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# Production and Purification of a Bioemulsifier and Flocculating Agent Produced by *Pseudomonas* sp.UBF 2

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**Abstract:** The emulsifying and flocculating activities of several biopolymers produced from different bacterial isolates were examined comparing with other pure gums. The highest biopolymer concentration (8.6 $gl^{-1}$) was obtained by an isolate which identified as *Pseudomonas* sp. UBF 2. This biopolymer exhibited the highest lipid emulsifying capacity (100 %) as compared with that xanthan gum (90 %). The minimum concentration of biopolymer solution to obtain a 100 % emulsion stable for 10 days against all oils was 0.2%, while against cotton seed oil was 0.5%. Moreover, the biopolymer exhibited high flocculating activity against activated carbon after 5 min. The rheological study suggested that the biopolymer has characteristics of the pseudoplastic fluid. The biopolymer was precipitated by cetyl-trimethyl-ammonium bromide suggesting the biopolymer as an acidic biopolymer. Carbohydrate analyses using various color reactions revealed that the biopolymer is a polysaccharide.

**Keywords:** Bioemulsifier, flocculating agent, extracellular polysaccharide, *Pseudomonas* sp

## INTRODUCTION

Nowadays, microbial biopolymers are considered as important sources of polymeric materials that have a great potential for commercialization. Due to their diversity in structure and unique properties, they have a wide range of food, pharmaceutical, and industrial applications. They can modify the flow characteristics of fluids, stabilize suspensions, flocculate particles, encapsulate materials and produce emulsions. Consequently, they are now widely used as thickener, stabilizer, emulsifier, gelling agent and water-binding agents in the food, cosmetics, bioplastics and oil industries[16]. Moreover, some polysaccharides have unique physiological activities as anti-tumour, anti-viral and anti-inflammatory agents as well as an inducer for interferon, platelet aggregation inhibition and colony stimulating factor synthesis[19].

Recently, increasing attention has been paid to microbial polysaccharides and a great number of microbial strains have been shown to produce polysaccharides with various compositions and functionalities[6]. Among them, are *Xanthomonas*, *Pseudomonas*, *Rhizobium, Erwinia, Aureobasidium, Leuconostoc* and *Vibrio* spp.[16,3,14,19,5,10,18,2] and a number of lactic acid bacteria[20,4,13].

The screening of novel microbial polysaccharides is promising because of the enormous range of microbial polysaccharides that have yet to be adequately explored. Many microbial polysaccharides have been extensively characterized and developed for commercial applications. This is due to the possibility of easy and quick mass production. In order to understand possible applications of microbial polysaccharides, studies on their chemical structure and physicochemical properties are essential[15].

In the present work, the ability of some local bacterial isolates to produce extracellular polysaccharides were determined, produced polysaccharides were purified and some of their physical and chemical properties were studied, including stabilizing effects on oil–water emulsions with a variety of vegetable oils as well as flocculating effects against activated carbon powder.

## MATERIALS AND METHODS

**Bacterial Isolates and Biopolymer Production Conditions:** Five viscid bacterial isolates were obtained from the soil of the Faculty of Agriculture farm, Ain Shams University, Shoubra El-Kheima, Cairo, Egypt, using striking plate method on medium containing 2 % glucose, 0.3 % Bacto-peptone, 0.05 % $MgSO_4.7H_2O$, 0.03 % $KH_2PO_4$ and 0.07 % $K_2HPO_4$[21].

For batch biopolymers production, 100 ml liquid medium containing 2 % glucose, 0.3 % Bacto-peptone,

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0.025 % $MgSO_4.7H_2O$, 0.01 % $K_2HPO_4$, and 0.002 % NaCl, in 250 ml Erlenmeyer flask was inoculated with each of tested isolates. Growth was maintained on a rotary shaker at 150 rpm for 5 days at 30°C[22].

Viscosity of cultures as an index of biopolymer production was measured daily for five days using a rotational viscometer (Cole – Parmer, USA) at a constant speed of 0.6 rpm using spindle number 5. Different biopolymers were precipitated, purified, dried and then tested for lipid emulsifying using olive oil. The bacterium producing highly emulsifying activity biopolymer was identified using the biolog instrument according to physiological characteristics.

**Purification and Determination of Biopolymers:** The amount of biopolymer was purified from different cultures broth by the method described by Yun & Park[21]. The culture broth (after diluted with distilled water as needed) was centrifuged at 14000 rpm for 30 min, to remove the cells. The biopolymer in the supernatant was harvested by acetone precipitation (1 culture: 3 acetone) and centrifugation. The sediment was washed with 70% ethanol and re-dissolved in distilled water. The biopolymer was further purified by cetyl-trimethyl-ammonium bromide (1:1) precipitation followed by ethanol precipitation in 10 % NaCl solution. After washing with 70 % ethanol, the precipitated biopolymer was lyophilized to obtain a purified biopolymer fraction then weighed and expressed as dry weight (g) per liter of culture broth.

**Lipid Emulsifying Test:** Emulsifying effect of the polysaccharide was tested using the method of Kurane & Nohata[11]. Equal volumes of olive oil and 0.5% biopolymer solution in distilled water were shaken for 10 min at 150 rpm on a rotary shaker to make a lipid emulsion. After the emulsion was centrifuged at 2000 *g* for 5 min, the height of the emulsified layer was measured. The lipid emulsifying activity was expressed as percentage of the height of emulsified layer per the height of whole layer. Other pure gums, i.e., dextran, pullulan, rhizobial exopolysaccharide[8], xanthan (Sigma Co.) and arabic gum were also tested. Moreover, the emulsifying activity of both the biopolymer and pure gum (which gave the highest emulsifying activity), against various oils like olive, corn, sunflower and cotton seed oil were tested using different concentrations ranging from 0.05 – 0.5 %.

**Biopolymer Analyses:** Hydrolysis of the selected purified biopolymer was carried out in a boiling water bath for 4 h using 2.5 M HCl. After being neutralized with $Na_2CO_3$ to pH 7.0, the hydrolyzed solution was concentrated using a vacuum evaporator, and filtered through a membrane with 220 nm pore size. The filtrates were used as the biopolymer hydrolyzate. Various colorimetric analyses of biopolymer and its hydrolyzate were performed according to the method of Dubois *et al*,[7].

**Morphology of the Purified Polysaccharide:** This was observed using a scanning electron microscope (JEOL, JSM, T330 A, Tokyo, Japan), Centeral Laboratory, Faculty of Agriculture, Ain Shams University, Shaubra El-Khiema, Cairo, Egypt.

**Rheological Characteristics of the Purified Polysaccharide:** Effects of shear rate (rotation speed rpm) and biopolymer concentrations of 0.02, 0.04, 0.06, 0.08 and 0.1% in water on the shear stress (cp) were investigated using a rotational viscometer (Cole–Parmer, USA) using spindle number 5[22].

**Flocculating Test:** Flocculating test was carried out in a test tube containing a mixture of 10 ml of 0.5 % activated carbon and 100 $\mu$l of 1 % $CaCl_2$ by the method of Kurane & Nohata[11]. To the test tube, 100 $\mu$l of 0.02 % biopolymer solution was added and mixed well to make a suspension. The resulting suspension was observed during incubation at room temperature. Flocculation activity of water, xanthan, dextran, pullulan, rhizobial exopolysaccharide, and arabic gum served as controls.

## RESULTS AND DISCUSSIONS

**Characterization of Bacterial Isolates:** Five bacterial isolates which showing slimy and viscid colonies were chosen. Cells were straight rods (one isolate was Gram positive and four isolates were Gram negative), motile, obligately aerobic.

**Production of Biopolymers:** During the growth of different isolates in the biopolymer production medium containing 20 $gl^{-1}$ glucose, apparent culture viscosity and biopolymer concentration were daily determined (Fig.1). Maximum levels of biopolymer concentration and viscosity were obtained by isolate No. 2 (Gram negative short rod) being 8.6 $gl^{-1}$ and 7452 cp, respectively, while the minimum levels were obtained by isolate No. 5 (Gram positive bacilli) being 2.1 $gl^{-1}$ and 2113 cp, respectively, after 96 hours.

**Emulsifying Capacity of Purified Biopolymers:** Microbial and plant gums as well as some plant and animal proteins have been known to possess lipid

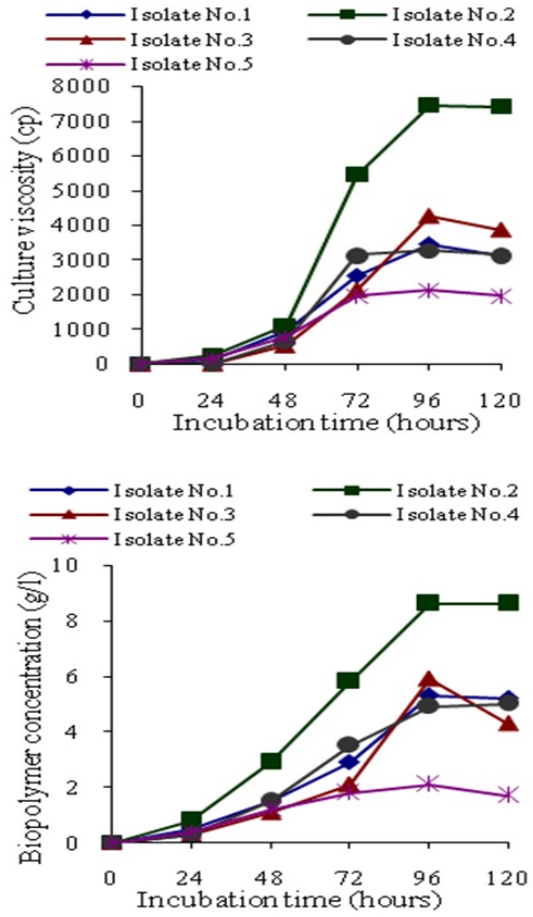


**Fig. 1:** Culture viscosity and biopolymer concentration of bacterial isolates during 120 hours at 30°C using shake flask as a batch culture.

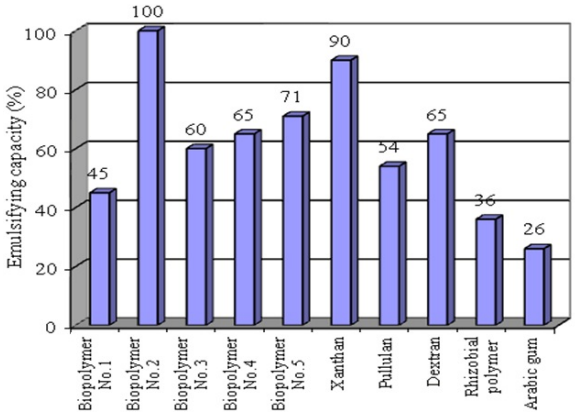


**Fig. 2:** Lipid emulsifying capacity of purified biopolymers produced by bacterial isolates compared with some pure polymers.

emulsifying effects. Especially, xanthan gum with microorganism origin has been widely used in the food industry because of its high emulsifying activity[16]. The emulsifying effect of the purified biopolymers was examined against olive oil. The biopolymer obtained from isolate No. 2 (biopolymer No. 2) showed the highest emulsifying capacity among all biopolymers tested (Fig. 2 & 3). The emulsifying capacity of the purified biopolymer was 100 %, representing that the whole layer of the lipid emulsion remaining stable after centrifugation at 2000 *g* for 5 min. Addition of the biopolymer resulted in the stability of the oil–water emulsion for 10 days. The lipid emulsifying capacities of xanthan, pullulan, dextran, rhizobial exopolysaccharide and arabic gum were 90, 54, 65, 36 and 26%, respectively. Sutherland[15] reported that, several polysaccharides from Gram-negative bacteria have been commercialized but currently only a very limited number are excellent viscosifying or suspending agents with high stability under a range of pH and temperature conditions. Xanthan and pullulan produced by *Xanthomonas campestris* and *Aureobasidium pullulans* have been most widely used in the food and other industrial fields, because of their high gelling and emulsion stabilizing functions. Their applications cover the production of salad dressings, relishes, tart sources, gelled meats, puddings, dense syrups, ice cream, and toothpaste[16,10].

**Emulsifying Activity of Biopolymer No. 2 Against Various Vegetable Oils:** The results of after mentioned experiment reveled that biopolymer No. 2 gave the highest emulsifying activity within tested isolates, while, xanthan gum was the best between tested pure gums in this respect. Therefore, the emulsifying activity of both biopolymer No. 2 and xanthan gum at different concentrations against various vegetable oils were examined (Fig. 4). Data show that the biopolymer No. 2 showed higher emulsifying activity than that of xanthan gum against all vegetable oils. The emulsifying activity of biopolymer No. 2 at 0.2 % was approximately equal to that obtained at 0.5 % against olive oil, sunflower oil and corn oil, for cotton seed oil, 0.5 % of both biopolymer No. 2 and xanthan gave highest results. Therefore, the biopolymer No. 2 produced by isolate No. 2 at 0.2 % is expected to have a great potential as a bioemulsifier.

**Flocculating Effect:** Many studies have been reported on the flocculating effect of microbial polysaccharides to replace synthetic flocculants, which are industrially used[17]. Therefore, flocculating effect of the purified biopolymers against a suspension of activated carbon in water was investigated (Fig. 5). Other pure gums including xanthan, pullulan, dextran, rhizobial exopolysaccharide and arabic gum were also examined as flocculating agents. The highest flocculating activity

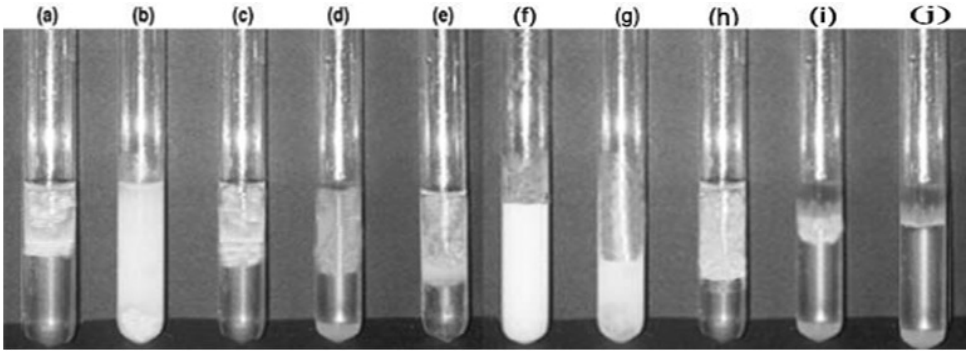


**Fig. 3:** Lipid emulsion stabilizing effect of the purified biopolymers on olive oil. The resulting emulsion was observed after incubation at room temperature for 10 days. Each tube represents the emulsion containing (a) biopolymer No. 1, (b) biopolymer No. 2, (c) biopolymer No. 3, (d) biopolymer No. 4, (e) biopolymer No. 5, (f) xanthan gum, (g) pullulan, (h) dextran, (i) rhizobial biopolymer and (j) arabic gum.

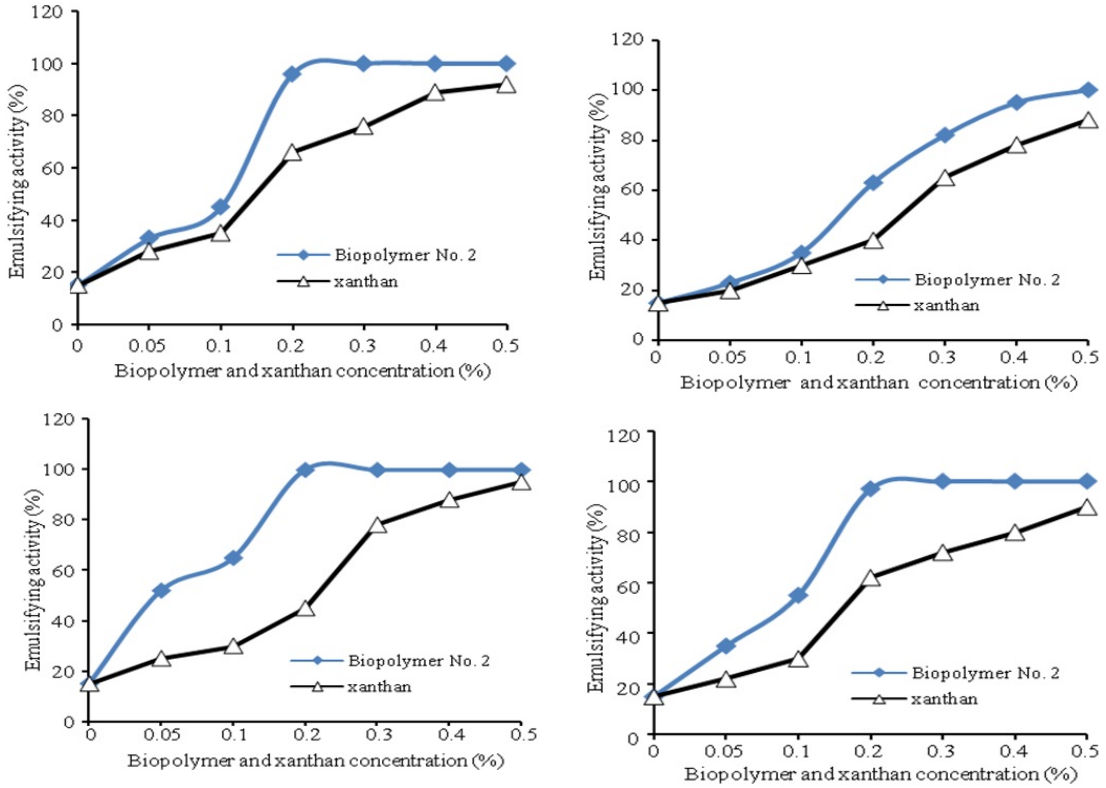


**Fig. 4:** Lipid emulsifying activities of different concentrations of biopolymer No. 2 and xanthan gum on various vegetable oils.

was observed by biopolymer No. 2 followed by xanthan gum, biopolymer No. 1, biopolymer No. 5 and pullulan (Fig.5 c & g). However, dextran, rhizobial exopolysaccharide and arabic gum showed poor flocculating effect (Fig.5 j & k). The unique flocculating activity of biopolymer No.2 suggests that it has a great potential as a flocculating agent.

**Rheological Characteristics of the Biopolymer:** Using various concentrations of purified biopolymer No. 2, the shear stress {culture viscosity (cp)} was determined according to the increase of the shear rate (Fig. 6). Its shear stress increased according to the decrease of the shear rate. However, the degree of the increase was not linear and declined at a higher shear rate, suggesting that the biopolymer showed characteristics of a typical

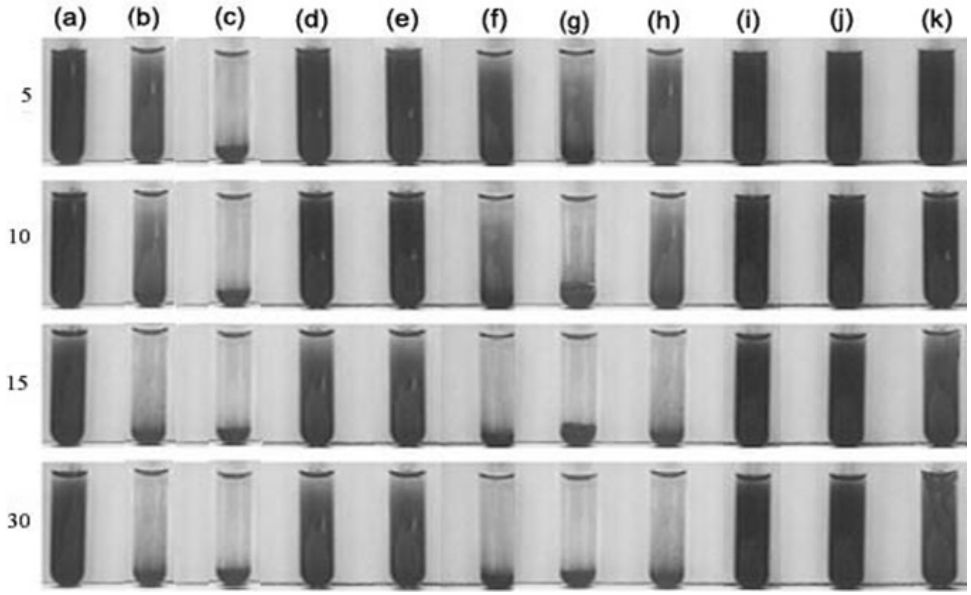


**Fig. 5:** Flocculating effect of the purified polysaccharides against activated carbon. Each tube represents the suspension containing (a) no polymer as a negative control, (b) biopolymer No. 1, (c) biopolymer No. 2, (d) biopolymer No. 3, (e) biopolymer No. 4, (f) biopolymer No. 5, (g) xanthan gum, (h) pullulan, (i) dextran, (j) rhizobial biopolymer, (k) arabic gum. Numbers on the left of the figure indicate the incubation time (min).

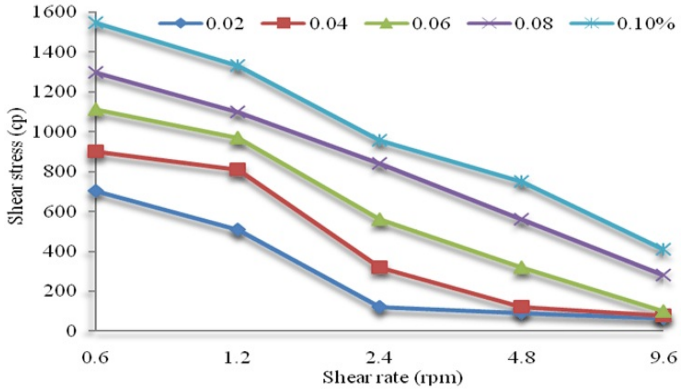


**Fig. 6:** Shear stress of different concentration of biopolymer No. 2 (i.e., 0·02, 0·04, 0·06, 0.08 and 0.1% in water) based on the shear rate using a rotational viscometer.

non-Newtonian pseudoplastic fluid[12]. Its shear stress also increased in the solution with a higher concentration of the biopolymer.

**Identification of Isolate No. 2 and Analyses of its Biopolymer:** Isolate No.2 (Gram negative short rods, Fig. 7a) the most efficient bioemulsifier producer, was identified as a strain of *Pseudomonas* sp. UBF 2. The biopolymer produced by *Pseudomonas* sp.UBF 2 was purified with several precipitation steps using acetone, ethanol and cetyl-trimethyl-ammonium bromide. As shown in Fig.(7b & c), a number of bacterial cells were found to stick to the biopolymer fibers obtained from the first step of precipitation (acetone precipitation). When the biopolymer was extracted with several precipitations of diluted culture broth, few bacteria were found attached to the biopolymer fibers as shown in Fig. (7c). During the purification, the biopolymer was precipitated by cetyl-trimethyl-ammonium bromide, suggesting that it is an acidic biopolymer[21]. The anthrone and Seliwanoff tests, suggested that the biopolymer is composed of sugars or their derivatives containing ketone group(s). Benedict and Fehling reactions for the detection of reducing sugars, in addition to Barfoed reaction for the detection of monosaccharides, were negative for the biopolymer and positive for the hydrolyzate.

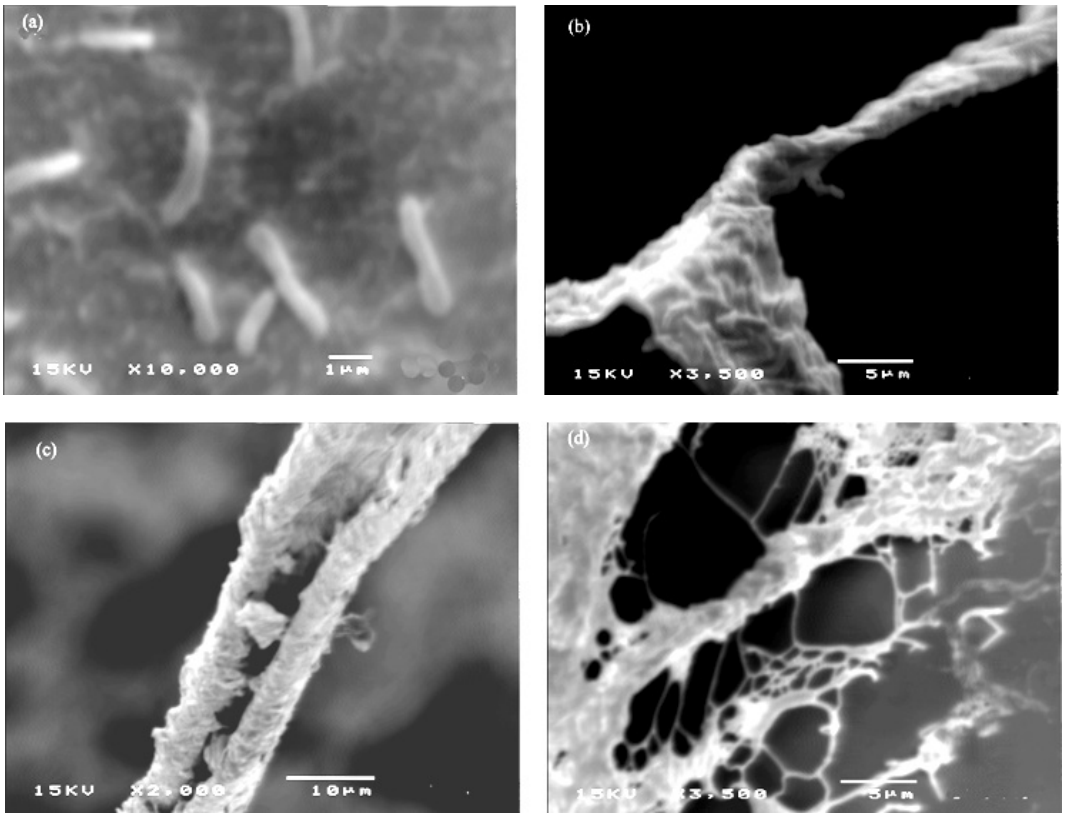


**Fig. 7:** Scanning electron microphotographs showing Pseudomonas sp. UBF 2. cells (a) and fibers of the biopolymer No. 2. The biopolymer was purified with acetone precipitation (b & c) and with absolute ethanol, cethyl trimethyl ammonium bromide and ethanol again of the five-fold diluted culture broth with distilled water (d).

The present data reveal that the exopolysaccharide produced by *Pseudomonas* sp. UBF 2 showed high emulsifying activity and flocculating effect as compared with pure xanthan gum (showing the highest emulsifying and flocculating effect among the tested pure gums). Therefore, it is expected that the produced exopolysaccharide may have great industrial applications.

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