Production, partial purification and characterization of amylasefrom *Paenibacillus lactis* PEL6, an endophytic bacteria from *Mitrephora heyneana*

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Abstract

In recent years, researchers are keenly focusing on discovering antifungals, enzymes, immune suppressants and anticancer compounds produced by endophytic microorganisms, aiming to improve human well-being. The objective of this study is to produce, purify and characterize amylase produced by Paenibacillus lactis PEL6, an endophyte isolated from Mitrephora heyneana. The enzyme screening study in starch agar plate demonstrated the activity of amylase produced by Paenibacillus lactis. In ammonium sulphate precipitation study, the optimal saturation level for precipitating amylase was determined as 40-50%. Ammonium sulphate fractionation increased specific activity of amylase in crude enzyme from 0.347 to 0.556 and increased the purity of amylase to 1.6-fold.

The study on the effect of pH and temperature on amylase revealed that the optimum pH and temperature for the action of amylase on starch are pH 6.5 and 22°C respectively. The combination of amylase with commercial detergent was more effective in removing stains from fabrics than using the commercial detergent alone. In summary, this study validates that Paenibacillus lactis can act as a good source of amylase enzyme, which has great potential to be utilized in detergent industries.

Keywords: Enzymes, Amylase, Endophytes, Detergent, Stain removal, *Paenibacillus lactis*.

Introduction

Bioprospecting is the methodical search for valuable products derived from biological resources such as plants, microorganisms, animals and the development of commercial products for pharmaceuticals, agriculture and industry, which benefit society as a whole[16]. Endophytic microorganisms are present in almost every plant on earth. Bioprospecting of these microbial endophytes holds an extraordinary potential for discovering new drugs and enzymes that could effectively treat emerging diseases in humans, plants and animals[16].

Plants have been considered as a source of medicinal compounds for centuries, but recently, microorganisms associated with plants, known as endophytes, have shown significant therapeutic potential. These endosymbiotic bacteria or fungi live within plants without causing disease, exhibiting mutualistic, antagonistic, or rarely parasitic interactions. Endophytes enhance plant growth, nutrient uptake, stress tolerance, resistance to pests, while producing useful bioactive compounds such as enzymes and pharmaceutical drugs[9]. Endophytes are microorganisms that live inside plants and can be isolated from plant or microbial growth media. They produce bioactive secondary metabolites with potential antimicrobial, anti-insect, anticancer and other therapeutic properties, making them important for drug discovery[9].

Endophytes help plants through promotion of plant growth and yield, suppression of pathogens, removal of contaminants, solubilization of phosphate and contribution of assimilable nitrogen to plants[19]. Plant-associated bacteria have the capacity to confer plant growth promotion and to increase resistance towards various diseases as well as abiotic stresses[6]. A study indicated that the seed-inhabiting bacterial endophytes enhance seedling growth by stimulating root hair formation, increasing root and shoot length, boosting photosynthetic pigment content and improving disease resistance[22].

Endophytes are asymptomatic microbes (mostly bacteria and fungi) in plants. They carry nutrients from soil to plants, modulate plant development, increase stress tolerance, suppress pathogen virulence, enhance disease resistance and inhibit competitor plant species[23]. Endophytic microbes reside in plant roots either intracellularly or intercellularly, depending on the host species and bring nutrients to plants[17]. Endophytic microorganisms live inside the plant tissues without having any negative impact to the host plant. Medicinal plants harbour a vast diversity of endophytic actinobacteria of economic importance. Therefore, screening and isolation of promising endophytes with antimicrobial properties and employing them in the agricultural, medical, pharmaceutical and other fields is needed[8].

Bacterial endophytes produce biologically active compounds such as insecticides, antioxidants, antimicrobial agents, anticancer, antidiabetic compounds, immune-suppressive compounds, plant growth promotors and antibiotics among others[4].

Also they produce various secondary metabolites, hydrolytic enzymes such as proteases, pectinases, cellulases, amylases and chitinases, which have been used for the manufacture of

industrial products including food and food supplements, biofuels, pharmaceuticals, detergents[7].

Endophytic bacteria are found virtually in every plant species on earth[15]. The endophytic bacteria isolated from *P. cuspidatum*, a traditional chinese medicinal plant, showed inhibitory activity against pathogenic fungi and bacteria[14]. About, 244 prokaryotic endophytes have been isolated and identified from *P. cuspidatum* and they belong to five orders, namely, *Actinomycetales, Bacillales, Rhizobiales, Pseudomonadales and Enterobacteriales*. At the genus level, 244 strains were identified including *Paenibacillus, Streptomyces, Providencia, Rhizobium, Brachybacterium*[21].

For the identification of novel bioactive microbial metabolite and natural products such as antimicrobial and anticancer compounds, research should focus on mining inexhaustible natural microbial sources, including endophytic microbes[4]. Fungal endophytes are widespread and are increasingly studied for their potential to enhance plant health and protect against diseases. Hence, fungal endophytes offer advantages of using them as plant protection products[11].

The genus *Paenibacillus* includes bacterial species important to humans, animals, plants and the environment. Many species promote crop growth through nitrogen fixation, phosphate solubilization, production of the phytohormone IAA and siderophores for iron acquisition[13]. They also protect plants from insect herbivores, pathogens by producing antimicrobials and insecticides and by inducing systemic resistance in plants. *Paenibacillus*-derived antimicrobials like polymyxins and fusaricidins have medical applications. Additionally, they produce useful molecules such as exo-polysaccharides and various enzymes such as amylase, cellulase, hemicellulase, lipase, pectinase, oxygenase, dehydrogenase, lignin-modifying enzyme and mutanase with potential uses in detergents, food, feed, textiles, paper, biofuel and healthcare[10].

Amylases are the key enzymes in industry, responsible for hydrolyzing starch molecules into glucose units. They have broad applications in food, fermentation and pharmaceutical industries. While amylases can be derived from plants, animals, microorganisms, those from fungal and bacterial sources are most commonly used in industrial applications. The production of amylase is crucial for converting starches into oligosaccharides. Amylases are used to produce maltodextrin, modified starch,glucose and fructose syrups. Microbial amylases are widely applied in food, textile, paper and detergent industries[5].

Starch-hydrolysing enzymes, known as amylases, are vital industrial biocatalysts produced by microorganisms on a commercial scale. There are three types of amylases based on their hydrolytic actions: alpha-amylase, beta-amylase and glucoamylase. Owing to their versatile enzymatic activities, amylases are widely used in baking, brewing, pharmaceuticals, laundry, paper, textile and biofuel industries[1].

Amylase was the first enzyme to be characterized and for the past 200 years, its clinical role has been primarily as a diagnostic aid. However, recent interdisciplinary research has significantly expanded its role into novel, viable diagnostic and therapeutic applications in cancer, infection and also for wound healing[2]. Therefore, in this study, we aimed to produce, partially purify and characterize amylase from *Paenibacillus lactis* PEL6, an endophytic microorganism isolated from *Mitrephora heyneana*, a small tree native to India and Sri Lanka. Objectives in this study include production of extracellular amylase from *Paenibacillus lactis*, partial purification of amylase using ammonium sulphate precipitation, characterization of the amylase and evaluation of theamylase's destaining property for potential application in detergents.

Material and Methods

Isolation of microorganism: The soil sample was serial diluted by the addition of 1.0 g of sample in 10 ml of distilled water, mixing thoroughly for 15 minutes, then vortexed. About 0.1 mL of culture was streaked on to potato dextrose agar (PDA) and nutrient agar media (NAM) with dilutions ranging from10^{-9} to 10^{-1} of which 10^{-6} to10^{-1}were considered for this research.

Screening for amylase activity: For primary screening of amylase activity, the bacterial strains *Paenibacillus lactis* PEL6, isolated from *Mitrephora heyneana*, were streaked onto starch agar plates and the plates were incubated at 37 °C for 48 h. After incubation, the plates were flooded with iodine solution. A clear zone formed around the bacterial colony indicates a positive test result for the production of amylase enzyme by *Paenibacillus lactis*.

Production of amylase enzyme: The isolated *Paenibacillus lactis* PEL6 was inoculated and incubated in a flask containing nutrient broth for about 48 h. After the growth of bacteria, the culture was transferred to centrifuge tubes and centrifuged at 10,000xg for 10 mins. The resulting supernatant was used as crude enzyme for further enzymatic assays[20].

Protein quantification by Lowry's method: Protein concentration was measured by Lowry's method[12] using Bovine serum albumin as standard.

Estimation of amylase activity by DNSA method: This involves mixing of dinitrosalicylic acid (DNSA) reagent with the sample containing reducing sugars. Upon heating the mixture, a reduction reaction occurs, leading to the formation of 3-amino-5-nitrosalicylic acid, which gives rise to an orange-red colour.

The intensity of colour change is directly proportional to the concentration of reducing sugars present in the sample. By

measuring the absorbance of the solution at 540 nm, amount of reducing sugars present in the sample can be quantified[3].

To determine the concentration of reducing sugars in an unknown sample using this method, standard solutions were prepared using known concentrations of reducing sugar. The absorbance values obtained from these standards were plotted against their respective known concentrations to generate the standard curve. Then, the concentration of unknown sample was interpolated from the standard curve based on absorbance value of unknown sample.

The following reagents were used for the measurement of amylase activity:

1. Substrate for amylase: 1 g of soluble starch was mixed in 200 ml of 0.1M phosphate buffer (pH 6.8), boiled for 3 minutes, cooled to room temperature and filtered as necessary.
2. Amylase source: Culture Supernatant.
3. 1% Sodium chloride
4. DNSA (Dinitro Salicylic acid) solution about 1.6 g of NaOH was dissolved in 20 ml of distilled water. 1 g of 3,5 DNSA was added to the above solution. 30 g of sodium potassium tartrate was dissolved in 60 ml of distilled water. Sodium potassium tartrate solution was mixed with DNSA solution and the volume was finally made up to 100 ml with distilled water.
5. Standard Maltose Solutions: It was prepared by dissolving 200 mg maltose in 100 ml of water.

The following procedure was followed for preparing the standard solutions for DNSA method of estimating reducing sugars: A series of test tubes were labelled as follows: Blank, S1, S2, S3, S4, S5. To tubes S1, S2, S3, S4 and S5, 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml of working standard maltose solution were added respectively. The final volume in each tube was made up to 1 ml with distilled water. A tube containing 1 ml of distilled water was used as blank. To all tubes (Blank, S1, S2, S3, S4, S5), 1 ml of DNSA (Dinitrosalicylic acid) reagent was added. All the tubes were incubated at 65°C for 30 minutes to allow the reaction between DNSA and reducing sugars. The disappearance of yellow colour and the appearance of orange or red colour after incubation is due to the formation of 3-amino-5-nitrosalicylic acid. The tubes were allowed to cool to room temperature and absorbance of the solutions in each tube was measured at 540 nm. A standard curve of absorbance (OD) against the concentration of maltose was plotted.

The following procedure was followed for the preparation of the test solutions for DNSA method and the estimation of reducing sugars released from starch by amylase enzyme:0.5 ml of starch and 0.2 ml of 1% NaCl were added to a test tube and incubated at 37°C for 10 minutes.1 ml of enzyme (supernatant) was added to the tube and it was incubated at 37°C for 15 minutes. Then, 1 ml of DNSA reagent was added to it and mixed well. The tubes were incubated at 65°C for 30 minutes in the water bath. After incubation, the tubes were cooled down and the red colour that developed was colorimetrically read at 540 nm. The standard graph and optical density readings of the unknown samples were used to determine the concentration of reducing sugars in the unknown samples.

One unit of amylase (U) is defined as the amount of enzyme that catalyses the formation of one micromole of reducing sugar per minute under specified conditions of the assay method (μmol/min).

Partial purification of amylase by ammonium sulphate precipitation: The enzyme purification in this study was carried out at 4 °C. In order to partially purify amylase, the crude enzyme was subjected to ammonium sulphate fractionation over a range of saturated ammonium sulphate solutions, specifically at 30%, 40%, 50%, 60% and 70%. At each fractionation step, the culture supernatant was brought to the required percentage of saturation of ammonium sulphate by adding the required quantity of salt and stirring continuously on ice for 3 hours. The solution was centrifuged at 10,000xg for 10 minutes at 4°C. The supernatant was saved for next round of fractionations. The precipitated proteins from each fractionation were collected and dissolved in minimum volume of 0.1 M phosphate buffer, pH 6.8. Subsequently, enzyme solutions were dialyzed against the same buffer with three changes of buffer. Amylase activities in different dialysates obtained from all fractionations were analysed.

Analysis of the effect of pH on the activity of partially purified amylase: To study the effect of temperature on amylase, assay mixtures were incubated at various pH ranges *viz.*, 5.5, 6.0, 6.5, 7.0, 7.4 and enzyme activity was assayed using DNSA method.

Analysis of the effect of temperature on the activity of partially purified amylase: To study the effect of temperature on amylase, assay mixtures were incubated at various temperature ranges *viz.*,15 °C, 22 °C, 37 °C, 45°C, 55 °C and enzyme activity was assayed using DNSA method.

Evaluating the effectiveness of stain removal by partially purified amylase: To test the stain removal efficacy of the amylase, the following procedure was used. Pieces of white cloth of size 5x5 cm were used for this experiment. Cloth pieces were stained with chocolate and allowed to sit overnight. For reference, unstained cloth and stained cloth are shown in figure 4. For this experiment, stained cloth pieces were taken in a flask and following procedure was carried: (i) For “control” purpose, a piece of stained cloth was incubated in 100 ml distilled water. (ii) For carrying out treatment with commercial detergent, a piece of stained cloth was incubated with 98 mL distilled water and 2 mL Surf excel detergent (1% w/v). (iii) For carrying out combinatorial studies, a piece of stained cloth was incubated

with 96 mL distilled water along with 2 mL detergent (1% w/v) and 2 mL enzyme extract. The above flasks were incubated at room temperature for 2 hours in a rotatory shaker. After incubation, cloth pieces were taken out, rinsed with cold tap water, dried and the results were compared to evaluate the effectiveness of the enzyme in removing the chocolate stain completely.

Results and Discussion

Screening of amylase activity: Bacterial strain *Paenibacillus lactis* was cross-streaked across the starch agar plate and incubated at room temperature for 48 hours. After growth, plate containing the bacterial colony was flooded with iodine solution and observed for zone formation. Plates with the bacterial colony produced a clear zone around the colony, indicating the presence of activity of extracellular amylase released by bacterial colony, while plates without the bacterial colony showed no clear zone, indicating the absence of amylase activity (Figure 1). This indicates that *Paenibacillus lactis* PEL6, an endophyte from *Mitrephora heyneana,* is a very good source of extracellular amylase enzyme.

Production of amylase enzyme: The endophytic bacteria, inoculated in a flask containing nutrient broth, was cultured for about 48 h. Then, the culture was centrifuged at 10,000x g for 10minutes.The supernatant was used as enzyme source for subsequent enzyme characterization studies.

Estimation of amylase activity in crude enzyme by DNSA method: The concentration of reducing sugars, such as glucose and maltose, in various sample is precisely estimated using the 3,5-Dinitrosalicylic acid (DNSA) reagent method[18]. This method is widely employed to measure enzyme activities that produce reducing sugars. It is favoured for its speed, cost-effectiveness, precision compared to earlier methods and it eliminates the necessity for sample dilution. Amylase activity was determined by DNSA method and protein quantification was done by Lowry's method. The activity and specific activity of amylase in the crude enzyme were 0.146 U/mL and 0.347 (U/mg protein) respectively (Table 1).

Partial purification of amylase by ammonium sulphate precipitation method: The level amylase activities in different dialysate were prepared from protein precipitates formed at 30%, 40%, 50%, 60% and 70% saturations. Highest amylase activity was found in dialysates pertaining to 40-50% ammonium sulphate saturation levels. The activity and specific activity of partially purified amylase were 0.232 U/mL and 0.556 (U/mg protein) respectively (Table 1).

To determine the optimum temperature, pH of purified amylase and to determine stain removal activity of purified amylase, partially purification of amylase was carried out using ammonium sulphate precipitation method. The highest enzyme yield was obtained in protein precipitates formed at 40-50% ammonium sulphate saturation. Ammonium sulphate fractionation increased specific activity of amylase in crude enzyme from 0.347 to 0.556 (U/mg protein) and hence, increased the purity of amylase 1.6-fold.

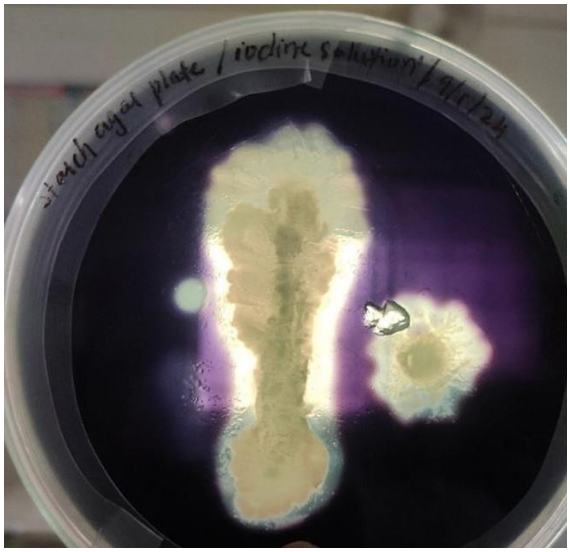


Figure 1: Formation of clear zone in starch agar plate by amylase of *Paenibacillus lactis*

Table 1
Purification of amylase from *Paenibacillus lactis*

Treatment	Amylase activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg protein)	Purification fold
Crude enzyme	0.146	0.42	0.347	1
Partially purified enzyme	0.232	0.417	0.556	1.6

Effects of pH on amylase enzyme activity: The activity of enzyme was assayed at different pH ranges viz., 5.5, 6.0, 6.5, 7.0, 7.4. The results showed that the highest enzyme activity was found at pH 6.5 and the enzyme activities at other pH levels were relatively lower than the activity observed at pH 6.5 (Figure 2).

Effect of temperature on amylase enzyme activity: The activity of enzyme was assayed at different temperature ranges, viz., 15 °C, 22 °C, 37 °C, 45°C, 55 °C. The results showed that the highest activity of enzyme was found at 22°C and enzyme activities at other temperature levels were relatively lower than the activity seen at 22°C (Figure 3).

The rate of all enzymatic reactions was influenced by temperature and pH. Each enzyme has its own characteristic optimum temperature and pH at which it exhibits maximal activity. Hence, for finding the optimum temperature and pH of amylase, the activity of enzyme was assayed at different pH and temperature ranges. The results showed that the optimum pH and temperature of amylase on hydrolysis of soluble starch was 22°C and pH 6.5 respectively.

Performance of amylase in the removal of stain: To evaluate how well amylase functions in the role of a stain remover, cloth pieces stained with chocolate were soaked either in detergent or in enzyme-detergent combination for 2 hours at room temperature. The results revealed that washing performance of amylase-detergent combination was better than that of commercial detergent alone (Figure 4).

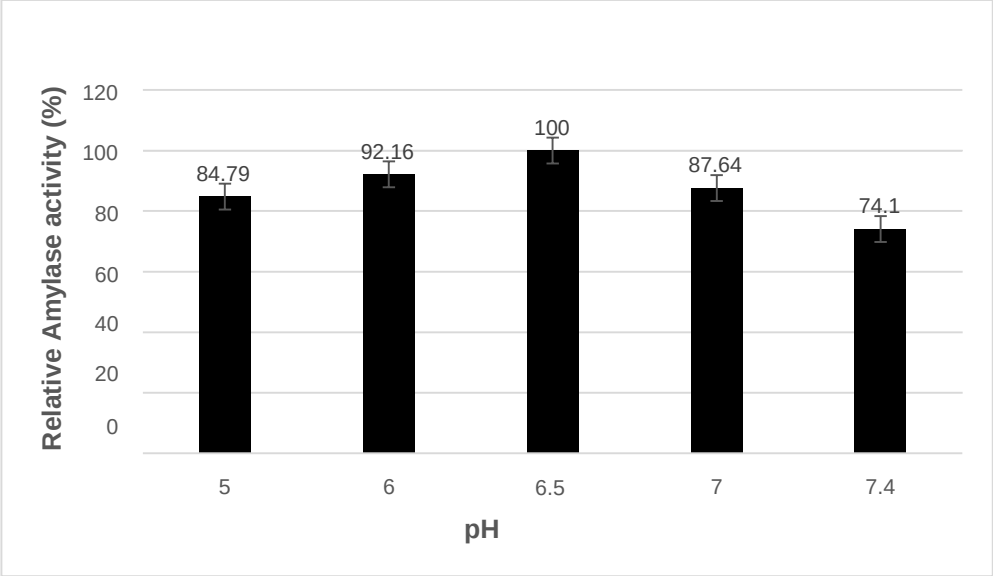


Figure 2: Effect of pH on amylase activity.
Error bars represent standard deviation of three independent experiments

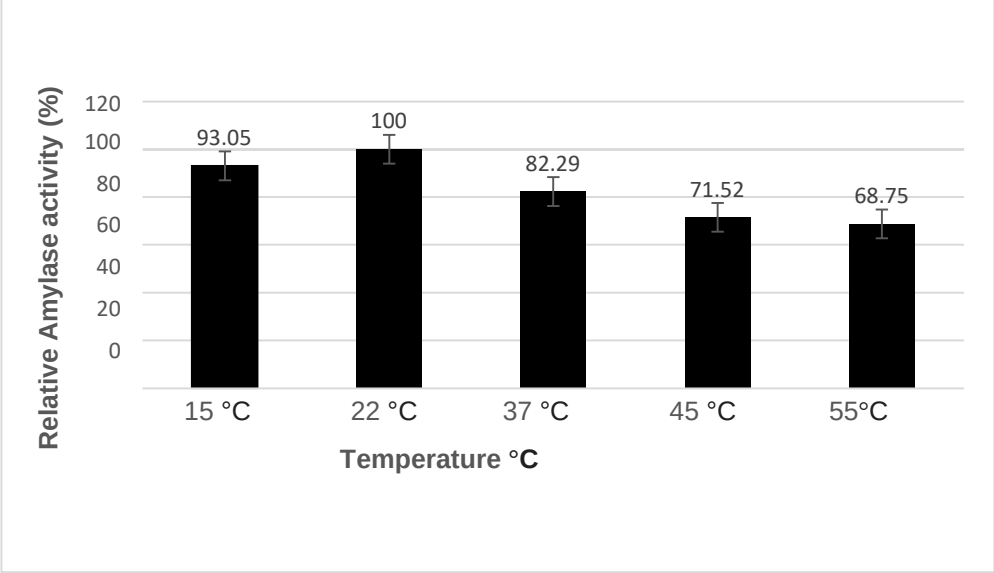
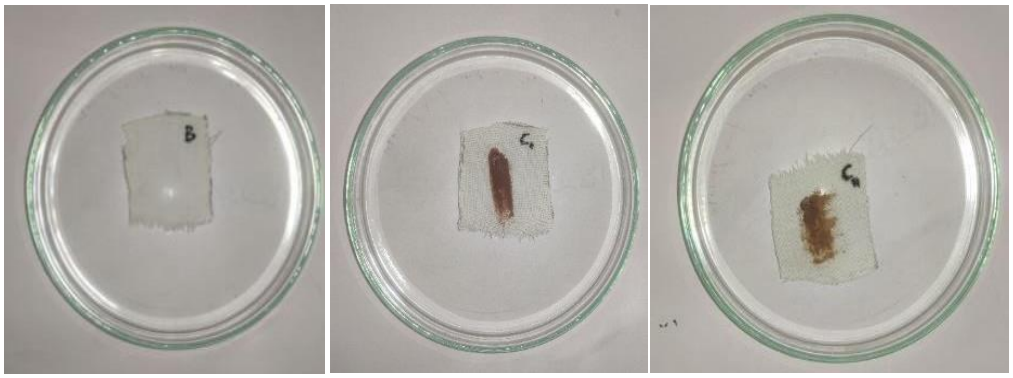
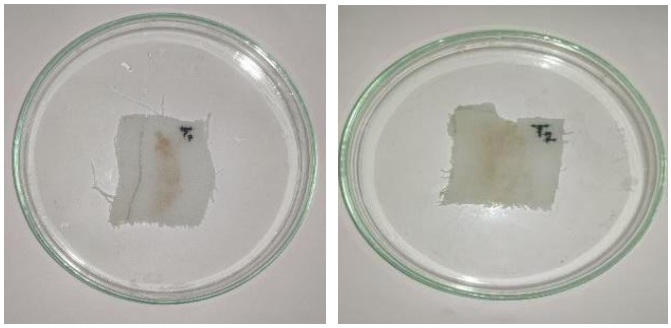


Figure 3: Effect of temperature on amylase activity.
Error bars represent standard deviation of three independent experiments



(i) Unstained Cloth **(ii) Stained Cloth** **(iii) Control**



(iv) Treatment with detergent only **(v) Treatment with both detergent and Amylase**

Figure 4: Effective removal of stain in cloths by detergent combined with amylase

Amylase enzyme has been employed in detergent industries for the removal of stains. Our stain removal study showed that using the combination which contains both amylase and commercial detergent was more effective for stain removal than using commercial detergent alone. This indicates that amylase was effective in the removal of stain, amylase improves the stain removal efficiency of commercial detergent through synergistic effect. These results clearly indicated that the amylase from *Paenibacillus lactis* PEL6 has a great potential in enzyme-based detergent industries. The use of enzyme as detergent offers several advantages over commercially available detergents such as being pollution-free, since it is a biological sample and working efficiently at room temperatures.

Tallapragada et al[20] have opined that the improved efficiency of amylase-detergent combination in washing experiment might be due to the fact that amylase provides improved starch hydrolysis, resulting in better stain removal efficacy. Also, they had suggested that demand for microbial-enzyme based detergents is on the rise due to that fact that chemical-detergents, commonly employed in laundry and dishwashing, are very harsh towards cloth and hands[20]. Use of amylase enzyme in various detergents is due to its ability to degrade the residues of starchy foods such as gravies, chocolate etc. into smaller moieties such as dextrins, oligosaccharides and monosaccharides[20].

Recently, Ugwuoji and coworkers[21] have reported a similar finding. They have isolated a novel isolate, *Paenibacillus lactis OPSA3*, which produces thermostable, alkaliphilic and detergent-tolerant amylase. In their stain removal analysis, they have found that enzyme- commercial detergent combination effectively removed the stain and hence, suggested that the amylase can be exploited as a component of green detergent[21].

Conclusion

In conclusion, *Paenibacillus lactis* is a valuable source of amylase enzyme that offer significant implications for both academia and industries alike. The addition of enzymes to the detergent will drastically reduce the dependency on detergents. Additionally, consumers who are aware of this can reduce their monthly household expenses on detergents. This reduced usage of detergents will, in turn, protect our surrounding environment and nature.

Acknowledgement

The authors would like to thank FIST grant from DST and the facilities from PSG College of Arts & Science for carrying out this work.

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(Received 09th August 2024, accepted 10th October 2024)