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Journal of Microbial Biology
E-ISSN: 2322-5173
12rd Year, Vol. 12, No. 48, Winter 2023 pp. 41-50
Received: 12-08-2023 Accepted: 07-11-2023

(Research Paper)

Isolation and Identification of Lipase-producing Bacteria from Effluents Contaminated with Oily Compounds

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Abstract

Introduction: Lipases play a crucial role as biocatalysts, specifically in breaking down lipid ester bonds. Among these biocatalysts, bacterial lipases stand out for their ability to hydrolyze a wide range of substrates, while also being more stable and cost-effective to produce. Unfortunately, the discharge of effluents from oil refineries into surface waters leads to significant environmental pollution. In light of this, the objective of this study is to isolate and identify lipase-producing bacteria from effluents contaminated with oily compounds.

Materials and Methods: To identify lipase-producing strains, specific culture media such as tributyrin agar were used, and the secretion of lipase around the bacterial colonies was observed. The isolates were then subjected to biochemical evaluation to determine the strains with the highest enzyme activity. To identify the bacterial strains accurately, PCR was performed targeting conserved 16S rRNA sequences. The PCR products were sequenced and analyzed using the BLAST method for further analysis and identification.

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Moravej R. and Dibanezhad N. Isolation and Identification of Lipase-producing Bacteria from Effluents Contaminated with Oily Compounds. *Journal of Microbial Biology* 2023; 12 (48): 41-50. http://dx.doi.org/10.22108/bjm.2023.138739.1556

Results: To identify lipase-producing strains, different dilutions of contaminated effluent and soil samples were cultured. Four colonies with the widest transparent halo around them were isolated and purified (R1-R4). Strain R2 exhibited the highest lipase activity with a value of 3.452u/ml, whereas strain R4 displayed the lowest activity at 1.996u/ml. After conducting the molecular evaluation of the isolated strains, it was revealed that strain R1 belonged to *Acinetobacter junii* strain B2, strain R2 was identified as *Rummeliibacillus pycnus* strain NBRC 101231, and strain R3 was categorized as *Pseudomonas aeruginosa* strain HX-2.

Discussion and Conclusion: The study identifies a promising local bacterial candidate that holds potential for industrial-scale production of lipase.

Key words: Bacteria, Isolation, Lipase, Oil**,** Para-nitrophenyl Palmitate (P-npp)

Introduction

Following the increase in population and the subsequent rise in food demand, the production of industrial effluents and the associated challenges related to their discharge into the environment have become significant issues. Oily wastewater, in particular, contains numerous organic compounds that contribute to high levels of chemical oxygen demand (C.O.D.), typically ranging from 30,000 to 100,000 mg/L. Discharging such wastewater into the environment and surface water leads to a significant depletion of oxygen and harmful effects on the aquatic ecosystem. These effluents contain approximately 1,500 to 15,000 mg/L of fats and oils, which can pose severe problems for wastewater treatment systems, including sudden sludge removal, reduced oxygen availability for microorganisms, accumulation, and the development of strong odors (1, 2).

Currently, the enzymatic treatment of this wastewater type is the focus of several studies conducted by various researchers. The enzymatic purification process of fatty compounds necessitates the presence of the enzyme lipase. Lipase works to break down oils and fats into fatty acids and glycerol. This transformation allows these compounds, unlike long-chain oily compounds, to be readily utilized by microorganisms (3).

Under normal conditions, this enzyme catalyzes the hydrolysis of ester bonds on the surface between the insoluble phase of the substrate and the aqueous phase, where the substrate can be triglyceride. Under certain laboratory conditions in the absence of water, the reverse reaction by these enzymes leads to esterification and formation of glycerides from fatty acids and glycerol, as well as interesterification (4).

Unlike chemical catalysts, lipases have a particular function, milder reaction conditions, and are more environmentally friendly; lipases can act specifically on the length of the chain, the double bond position in the fatty acid, or the type of fatty acid. Among plant, animal, and microbial sources of lipase, bacterial types are the most popular and widely-used ones. Because microbes are easily cultured, the lipase extracted from them can be used in a wide range of hydrolytic and synthetic reactions (4, 5).

Sewage and soils contaminated with oily compounds serve as potential sources for the isolation of lipase-producing microorganisms. The biocatalytic capabilities of microbial lipases in both aqueous and non-aqueous environments, coupled with their remarkable resistance to alkaline and high-temperature conditions,

have led to their widespread utilization in various industries, such as detergent manufacturing, food production, cosmetics, pharmaceuticals, and the synthesis of fatty compounds (6, 7). The objective of the present study is to isolate and identify lipase-producing bacteria from oil-contaminated effluent and soil.

Materials and Methods

Bacteria Isolation from Oil-Contaminated Effluents: Sampling was conducted from the effluents of two factories, which produce dairy products and vegetable oils. To achieve this, samples were collected from the factory's effluent prior to entering the effluent treatment system, as well as from various sections within the treatment system. In order to sample effluent-contaminated soils, samples were taken from a depth of 30 cm at the effluent outlet of the factories. All samples were promptly transferred to sterile containers (6).

Serial Dilution Preparation and Colony Counting: For pure cultures, serial dilutions of the samples were prepared, ranging from 10^{-1} to 10^{-5}. To count the bacteria, all dilutions were cultured on plate count agar (consisting of 0.5% peptone, 0.25% yeast extract, 0.1% glucose, and 1.5% agar). Additionally, the pour plate method was employed to determine the number of colony-forming bacteria present in liquid samples (6).

Screening of Lipase-producing Strains: After culturing the bacteria on a nutrient agar medium, the colonies were cultured on the differential medium of Tributyrin agar. This medium contains peptone, yeast extract, glyceryl tributyrate, and agar. Additionally, the colonies were cultured on a medium containing Tween 80 and olive oil as a carbon source. The cultures were then incubated at 37°C for 1 day. If a clear halo was observed around a colony, it indicated the secretion of the lipase enzyme by the bacteria. Lipolytic colonies with a clear halo were isolated and purified to obtain a single colony. These single colonies were then preserved by freezing them in a medium containing glycerol in the freezer for future use.

Bacterial Identification Using Biochemical Tests: Firstly, the lipase-producing bacterial isolates were grown in 10mL of broth medium at 37°C overnight at 190 rpm and later subjected to Gram staining. Then, in order to identify the bacteria, the cultures were subjected to biochemical tests.

Lipase Production and Activity Assessment: The isolated strains were grown in a specific liquid medium containing (g/L): peptone, 0.2; $NH_4H_2PO_4$, 0.1; NaCl, 0.25, $MgSO_4.7H_2O$, 0.004; $CaCl_2.2H_2O$, 0.004; Olive oil, %2(v/v); Tween 80, 0.001 (v/v)) under 150 rpm, and 25°C condition for 48 hours. The cultured were then centrifuged at 10000 rpm at 4°C for 30 minutes. The enzyme-containing supernatant was then collected for further analysis. In order to determine the lipolytic activity of the enzyme, using p-nitrophenyl palmitate (Pnpp), a standard curve was prepared. For this purpose, Pnpp was dissolved in different concentrations (in µg) in distilled water, and their light absorption was measured at 410 nm, and then the curve was plotted. Using this curve and comparing the light absorption of the samples at 410 nm, the enzymatic activity was quantified. According to the following equation, the lipase activity of selected samples was calculated precisely (8).

For this purpose, a mixture of 700 µL pnpp solution and 300 µL of lipase-containing supernatant was used. The Pnpp solution consisted of 0.001 g of Pnpp in isopropanol, to which 0.01 g of gum Arabic, 0.02 g of sodium deoxycholate, and 50 µL of Triton X-100 in 9 ml of Tris HCl 50 mM buffer were added. The final solution was then adjusted to pH 8. To quantify lipase activity for each sample, a culture medium containing the enzyme and the stopper solution was added simultaneously with the

http://dx.doi.org/10.22108/bjm.2023.138739.1556

reaction solution to halt the enzymatic activity. Using the OD obtained at a wavelength of 410 nm and interpolated on the standard curve, the amount of released P-npp was determined and reported as an enzyme unit. One lipase unit is defined as the amount of enzyme that releases 1 µg of P-npp per milliliter per minute under standard conditions. According to the following equation, the lipase activity of selected samples was calculated precisely (9).

$$Lipase\ Activity\ (\frac{unit}{ml}) = \frac{para\ nitrophenol\ released(standard\ curve) \times total\ in\ assay}{\text{time in assay} \times \text{ml lipase used} \times \text{ml put in spectrophotometer}}$$

Characterization of Isolated Bacteria Using PCR: First, the isolated bacteria were cultured on a nutrient agar medium to prepare a new culture of bacterial cells for extraction. To do this, a loopful of bacterial storage medium was streaked onto a nutrient agar medium and incubated at 37°C for 24 hours. Then, the previously prepared L.B. medium was brought to room temperature. A loopful of bacterial colonies cultured on agar in the previous stage was then transferred to the L.B. liquid medium and shaken. The L.B. medium containing the bacteria was incubated at 37°C for 24 hours in a shaker incubator at 150 rpm. In later stages, this culture was used for DNA extraction. Axygen™ (AxyPrep Bacterial Genomic DNA Miniprep, U.S.A.) was used for DNA extraction according to the manufacturer's protocol. The primers used to identify the conserved region of the 16SrRNA gene are provided in Table 1. They were sourced from a previous study (9).

Table 1- Primers Used in the Present Study

Primer type	Primer sequence	PCR product length
Forward	5'-AGAGTTTGATCCTGGCTCAG-3'	1500 bp
Reverse	5'-TACGGTTACCTTGTTACGACT-3	

PCR was carried out in a Thermocycler (Eppendorf, Mastercycler®) using a Taq polymerase (Cinnagen, Iran). The PCR procedure consisted of initial denaturation at 95°C for 7 min, followed by another denaturation step at 94°C for 1 min, then a set of 35 cycles each of 94°C for 1 min, 51°C for 1 min, 72°C for 1 min; 72°C for 10 min; and the final incubation at 4°C for 15 min. The DNA extracted from the gel was prepared for sequencing using the ABI Capillary System by Macrogen Research in Seoul, Korea. The resulting sequence was analyzed and checked using Chromas, MEGA software (ver.7), and BLAST, with searches conducted in the EMBL/GEN BANK databases. Software that included previously reported bacterial strain sequences from the GenBank was used for these analyses. Finally, the phylogenetic tree for each sequence was constructed using the Neighbor-joining method.

Gel Electrophoresis of PCR Products: Gel electrophoresis was performed to analyze the PCR products. To prepare a 1% agarose gel, 0.3 g of agarose powder was added to 30 ml of 0.5X T.B.E. buffer and heated for 30 seconds. After cooling, 0.5 µL of cyber green was added, and the gel was poured. Next, 3 µL of the PCR product was mixed with 2 µL of loading dye and carefully loaded into the wells. Additionally, 2 µL of DNA ladder (without loading dye) was added to a separate well to determine the approximate size of the PCR fragments. The gel was then subjected to an applied voltage of 80 volts. After the electrophoresis was complete, the gels were exposed to UV light using gel documentation, and gel images were captured (9).

http://dx.doi.org/10.22108/bjm.2023.138739.1556

Results

Culture and counting of samples: The isolated samples were used to prepare serial dilutions to inoculate in the pour-plating method. The primary purpose of the pour plate method is to separate pure culture from a mixture of different bacterial populations and show the characteristics of bacterial culture. The results of counting bacteria in the sample collected are shown in Fig. 1. The findings suggest that the bacterial population in the oily wastewater sample is relatively higher compared to the dairy factory wastewater sample, although this difference was not statistically significant ($p > 0.05$).

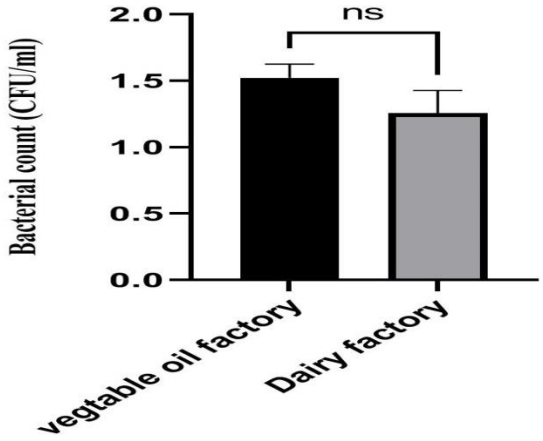

Fig. 1- The average count of bacteria in terms of CFU at sampling sites (×10000). Bacterial counts are represented by mean value ± standard deviation (n = 3). ns: not significant.

Lipase Producing Isolates: Colonies that were able to grow on a selective culture medium of tributyrin agar and produce a halo around them were considered positive colonies for lipase production (Fig. 3). The halo formation around the colony was evaluated with three replicates, and the results are depicted in Fig. 2. Out of all the positive colonies, we selected four colonies with the largest halo diameter (R1 = 7.3 mm, R2 = 9 mm, R3 = 5.1 mm, R4 = 3.6 mm) for further analysis. Among the isolates examined, the R2 isolate exhibited the largest diameter of the inhibition zone, followed by R1, R3, and R4 ($p<0.0001$). Samples R1, R2, and R4 were obtained from the effluent and soil of the vegetable oil-processing factory, while sample R3 originated from the effluent of the dairy factory. A specific culture medium containing tween 80, as the only available carbon source, was used to promote the growth of lipase-producing strains and facilitate the separation based on the decomposition halo characteristics. Interestingly, the colonies that grew and formed a decomposition halo in the Tween 80 medium also exhibited the same halo size in the tributyrin agar medium. This indicates that the strains with the largest halo diameters were consistent in both media.

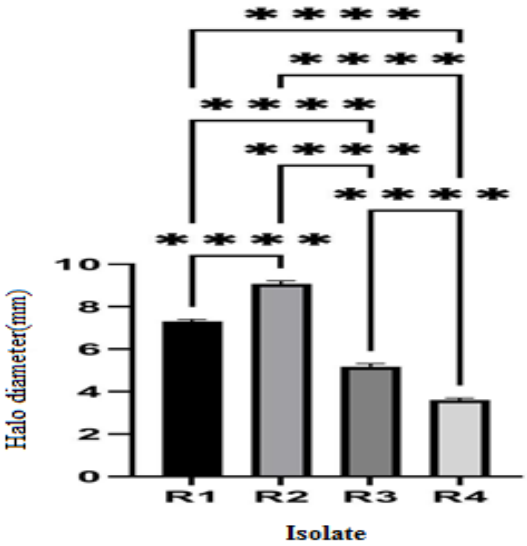

Fig. 2- The Average Diameter of the Clear Zone for Lipase-Producing Strains (**** $p<0.0001$)

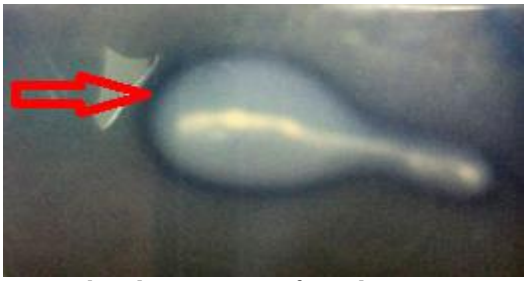

Fig. 3- The Clearing Zone of Lipolytic Activity on Tributyrin Agar

The enzymatic activity of these isolated samples was evaluated by spectrophotometry. After preparing the standard curve of P-npp, the amount of enzymatic activity and light absorption of the strains isolated by spectrophotometry at 410

http://dx.doi.org/10.22108/bjm.2023.138739.1556

nm were evaluated. The values were adapted to the standard curve and used in a formula, the amount of lipase enzyme activity was determined. The analyses showed that isolate R2 has the highest lipase activity with a value of 3.452 units/ml, which was significantly different from other isolates ($p<0.05$). Isolates R1, R3, and R4 followed by lipase activity values of 3.05, 2.736, and 1.996 units/ml, respectively. However, there was no significant difference in lipase production between R1 and R3 isolates ($p>0.05$) (Fig. 4).

When comparing the diameter of decomposition colonies on the tributyrin agar and tween80 culture mediums with the measured values of lipase activity, it was discovered that there is a direct relationship between the size of the decomposition halo diameter and the amount of lipase activity. This means that as the halo diameter increases, there is an accompanying increase in enzymatic activity.

Biochemical and Molecular Identification: The biochemical characteristics of the isolates are shown in Table 2. Based on primary screening and information obtained from PCR followed by the BLAST procedure, three selected strains were identified with a similarity index of 100% (Fig. 5). As a result, strain R1 was identified as *Acinetobacter junii* strain B2w, and its sequence was subsequently registered in Genbank. Similarly, the R2 sample was identified as *Rummeliibacillus pycnus* strain NBRC 101231, and the R3 sample was identified as *Pseudomonas aeruginosa* strain HX-2 (Fig. 6).

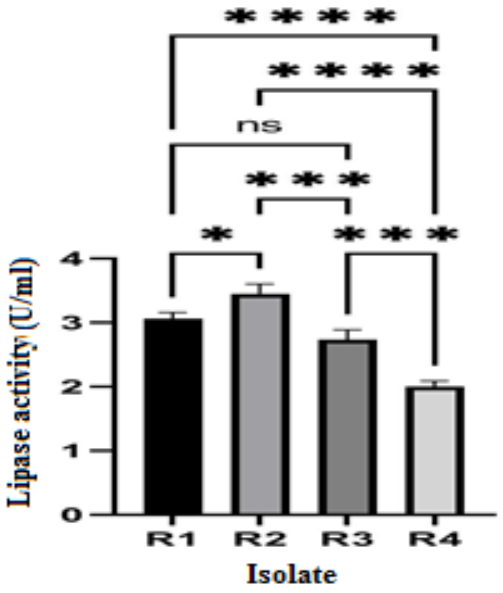

Fig. 4- Enzyme activity of 4 isolates that tested positive in qualitative analysis. Lipase activities are represented by mean value ± standard deviation (n = 3), (**** $p<0.0001$, *** $p<0.001$, * $p<0.05$, and ns: not significant.)

Table 2- Gram Staining and Biochemical Characterization of the Bacterial Strains

	Catalase	Oxidase	Citrate	Indole	Motility	H_2S	MR	VP	TSI	Gram	Lactose
R1	+	–	–	–	–	–	–	+	ALK/A	–	–
R2	+	–	–	–	–	–	+	–	ALK/Agas	+	–
R3	+	+	+	+	+	–	–	+	ALK/A	–	–
R4	+	–	–	+	–	–	+	–	A/Agas	–	+

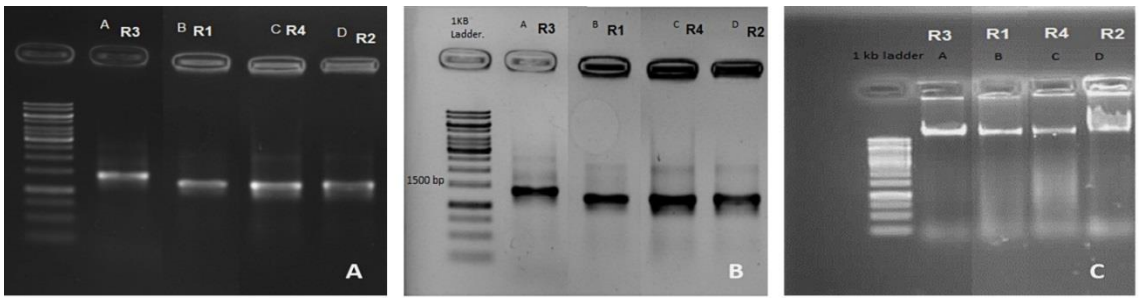

Fig. 5- Gel electrophoresis results of PCR products (A, B) and extracted DNA (C) of the four bacterial strains for the conserved 16S rRNA sequence

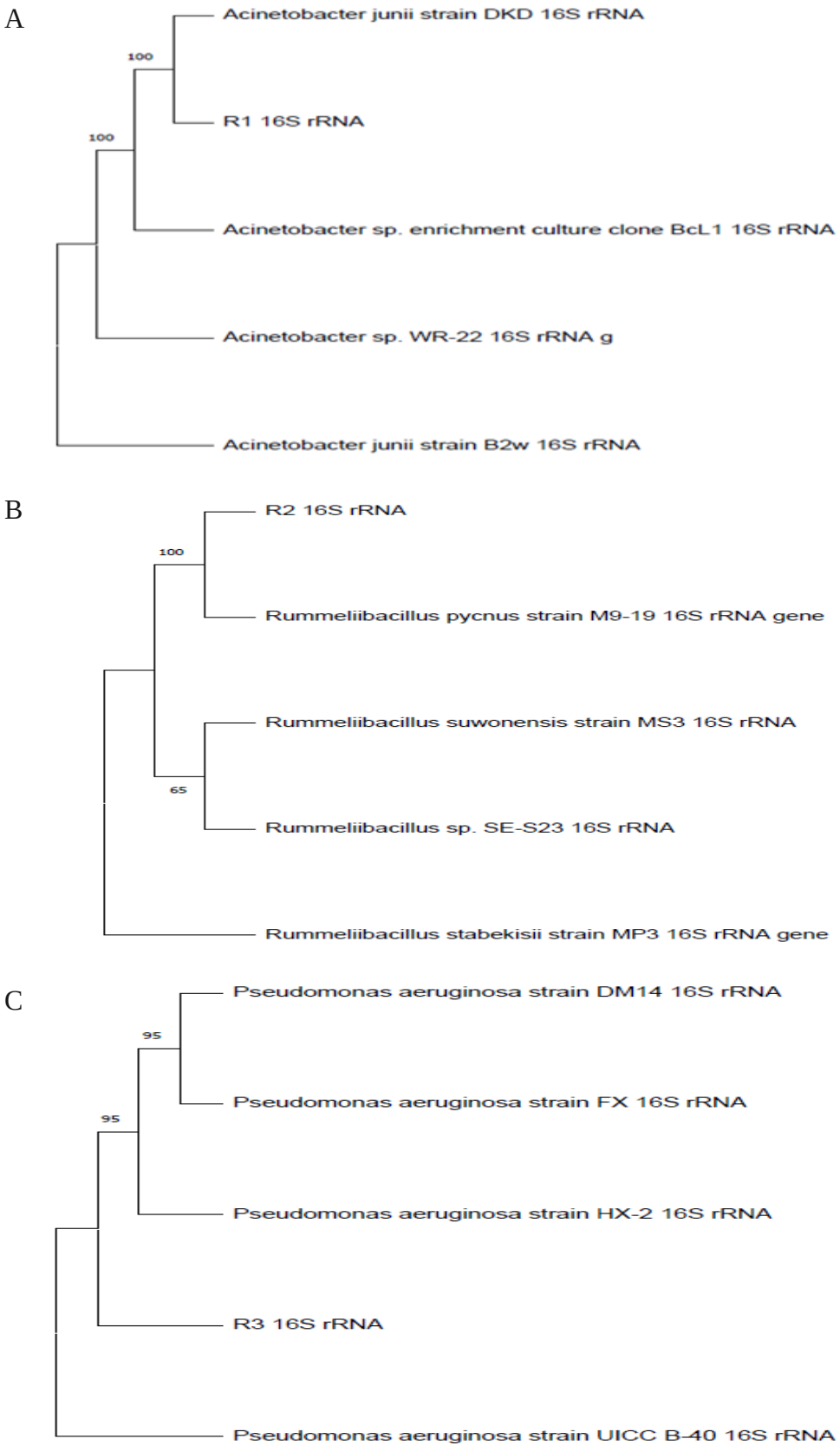

Fig. 6- The Inferred Evolutionary History Using the Neighbor-Joining Method

http://dx.doi.org/10.22108/bjm.2023.138739.1556

The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 5 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1409 positions in the final dataset. Evolutionary analyses were conducted in MEGA11: (A) R1 isolate, (B) R2 isolate, and (C) R3 isolate.

Discussion and Conclusions

In recent years, carbohydrates, proteases, lipases, and cellulases have become some of the most important types of commercial enzymes. Among them, lipases and proteases have garnered significant attention due to their critical applications in various industries. Lipases are a group of enzymes that play a key role in breaking down lipids. These lipolytic enzymes are present in most organisms and are essential for cellular growth and differentiation. Today, lipases have become indispensable in numerous aspects of human life, finding applications in industries such as food, medicine, pharmaceuticals, cosmetics, and detergents, as well as in processes like wastewater treatment and biodegradation (4).

The significance of lipase-producing bacteria in industry has led to extensive research conducted by numerous research groups worldwide. Meanwhile, the remarkable advantages of lipase enzymes derived from bacteria, such as simpler culture conditions, easier screening processes, genetic engineering of productive bacteria, cost-effectiveness in industry, and minimal negative impact from environmental conditions, have further amplified their importance (4).

Bacterial lipases are mostly extracellular, and their production is primarily influenced by physicochemical factors such as pH, temperature, and insoluble oxygen. Also, carbon sources have generally been reported as an essential factor in the expression of lipase activity. When effluent and soil microorganisms are tested, sampling may vary depending on the sampling site and the type of lipase studied (10). The present study sought to isolate bacteria from a sample of effluent whose microorganisms produce high levels of the enzyme lipase.

Scientists today do not consider the results of phenotypic and molecular identification tests alone as sufficient criteria for confirming the accurate identification of a microbial species. Instead, they rely on both methods together to support and reinforce each other (11). Therefore, in this study, both phenotypic and genotypic identification (16SrRNA) was used to identify bacteria.

In order to conduct this research, oil-contaminated effluent and soil samples were collected from the wastewater outlet and treatment system of an oil-processing factory and a dairy factory. The entry of oil effluents into the surface water of these factories causes much environmental pollution. These areas are among the most oil-contaminated regions in food industries and, the alleviation of wastewater mentioned above, especially in food industries, is of high importance. The bacteria-producing lipase was isolated and identified from the effluent contaminated with oily compounds. The samples were subjected to cell counting followed by culturing in an enriched medium to enhance the growth of lipase-producing bacteria.

In this study, after phenotypic and biochemical identification, the molecular

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method was employed to accurately recognize the species. This procedure involved utilizing 16S rRNA sequencing, which has now become the gold standard for bacterial species identification (9). Compared to a finding of previous studies, there are notable items about the identified strains to contemplate.

In a study conducted by Mehta et al., (12) isolated bacteria were identified as *Bacillus* and *Pseudomonas* species. In another study conducted by Ramani, K. et al., the isolated strain was identified as *Pseudomonas gessardii* (13). Whereas in another study, isolated strains were identified as *Bacillus* strains (14). In another study conducted by Mobarak Qamsari et al. (9) and Gao (15), the *Pseudomonas aeruginosa* KM110 strain was identified as a lipase-producing strain. In the investigation carried out by Bisht in 2011(16), isolated strains were identified as *Acinetobacter sp., Brevibacillus borstelensis*, and *Porphyrobacter cryptus*. The findings of Qamsari (9) and Bisht (16) regarding the isolation of *Pseudomonas* and *Acinetobacter* strains are consistent with this study.

As mentioned earlier, in this study, isolated strains were identified as *Acinetobacter junii strain B2*, *Rummeliibacillus pycnus* strain NBRC 10123, *and Pseudomonas aeruginosa* strain HX-2.

Except for *Rummelibacillus*, all the genera reported in this study have been reported in previous similar studies. The high concentration of enzyme produced by this strain doubles its importance. Moreover, Junpadit et al. suggested that *Rummeliibacillus pycnus Strain TS8* could potentially be used to produce PHA polymer and intracellular lipid from POME as a cost-effective substrate, which could be used as a raw material in biodiesel production. This could expand the applications of the newly discovered species, and in the case *of Rummeliibacillus pycnus* NBRC 101231, in addition to reducing environmental pollution, its usefulness in producing biodiesel as an Eco-friendly fuel could be considered. On the other hand, this bacterium is found in water and soil and is involved in the breakdown of organic materials (17). The results indicated that strains R1 and R3 exhibit a closer phylogenetic relationship to each other, while strain R2 is more distantly related. These findings are further supported by the results of morphological studies and Gram staining.

The 16S rRNA sequence of *Acinetobacter junii* strain N.D. has been submitted to GenBank with the accession ID KY848852.1. [https://www.ncbi.nlm.nih.gov/nuccore/KY848852].

Considering the significant need for industrial enzymes in the country, and based on the data obtained from this study, it appears that utilizing local enzyme-producing strains can effectively lead to high production of the lipase enzyme and eliminate the need for the country to import this product.

Acknowledgments

The authors of this study would like to thank the staff of the Department of Biology, Sanandaj branch, Islamic Azad University, Sanandaj, Iran.

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