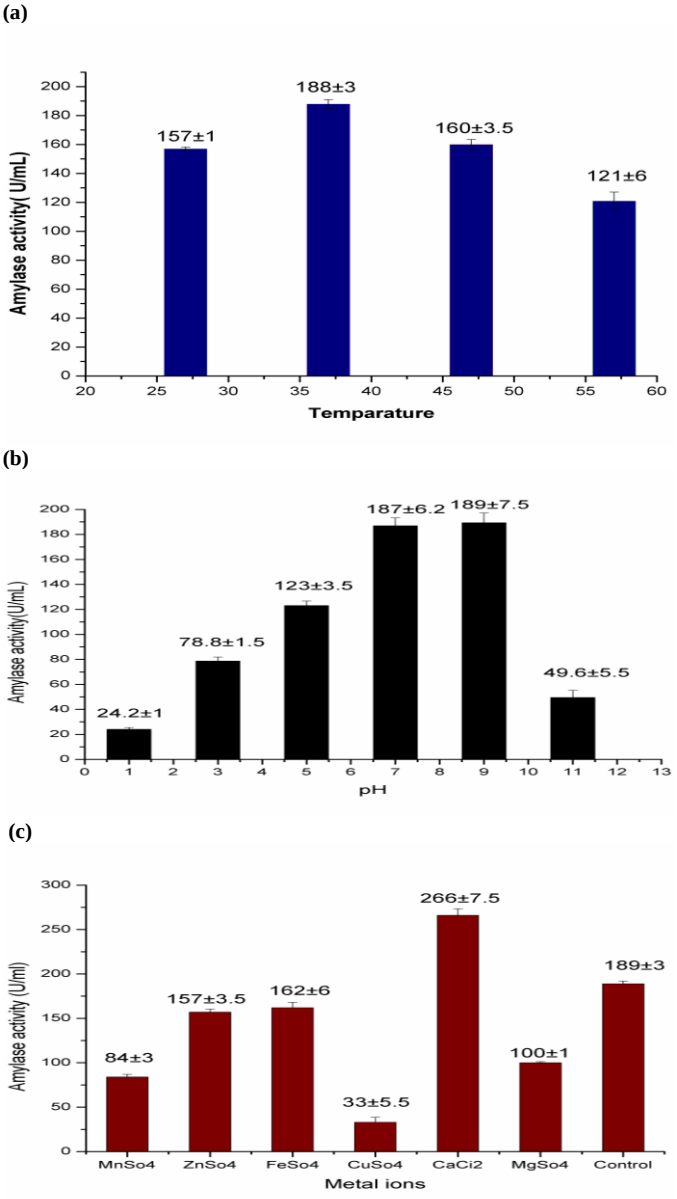

Figure 3 (a) The standard plot of reducing sugar (maltose), (b) Effect of Temperature on amylase activity, (c) Effect of pH on amylase activity, and (d) Effect of Metal ions on amylase activity

Demonstration of amylase in the removal of textile starch

The textile fabric in distilled water without enzyme (control) turned blue colour when iodine solution was added, while the fabric remained white in the enzyme-containing solution. The weight of the fabric in enzyme enzyme-free environment was noticed as 0.23g and that in the presence of an enzyme was detected as 0.18 g (Figure 4).

Figure 4 Isolated amylase removed the starch from cotton cloth. The Violet color cloth in the tube indicates the starched cotton (water only) and the white color indicates the starch hydrolyzed cotton by isolated amylase (water + CFS).

DISCUSSION

The present study evaluated rhizosphere soil samples of mangroves from different areas of Kadalundi, Calicut, for isolating the bacteria with amylase activity. The current work isolated 12 morphologically distinct bacterial isolates with amylase activity. Out of these, one isolate isolated from mangrove soil showed different morphological characteristics and potential activity was selected for further studies. The bacteria were further subjected to various morphological and biochemical characterizations, which appeared to a Gram-positive and endospore-forming bacilli. For biochemical reactions, the bacteria only showed positive reactions in the nitrate reduction test, catalase test, and methyl red test. All the other reactions exhibited negative reactions, including the carbohydrate fermentation test. The appearance of long Gram-positive bacilli, endospores, and the results reveal that the isolate belongs to *Bacillus* sps. The studies also suggest that most of the amylase-producing bacteria are from the *Bacillus* species **(Luang-In *et al*., 2019 ; Mishra & Behera, 2008)**. The results of 16S rDNA sequencing and further sequence analysis revealed that the isolate was included in the *Bacillus subtilis* species. The combined sequence was further submitted to Genebank as *Bacillus subtilis* EMEA 22 and provided an accession number: ON908837. The same species, *Bacillus subtilis* C10, was found to produce the amylase in previous studies **(Kalyani & Rajesh, 2018; Loc *et al*., 2010)**.

Furthermore, the study collected the cell-free supernatant and evaluated for the amylase activity, the results of which revealed that the amylase was released as an extracellular enzyme. The present study assessed the amylase activity of cell-free supernatant in two ways: one was by the well diffusion method, and the one by evaluating the release of reducing sugar by the DNS method. Moreover, the study also focused on the effect of various parameters such as temperature, pH, and metal ions on the activity of enzymes. Here, the results of the effect of pH and temperature on enzyme activity indicate that the optimum pH for enzyme action is pH 9 and the temperature is 37 °C and these results were matched with a previous study, in which the authors isolated and purified the amylase from *Streptomyces sp*. Al-Dhabi *et al* found that the optimum pH and temperature for amylase activity are 7 and 37 °C, respectively **(Al-Dhabi *et al*., 2020; Raul *et al*., 2014)**. Another study found that the optimum pH and temperature for the amylase from *Bacillus subtilis* KIBGE were observed at pH 7.5 in 0.1 M phosphate buffer at 50 °C **(Bano *et al*., 2011)**. Thermo-stable amylases were isolated from *Bacillus* species and showed a temperature tolerance of up to 75 °C. This bacterial amylase showed a wide range of pH tolerance, also (5-9) **(Yassin *et al*., 2021).** In the case of metal ions, the presence of 1 mM concentration of CaCl2 enhanced the enzyme activity from 195 U/ml to 266±7.5 U/ml. All the other tested metal ions decrease the enzyme activity. Therefore, the results conclude that there is an elevated activity of amylase in the presence of 1 mM concentration of CaCl2.The study of **Yadav *et al*., (2012)** found that amylase delivers an optimum activity at 1-2 mM concentration of CaCl2, upon increasing the concentration up to 10 mM turns the enzyme to a compact, thermodynamically less active enzyme.

A study of the application of amylase on textile de-sizing by removing the starch on white cloths was also conducted in this study. Upon adding the iodine solution, the cloth appeared white while the control appeared violet. The results indicate that isolated amylase effectively removes the starch in textile fabrics. The test appeared white, is might be due to the starch in the cloth being hydrolyzed by the amylase present in the cell-free supernatant. At the same time, the study also demonstrated the weight loss of fabric with and without enzyme presence, and the outcome of which observed that there was a drastic reduction (38%) in the weight of fabric in the presence of enzyme. An effective desizing of fabrics was observed in a previous study using a partially purified extracellular amylase from *Aspergillus* sp. **(Aggarwal *et al*., 2019).**

CONCLUSION

The study isolated a potent amylase producer named *Bacillus subtilis* EMEA 22. The findings revealed that the optimum temperature and pH for enzyme actions is 37 °C and 9, respectively. Moreover, the study also revealed the activity enhancement property of CaCl2. The study of amylase application revealed that the isolated amylase acts as an efficient starch remover or desizing component in textile cotton materials. Future applications of these microorganisms locally and nationally in agriculture, food processing, and the textile industry for the manufacture of amylase are possible. As a result, the cost of importing industrial enzymes from other nations will decrease, local enzyme manufacturing will be sustainable, and the country's economy will benefit. The purification of the amylase is bit complicated, for reducing the cost and the complications in purification the cloning of the enzyme can be done as future work, and the structural and the amino acid sequence similarity can be studied as a future perspective.

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