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STUDY OF EXOPOLYSACCHARIDE EXTRACTED FROM *KLEBSIELLA SPP.* ISOLATED FROM THE GUT OF *POECILIA SPHENOPS*

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ABSTRACT

The normal microflora of aquatic organisms occupies vital niches on the surface of the body and in the digestive tract, thus preventing intrusion of pathogenic microorganisms. Microflora of the digestive tract of fish and shellfish plays an important role in the formation of resistance to infectious diseases. Intestinal microbes help maintain host health. Recent studies have shown tight connection between microbial population in gut and different health disorders. The microbial diversity of the digestive tract of fish participates in the secretion of enzymes, polysaccharides, vitamins, amino acids and other physiologically active materials and, therefore, is necessary for normal metabolism of an organism. Gastrointestinal microorganisms feed on the food of the macroorganism which is digested by the enzymes produced by them and by the latter. As a result, chymous is formed, the composition of which decides the abundance and qualitative composition of communities of gastrointestinal microorganisms. The present study is aimed at extracting Exopolysaccharide from *Klebsiella spp* from the gut of *Poecilia sphenops* by precipitating it in isopropanol and by identifying it using staining and biochemical tests. Furthermore Total sugars by Phenol-sulphuric acid method, Total protein content by Lowry's protein estimation method, Exopolysaccharide Characterization by FT-IR spectroscopy forms the other components of the study. The EPS extracted is utilised for emulsification of hydrocarbons and water and also for immobilisation of the mother organism.

KEYWORDS: EPS, *Klebsiella spp, Poecilia sphenops*, Immobilization and Emulsification.

INTRODUCTION

Poecilia sphenops is commonly sold in local market by the name '**molly**'. *Poeclia sphenops* belongs to the family Poeciliidae. It is classified under Order Cyprinodontiformes (Tooth-carps) and Class Actinopterygii (Ray-finned Fish). The wild variety of the common or short-finned molly has dull silvery colouration, with a few black dots on its skin and a bright yellow fringe on the ends of its rounded dorsal and caudal fins **(Uchechukwu U, *et. al.*, 2012)**. Individuals of this species exhibit wide ranges of colour variation that mainly incorporates silver, black and yellow-orange. The body is oblong with a round caudal peduncle and a small dorso-ventrally flattened head with protruding jaws that function as a scraping tool, ideal for rasping algae from benthic surfaces. Its mouth also possesses many rows of very small teeth, with the outer row being the largest and reducing in size with each successive row (**Figure 1**).

Figure 1: *Poecilia sphenops*

Fish gastro-intestinal tract is a habitat for bacteria just like that of human beings. Fishes are continuously exposed to the microorganisms present in water. These organisms will undoubtedly influence the microflora on external surfaces, including the gills of fish. Similarly, the digestive tract of fishes will continuously receive water and food that is populated with microorganisms. Thus microbial colonisation may well start at the egg and/or larval stage and continue with the development of the fish **(Aparna Balakrishna, 2011)**. Thus, the numbers and range of microorganisms present in the eggs, on food and in the water, will influence the microflora of the developing fish. Composition of intestinal microflora differs with organism as well as the environment it is exposed to (**Muthusamy Ashok Kumar, *et. al.*, 2011)**.

Klebsiella refers to a group of gram negative rod-shaped bacteria belonging to the family Enterobacteriaceae. *Klebsiella* organisms are categorized microbiologically as facultative anaerobic, non-motile bacteria (**Sunil T, *et. al.*, 20**13). *Klebsiella* organisms occur in soil and water and on plants and some strains are considered a part of the normal flora of the human gastrointestinal tract (**Magbooljan Noornissa Begum and Kasturi Revathi, 2014)**. These opportunistic pathogens is found in other vertebrates also. The genus is named for German physician and bacteriologist Edwin Klebs. In 1883 Friedlander isolated a capsulated *Bacillus* from the lungs of patient who died of pneumonia. This was named after him as Friedlander's *Bacillus*. Later on this organism was given the generic name of *Klebsiella*, which is ubiquitously present and reported worldwide. Strains of *Klebsiella* are responsible for a wide variety of diseases in humans (**Joana Azeredo and Rosario Oliveira, 1996)**. Traditionally, the bacteria *K. ozaenae* and *K. rhinoscleromatis* were recognized as separate species, but DNA studies indicate that they should be classified as subspecies of *K. pneumoniae*; for medical purposes, the species distinctions are still observed, however. Other *Klebsiella* species include *K. oxytoca* and *K. planticola*, which along with *K. pneumoniae* can cause human urinary tract and wound infections. *K. planticola* and certain strains of *K. pneumoniae* have been isolated from the roots of plants such as wheat, rice and corn (maize), where they act as nitrogen-fixing bacteria. *K. variicola*, which was discovered in 2004, also occurs on various plants, including rice, banana and sugar cane. This species of bacteria has also been isolated from hospital settings, where it may act as an opportunistic pathogen, similar to other *Klebsiella* organisms. The growth of bacteria in different habitats involves production and secretion of polysaccharides which are found outside its cell wall. These polysaccharides may be found outside as capsule attached to the cell wall or they may be secreted to the external medium as slime. Some produce capsule and still secrete polysaccharides to the medium. Polysaccharides are large chains of monosaccharide attached to each other by glycoside linkages. They can be linear or branched high molecular weight moieties (**Dlamini Abednego M, *et. al.*, 2007; P. Vijayabaskar, *et. al.*, 2011)**.

A polysaccharide might be important for the bacteria for adhesion to the substratum, protection or even infection. It has commercial values as well. These polysaccharides which are found external to the cell wall of the bacteria is commonly called Exo-polysaccharide or EPS. EPS's of microbial origin are ubiquitous in nature, have unique properties and can be isolated from the bacteria in fresh water, marine environment, extreme conditions and soil ecosystem. It usually consists of glucose, rhamnose, glucuronic, mannose, xylose etc. Exopolysaccharides are comprised of repeated units of sugar moieties, attached to a carrier lipid and can be associated with proteins, lipids, organic and inorganic compounds, metal ions and DNA. Specific functions and precise role of EPS's depend on structural units and ecological niches of the host microorganisms (**Maria C, *et. al.*, 1996**). EPS's produced by bacteria have great potential. Exo-polysaccharides are environment friendly natural polymers. Due to the presence of unique structural composition, EPS shows diverse applications such as in food formulations, pharmaceutical and cement based construction industry, etc. The application of exo-polysaccharides depends on the composition of the polysaccharide and the quantity of production (**Varinder Kaura, *et. al.*, 2013**). Their composition and structure is very varied: they may be either homo- or heteropolysaccharides and may also contain a number of different organic and inorganic constituents. Microbial EPS's occur naturally in many habitats. They are important in the formation of biofilm, a structure involved in the adherence to surfaces and in the protection of bacteria against noxious influences of the environment. Exopolysaccharides can be readily prepared in the laboratory by fermentation. Increasing attention is being paid to these molecules because of their bioactive role and their extensive range of potential applications (**Zambou Ngoufack François, *et. al.*, 2004)**.

MATERIALS AND METHODLOGY

Sample Collection

Poecilia sphenops fish samples were obtained from the local aquarium market in Chennai. Kolathur in North Chennai is the hub for ornamental fish trading in India. Healthy fishes of both sexes were selected for the work (**Figure 2**).

Figure 2: *Poecilia sphenops*

ISOLATION OF GUT MICROORGANISM - DISSECTION OF FISH

The fish was sacrificed and dissection was done just after the fish was killed. The fish was surface sterilised with ethanol in a sterile petriplate and placed on a dissection board. The fish was held at the petriplate with sterile forceps and fins were removed. Fins on both sides were removed and using sterile scalpel a cut was made near the gills and the cut was opened up. The gut was taken using forceps and was transferred to a sterile dish (**Figure 3**). The next step should be done immediately after dissection.

Figure 3: Dissection of fish for obtaining gut sample

SAMPLE PREPARATION

Gut was homogenised with minimum volume of sterile distilled water using mortar and pestle. The sample was transferred to a micro-centrifuge tube using micropipette and centrifuged at 1000 rpm in desktop centrifuge.

SERIAL DILUTION OF THE SAMPLE

99ml of distilled water was taken in a 100ml conical flask and was labelled as master dilution. 9ml of sterile distilled water was taken in 5 test tubes and the tubes were marked as 10^{-1}, 10^{-2}, 10^{3}, 10^{-4} and 10^{-5} representing the concentrations after adding the sample. From the micro-centrifuge tube, after centrifugation, 1ml of aqueous phase of the sample was transferred to the conical labelled master dilution and mixed well. From the above conical flask, 1ml is pipetted to the test tube labelled 10^{-1}. From 10^{-1} test tube, 1ml was transferred to 10^{-2} and this was repeated till 10^{-5}.

CULTURING OF MICROBES PRESENT IN THE SAMPLE – ISOLATION OF *KLEBSIELLA*

Gut sample of fish contains microorganism in it. When plated to a media, these microorganisms grow utilising the nutrients in the media. Nutrient agar is the most commonly used media. Many organisms grow better in specific media like Eosin Methylene-Blue Agar or Mannitol Salt Agar. Agar is used for the solidification of media.

PLATING OF SAMPLE IN NUTRIENT AGAR

Plating of sample allows the microorganisms to grow on solid support media in petriplates. Nutrient agar is the most commonly used media. It supports the growth of less fastidious microbes. It constitutes of peptone, yeast extract, beef extract, sodium chloride, agar-agar and water. It should be autoclaved for sterilisation at 121°C for 15 minutes. 100ml distilled water was taken in a 250ml conical flask. To that 0.5g of Peptone, 0.5g of Sodium Chloride, 0.15g of Beef extract, 0.15g of Yeast extract and 2g of agar were added and was heated in Microwave oven to dissolve the components. The conical flask was cotton plugged and kept for autoclaving along with four petriplates. Non-absorbent cotton was used for making the cotton plug. After autoclaving at 121°C and 15lbps pressure for 15 minutes the agar was poured equally to the petriplates inside the Laminar Air Flow cabinet. Laminar Air Flow cabinet was wiped with 70% ethanol and kept with UV for 20 minutes prior use. The agar was allowed to solidify. After solidification, serial dilutions 10^{-5}, 10^{-4} and 10^{-3} were quadrant streaked using inoculation loop on one plate each. The inoculation loop was heated red hot at the flame of a Bunsen burner and then cooled before using. One plate was kept as control. Plates were incubated for 24 hours at 37°C. Absence of growth in the control plate indicated the absence of contamination during the procedure. The subculture and second subculture was obtained by culturing on EMB Agar plate.

IDENTIFICATION OF MICROORGANISM

Bacterial identification was done by Gram staining, Capsule staining and various Biochemical tests which allows preliminary identification of microorganism. Biochemical tests are more specific than staining techniques. These tests involve chemicals and each genus of microbe will have specific results with these chemicals. The tests include Catalase test, Oxidase test, Indole test, MR –VP test, Voges Proskauer test, Citrate test and Urease test.

EXTRACTION OF EXOPOLYSACCHARIDE

150ml LB broth was prepared in two 250ml conical flasks each as per instructions in the hi-media bottle. The flasks were autoclaved at 121°C and 15lbps for 15 minutes. One of the flasks was marked as sample and the other was marked as control. One loop full of culture was inoculated to the conical flask marked sample and mixed well using a pre-sterilised Nichrome inoculation loop. The flasks were incubated for 24 hours inside a rotary shaker at 120rpm and was left for two days at 37°C without shaking (**Figure 4**).

Figure 4: LB broth kept in room temperature after 24 hours of shaking.

EXTRACTION OF EXOPOLYSACCHARIDE

From the 150ml of microbial culture in sample conical flask, 20ml each was poured to centrifuge tubes and were centrifuged in a 4°C cooling centrifuge at 9500 rpm for 20 minutes. The cell pellet was discarded and the supernatant obtained was transferred back to two conical flasks equally. 250ml of isopropanol was poured to both the conical flasks and vortexed well. Flasks were incubated at 4°C overnight. After 24 hours, the conical flasks were taken out and kept at room temperature for 20 minutes. The contents were poured to centrifuge tubes and centrifuged at 2000 rpm for 5 minutes and the supernatant were discarded. Pellet obtained contained exopolysaccharide which was transferred to sterile micro-centrifuge tubes and stored at 4°C for future use.

STUDY OF PROPERTIES OF POLYSACCHARIDE

Two properties were tested here namely the ability of the sample to form gel and the ability of the sample to coagulate blood.

TESTING FOR GEL FORMATION

Abilities of samples to form gels were tested by heating the sample solutions at different temperatures (50, 85, 100, 110 and 121°C). After heating, samples were allowed to cool and were examined for gelling.

TESTING FOR BLOOD COAGULATION

The ability of the sample to coagulate blood was tested based on a simple method. A clean glass slide was obtained and two drops of blood was placed on the slide. To one drop, the crude extract sample was added immediately and was mixed using a glass rod.

Qualitative analysis of carbohydrates in the sample was done by Molisch's test. Exopolysaccharides were estimated for total carbohydrates by phenol-sulphuric acid method. Test for total protein was done following Lowry's method for protein estimation. FTIR Analysis and Antioxidant study using Ammonium Molybdate Reduction Assay was carried out. The percentage was calculated using the formula

% Scavenging = (Ao – Ai)/Ao X100

where, **Ao is the O.D value of Ascorbic acid standard and Ai is the O.D value of the sample**.

APPLICATIONS OF EXPOLYSACCHARIDE

EMUSIFICATION USING EXOPOLYSACCHARIDE

Some microorganisms are able to produce biosurfactants and may enhance hydrophobic substrate utilization and detoxification. These microbial products are divided into low-mass molecules that lower the surface and interfacial tensions and high-molecular-mass polymers (e.g. polysaccharide–protein surfactants), which are more effective at stabilizing oil-in-water emulsions (**Rosenberg and Ron, 1999**). A number of bioemulsifiers contain a polysaccharide moiety attached to lipid and/or protein. Emulsan is a commercial emulsifying agent of biological origin.

10ml of sample was prepared for the test at 5mg/ml concentration. Four clean test tubes were obtained and 2ml of sample was added to each test tube. 2ml of gingelly oil was added to two of the tubes with sample and to the other two tubes coconut oil was added. Tubes were mixed thoroughly by vortexing. Tubes were incubated at room temperature for 96 hours. Tubes were observed at every 24 hours. The oil, emulsion and aqueous layers were measured at every 24 hr interval and an emulsification index (E) was calculated as the {(volume of the emulsion layer × total volume-1) × 100}.

IMMOBILISATION USING EXOPOLYSACCHARIDE

The use of immobilized cells has found applications in a wide range of biological processes. Industrial methods for cell immobilization are classified under two broad categories: immobilized-"free"-cell method and "modified"-cell methods. In the free-cell method, cells are immobilized by confining them behind membranes. Entrapment inside a matrix has limitations of a high degree of mass transfer resistance between the cell and the surroundings (**Pilkington, P. H *et. al* 1998**). Additionally, the use of entrapment sometimes requires harsh conditions and may result in damage or loss of viability. Some polysaccharide forms a gel in presence of multivalent ions like calcium or aluminium. The bacterial culture used was of the exopolysaccharide producing *Klebsiella spp.* is revived in nutrient broth the day before testing. 4% and 10% of the sample was prepared in two test tubes and 1000µl of the broth was mixed with 2ml of the sample. Meanwhile 1.5% calcium chloride solution was prepared and kept at 4°C. The mixture of sample and culture broth was loaded to a 5ml syringe and was added drop-wise to the chilled calcium chloride solution.

RESULTS AND DISCUSSION

ISOLATION OF *KLEBSIELLA* SPECIES

Isolated colonies were observed after 24 hours of incubation on nutrient agar plate when the gut sample was plated in nutrient agar (**Figure 5**). The colonies were isolated and they were light in colour and control plate showed no growth.

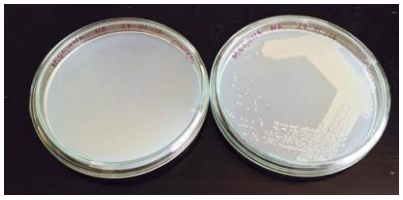
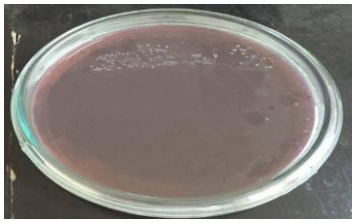
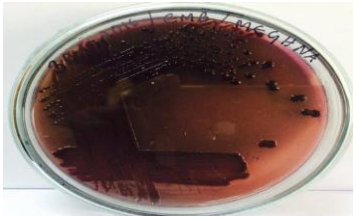


Figure 5: Sample plated on nutrient agar. Control plate showed no growth.

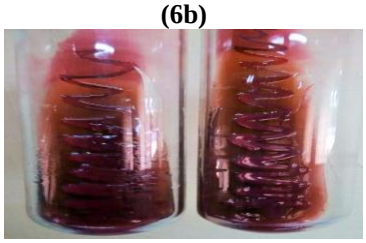
From nutrient agar when the isolated colonies were subcultured in EMB agar plated pinkish purple colonies developed. The colonies were faded pink as they developed and showed shiny violet colour after two days (**Figure 6(a)** and **6(b)**. The plates did not show greenish sheen indicating absence of *E.coli.* Control did not show any growth indicating no contamination. Subculture tubes also showed purplish pink growth (**Figure 6(c)**.



(6a)



(6b)



(6c)

Figure 6 (a): Initial growth on EMB plate after 36 hours of incubation
Figure 6 (b): Fully developed colonies on EMB plate after 48 hours
Figure 6 (c): Subculture tubes in EMB agar

IDENTIFICATION OF THE BACTERIA
GRAM STAINING
Gram staining results showed pink rods. The bacterium was identified to be gram negative rod (**Figure 7**).

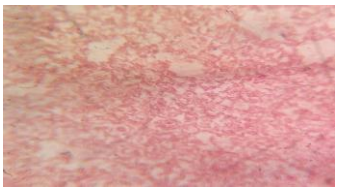


Figure 7: Gram negative rods

CAPSULE STAINING
Capsules were clearly seen transparent against dark colour of the cell (**Figure 8**).

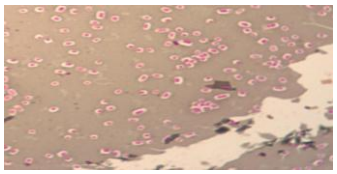


Figure 8: Capsule staining of the organism

BIOCHEMICAL TESTS
CATALASE TEST
The organism was found to be catalase positive. The organism was able to convert hydrogen peroxide to water and oxygen indicating catalase enzyme production by the organism (**Figure 9**).

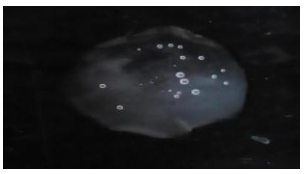


Figure 9: Production of bubbles by the organism in liquid hydrogen peroxide

OXIDISE TEST
The organism was negative for oxidase test (**Figure 10**). The organism showed no result on oxidase disc and thus inferring the lack of enzyme Cytochrome oxidase in the organism.

Figure 10: Negative result for oxidase test

INDOLE TEST
The organism showed indole negative (**Figure 11**). There was no colour change to pink on addition of Kovac's reagent to the indole tubes showing absence of tryptophanase enzyme which can convert amino acid tryptophan to pyruvate and indole.

Figure 11: Negative result for indole test

METHYL RED TEST
The organism was able to give acidic end product and was indicated by red colour of the MR-VP broth on addition of Methyl red reagent (**Figure 12**).

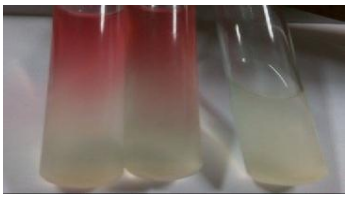


Figure 12: Positive result in MR-VP broth indicated by red colour

VOGES PROSKAUER TEST
The organism showed negative result in VP tubes. The broth changed brown in colour on addition of Baritt's reagent (**Figure 13**).

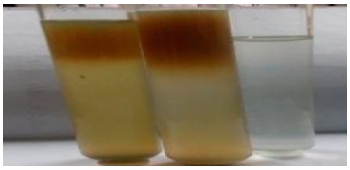


Figure 13: Negative result in Voges Proskauer tubes

CITRATE TEST
The organism showed positive for citrate test (**Figure 14**). The blue Simmon's citrate agar slants turned blue after 24 hours of incubation with organism. The

organism is able to utilise citrate as the only carbon source.

Figure 14: The first two tubes shows positive result. The third tube is the control tube

UREASE TEST

The only biochemical test which can show a distinction between *Enterobacter spp* and *Klebsiella spp* is urease test. The organism was able to grow in urea broth, changing the colour of the broth to a darker shade of pink (**Figure 15 & Table 1**).

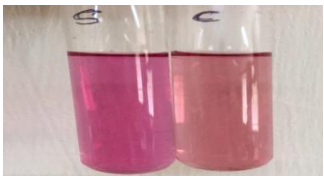


Figure 15: The sample tube shows positive and the other tube was control for urease test

Table 1: Identification of Bacteria

TEST FOR IDENTIFICATION	RESULT
Gram staining	Gram negative rod
Capsule staining	Capsule positive
Catalase test	Positive
Oxidase test	Negative
Indole test	Negative
Methyl red test	Positive
Voges proskauer	Negative
Citrate	Positive
Urease	Positive

From Table 1, the results of the biochemical tests and staining make it clear that the isolated microorganism belongs to *Klebsiella* genus.

EXTRACTION OF EXOPOLYSACCHARIDE

Luria bertani broth inoculated with the organism showed good growth after 72 hours. The broth turned turbid to a very high extend as shown in **Figure 16**, which was then used for centrifugation and extraction of exopolysaccharide.

Figure 16

Figure 17

Growth of Organism in LB Broth Micro-centrifuge tube containing Exopolysaccharide sample

The culture was centrifuged and precipitated overnight using isopropanol at 10°C. There was significant quantity of precipitate which was light brown in colour as shown in Figure 17.

STUDY OF PROPERTIES OF POLYSACCHARIDE

The polysaccharide did not show any gelling properties. It was also not easily dissolving in water. The polysaccharide also did not show coagulation of blood (**Figure 18**).

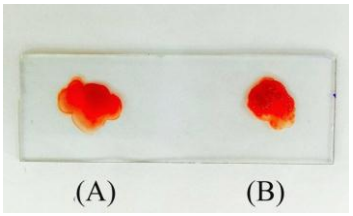


Figure 18: (A) Control blood (B) Blood sample without coagulation

COMPOSITION ANALYSIS OF THE EXTRACT

Compostion of the sample was analysed by Molisch's test, total protein estimation, total sugar estimation and FT-IR spectroscopy.

QUALITATIVE IDENTIFICATION OF SUGARS

Violet ring formation for Molisch's test indicates presence of sugars. This preliminary test was answered by the sample. A violet ring was formed at the junction of acid and α-naphthol indicating presence of sugars (**Figure 19**).

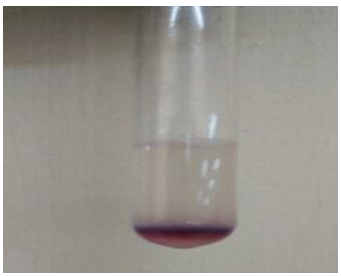


Figure 19: Positive result for Molisch's test indicating presence of sugars in the sample

TEST FOR TOTAL SUGARS

Phenol sulphuric acid method was used for analysis of total sugar content in the sample. Different grades of

yellow was developed in control tubes as well as in the sample tube, according to the concentration of sugars present, after incubation period and the absorbance was read at 490nm for each tube (**Figure 20 & Table 2)**.

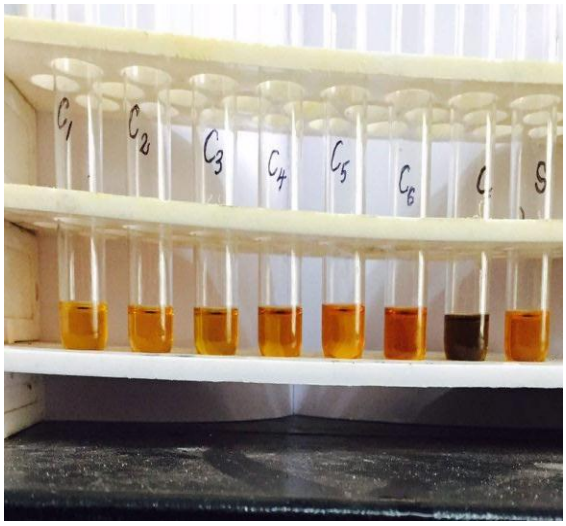


Figure 20: Test tubes 'C1' to 'C7' represents different concentration of Glucose from 10µg to 70µg respectively. The test tube marked 'S' shows the sample.

Table 2: Estimation of total sugars in the sample

SL. NO.	TEST TUBE NO.	CONCENTRATION (µG)
1	C1	10
2	C2	20
3	C3	30
4	C4	40
5	C5	50
6	C6	60
7	C7	70
8	S	50

The concentration of sugars present in the sample was 50µg/ml.

TEST FOR TOTAL PROTEINS

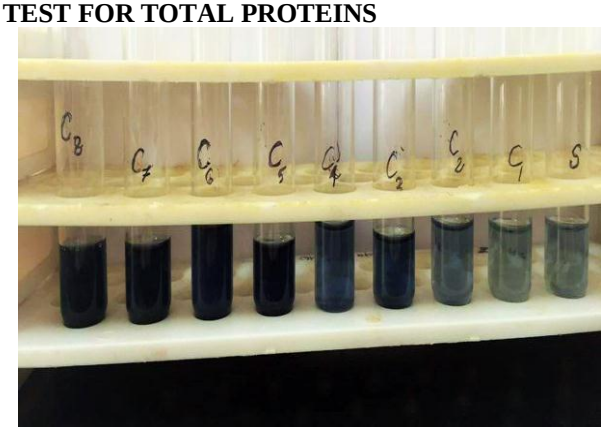


Figure 21: Test tubes 'C1' to 'C8' representing various concentrations of BSA from 10µg to 200µg respectively. Tube marked 'S' represents the sample.

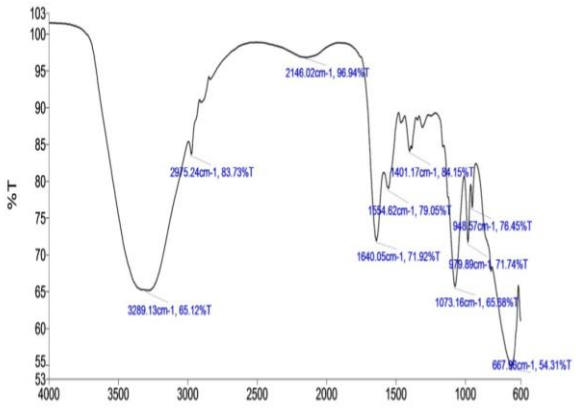
Different gradients of blue was observes in the control and sample test tubes which developed over the incubation period (**Figure 21**). Absorbance measured at 660nm is tabulated below in **Table 3**. The values were correlated and the concentration of proteins in the sample was estimated.

Table 3: Estimation of total proteins in the sample

SL. NO.	TEST TUBE NO.	CONCENTRATION IN µG
1	C1	10
2	C2	25
3	C3	50
4	C4	75
5	C5	100
6	C6	125
7	C7	150
8	C8	200
9	S	Unknown

The total protein content in the sample was determined to be 20µg/ml.

FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY



Graph 1: FT-IR spectra of the sample

FT-IR spectrum of *Klebsiella* exopolysaccharide was analysed and absorption bands were assigned to reveal typical polymeric structure of the carbohydrate as shown in **Graph 1**. Absorption bands in the range 850 – 1200 cm^{-1} corresponds to carbohydrates. A broad peak in the region 3289 cm^{-1} was observed which represented the stretching vibrations of the hydroxyl groups of the carbohydrate. This is the characteristic absorption band of carbohydrate responsible for hydrophilicity of the carbohydrate. The absorption at 1200-900 cm^{-1} was observed and is due to stretching vibrations of C-C, C-O-C and CO which is characteristic of carbohydrates. The absorption band at 2975 cm^{-1} represented C-H stretching bonds of methyl and methylene group. The absorption band at 1640 cm^{-1} usually represents the stretching vibrations of enol and amide groups. A sharp absorption

band at 1073 cm^{-1} indicated –C-O stretching vibration. The absorption band at 1073 also suggests the monomer mannose. The absorption band at 979 cm^{-1} shows the presence of glucose and galactose in *Klebsiella* exopolysaccharide. The spectra showed bands around 1000, 1400, 1500 and 1600 cm^{-1} revealed the (1,3) –ß-glucan linkages in addition to band at 2900 cm^{-1}. The IR spectrum of polymer proved the presence of carboxyl group which may serve as a binding site for divalent cations. This carboxyl group may also work as a functional moiety to generate an innovative new or modified polymer, by using different novel approaches like synthetic polymers.

TEST FOR ANTIOXIDANT ACTIVTY OF THE SAMPLE

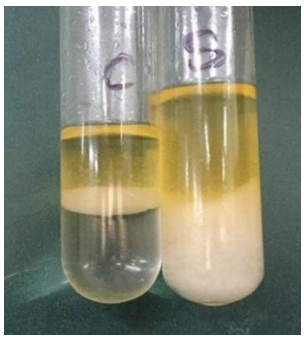
The absorbance of the sample was measure at 490nm and the value for sample was **0.12 O.D.**

APPLICATIONS OF EXPOLYSACCHARIDE

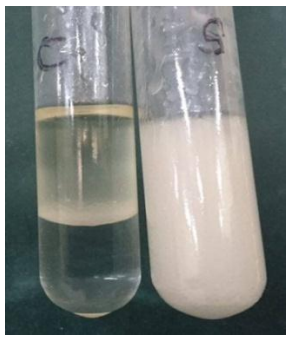
The exopolysaccharide was used for emulsification and immobilisation applications.

EMUSIFICATION USING EXOPOLYSACCHARIDE

Gingelly oil and coconut oil was used for testing emulsification. The levels of two solutions were measured daily and the level was learned to lower in every 24 hours. Coconut oil emulsion took a more time than that of gingelly oil. After 4 days the following results were observed. Figure 22 (a) shows the result in gingelly oil and 22 (b) in coconut oil. 'C' tubes in both the figures correspond to the hydrocarbon in distilled water without exopolysaccharide and 'S' tubes show hydrocarbon in aqueous solution of the *Klebsiella* exopolysaccharide. The *Klebsiella* exopolysaccharide produces good emulsification of hydrocarbons like gingelly oil and coconut oil. The emulsification index for gingelly oil was found to be 36.98 ±1.5 and the index for coconut oil was determined as 71.55 ±2.2.



22(a)

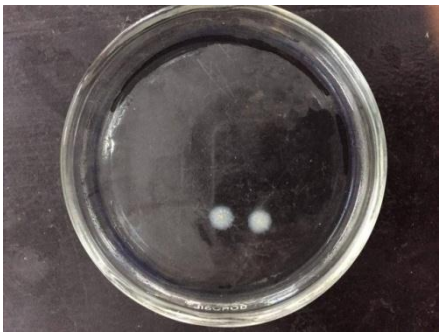


22(b)

Figure 22(a): Emusification of gingelly oil.
Figure 22(b): Emulsisfication of coconut oil.

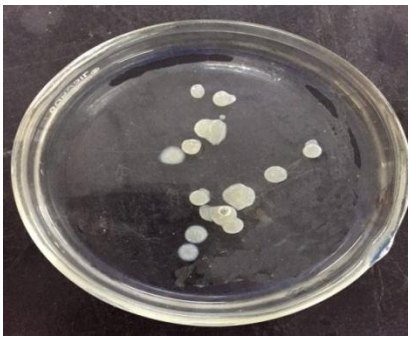
IMMOBILISATION USING EXOPOLYSACCHARIDE

Immobilisation of the mother organism was attempted at two concentrations. 4% and 10% of the exopolysaccharide showed immobilisation. At 4% the exopolysaccharide was not able to form exact bead structures but managed to form a flat structure (**Figure 23(a)**. However, 10% of exopolysaccharide showed proper immobilisation as beads, as shown in **Figure 23(b).**



23(a)

Figure 23(a): Immobilisation in 4% of polysaccharide



23(b)

Figure 23(b): Formation of immobilised beads by 10% of the exopolysaccharide

CONCLUSION

Summarising the work, bacteria was isolated from the gut of *Poecilia sphenops* and was identified as *Klebsiella spp.* by staining and biochemical tests. Exopolysaccharide was extracted by precipitating in isopropanol. Total sugars were estimated by using phenol-sulphuric acid method. The total protein content was determined by Lowry's protein estimation method. The exopolysaccharide was characterised by FT-IR spectroscopy and the absorbance peaks were analysed. The EPS was then utilised for emulsification of hydrocarbons and water. The polysaccharide was also used for immobilisation of the mother organism.

In conclusion, *Klebsiella spp.* showed good growth in LB broth and exopolysaccharide was successfully extracted. The total sugar content of the sample was found to be 50µg/ml and the total protein content was found to be 20µg/ml. The exopolysaccharide was partially characterised by FT-IR spectroscopy which showed the presence of C-H and O-H vibrations. Glucose, galactose and mannose were found to be the monomers of *Klebsiella* Exopolysaccharides. The Exopolysaccharides did not show any antioxidant activity. The *Klebsiella* EPS did not show gelling or

coagulation of blood. The extra cellular polysaccharide was able to emulsify hydrocarbons like gingely oil and coconut oil with water. At 10% concentration, the Exopolysaccharides was able to form bead like structure for immobilisation. The structural, physiochemical and morphological characters revealed the possible industrial applications of the exopolysaccharide. It has functional properties like good emulsification activity and ability to immobilise. These promising results can be regarded as an initiatory step towards future modification and utilisation of the exopolysaccharide as future cheap source for industrial applications. Further work is to be carried out to investigate the in-vitro and in-vivo activity and ecological role of the polymer.

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