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Biochemical Study of Exopolysaccharide from *Klebsiella Spp*. isolated from the Gut of *Poecilia Sphenops*

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A B S T R A C T

Exo-polysaccharides (EPS) are environment friendly natural polymers secreted by microorganisms to the surrounding medium. Due to the presence of unique structural composition, EPS shows diverse applications such as in pharmaceutical, and cement based construction industry, etc. Microbial derived EPS have advantages over traditionally used polysaccharides from plant source and seaweed-derived gums as the later are easily affected by environmental factors. EPS show different properties like, thickening, gelling, emulsifying, and ability for immobilisation. Bacteria offers wide diversity of exo-cellular substances with characteristic composition and properties, that provides opportunities for the development of new commercial product with improved properties. The present study is aimed at isolating EPS from the gut of *Poecilia sphenops* and conduct biochemical analysis. The extra cellular polysaccharide polymer produced from *Klebsiella spp*. can find its application for emulsification at the industrial level.

Keywords: EPS, *Klebsiella spp, Poecilia sphenops*, Biochemical Study and Emulsification

A R T I C L E I N F O

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1. Introduction

Poecilia sphenops is a common aquarium fish variety which lives both in fresh and marine water commonly encountered in Central America. It has been introduced to Asian countries including India and has been a very successful ornamental fish in the country since then. *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*

Many people find it very satisfying to keep a wide variety of species and types of ornamental fish, not only for their beauty but also to have a small part of 'another world' in their home. Ornamental fish keeping is a hobby which gives pleasure to the young and old alike. Aquarium creates an excellent opportunity to watch the glittering colours and graceful movements of fishes besides being a rewarding feast to the eyes and relieves the mind off stress **(Aparna Balakrishna, 2011)**. In ornamental fishes intestinal micro flora has been reported to aid in the digestion of algal cells and other products for the production of amino acids and the secretion of inhibitory substances that prevent colonization by bacterial pathogens (**Magbooljan Noornissa Begum and Kasturi Revathi, 2014)**. Ornamental fish culture has shown a rapid progress during the past few years due to its high demand in the market. Same like other fishes ornamental fishes also harbour a large variety of microorganism in them (**Muthusamy Ashok Kumar, *et. al.*, 2011)**.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 (**P. Vijayabaskar, *et. al.*, 2011**). 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 (**Varinder Kaura, *et. al.*, 2013**).

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.*, 2013**). *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. These opportunistic pathogens are 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* are 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 & 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).**

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. 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. The application of exo-polysaccharides depends on the composition of the polysaccharide and the quantity of production. 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)**.

2. Materials and Methods

Sample Collection

Poecilia sphenops fish samples were obtained from the local aquarium market in Kolathur in North Chennai which is a 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 carried out. The fish was surface sterilised with ethanol in a sterile petriplate and placed on a dissection board. Fins on both sides were removed and using sterile scalpel a cut was made near the gills and the gut was taken using forceps and was transferred to a sterile dish (**Figure 3**).

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 utilizing 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.

3. Results and discussions

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.

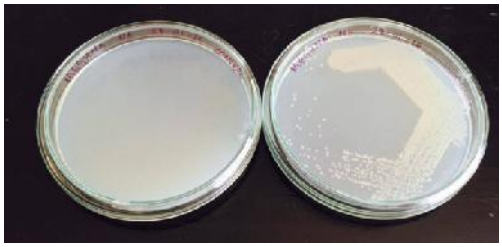

Figure 5: Sample plated on nutrient agar. Control plate showed no growth.

From nutrient agar when the isolated colonies were subcultured in EMB agar plate, 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)**).

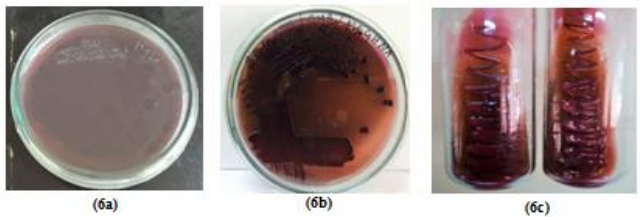

Figure 6(a): Initial growth on EMB plate after 36 hours of incubation. **Figure 6(b):** Fully developed colonies on EMB plate after 48 hours. **Figure 6(c):** Subculture tubes in EMB agar.

Identification of the bacteria

Gram staining: Gram staining results showed pink rods. The bacterium was identified to be gram negative rod (**Figure 7**).

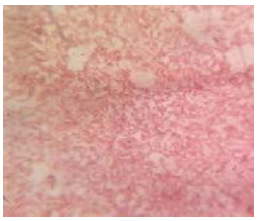

Figure 7: Gram negative rods

Capsule Staining: Capsules were clearly seen transparent against dark colour of the cell (**Figure 8**).

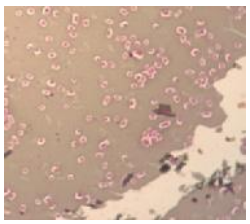

Figure 8: Capsule staining of the organism

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**).

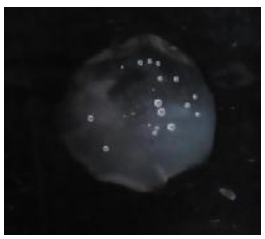

Figure 9: Production of bubbles by the organism in liquid hydrogen peroxide

Oxidase 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.

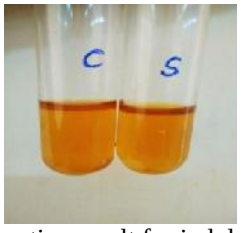

Figure 11: Negative result for indole test negative

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 were inoculated and 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**).

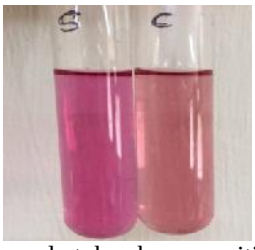

Figure 15: The sample tube shows positive and the other tube was control for urease test

Table 1: Identification of Bacteria

Tests 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: Growth of Organism in LB Broth
Figure 17: 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.

4. 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. In conclusion, *Klebsiella spp.* showed good growth in LB broth and exopolysaccharide was successfully extracted. Further work can be carried out to study the structural, physiochemical and morphological characters for possible industrial applications of the exopolysaccharide. Investigations can also be carried out to study the in-vitro and in-vivo activity and ecological role of this polymer.

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