

Original Research Article

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Isolation and Characterization of Larvicidal Extracellular Polysaccharide (EPS) from *Pseudomonas aeruginosa* B01

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ABSTRACT

In the present study extracellular polysaccharide (EPS) was characterized from *Pseudomonas aeruginosa* B01. EPS was purified using ethanol precipitation and dialysis. The purified EPS from *P. aeruginosa* B01 was analyzed using a High Performance Liquid Chromatography (HPLC). The infrared spectra of EPS from *P. aeruginosa* B01 clearly showed the presence of carboxylate groups. The FT-IR spectrum indicated an intense absorption band in the region of 3296 cm^{-1} on the stretching vibration of hydroxyl groups (OH). The bands in the region of 2941 cm^{-1} and 1627 cm^{-1} are due to stretching vibrations of C-H bonds. The absorption in the region of 1029.99 cm^{-1} indicated the presence of glycoside link in the alpha (α) conformation. A C=O stretch between at 1730 and 1700 indicated the presence of carboxylic acid group. The monosaccharide composition of hydrolyzed EPS was analyzed using Gas Chromatography – Mass Spectrophotometry (GC-MS). GC-MS analysis revealed the presence Octasiloxane, 1, 1, 3, 3, 5, 5, 7, 7, 9, 9, 11, 11, 13, 13, 15, 15 – Hexadecamethyl, D-mannose, N-Hexadecanoic acid, Cyclohexane, 1-(1,5, Dimethylhexyl)4-(4-methylpentyl)-, D-galactose, Cyclotrisiloxane, hexamethyl-, and D-glucose were detected. Nuclear Magnetic Resonance (NMR) spectrum of EPS from *P. aeruginosa* B01 revealed two peak signals at 3.6 ppm and 6 ppm. The ^1H NMR spectrum of EPS from *P. aeruginosa* B01 showed that protons appeared at 6.131, 6.113, 6.096, 6.078, 3.651, 3.633 and 3.615 ppm. The anomeric protons of residues and their chemical shift at these ppm suggest these residues are α and β linked. Although, NMR peaks are poorly resolved because of viscosity, the characteristic signals for glucan (a branched polysaccharide chain with either 1, 4 or 1, 3 and 1, 6 linkage) anomeric protons could be detected. The ^1H NMR spectra of EPS extracted from *P. aeruginosa* B01 exhibited characteristic chemical shifts (ppm) and corresponding functional groups. Acid hydrolysis of EPS was carried out using H_2SO_4 and yielded hydrolyzed product of polysaccharide monomers. The purified EPS showed potent activity against mosquito larvae.

Keywords

Pseudomonas aeruginosa,
Extracellular
polysaccharides,
Larvicidal activity

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Introduction

Bacterial polysaccharides represent a diverse range of macromolecules that include capsular

polysaccharides, extracellular polysaccharides (EPS) and peptidoglycan (Poli *et al.*, 2008). The structure and the function of these polysaccharides varied widely. The EPS is

highly hydrated, gel-like and charged matrix. The EPS mainly consists of lipids, proteins, extracellular DNA and polysaccharides (Flemming *et al.*, 2007). Recently, more attention has been paid in exploring EPS from many sources because of their applications in various industries. The wide physical and structural diversity and other properties of EPS produced by the biofilm-forming bacteria make it biotechnologically and industrially significant (Vu *et al.*, 2009).

EPSs are secreted by various microorganisms such as, bacteria, fungi, blue-green algae and macro algae (Amjres *et al.*, 2014). Bacterial species such as, *Vibrio parahaemolyticus* (Muralidharan and Jayachandran, 2003), *V. harveyi* (Bramhachari and Dubey, 2006), *V. alginolyticus* (Bramhachari *et al.*, 2007), *Oenococcus oeni* I4 (Ibarburu *et al.*, 2007) *V. furnissii* (Kavita *et al.*, 2011), *Pediococcus parvulus* (Duenas-Chasco *et al.*, 1997), *Lactobacillus* sp. G77 (Dueñas-Chasco *et al.*, 1998), *Lactobacillus* (Costa *et al.*, 2010), *Pediococcus* sp. (Ramchandran and Shah, 2010), *Lactococcus* (Song *et al.*, 2013), *Bifidobacterium* (Zhang *et al.*, 2013), *Cordyceps gracilis* (Grev.) (Sharma *et al.*, 2015), *Bacillus* sp. (Orsod *et al.*, 2012) was reported to produce EPSs.

Some of the microbial EPS can replace polysaccharides extracted from plants for example pectin or guar gum, or alginate, or carrageenan extracted from algae in traditional applications, due to their physical properties. The microbial EPS possesses superior and unique properties that enable the development of new commercial entities (Freitas *et al.*, 2011). Because of this reason, in recent years, there has been an increasing demand for the isolation and identification of new microbial polysaccharides that can compete with traditional polymers because of their improved chemical and physical properties, higher flocculating and emulsifying activities,

biological activity and resistance to solvents (Kumar *et al.*, 2007). In addition, microbial polysaccharides production is more advantageous comparing with algae-derived and plant polysaccharides, because usually its productivity is high in control product composition, productivity and yield (Moreno *et al.*, 1998).

Microbial polysaccharides have pesticidal activities. Over the past few decades, chemical pesticides were widely used in agricultural practices. However, the widespread and prolonged use of these chemical pesticides resulted in biomagnifications of insecticides and insecticide resistance, which in turn resulted baring on export of agricultural products. It was estimated that the damage to the social economy and environment was caused by chemical pesticides about \$ 8.1 billion a year (Shen and Zhang, 2000). Further, the use of organic insecticides in pest control programs in agricultural crop throughout the world had caused major damage to the environment, pest resistance to insecticides, pest resurgence, and lethal effects on non-target organisms (Abudulai *et al.*, 2001). The main advantages of biopesticides are less harmful, designed to affect only one specific pest, very effective in very small quantities, degrade quickly and very less pollution. In this study, an attempt was made to characterize EPS from *P. aeruginosa* B01.

Materials and Methods

Isolation of EPS producing bacterial isolate

EPS producing bacterial isolates were isolated from the Municipal wastewater. A total of one ml of sewage water was aseptically added in physiological saline (0.9% NaCl) and serial dilutions were made upto 10^{-7} and incubated in nutrient agar medium (Himedia, Mumbai, India). Bacteria that produce EPS characterized by colonies of bacteria that form

very thick slime subsequently were selected and purified by streaking the four quadrants to obtain single colonies (Fusconi and Godinho, 2002).

Characterization of EPS producing bacteria

Based on the morphological, biochemical tests and 16S rRNA analysis, the bacterial isolates were identified. Gram staining, motility test, indole production, starch-, gelatin-, casein - hydrolysis, hydrogen sulphide production, urease-, citrate utilization, nitrate reduction, catalase test, oxidase and o fermentation test were carried out (Holt *et al.*, 1994). The bacterial isolate was further subjected for 16S rRNA sequencing.

The universal primer (P1: 5'-AGAGTTTGATCMTGGCT AG-3' (forward) and P2: 5'-ACGGGCGGTGTGTRC-3' (reverse) was used to amplify the 16S rRNA. DNA was amplified by using a Thermal Cycler with DNA polymerase enzyme (Sigma-Aldrich, USA). The amplified PCR product was purified by standard method and sequenced. Sequence comparison with databases was performed using BLAST through the NCBI server (Altschul *et al.*, 1997). The sequence was submitted to the GenBank database and an accession number was assigned.

Inoculum preparation

The candidate organism, *P. aeruginosa* B01 was inoculated into the nutrient broth medium composed of (g/l) peptone digest of animal tissue 5.0, sodium chloride 5.0, beef extract 1.5, and yeast extract 1.5. The medium pH was adjusted to 7.0. Then, the Erlenmeyer flask was inoculated with a loopful culture of *P. aeruginosa* B01 and incubated at 37 °C for 18 h. It was stored at 2 – 8 °C and used as the inoculum for further studies.

Production and extraction of EPS

EPS production was performed in culture containing nutrient broth medium. The potent EPS producing bacterial isolate was incubated at 37 °C for 5 days. EPS was precipitated from the cell free extract by adding double volumes of ice cold ethanol. The mixture was kept in ice for 2 h and the precipitate was collected by centrifugation (10,000 rpm, 15 min, 4 °C). It was partially purified by conducting three successive washes in double distilled water, followed by reprecipitation in ice cold ethanol. The EPS precipitates were dried in an oven at 60 ± 2 °C to obtain constant weight. The extracted EPS was quantified using phenol-sulphuric acid method (Dubois *et al.*, 1956) and glucose was used as standard.

Purification of EPS from *P. aeruginosa* B01

This method is mainly based on the elimination of proteins by precipitation with trichloroacetic acid (TCA) (12%, w/v) and subsequent precipitation of EPS was done by using double volumes of ice cold ethanol. Further, EPS was purified by following the combination of ethanol precipitation and dialysis. Ice cold ethanol was added to supernatant ratio of 3:1 (v/v). The precipitated EPS was further dialyzed against double distilled water with a molecular cut-off weight of 12-14 kDa for 24 h. The sample was then centrifuged at 10,000 rpm for 15 min and the precipitate was recovered. The precipitated product (EPS) was dried at 40 ± 2 °C until a constant weight was obtained. The protein content and carbohydrate content of purified EPS were analyzed.

High Performance Liquid Chromatography (HPLC) analysis

HPLC analysis was performed to check the purity of the sample. This was performed using a HPLC-system (Cyberlab, U.S.A). The

mobile phase (acetonitrile and water, 65:35 ratio) and sample were degassed using a Sonicator. 10 μ l sample was injected on C18 column (7.8 \times 300 mm) under optimized chromatographic conditions.

Fourier-Transform Infrared (FT-IR) spectroscopy

FT-IR spectrum of the EPS was measured. The purified EPS was further allowed to dry 12 h on stainless steel before FT-IR analysis. The sample pellets were prepared by mixing the fine dried EPS (2 mg) with 200 mg KBr. Spectrum was recorded using Shimadzu FT-IR spectrophotometer (IR Prestige-21). The FT-IR spectrum was recorded in the region of 4000–500 cm^{-1} and KBr pellet was used as a background reference (Mishra and Jha, 2013).

Gas Chromatography - Mass Spectrometry (GC-MS) analysis

The GC-MS analysis of EPS was carried out using Thermo GC-trace standard non-polar column (Dimension: 30 meters, film: 0.25 μ M). The injector temperature was set at 260 $^{\circ}\text{C}$ during the chromatographic run.

The 1 μ l of sample was injected into the instrument. The oven temperature was as follows: 60 $^{\circ}\text{C}$ (2 min); followed by 300 $^{\circ}\text{C}$ at the rate of 10 $^{\circ}\text{C min}^{-1}$; and 300 $^{\circ}\text{C}$, where it was held for 6 min. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 50-650 (m/z).

The ionization voltage was 70eV. In this chromatography hydrogen was used as the carrier gas. This column (30 m \times 0.25 mm) was closely fitted to a Perkin Elmer gas chromatography which was equipped with a flame ionization detector. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

Nuclear magnetic resonance (NMR) analysis of EPS

^1H -NMR spectra of the polysaccharide solution was recorded at room temperature (30 \pm 2 $^{\circ}\text{C}$) using a Bruker Avance II-500 spectrometer. The polysaccharide was dissolved and analyzed in 99.96% D_2O yielding a clear solution at 5 mg/500 μ l concentration. Tetramethylsilane (TMS) was used as an internal standard. For ^1H NMR spectra, the sample was submitted to a delay (D1) and acquisition time (AQ) of 1.00 s and 3.17 s respectively. Chemical shifts were measured in part per million (ppm) with signal of D_2O as reference.

Acid hydrolysis of EPS

The purified EPS (0.5 g) was hydrolyzed by adding 1.25 ml of concentrated sulphuric acid (72%) and was incubated at 30 $^{\circ}\text{C}$ for 60 min. To this 13.5 ml of double distilled water was added and kept it in a water bath for 4 h. Then it was cooled and 3.1 ml of 32% (w/v) sodium hydroxide solution was added. The hydrolyzed sample was dissolved in methanol as described by Vijayabaskar *et al.*, (2011). Finally, the acid hydrolyzed EPS sample was analyzed with a Thin Layer Chromatography (TLC). The mixture of acetic acid: chloroform: ethanol: water (11:3:11:1) was used as the mobile phase. The spots migrated on the TLC plate were detected by spraying with an alcoholic solution of α -naphthol and sulphuric acid. Then, the plate was heated at 120 $^{\circ}\text{C}$ for 3 min. The development of purple colour

Insecticidal properties of EPS

Purified EPS was used to evaluate the insecticidal properties. For this experiment, the mosquito larvae were collected from the stagnant wastewater. These larvae were counted and distributed 20 numbers each in

plastic containers. Experiment was carried out by applying 10 mg of EPS/dL water. It was continued upto 96 h and the mortality rate of the larvae was registered.

Results and Discussion

EPS was isolated and characterized from various *Pseudomonas* sp. for eco-friendly applications. The monosaccharides isolated from *P. aeruginosa* B01 in this study was similar to that of sugar components in *P. putida* G7 (Kachlany *et al.*, 2001) and in *P. caryophylli* CFR1705 (Sudhamani *et al.*, 2004).

Lipopolysaccharies with phosphate groups have been isolated from various *Pseudomonas* sp. (Kachlany *et al.*, 2001). In *P. fluorescens* Biovar II, the EPS consists of fucose, rhamnose, ribose, xylose, arabinose, mannose, glucose, galactose and low level of phosphate and sulphate (Hung *et al.*, 2005). The types of EPS produced by *P. aeruginosa* and *B. subtilis* were significantly differed each other (Harimawan and Ting, 2016).

Purification of EPS

Purification of the EPS can be obtained by precipitation with the addition of organic solvents such as ethanol, acetone or ethyl acetate. In the present investigation ethanol was used for the precipitation of EPS. The results obtained in this study were in agreement with the previous results. Recently, Yang *et al.*, (2015) and Bajpai *et al.*, (2016) used ethanol to precipitate EPS from various sources and showed better results than other organic solvents. The EPS of *P. aeruginosa* B01 showed one major and two minor peaks on the HPLC spectrum and these were detected at the retention time of 1.54, 2.12 and 6.18 min, respectively. The height of the major peak was 174, 58 and 65 mv, respectively (Fig. 1).

FT-IR spectrum of EPS from *P. aeruginosa* B01

The infrared spectra of EPS from *P. aeruginosa* B01 clearly showed the presence of carboxylate groups. These carboxylate groups may serve as binding sites for divalent metal ions. The FT-IR spectrum indicated an intense absorption band in the region of 3296 cm^{-1} on the stretching vibration of hydroxyl groups (OH). The bands in the region of 2941 cm^{-1} and 1627 cm^{-1} are due to stretching vibrations of C-H bonds. The absorption in the region of 1029.99 cm^{-1} indicated the presence of glycoside link in the alpha (α) conformation. A C=O stretch between at 1730 and 1700 indicated the presence of carboxylic acid group (Fig. 2).

In this study, a vibration peak was detected at 1373 cm^{-1} and this peak indicated the presence of carboxy groups. The spectrum peak at 3296.35 cm^{-1} indicated the presence of OH group and NH_2 group in the EPS molecule (Desouky *et al.*, 2008). In this study, a medium-broad C=O stretching was observed about 1029 cm^{-1} due to the ether linkages present within oligomers (Wang *et al.*, 2010). This stretching shows the good flexibility of the chain of EPS as studied by Shingel (2002). The peak between 1200 and 900 cm^{-1} was mainly attributed to polysaccharide composition of EPS.

The band vibrations at 1627 cm^{-1} are generally indicative of amide bonds. The stretching vibration of OH groups in the EPS was described earlier (Liu *et al.*, 2007). The bands in the region of 2941.44 cm^{-1} were due to stretching vibrations of C-H bonds, in accordance with the observations made previously with EPS from various bacterial species (Liu *et al.*, 2007). In EPS analysis using IR spectra, β -configuration was indication of vibration around 890 cm^{-1} (Yang *et al.*, 2010).

Fig.1 High Performance Liquid Chromatography (HPLC) chromatogram of EPS

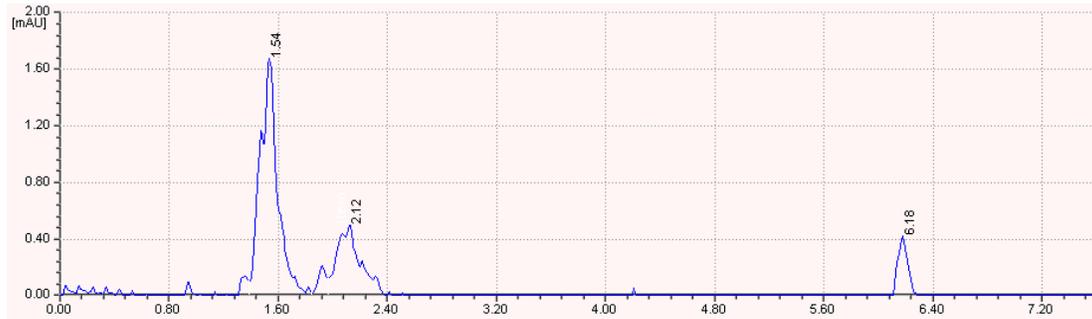


Fig.2 FTIR spectra of extracellular polysaccharide produced by *P. aeruginosa* B01

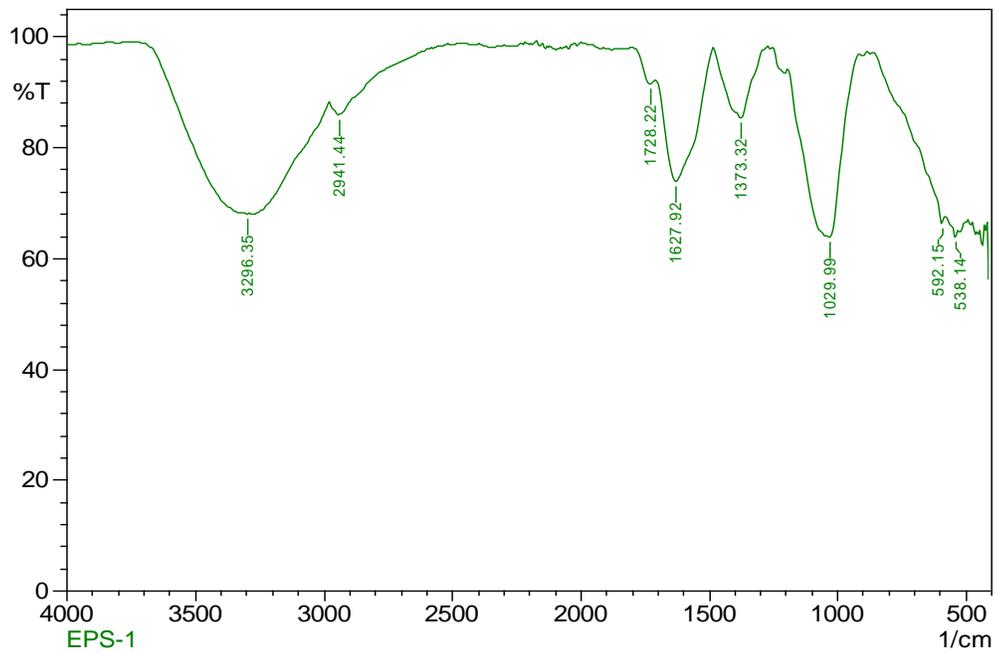


Fig.3 Gas Chromatography – Mass Spectrophotometry profile of EPS obtained from *P. aeruginosa* B01

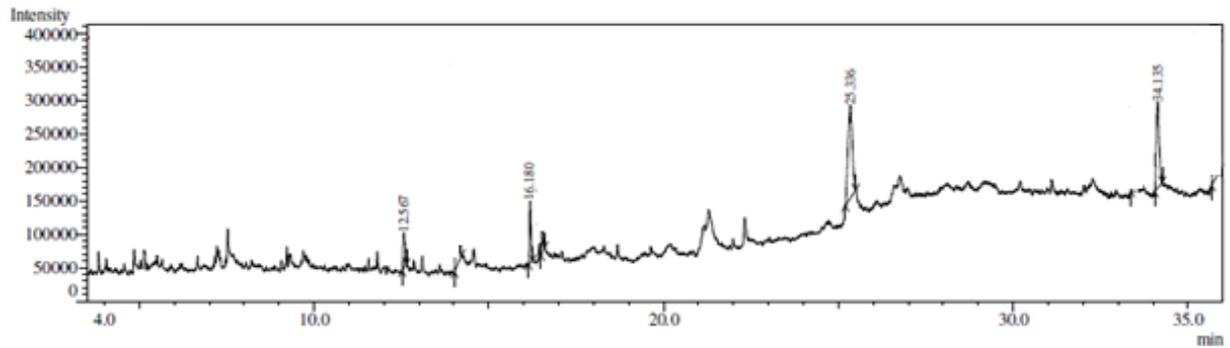


Fig.4 Proton nuclear magnetic resonance (^1H NMR) spectra of EPS obtained from *P. aeruginosa* B01

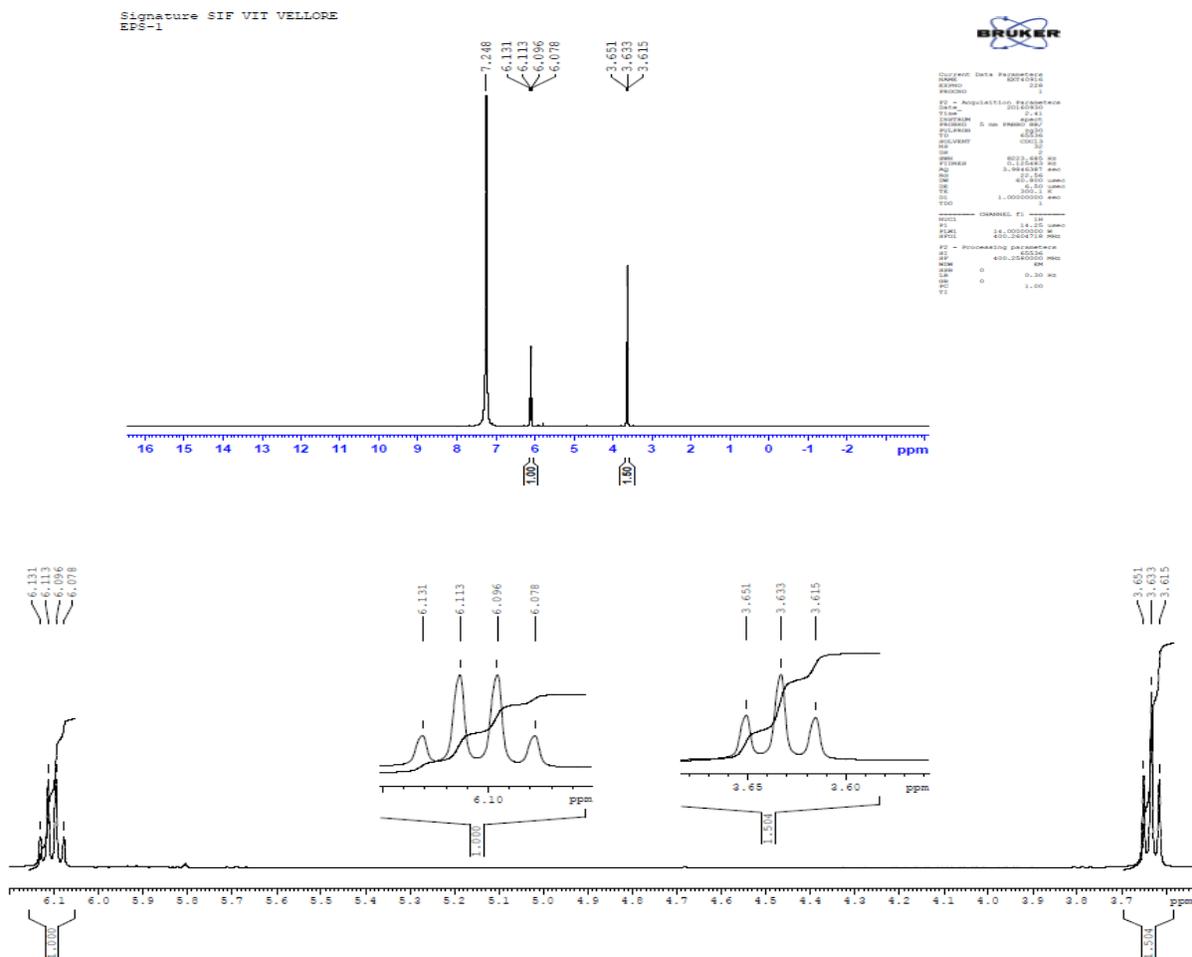


Fig.5 Acid hydrolysed EPS separation using Thin Layer Chromatography [1 – non treated samples; 2 – 50 μg acid hydrolyzed sample; 3 – 75 μg acid hydrolyzed sample)



Gas Chromatography – Mass Spectrophotometry (GC- MS) analysis of EPS

The monosaccharide composition of hydrolyzed EPS was analyzed using GC-MS. GC-MS analysis revealed the presence of three monosaccharides and other compounds (Fig. 3). The compounds such as, Octasiloxane, 1, 1, 3, 3, 5, 5, 7, 7, 9, 9, 11, 11, 13, 13, 15, 15 – Hexadecamethyl, mannose, N-Hexadecanoic acid, Cyclohexane, 1-(1,5, Dimethylhexyl)4-(4-methylpentyl)-, galactose, Cyclotrisiloxane, hexamethyl-, and glucose were detected. The multiple combinations of monomeric units along with glucosidic linkages, leading to complex structure of EPS are very difficult to resolve the chemical characteristic features. The linkage pattern of the monomer is evaluated by methylation of all free hydroxyl groups. The combination of EPS from *P. aeruginosa* and *P. fluorescens* was studied by various research groups (Tian, 2008; Torino *et al.*, 2015). Mono-carbohydrate constituted exopolysaccharides are often D-galactose, L-fucose, L-rhamnose, D-mannose, N-acetyl- D-glucose amine, L-arabinose and N-acetyl-D-galactose amine as well as the uronic acids D-glucuronic acid, D-galacturonic acid, D-manuronic acid and L-guluronic acid. Other sugar monomers less frequently occurring are D-xylose, D-ribose, 3-keto-deoxy-D-mannooctulosonic acid and several hexoseamineuronic acids (Andersson *et al.*, 2009). In another study, fucose was characterized from the EPS of *Enterobacter* (Pawlicki-Jullian *et al.*, 2010).

NMR spectrum of EPS from *P. aeruginosa* B01

In this spectrum two peak signals were detected at 3.6 ppm and 6 ppm. The strong water solvent signal was detected at 7.248 ppm. The ¹H NMR spectrum of EPS from *P. aeruginosa* B01 showed that protons appeared

at 6.131, 6.113, 6.096, 6.078, 3.651, 3.633 and 3.615 ppm. The anomeric protons of residues and their chemical shift at these ppm suggest these residues are α and β linked. Although, NMR peaks are poorly resolved because of viscosity, the characteristic signals for glucan (a branched polysaccharide chain with either 1, 4 or 1, 3 and 1, 6 linkage) anomeric protons could be detected. The ¹H NMR spectra of EPS extracted from *P. aeruginosa* B01 exhibited characteristic chemical shifts (ppm) and corresponding functional groups. The ¹H NMR spectroscopy of the EPS was complex due to the signals in the region of 3.6 to 6.1 ppm. Nuclear magnetic resonance (NMR) spectroscopy has been widely used to study the structure of EPS (Cui *et al.*, 2000). The signals obtained in the present study are in agreement with the previous reports. The β -1, 6 glycosidic linkage observed in EPS from *S. commune* AGMJ-1 with spectrum of protons at H1-H6 was 4.25, 3.04, 3.1, 3.2, 3.32, 4.01a and 3.61b. The presence of β -glucan with β -1-6-glycosidic linkage has been reported by Kao *et al.*, (2012) in the polysaccharide isolated from the fruiting body of *G. lucidum*. Singh *et al.*, (2005) reported that anomeric signals for (1-3)- β -D-glucan in a ¹H NMR spectrum in the liquid state were between 4.7-4.8 and 3.0-3.2 ppm, whereas those of (1-6)-D-glucan appear between 4.4 and 4.6 ppm. The composition of EPSs produced by *Rhizobium* was reduced to a few monosaccharides including galactose, mannose, glucose, glucuronic acid, rhamnose, and galacturonic acid (Castellane *et al.*, 2015) (Fig. 4).

Hydrolyzed product of EPS from *P. aeruginosa* B01

In the present study, TLC was used to determine the hydrolyzed product of EPS from *P. aeruginosa* B01. The composition of EPS varies depending upon several factors, including bacterial strain and kind of metal

exposure (Aquino and Stuckey, 2008). In the present study, the sugar units such as glucose and galactose were detected from the hydrolysis product of EPS (Fig. 5). This result was in agreement with that of previous reports (Kavita *et al.*, 2013). The EPS of biofilm forming bacterium *Aureobasidium pullulans* mainly composed of galactose and glucose (Lee *et al.*, 2001). In *Lactococcus lactis* sub sp. *lactis*, the sugar units such as rhamnose and fructose were determined (Pan and Mei, 2010) in TLC. The monomers such as galactose and glucose were detected from *L. fermentum* TDS030603 in TLC (Fukuda *et al.*, 2010). In *L. rhamnosus* JAAS8, the sugar units such as glucose, galactose and N-acetylglucosamine were detected (Yang *et al.*, 2010). The EPS isolated from *L. plantarum* KF5 composed of glucose, mannose and galactose (Wang *et al.*, 2010).

Insecticidal activity of EPS

EPS showed insecticidal activity against the mosquito larvae. After 24 h of incubation, 100% mortality was registered. The bacterial strains such as, *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas protegens* strains CHA0 and Pf-5 were shown to have insecticidal activity (Ruffner *et al.*, 2013; Péchy-Tarr *et al.*, 2008). Microbial insecticides are the important component of the bio-pesticide industry in recent years (Shi 2000). Xiong *et al.*, (2004) extracted novel compound from *Streptomyces* sp.173 and showed potent insecticidal activity. The isolated *P. aeruginosa* strain B01 has potent insecticidal activity. This work indicated that micro-organism from wastewater could be an important source of insecticidal EPS.

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