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ASSESSMENT OF EMULSIFICATION AND ANTIMICROBIAL POTENTIALS OF EXTRACELLULAR POLYMERIC SUBSTANCES EXTRACTED AND PURIFIED FROM BIOFILM FORMING *KLEBSIELLA PNEUMONIAE* STRAIN MB398

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ABSTRACT

Extracellular polymeric substances (EPS) from microbial sources have gained considerable attention due to their unique and novel properties. Therefore, in present investigation purified EPS extracted from *Klebsiella pneumoniae* MB398 were characterized morphologically and biochemically, and also assessed for emulsification and antimicrobial activities. Colorimetric analysis revealed that EPS was comprised of uronic acids (15.48±0.013 µg mg^{-1}), pyruvic acids (12.86±0.061 µg mg^{-1}), carbohydrates (10.13±0.030 µg mg^{-1}) and proteins (1.85±0.044 µg mg^{-1}). FTIR spectroscopic analysis depicted the presence of carbohydrates, proteins, alkyl halides and nucleic acids, various hydrophilic and hydrophobic functional groups. SEM analysis revealed compact nature of EPS with particle size ranging between 3.35 to 26.68 µm. XRD profiling of EPS indicated crystalline nature of biopolymer. EPS formed 60.46±2.88, 58.06±0.94, 55.97±4.03 and 51.36±1.97 % stable emulsions against Kerosene, Diesel, Canola oil and n-Hexane, respectively. Quantitative assessment for emulsification activity showed that EPS from

MB398 gave highest emulsion retention values against Canola oil (1.330±0.045) following Kerosene oil (1.261±0.251), Diesel (1.051±0.006) and n-Hexane (0.794±0.120). Moreover, EPS demonstrated discernible antimicrobial activity against *Microbacterium oxydans* ES-CA1, *Bacillus pumilus* PB09, *Pseudomonas geniculata* ES-EC2, *Bacillus cereus* PB28, *Serratialaurentii* RE14 *and Enerococcus faecium* OGRIF. EPS extracts (5.0 mg L^{-1}) exhibited fairly good antimicrobial activities against target bacteria with zones of inhibition ranging between 1.10-1.91 cm. Results of antimicrobial activities will be helpful in elucidating the antimicrobial components present in EPS and their mechanisms in *Klebsiella sp.* All these features suggested extracellular polymeric substances from *Klebsiella pneumoniae* MB398 as promising candidates for being applied as biological agents, and in detoxification/bioremediation activities.

Keywords: Extracellular polymeric substances; *Klebsiella pneumoniae*; Morphological and biochemical analysis; Emulsification; Antimicrobial activity and components

INTRODUCTION

Production of extracellular polymeric matrix is one of the unique features of microorganisms among various chemical, physiological and biological characteristics. It also hallmarks the degree of microbial cell attachments with one another and on to a substratum or surface [1]. The term extracellular polymeric substance (EPS) was used for the first time by Sutherland [2], who described them as high molecular weight (Mw >10,000) carbohydrate containing biopolymers, which constitute the intracellular spaces of microbial aggregates in order to assemble the structure and architecture of biofilm matrix [3-6]. These metabolic products often surround or accumulate on both prokaryotic and eukaryotic microbial cell surfaces and facilitate microbial entrapment and immobilization thus participate in biofilm formation and comprise about 50-90% of total organic matter in biofilms [4, 7-10]. They remain closely associated with cell surfaces as discrete capsules (bound EPS) or else get released and excreted in the form of unattached loosely bound slimesaround the microbial cell surfaces (free EPS) or found in extracellular medium [1, 5,10]. Extracellular polymeric substances are comprised of wide variety of organic and inorganic components [8, 11]. These substances exist in complex structural diversity consisting either of homopolysaccharides composed mainly of D-glucose such as levans, dextrans and

mutans etc. or heteropolysaccharides with repeating units ranging from disaccharides to octasaccharides having unusual branches or chains [11, 12]. EPS matrix also contains nucleic acids, lipids, uronic acids, pyruvic acids and humic substances [1, 4, 8,-9, 13].

Exopolymeric substances play vital roles in various processes like biofilm formation, maintenance of primary cellular functions and enzymatic activities, plasma substituting capacity protection of microbial cells from desiccation, and harsh environmental conditions and stresses e.g. toxins and antimicrobial agents, pollutant degradation and bioremediation activities [11, 14]. They also function as carbon and energy reserves during nutritional deficiency or starvation, facilitate communication among microorganisms via physical and chemical signals and symbiosis [11, 15]. Moreover, EPS secreted by many microorganisms produce antimicrobial components which impart antimicrobial activities against predators and control microbial attachment [6].

Many microorganisms secrete exopolymeric substances (EPS) as a strategic part of their growth to facilitate adhesion to various surfaces and survival against adverse environmental conditions. These biopolymers offer numerous advantages, have unique physical or chemical properties, and have been applied extensively in various industrial setups. During couple of years, the demand and interest in production and applications of natural polymers in food, pharmaceutical and many other industries has remarkably increased, therefore, exopolymers produced by microorganisms have gained much attention [5, 12]. Microbial EPS are usually more preferred in industry because of their interesting and novel functions, continuous supply, structural variability, reproducibility, affordability and stable costs, unique rheological, physical, chemical and many other features [4, 9, 12]. Hence, they have been applied extensively in biomedical, pharmaceutical and food industries as well as in bioleaching, bioremediation and wastewater treatment [11].They have been applied as stabilizers, emulsifiers, adhesives, gelling and coagulating agents etc. They are also useful in oil recovery, reclamation of heavy metal pollutant compartments, detergents, textile, beverages and food industries. All these aspects have motivated the concerns in isolating new EPS producing microorganisms from different environmental settings [5, 12]. Therefore, this study involved the biochemical characterization of EPS extracted from

biofilm forming bacteria *Klebsiella pneumoniae* MB398 by quantification of carbohydrate and protein contents, pyruvic acid and uronic acids. Structural determination was performed by FTIR spectroscopy, surface morphology and elemental composition by SEM and EDX/EDS analysis and XRD technique was applied to study the nature of EPS. Antimicrobial activities of EPS against pathogenic bacteria and potential to form stable emulsions with different hydrophobic substrates were also evaluated.

MATERIALS AND METHODS

Microorganism used

Klebsiella pneumoniae MB398 (GenBank accession number KP886827) was used for extraction and purification of extracellular polymeric substances (EPS) following Verhoef et al. [16].

Structural and biochemical characterization of EPS

Extracted EPS matrix was analyzed using UV-VIS spectroscopy, SEM and IR techniques.

Quantification of total carbohydrate content

Total carbohydrate content was determined using phenol-sulfuric acid assay with glucose (0.1 mg ml^{-1} of PBS) as calibration curve ranging from 1-11 µg ml^{-1}[17] at 490 nm.

Quantification of protein content

Amount of proteins present in EPS were quantified using Bradford method [18] at 595 nm with bovine serum albumin (0.1 mg ml^{-1} of PBS) as calibration curve ranging between 1-12 µg ml^{-1}.

Quantification of pyruvic acids

Quantification of pyruvic acid content was performed following Slonecker and Orbntas[19] at 375 nm using pyruvic acid (100 µg ml^{-1}) ranging from 1-15 µg ml^{-1} as calibration standard.

Quantification of uronic acids

Uronic acid content was determined by Filisetti-Cozzi and Carpita method [20] at 525 nm with galacturonic acid (0.05 µg ml^{-1} PBS) ranging from 1-16 µg ml^{-1} as calibration curve.

Fourier transform infrared (FTIR) spectroscopic analysis for identification of functional groups

Major functional groups present over the entire surface of EPS were detected through Fourier transform infrared spectroscopy. Briefly, spectroscopic grade KBr was added to dried EPS samples (5:95 ratio), ground till appearance of fine powdered form. The sample was then pressed using hydraulic presser for obtaining IR transparent pellets. IR spectrum of the samples was taken using FTIR-8400 Schimadzu spectrophotometer.

FTIR analysis was performed in IR region of 4000-250 cm-1 with 15 scan speed.

Scanning electron microscopic (SEM) analysis of EPS matrix

The structure, morphology and particle size of EPS was observed by scanning electron microscope (MIRA 3 TESCAN). Dried EPS sample mounted on stainless steel stubs with double coated adhesive tape was placed in sputter coater chamber and carbon coated in presence of argon (8-10 psi). The specimen was then examined under SEM.

Elemental analysis of EPS matrix extracted from biofilm forming bacteria

Energy dispersive X-ray spectroscopy (EDX/EDS) coupled with SEM was performed for elemental analysis of EPS. X-rays emitted from EPS matter revealed the weight and atomic percentages of different elements present in EPS.

X-ray diffraction analysis

Powdered EPS was analyzed by X-ray diffraction using PAN alytical X-ray diffractometer (XPERT-PRO) (Dusseldorf, Germany), with Cu-Kα X-rays of wavelength 1.5406 ˚A. 2θ data was extracted at scan range from 5 to 80˚, at scan step size of 0.02˚ a;;nd time per step of 0.4 sec by providing voltage of 40 Kv and 30 mA beam current. d-spacing was calculated by Bragg's equation.

$$d = \lambda/2\sin\theta$$

Emulsification index

Emulsifying stability of EPS was determined against hydrophobic substrates (Canola, Kerosene, Diesel and n-Hexane) by measuring emulsification index [21]. Substrates were added to test tubes containing purified EPS suspension (1 mg EPS dissolved ml^{-1} of PBS) in equal volumes (i.e. hydrophobic substrate:EPS in a ratio of 2:2). Tubes were agitated vigorously for 2 min and left to stand for 24 hours at room temperature. After 24 hours height of stable emulsion layer and aqueous layer was measured. Emulsification index (E24) was calculated as:

$$\text{Emulsification index} = \frac{\text{Height of emulsion layer (cm)}}{\text{Total height of liquid (cm)}} \times 100$$

Emulsification activity of extracellular polymeric substances

Emulsification activity of EPS was evaluated as described by Patel and Desai [22]. About 0.5 ml of EPS solution (0.1 g ml^{-1} in PBS) was poured in screw-capped test tube. 7.5 ml of Tris-Mg buffer and 0.1 ml of hydrophobic substrate (Canola, Kerosene, Diesel and n-Hexane) was added to the tubes. The mixture was thoroughly vortexed at high speed and allowed to equilibrate for 1 hour at room temperature. After one hour its absorbance was measured at 540 nm using UV-VIS spectrophotometer against the blank (buffer+respective hydrophobic substrate).

Antimicrobial activities of purified EPS

Antimicrobial activities of EPS were assessed for using 10 pathogenic strains (*Microbacteriumoxydans* ES-CA1, *Bacillus pumilus* PB09, *Klebsiella pneumoniae* PB08, *Pseudomonas geniculata* ES-EC2, *Bacillus cereus* PB28, *Alcaligenes faecalis* MB90, *Serratialaurentii* RE14, *Enterococcus faecalis* JH22, *Klebseilla pneumoniae* MB81, *Enterococcus faecium* OGRIF) as test organisms. Antimicrobial activity was assessed by well diffusion method. Purified EPS (100 mg ml^{-1}) was dissolved in PBS to get a homogenous solution. 25 µl of each target pathogenic strain (suspended in 1ml of autoclaved distilled water) was grown on nutrient agar plate using spread plate method. The plates were left for 20 minutes and then wells were bored into the plates using sterilized borer (3 mm in diameter). 10 µl of molten agar was poured at the bottom of the wells and allowed to solidify. After solidification 25 and 50 µl of EPS suspension (in duplicate) was poured in the wells and incubated at 37˚C for 24 hours. Antibacterial activity was evaluated through measurement of clear zones of inhibition around the wells.

RESULTS AND DISCUSSION

The extracellular matrix extracted from *Klebsiella pneumoniae* MB398 was rich in uronic acid content compared to that of pyruvic acid, carbohydrates and protein contents (Table 1). Occurrence of uronic acids in higher quantities is an indicative of acidic nature of EPS which plays a crucial role in emulsification activity (Table 4, 5) as reported by Ismail et al. [12]. Structural analysis revealed two sharp peak bands (at 617.24 and 1116.82 cm^{-1}) which represented C-O-C and P-O-C bonds associated with phospholipids, RNA and aromatic compounds, and ring vibrations of P=O, C-O-C & C-O-P associated with phosphodiesters and polysaccharides (Fig 1). Broad peak at 3419.90 cm^{-1}position showed O-H stretching vibrations in polymeric structure, whereas, weak visible peaks recorded at 1396.51, 1456.30, 1558.54, 1647.26, 2852.81 and 2922.25 cm^{-1} were characteristics of amino acids (C-O stretching of COO^- groups), proteins (C-O, $C-H_3$ and $C-H_2$ stretching), amide II stretching vibrations (C-N and N-H), C=O stretching vibrations in proteins (amide I), $N-H_2$ of primary amines, membrane lipids & fatty acids, asymmetric stretchings of $C-H_2$ related to aliphatic chains of proteins, carbohydrates and lipids etc. Hence, FTIR finger printing of EPS biomass showed the presence of different functional groups arising from diverse polysaccharide structures, alkyl halides, proteins, membrane

lipids and fatty acids, uronic acids and nucleic acids as well as the humic substances, thereby suggested complex nature of EPS matrix both with hydrophilic and hydrophobic properties (attributed to presence of hydroxyl groups and aliphatic C-H_2 groups). Presence of these biomolecules in EPS matrix was supported by reported literature [7-10, 13, 23-25]. Furthermore, these functional groups within the molecular chains of exopolymer are one the important determinants of emulsification activity [12].

Morphological appearance of EPS matrix was analyzed through scanning electron microscopy (SEM). SEM analysis provided information about the surface morphology and particle size of EPS matrix. EPS from MB398 appeared as rod shaped which were long and broad, with compact nature and had constricted interspaces between them. Particle size was recorded between 3.35 to 26.68 µm (Fig 2). Almost similar findings have been reported for EPS matrix extracted from *Cellulomonas* sp., *Sinorhizobium meliloti* and *Oceano bacillu siheyenessis* [13, 24, 26]. Compactness with porosity has also been reported in EPS extracted from *Azotobacter* sp. and *Vibrio parahaemolyticus* [9, 27]. While the elemental analysis through EDX/EDS showed that EPS biopolymer from MB398 was comprised of variable proportions (expressed in weight and atomic percentages Table 2) of carbon, oxygen, sodium, phosphorous, sulfur and potassium (Fig 3).Likewise, EPS containing high ratios of carbon, nitrogen and oxygen in addition to sulfur and phosphorus have also been reported from *Cellulomonas* sp. [26].

X-ray diffraction (XRD) is a widely applied technique that facilitates phase identification of polymeric substances (Kavita *et al.* 2011). Hence, this technique was applied for studying the basic characteristics and nature of extracellular polymeric substances. X-ray diffraction profile of EPS from MB398 displayed characteristic peaks at 23.76˚, 30.46˚, 31.49˚ and 466.4˚ with interplanar spacings (d-spacings) at 3.75 ˚A, 2.93 ˚A, 2.84 ˚A and 1.95 ˚A, respectively (Table 3). Sharp peaks in the diffractrogram indicated crystalline nature of EPS biopolymer (Fig 4). On the other hand Chowdhury et al., [28] and Kavita et al. [9] reported amorphous and/or non-crystalline nature of EPS matrices extracted from *Bacillus megaterium* RB-05 and *Vibrio parahaemolyticus*.

Emulsification indices of EPS from MB398 with different hydrophobic substrates are shown in Table 4 and Fig 5. Results showed that the biopolymer possessed the capability of stabilizing the tested hydrophobic substrates. Most stable emulsification index

was recorded as 60.46±2.88% against Kerosene oil. These indices were complimented by the findings of Kavita et al., [9] for EPS extracted from *Vibrio parahaemolyticus*. Around 66.47% stable emulsions have been reported to be produced by EPS of *Oceanobacillus iheyensis* against hexadene [13]. The emulsification activity of EPS polymer from MB398 against various hydrophobic substrates was also determined by its strength in retaining the emulsion breaks quickly within one hour of incubation. Absorbance readings provided a fairly good indication about the stability of exopolymer emulsion with most efficient emulsification activity against Canola oil (1.330±0.045), following Kerosene oil and Diesel, respectively (Table 5). Ismail et al., [12] reported that EPS obtained from *Pseudoalteromonas* sp. displayed fairly good stability against Paraffin oil (0.289), n-Hexadecane and Tween 80 (0.165), and Kerosene oil (0.149). These emulsification properties can also be attributed to the presence of hydroxyl (hydrophilic) and aliphatic C-H_2 (hydrophobic) functional groups within the extracellular polymeric substances [13].

Table 1: Various components present in EPS matrix extracted from *Klebsiella pneumoniae* MB398

MB398	Carbohydrates	Proteins	Pyruvic acid	Uronic acid
Content (µg mg^{-1})	10.13±0.030	1.85±0.044	12.86±0.061	15.48±0.013

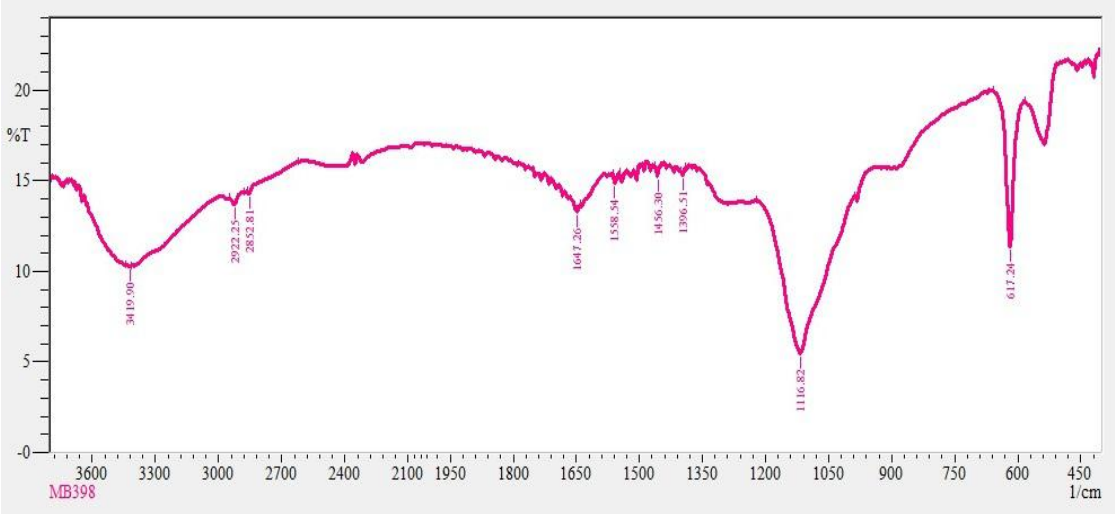


Figure 1: FTIR spectrum of EPS matrix extracted from MB398 showing major functional groups

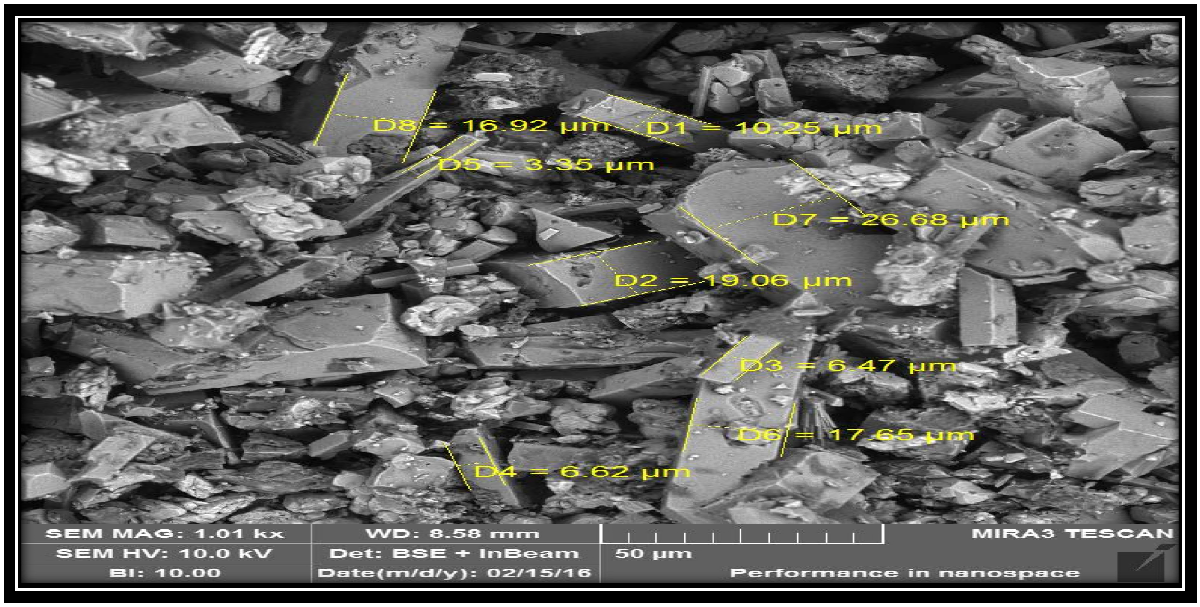


Figure 2: Scanning electron micrographs of EPS matrix extracted from *Klebsiella pneumoniae* MB398

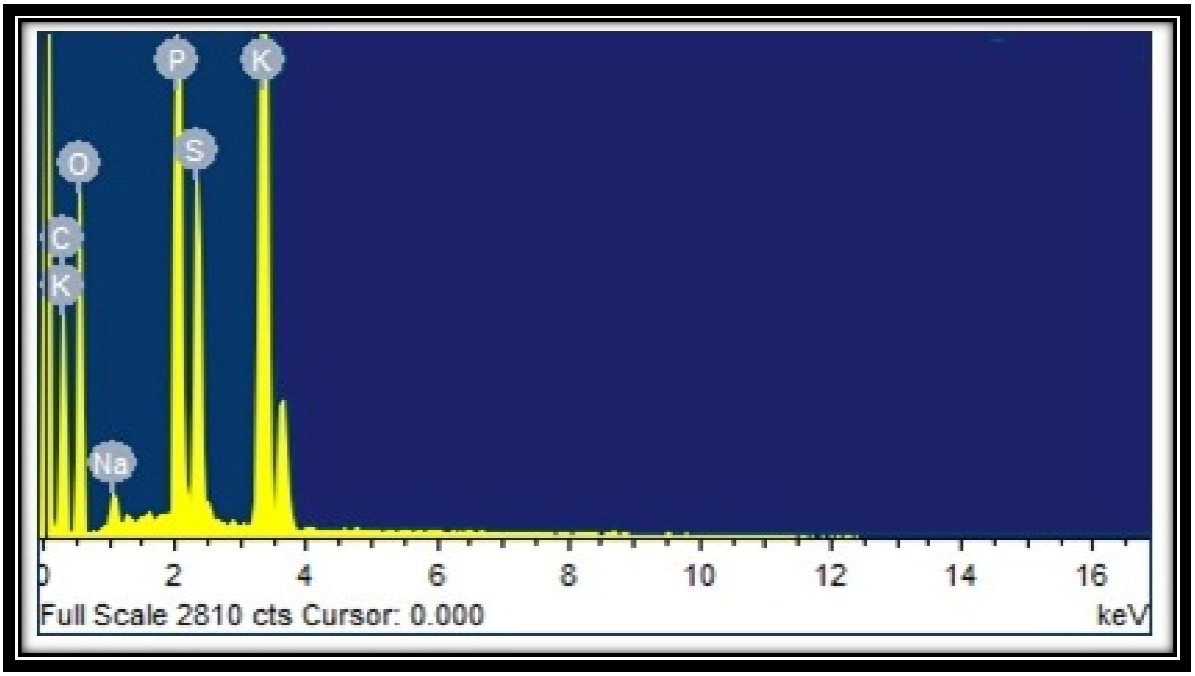


Figure 3: EDX/EDS analysis representing elemental composition of EPS extracted from *Klebsiella pneumoniae* MB398

Table 2: Results of elemental analysis performed through EDX/EDS for EPS extracted from *Klebsiella pneumoniae* MB398

Elements	Composition	Source of EPS
		MB398
Carbon	Weight (%)	31.29
	Atomic (%)	46.15
Oxygen	Weight (%)	31.96
	Atomic (%)	35.39
Sodium	Weight (%)	0.59
	Atomic (%)	0.45
Magnesium	Weight (%)	0.47
	Atomic (%)	0.34
Phosphorous	Weight (%)	7.24
	Atomic (%)	4.14
Sulfur	Weight (%)	6.42
	Atomic (%)	3.55
Potassium	Weight (%)	22.02
	Atomic (%)	9.98

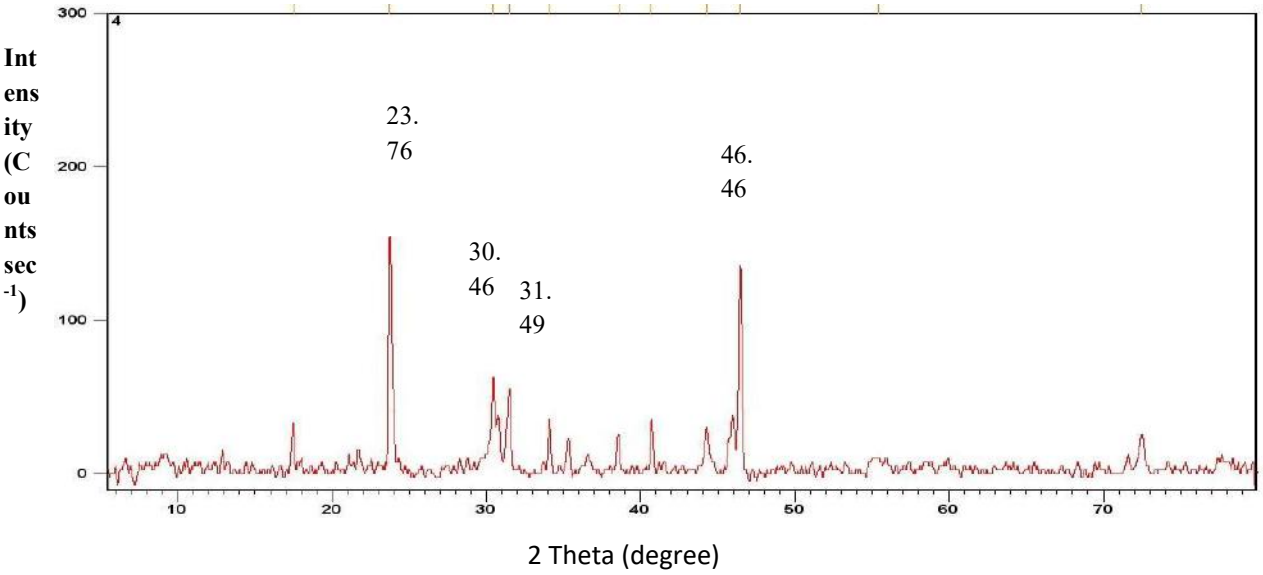


Figure 4: X-ray diffraction pattern of EPS obtained from *Klebsiella pneumoniae* MB398

Table 3: X-ray diffraction data of EPS extracted from *Klebsiella pneumoniae* MB398

Source of EPS	2 theta position (degree)	d-spacing (˚A)	Relative intensity (%)
MB398	23.76	3.75	100
	30.46	2.93	41.08
	31.49	2.84	37.65
	46.46	1.95	94.14

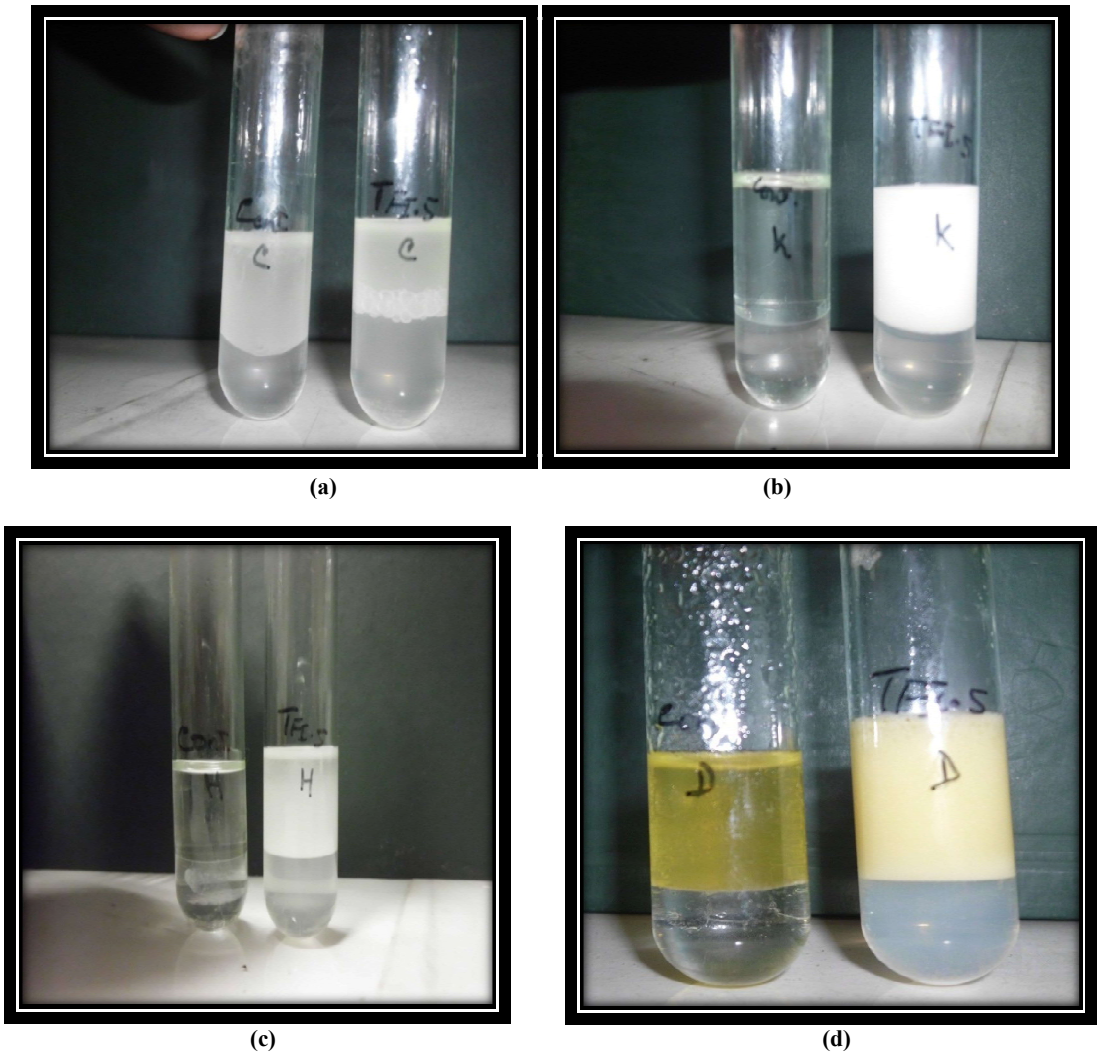


Figure 5: Emulsification index of EPS extracted from *Klebsiella pneumoniae* MB398 with hydrophobic substrates (a) Canola oil, (b) Kerosene oil, (c) n-Hexane and (d) Diesel

Table 4: Results of emulsification index of extracellular polymeric substances extracted from *Klebsiella pneumoniae* MB398

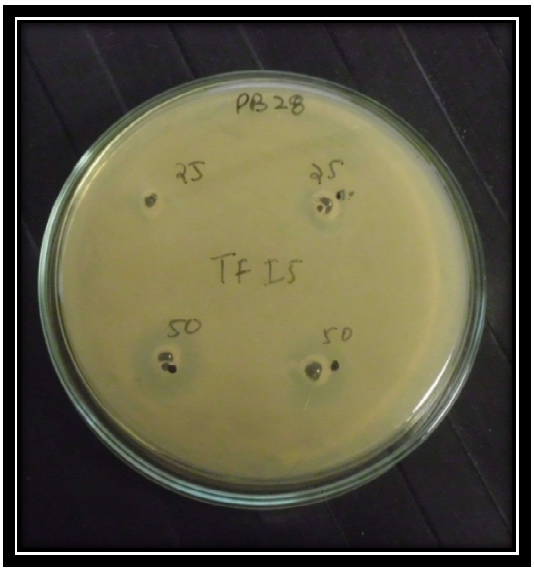
Source of EPS	Emulsification index (%)			
	Canola oil	Kerosene oil	Diesel	n-Hexane
MB398	55.97±4.03	60.46±2.88	58.06±0.94	51.36±1.97

Table 5: Results of emulsification activity ofextracellular polymeric substances extracted from *Klebsiella pneumoniae* MB398

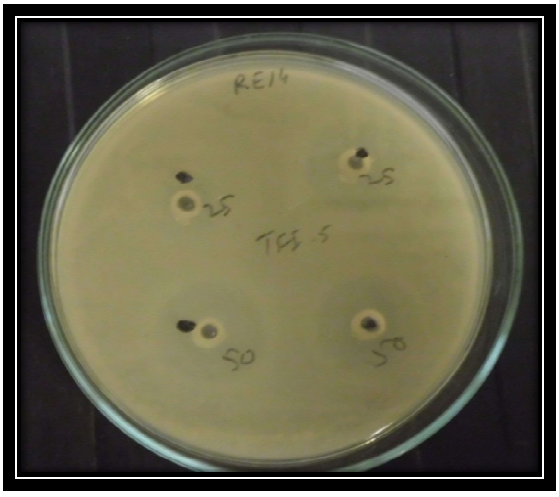
Source of EPS	Emulsification activity (540 nm)			
	Canola oil	Kerosene oil	Diesel	n-Hexane
MB398	1.330±0.045	1.261±0.251	1.051±0.006	0.794±0.120

Table 6: Antimicrobial activity of EPS (25 µl and 50 µl) extracted from *Klebsiella pneumoniae* MB398

Test organisms	Zone of inhibition (cm)	
	25 µl	50 µl
ES-CA1	-	1.10
PB09	1.25	-
PB08	-	-
ES-EC2	-	1.25
PB28	1.05	1.6
MB090	-	-
RE14	1.35	1.91
JH22	-	-
MB081	-	-
OGRIF	-	1.25



(a)



(b)

Figure 6: Representative inhibitory zones on the agar plates depicting antimicrobial activity of EPS extracted from *Klebsiella pneumoniae* MB398 against target strains

CONCLUSIONS

All these observations reinforced complex biochemical nature of EPS matrix extracted from *Klebsiella pneumoniae*MB398. Biopolymer was rich in uronic acids. FTIR analysis revealed that EPS was comprised of diverse sugar groups, proteins, uronic acids, lipids and fatty acids etc. and also harbored discernible antimicrobial activities. The biopolymer exhibited fairly good potential for forming stable emulsions with the provided hydrophobic substrates, due to the presence of hydrophobic and hydrophilic functional groups. This suggested its potential as suitable bioemulsifying candidate for treatment of hydrocarbon contamination. While the results of antimicrobial activity against target bacteria will be helpful in elucidating the antimicrobial components present in EPS and providing an insight into their mechanisms in *Klebsiella* sp. In short the properties contained in EPS make it a promising and potential candidate as biodetoxifier and for commercial, biotechnological exploitations.

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CONFLICT OF INTERESTS

The authors declare no competing/conflict of interests.

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