Screening isolation and characterisation of EPS producing Bacteria

An industrial training report submitted for the partial fullfilment of Degree of Bachelors of science

By

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[B.Sc (Biotechnology), sem-6]

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Summer Research Internship Program

By

Gujarat State Biotechnology Mission

Project Report

On

"Screening, Isolation and Characterisation of EPS Producing Bacteria"

CSIR-IMTECH

GSBTM

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B.Sc. Biotechnology (2nd year)

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DECLARATION

I hereby declare that I contributed a small bit to a project entitled "Screening, Isolation and Characterisation of EPS Producing Bacteria" from extreme conditions which was carried out at Biochemical Engineering Research & Process Development Centre, Institute of Microbial Technology, Council of Scientific and Industrial Research, Sector 39A, Chandigarh, 160036, India under the supervision of Dr. Anirban Roy Choudhury, Senior Principal Scientist, CSIR-IMTECH, Chandigarh. This report is being submitted as mark of successful completion of 1 month Summer Research Internship Program by GSBTM (Gujarat State Biotechnology Mission).

I further declare that the finding/data of the experiments performed under this project are preserved by CSIR-IMTECH, Chandigarh and can be published in part or full only with their consent. It is being submitted to the GSBTM office under the knowledge of CSIR-IMTECH, Chandigarh.

Arundhatiba Jethwa

Acknowledgement

On completion of this one month internship I would like to express my gratitude towards Government of Gujarat for providing this opportunity, and selecting me for this internship, which has added value to my Bachelor's degree in Biotechnology. Due to this opportunity I've managed to gain skills and exposure which would be helpful in my future studies.

It is my pleasure to show my heartiest gratitude to Dr. Sanjeev Khosla, Director, CSIR-Imtech, Chandigarh, for permitting me to work in a research atmosphere and providing facilities in the institute.

I am grateful to my project supervisor Dr. Anirban Roy Choudhury, Senior Principal Scientist, CSIR-IMTECH, for his valued guidance, inspiration, support and encouragement throughout the work and allowing me to work in his lab. His guidance motivated me to translate my acquired knowledge to actions.

While pursuing my Bachelor's degree at Atmiya University, Rajkot, Gujarat, I would like to express my very great appreciation to Dr. Shivani Patel (Head of Biotechnology and Microbiology Department) and Atmiya University for allowing me to work at CSIR-IMTECH, Chandigarh.

This work would not have been possible without support and guidance of Ms. Sheetal Katiyar (Ph.D. Research Scholar at CSIR-IMTECH). She was always available whenever I ran into a trouble spot or had a question about my work or writing. Her kind and humble behaviour towards me allowed to work with an ease and better understanding. It is a matter of diligence to convey my thanks to Ms. Nandita Srivastva for adding knowledge to my work and drafting report. I would like to thank Mr. Digendra Kumar for co-operating and morally supporting during this entire period of my internship.

Nobody has been more important to me in the pursuit of this work than the members of my family. I would like to thank my parents for their love and guidance. They are the ultimate role models.

Arundhatiba Jethwa

Background of the Host Institute

The Council of Scientific & Industrial Research (CSIR), covers a broad spectrum of science and technology from radio and space physics, oceanography, geophysics, chemicals, drugs, genomics, biotechnology and nanotechnology to mining, aeronautics, instrumentation, environmental engineering and information technology.

CSIR-IMTECH is a hub of scientists proficient in the R&D areas of:

- Proteins - engineering, cloning, manipulating, crystallography
- Molecular biology of pathogens-genetics, drug resistance and vaccine development, immunology of infectious diseases, microbial taxonomy and metagenomics.
- Yeast genetics, Bioinformatics, screening microorganisms for novel enzymatic activities and strain improvement.
- Biochemical Engineering Research and Process Development Center (BERPDC)

I've worked in the BERPDC department under Dr. Anirban Roy Choudhury, Senior Principal Scientist. BREPDC is one of the largest and most sophisticated centers for research in technology development, process optimization and scale-up in the field of biochemical engineering in India.

1. Introduction

Polysaccharides are the class of biopolymers having simple monosaccharides joined by glycosidic bond in an explicit stoichiometry [1, 2]. Polysaccharides occupy almost 50% carbon source in nature and are originated from almost all type of organisms like plants (cellulose, pectin), animals (chitin, hyaluronic acid), algae (agar) and microbial origin (xanthan, pullulan). As per recent studies, polysaccharides from microbial sources have been reported to produce unique polysaccharides [3].

Polysaccharides derived from microorganisms can be further classified into lipopolysaccharides, capsular polysaccharides, and exopolysaccharides. Capsular polysaccharides are found to be associated with promotion of pathogenicity in microorganisms [4]. Likewise, pathogenicity and virulence is observed in lipopolysacccharides. Exopolysaccharides are biomacromolecules composed of carbohydrate residues which are secreted by a microorganism into the surrounding environment as a strategy to adapt to the extreme environment conditions [5]. EPS work as a functional and structural unit of biofilms. Most of the functional properties depicts protective nature of EPSs. They areoftenly biodegradable, non-immunogenic, and abundantly present in the environment. EPSs are well known for providing cell adhesion to surfaces and also act as reservoir of carbon sources [6].

On the basis of composition EPSs are classified into two main categories,

- Homopolysaccharides – typically composed of one type of monomer which contributes to the backbone structure, for example pullulan.
- Heteropolysaccharides – composition of this category consist of two or more different types of monosaccharides for example gellan.

EPS produced by microorganisms show unique properties for which they are used across pharmaceuticals, food and beverage, cosmetics and textile industries quite extensively. For instance, they are used as texture enhancer and moisture retainer in food industries, as emulsifier, stabilizer, and anticoagulant agent in biomedical sectors [7]. Some of the important EPSs producing bacteria isolated from various niches, their properties and applications are mentioned in table (1)

Table (1) properties and functional attributes of some bacterial exopolysaccharides [9]

Bacterial Exo-polysaccharide	Bacterial strains	Niches	Properties	Applications
Dextran	L. mesenteriodes	sugary niches and pickled vegetable products	Non-ionic, good stability Newtonian, fluid behaviour	Foods, Pharmaceutical industry (Blood volume expander) and Chromatographic media
Alginate	P. aeruginosa and A. vinelandii	Isolated from the skin, throat, and stool of healthy persons. Often colonise hospital food, sinks, taps, mops, and respiratory equipment	Gelling capacity, film forming	Food hydrocolloid and medicine (surgical dressings, wound management and controlled drug release)
Xanthan	Xanthomonas spp.	Bacterial leaf spots.	High viscosity, Stable over a wide temperature, pH and salt concentrations ranges	Foods, petroleum industry, pharmaceuticals, cosmetics and personal care products
Curdian	Rhizobium meliloti and Agrobacterium radiobacter	fixing nitrogen in leguminous plants	Gel-forming ability, water insolubility, edible and non-toxic has biological activity	Foods, pharmaceutical industry, heavy metal removal and concrete additive
Cellulose	Acetobacter spp.	Rotten fruits, vegetables, sour juices, vinegar, and beverages.	Not soluble in most solvents and high tensile strength	Foods (indigestible fibres), biomedical (wound healing, tissue engineered blood vessels) and audio speaker diaphragms
Succinoglycan	Alcaligenes faecalis var. myxogenes	hospital settings, such as in respirators, haemodialysis systems, and intravenous solutions	High viscosity and acid stability	Food and oil recovery

2. Objective

As it is seen in table (1) that EPS producing bacteria are isolated and purified from various niches, however there are limited reports for extreme locations.

Therefore, the objective of my training were:

- Hands-on training in different techniques of microbiology and fermentation lab
- Isolation, purification and screening for bacteria producing EPSs.

3. Methodology

3.1 Collection of samples

Soil samples from extreme conditions were collected and inoculated in desirable media.

3.2 Media preparation

Luria Bertani (LB) agar was used as media for spreading the sample as it is most preferable for the growth of bacteria. The media was prepared by suspending 35.0 g of LB agar in 1000 ml of distilled water. Further, it was sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes. Along with media preparation, 100 ml normal saline [0.85% (w/v)] was also prepared and autoclaved for serial dilutions. The autoclaved media was then poured into sterilized petri plates and left for solidification. fig. 1.

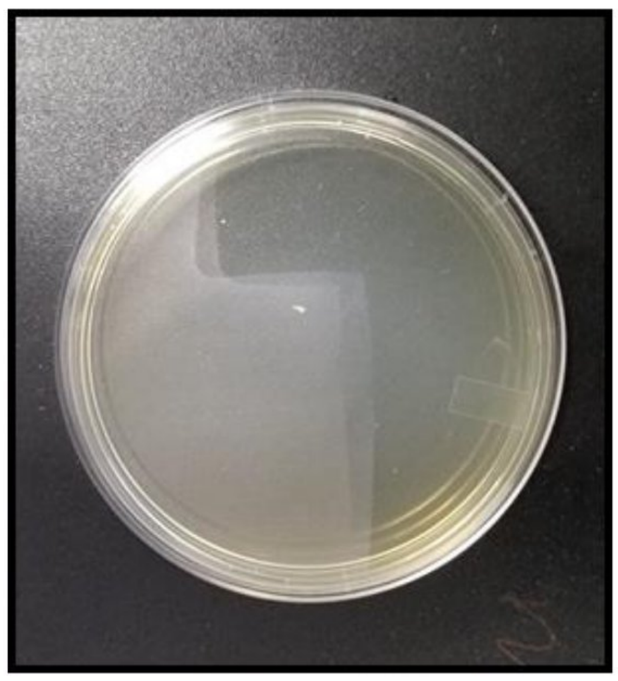


Fig. (1) Fresh LA plate

3.3 Isolation and Purification of Bacteria producing EPS

Samples of about 1 ml were serially diluted to reduce the number of bacterial colonies for conventional isolation. 0.1 ml of sample suspension was serially suspended into MCTs (Micro

centrifuge tubes) containing sterilized normal saline [0.85% (w/v)] of 0.9 ml with the help of a micro-pipette of 1000µl up to 10^{-6} dilution factor. fig. 2.

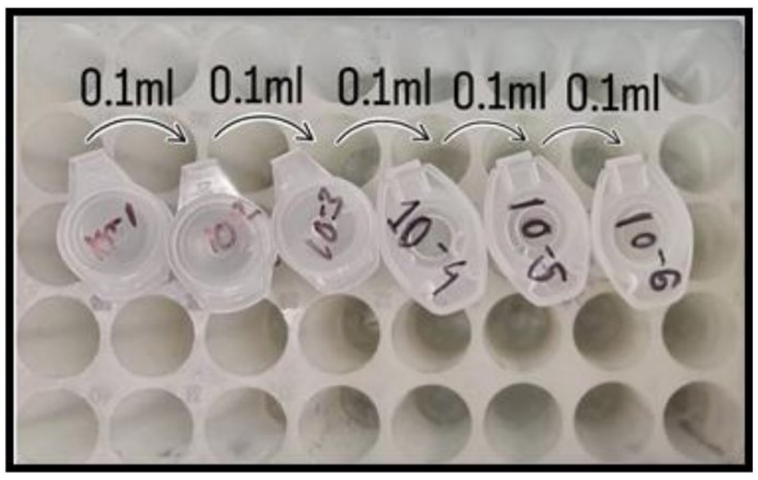


Fig. (2) Serial dilutions up to 10^{-6} dilution factor

3.4 Spreading

Further, 0.1 ml serially diluted bacterial samples were spread on LB agar plates with the help of a sterile glass spreader. The plates were inverted and incubated at 25°C till the growth was observed. After incubation at suitable temperatures and time, different colonies of bacteria were observed, these were further purified based on their morphology. These colonies were then subcultured for favorable isolation and purification of EPS producing bacteria.

3.5 Subculturing techniques

Subculturing is done for isolation and purification of the desired bacteria. For this purpose streaking was performed. In brief, bacterial colonies were directly streaked with the help of a loop on a fresh plate, to avoid contamination the wire loops were first sterilized on blue flame till the loop turns hot red. Further, the loops were allowed to cool down before streaking the bacteria to prevent their lysis. After streaking the plates with the quadrant method they were inverted and kept for incubation at 25°C till growth was observed. Finally, after the growth was observed, plates were screened for EPS production.

3.6 Screening for EPS producing bacteria

The production media was prepared containing 2% (w/v) glucose along with 20 g of LB suspended in 1000 ml of distilled water. The media prepared was divided into 15 different Erlenmeyer flasks. The media was then autoclaved and further inoculated with the bacterial colony. Additionally, the broth with different colonies was incubated at 25°C for 72hrs fig. 3. After the incubation period was over, the following parameters were observed for it.

Fig. (3) LB broth containing bacterial colonies after incubation

pH

pH of the culture was measured with the help of pH meter (Eutech, PC 2700).

Optical density

Initially the samples were 10 times diluted and optical densities at 600nm of these samples were measured with the help of Eppendorf Biospectrometer.

Centrifugation

Centrifugation was done to remove the cell mass from media and to obtain cell free soup. The culture was poured into the centrifuge tubes, keeping a check on the uniform weight of each tube. The tubes were placed oppositely in the centrifuge (Eppendorf, 5804 R) to balance. Further, they were centrifuged at 10000x rpm. for 15 minutes at 4°C. After completion of centrifugation, the supernatant was separated from the pellet as observed in fig. 4.

Fig. (4) Pellet separated from supernatant after centrifugation

Residual sugar percentage

For the determination of residual sugar percentage, first a glucose standard was prepared by the DNSA (2-hydroxy-3, 5-dinitrobenzoic acid) method. The chemical equation of this method is shown in the fig. 5. DNSA method is used to determine the presence of reducing sugar in the sample. When

DNSA which is yellow in color reacts with the reducing sugar in the sample it turns dark orange by forming 3 amino 5-nitro salicylic acid.

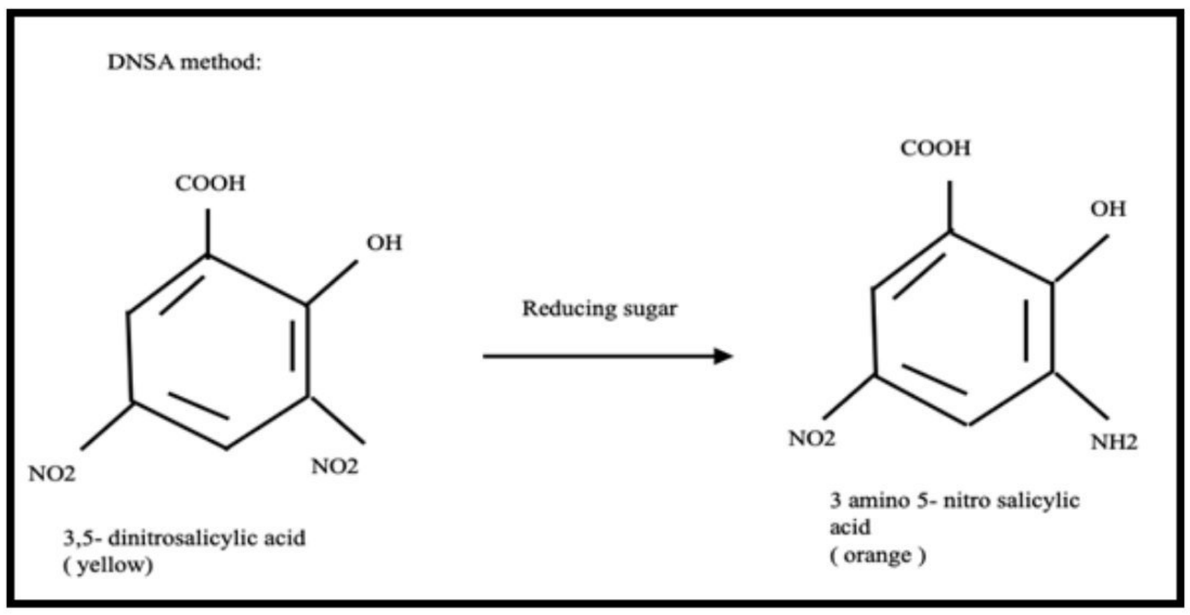


Fig (5) Chemical reaction of DNSA method

A glucose standard was prepared as shown in the table 2. Initially, a stock solution of glucose was prepared with a concentration of 0.2 mg/ml. Glucose and water was added to the tubes in different volumes as mentioned in table 2 to make up the total volume of 1 ml. Then, 3 ml DNSA was added to each tube. Further, they were incubated in boiling water for 10 minutes and then kept in ice for 10 minutes. After completion of the incubation, absorbance of these samples were measured at 540 nm. The values are present in the table 2.

Same procedure was performed with the cell free supernatant of bacterial samples. The glucose concentration (mg/ml) of each sample was determined using the linear equation obtained from the graph of glucose standard.

Table (2) DNSA method

Sample	Glucose in µl	Water in µl	DNSA in ml	Incubation	Incubation	Absorbance at 540nm
Blank	0	1000	3	Incubation in boiling water for 10min	Incubation in ice for 10min	0.000
S1	100	900	3			0.012
S2	200	800	3			0.013
S3	300	700	3			0.040
S4	400	600	3			0.201

Sample	Glucose in µl	Water in µl	DNSA in ml	Incubation	Incubation	Absorbance at 540nm
S5	500	500	3			0.301
S6	600	400	3			0.398
S7	700	300	3			0.486

3.7 Precipitation of EPS

For obtaining EPS, 1000 µl of supernatant collected after centrifugation was added to the test tube and chilled ethanol (two fold volume of supernatant) was added to precipitate the EPS.

Further, the bacterial sample with EPS production were selected and inoculated into LB media for preparing inoculum and were kept for incubation overnight at 25°C at 200 rpm for 72 hrs. After the bacterial growth was observed, 5% (v/v) of this inoculum was added to the production media containing 5% (w/v) glucose. This culture was incubated for 48hrs at 25°C at 200 rpm. The pH and optical density of this culture was measured according to the protocol mentioned in section 6. Moreover, the residual sugar of supernatant was measured using biochemistry analyzer (YSI 2900D). For EPS production, 1000µl supernatant was pipetted into a test tube and two fold chilled ethanol was added. To isolate EPS, the sample which showed precipitated EPS was further centrifuged at 4°C at 10000xg rpm for 15 minutes. After centrifugation, the supernatant was separated from pellet (EPS). The EPS was kept for drying in hot air oven at 70°C for measuring the dry weight.

3.8 Gram staining

EPS forming bacteria were further identified using gram staining. With the help of a sterilized loop, the sample was added to the slide and a smear was formed which was heat fixed. Crystal Violet was applied to the smear followed by gram's iodine which was used as a mordant. Rapid decolonization with ethanol was done. Lastly, counterstain safranin was added. Observation under a phase contrast microscope at 100x magnification was done. Prior to this immersion oil was added on top of it before observing to avoid refraction of light.

3.9 Growth kinetics

The sample of bacteria which showed EPS precipitation, was inoculated into a fresh LB medium for growth kinetics. A loopful of bacteria was inoculated from plate to broth under a laminar hood and kept for incubation at 25°C. Optical densities at 600nm of this sample were measured at an interval

of two hrs up to 22hrs, for plotting the growth curve. The growth curve obtained determined the log phase suitable for fermentation kinetics.

4. Results

4.1 Isolation and purification

For isolation of bacterial colonies, initially, serial dilutions up to 10^{-6} dilution factor were done. Further, these diluted samples were spread on fresh petri plates containing solidified LB agar. Numerous colonies were observed on these plates after incubation as shown in the fig. 6. It was observed that variety of morphologically differing colonies were present on a single plate. These colonies were differing on the basis of following criteria:

Color-some colonies were yellow, peach, pink, orange, neon and most of them were white or translucent.

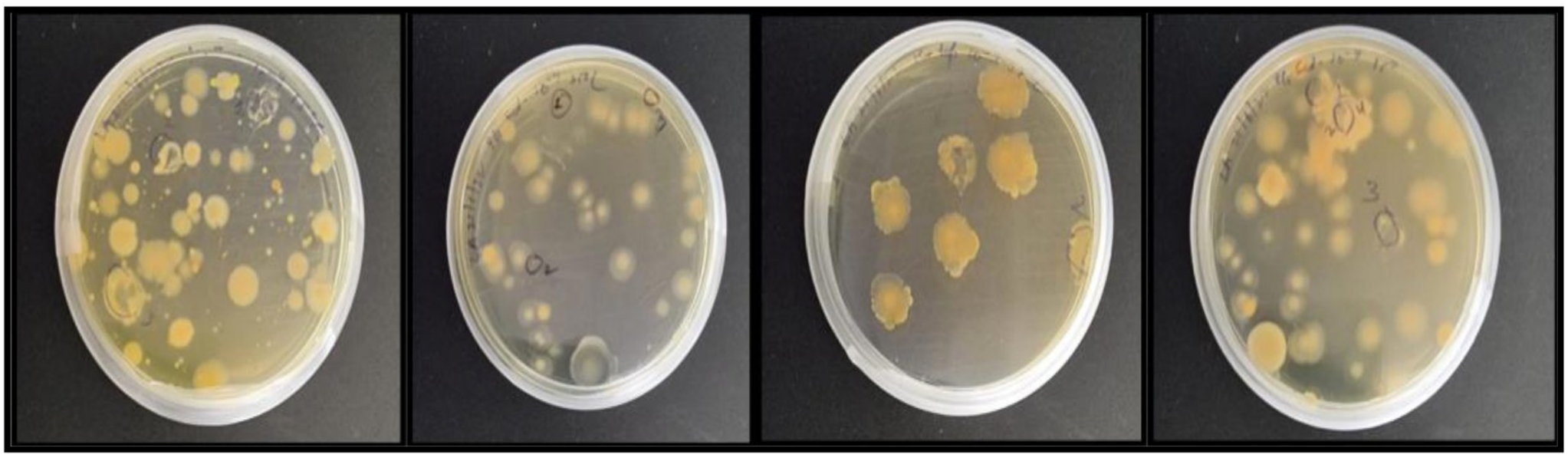
Shape-some were round, irregular, filamentous, rhizoid, curled.

Opacity-transparent (clear), opaque (not transparent or clear), translucent (almost clear, but distorted vision–like looking through frosted glass.

Texture-Butyrous (buttery), viscid (sticks to loop, hard to get off), brittle/friable (dry, breaks apart), mucoid (sticky, mucus-like) Texture, Smooth, glistening, rough, dull (opposite of glistening), rugose (wrinkled).

Elevation-some were raise, flat and convex.

Next, the colonies from these plates were streaked on fresh LB agar plates with the help of inoculating loops for obtaining pure colonies of bacteria. After the incubation of streaked plates a wide range of colonies were obtained as depicted in fig. 7.



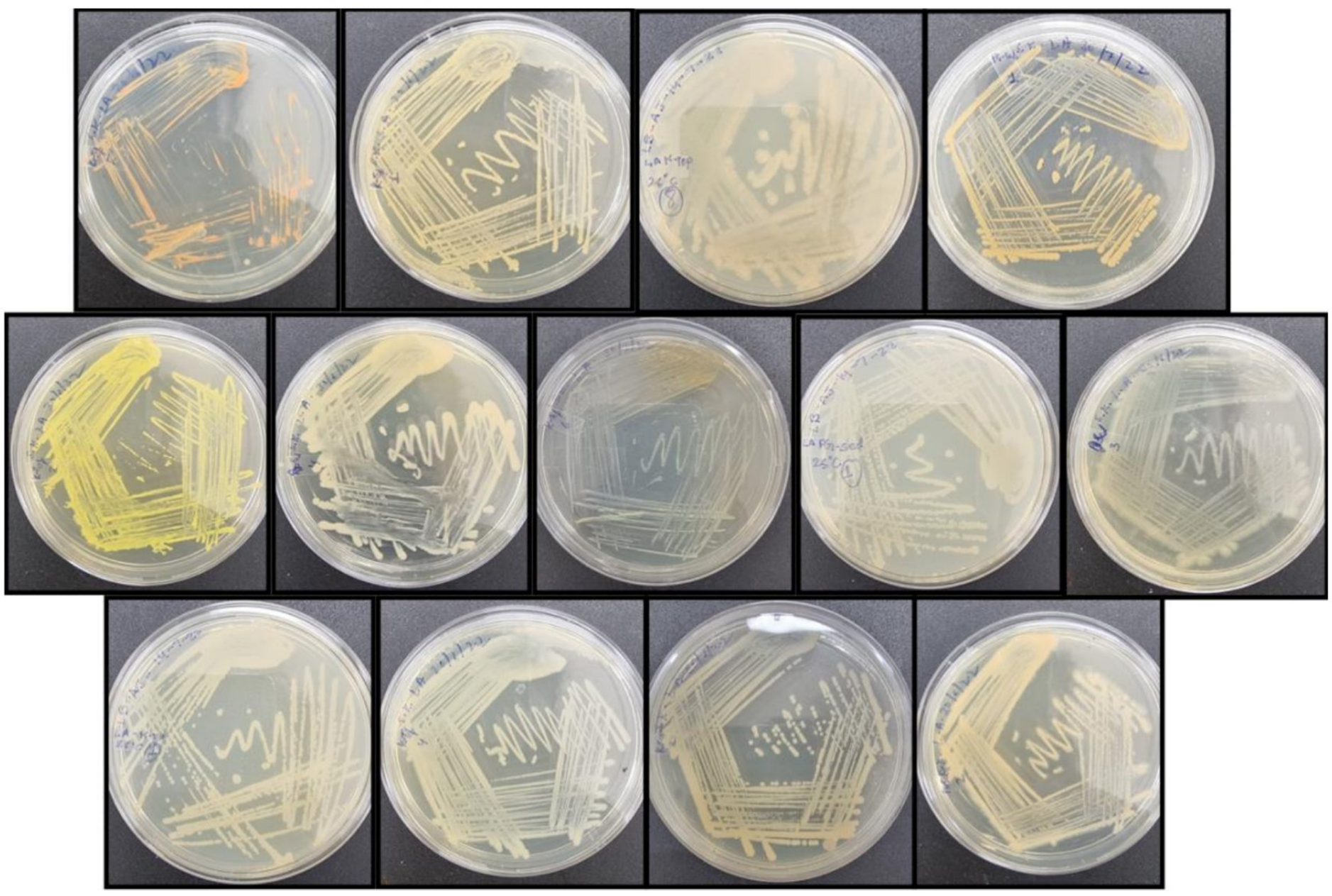


Fig (6) Spreading

Fig. (7) Streaking of different bacterial colonies on LB agar

4.2 Screening of eps producing bacteria

Obtained pure bacterial colonies were inoculated into LB media enriched with glucose and incubated at 25°C. After the incubation period, serval analytical tests were performed to analyze the fermentation characteristics of the obtained bacterial cultures. Glucose standard with linear equation y=4.665x-0.1619 fig.8 was used to find the concentration for the residual sugar. Further, the cell free soup was analyzed for the presence of EPS, if any. The results of the parameters observed by performing different analytical tests on the samples are provided in the table (4). There were different trends observed in the pH of samples owing to the various metabolites produced by bacteria during growth. Optical densities help in measuring the turbidity of bacterial culture. The more the optical density, more is the growth of culture. These parameters are of the preliminary test which were performed. Out of which only 5 samples (highlighted in the table) showed precipitates which might be EPS fig. 9. To know if the precipitates observed were EPS or not. The isolated and purified bacterial colonies were used to form inoculum and then this inoculum is added to liquid media

consisting 5% glucose. After incubating them at favorable conditions ethanol was added which resulted in one [highlighted in the table (3)] out of five samples showing EPS precipitations fig. 10. The precipitated EPS was collected and dried in hot air. Dry mass of 0.32g/l was obtained.

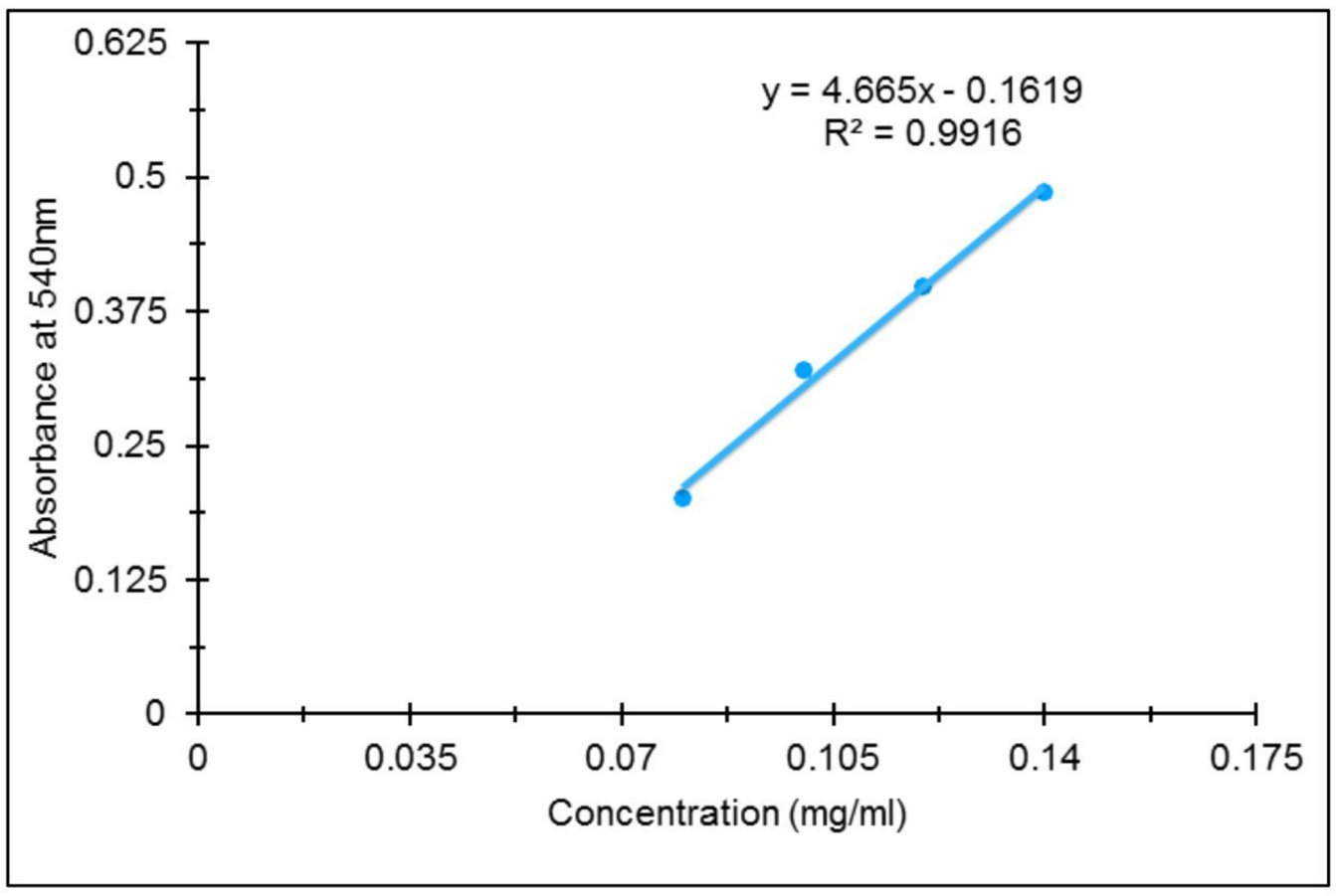


Fig (8) Graph depicting glucose standard curve

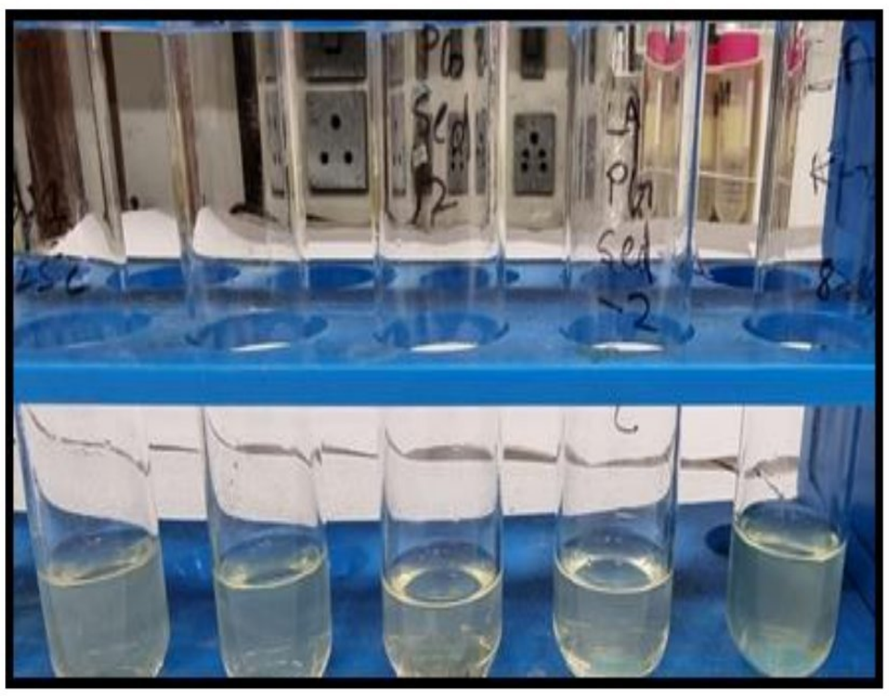


Fig (9) samples in table (4) which showed precipitates after preliminary test

Fig (10) LA-K-T-8-A precipitating EPS on addition of chilled ethanol

Table 3 preliminary test for EPS

Age in days	Sample name	pH	Sample OD (600nm)	DNSA method	Vale after multiplying With dilution factor	x=(y+0.1619)/4.665	Residual sugar %
				Absorbance at 540nm		Concentration (mg/ml)	
3	LA-K-T-4	9.04	8.27	0.653	6.53	1.434	0.1434
3	LA-P-2-A	6.14	4.79	0.369	3.69	0.825	0.0825
3	LA-K-T-8	8.25	2.27	0.235	2.35	0.538	0.0538
3	LA-K-T-1	8.48	1.09	0.526	5.26	1.162	0.1162
3	LA-P-3	6.93	3.46	0.45	4.5	0.999	0.0999
3	LA-K-1-A	3.14	19.03	0.11	1.1	0.270	0.027
3	LA-K-T-8-A	8.51	7.46	0.546	5.46	1.205	0.1205
3	LA-K-T-3	8.13	13.9	0.646	6.46	1.419	0.1419
3	LA-P-2	5.88	6.22	0.352	3.52	0.789	0.0789
3	LA-K-T-6	8.25	0.75	0.644	6.44	1.415	0.1415
3	LA-K-T-2	8.51	0.81	0.549	5.49	1.211	0.1211
3	LA-P-1	6.16	6.18	0.351	3.51	0.787	0.0787
3	LA-P-4	7.83	4.11	0.533	5.33	1.177	0.1177
3	LA-K-T-5	3.25	3.29	0.605	6.05	1.331	0.1331

Table (4) secondary test for bacteria producing EPS

Age in days	Sample name	pH	Sample OD at 600nm	Residual sugar%	EPS g/l
2	La-k-top-5	6.93	0.02	4.83%	0
2	La-k-top-8-A	8.25	1.1	4.14%	0.32
2	La-pg-sed-2-A	5.59	3.34	4.14%	0

2	La-pg-sed-2	5.74	4.36	3.34%	0	
2	La-k-top-8	6.93	0	4.12%	0	

4.3 Identification for bacteria producing EPS

The sample was observed under phase contrast microscope after performing gram staining. After observing under microscope at 100x magnification gram negative rod shaped (bacilli) bacteria were observed fig. 11.

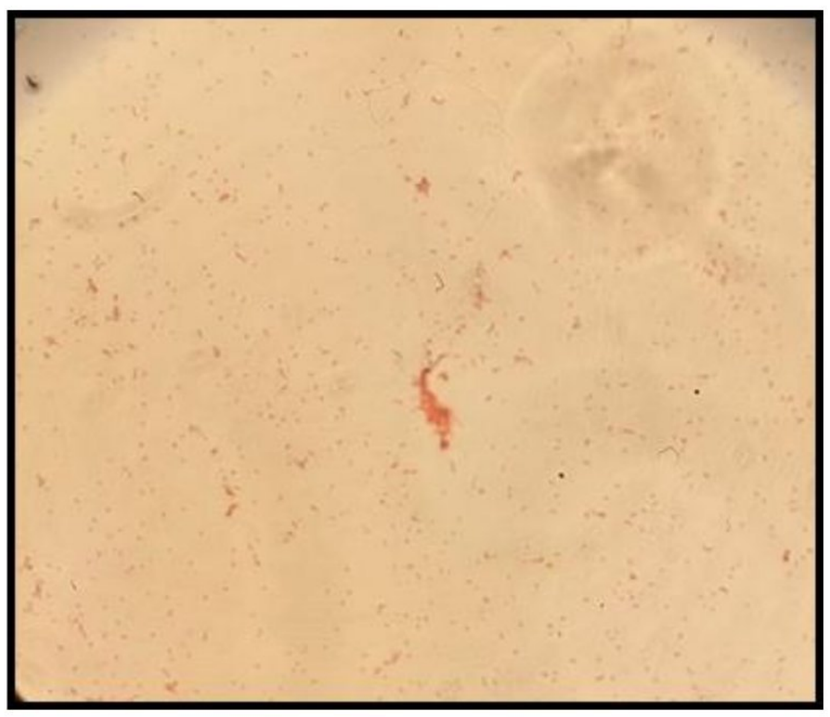


Fig (11) Pink colored gram negative rod was observed

4.4 Growth kinetics

For obtaining a growth curve of the bacteria producing EPS, growth kinetics was performed on La-K-top-8-A.The bacterial colony was inoculated in the liquid media and optical densities at an interval of 2 hrs. from 0hr to 22hr were measured at 600nm. After getting the values a growth curve was plotted fig. 12. In the time interval of 0 h-5hrs the bacteria is in lag phase where they are active but not divining. They are still adjusting to the new environment. The time interval of the log phase was 5 hrs-14 hrs which shows the rapid increase in the growth of the bacteria. This time is the best time at which the inoculation for production media should be done to have maximum productivity. The suitable time interval in log phase is 8hrs-10hrs, here the bacteria are showing maximum activity and are diving actively. After 15hrs of incubation, the bacteria attained stationary phase.

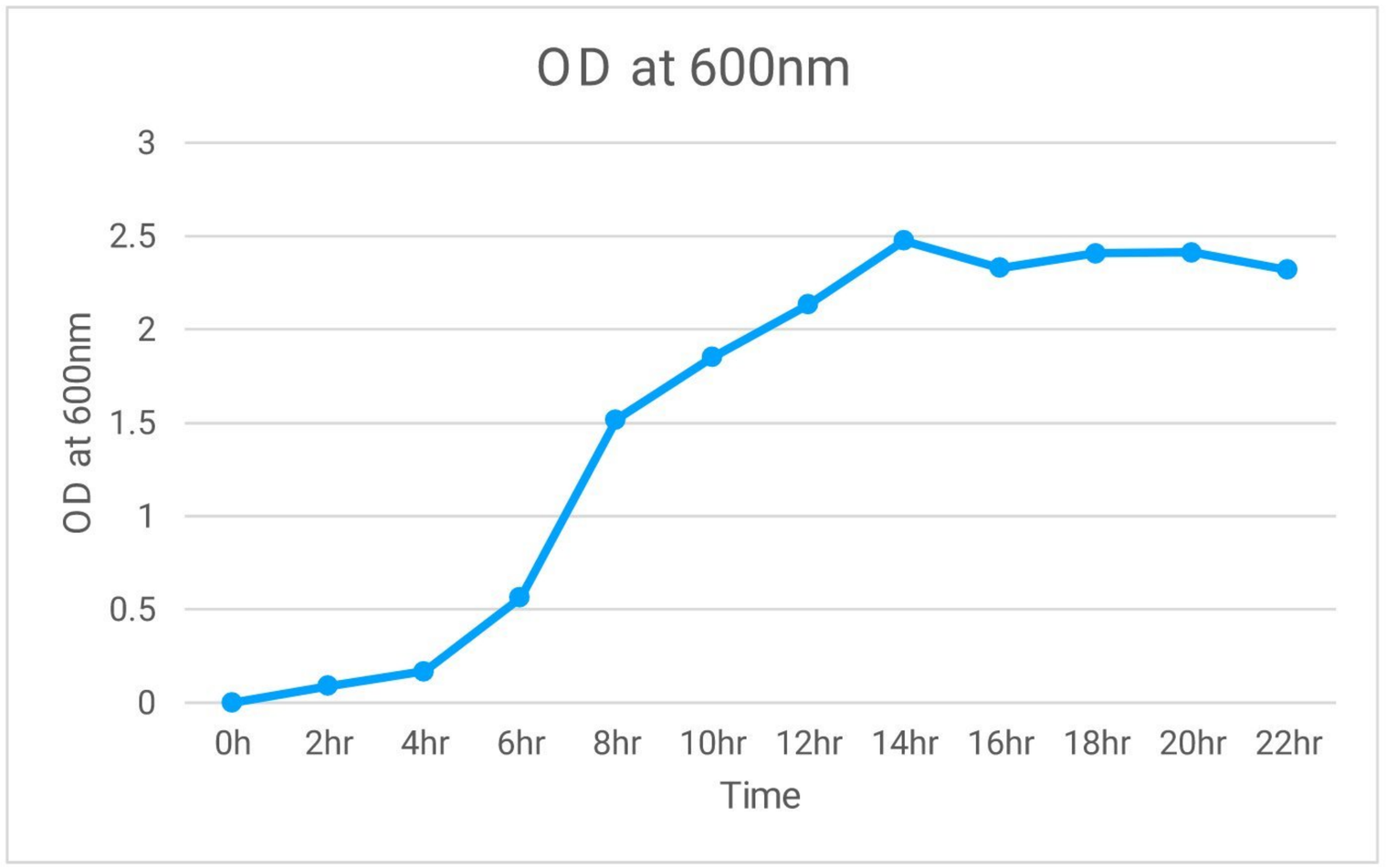


Fig. (12) Growth curve of the bacteria producing EPS

5. Conclusion

Exopolysaccharides are biomacromolecules which holds a very high potential for industrial use within its domain. They are an important component of biological systems. However, the physiological role of EPSs are still unexplored. (8)

From these series of experiments, we can conclude that the EPS was precipitated from the bacteria isolated from extreme conditions. The EPS produced were fibrous in morphology and the yield was 0.32g/l.

There were many techniques used for the isolation and purification of the bacteria producing EPS such as, centrifugation, sterilization, spectrophotometry, biochemistry analysis, culturing techniques, and microscopy.

6. Future prospective for experiment of LA-KT-8--A

- Fermentation kinetics of the bacteria
- Complete characterization of EPS using techniques like HPLC, NMR, and TLC etc.
- DNA sequencing of the bacteria producing EPS
- Cell toxicity of EPS

7. Troubleshooting

- When sample with dilution factor 10^{-4} was used for isolation and purification a large number of undefined colonies were obtained. Therefore, the sample with dilution factor 10^{-6} was used for isolation and purification of bacteria producing EPS.

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9. Feedback

Feedback for Host Institute

My experience at CSIR-IMTECH was wonderful. It has well equipped labs with an easy access. This helped me to get excellent hands-on training in my work. Along with a wide variety of labs it is rich in flora and fauna. Scientists and research scholars around the institute are very humble and helpful. They have a remarkable co-ordination amongst them.

Feedback for SRIP and GSBTM

It was a great opportunity provided by Government of Gujarat. This opportunity pushed me out of my comfort zone and allowed me to explore the real side of the research field. This experience has added value to my skills.

Signature of Intern

Signature of Project supervisor

Date:

Place: CSIR-IMTECH, Chandigarh.