An innovative extracellular polysaccharide produced by Egyptian Marine *Bacillus licheniformis,* and its applications as an antibacterial agent.

Sokara Ali[1,*], Mohamed Amer[2], Hanafy Ahmed Hamza[1], Rateb Nabil Abbas[1]

[1]Department of Microbial Biotechnology, Genetic Engineering & Biotechnology Research Institute, Univeristy of Sadat City, Egypt

[2]Microbiology Laboratory, Environment Division, National Institute of Oceanography and Fisheries, Alexandria, Egypt.

DOI: 10.21608/RJAB.2023.198462.1027

****Corresponding author: Sokara Ali***
E-mail: **sokaraabdelaziz@yahoo.com**

ABSTRACT

Twenty-two marine bacterial isolates were collected from the Eastern Harbor (29885 E longitude and 31205 N latitude) located at Alexandria, Egypt selected to produce exopolysaccharides, using the Shene extraction method in 2008. The most potent marine bacterium was identified as ***Bacillus licheniformis SMRH1*,** accession number ON777836 by using 16s rRNA as a molecular identification technique. According to the chemical analysis, the obtained polysaccharide content is estimated by using Fourier Transform Infrared spectra FTIR spectrum of the extracted exopolysaccharide from ***Bacillus licheniformis*** which showed that it contains νOH at 3420cm^{-1}, νC-H aliphatic at 2952cm^{-1}, νC=O in range 1660-1630cm^{-1} and Nuclear magnetic resonance spectra NMR showed signals δ'^{s} ppm at 1.22 (s, -CH_3), 2.07 (s, -$CH_{2\ chain}$) and 2.35(m, -CH_2) and Scanning electron microscope SEM which confirmed the amorphus shaped uneven texture polysaccharide at higher magnification of ×5000. The exopolysaccharide that was generated has antibacterial activity by forming a free hollow zone around it against some gram positive and gram-negative bacteria according to disc diffusion method. The innovatively produced exopolysaccharide also has anticoagulant activity. A recent study proved that the generated exopolysaccharide induced β-hemolysis. The high yield of exopolysaccharide was "0.7 g/l" by using different culture media such as nutrient broth, peptone water, and king's B broth.

Keywords: Marine bacteria, Bacillus licheniformis, Exopolysaccharide, chemical characterization, Antibacterial.

1. INTRODUCTION

Marine microorganisms are an innovative source of novel bioactive components with potential human utility (Ghosh *et al*., 2022). These microorganisms produce complex compounds with distinctive biologically intriguing features for a wide range of industrial and biotechnological uses since some of them can thrive in harsh maritime settings. As a result, various marine microorganisms (fungi, myxomycetes, bacteria, and microalgae) have previously been discovered that produce chemicals with antioxidant, antibacterial, apoptotic, antitumoral, and antiviral activity (Ameen *et al*., 2021). Bacteria found in the sea have a wealth of useful products and functions. The quest for novel physiologically active chemicals has expanded to include creatures

found in less-studied habitats (Núñez-Pons *et al*., 2015). Bacteria found in the sea have a wealth of useful products and functions. The quest for novel physiologically active chemicals has expanded to include creatures found in less-studied habitats (Yadav *et al*., 2019). A good source of microbial variety that can be leveraged to create new secondary metabolites like exopolysaccharides is the marine environment (EPSs). In the marine environment, where they are necessary for survival and defense, microbial EPSs are commonplace. EPSs produced by marine bacteria demonstrate a wide range of biotechnological uses. Because of the variety in their physicochemical makeup and structure (Shukla *et al*., 2023). Marine organisms are becoming more and more significant as a source of unique bioactive compounds. Because marine animals make up more than half of the world's biodiversity, oceans and seas are thought to be the largest remaining store of beneficial natural compounds that might be used as functional ingredients in the food industry. This study refreshes our understanding of the functional seafood ingredients we already use, with an emphasis on their potential uses and health benefits. Proteins, peptides, amino acids, fatty acids, sterols, polysaccharides, and oligosaccharides are some of these substances (Rocha *et al*., 2011). By using infrared spectra, nuclear magnetic resonance spectra, and scanning electron microscopy, numerous novel and intriguing bioactive compounds, such as exopolysaccharides, have been discovered and described. These molecules have a variety of medical, industrial, and agricultural applications. Microbial populations may thrive in many kinds of extreme environmental conditions, including hot or low temperatures, acidic or alkaline conditions, saline conditions, and water scarcity or stress. These animals, known as extremophiles, have adaptive traits that have evolved to allow them to survive in one or more extreme environments, in contrast to polyextremophiles, who survive in a variety of environments. Polyextremophiles may adapt to abiotic stresses such poor water availability, high salinity and temperature, low PH, and high pressure. habitats that are subject to one or more additional environmental factors, such as salinity, osmolarity, dehydration, and UV radiation (Yadav *et al*., 2021). These microorganisms produce complex chemicals with distinctive biological features that can be exploited in a variety of industrial and biotechnological applications. Some of these microbes can survive in severe maritime environments (Ghosh *et al*., 2022). The primary goal of this article is to produce exopolysaccharide as a bioactive compound from the most potent marine bacteria and its applications like; antibacterial, anticoagulant and hemolysis activities and other biotechnological applications.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1.Chemicals

All chemicals used for biochemical tests and extraction of polysaccharides were of pure grade and were purchased from TECHNO PHARMCHEM, BAHADURARH, HARYANA, India. AN ISO 9001: 2008 certified company. All other chemicals and reagents were bought locally and were of analytically reagent grade from Algomhoryia Company for Chemicals, Cairo, Egypt. PCR (polymerase chain reaction) and sequencing chemicals and DNA/RNA extraction kit provided by the Egyptian Center for Identification of Microbes at the Institute of Genetic Engineering and Biotechnology research institute, Sadat city, Egypt.

2.1.2. Medias

2.1.2.1. Culture media

Sea water agar is used for determining viable bacteria (Zobell *et al*., 1946), All constituents are given in g/L. Peptone 5.0, Ferric phosphate 0.1, and Agar 15.Nutrient broth medium (Atlas *et al*., 1997 Yeast extract 2.0, Beef extract 1.0, Peptone 5.0). peptone water medium (PW) Peptone 5.0, Tryptone 5.0. Sodium chloride 5.0. King's B medium (KB) (Murray *et al*., 2003) Glycerol 30.0, Protease peptone 10.0 K2HPO4 0.5. MgSo4.7H2O 0.5 and the PH was adjusted at 7.0 by using a buffer solution.

2.2. Methods

2.2.1. Collection of samples

Sea samples were collected from the Eastern Harbor (29885 E longitude and 31205 N latitude) located at Alexandria, Egypt (Fig. 1). Seawater samples were collected using 500 ml sterile blue screw-caped bottles according to the standard methods published by American Public Health Association (Apha *et al.*, 1995). Serial dilutions were made using filtered sterilized seawater (from 10-2 to 10-6). A portion (0.1 ml) from each diluted sample was spread on a seawater nutrient agar plate medium (5 g peptone, 3 g beef extract, 20 g agar, 1000 ml seawater). Plates were incubated at 30°C for 24 h. Purification of the obtained bacterial colonies was carried out by streaking technique.

2.2.2. Isolation and purification of bacterial isolates

Sea water samples were collected in 500 ml sterile screw-caped bottles as previously described by (Austin *et al*., 1988). Seril dilutions from10^{-2} through superscript were made using filtered sterilized seawater. A portion (0.1ml) from each appropriately diluted sample was used to inoculate plates prepared with seawater agar for counting aerobic heterotrophs. Plates were incubated at 30°C for 24-48 h. purification of bacterial colonies was carried out by streaking on agar plates of the same medium. The pure colonies obtained were transferred to fresh slants. Subcultures were kept under refrigeration for further investigations.

2.2.3. Production and Extraction of Exopolysaccharide (EPS)

In a separate liquid medium made up of the following ingredients (g/L): peptone 4.0, yeast extract 2.0, and sucrose 20 isolates were tested for the ability to produce EPSs (Jiang *et al.*, 1999). 750 mL of seawater was used to dissolve the components. After correcting the pH, distilled water was used to get the final volume to 1 L. The culture medium was centrifuged at 5000 rpm for 20 min to remove bacterial cells after being incubated at 37 °C for 3 days. 5% trichloroacetic acid was added, let to sit at 4 °C for the night, and then centrifuged one more at 5000 rpm. With the help of

10 M NaOH solution, the pH of the clear solution was brought down to 7.0 before being dialyzed three times against distilled water. Absolute ethanol was used to dilute the supernatant to four volumes before leaving it at 4 °C overnight. Precipitated polysaccharides were separated by centrifugation at 5000 rpm, twice washed with acetone, dehydrated with ether, and then dried under vacuum at 40 °C (Shene *et al.*, 2008).

2.2.4. Characterization of the most potent bacteria

2.2.4.1. Molecular identification

Total DNA content was extracted from an overnight pure culture of the most bioactive marine bacterial isolate using path-gene-spin DNA/RNA extraction kit provided by the Egyptian Center for Identification of Microbes at the Institute of Genetic Engineering and Biotechnology research institute, Sadat City, Egypt, was used the procedure was identical to that recommended by the manual instructions (Sambrook *et al.*,

1989). PCR was performed using two universal primers namely 27F (5'-AGAGTTTGATCC TGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The purified PCR products (amplicons) were reconfirmed using a size nucleotide marker (100 base pairs) by electrophoreses on 1% agarose gel. Purified amplicons were sequenced in the sense and antisense directions using 27F and 1492R primers with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture (White *et al*., 1990). Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done using MegAlign (DNA Star) software version 5.05 and compared to the database presented at GenBank.

2.2.4.2. Morphological characterization

Morphological characterization of the most potent bacteria was done by gram stain and the colony's shape and color.

2.2.5. Partial chemical characterization of the extracted exopolysaccharide from marine bacterial strain *Bacillus licheniformis.*

2.2.5.1. Infrared spectra (IR)

At Nawah Scientific Inc., (Mokatam, Cairo, Egypt), both native and hydrolyzed samples underwent infrared spectral analysis using a Perkin Elmer spectrum-I Pc spectrometer, with spectra acquired in the wave numbers (cm−1) 400 to 4000 cm−1. Using a peak find-memory-27 spectrophotometer, the molecular structure of the exopolysaccharide was partially identified (Marcotte *et al*., 2007).

2.2.5.2 . Nuclear magnetic resonance spectra (NMR)

At Nawah Scientific Inc., (Mokatam, Cairo, Egypt), using a Varian Inova 400 spectrometer set at 9.4 T, solid-state 13C NMR spectra were collected. Using a Variable Amplitude Cross Polarization Magic Angle Sample Spinning sequence, the solid-state spectra were obtained (VACP-MAS). The 1H /2 pulse had a pulse width of 4 ms, a contact time of 1 ms, an acquisition time of 13.8 ms, a recycle time of 3 s, a decoupling bandwidth of 60 KHz, and a spectral width of 40 KHz. 200 mg worth of samples were placed within a 5 mm zirconia rotor and spun at a magical angle of 9 KHz. Chemical shifts are expressed in ppm, and an exponential function was used to filter the data (line broadening factor of 20 Hz) (Gadian *et al*., 1995).

2.2.5.3. Scanning electron microscopy (SEM)

At Nawah Scientific Inc., (Mokatam, Cairo, Egypt), the sample was scanned with an electron beam to produce a two-dimensional magnified image for analysis, including external morphology (texture) (Cheng *et al.*, 2013).

2.2.6. Applications of bioactive exopolysaccharides from *Bacillus licheniforms*

2.2.6.1. Antibacterial activity of the extracted polysaccharide by disc diffusion method

At the National Institute of Oceanography and Fisheries, Alexandria, Egypt. antibacterial activity was done. On top of a plate containing nutrient broth media, one disc from each of the fish pathogenic bacterial strains (*Pseudomonas fluorescence-Streptococcus glacialis*) was individually placed. All plates were kept at 28 °C incubation until bacterial growth completely covered the control. The inhibitory zone diameter was measured three times, and the findings were represented in millimeters (Jeganathan *et al*., 2013).

2.2.6.2. Anticoagulant activity of the extracted polysaccharide

At the National Institute of Oceanography and Fisheries, Alexandria, Egypt, anticoagulant activity was performed. Activated partial thromboplastin time (APPT) and prothrombin time (PT) assays were used to assess the anticoagulant properties of EPS samples. Several EPS concentrations (0.05–2 mg/mL) were combined with control plasma samples, and the mixture was then incubated at 37 °C for 60 s. The mixture was incubated at 37 °C for 2 minutes with the preheated aPTT test reagent. Lastly, preheated (Imran *et al*., 2015.

2.2.6.3. Hemolysis activity of the extracted polysaccharide

At the National Institute of Oceanography and Fisheries, Alexandria, Egypt, hemolysis activity was performed. Exopolysaccharide sample of concentrations 100 ml and 200 ml, with replicates by using human blood agar plates with a 5% (v/v) concentration, then autoclaved at 37 °C for 48 h. An obvious halo encircling the colonies indicated hemolytic activity. (Henkelman *et al*., 2009).

3. RESULTS AND DISCUSSION

3.1. Isolation of marine bacteria from seas and extraction of exopolysaccharide

A preliminary analysis for exopolysaccharides from different marine bacterial strains isolated from the eastern harbor of Alexandria, Egypt were grown on a nutrient broth medium and then screened to select the most potent marine bacterial isolate acting bioactivity by adding ethanol, then centrifugation from the most promising strains. Then the production of exopolysaccharides is dried in the oven then weighted to detect the high production of exopolysaccharides and the most promising strain was *Bacillus licheniformis SMRH1* encoded MT3 as the same do Xu *et al*., (2019) by the extraction of polysaccharides from *Bacillus licheniformis* which produce a novel class of water-soluble exopolysaccharides (EPS). First, the ideal circumstances for EPS extraction were found using response surface methodology (RSM), which is based on a three-level, three-factor model. The greatest yield of EPS was 3.07 g/mL, and RSM analysis showed that the ideal conditions were at 8 °C for 10.44 hours with ethanol at a concentration of 79.22% (v/v).

3.2. Morphological characterization

We noticed morphological characteristics in the isolates produced on Nutrient agar media. The strain MT3 is a gram-positive and bacilli in form. The strain is a mucous, slimy colony, transparent in color (Fig.1**).** As the same A Gram-positive spore-forming bacterial species with significant biotechnological interest, *Bacillus licheniformis* is used to produce bioactive chemicals that are used in a variety of industries, including aquaculture, agriculture, food, biomedicine, and pharmaceuticals (Xu, Z *et al* ., 2019).

3.4. Partial chemical Characterization of exopolysaccharide produced from *Bacillus Licheniformis*

Exopolysaccharide from *Bacillus licheniformis* is characterized by Fourier Transform Infra-Red (FT-IR) and nuclear magnetic resonance NMR and scanning electron microscopy SEM. As the same marine species' microbial polysaccharides are essential to the food and cosmetic industries was isolated and structurally analyzed. The presence of several functional groups and primary aromatic compounds were detected using FTIR and 1H-NMR. (Abinaya *et al.*, 2018).

3.4.1. FT-IR

The IR spectrum of the extracted exopolysaccharide from *Bacillus licheniformis* showed that it contains νOH at 3420cm^{-1}, νC-H aliphatic at 2952cm^{-1}, νC=O in the range 1660-1630cm^{-1}.

3.4.2. NMR

^{1}H NMR spectrum (DMSO-*d6*) of extracted exopolysaccharide from *Bacillus licheniformis* showed signals δ'^{S} ppm at 1.22 (s, -CH_3), 2.07 (s, -$CH_{2\ chain}$) and 2.35(m, -CH_2).

3.4.3. Scanning electron microscope (SEM)

Scanning electron microscope of exopolysaccharide from *Bacillus licheniformis* show amorphous shaped uneven texture polysaccharide at higher magnification of ×5000. As the same Ibrahim *et al.,* (2022) statedThe semi-pure elvan's SEM micrographs showed that it had an amorphous, texture.

3.5. Applications of bioactivity of *Bacillus licheniformis*

3.5.1. Antibacterial activity

Exopolysaccharide from *Bacillus licheniformis* exhibited a wide spectrum of antibacterial activity against two fish pathogenic bacteria (*Pseudomonas fluorescence-Streptococcus glacialis)* has an antibacterial effect by forming free hollow zone around it Fig.2. As the same *B. licheniformis* MKU3 strain and has a low-molecular-weight bacteriocin-like protein that has a broad range of antibacterial action against numerous Gram-positive bacteria, several fungi, and yeast. The culture medium was improved using a fractional factorial design, leading to a 3–6-fold increase in the production of bacteriocin (Kayalvizhi *et al.,* 2008).

3.5.2. Anticoagulant activity

Bacillus licheniformis has anticoagulant activity by measuring the Partial thromboplastin time -PTT and Prothrombin - TimePT as the following

As the same Jouault *et al*., (2001) reported that the low-molecular-weight exopolysaccharide fractions have anticoagulant properties. When the fractions obtained from sulfation and depolymerization were compared to heparins, oversulfated fractions were shown to have anticoagulant activity, but native exopolysaccharide did not. Only the contact-activated assay showed a protracted lag phase in which the free radical depolymerized fraction hindered the production of thrombin in both contact-activated and thromboplastin-activated plasma. According to studies using affinity co-electrophoresis, only a small subpopulation of polysaccharide chains interacts strongly with heparin cofactor II, while the entire population of polysaccharide chains binds to antithrombin.

3.5.3. Hemolysis activity

β Hemolysis with free zones is showed to be an activity **of *Bacillus licheniformis* Fig.3** after using two concentrations of exopolysaccharide 100 ml and 200 ml, as the same *B. licheniformis* strains have various biotechnological uses, such as flocculation, biomineralization, biofuel generation, bioremediation, and food additive due to their lack of toxicity and anti-biofilm action (Muras *et al*., 2021).

As the same, Siavoshi *et al*., (2021) reported that after autoclaving, bacterial EPS was still hemolytically active. Heat-stable *Weissella confusa* and its hemolytic EPS were not adversely affected by boiling contaminated meat. Heat stress was reduced for *Weissella confusa* by thermostable hemolytic EPS.

3.6. polysaccharide from *Bacillus licheniformis* production at different cultural media

Using different culture media such as nutrient broth, peptone water, and king's B broth to detect the high yield production of exopolysaccharide from *Bacillus licheniformis*, the best culture media was nutrient broth media 0.7 g/l. for use in further optimization studies.

4. CONCLUSION

The current study has observed that the marine bacteria Bacillus *licheniformis* is a promising source of bioactive Compounds like exopolysaccharides which have various biotechnological like antibacterial, anticoagulant, and hemolysis activities which are very important in pharmaceutical and industrial fields.

5. ACKNOWLEDGMENT

I would like to thank Allah, for letting me through all the difficulties, experienced his guidance day by day. he is the one who let me finish my degree. I will keep on trusting Allah.

6. REFERENCES

Abinaya, M., Vaseeharan, B., Divya, M., Vijayakumar, S., Govindarajan, M., Alharbi, N. S., ... & Benelli, G. (2018). Structural characterization of *Bacillus licheniformis* Dahb1 exopolysaccharide—antimicrobial potential and larvicidal activity on malaria and Zika virus mosquito vectors. *Environmental Science and Pollution Research*, *25*, 18604-18619.

Ameen, F., AlNadhari, S., & Al-Homaidan, A. A. (2021). Marine microorganisms as an untapped source of bioactive compounds. Saudi Journal of Biological Sciences, 28(1), 224.

Apha, A. (1995). WPCF, Standard methods for the examination of water and wastewater. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Atlas, R.M. (1997). Hand book of Media For Environmental Microbiology.pp. 265,412.boca Raton, FL: CR Press.

Austin, B. (1988). The Marine Environment, In: Marine Microbiology,pp.1-11).Cambridge: Cambridge University Press.

Cheng, H., Feng, S., Jia, X., Li, Q., Zhou, Y., & Ding, C. (2013). Structural characterization and antioxidant activities of polysaccharides extracted from Epimedium acuminatum. Carbohydrate polymers, 92(1), 63-68.

Gadian, D. G. (1995). NMR and its applications to living systems (Vol. 7). Oxford: Oxford University Press.

Ghosh, S., Sarkar, T., Pati, S., Kari, Z. A., Edinur, H. A., & Chakraborty, R. (2022). Novel bioactive compounds from marine sources as a tool for functional food development. *Front Mar Sci*, *9*(832957), 10-3389.

Henkelman, S., Rakhorst, G., Blanton, J., & van Oeveren, W. (2009). Standardization of incubation conditions for hemolysis testing of biomaterials. Materials Science and Engineering: C, 29(5), 1650-1654.

Ibrahim, M. I., Amer, M. S., Ibrahim, H. A., & Zaghloul, E. H. (2022). Considerable Production of Ulvan from Ulva lactuca with Special Emphasis on Its Antimicrobial and Anti-fouling Properties. Applied Biochemistry and Biotechnology, 1-22.

Imran, M., Shafi, H., Wattoo, S. A., Chaudhary, M. T., & Usman, H. F. (2015). Analytical methods for determination of anticoagulant rodenticides in biological samples.

Forensic science international, 253, 94-102.

Jeganathan, P., Rajasekaran, K. M., Devi, N. A., & Karuppusamy, S. (2013). Antimicrobial activity and Characterization of Marine bacteria. Indian Journal of Pharmaceutical and Biological Research, 1(4), 38-44.

Jiang ZD, Jensen PR, Fenical W (1999) Lobophorins A and B, new antiinflammatory macrolides produced by a tropical marine bacterium. Biog Med Chem Lett 9(14):2003–2006

Jouault, S. C., Chevolot, L., Helley, D., Ratiskol, J., Bros, A., Sinquin, C., ... & Fischer, A. M. (2001). Characterization, chemical modifications and in vitro anticoagulant properties of an exopolysaccharide produced by Alteromonas infernus. Biochimica et Biophysica Acta (BBA)-General Subjects, 1528(2-3), 141-151.

Kayalvizhi, N., & Gunasekaran, P. (2008). Production and characterization of a low-molecular weight bacteriocin from *Bacillus licheniformis MKU3*. Letters in applied microbiology, 47(6), 600-607.

Marcotte, L., Kegelaer, G., Sandt, C., Barbeau, J., & Lafleur, M. (2007). An alternative infrared spectroscopy assay for the quantification of polysaccharides in bacterial samples. Analytical biochemistry, 361(1), 7-14.

Muras, A., Romero, M., Mayer, C., & Otero, A. (2021). Biotechnological applications of Bacillus licheniformis. Critical Reviews in Biotechnology, 41(4), 609-627.

Murray, P.R., Baron, E.J., Jorgensen, J.H., Pfaller, M.A. and Yolken, R.H .(Eds.) (2003).

Núñez-Pons, L., & Avila, C. (2015). Natural products mediating ecological interactions in Antarctic benthic communities: a mini-review of the known molecules. *Natural Product Reports*, *32*(7), 1114-1130.

Rocha, J., Peixe, L., Gomes, N., & Calado, R. (2011). Cnidarians as a source of new marine bioactive compounds—An overview of the last decade and future steps for bioprospecting. Marine drugs, 9(10), 1860-1886.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: a laboratory manual, 2^nd ed. Cold spring Harbor, NY: cold spring Harbor laboratory. 5:51-56.

Shene C, Canquil N, Bravo S, Rubilar M (2008) Production of the exopolysacchzrides by *Streptococcus thermophilus*: effect of growth conditions on fermentation kinetics and intrinsic viscosity. Inter J of Food Microbiol 124(3):279–284

Shukla, P. J., Vhora, S. B., Murnal, A. G., Yagnik, U. B., & Patadiya, M. (2023). Exopolysaccharide Production from Marine Bacteria and Its Applications. In *Marine Biochemistry* (pp. 337-368). CRC Press.

Siavoshi, F., Ebrahimi, H., & Sarrafnejad, A. (2021). Weissella confusa with thermostable β-hemolytic exopolysaccharide. Toxicon, 202, 67-74.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A guide to Methods and Applications (ed. M. A. Innis, D. H. Gelfand, J. J. Sninsky & T.J. White), pp. 315-322. Academic Press: San Diego, U.S.A.

Xu, Z., Chen, G., Xue, L., Zhang, H., Wang, J., Xiang, H., ... & Zheng, K. (2019). Isolation, structural characterizations and bioactivities of exopolysaccharides produced by *Bacillus licheniformis*. International journal of biological macromolecules, 141, 298-306.

Yadav, A. N. (2021). Microbial biotechnology for bio-prospecting of microbial bioactive compounds and

secondary metabolites. Journal of Applied Biology & Biotechnology, 9(2), 1-6

Yadav, A. N., Kour, D., Rana, K. L., Yadav, N., Singh, B., Chauhan, V. S., ... & Gupta, V. K. (2019). Metabolic engineering to synthetic biology of secondary metabolites production. In *New and future developments in microbial biotechnology and bioengineering* (pp. 279-320). Elsevier.

Zobell, C.E. (1946). Marine Microbiology. Waltham, MA: Chronica Botanica. 240 pp.

Table 1: Antibacterial activity of exopolysaccharide produced from *Bacillus licheniformis* against fish pathogenic bacterial strains, measuring diameter of inhibition zone in mm.

Strain number	Test organism	Size of inhibition zone (mm)			
1	pseudomonas fluorescence	14	15	16	18
2	Streptococcus glacialis	19	20	22	16

Table 2: show the anticoagulant activity of exopolysaccharide produced from *Bacillus licheniformis* according to Partial thromboplastin time -PTT and Prothrombin – TimePT

Sample / Test	Control	Exopolysaccharide from *Bacillus licheniforms*
PTT	**53 second**	**132 second**
PT		
1-T	**15 second**	**23.5 second**
2-A	**84%**	**42%**
3-INR	**1.16**	**2.11**

SHORT COMMUNICATION

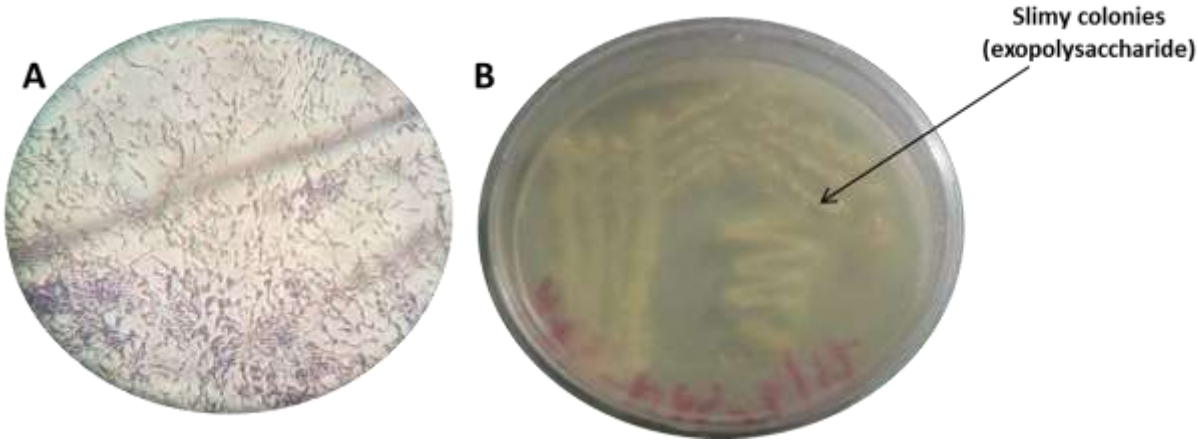

Fig.1 show (A) is a gram-positive *Bacillus licheniformis*, (B) show the slimy colonies which excrete exopolysaccharide around the colonies.

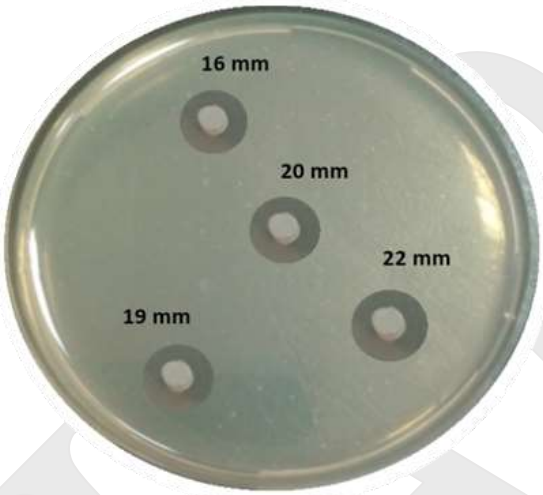

Fig.2 shows the antibacterial effect of exopolysaccharide produced from *Bacillus licheniformis* against fish pathogenic bacteria *Streptococcus glacialis* by forming zone around it and its size.

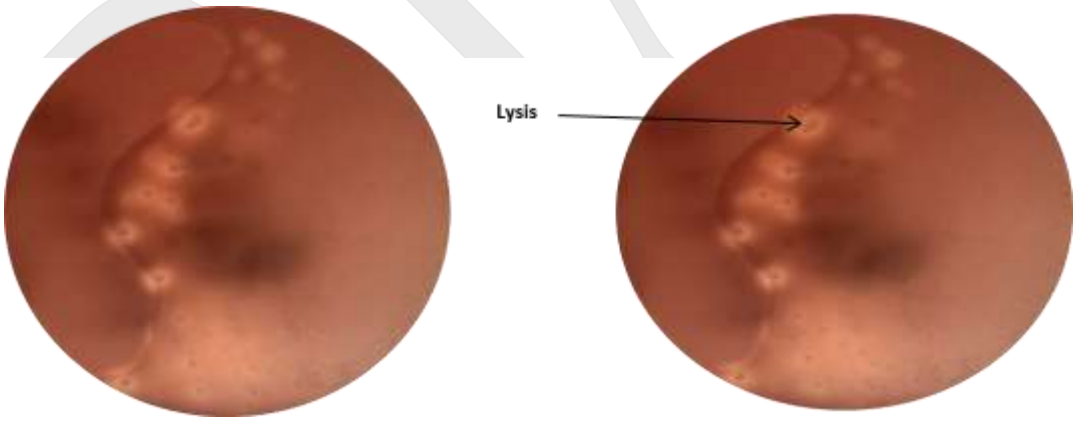

Fig.3. show β Hemolysis with free zones of the extracted polysaccharide from *Bacillus licheniformis*