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Production of Antibacterial and Larvicidal Extracellular Polysaccharide from *Pseudomonas aeruginosa* Strain b01 Isolated from Wastewater

N. Benit and A. Stella Roslin*

Department of Botany, Holy Cross College, Nagercoil, Kanyakumari District, Tamilnadu 629 001, India

Corresponding author

Abstract

The aim of this study is to optimize extracellular polysaccharide (EPS) production from *Pseudomonas aeruginosa* B01 with potent antibacterial and larvicidal activity. EPS producing *P. aeruginosa* B01 was isolated from wastewater. Process parameters were optimized to enhance the production of EPS. EPS was fractionated with two-, three-volume of various solvent and antibacterial and larvicidal activities were evaluated. The bacterial cell growth was maximum after 48 h fermentation period, however, EPS production was maximum after 72 h. EPS production was found to be high when this organism was grown in the medium containing 1% maltose, 1% ammonium sulphate and 0.1% Mn^{2+} ions. EPS production was maximum at pH 7.5 and at 30 °C. Ethanol precipitated fractions showed high activity than other tested solvents. It was further subjected to Thin Layer Chromatography. EPS showed potent larvicidal activity against mosquito larva and showed potent activity against various pathogenic bacteria.

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Keywords

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Extracellular polysaccharide,
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Antibacterial,
Larvicidal.

Introduction

Extracellular polysaccharides (EPSs) are a mixture of organic compounds that are released into the surrounding medium by microorganisms. These EPSs are considered as eco-friendly, cost effective and sustainable alternate to substitute the currently using chemical flocculants. These form a protective layer for the cells against the harsh environment such as salinity, temperature, pH, heavy metals, UV radiation, biocides and heavy metals and also serve as carbon and energy reserves during starvation (Flemming and Wingender, 2010). EPSs contain humic substance, uronic acid and deoxyribonucleic acids, however, information about their concentration are highly limited (Tsuneda *et al*., 2001). The majority of bacterial isolates secrete EPS and these mucoid type polysaccharides generally have very high molecular weight (Sutherland, 1977). These EPSs may either be attached to the surface of the cell in the form of capsular polysaccharides or completely separated from the cell in the form of exopolysaccharides. Bacterial polysaccharide species represent a diverse range of macromolecules that include peptidoglycan and EPS. The structure and the function of these polysaccharides varied widely (Sutherland, 1985).

Biofilm formation is believed play significant role in protection toward antimicrobial agents and infection immunity. The organisms such as, *Staphylococcus epidermidis* and *P. aeruginosa* are most prevalent pathogens involved in clinical chronic infections. The growth of these bacteria within a biofilm provides them a

host defense mechanism and protection from antibiotics by preventing penetration or by slowing down of different agents through the biofilm. The bacteria from the genera including, *Escherichia*, *Legionella*, *Streptococcus* and *Vibrio* also produced biofilm (Vu *et al*., 2009). EPSs have revolutionized the medical sectors and industrial due to their retinue of prospects and functional applications. These applications have been extensive in areas such as nutraceutical, pharmacological, cosmeceutical, functional food, insecticides and herbicides. The prospects of EPS include antithrombotic, anticoagulant, anticancer, immunomodulation and bioflocculants (Nwodo *et al*., 2012).

Bacterial based biopesticides are widely used in agricultural sector. *Pseudomonas* sp. synthesized several inhibitory compounds have broad spectrum antagonistic activity on plant pathogens (Cartwright *et al*., 2007). The genus *Pseudomonas* contains a large group of biocontrol strains and their ability to produce various active compounds was reported previously. The metabolites such as 2, 4-diacetylphloroglucinol, phenazine-1-carboxylic acid and pyrrolnitrin [3-chloro-4-(2'-nitro-3'chlorophenyl)-pyrrole], as well as the complex, macrocyclic lactone are actively involved in biocontrol (Ligon *et al*., 2000).

Pseudomonas sp. BRG100 inhibits the growth of certain agricultural pests and is a potentially useful biopesticide for weeds and plant diseases. In agricultural practice, the widely used microbial pesticides are strains and subspecies of *B. thuringiensis*, commonly known as *Bt* (Chattopadhyay *et al*., 2004). It has been frequently used to control pests important in forestry, medicine and agriculture (Mazid and Kalita, 2011).

Many commercial biopesticides were available from *Bacillus* species such as *B. pumilus*, *B. amyloliquefaciens*, *B. subtilis* and *B. licheniformis* as biofungicides in the market (Kumar *et al*., 2008). Many species of *Pseudomonas* are also widely used for preparing biopesticides that include *P. aeruginosa, P. fluorescence, P. syringae* and *P. aureofaciens* (Kloepper *et al*., 2004). In spite of the various types of extra cellular polysaccharides which are commercially available, researchers are continuing to search novel polysaccharides with improved characters to meet the demand of the various industries. Most of the studies focused on the isolation of EPS from the bacteria and fungi for various applications. However, few studies on EPS from the waste water have been studied. Research work has been carried out to optimize the process conditions to enhance the production of EPS in submerged fermentation and few studies were carried out to explore the characteristic features of EPS. Considering these points, the present study was carried out to use EPS from *P. aeruginosa* B01 as a biopesticide.

Materials and Methods

Isolation of bacterial isolate

The wastewater samples were collected from Nagercoil Municipal area, Kanniyakumari District, Tamilnadu. The samples were serially diluted and plated on nutrient agar plates [(in g/l), (peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5.0; and agar, 15)]. All plates were incubated at 37 °C for 48 h. The potent EPS producing isolate was retained by observing for good mucoid colony morphology (Fusconi and Godinho, 2002).

Morphological, biochemical ad 16S rDNA sequencing of the potent EPS producing bacterial isolate

Based on the morphological and biochemical tests the bacterial isolates were identified. The bacterial isolate which showed potent larvicidal activity was further subjected for 16S rDNA sequencing. The universal primer was used to amplify the 16S rDNA. The primers used were P1: 5′-AGAGTTTGATCMTGGCTAG-3′ (forward) and P2: 5′-ACGGGCGGTGTGTRC-3′ (reverse) (Rajiniemon *et al*., 2015). The DNA was amplified by using a Thermal Cycler Machine and DNA polymerase (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

A loopful culture of *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 with 1N HCl/NaOH. The Erlenmeyer flask was incubated at 37 °C for 18 h in an orbital shaker at 150 rpm. It was stored at 2–8 °C and used as the inoculums for optimization studies.

Effect of fermentation period on the growth and EPS production

About 0.5 ml of inoculum was introduced into a 250-ml Erlenmeyer flask containing basal medium consists of (g/l): glucose-0.5 g; yeast extract-0.1 g; peptone-0.25 g; KH_2PO_4-0.05 g; $MgSO_4$-0.01 g and NaCl-1.0 g. The culture was incubated at 37 °C in an orbital shaker at 150 rpm for 8 days. At every 24 h, 10 ml culture was withdrawn and the contents were centrifuged at 10,000 rpm for 15 min at 4 °C. The cell free extract was subjected to ice cold ethanol and EPS content was assayed.

Effect of carbon sources on EPS production

The effect of carbon sources on EPS production was studied by supplementing various carbon sources such as glucose, lactose, maltose, fructose, sucrose, trehalose, and galactose at 1% (w/v) level each in the basal medium inoculated with 500 µl of bacterial strain. After 3 days of incubation, the cells were harvested. The culture supernatant was precipitated with ethanol and EPS content was assayed.

Effect of nitrogen sources on EPS production

The effect of nitrogen sources on EPS production was studied by supplementing various nitrogen sources such as beef extract, yeast extract, peptone, casein, skimmed milk, urea, and ammonium sulphate at 1% (w/v) level each in the basal medium inoculated with 500 µl inoculum. After 3 days of incubation, the culture was centrifuged at 10,000 rpm for 10 min. The culture supernatant was precipitated with ethanol and EPS content was assayed.

Effect of carbon – nitrogen ratio on EPS production

To find out the suitable carbon, nitrogen ratio on EPS production, *P. aeruginosa* B01 was cultured in the medium with 20 g/l glucose as carbon source adjusting the ratio of nitrogen source (g/l) 5, 10, 15, 20, 25, and 30% respectively. After 3 days of incubation, the cells were harvested. The culture supernatant was precipitated with ethanol and EPS content was assayed.

Effect of ions on EPS production

The ionic sources on EPS production was studied by supplementing metalic ions such as Mg^{2+}, Mn^{2+}, Ba^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, and Hg^{2+} at 0.1% (w/w) level each in the basal medium inoculated with 500 µl inoculum. After 3 days of incubation, the cells were harvested. The culture supernatant was precipitated with ethanol and EPS content was assayed.

Effect of pH on EPS production

Effect of pH on EPS production by *P. aeruginosa* was studied by adjusting into various pH such as 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 using 1N HCl/NaOH. In the basal medium with various pH, 500 µl of inoculum was introduced into an Erlenmeyer flask. Further, these flasks were incubated at 37 °C for 3 days. After 3 days of incubation, the cells were harvested. The culture supernatant was precipitated with ethanol and EPS content was assayed.

Effect of temperature on EPS production

Effect of temperature on EPS production was studied by inoculating the basal medium at various temperatures over a period of 8 d. The temperatures maintained were 20, 25, 30, 35, 40, 45, and 50 °C. In the basal medium incubated at various temperatures 500 µl of inoculums was inoculated. After 3 days of incubation, the cells were harvested. The culture supernatant was precipitated with ethanol and EPS content was assayed.

Determination of EPS by phenol-sulphuric acid method

The EPS yield was measured by using phenol-sulphuric acid method (Chen *et al*., 2004). The amount of extracellular polysaccharide produced was calculated using an equivalent amount of glucose as the standard (Torino *et al*., 2001).

Fractionation of EPS by various solvents

For the extraction of EPSs, the crude EPS was mixed with the solvents such as chloroform, acetone, ethyl acetate, ethanol and methanol were used individually to precipitate the EPS. The pellet was removed and respective solvent was added (1:2 and 1:3 ratio) to the culture supernatant. It was stored at 4 ºC for 24 h and the precipitates were separated. The precipitates were washed three times with double distilled water and were dialyzed against double distilled water using a dialysis membrane with an exclusion limit of 15 kDa. EPS precipitates were finally dried in a hot air oven (40 ± 2

ºC) to get a constant weight. The EPSs in the solid form were stored in airtight container at 4 ºC for further analysis.

Thin Layer Chromatography (TLC)

The purified EPS was further loaded on TLC. The sample was placed on pre-coated silica gel (Merck, Bangalore) using benzene, acetic acid and methanol (10:10:30) as the mobile phase and iodine crystal was used to develop the spots.

The spots were scraped and equal volume of double distilled water was added. This sample was further vortexed for 10 min and centrifuged at 10000 rpm for 20 min. This sample was further used for characterization studies.

Evaluation of antibacterial activity of EPS against selected pathogens

Test microorganisms

For the antimicrobial activity studies, the bacteria such as *Escherichia coli* (MTCC 584), *Klebsiella pneumoniae* (MTCC 9544), *Proteus vulgaris* (MTCC 744), *Enterobacter* sp., (MTCC 11819) and *Staphylococcus aureus* (MTCC 9886) were used. These microorganisms were grown for 18 h at 37 ºC in nutrient broth medium [(g/l) peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5 and sodium chloride, 5.0] and used for antibacterial activity studies.

Antimicrobial activity of EPS

The EPS obtained from five different solvent precipitation was loaded on Mueller – Hinton agar plates and swabbed with pathogenic bacteria such as *Escherichia coli* (MTCC 584), *Klebsiella pneumoniae* (MTCC 9544), *Proteus vulgaris* (MTCC 744), *Enterobacter* sp., (MTCC 11819) and *Staphylococcus aureus* (MTCC 9886). The MHA medium was poured into the Petriplate and after solidification the inoculum was spread on the solid plates with sterile swab moistened with the bacterial suspension. Antibacterial activity of EPS was determined by well diffusion method on Mueller Hinton Agar (MHA) medium. The antibacterial substance loaded into the well prevents growth of bacteria in areas where active concentration is reached.

Elucidation of insecticidal activity of EPS

The mosquito larvae were collected from the stagnant waste water. Collected larvae were distributed in plastic containers. Toxicity assay was carried out by applying 1.0 – 5.0 mg of EPS to the larvae. The experiment was continued upto 96 h and the mortality of the larvae was registered.

Results and Discussion

The EPS from *Pseudomonas* sp. showed potent activity against mosquito larvae (100% mortality). Hence, this organism was selected for further studies. This result was in accordance with the observations made previously with other *Pseudomonas* sp. It was previously reported that the metabolites produced by *Pseudomonas* sp. have insecticidal activity (Grundmann *et al*., 2012). The secondary metabolites produced by bacteria such as *Photorhabdus* sp. and *Pseudomonas* sp. are active against variety of insects as well as against microbial pathogens of plants and animals (Seo *et al*., 2012). In the present study, the potent EPS producing *Pseudomonas* sp. was subjected to 16S rDNA sequencing. The BLAST analysis of the 16S rRNA sequence of *Pseudomonas* sp. has 95% sequence similarity with *Pseudomonas* sp. in the NCBI database. The accession number of isolated strain submitted to genbank (KX 986759).

Incubation time is an essential factor determining the enhancement of EPS synthesis in the culture medium. As EPS is highly synthesized during late exponential growth phase or in the stationary phase, decrease in incubation time may lower the production. Higher incubation time might affect the yield due to the production of certain enzymes by the bacteria. As shown in figure 1, the bacterial growth was maximum after 48 h of incubation at 37 ºC, however growth decreased thereafter. EPS production was found to be high after 72 h of incubation.

These results indicated that the production of EPS occurred in the stationary phase of the organism. The present finding was similar to that of previous studies. Previously, in *Staphylococcus epidermidis* and *P. aeruginosa*, EPS production was found to be high during the early stationary phase and late logarithmic phase of microbial growth (Evan *et al*., 1994). Therefore, following optimization steps, this organism was cultured for 72 h to enhance the EPS production.

Medium optimization helps to enhance the growth of organism and maximum EPS production. Optimization of EPS production was carried out previously to increase the EPS production from *Pseudomonas* sp., and *Rhizobium* sp. (Breedveld *et al*., 1993).

Table.1 Evaluation of larvicidal activity of EPS from the selected bacterial isolates

Bacteria	Mortality (%)		
	1 mg EPS	2 mg EPS	5 mg EPs
Bacillus sp.	17±1.0	21±1.8	39±2.1
Escherichia coli	3.6±0.5	5.8±1.2	17.3±1.6
Proteus sp.	2.3±0.3	7.3±0.55	49.2±1.6
Klebsella sp.	48±3.8	63±3.5	93.3±2.9
Pseudomonas aeruginosa	100±1.2	100±0.5	100±0.25
Salmonella typhimurium	3±0.06	4.9±0.09	16.4±1.1
Staphylococcus aureus	47±0.09	58.3±5.2	96.8±2.7
Bacillus cereus	0	0	3.5±1.0
Bacillus subtilis	78.3±4.8	89.1±1.0	98.3±0.5
Bacillus sp.	84±3.3	92±3.1	93±0.25

Fig.1 Bacterial growth and EPS production at various incubation time in submerged fermentation

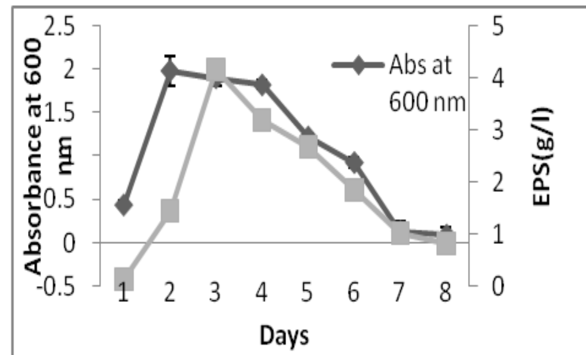

Fig.2a Effect of carbon sources on EPS production by *P. aeruginosa* B01

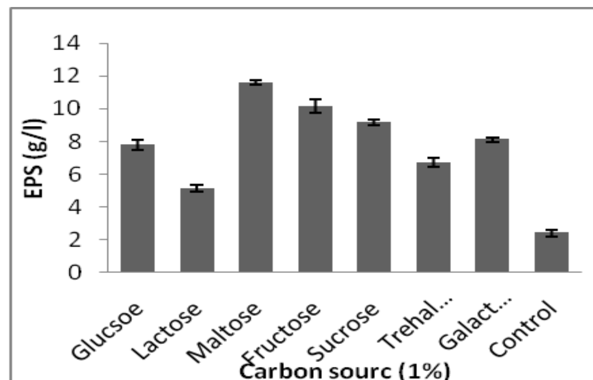

Fig.2b Effect of nitrogen sources on EPS production by *P. aeruginosa* B01

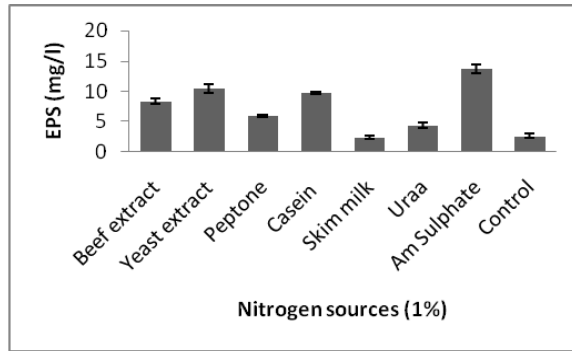

Fig.2c Effect of carbon and nitrogen ratio on EPS production by *P. aeruginosa* B01

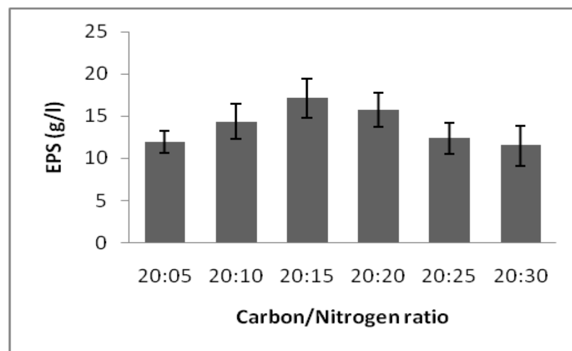

Fig.2d Effect of divalent ions on EPS production by *P. aeruginosa* B01

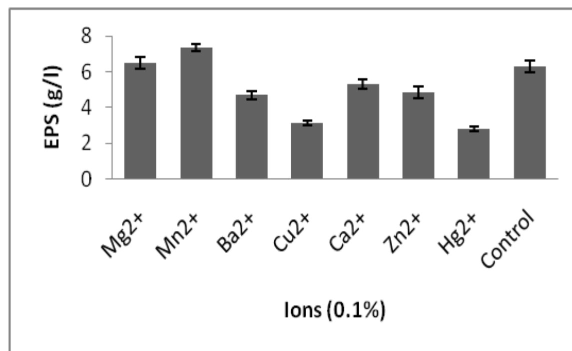

Fig.3a Effect of pH on EPS production by *P. aeruginosa* B01

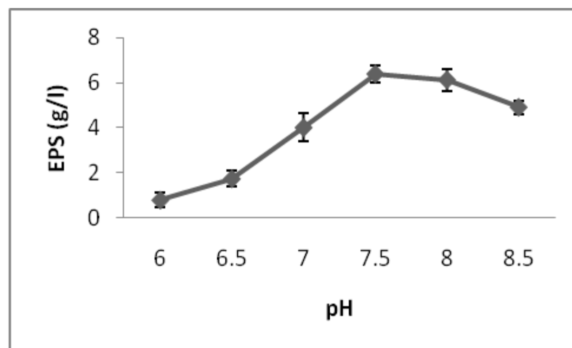

Fig.3b Effect of temperature on EPS production by *P. aeruginosa* B01

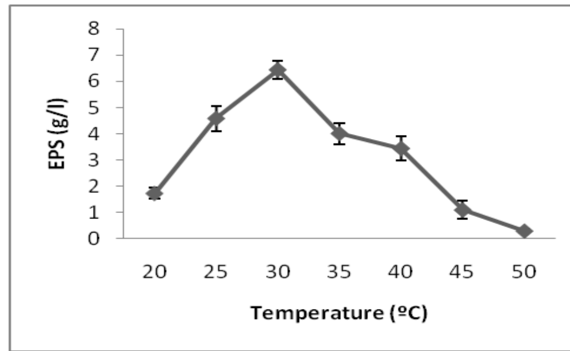

Fig.4 Precipitation of EPS using various solvent

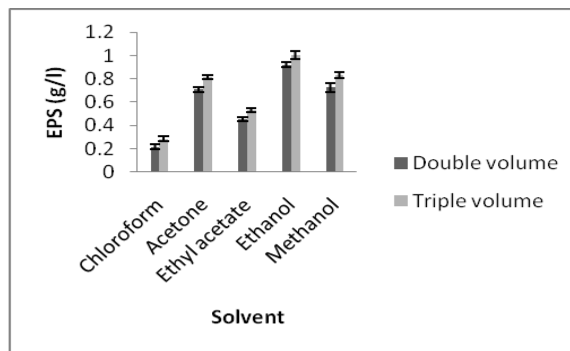

Fig.5 Antibacterial activity of EPS against *E. coli* (MTCC 584) (a); *K. pneumoniae* (MTCC 9544) (b); *P. vulgaris* (MTCC 744) (c); *Enterobacter* sp., (MTCC 11819) (d) and *S. aureus* (MTCC 9886) (e). EPS was precipitated with solvents such as chloroform, acetone, ethyl acetate, ethanol, methanol and loaded into the wells 1, 2, 3, 4 and 5, respectively

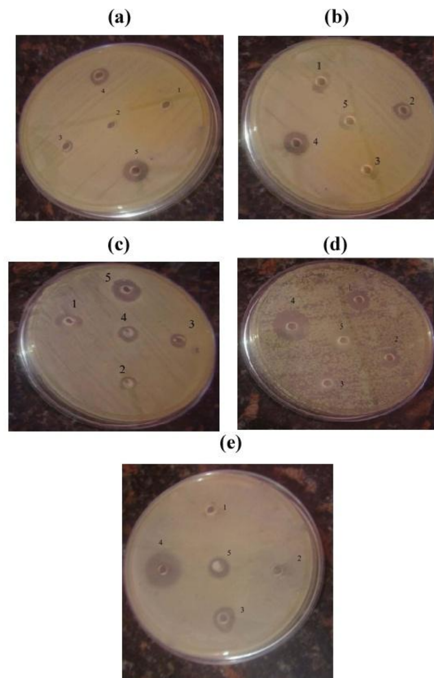

Fig.6 Thin Layer Chromatography of EPS from *P. aeruginosa* B01. The Rf value of EPS was found to be 0.83

The EPS production by microbial strains mainly depends on the availability of nutrients in the culture medium. Various studies were carried out to elucidate the effects of different carbon substrates on EPS production previously. In the present study, EPS production was found to be high in the medium containing maltose as the carbon source (11.61 ± 0.138 g/l) (Fig. 2a). Similarly, in *Cordyceps jiangxiensis,* EPS production was found to be high in the medium containing maltose (Xiao *et al*., 2004).

In the present study, ammonium sulphate was used as the nitrogen source for bacterial growth and it significantly influenced on EPS production (13.71 ± 0.728 g/l) (Fig. 2b). This result was in accordance the observations made previously with many other bacterial species. It was earlier reported that the bacteria utilize ammonium salts as their sole source of nitrogen for their growth and EPS production (Czaczy and Wojciechow, 2003). It was also reported that under limited ammonium salts availability in the culture medium, more than 50% of the glucose was converted into EPS in the strains of *Escherichia* spp. (Lee *et al*., 1999). In the present study EPS production was maximum in the medium containing maltose and ammonium sulphate at the rate of 20:15 (Fig. 2c). The effect of various metal ions was studied and Mn^{2+} showed significant effect on EPS production (7.34 ± 0.19 g/l) (Fig. 2d). This result was in accordance the observations made with *Pseudomonas elodea.* In *P. elodea*, EPS production was found to be maximum when this organism was cultured in the culture medium containing Mn^{2+} (6.25 ± 0.1 g/l) (Dlamini and Peiris, 1997). The ions influence the catalytic activities of enzyme involved in microbial growth and EPS production. It was previously reported that the ions such as Mn^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+} and K^{+} favour the growth and production of EPS (Petry *et al*., 2000).

The pH value of the culture medium might also be a one of the significant factors for the production of EPS by *P. aeruginosa*. In the present study, *P. aeruginosa* grown and produced EPS at range of pH values. In any microbial fermentation process, pH is one of a critical factor, because medium pH directly influences the physiology of a microorganism by affecting nutrient solubility and uptake, cell membrane morphology, EPS activity and product formation (Bajaj *et al*., 2009). In this study, EPS production was found to be high at pH 7.5 (6.38 ± 0.381 g/l) and this was found to be optimum (Fig. 3a). Generally, the pH of the culture medium induces morphologically changes of the bacterial cells. Lee *et al*., (1999) reported that the optimal pH value for the EPS production ranged between 5.5 and 6.5 for most of the bacterial species. Shu and Lung (2004) reported the EPS production of *Antodia* spp., and it was found to be high in the culture medium containing pH value of 5.0. In *P. polymyxa* EJS-3, the maximum production of EPS was found at pH 8.0 (Liu, 2009).

In bioprocess, incubation temperature is one of the critical factors which influence on EPS production. In this study, EPS production was found to be high when the organism was incubated at 30 °C (6.43 ± 0.34 g/l) (Fig. 3b). It was previously reported that the temperature plays critically on the growth and EPS production. Kandler and Weiss (1986) reported that the optimum

temperature for EPS production was found within the optimum growth the bacterial strain (37 °C to 45 °C). The search of new classes of antibiotics is an urgent need because of increased incidence of multiple resistances among pathogenic microorganisms to drug that is currently in clinical use. Mehta *et al*. (2014) characterized novel EPS from the osmotolerant marine strain of *Alteromonas macleodii* for various applications including biomineralization of silver. The antimicrobial EPS was also reported earlier from *Pseudomonas aeruginosa* B1 and B2 (Onbasli and Aslim, 2008). In the present study the EPS was precipitated by five different solvent (chloroform, acetone, ethyl acetate, ethanol and methanol). Among the solvent, ethanol precipitated EPS showed considerable amount than other solvent (Fig. 4) The precipitates were subjected to evaluate its biological activity against the human pathogens such as *Escherichia coli* (MTCC 584), *Klebsiella pneumoniae* (MTCC 9544), *Proteus vulgaris* (MTCC 744), *Enterobacter* sp., (MTCC 11819) and *Staphylococcus aureus* (MTCC 9886). The solvent precipitated EPS showed antibacterial activity against most of the selected pathogens. The methanol extract of EPS was highly active against *Enterobacter* sp., (MTCC 11819) and *K. pneumoniae* (MTCC 9544). The ethanol extract of EPS was highly effective against the pathogens such as *E. coli* (MTCC 584), *P. vulgaris* (MTCC 744), and *S. aureus* (MTCC 9886) (Fig. 5). Ethanol precipitated EPS fraction was loaded on Thin Layer Chromatography and it was separated (Fig. 6). The ethanol precipitated EPS showed larvicidal activity. The EPS produced by *P. aeruginosa* B01 showed 100% larvicidal activity, followed by *Bacillus* sp. (84 ± 3.3% to 93 ± 0.25% activity) (Table 1).

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