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Isolation of Salt Tolerant Enzyme Producing Bacteria from Marine Environments in Sri Lanka for Industrial Applications

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Abstract

Microbial enzymes have been used in our day-to-day lives since ancient times. In the current world, microbial enzymes are widely used in industries because of their environmental friendly nature and cost-effective production. Halophiles are industrially important bacteria due to their high stability in high concentrations of salts. Amylases, cellulases, proteases and lipases are the most widely used halophilic hydrolytic enzymes in food production, detergent industry, pharmaceutical industry, biofuel production, paper production, etc. Each of these enzymes plays a vital role in industrial processes and world enzyme market which represents a considerable percentage of world trades. Thus the current study focused on isolation of salt-tolerant enzyme-producing bacteria from marine environments in Sri Lanka for industrial applications. The samples were collected from Negombo Lagoon and Panadura Beach in Sri Lanka and screened for the production of amylases, cellulases, proteases and lipases using plate assays. Then, the optimization of growth temperature and salt concentrations was performed for enzyme-producing bacteria. A total of 44 morphologically different bacteria were isolated, and almost 95% showed at least one enzyme activity. The optimum salt concentration for bacterial growth was in a range of 3% to 12%, while the optimum growth temperature was in the range of 30^0C and 35^0C. Thus, marine environmental bacteria can be considered as potential halophilic enzyme producer, and may be utilized in industrial settings operating under high salt concentrations.

Keywords: Halophiles, Halophilic bacteria, Industrial applications, Marine bacteria, Enzyme producing bacteria

1. Introduction

Biological reactions are being catalysed by biological catalysts called enzymes.[1] Catalysts are essential for mediating the biological reactions in living organisms. It includes the biological use of catalysts as well as the industrial use of catalysts. Use of catalysts in industrial applications would increase the rate of reaction and hence result in increased production of the desired end product.[2]

Amidst the Industrial Revolution, emerging industries utilized chemical catalysts, which produce chemical wastes.[2] Since chemical wastes can drastically affect the ecosystem, modern industries are in search of environmentally friendly catalysts, preferentially biological catalysts, as an increasing environmental concern. The use of eukaryotic enzymes as biological catalysts is often complex and involves expensive procedures.[3] Cultivation and purification of microbial enzymes were proven effective compared to eukaryotic enzymes

due to their easy access, fast growth rate and inexpensive procedures.[4]

Mesophilic enzymes produced by common mesophilic bacteria are not likely to withstand the extreme conditions of the industries, including extreme temperatures, high salinity, extreme pH levels, oxidative stress, extreme pressure, and exposure to toxic chemicals.[5,6] Hence, extremozymes derived from extremophiles are favoured due to their adaptable metabolism and unique structural alterations of their biomolecules for extreme conditions.[7]

Halophiles are salt-loving micro-organisms, preferably in Eubacteria and Archea.[8] Halophiles predominantly occupy a hypersaline environment, with a minimal microbial diversity due to its multiple extreme conditions, including high salt concentration.[9] The halo tolerance of the halophiles can be classified as slight halophiles, moderate halophiles and extreme halophiles. Slight halophiles prefer a salt concentration of 1% - 3%, which is 0.2 M to 0.5 M of NaCl, while the moderate halophiles prefer a salt concentration of 3% - 15%, which is 0.5 M to 3.5 M NaCl. Extreme halophiles are present in environments with a salt concentration of 15% - 30%, which is approximately 2.5 M to 5 M NaCl.[10,11] Halophiles have adapted to tolerate high external salt concentrations, such as accumulating salts inside the cytoplasm.[12]

The most widely wanted enzyme in the industries is the hydrolases. The major hydrolases commonly used in industries include amylases, cellulases, proteases, lipases, pullulanases, xylases, esterases, pectinases and laccases.[13] Hydrolases are preferred over chemical catalysts due to the highly specific, ecologically friendly and clean processing of the enzyme.[14]

Amylase belongs to the group of hydrolases. The primary function of amylase is to cleave glycosidic bonds of starch by using H_2O, resulting in simple sugars.[15] Amylase has a crucial role as an enzyme in most industries, including food production, paper production, textile industry, etc. For industrial purposes, amylase synthesized from fungi and bacteria is widely used.[16] Bacterial amylase is favoured due to its cheap and fast methods of extraction. Halophilic amylases are preferred due to their ability to withstand extreme conditions that occur during the process of synthesis.[17] The principal purpose of halo-tolerant amylase in industries include starch saccharification for fructose syrup production, antistaling agents in baking, laundry detergent production, textile industry, biofuel production and paper production.[18]

Cellulases are a diverse class of enzymes belonging to the group hydrolases.[19] The primary substrate for cellulase is cellulose, which is the major component of plant cell wall and is the most abundant carbohydrate. Cellulase degrades cellulose by hydrolysing the β-1, 4-glycosidic bonds.[20] Halotolerant cellulases have a wide range of applications, including the food and beverage industry, textile industry, pharmaceutical industry, detergent industry, paper processing industry, biofuel refining industry and olive oil extraction.[21-23]

Proteases, also known as proteolytic enzymes and peptidases, are a class of hydrolases that catalyse hydrolysis of peptide bonds of proteins and polypeptides forming amino acids.[13] The halophilic proteases possess stable activity at high temperatures and ionic strength in the presence of organic solvents.[24] Halotolerant proteases is widely used in the food industry involved in fish sauce fermentation, the detergent and leather industry, and the pharmaceutical industry.[25]

Lipases are a group of enzymes that catalyse the hydrolysis of lipids into fatty acid and glycerol.[26] Halotolerant lipases are efficiently utilized in food, dairy, pharmaceutical, cosmetic, agrochemical, bio-surfactant, detergent, paper industries and biofuel synthesis.[14,27,28]

Thus, the current study focuses on the isolation of salt-tolerant enzyme-producing bacteria from marine environments in Sri Lanka for industrial applications.

2. Methodology

2.1 Sampling and Enrichment of the marine bacteria. Seawater was collected from two sites: Panadura Beach (6.710 N, 79.901 E) and Negombo Lagoon (7.1584 N, 79.832 E). Approximately 250 mL of sample was collected

from each site into sterilized glass bottles. The collected samples were transported to the laboratory in ice boxes. The samples were then enriched in a quarter-diluted nutrient broth medium supplemented with 3% NaCl at room temperature for 48 hours.

2.2 Serial Dilution. After incubation, each sample was serially diluted up to 10^{-6} by adding autoclaved 0.9% NaCl solution. Briefly, 1 mL of incubated broth was transferred to a test tube with 9 mL of 0.9 NaCl solution, mixed well and labled as 10^{-1}. Then, from the first tube, 1 mL was transferred to the next tube of 9 mL of 0.9% NaCl solution and labled as 10^{-2}. The procedure was repeated sequentially up to 10^{-6}.

2.3 Isolation of Bacteria. Bacteria were isolated using the spread plate technique on Nutrient Agar plates supplemented with 3% NaCl. The autoclaved Nutrient Agar was poured into sterilized petri plates and allowed to solidify. Then 100 µL of each serially diluted sample was added onto the Nutrient Agar supplemented with 3% NaCl plates and evenly spread using a sterilized glass spreader. The inoculated plates were then incubated at room temperature for 24 hours.

Morphologically distinct bacterial colonies were isolated and were streaked onto steriled Nutrient Agar plates supplemented with 3% NaCl plates. The pure cultures were obtained by further streaking on Nutrient Agar supplemented with 3% NaCl plates. The isolated pure bacteria were used for further studies.

2.4 Primary screening for production of Amylase enzyme. The activity of the amylase enzyme was screened using starch as the substrate. Starch agar was prepared using 28 g/L of Nutrient Agar, 30 g/L of NaCl and 10 g/L of soluble starch topped with distilled water. and the media was autoclaved. Afterwards, the bacterial colonies were spotted on the starch agar plates and incubated at room temperature for 36 hours. After incubation, the colonies were flooded with Potassium Iodide (KI) reagent and observed for the formation of a clear zone around the colony, which indicates the production of amylase enzyme.

2.5 Primary screening for production of Cellulase enzyme. Carboxy Methyl Cellulose (CMC) agar was used to screen for activity of cellulase. CMC agar was prepared using 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4.7H_2O$, 1.0 g/L of $CaCl_2.2H_2O$, 1.0 g/L of K_2HPO_4, 0.2 g/L of $FeCl_3$, 2.0 g of Tryptone, 15.0 g/L of CMC powder, 15.0 g/L of Bacteriological agar and 30.0 g/L of NaCl topped with distilled water and the media was autoclaved. Then, the autoclaved CMC agar was poured into petri plates and allowed to solidify. Following this, the bacterial colonies were spotted on the petri plates and then incubated at room temperature for 36 hours. After the period of incubation, the cellulase activity was screened using 0.1% Congo Red which was flooded in the CMC agar plate and was left for 30 minutes. Then the excess stain was washed off, and 1 M NaCl solution was flooded on the plate, and after 5 minutes, the excess NaCl solution was removed, and 5% acetic acid was poured and observed for the formation of a clear zone around the colony which indicates the production of cellulase enzyme.

2.6 Primary screening for production of Protease enzyme. Protease activity was screened using Skimmed Milk Agar (SMA). Two flasks were prepared: one with 28.0 g/L non-fat milk powder in one-third of the distilled water, and the other with 2.5 g/L yeast extract, 1.0 g/L dextrose, 5.0 g/L casein hydrolysate, 15.0 g/L agar, and 30.0 g/L NaCl in the remaining water. Both were autoclaved and then mixed to form the final agar and poured into petri plates, and allowed to solidify. Subsequently, the bacterial colonies were spotted on the SMA plates and were incubated at room temperature for 36 hours. After incubation, the colonies with the enzyme protease showed a zone of clearance due to the lysis of proteins.

2.7 Primary screening for production of Lipase enzyme. Phenol Red Agar (PRA) with pH 7.4 was prepared to screen the activity of lipase activity by adding 5 g/L of peptone, 3 g/L of yeast extract, 15.0 g/L of bacteriological agar, 1.0 g/L of $CaCl_2$. 0.1 M NaOH was added to adjust pH and 30.0 g/L of NaCl was added as supplement, and the final mixture was autoclaved. Then 10 ml/L of olive oil (substrate) and 10 mg/L of phenol red dye was added. Then, it was poured into petri plates

and allowed to solidify. Afterwards, the bacterial colonies were spotted on the PRA plates and incubated at room temperature for 36 hours. The presence of lipase would break down the lipid (olive oil) into fatty acid and glycerol, changing the pH, which was depicted by the change of colour from orange to red.

2.8 Optimization of the Bacteria to deduce the optimum salt concentration required for the growth. Nutrient broth supplemented with 3% NaCl, 6% NaCl, 9% NaCl, 12% NaCl and 15% NaCl was prepared with 50 ml of Nutrient broth for each NaCl concentration using 13.0 g/L of Nutrient broth powder and appropriate NaCl, and the media was autoclaved. In the Nutrient Broth containing flasks with different concentrations, 200 µL of the equalized bacterial solutions were inoculated. Then, the flasks were incubated at room temperature for 24 hours. Then, the growth of the bacteria was identified using the absorbance obtained using a spectrophotometer at a wavelength of 600 nm.

2.9 Optimization of the Bacteria to deduce the optimum incubation temperature required for the growth. Nutrient broth of 50 mL supplemented with the respective NaCl concentration of the bacteria determined earlier was prepared and autoclaved. In the Nutrient Broth containing flasks, 200 µL of the equalized bacterial solutions were dispersed and incubated at temperatures 30^0C, 35^0C, 40^0C and 45^0C for 24 hours. Then, the growth of the bacteria was identified using the absorbance obtained using a spectrophotometer at a wavelength of 600 nm.

3. Results

3.1 *Isolation of morphologically different bacterial colonies.* A sum of 44 morphologically different bacterial colonies were isolated from both the sample sites (Figure 1). A total of 25 bacterial colonies were isolated from the Negombo Lagoon sample, and the remaining 19 bacterial colonies were isolated from the Panadura Beach sample.

3.2

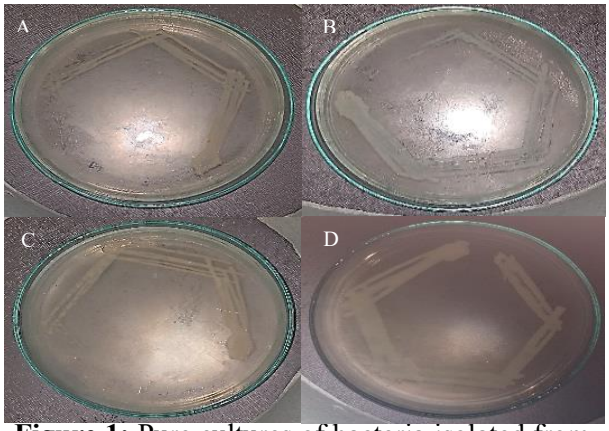
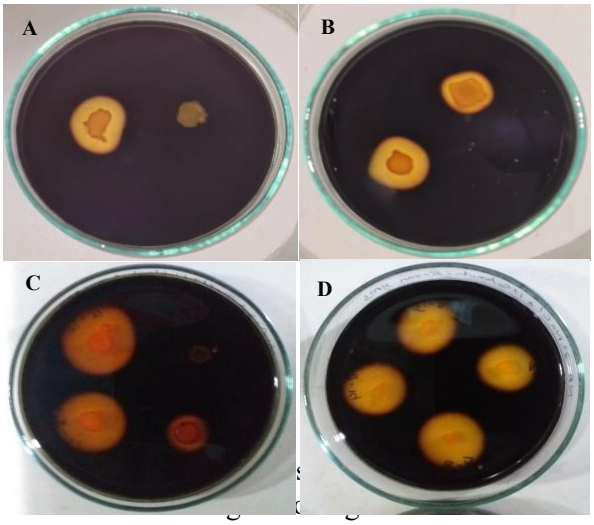


Figure 1: Pure cultures of bacteria isolated from Negombo lagoon and Panadura beach. A). NL-05 isolated from Negombo Lagoon: B).NL-16 isolated from Negombo Lagoon: C).PB-02 isolated from Panadura Beach: D). PB-18 isolated from Panadura Beach.

3.2 Screening for Salt-tolerant Amylase enzyme-producing bacteria using starch agar plate assay. A total of 21 bacterial isolates were positive for amylase production, 3 from Negombo lagoon and 18 from Panadura beach samples (Figure 2).



beach. A). NL-12 isolated from Negombo Lagoon: B).NL-09 and NL-10 isolated from Negombo Lagoon: C). PB-16, PB-17 and PB-18 isolated from Panadura Beach: D). PB-11, PB-12, PB-13 and PB-14 isolated from Panadura Beach.

3.3 Screening for Salt-Tolerant Cellulase enzyme-producing Bacteria using Congo-Red assay. A total of 26 bacterial isolates were positive for cellulase enzyme production, 9 from Negombo lagoon and 17 from Panadura beach samples (Figure 3).

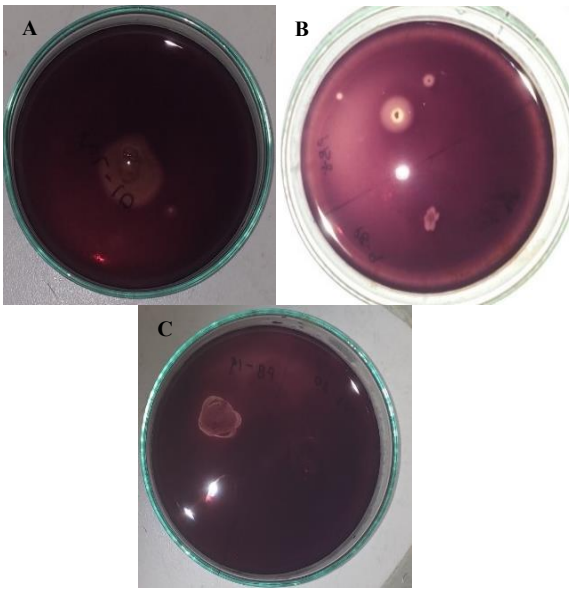
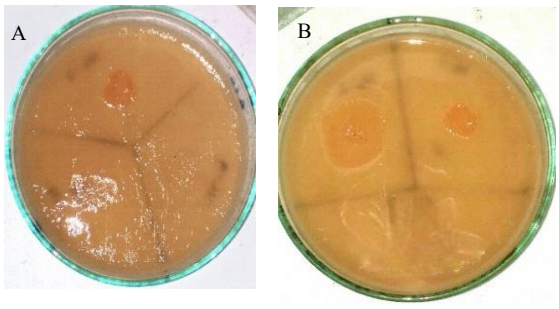


Figure 3: Cellulase positive bacterial colonies isolated from Negombo lagoon and Panadura beach. A). NL-10 isolated from Negombo Lagoon: B). PB-08 isolated from Panadura Beach: C).PB-19 isolated from Panadura Beach.

3.4 Screening for Salt-Tolerant Protease enzyme-producing Bacteria using Skimmed Milk Agar assay. A total of 30 bacterial isolates were positive for protease enzyme production, 25 from Negombo lagoon and 5 from Panadura beach samples (Figure 4).



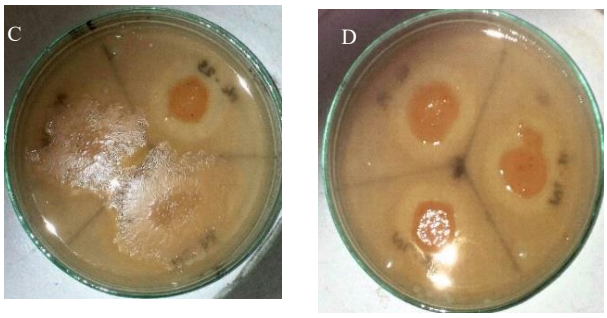


Figure 4: Protease positive bacterial colonies isolated from Negombo lagoon and Panadura beach. A). NL-07 isolated from Negombo Lagoon: B).PB-07 isolated from Panadura Beach: C). NL-25 isolated from Negombo Lagoon: D). NL-10, NL-11 and NL-12 isolated from Negombo Lagoon.

3.5 Screening for Salt-Tolerant Lipase enzyme-producing Bacteria using Phenol Red Agar assay. A total of 22 bacterial isolates were positive for lipase enzyme production, 14 from Negombo lagoon and 8 from Panadura beach samples (Figure 5).

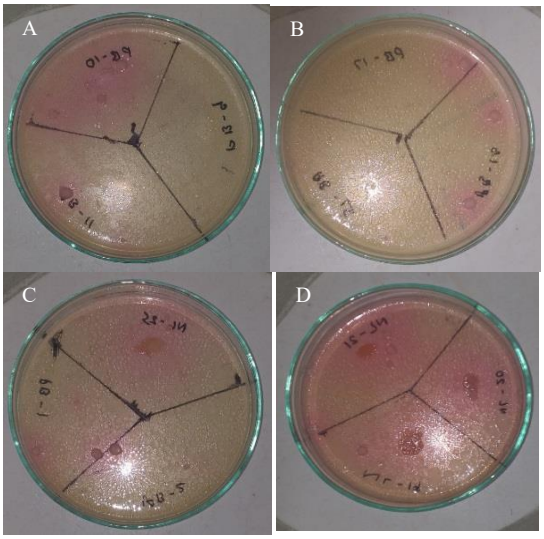
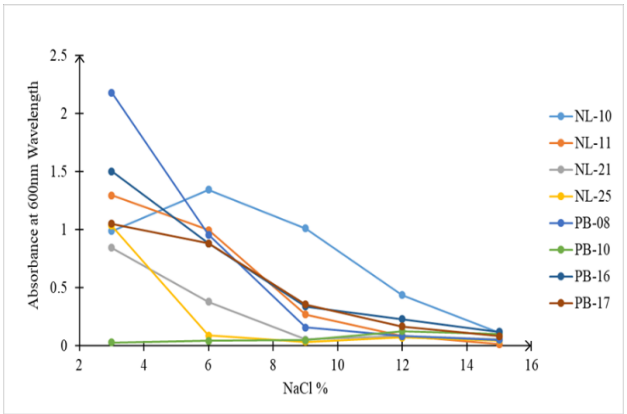


Figure 5: Lipase positive bacterial colonies isolated from Negombo lagoon and Panadura Beach. A). PB-10 isolated from Panadura Beach: B).PB-15 and PB-16 isolated from Panadura Beach: C). NL-25 isolated from Negombo Lagoon: D). NL-19, NL-20 and NL-21 isolated from Negombo Lagoon.

3.6 Optimum Salt- Concentration for the Selected Bacterial Samples. NL10 had the best growth at 6% NaCl concentration. PB-10 had an optimum NaCl concentration of 12%. NL-11, NL-21, NL-25, PB-08, PB-16 and PB-17 had the best growth at 3% NaCl concentration (Figure 6).



different salt- concentrations.

3.7 Optimum Incubation Temperature for the Bacterial Colonies at their Optimum Salt Concentration. The optimum growth temperature of NL-21 and PB-17 at 3% NaCl concentration was 35^{0}C and PB-10 also had the same optimum growth temperature at 12% NaCl concentration. The bacterial isolates PB-08, NL-11, PB-16 and NL-25 showed the optimum growth at 30^{0}C at 3% NaCl concentration and NL-10 the optimum growth at 35^{0}C at 6% NaCl concentration (Figure 7).

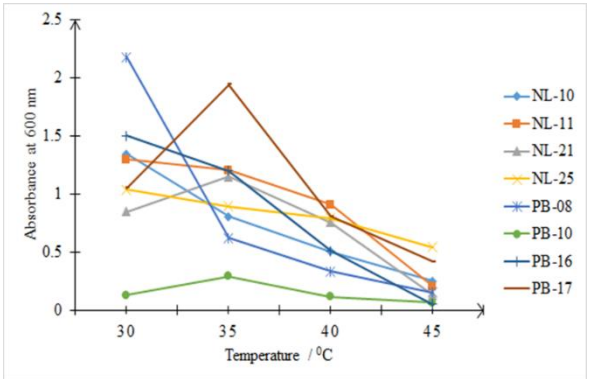


Figure 7: Growth of bacterial isolates at different incubation temperatures.

4. Discussion

Bacteria are classified in various categories due to their variations. Bacteria differ from one another in several ways including their shape, size, composition of cell wall, environment they live in, antibiotic resistance, functional enzymes produced, etc.[29] The above research determines the availability of certain enzymes in halophilic bacteria to be used in industrial applications. Even though all bacteria produce enzymes, halophilic bacteria are mainly used in enzyme assay not only due to their high concentration stability but also because of their high stability in high temperatures and organic solvents.[30]

A moderate number of bacterial isolates from the Negombo lagoon have activity of enzymes lipase and protease still, only a very few isolates from the Negombo lagoon have the enzymatic activity for amylase and cellulase. Meanwhile, the enzyme activity of amylase and cellulase were shown by almost all the bacterial isolates isolated from Panadura Beach, but only about 50%-60% of the bacterial isolates of Panadura Beach had protease and lipase enzyme activity. These differences can prove that the environment influences the growth and functionality of the bacteria.[31] The majority of the bacteria produce more than two hydrolases, while some bacteria do not produce at least one type of enzyme. Similar findings were reported in previous studies, where the majority of halophilic bacteria were found to produce multiple hydrolases.[11,32] Optimization of bacteria determines the best condition at which the bacteria grows the most. The optimum salt concentration of the bacteria is helpful in identifying the type of halophile, while the optimum incubation temperature determines the best temperature for the growth of the bacteria. Out of the isolated 44 bacterial colonies, the colonies with the most prominent enzyme activity were chosen for the optimization. Through optimization, the most promising bacteria at their optimum conditions were identified for the biotechnological usage of the enzymes for industrial applications.

5. Conclusion

Halophilic bacteria which produce amylase, cellulase, proteinase and lipase at room temperature were isolated. Hence, the marine environments can be considered as a rich source of enzyme producing bacteria. These enzymes may be used in industrial settings operates under high salt concentrations as biological catalyst and will be a better alternative for high cost environmental hazardous chemical catalysts.

Further studies are needed to determine the characteristics and suitability of these enzymes for specific industrial settings. Reaction mixture condition optimization can be carried out to determine the optimum conditions for enzyme activity.

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