Production and characterization of Exopolysaccharide from *Bacillus* sp.

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I hereby declare that the data presented in this Dissertation report entitled, "**Production and Characterization of Exopolysaccharide from *Bacillus* sp.**" is based on the results of investigations carried out by me in the Discipline of Marine Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of **Prof. Sanjeev C. Ghadi** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Production and Characterization of Exopolysaccharide from *Bacillus* sp.**" is a bonafide work carried out by **Mr. Anurag Kumar** under my supervision in partial fulfilment of the requirements for the award of the degree of Masters of Science in the Discipline of Marine Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

This dissertation represents an exploration of the production and characterization of exopolysaccharides (EPS) derived from a *Bacillus* sp. The study delves into various aspects of EPS extraction, quantification, and characterization, aiming to elucidate the properties and potential applications of EPS produced by "*Bacillus* sp. Strain LCR1". Through a combination of experimental approaches and analytical techniques, this research endeavor seeks to contribute to the understanding of bacterial EPS and its significance in biotechnological and environmental contexts.

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ABBREVIATIONS

°C	Degree Celsius
mg	Micro gram
g	Gram
ml	Milli litter
M	Molar
%	Percentage
H_2SO_4	Sulfuric acid
HCL	Hydrochloric acid
O.D	Optical density
ZMA	Zobell marine agar
ZMB	Zobell marine broth
DNSA	Di nitro salicylic acid
TLC	Thin layer chromatography
EPS	Exopolysaccharide
LAF	Laminar airflow
SVI	Sludge volume index
MLTS	Mixed liquor total solid
MLDS	Mixed liquor dissolved solids
ASW	Artificial seawater
PCR	Polymerase chain reaction
DNA	Deoxy ribonucleic acid
MLSS	Mixed liquor suspended solids
hours	Hours
bp	Base pair

ABSTRACT

This study investigates the production and characterization of exopolysaccharides (EPS) from the *Bacillus* sp. strain LCR1, focusing on its potential applications in environmental remediation. Bacterial identification reveals LCR1 as a Gram-positive rod proficient in polysaccharide degradation and carbohydrate utilization. Molecular identification through DNA isolation, PCR amplification, sequencing, and the NCBI Nucleotide Blast reveals LCR1 as a *Bacillus* species strain. This study suggests that the *Bacillus* sp. strain LCR1 shows maximal EPS production during the logarithmic bacterial growth phase. EPS extraction and quantification demonstrate a significant yield, particularly in the log phase. Characterization studies encompass total carbohydrate analysis by the Anthrone method, reducing sugar analysis by the DNSA method, uronic acid analysis by the carbazole method, and protein quantification by the Folin Lawry method, revealing elevated levels of carbohydrates and proteins during the log phase. Raman spectroscopy confirms the functional group composition, while solubility tests indicate the hydrophilic nature of the EPS. Purification methods result in decreased protein content, with hydrolysis showing increased concentrations of reducing sugars. The application of purified EPS in wastewater treatment reduces the sludge volume index, highlighting its potential for environmental remediation. Further evaluation demonstrates EPS adsorption capabilities for heavy metals. This study elucidates the versatility and promise of EPS from *Bacillus* sp. strain LCR1, offering insights into its biotechnological and environmental applications.

CHAPTER - I

1. INTRODUCTION

1. INTRODUCTION

1.1 BACKGROUND

Extracellular polymeric substances (EPS) represent a class of polymers synthesized and released by microorganisms, constituting a pivotal aspect of microbial physiology and exerting profound influences on various environmental processes (Lin et al., 2014). Initially, only Polysaccharide was thought to be the main component but further research has revealed that a wide range of elements make up the EPS (Extracellular Polymeric substances), such as uronic acids, humic acids, nucleic acids, lipids, proteins as well (Lin et al., 2014; Zhao et al., 2019; Guibaud et al., 2012).

The inception of EPS discovery dates back to the 1800s with the identification of dextran, a prominent exopolysaccharide, in *Leuconostoc mesenteriodes*, marking the initiation of extensive research into various other exopolysaccharides such as cellulose, alginate, and xanthan (Roberson et al., 1993).

The intrinsic material properties of EPS have garnered significant attention across diverse industrial and medicinal domains, owing to their multifunctional characteristics and potential applications (Nwodo et al., 2012). Within bacterial ecosystems, EPS plays crucial roles in fundamental biological processes, including coordination, adhesion, and protection, thus underscoring their indispensability across sectors like food and medicine (Tahir et al., 2020).

These complex extracellular polymers, characterized by large molecular weights, are produced as metabolic byproducts by microorganisms through the enzymatic hydrolysis or microbial lysis of organic substrates, contributing to the intricate network of biogeochemical cycling (Sun et al., 2020).

EPS possesses a distinctive three-dimensional structure reminiscent of a gel, comprising a highly hydrated matrix that effectively immobilizes microorganisms within it, thereby facilitating the formation of microbial aggregates and biofilms (Lin et al., 2014). This unique structural framework exerts significant influences on the physicochemical properties of microbial aggregates, impacting crucial processes like dewatering, settling, and flocculation, thus bearing implications for wastewater treatment and bioremediation applications (Jorand et al., 1995). Moreover, EPS serves as an indispensable defense mechanism for bacteria during desiccation events, by serving as reservoirs for essential nutrients and water, thereby ensuring their survival under adverse conditions (Roberson et al., 1993).

Within microbial aggregates, EPS function as mediators of intercellular interactions, employing diverse forces such as van der Waals, hydrogen bonding, and ion bridging to enhance the stability and cohesion of microbial aggregates, thereby modulating the resilience and adaptability of microbial communities to dynamic environmental conditions (Mikkelsen & Nielsen, 2001). However, the intricate matrix of EPS may also pose challenges to industrial processes, by impeding dewatering processes through the formation of impermeable barriers that restrict water flow through the pores in flocs, thus diminishing the efficacy of sludge treatment processes (More et al., 2014).

The vast marine biosphere harbors a plethora of microbial ecosystems, offering a treasure trove of unexplored polysaccharides with unique structural features and functional properties, thereby presenting promising avenues for innovative biotechnological applications (Nwodo et al., 2012). Notably, marine bacteria such as *Vibrio diabolicus* have been found to synthesize polysaccharides closely resembling hyaluronic acid, with potential commercial applications in skincare formulations under the brand name "Hyalurift" (De Morais et al., 2010).

The categorization of biopolymers into extracellular or intracellular types is determined by where they are located within cells. Since EPS is very diverse so it has very diverse applications in various industries, from biotechnology to materials science. This underscores the need for more research and exploration in this rapidly growing field (Cerning, 1995)

The exploration of marine biospheres holds immense promise for uncovering novel polysaccharides with unexplored functionalities and applications, thus underscoring the critical need for continued research efforts in this domain, aimed at harnessing the full potential of microbial resources for sustainable biotechnological innovations (Nwodo et al., 2012).

1.2 AIM AND OBJECTIVES

Aim: Production and characterization of EPS from *Bacillus* sp. strain LCR1.

Objectives:

1. Growth curve and production of Exopolysaccharide from *Bacillus* sp. strain LCR1.
2. Characterization of the Exopolysaccharide produced by the *Bacillus* sp. strain LCR1.
3. Application of the Exopolysaccharide produced by the *Bacillus* sp. strain LCR1.

1.3 RESEARCH HYPOTHESIS

It is hypothesized that the *Bacillus* sp. strain LCR1 possesses the ability to produce significant quantities of exopolysaccharides during its growth phase, particularly in the logarithmic phase. Furthermore, it is postulated that the EPS produced by *Bacillus* sp. strain LCR1 exhibits favorable characteristics for applications in wastewater treatment and heavy metal adsorption. Additionally, it is hypothesized that the *Bacillus* sp. strain LCR1 demonstrates distinct capabilities for polysaccharide degradation and carbohydrate utilization, reflecting its potential ecological niche and metabolic versatility.

1.4 SCOPE

The scope of this study encompasses the entire process of EPS production and characterization from the *Bacillus* sp. strain LCR1. Key components of the research include growth curve analysis, EPS extraction, quantification, and comprehensive characterization using biochemical assays and spectroscopic techniques. The investigation extends to the evaluation of EPS properties, including solubility, functional group composition, and physical characteristics. Furthermore, the study explores the efficacy of purified EPS in wastewater treatment and heavy metal adsorption, highlighting potential applications in environmental remediation. Molecular identification of *Bacillus* sp. strain LCR1 through DNA isolation, PCR amplification, sequencing, and 16S rRNA analysis further elucidates its taxonomic identity.

CHAPTER - II

2. LITERATURE REVIEW

2. LITERATURE REVIEW

The EPS matrix is made up of a wide range of biochemicals secreted by microorganisms, encompassing organic substances found in the surrounding medium and potentially cellular components or products released by cell lysis (More et al., 2014). The contents of EPS can be closely related to the phase of growth of bacteria, usually increasing with longer incubation times when the phase of development is exponential. Notably, though, EPS quantities may decrease as the incubation period extends toward the stationary phase (Liu et al., 2018).

Exopolysaccharide production usually involves a number of important stages. First, sugar nucleotides are organized into monosaccharide molecules on a lipid carrier. GTFs, or glycosyltransferases, are a class of enzymes that are essential for catalyzing the transfer of sugar molecules, and they aid in this assembly process. These united monosaccharide units then proceed through a series of consecutive polymerization steps that result in the production of polysaccharide chains. The biosynthesis process is ultimately completed when these polysaccharides are transferred outside of the cell (Van Kranenburg et al., 1999).

Enzymes linked to the cell wall of bacteria enhance extracellular production, which produces exopolysaccharides (EPS), which are polysaccharides secreted into the external environment. Because bacterial polysaccharides are so diverse, they can be categorized according to their molecular weight, functionality, linkage bonds, and chemical structure (Ruas-Madiedo et al., 2002) The monomeric makeup of EPS allows for chemical compositional differentiation. Based on that Homopolysaccharides and Heteropolysaccharides are the two primary groupings that

are understood. While repeated units of a single type of monosaccharide makeup homopolysaccharides, repeating units of different sizes, ranging from disaccharides to heptasaccharides, make up heteropolysaccharides. (Faber et al., 2001)

The four primary families of homopolysaccharides are fructans, polygalactans, b-D-glucans, and a-D-glucans. They show a wide range of characteristics. Conversely, heteropolysaccharides are repeating units made up of different monosaccharides such as L-rhamnose, D-galactose, glucuronic acid, N-acetylgalactosamine, and N-acetylglucosamine (Nwodo et al., 2012).

There are four pathways used by bacteria to produce EPSs, which are listed in Table No. 01.1:

Table No. 01.1: Different pathways used by bacteria to produce EPS

Pathways	Types of Polysaccharides	Enzymes evolve	Authors.
Synthase pathway	Homopolysaccharides	Synthase protein	(Rehm, 2010)
Sucrase pathway	Homopolysaccharides	Glycansucrase enzymes	(Rana & Upadhyay, 2020)
The ATP-Binding cassette (ABC)	Heteropolysaccharides	Glycosyltransferases	(Nwodo et al., 2012)
Wzx/Wzy pathway	Heteropolymer	WZx flippase	(Vorhölter et al., 2008)

Numerous factors, such as the stage of cell growth, the availability of nutrients, and exposure to environmental stressors, are important in the formation of extracellular polymeric substances (EPS) by bacteria. As mentioned above, EPS generation usually happens during the stationary

phase of development or near the conclusion of the exponential growth phase (Sengupta et al., 2018) in that the balance of carbon and nitrogen is one of the two main elements influencing EPS production; optimal production is generally found when there is a surplus of carbon and a deficiency of nitrogen (Antunes et al., 2017). The carbon source is used in the stationary phase to produce EPS, and a higher yield is usually obtained with increasing carbon availability. Simple sugars are broken down by bacterial enzymes more quickly than complex ones, making utilization easier and yield higher (Celik et al., 2008). A supply of nitrogen is necessary, but too much nitrogen might encourage cell growth at the price of EPS yield, as seen in *Enterobacter A47*'s production of FucoPol (Baptista et al., 2022). Additional necessary components for bacterial growth and EPS generation include magnesium, potassium, phosphorus, and different metal cations (Antunes et al., 2017).

The generation of EPS molecules is significantly influenced by the pH level of the culture media, which also affects the morphological properties of the cells. In addition to impeding microbial development, extreme pH conditions in the medium, such as pH 2.0–3.0 or pH levels equivalent to or higher than 10, also limit the production of extracellular polymers (Valepyn et al., 2012).

The temperature showed a remarkable effect during the production of exogenous proteins and exopolysaccharides by microorganisms. The bulk of EPS molecules were found to be produced best at temperatures between 26 and 31°C during cultivation; however, species-to-species differences in temperature and pH requirements may also be present (Çam & Bicek, 2023). Some of them are in Table No. 01.2:

Table No. 01.2: A Study on EPS Yield reported in different journals.

Bacterial species.	EPS production (g/L)	Carbon and Nitrogen Sources provided	pH	Temperature	Authors
Lactobacillus sp. KPB-167B.	2.04	10% lactose (Carbon source) 4% Yeast Extract (Nitrogen source)	5	30ºC	(Yokoi & Watanabe, 1992)
B.lichaniformis MS3.	2.9 – 4.8	Mango peel (5 – 10 g/L) Yeast Extract (0.3 – 0.8 g/L)	7	30 ºC	(Asgher et al., 2020)
Bacillus strains	4.6 – 7.2	Glucose (25 g/L) Yeast extract (0.01 g/L)	7	28 ºC	(Bala Subramanian et al., 2010)
Lactobacillus helveticus ATCC 15807	4.5 – 5.5 (mg/L)	Glucose (10 g/L) And Vitamins	6.5	37 ºC	(Torino et al., 2005)
Providencia sp.	3.92	Carbon source (30 g/L) Yeast Extract (4 g/L)	7	37 ºC	(Kopperi et al., 2021)
Lactobacillus spp.	0.71 – 2.38	Maltose and yeast extract	7	37 ºC	(Fuso et al., 2023)
Enterobacter Sp.	8.6	15% Sucrose 0.5% peptone	7.5 - 8	37 ºC	(Almutairi & Helal, 2021)
E. meliloti strains	4.12	Soybean molasses	7	30 ºC	(Oliveira et al., 2020)

Purification and impurity removal are the last steps in the bacterial EPS (extracellular polymeric substances) manufacturing process. Bacterial cells, proteins, minerals, other polymers, and contaminants—all of which are usually undesirable in the finished product—are included in the ultimate broth. The first stage usually involves filtering or centrifuging the bacterial cells out

of the solution (Antunes et al., 2017). The EPS can be separated from the supernatant after the bacterial cells have separated. Adding water-miscible polar solvents, such as acetone, isopropanol, or ethanol, is usually how this is accomplished (Antunes et al., 2017). However, precipitation is not unique to the polysaccharide that is being targeted, which can cause other polysaccharides and proteins to precipitate together. Additionally, it may lead to lower yields (Ziadi et al., 2018). In addition to centrifugation, chemical methods can also be used to remove proteins. Some techniques, such as the trichlorotrifluorethane and trichloroacetic procedures, cause protein precipitation, while others, like the Sevag method, use chloroform to denature proteins. (Chambi et al., 2021). In addition to removing contaminants, mixed polysaccharides can be purified into several homogenous polysaccharides using HPLC (Asgher et al., 2020), Metallic coordination compounds can be formed by introducing different metal ions like copper, barium, calcium, or lead to precipitate alongside the polysaccharide. Subsequently, the complex can be decomposed using an acid (Shi, 2016). Functional group characterization techniques like FT-IR and Raman spectroscopy further contribute to understanding EPS composition and structure (Asgher et al., 2020; Carter, 2011)

Many functional groups, such as carboxyl, phosphate, amine, sulfhydryl, phenol, and hydroxyl groups, are present in EPS. These functional groups are mostly negatively charged at neutral pH and can form organometallic complexes with metal ions, however, since carboxyl and hydroxyl functional groups are so common. The precise ratios of these groupings change based on the EPS's makeup (Ding et al., 2018). As such, EPS is useful as biosorbents in the removal and recovery of heavy metals from certain streams of industrial effluent (Sheng et al., 2010). When it comes to Microbial EPS, exhibits the capacity to sorb and bind different soluble and insoluble metal species within soils. Additionally, EPS interacts with oxides, colloids, and clay

minerals—all of which can bind metals—to help contaminated ecosystems recover (Gadd, 2010).

The interactions between metals and EPS depend on metal sorption mechanisms including chelation, ion exchange, proton exchange, coordination, and precipitation (Guibaud et al., 2012).

The binding sites or their chemical properties are the main variables influencing metal biosorption by EPS. These consist of the molecular weight and degree of branching of the EPS in addition to pH, metal content, ionic strength, and surface characteristics (Fukushi, 2012). A study has been done on *Paenibacillus jamilae* - derived EPS has remarkable adsorption capacity for a range of heavy metals, such as Pb, Cd, Co, Ni, Zn, and Cu. Pb exhibits a particularly robust interaction among these metals, with a maximal binding capacity of 303.03 mg/g (Morillo Pérez et al., 2008). In contrast, Cu (II) is firmly bound to proteins and Humic compounds found in EPS. After more investigation, it was shown that Cu(II) ions primarily attach to oxygen atoms in the carboxyl groups of EPS. (Sheng et al., 2010)

The production of EPS by microorganisms plays a crucial role in the treatment of wastewater, especially in terms of its ability to lower the Sludge Volume Index (SVI) (Bala Subramanian et al., 2010). It promotes the process known as bio flocculation, in which organic matter and suspended particles aggregate into denser, bigger flocs through the action of EPS as a bonding agent (Nouha et al., 2018). Because of EPS's adhesive qualities, these flocs settle in sedimentation tanks more effectively, improving solid-liquid separation. As a result, there is a drop in the Sludge Volume Index (Wilén et al., 2003), which suggests improved settling

efficiency. This emphasizes how important bacterial EPS is for treating wastewater since it promotes sludge settling, helps with bioflocculation, and helps with treatment optimization in general.

As indicated by Meng et al., 2010, ribose, mannose, glucose, and Uronic Acids were discovered to be the main monomeric components that made up the polysaccharides in the EPSs separated from sludge flocs but Apart from carbohydrates, structural proteins, enzymes, and extracellular DNA (exDNA) in EPS have garnered increasing attention due to diverse nature of EPS as well as ExDNA from the EPS of different strains has been analyzed in studies, and the results shows that DNA is involved in more processes than just passive agglomeration. It is a major structural component and facilitates genetic exchange, signaling, and adhesion (Flemming & Wingender, 2010).

CHAPTER –III

3.METHODOLOGY

3. METHODOLOGY

3.1 Bacterial Strain Revival:

The *Bacillus* sp. strain LCR1 of bacteria used in this study was revived from our research group's culture collection.

The *Bacillus* sp. strain LCR1 was inoculated on Zobell Marine Agar (ZMA) from glycerol stocks stored at -80ºC. The inoculated plates were incubated at 30ºC for 24 hours. The bacterial culture was maintained by regular subculturing on the ZMA medium.

3.2 Gram Characterization:

Gram staining was carried out using a kit “HiMedia K001-1KT” (HiMedia Pvt. Ltd). provided, adhering to the manufacturer's standard technique. The process included making bacterial streaks on glass slides and then fixing them with heat. Following the manufacturer's directions, the slides were successively inundated with solutions of crystal violet, gram’s iodine, gram’s decolorizer, and safranin. The slides were cleaned with distilled water following each stage. After staining, the slides were allowed to air dry and then inspected at 1000x magnification using a light microscope.

3.3 Growth Curve of *Bacillus* sp. strain LCR1:

For the growth curve analysis, bacterial inoculation and incubation followed a modified protocol (Kacena et al., 1999), a loopful of *Bacillus* sp. Strain LCR1 bacterial cells was

inoculated into Zobell Marine Broth (ZMB) and uninoculated st. ZMB medium was kept as blank. All flasks were placed in a shaker incubator (Orbital incubator shaker, Remi Elektrotechnik Limited), set at a constant temperature of 30°C and 120 rpm (rotations per minute). 1 ml of the sample was pipetted out from each flask into a sterile centrifuge tube in a laminar hood after every few hours, till 93 hours and Optical density (OD) measurements were conducted at a wavelength of 600 nanometers (nm) using a UV-VIS spectrophotometer (UV mini-1240, Shimadzu), with baseline correction achieved by referencing readings from the control flask (blank).

3.5 Carbohydrate Utilization Tests:

Using the HiCarbo™ Kit from HiMedia Pvt. Ltd, which consists of Parts A, B, and C, the test for carbohydrate utilization was conducted. By following the manufacturer's standard methodology First, 50 µl of 24 hours grown bacterial culture were inoculated into the wells of the kit covering a variety of carbohydrates After the incubation period of 24 hours, color changes were noted. The KB009 kit's guidance was used to interpret the results.

3.6 Polysaccharide degradation:

This methodology employed a dye-based plate assay described by "Poduval (2019)". In brief, the *Bacillus* sp. strain LCR1 was grown for 48 hours on the plates supplemented with only Agar, only Carrageenan, and only Alginate.

1. Agar Plate Preparation:

The agar plates were prepared by supplementing Artificial Sea Water (ASW) media with 2% agar.

2. Carrageenan Plate Preparation:

Carrageenan plates were prepared by dissolving 2X of 1% carrageenan in 2X Artificial Sea Water (ASW), which was autoclaved separately. Subsequently, 2X of 1% Gelrite dissolved in distilled water was autoclaved separately. The carrageenan solution was then mixed with the Gelrite solution to prepare the carrageenan-supplemented plates.

3. Alginate Plate Preparation:

For alginate plates, 2X of 1% alginate was dissolved in 2X Artificial Sea Water (ASW) and autoclaved separately. Similarly, 2X of 1% Gelrite dissolved in distilled water was autoclaved separately. The alginate solution was then mixed with the Gelrite solution to prepare the alginate-supplemented plates.

For the detection of the zone of clearance, the Agar, carrageenan, and alginate plats were flooded with Lugol's iodine (Appendix 2), 0.1% Phenol red, and 10% Sulfuric acid respectively.

3.7 16S rRNA Sequencing:

A. DNA isolation:

Using the HiPurA® Bacterial Genomic DNA Purification Kit from HiMedia Pvt. Ltd. and the manufacturer's recommended technique, the bacterial strain's DNA was isolated. First,

overnight-grown bacterial cells were taken out and lysed to extract genomic DNA. Purified genomic DNA was obtained by treating the sample with the proper buffers and enzymes supplied in the kit, which eliminated RNA, proteins, and other impurities. Agarose gel electrophoresis was used after isolation to verify the presence of the DNA.

B. PCR:

PCR was conducted using primers '27F' (AGATTTGATCCTGGCTCAG) and '1492R' (TACGGTTACCTTGTTACGACTT) in a thermal cycler (SureCycler 8800), following specified parameters (Table No. 02.1) and PCR reaction conditions (Table No. 02.2). Following PCR completion, the PCR bands were confirmed by performing gel electrophoresis with agarose gel and a DNA Marker. After that sample was sent to "Eurofins Scientific Pvt. Ltd." for sequencing.

Table No. 02.1: PCR parameters

Steps	**Temperature**	**Time**	**Total number of cycles**
Initial denaturation	95°C	2 minutes	1 Cycle
Denaturation	95°C	30 seconds	30 cycle
Annealing	51.9°C	30seconds	
Elongation	72°C	1 minute and 33 seconds	
Final Elongation	72°C	8 minutes	1 cycles

Table No. 02.2: PCR reaction

Ingredients	Volume (in µl)
1. Nuclease-free water	40
2. Taq Buffer	5
3. dNTP mix	1
4. Templet DNA(50µg)	1
5. Forward primer	1
6. Reverse primer	1
7. Taq polymerase	1
Total volume	50

C. 16S rRNA Taxonomic analysis

The taxonomic analysis of the *Bacillus* sp. strain LCR1 was done by doing Blast analysis of the 16S rRNA gene Sequence of *Bacillus* sp. strain LCR1 by using NCBI nucleotide blast tool.

3.8 Exopolysaccharide Production:

3.8.1 Cultivation of Bacteria for EPS Production:

Bacterial cultures were inoculated into three separate 100 ml Erlenmeyer flasks, each containing 50 ml of ZMB medium, pH -7. These flasks were then incubated at 30°C and 120 rpm in a shaker incubator (Orbital incubator shaker, Remi Elektrotechnik Limited). After incubation periods of 24 hours (late Lag phase), 48 hours (Log phase), and 72 hours (stationary phase), one flask at a time was removed from the incubator for EPS extraction

3.8.2 EPS Extraction:

The EPS extraction protocol outlined by “Bala Subramanian et al. (2010)” was adopted for this study. Initially, 10 ml of bacterial culture was sampled from each flask and subjected to centrifugation for 15min at 6000 × g, room temperature (RT), to separate the cell pellet. Subsequently, 2 volumes of ice-cold ethanol were added to the supernatant to induce precipitation of the extracellular polymeric substances (EPS). Following a 1-hour incubation period at -20°C, the mixture was centrifuged for 15min at 6000 × g, 4°C, to collect the precipitated EPS. The EPS precipitate was further subjected to lyophilization using a lyophilizer (Scanvac coolSafe) operating at -109°C, resulting in the formation of a powdered product.

3.8.3 Quantification of EPS Yield:

The lyophilized powder obtained from samples extracted at all three flasks was quantified using a precision weighing balance (Shimadzu ATX224).

3.9 Purification of EPS:

For the purification of EPS, the reprecipitation method described by “Pham et al. (2000)” was employed. Briefly, the precipitated EPS was initially dissolved in deionized water and thoroughly mixed. Subsequently, the solution was subjected to reprecipitation by adding two volumes of ethanol. This reprecipitation process was repeated twice to enhance purification efficiency and ensure the removal of impurities.

3.10 EPS Characterization:

3.10.1 Total Carbohydrate Analysis:

The total carbohydrate concentration in each sample was determined using the Anthrone method described by "Loewus (1952)". A working standard solution ranging from 20 to 200 µg/ml of glucose was prepared from a 0.2 mg/ml stock solution of glucose. Absorbance readings were taken at 620 nm using a UV-VIS spectrophotometer (UV mini-1240).

For sample preparation, 25 mg of lyophilized EPS was dissolved in 5 ml of distilled water and thoroughly mixed. Aliquots of the sample solution were then pipetted into cuvettes, and absorbance measurements were recorded at 620 nm. The obtained absorbance values were compared to the standard curve generated from glucose standard solutions to determine the total carbohydrate concentration in the EPS samples.

3.10.2 Reducing Sugar Analysis:

Reducing sugar concentration in each sample was determined utilizing the DNSA (3,5-dinitrosalicylic acid) method as described by "Jain et al. (2020)". A working standard solution ranging from 0.1 to 1 mg/ml of glucose was prepared from a 1 mg/ml stock solution of glucose. Absorbance readings were taken at 540 nm using a UV-VIS spectrophotometer (UV mini-1240).

For sample preparation, 25 mg of lyophilized EPS (Extracellular Polymeric Substance) was dissolved in 5 ml of distilled water and thoroughly mixed. Aliquots of the sample solution were then pipetted into cuvettes, and absorbance measurements were recorded at 540 nm. The obtained absorbance values were compared to the standard curve generated from glucose standard solutions to determine the reducing sugar concentration in the EPS samples.

3.10.3 Uronic Acid Concentration Analysis:

The determination of Uronic acid concentration in each sample followed the carbazole assay method, initially outlined by “Taylor & Buchanan-Smith (1992)”, with certain modifications. A working standard solution, ranging from 0.1 to 1 mg/ml of D-galacturonic acid, was prepared from a 1 mg/ml stock solution of D-galacturonic acid. Absorbance readings were collected at 525 nm utilizing a UV-VIS spectrophotometer (UV mini-1240).

For sample preparation, 25 mg of lyophilized EPS (Extracellular Polymeric Substance) was dissolved in 5 ml of distilled water and thoroughly homogenized. Subsequently, aliquots of the sample solution were pipetted into cuvettes, and absorbance measurements were conducted at 525 nm. The resulting absorbance values were plotted against the standard curve derived from D-galacturonic acid standard solutions to ascertain the uronic acid concentration in the EPS samples.

3.10.4 Protein Concentration Analysis:

The determination of protein concentration in each sample was conducted following “Folin's Lowry” method, initially outlined by “Waterborg (2009)”, with certain modifications. A working standard solution ranging from 0.1 to 1 mg/ml of Bovine Serum Albumin (BSA) was prepared from a 1 mg/ml stock solution of BSA. Absorbance readings were obtained at 620 nm using a UV-VIS spectrophotometer (UV mini-1240).

For sample preparation, 25 mg of lyophilized EPS (Extracellular Polymeric Substance) was dissolved in 5 ml of distilled water and thoroughly mixed. Subsequently, aliquots of the sample solution were pipetted into cuvettes, and absorbance measurements were taken at 525 nm. The

resulting absorbance values were then compared to the standard curve derived from BSA standard solutions to determine the protein concentration in the EPS samples.

3.10.5 Functional Group Identification by Raman Spectroscopy:

For the detection of functional group vibrations via Raman spectroscopy (using LabRAM Evolution Raman microscope), the lyophilized EPS were kept on a glass slide wrapped with aluminum foil and a 785 nm laser were used for the excitation. Peak seeker generates a 50 mW at the surface of the sample. A 40X objective lens were utilized to focus the laser at the sample. Upon receipt of the data, processing, and analysis were performed using Origin 8 software version 8E.

3.10.6 Solubility:

According to the protocol outlined by Asgher et al. (2020), the solubility evaluation of EPS included dissolving in a variety of solvents, including aqueous and several organic media (methanol, ethanol, isopropanol, chloroform, and acetone). Initially, 5 mg/mL lyophilized EPS samples were made and vortexed in all solvents, including deionized water, to dissolve them. To separate the undissolved EPS pellets, the solutions were centrifuged for 15 minutes at 10,000 rpm, RT. To quantify the separated, undissolved EPS pellets, they were then dried at 60°C in a hot air oven (i-therm AI-7981). Solubility percentages were calculated with the help of the following equation.

$$Solubility\ (\%) = \frac{(Initial\ weight\ of\ tube+EPS)-(\ Final\ weight\ of\ tube+EPS)}{(Initial\ weight\ of\ tube+EPS\ )-(Final\ weight\ of\ tube\ )} \times 1oo$$

3.11 EPS Hydrolysis:

3.11.1 Hydrolysis Procedure:

The EPS (purified) hydrolysis was carried out in accordance with the "Felz et al (2019)". procedure. In short, 1M HCl was used to hydrolyze a 10 g/L sample in an oven (i-therm AI 7921) at 105°C for 8 hours. The sample was centrifuged at 13,300× g for 5 minutes,RT after hydrolysis, and then it was neutralized with 1M NaOH. After neutralizing the solution, any particles or insoluble residues were eliminated by filtering it using a 0.22 µm syringe filter.

3.11.2 Analysis of Increase in Reducing Sugar Concentration:

Analysis of the increment in reducing sugar after hydrolysis was performed using the DNSA (3,5-dinitrosalicylic acid) method, as previously described. This experiment was conducted to determine the difference in concentration of reducing sugar before and after hydrolysis.

3.11.3 Thin Layer Chromatography (TLC) for Composition Analysis:

The hydrolyzed EPS underwent TLC (thin-layer chromatography) for saccharide determination, with modifications to the protocol described by Takács et al. (2011). The TLC chamber utilized was grease-free, and the solvent system consisted of propanol, water, and butanol in a ratio of 92:32:24. Standard solutions of glucose, maltose, and raffinose, each at a concentration of 1 mg/ml, were spotted alongside the hydrolyzed sample onto the TLC plate. The TLC plate was then developed using a developer solution composed of 0.2 g of resorcinol dissolved in 15% v/v sulfuric acid in absolute ethanol.

3.12 Application Studies:

3.12.1 Wastewater Treatment Experiment:

The extracted EPS was utilized for treating wastewater, assessed through Sludge Volume Index (SVI) determination, following the protocol outlined by "Bala Subramanian et al. (2010)", with modifications. Activated sludge was obtained from the wastewater treatment plant at Goa University. The wastewater was transferred into two 100 ml measuring cylinders, one serving as the control and the other treated with EPS at a concentration of 1 mg/ml and mixed with 10% v/v of EPS to the wastewater. Both cylinders were left undisturbed for 30 minutes to allow for sludge settlement, following which the sludge volume was recorded. Subsequently, both cylinders were thoroughly mixed, and the mixture was filtered through pre-weighed grade 1 filter paper. The filter paper was dried in a hot air oven at 105°C for 1 hour and then weighed to determine Mixed Liquor Total Solids (MLTS). Additionally, 25 ml of the filtrate was transferred into pre-weighed crucibles and kept in a hot air oven at 105°C overnight, followed by weighing to determine Mixed Liquor Dissolved Solids (MLDS). SVI was calculated using the implemented formula.

$$\text{SVI (mg/g)} = \frac{volume\ of\ settled\ sludge \times 1000}{Mixed\ Liquor\ suspended\ solid\ (MLSS)}$$

MLSS (mg/L) = Mixed liquor total solid (MLTS) – Mixed liquor dissolved solid (MLDS)

3.12.2 Heavy Metal Adsorption Experiment:

The extracted EPS was employed for adsorbing heavy metals by implementing the protocol given by "Salehizadeh et al., (2003)" with some modifications. In brief Cu^{2+} and $Pb^{2+,}$ utilizing a standard solution prepared by dissolving Cu $(NO3)_2$ and Pb $(NO3)_2$ at a concentration of 10 mg/L. Subsequently, 50 ml of each standard solution was transferred into separate 100 ml flasks, followed by the addition of 50 mg of EPS. The pH was adjusted to 6, and the mixture was incubated for 20 minutes. Next, 2 volumes of ethanol were added to precipitate the EPS, after which the samples were filtered through Whatman filter paper grade 1. Concurrently, controls were prepared with the same treatment, excluding the addition of EPS. Both samples and controls were then sent to the ITALAB (GOA) Pvt. Ltd. Margao, Goa, for metal concentration analysis using Atomic Absorption Spectroscopy.

CHAPTER – IV

4. ANALYSIS AND CONCLUSION

4.1 Colony morphology:

The *Bacillus* sp. strain LCR1 Streaked on ZMA plate exhibits the following morphology (Table No. 03.1)

Table No. 03.1: Colony morphology of the *Bacillus* sp. strain LCR1

Size	Color	Shape	Margin	Elevation	Opacity	Texture
5mm	Yellow - white	Circular	Undulate	Flat	Opaque	Mucoid

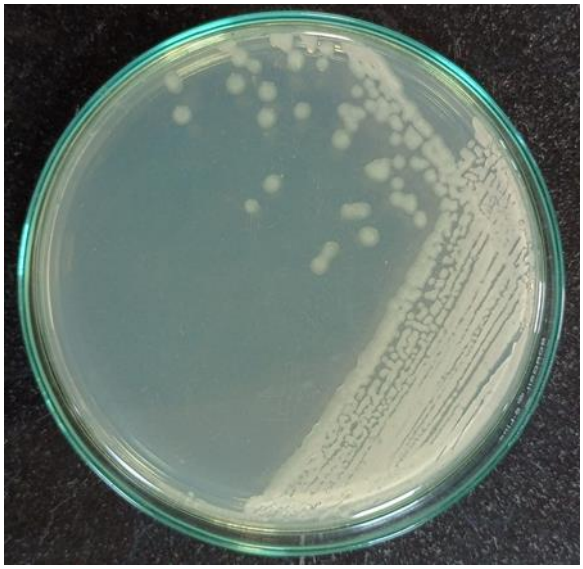


Figure No. 02: Isolated colonies of *Bacillus* sp. strain LCR1.

4.2 Gram staining:

The Gram stain technique was carried out and the result was found to be Gram-positive rods bacteria (Figure No. 4.2). Therefore, the isolate was selected for further process.

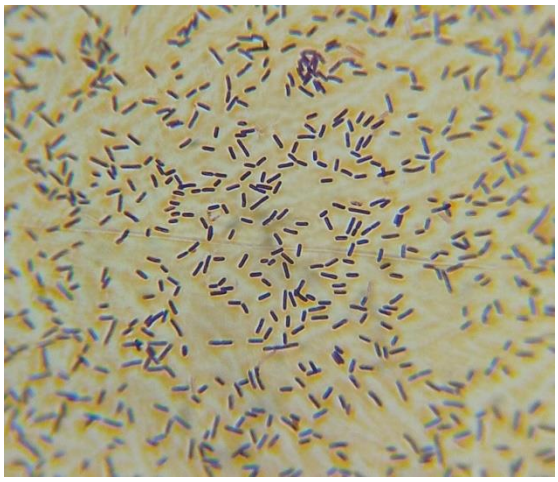


Figure No. 4.2: *Bacillus* sp. strain LCR1 at 1000X, magnification after Gram staining.

4.3 Growth curve:

The bacterial population's growth stages and kinetics were determined by analyzing the data, which included hours elapsed and associated OD values. Different phases of the growth curve were identified by the analysis: a lag phase (0-12 hours) indicating preparation for growth, a logarithmic phase (10-48 hours) marked by rapid proliferation, a stationary phase (48-72 hours) indicating a stable population size and a subsequent decline phase (after 72 hours) suggestive of population reduction. (Kacena et al., 1999) (Figure No. 4.3).

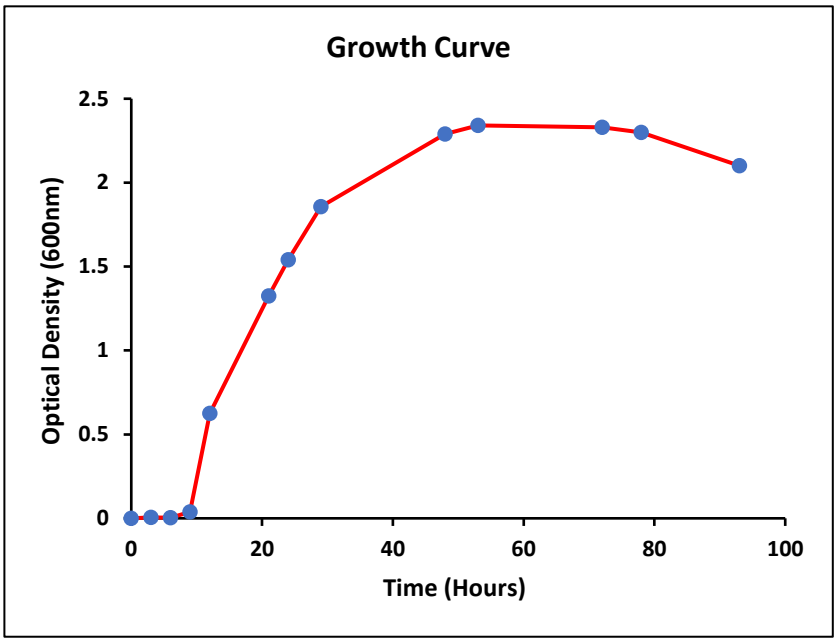


Figure No. 4.3: Growth pattern of the *Bacillus* sp. strain LCR1

4.5 Carbohydrate utilization test:

This test was carried out by using HiCarbo™ Kit and the carbohydrate utilization was confirmed by observing the wells for the color change (Figures No. 04 and 05). The *Bacillus* sp. strain LCR1 given a positive test for those carbohydrates indicated as '+' and the negative test indicated as '-' and the intermediate result indicated as '±' (Table No. 03.2).

Figure No. 4.4: HiCarbo™ Kit before inoculation of bacterial culture (A- part B1, B- Part C, C- Part A).

Figure No. 4.5: HiCarbo™ Kit after inoculation of bacterial culture showing color change (A- Part A, B- Part B1, C- Part C).

Table No. 03.2: Carbohydrate utilization by the *Bacillus* sp. strain LCR1.

S. No	**Test**	**Observation**
1	Lactose utilization	-
2	Xylose utilization	-
3	Maltose utilization	-/+
4	Fructose utilization	+
5	Dextrose utilization	+
6	Galactose utilization	-
7	Raffinose utilization	-
8	Trehalose utilization	+
9	Malibiose utilization	-
10	Sucrose utilization	+
11	L- Arabinose utilization	-/+

12	Mannose utilization	+
13	Inulin utilization	+
14	Sodium gluconate utilization	-/+
15	Glycerol utilization	-
16	Salicin utilization	+
17	Dulcitol utilization	-
18	Inositol utilization	-/+
19	Sorbitol utilization	-/+
20	Mannitol utilization	+
21	Adonitol utilization	-
22	Arabitol utilization	-
23	Erythritol utilization	-
24	Alpha – Methyl – D glucoside utilization	-
25	Rhamnose utilization	-
26	Cellobiose utilization	+
27	Melezitose utilization	-
28	Alpha – Methyl – D Mannoside utilization	-
29	Xylitol utilization	-
30	ONPG activity	-
31	Esculin hydrolysis	+
32	D – Arabinose utilization	-
33	Citrate utilization	-
34	Malonate utilization	-
35	Sorbose utilization	-
36	Control	-

4.6 Polysaccharide degradation:

Different polysaccharide supplements were added to the plates in the polysaccharide degradation test for *Bacillus* sp. strain LCR1, resulting in distinct observations and a definite zone of clearance was observe after the agar plate was flooded with Lugol's iodine solution, indicating the presence of *Bacillus* sp. strain LCR1's agarolytic activity (Figure No. 4.7 A). Similarly, a noticeable zone of clearing was seen on the carrageenan plate that was submerged in phenol red solution, indicating pH alterations brought on by the bacterial strain's breakdown of the carrageenan (Figure No. 4.7 B). However, no zone of clearance was seen on the plate with alginate that had been inundated with 10% sulfuric acid. This lack of clearance suggests that, given the circumstances of the experiment, *Bacillus* sp. strain LCR1 is not degrading alginate (Figure No. 4.7 C).

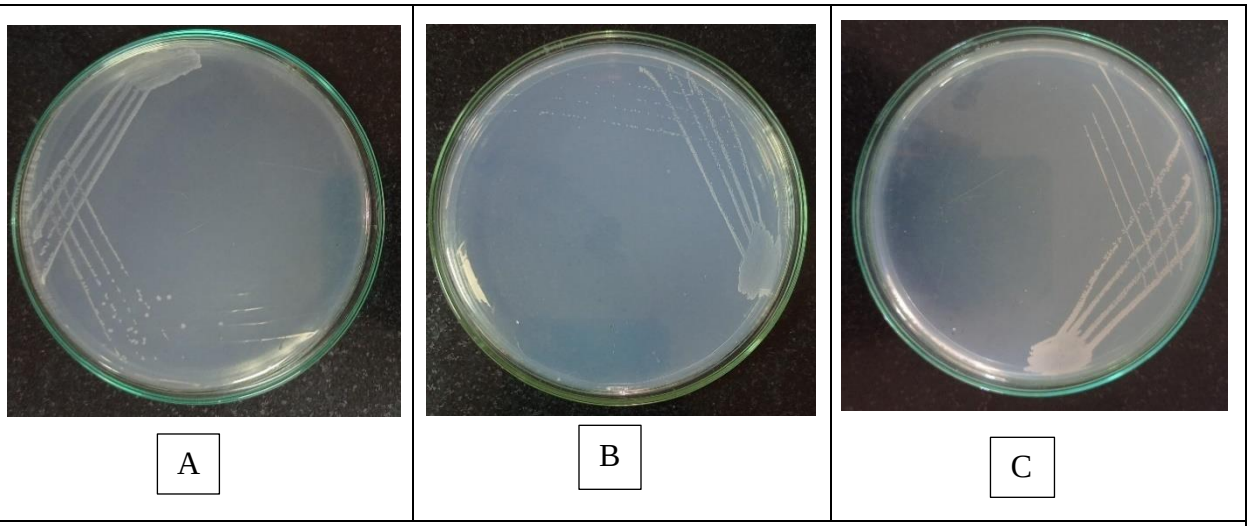
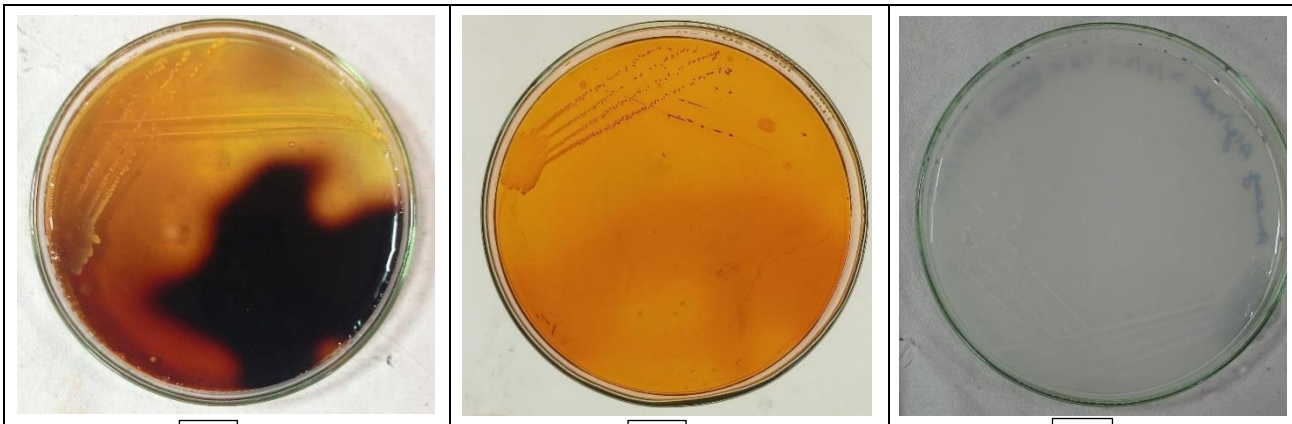


Figure No. 4.6: Plates before staining with specific stain (A – Agar supplemented, B- Carrageenan supplemented, and C – Alginate supplemented)



A B C

Figure No. 4.7: Plate after staining with specific stain (A –Agarolytic activity shown by the *Bacillus* sp. strain LCR1 after staining with lugol's iodine, B – Carrageenolytic activity shown by the *Bacillus* sp. strain LCR1 after staining with phenol red, and C – no changes seen after adding 10% of Sulphuric Acid).

4.7 DNA isolation and PCR:

DNA isolation was done by using the HiPurA® Bacterial Genomic DNA Purification Kit and the genomic band was observed on agarose gel after electrophoresis (Figure No. 4.8) and subsequently PCR was run by using the Primers '27F' and '1492R' in a thermal cycler (Agilent Technologies, SureCycler 8800). After completing PCR, the amplified band was observed on agarose gel again with the 500bp ladder after gel electrophoresis (Figure No. 4.9).

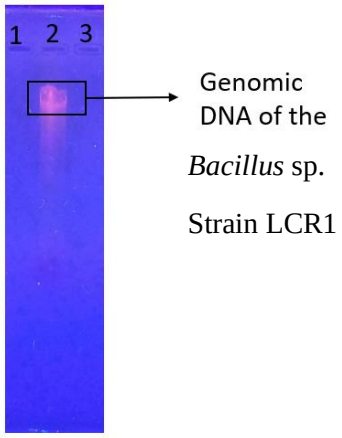


Figure No. 4.8: Genomic DNA band of the *Bacillus* sp. strain LCR1 on Gel (Lane 2)

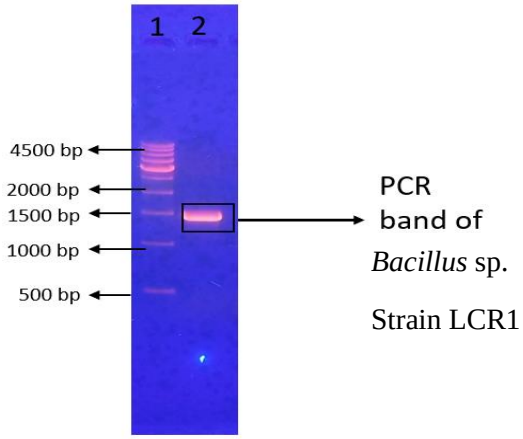


Figure No. 4.9: PCR amplified Band of the *Bacillus* sp. Strain LCR1 on Gel (Lane 1 – 500bp Ladder, Lane 2- Amplified DNA band).

4.8 Molecular identification:

NCBI Nucleotide Blast analysis of the 16S rRNA gene Sequencing of *Bacillus* sp. strain LCR1 suggested homology with various *Bacillus* species. The top hits included *Bacillus subtilis*, *Bacillus stercoris*, and *Bacillus rugosus*, among others, with sequence identities ranging from 86% to 87%. (Figure No. 4.9.1)

The sequence similarity between strain LCR1 and *Bacillus* species suggests its taxonomic affiliation within the *Bacillus* species. The conserved genetic features shared between strain LCR1 and *Bacillus species* support the molecular identification of LCR1 as a *Bacillus* species strain.

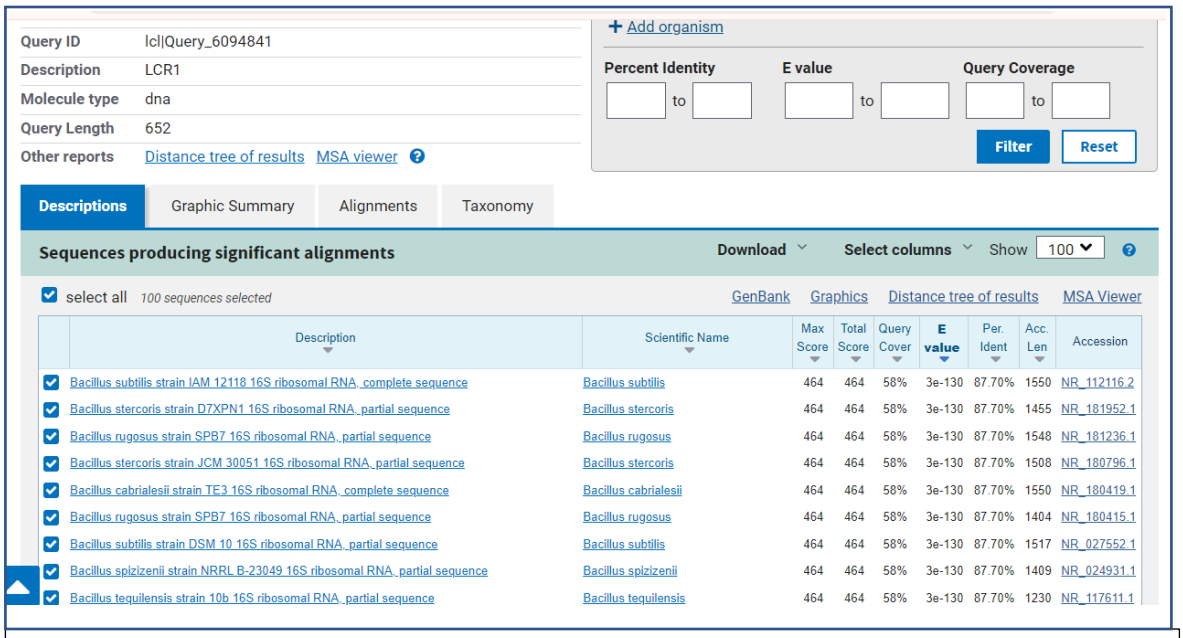


Figure No. 4.9.1 – NCBI Nucleotide Blast Hits

4.9 EPS yield:

Extracellular polymeric substance (EPS) yields during the late lag (12 hours), log (48 hours), and stationary (72 hours) phases were examined in this work. The relative EPS yields were 4.53 ± 0.30 g/L, 6.43 ± 0.49 g/L, and 5.5 ± 0.26 g/L (Figure No. 4.10). Variability was evident when comparing the results to earlier research by "Fuso et al. (2023)" reporting 0.71 to 2.38 g/L, "Asgher et al. (2020)" finding 2.9 to 4.8 g/L, and "Yokoi & Watanabe (1992)" reporting 2.4 g/L. The output of EPS fluctuated throughout the growth phases and peaked in the log phase. The late lag phase showed a moderate generation of EPS, suggesting that synthesis had begun. Active metabolism caused a significant increase in EPS during the log phase. Depletion of nutrients was the reason for the modest drop observed in the stationary phase. (Liu et al., 2018) Variability in research demonstrates the impact of bacterial strains and environments.

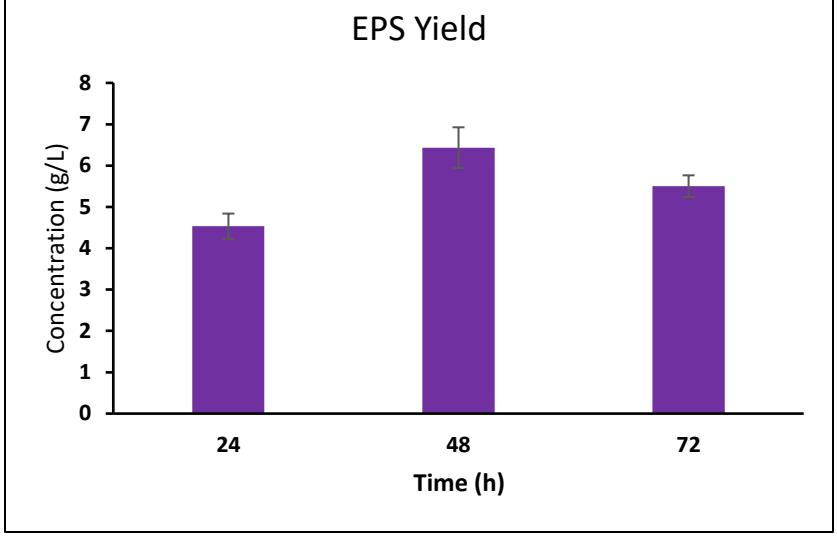


Figure No. 4.10: EPS yield in different growth phases of the *Bacillus* sp. strain LCR1

4.10 EPS characterization:

4.10.1 Solubility:

The solubility of the extracted extracellular polymeric substances (EPS) was assessed in various solvents. Water exhibited the highest solubility, with 93.96%, while organic solvents showed lower solubility: methanol (14.13%), ethanol (19.96%), isopropanol (11.84%), chloroform (4%), and acetone (5.96%) (Figure No. 4.11)

The recovered EPS's great solubility in water and its observed solubility profile highlight its hydrophilic character. The EPS matrix contains hydrophobic components, as evidenced by the decreased solubility in organic solvents. This result is consistent with earlier research done by

"He et al., (2023)", the intricate structure of EPS, which is made up of both hydrophilic and hydrophobic moieties.

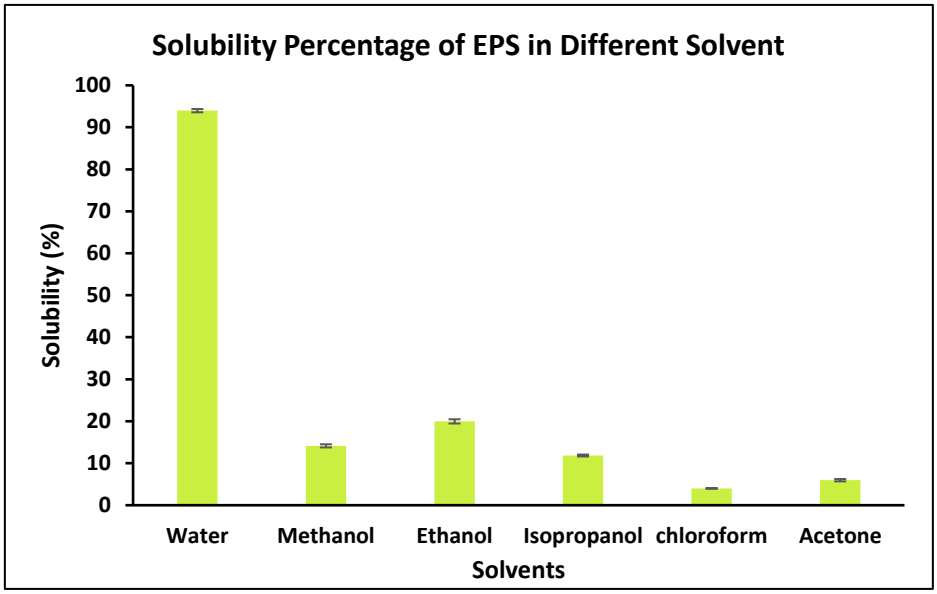


Figure No. 4.11: solubility % of EPS in different solvents

4.10.2 Total carbohydrate concentration:

Total carbohydrate concentrations were assessed across different growth phases of the bacterial strain using a glucose standard curve with a high coefficient of determination (R^2 = 0.997). The concentrations were found to be 0.31 g/L (319.59 ± 12.71 µg/ml) during lag phase (12 hours), 0.43 g/L (431.71 ± 11.27 µg/ml) in the log phase (48 hours), and 0.40 g/L (404.64 ± 8.87 µg/ml) in the stationary phase (72 hours).

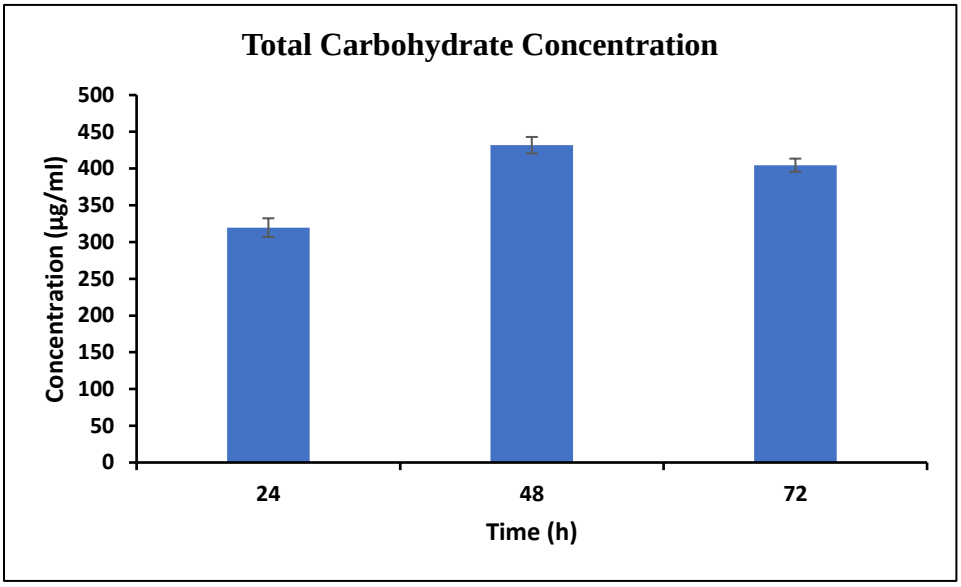


Figure No. 4.12: Total carbohydrate concentration in EPS of different growth phases of the *Bacillus* sp. strain LCR1

4.10.3 Reducing sugar concentration:

Reducing sugar concentrations were assessed across different growth phases of the bacterial strain using a glucose standard curve with a high coefficient of determination ($R^2 = 0.991$). The concentrations were found to be 0.11 ± 0.009 g/L during the lag phase (12 hours), 0.15 ± 0.01 g/L in the log phase (48 hours), and 0.09 ± 0.005 g/L in the stationary phase (72 hours).

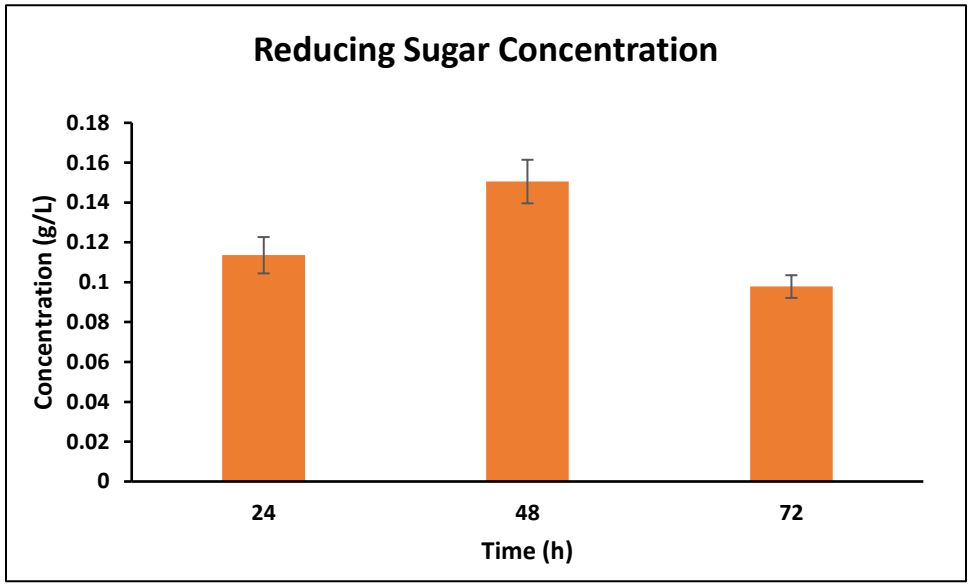


Figure No. 4.13: Reducing sugar concentration in EPS of different growth phases of the *Bacillus* sp. strain LCR1

This investigation focuses on the physiological alterations and metabolic activity that take place in the bacterial culture during its growth cycle and the findings support the research done by "Liu et al., (2018)". Carbohydrate levels were comparatively low during the late lag phase, which was marked by modest growth and limited biosynthetic activity. On the other hand, the exponentially growing log phase showed a rise in the content of carbohydrates, suggesting increased metabolic activity to facilitate fast cell division. Carbohydrate levels stayed constant during the stationary phase despite growth stabilizing, indicating the continuous metabolic activity needed for cellular upkeep.

4.10.4 Uronic acid concentration:

Uronic acid concentration was assessed across different growth phases of the bacterial strain using a D-galacturonic acid standard curve with a high coefficient of determination (R^2 = 0.987). The concentrations were found to be 0.31 ± 0.013 g/L during the lag phase (12 hours), 0.52 ± 0.015 g/L in the log phase (48 hours), and 0.49 ± 0.013 g/L in the stationary phase (72 hours).

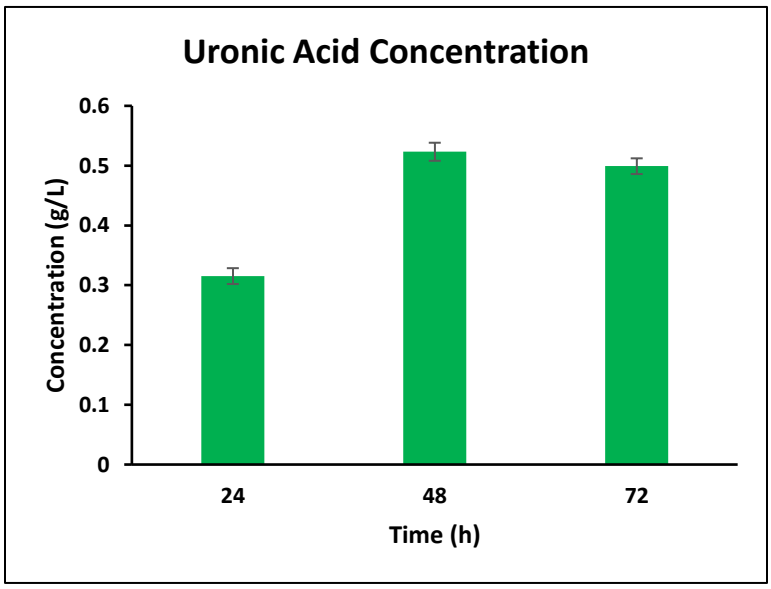


Figure No. 4.14: Uronic acid concentration in EPS of different growth phases of the *Bacillus* sp. strain LCR1

4.10.5 Protein Concentration:

Protein concentration was assessed across different growth phases of the bacterial strain using a Bovine Serum Albumin (BSA) standard curve with a high coefficient of determination (R^2 = 0.998). The concentrations were found to be 2.6 ± 0.017 g/L during the lag phase (12 hours), 2.91 ± 0.05 g/L in the log phase (48 hours), and 1.93 ± 0.01 g/L in the stationary phase (72 hours).

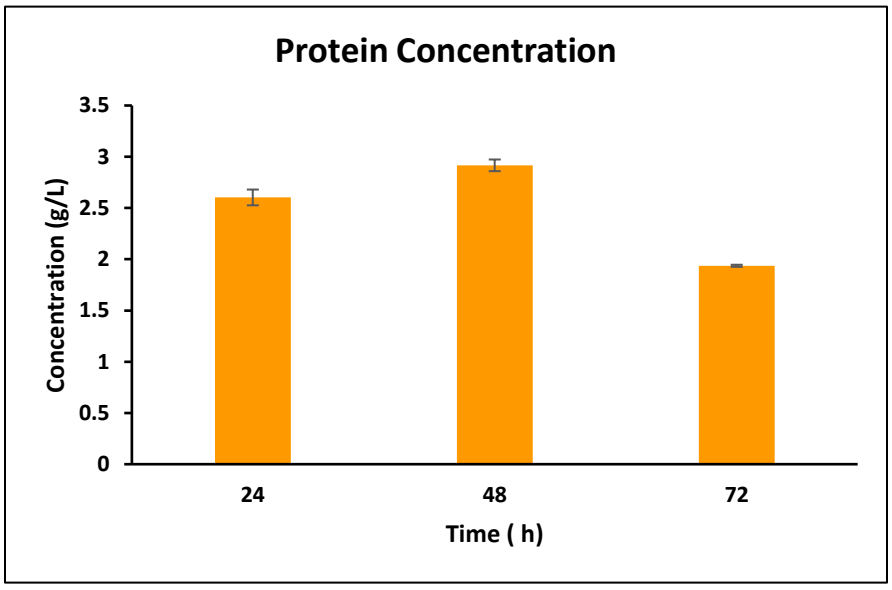


Figure No. 4.15: protein concentration in EPS of different growth phases of the *Bacillus* sp. strain LCR1

4.10.6 Comparative study:

A comparative analysis was conducted to assess the composition of extracellular polymeric substances (EPS) in different growth phases. Table No. 03.3 presents the concentrations of total carbohydrates, reducing sugar, uronic acid, and protein content in milligrams per milliliter (mg/ml) at 24, 48, and 72 hours.

Table No. 03.3: composition of extracellular polymeric substances (EPS) in different growth phases

Time (In Hours.)	total carbohydrate (g/L)	Reducing sugar (g/L)	Uronic acid (g/L)	protein content (g/L)
24	0.319	0.113	0.315	2.603
48	0.431	0.15	0.523	2.916
72	0.404	0.097	0.499	1.936

The data (Table No. 03.3) indicate variations in EPS composition across different growth phases. Notably, total carbohydrate and uronic acid concentrations show fluctuations over time, with the highest values observed at 48 hours. In contrast, reducing sugar content remains relatively consistent, while protein content decreases from 24 to 72 hours.

These findings suggest dynamic changes in EPS composition throughout bacterial growth (Figure No. 4.16), potentially reflecting shifts in metabolic activity and resource utilization.

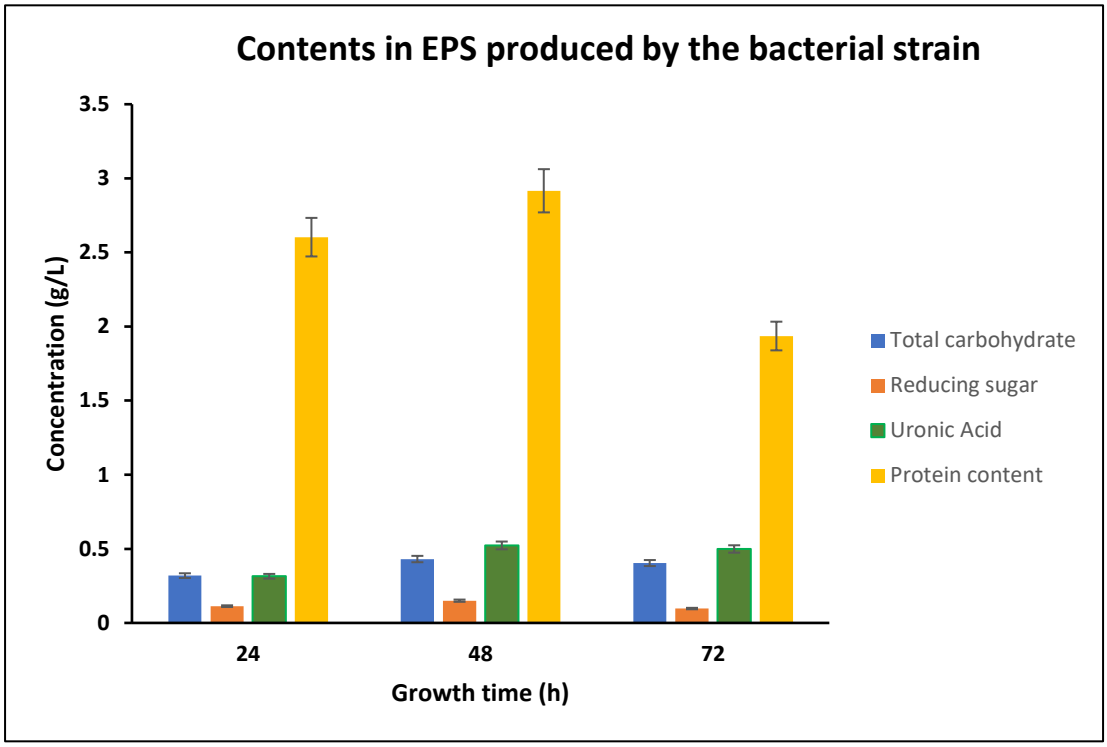


Figure No. 4.16: Overall comparative study of the composition of EPS in different growth phases of the *Bacillus* sp. strain LCR1

4.10.7 Raman spectroscopy:

Raman spectroscopy employed with a Raman Microscope (LabRAM Evolution Raman microscope) with 785nm laser, to identify the functional groups present in extracellular polymeric substances (EPS). The graph of Raman shift vs intensity has been plotted (Figure No.17.) using Origin 8 software version 8E and the results were compared with a study conducted by Tahir et al. (2020). The obtained Raman shifts (in cm^{-1}) and corresponding vibrations are presented in Table No. 03.4.

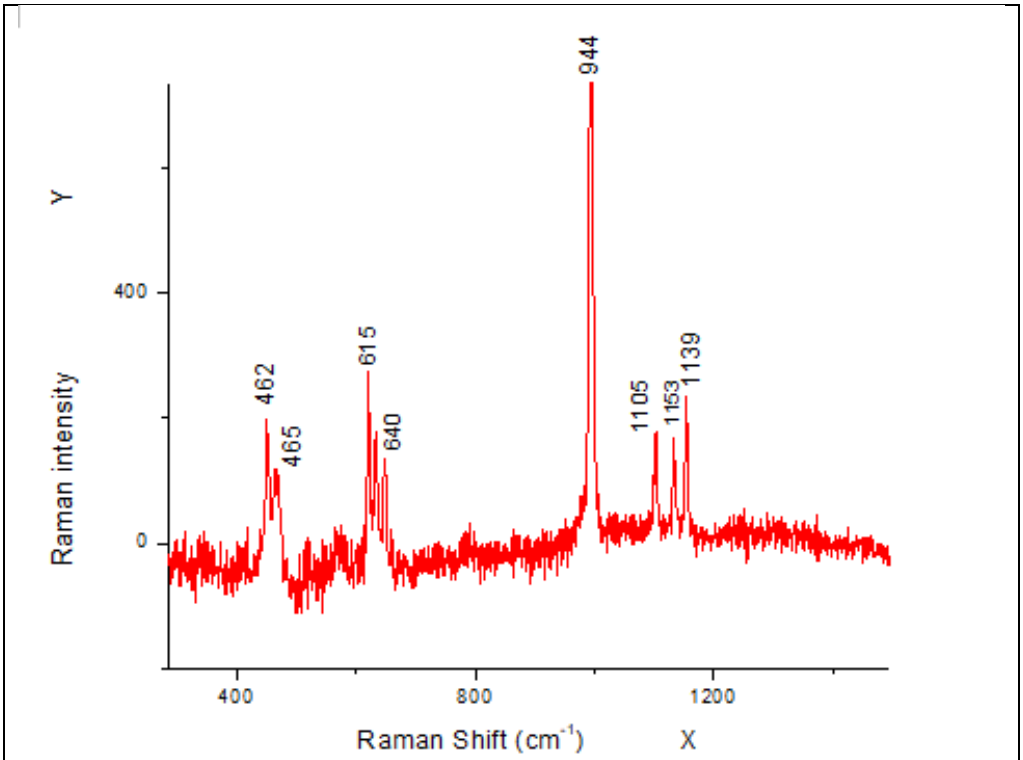


Figure No. 4.17: Raman spectroscopy of Lyophilized EPS at 785nm

Table No. 03.4: Raman shifts depicting corresponding functional groups.

Serial no.	Raman Shift (In cm^{-1})	Vibrations at 785nm
1.	462 cm^{-1}	δ (C-C-C) vibrations in the furanoid ring
2.	465 cm^{-1}	δ (C-C-C) vibrations in the furanoid ring
3.	615 cm^{-1}	Hydroxyl group
4.	640 cm^{-1}	δ (C-C-O) Exocyclic vibrations
5.	944 cm^{-1}	vibrations, (C-C + C-O stretching)
6.	1139 cm^{-1}	ν (C-C + C-O) Vibrations
7.	1105 cm^{-1}	Unidentified
8.	1135 cm^{-1}	Unidentified

4.11 Purification of EPS:

To mitigate protein contamination in the extracted extracellular polysaccharide (EPS), a purification step was implemented using ethanol reprecipitation. The concentrations of various components before and after purification are summarized in Table No. 03.50.1 and From Figure No.18, it is clear that after purification the protein concentrations decreased drastically while other component showed slight variations.

Table No. 03.5: Concentration of contents in EPS, before and after Purification

Contents	Concentration before Purification (g/L)	Concentration After purification (g/L)
Total carbohydrate	0.431	0.333
Reducing Sugar	0.15	0.12
Uronic acid	0.523	0.436
Protein	2.916	0.08

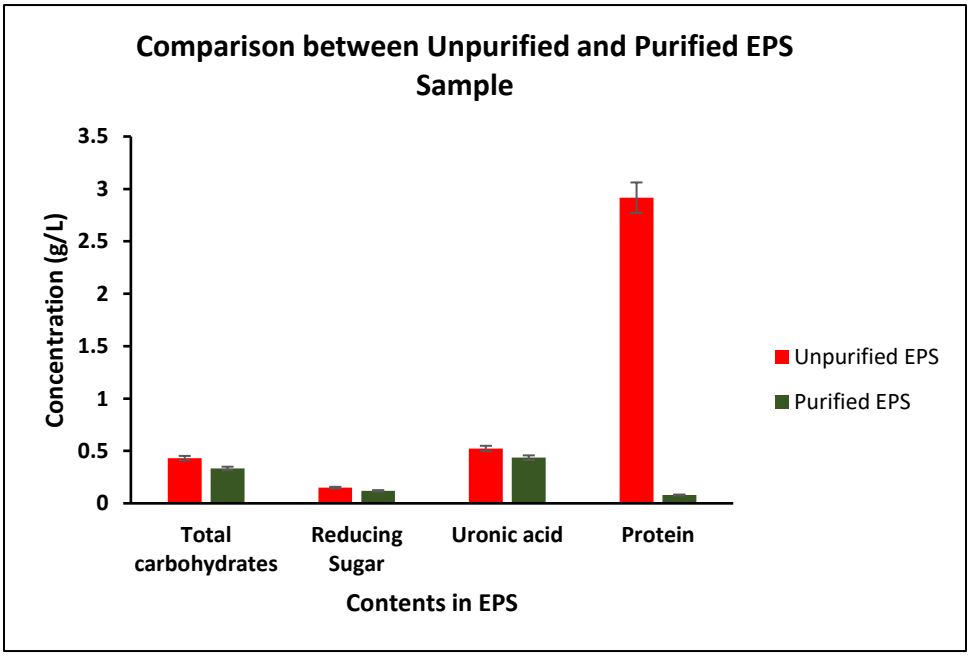


Figure No. 4.18: comparison of the composition of EPS before and after Purification.

4.12 Hydrolysis of EPS:

Following the hydrolysis of extracellular polysaccharides (EPS), the concentration of reducing sugar in the EPS sample increased from 0.126 g/L to 0.21 g/L. This observed elevation in reducing sugar content (Figure No. 4.19) indicates the breakdown of complex polysaccharides into simpler sugar units as a result of the hydrolytic process. A crucial stage in the breakdown of polysaccharides is hydrolysis, which releases mono- and oligosaccharides, which are usually recognized as reducing sugars. (Felz et al., 2019)

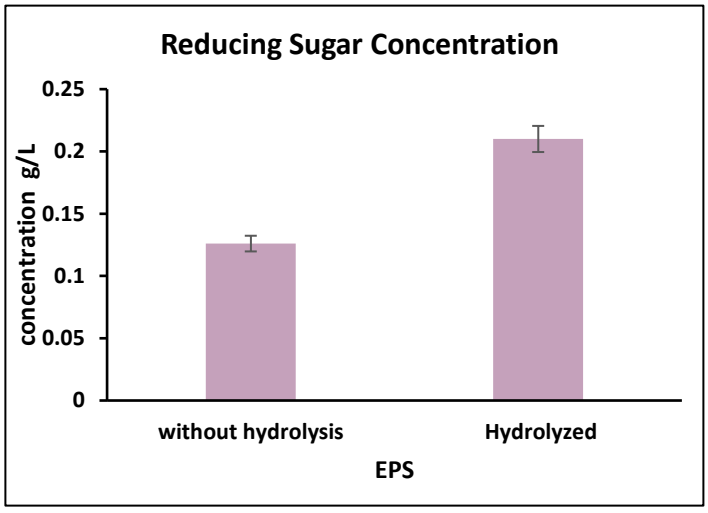


Figure No. 4.19: Reducing sugar concentration before and after hydrolysis

4.13 Thin Layer Chromatography (TLC):

The hydrolyzed sample of extracellular polysaccharides (EPS) was subjected to Thin Layer Chromatography (TLC) alongside standard solutions of glucose, maltose, and raffinose. However, the TLC analysis revealed a smear-like pattern for the hydrolyzed EPS sample, indicating that the separation did not achieve the desired level of resolution (Figure No. 4.20).

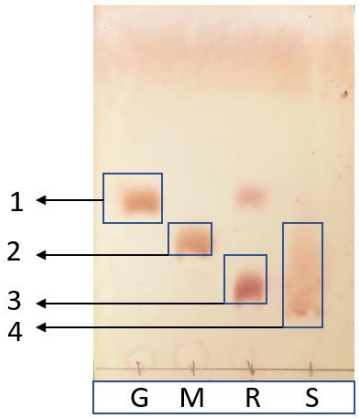


Figure No. 4.20: TLC Chromatogram obtained using resorcinol for spot visualization {from left to right, 1- Glucose(G), 2- Maltose(M), 3- Raffinose(R) and 4 – sample(S)}

4.14 Application of the extracted EPS:

4.14.1 In wastewater treatment:

The Sludge Volume Index (SVI) was used as a measure of how well extracted extracellular polymeric substances (EPS) from *Bacillus* sp. strain LCR1 treated wastewater. The untreated wastewater showed a reasonably strong tendency for sludge to settle, with an SVI of 16.61 ml/g.

The SVI dropped to 13.89 ml/g after receiving EPS treatment, showing better-settling properties and a decrease in sludge volume. This decrease is equivalent to a 16.37% drop in SVI, indicating how well EPS works to improve sludge settlement and decrease sludge volume in wastewater.

These findings support the "Bala Subramanian et al. (2010)" study, which found that EPS may increase sludge compaction and settling, thereby increasing the efficacy of wastewater treatment procedures.

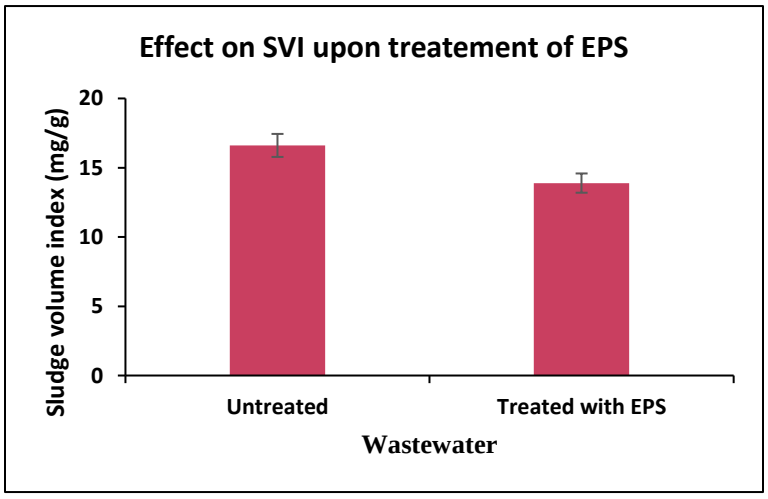


Figure No. 4.21: Comparison of sludge volume index upon treatment of EPS.

A B

Figure No. 4.22: Wastewater in measuring cylinder initially (A – untreated wastewater, B – treated wastewater).

A B

Figure No. 4.23: Wastewater in measuring cylinder after 30min (A – untreated wastewater, B – treated wastewater with a higher sludge volume than the untreated one).

4.14.2 In heavy metal adsorption:

The experimental findings show that Extracellular Polymeric Substances (EPS) are effective at removing lead (Pb) and copper (Cu) from solutions by adsorbing them. Following EPS treatment of 1mg/ml, the Pb concentration in the control samples dropped from 0.18 ppm to 0.12 ppm, and Cu concentration dropped from 0.26 ppm to 0.22 ppm respectively (Figure No. 4.24). The following study suggested that EPS have potential to adsorb Pb^{+2} up to 0.060ppm and copper up to 0.04ppm., which was higher as compared to a study conducted by "Salehizadeh et al., (2003)" showing maximum adsorption of Pb^{+2} and Cu^{+2} up to 0.055ppm and 0.03ppm respectively. According to these results, EPS appears to have a considerable capacity for adsorbing heavy metals.

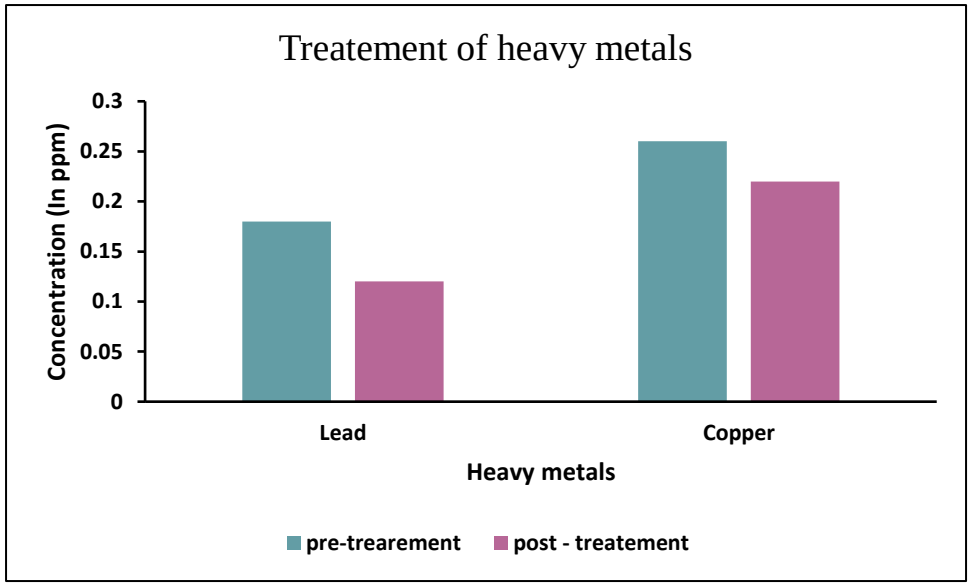


Figure No. 4.24 – Concentration of heavy metals pre-treatment and post-treatment with the EPS

CONCLUSION

The findings of this study underscore the significance of Exopolysaccharide (EPS) derived from the *Bacillus* sp. strain LCR1 in biotechnological applications and environmental remediation. The research demonstrates that EPS production is maximal during the logarithmic growth phase, with distinct characteristics observed in terms of carbohydrate composition and functional groups. Purification of EPS results in a reduction in protein content and an increase in the concentration of reducing sugars, indicative of enhanced purity and utility. The efficacy of EPS in wastewater treatment and heavy metal adsorption underscores its potential as a sustainable solution for environmental challenges. Furthermore, Gram identification confirms *Bacillus* sp. LCR1 as Gram-positive rods and 16S rRNA sequence confirms strain LCR1 as a *Bacillus* species strain with notable capabilities for EPS production and polysaccharide utilization. Overall, this study highlights the versatility and promise of EPS derived from LCR1, contributing to the advancement of biotechnological research and environmental science

FUTURE PROSPECTS

- Increment in the yield of Exopolysaccharides (EPS) by altering the Carbon and Nitrogen source.
- Purification and separation of saccharides from the mixture of Exopolysaccharides (EPS). using HPLC.
- Determination of the Zeta potential of the Exopolysaccharides (EPS).

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APPENDIX

1. **Anthrone reagent**

   Dissolve 0.2g of Anthrone in 100ml of concentration H_2SO_4.

2. **Lugol's Iodine:**

   Potassium Iodide: 1.66 (0.1M)

   Iodine (Crystal): 1.26 (0.05M)

   Add to 100 ml distilled water in an amber bottle and stir at RT vigorously till Iodine

   Crystals dissolve.

3. **DNSA:**

   A - 1g of DNSA Dissolve in 20 ml of NaOH

   B – 30g of Sodium Potassium Tratarate dissolve in 50ml of water

   Mix 'A' and 'B'

4. **Carbazole assay:**

   Reagent A- Dissolve 0.9 g of sodium tetraborate decahydrate in 10 ml of water and add 90 ml of ice-cold 98% concentrated sulfuric acid carefully to form a layer. Leave undisturbed overnight to mix without excessive heat production. Check it thoroughly mixed and at room temperature before use.
   Reagent B- Dissolve 100 mg of carbazole (recrystallized from ethanol) in 100 ml absolute ethanol.

5. Zobell marine broth (pH – 7.6 ± 2)

Ingredients	**Amount (g/L)**
Peptone	5.000
yeast extract	1.000
Ferric citrate	0.100
sodium chloride	19.450
$MnCl_2$	8.800
Na_2SO_4	3.240
$CaCl_2$	1.800
KCl	0.550
Na_2HCO_3	0.160
KBr	0.080
$SrCl_2$	0.034
Boric acid	0.022
Sodium silicate	0.004
Ammonium Nitrate	0.0016
Disodium Phosphate	0.008
Sodium fluorate	0.0024

6. Artificial seawater (pH-7):

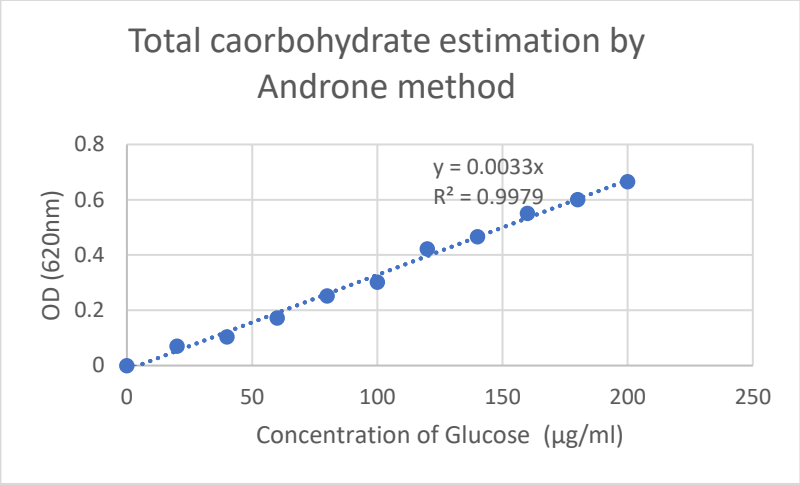
Ingredients	**Amount (g/L)**
Tris base	6.05
$MgSO_4$	12.32
KCl	0.74
NaCl	17.52
$CaCl_2$	0.14
$(NH)_2HPO_4$	0.13

7. Instruments:

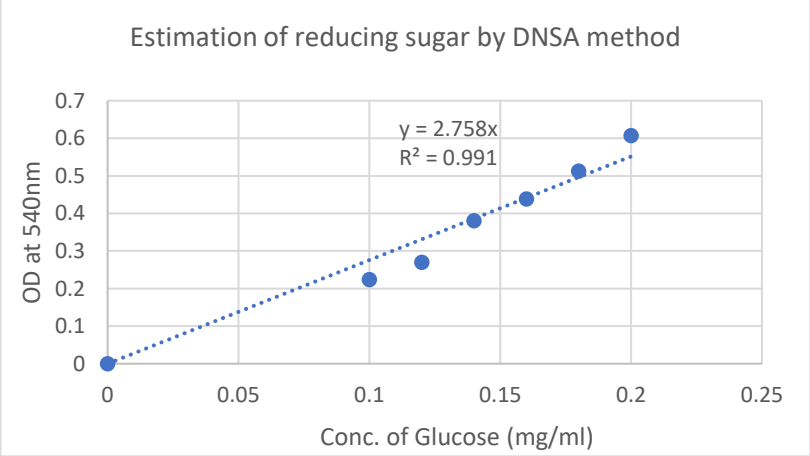
- SORVALL ST 8R refrigerated bench-top centrifuge
- UV mini 1240 UV-Vis spectrophotometer
- pH meter (pH 700, Eutech Instruments, Thermo Fisher Scientific, India)
- Autoclave
- Laminar Air Flow
- Hot air oven (i-therm AI-7981)
- Refrigerator
- Incubator shaker (Orbital incubator shaker, Remi Elektrotechnik Limited)
- Lyophilizer (Scanvac coolSafe)
- Weighing balance (Shimadzu ATX224)
- Thermal cycler (SureCycler 800)
- Light microscope
- Raman microscope (LabRAM Evolution Raman microscope)

8. Standard Curves:

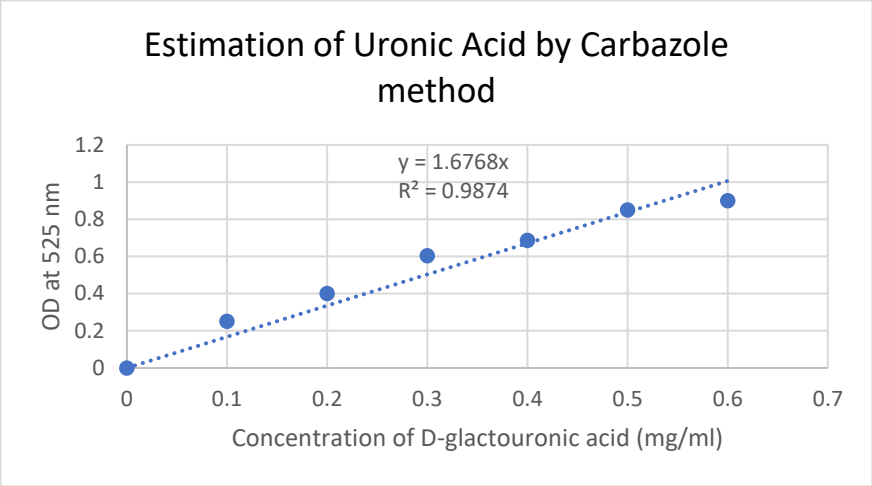
I. For total carbohydrate concentration (glucose as standard)



II. For reducing sugar concentration (Glucose as standard)



III. For uronic acid concentration (D-galacturonic acid standard)



IV. For protein concentration (Bovine serum albumin standard)