CHAPTER 1

Chapter 1

Screening and Identification of EPS-producing Bacteria from River Water Samples

1.1. Introduction

Microorganisms (like bacteria, phytoplankton and flagellates) during their life-cycle may produce hydrated polymer of high molecular weight compounds called exopolysaccharides (EPS) (Ducklow & Mitchell, 1979). Literature reveals that EPS may exist as capsules, sheaths, slimes (loosely attached to the cell wall), apical pads or mesh-like fibrils in the natural environment (Beveridge & Graham, 1991; Takeda et al., 1998). Capsules by means of non-covalent linkages are tightly bound to the cell wall where as sheaths are linear EPS-containing structures surrounding chain of cells. It was reported earlier that slime layer is a less organized form of capsule or sheath that diffuses into the surrounding environment (Wingender et al., 1999). To execute diverse functions, microorganisms produce various forms of EPS. Although not an essential feature, EPS does provide structural and functional stability to microbial assemblages in the natural environment (Decho, 2000). Most microorganisms produce EPS either for attachment to substratum (adhesion), formation of micro-consortium/biofilms or binding to other particulate matter (cohesion or aggregation). EPS produced for attachment by microorganisms may influence biofouling by conditioning the substratum (Characklis & Escher, 1988). Other functions like protection against osmotic shock, predation, gliding motility, desiccation and detoxification of toxic compounds, nutrient sequestering, chelation of metals, horizontal transfer etc. have also been accredited to microbial EPS

(Decho, 1990; Hoagland et al., 1993). In the natural environment EPS is generally heteropolymeric (made of different monomeric units) (Decho, 1990). EPS contain non-sugar components like uronic acid, methyl esters, sulphates, pyruvates, proteins, nucleic acids and lipids (Corpe, 1980; Hoagland et al., 1993). Divalent metal cations which are generally adsorbed on the EPS may act as ionic bridges linking adjacent polysaccharide chains (Fletcher, 1980). The presence of side-linkages and organic molecules influence the overall charge, stability, binding capacity, rheology and solubility of the polymer (Hoagland et al., 1993). Many bacterial cultures produce different types of EPS during its life-cycle. For example, most bacteria produce capsular form of EPS during the exponential growth phase and slime type EPS during the stationary growth phase (Decho, 1990). Similarly, the chemical characteristics of EPS changes with the age of the culture (Gloaguen et al., 1995), nutrient levels (De Philippis et al., 1991) and growth conditions (Underwood & Smith, 1998). Heissenberger and Herndl (1994) have shown that metabolically active bacteria produce EPS throughout their living period and may contribute to the EPS concentrations in marine waters, especially in oligotrophic growth conditions. Based upon the information available on the ubiquitous distribution of bacterial EPS in the oceans and its contribution to the organic carbon pool (Stoderegger & Herndl, 1999), its clear that bacterial EPS might play an important role in regulating various marine processes and fuelling the marine food-web (Decho, 1990). However, most of the laboratory and in situ studies on the factors regulating important marine processes like aggregation, flux and trophic web have focused on the role of phytoplankton and its extracellular polymers (Decho, 1990).

Thus, very little is known about the behavior of bacterial EPS in the marine environment despite its wide distribution and high abundance. Some forms of EPS showed good correlation with bacterial production suggesting its utility as a possible organic carbon source. Keeping in mind the ecological significance of the EPS in general and the limited information available on the role of bacterial EPS in marine processes, a study was carried out on screening and identification of river bacteria. Furthermore, experiments were also carried out to assess the effect of bacterial growth conditions on EPS production.

1.2. Materials and methods

1.2.1. Screening of the test strain

Composite water samples were collected using standard methodology (APHA, 1989) from a single sampling station on River Mahananda underneath the Mahananda Bridge, Siliguri, India. Serial dilutions of water samples were made in filtered (water passed through 0.2 mm filter, Millipore, Sydney, Australia) and autoclaved river water and plated on diluted (10^{-3} or 0.001x) Luria Bertani (LB) agar plates (HiMedia M575, India) (nutrient poor). After incubation at 30 °C for 72 h, culturable oligotrophic bacterial colony-forming units (CFU) were obtained. Purification of single colonies was done by dilution streaking on 0.001x LB agar plates. Single colony cultures were maintained on R2A agar (HiMedia M1687) which is a standard environmental cultivation medium. Master plate made up of R2A agar was constructed with purified single colonies. Each master plate was replicated separately in triplicate on LB, 0.001x LB, and R2A agar plate. Colonies that had grown on 0.001x LB agars but not on LB agar were termed as obligate oligotrophs, whereas colonies that were able to grow on all the three different

plates were termed as facultative oligotrophs (Kumar et al., 2010). Bacterial colonies with mucoidal appearance or colonies that had a sticky surface were picked up and purified by repeated streaking on R2A agar plates. On an average 5 to 7 colonies were randomly selected from each plate during every sampling for further study. The purified cultures were then transferred to R2A slants and stored at 4 °C. Pure colonies of each facultative oligotrophic isolates were then inoculated into 50 ml of screening medium (R2A) in 250 ml Erlenmeyer flask, incubated at 30 °C in a rotary shaker at 160 rpm for 48 h. Flocculating properties of culture broth from different isolates were examined using two different suspensions (kaolin and activated carbon) following standard method (Kurane et al., 1994; Suh et al., 1997). High concentration of Ca^{2+} (50 mM) was required for flocculation of kaolin suspension, whereas only 4 mM Ca^{2+} was required for flocculation of activated carbon. Hence, one isolate, named PB12, with high flocculating rate for activated carbon suspension was selected as test strain for further study. Viability of the test strain in different media was also performed at 30 °C to examine the nature of oligotrophic growth. Briefly, inoculum was prepared by transferring a single colony of 24 h old culture of PB12 into 10 ml sterile R2A (pH 7) in 100 ml Erlenmeyer flask. The inoculated medium was incubated at 30 °C for 12 h with agitation (160 rpm). The culture was harvested by centrifuging at 9587.5 x *g* for 5 min at 4 °C and washed twice with sterile saline (0.85% NaCl) water to remove traces of media. The washed pellet was finally suspended in 3 ml sterile saline water. Aliquot of 1.0 ml of concentrated ($1x10^7$ CFU ml^{-1}) cell suspension(s) was added to 25 ml of sterile LB or R2A or river water or 0.001x LB in 250 ml Erlenmeyer flask. The flasks were kept at 30 °C (with shaking; 160 rpm) throughout the period of investigation. Survivability of PB12 cells in each tested

medium was assessed through dilution-plating at different time intervals on fresh LB agar plates.

1.2.2. Identification of the test strain

The selected EPS producing bacterium was identified using both conventional physiological and biochemical tests followed by the molecular identification method based on 16S-rRNA sequencing.

a) *Conventional identification methods*- The bacterial isolate was studied for morphological, physiological and biochemical characteristics (like catalase, oxidase, caseinase, gelatinase, urease, amylase, indole, citrate, MR, VP, nitrate reduction, lipase, and carbohydrate fermentations) following standard methods described earlier (Gerhardt et al., 1981). The culture characteristics and biochemical characteristics were compared with those given in Bergey's Manual of Systematic Microbiology. Using these methods, the culture could be identified up to its family.

b) For further identification, phylogenetic method (16S-rRNA gene sequencing procedure) was used. It was done in three major steps: i) extraction of the chromosomal DNA; ii) gene amplification by polymerase chain reaction (PCR) followed by sequencing of the cloned amplicon; (iii) construction of phylogenetic tree to ascertain the position of the isolate.

i) *Chromosomal DNA extraction*

The chromosomal DNA was extracted by repeated freezing (-80 °C) and heating (at 95 °C) of the bacterial cells for 30 min each in Tris buffer at pH 8. The cells were centrifuged at 9587.5 x *g* for 10 min at 4 °C to separate the cells. To the supernatant, few microlitres of proteinase K, DNAase free RNAase and SDS were added and incubated at

37 °C for half an hour. Further extraction and precipitation was done as mentioned earlier (Sambrook et al., 1989).

ii) The amplification of 16S rRNA gene sequence, purification of PCR product and cloning were done as described earlier (Kumar et al., 2010). Briefly, 1504 bp-segment of the 16S-rRNA gene was amplified by primers 27F (5´-AGAGTTTGATCCTGGCTCAG3´) and 1492R (5´-TACGGTTACCTTGTTACGACTT3´) using BDT v3.1 cycle sequencing kit on ABI 3730x1 Genetic Analyzer following the manufacturer's recommendations. The PCR cycle involved denaturing of the strand at 95 °C for 5 min followed by 30 cycles of annealing (for 1 min at 40 °C), extension (for 3 min at 72°C) and denaturing at 95 °C (for 1 min). A final extension for 10 min at 72 °C was carried out before the amplified sequences were loaded on 0.8% agarose gel and separated by gel electrophoresis. The gel was then immersed in ethidium bromide for 2 h. and the products were detected using a transilluminator. The PCR products were then purified using QIAquick Purification Kit (Qiagen, USA). 16S rRNA gene sequence was used to carry out BLAST with the database of National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/ Blast.cgi).

1.2.2.1. Sequence analyses

An almost complete continuous stretch of sequence represented by 1504 nucleotides of 16S rRNA gene from the strain PB12 was used for sequence analyses. The sequence was subjected to analyses at EzTaxon server (Chun et al., 2007), RDP (Cole et al., 2014) and BLAST (Altschul et al., 1990). RDP has several online tools for sequence analyses. For present study two among these were used. This includes CLASSIFIER and

SEQMATCH. The former gives a specific conclusion as to what hierarchal level the query sequence (from a prokaryotic isolate) belongs with percent value, indicative of confidence limit. While the latter gives idea of near relatives that are stored in RDP database. BLAST is the most common search tool available in the web. However, there are some strong demerits as far as results of BLAST are concerned, especially with reference to 16S rRNA gene based taxonomic analysis. Sometimes, the closest relative is missed out. Moreover, the results are not always with respect to type strains. The worker has to carry out additional search. Analyses carried out at EzTaxon displays results with respect to near types strain relative representing the closest spp. Moreover the database displays extent of sequence identity, strain and sequence information as well as link for visualizing pair wise alignment. This server has become an invaluable tool for taxonomists over past few years. Due to several advantages, this server has become an ultimate choice for taxonomic 16S rRNA gene based sequence analyses.

1.2.2.2. Phylogenetic tree construction

In order to construct phylogenetic tree, first of all 16S rRNA gene sequences, representing type strains of all near relatives of the strain in the question were retrieved following searching and retrieving using EzTaxon server, RDP and BLAST search aligned with CLUSTAL_X (Thompson et al., 1997) and edited manually. The edited sequences were then saved in PHYLIP interleaved format. This file was then use as input for construction of distance based neighbour-joining (NJ) trees; trees were also constructed by TREECON software (Van de Peer & De Wachter, 1997) using 100 replications and by using both Kimura's (Kimura, 1980) correction.

1.2.3. Production of EPS

Time course of EPS production was performed in 500 ml flasks containing 100 ml of R2A (pH 7.0) with 160 rpm agitation at 30 °C. Samples were taken every 12 h to measure growth (O.D. at 600 nm), and EPS yield (determined as dry weight of EPS). Isolation of EPS was done after incubating the test strain at 30 °C for 48 h followed by centrifugation of the culture broth, precipitation of supernatant in double volume of cold 95% ethanol followed by dialysis in a dialysis tubing cellulose membrane (D9652, Sigma-Aldrich, retaining MW >12,400 Da) against distilled water for 24 h. The dialyzed material was again centrifuged at 9587.5 × g for 40 min at 4 °C and the supernatant was freeze-dried to obtain EPS.

To study the effect of various carbon sources on EPS production, R2A medium was supplemented singly with glucose, lactose, sucrose, mannose and arabinose (1% w/v). R2A medium without any supplementation was taken as control. Stock solutions of different carbon sources were filter-sterilized and aseptically added to the sterile medium before inoculation.

1.2.4. Effect of pH and temperature on EPS production

The effect of different pH on EPS production was investigated by growing PB12 in R2A medium adjusted to different pH (within the range of 4-9) using buffer systems. Temperature was kept constant at 30 ºC. To investigate the effect of temperature, PB12 was grown in R2A broth at 20 °C, 30 °C, 37 °C and 42 °C with its optimum pH. After an optimum period of incubation (time for maximum EPS production), both growth (measured as absorbance at 600 nm) and EPS yield was determined.

1.3. Results

1.3.1. Characterization of the test strain, PB12

The strain PB12 showed highest flocculating rate (98%) amongst one fifty exopolysaccharide producing isolates. Hence, the test strain used in this study was PB12. The optimum temperature and pH of the test strain was found to be 30 °C ± 2 °C and 7.0 ± 0.1 respectively .The results of biochemical tests have been shown in Table 1.1. It was straight rod 1-2 µm (when grown in LB and observed under light microscope), gram-negative, non-motile, facultative anaerobic belonging to class γ-Proteobacteria of family Enterobacteriaceae. Colonies were circular, convex, translucent, mucoid, sticky and offwhite in color with diameters of 2.0-3.0 mm after 3 days at 30 °C on R2A agar.

>gi|523328777|gb|KF192506.1| *Klebsiella pneumoniae* strain PB12 16S rRNA gene, partial sequence

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAG
CACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGA
TGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGG
ACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTC
ACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATG
CCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAGGTT
AATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCG
GTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTC
AAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCT
TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTG
GCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCT
TCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC
CTGGTCTTGACATCCACAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTGTGAGACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATCCTTTGTTGCCAGCGGTTGGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGA
AGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCA
TATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTG

GAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGG
TAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAG
GTAACCGTA

Fig. 1.1. Partial 16S rRNA gene sequence of the isolate PB12 (Accession no. KF192506)

Table 1.1. Biochemical characteristics and carbon source utilization of the test strain *Klebsiella pneumoniae* PB12.

+, positive; -, negative; A, acid; G, gas

S. No.	Recipe	Observations
1.	Gram's reaction	'-ve' rod
2.	Oxidase test	-
3.	Catalase test	+
4.	Indole test	-
5.	MR test	-
6.	VP test	+
7.	Citrate	+
8.	Urease	+
9.	Nitrate reduction	+
10.	Starch hydrolysis	-
11.	Glucose	AG
12.	Lactose	AG
13.	Sucrose	AG
14.	Mannitol	AG

When the16S rRNA gene sequence (1504 nucleotides; Fig. 1.1) of PB12 was used to search for similar sequences in the GenBank database, it showed closest sequence similarity (100%) with an uncultured bacterial clone SJTU_D_02_05, a representative sequence from 16SrRNA library of "human fecal sample from subject GGM"(http://www.ncbi.nlm.nih.gov/nucleotide/126111133?report=genbank&log$=nucl align&blast_rank=2&RID=VPKCM0KZ01R). Among the type strains, it showed closest sequence similarity (99.87%) with *Klebsiella pneumoniae* subsp. *rhinoscleromatis* ATCC13884^{T} (ACZD01000038), followed by *K. quasipneumoniae* subsp. *similipneumoniae* 07A044^{T} (HG933295) (99.86%), *K. quasipneumoniae* subsp. *quasipneumoniae* 01A030^{T}(HG933296) (99.86%), *K. pneumoniae* subsp. *ozaenae* ATCC 11296^{T} (Y17654) (99.5%), *K. pneumoniae* subsp. *pneumoniae* DSM 30104^{T} (AJJI01000018) (99.3%) and *Klebsiella variicola* F2R9^{T} (AJ783916) (99.2%). Such high extent of sequence identity is a strong indication that the strain PB12 is a strain within *K. pneumoniae* species. In the phylogenetic tree (Fig. 1.2), strain PB12 forms a clade along with the uncultured bacterial clone, showing very high bootstrap value of confidence. It may therefore be inferred that the isolate PB12 belongs to the *Klebsiella pneumoniae* subsp. *pneumoniae* cluster.

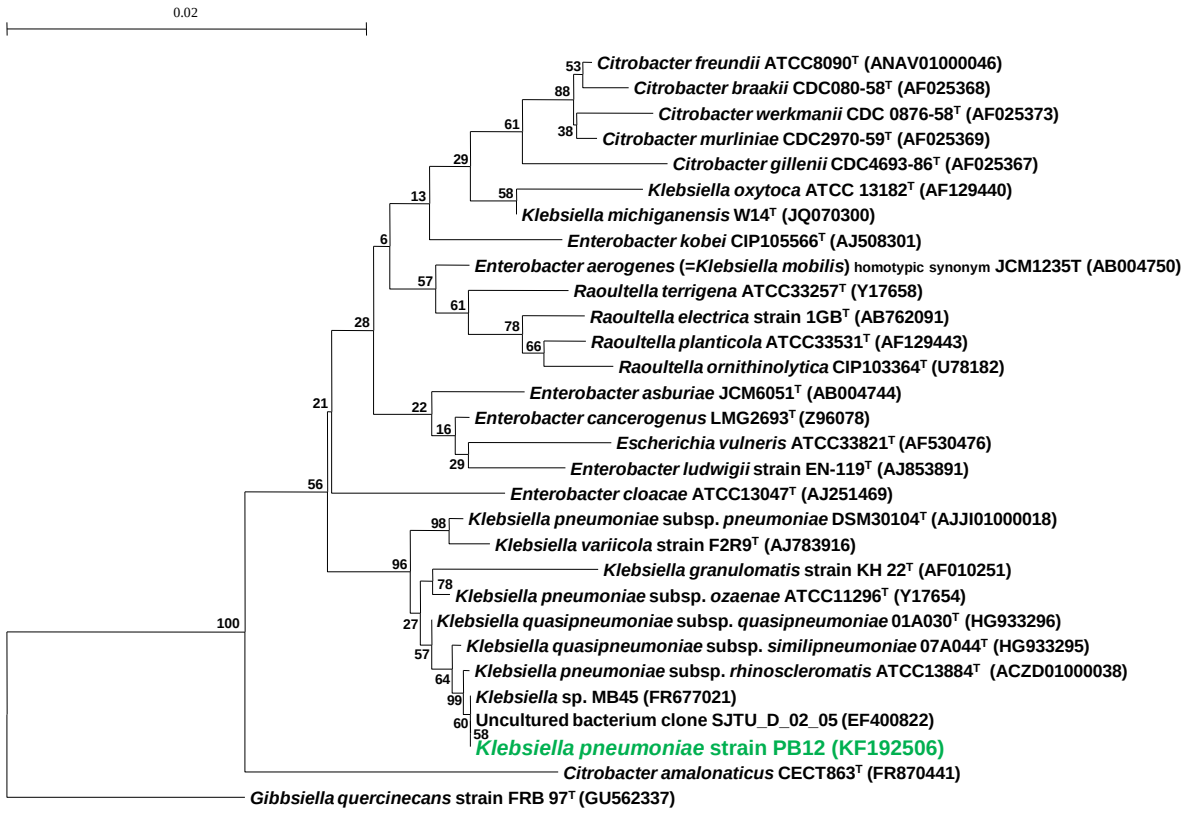

Fig. 1.2. 16S rRNA gene sequence based neighbor-joining tree, showing the position of *Klebsiella pneumoniae* PB12 (green color) among the members of genus *Klebsiella*. Bootstrap percentages are given at the branching nodes. *Gibbsiella quercinecans* strain FRB 97T (GU562337) was used as outgroup. EMBL/GenBank accession numbers are given in parentheses.

1.3.2. Demonstration of oligotrophic trait of PB12 and time course of EPS production

The cells of PB12 were able to grow in 0.001x LB broth as well as sterile river water without supplementation of any carbon or nitrogen source or growth factors. An

increment of nearly 13 times the initial cell number was noted in a span of 3 days in 0.001x LB (Fig. 1.3a). The ability of PB12 to survive without any reduction in viable cell number from the input cells explains the oligotrophic nature.

Yield of EPS in R2A varied with time and was maximal (1.3 g l^{-1}) at 48 h of incubation at 30 °C (Fig. 1.3b). The depletion in the amount of EPS was noticed after 48 h indicating probable utilization of the same as nutrient source for cell growth and viability.

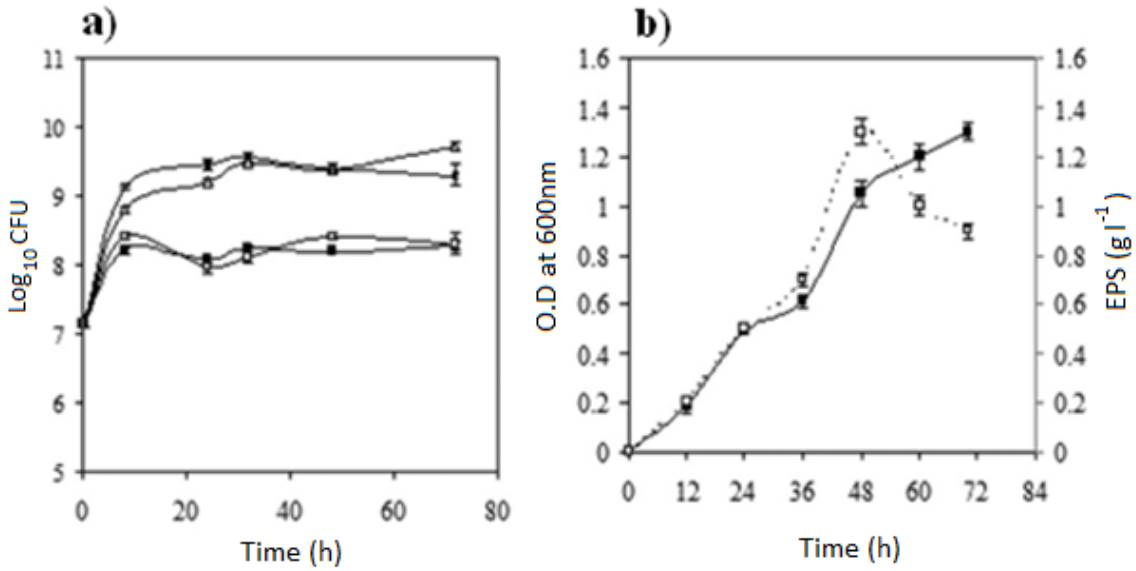

Fig. 1.3. a) Growth pattern (in terms of viability) of PB12 in LB (closed triangles), R2A (open triangles), river water (open squares) and 0.001x LB (closed squares) at 30 °C; **b)** Growth (closed squares; O.D at 600 nm) and EPS production (open squares; g l^{-1}) of *Klebsiella pneumoniae* PB12 in R2A (nutrient poor) broth at 30 °C, pH 7.2 ± 0.2. Data are the mean of triplicates ± S.E.

1.3.3. Effect of supplementation of carbon source in R2A medium on growth and EPS production

Growth and production of EPS in R2A was enhanced maximally by 62% and 38.4%, respectively, when supplemented with 1% glucose. When supplemented with 1% lactose, growth and production was enhanced by 43% and 30%, respectively. In sucrose and rhamnose supplemented R2A, the growth was negligibly affected but the EPS production was enhanced roughly by 18%. Growth was enhanced by 43% with only 8% increase in EPS production when grown in arabinose supplemented R2A medium (Fig. 1.4). Least alteration in growth or EPS production occurred in mannose supplemented R2A medium.

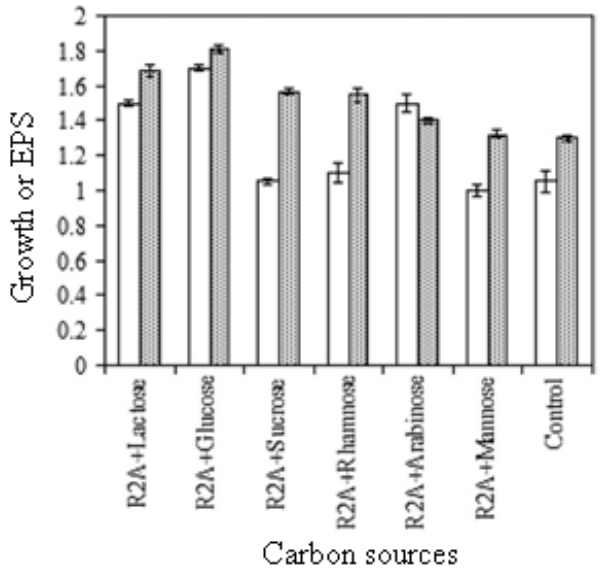

Fig. 1.4. Effect of various carbon source (1%) on growth (open columns) and EPS production (solid columns) at 30 °C. Data are the mean of triplicates ± S.E.

1.3.4. Effect of temperature and pH on EPS production

The highest productivity (1.3 g l^{-1}) of EPS was obtained at 30 ºC (Fig. 1.3b). Above 30 ºC, the productivity of EPS was reduced. Furthermore, maximum growth in terms of optical density was also noticed at 30 ºC, below and above this temperature, both growth and EPS production decreases. The high productivity of EPS (1.3 g l^{-1}) was observed at pH 7. The yield of EPS as well as growth decreased below and above pH 7.

1.4. Discussion

The results obtained from biochemical (Table 1.1) and physiological studies revealed that it belongs to genus *Klebsiella*. As evident from the phylogenetic tree (Fig. 1.2), the strain PB12 forms a clade along with the uncultured bacterial clone, with very high bootstrap value of confidence and it is located within the *Klebsiella pneumoniae* subsp. *Pneumoniae* cluster. The cluster contains four more subspecies of *Klebsiella pneumoniae*, *Klebsiella variicola* and *Klebsiella granulomatis*. Since, during RDP manalyses (CLASSIFIER), the conclusive report of CLASSIFIER tool suggested affiliation of strain PB12 to *Klebsiella* with 86%. In order to validate and confirm its taxonomic status a more extensive phylogenetic analyses of the strain PB12 with closest four genera (*Klebsiella*, *Enterobacter*, *Raoultella* and *Citrobacter*), within the family Enterobacteriaceae was undertaken.

As evident, different representative spp. of the two genera namely, *Citrobacter* and *Raoultella* distinctly occupied two different monophyletic clusters. Majority of the members of Enterobacter-*Escherichia* and *Klebsiella* were positioned in way that reflects monophyletic nature. The *Klebsiella michiganensis- K.oxytoca cluster* was positioned in

between two distinct monophyletic clusters constituted by *Citrobacter freundii- C. gillenii* and *Raoultella terrigena- R. Ornithinolytica*. This cluster constitutd by 2 *Klebsiella* spp. (i.e. *K. michiganensis- K. oxytoca*) looks away from basic monophyletic cluster constituted by other *Klebsiella* spp. This is similar to what has been reported by Drancourt et al (2001), where the genus *Klebsiella* has been suggested to be polyphyletic and heterogenous in nature.

The genus is composed of species that forms three different clusters and the strain PB12 falls within cluster I (that contains *K. granulomatis*, three sub spp of K. *pneumoniae*). Isolates identified as *K. pneumoniae* has been phylogenetically placed under three groups Kp-I, Kp-IIA, Kp-IIB and Kp-III (Brisse & Verhoef, 2001; Brisse et al., 2004; Fevre et al., 2005). No species under *K. granulomatis* is under axenic culture (Carter et al., 1999). Based on genetic and phenotypic characteristics, the names *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* subsp. nov. and *K. quasipneumoniae* subsp. *similipneumoniae* subsp. nov. has been proposed very recently for strains of KpII-A and KpII-B, respectively (Brisse et al., 2014).

The strain PB12 showed gas production from lactose like the three sub spp. of *K. pneumoniae* and like K. *pneumoniae* sun sp. *pneumoniae*, it is positive for VP test and urease activity but negative for MR test (Li et al., 2004). Thus, based on phenotypic and phylogenetic analysis, the strain PB12 was identified as *Klebsiella pneumoniae*.

Fig. 1.3a shows the viability of PB12 in 0.001x LB, river water, R2A and LB broth. In our earlier reports, facultative oligotrophic strains, *Acinetobacter johnsonii* MB52 and *Klebsiella pneumoniae* MB45 showed an increase of 2.5 and 4.6 times the initial cell number when grown in 0.001x LB in the span of 8 and 2 days, respectively

(Kumar et al., 2010; Kumar et al., 2011). Fig. 1.3b shows reduction in EPS amount after 48 h which was probably due to utilization of the EPS by the bacterium. Similar utilization of EPS as carbon source was also observed by earlier authors (Gauri et al., 2009). Microorganisms are known to produce more EPS when grown in carbon enriched medium (high C:N ratios) (Souza & Sutherland, 1994). Nitrogen limitation is known to trigger increased EPS production by microorganisms (Fajon et al 1999). Similarly, increase in EPS yield was observed for *Pseudomonas* PB1 under minimal nitrogen and phosphate concentrations (Williams & Wimpenny, 1977). Although very little is known about the mechanism regulating the production of EPS in nutrient depleted conditions, a shift in the biosynthetic pathways might explain the observed changes. The production of the EPS during the growth of PB12 had certain distinct features. EPS production increased rapidly within 24 to 48 h of culture growth. The EPS production was maximal as the culture approached stationary phase. Bacteria produce EPS during their log-phase depending upon the growth conditions and age of the culture (Decho, 1990). *Klebsiella pnemoniae* PB12 in the present study showed decrease in approximately 31% of EPS during further incubation from 48 h to 72 h in the same batch culture was associated with two times increment in viable cell number. Results showed that supplementation of glucose in R2A support both growth and EPS production in PB12. Similar observation was noted where the supplementation of glucose in nitrogen-free Burk's medium was found to be the best for EPS production by *Azotobacter* sp. SSB81 (Gauri et al., 2009). In the case of *Chryseobacterium daeguense* W6, supplementation of mannose or maltose or glucose in low nutrient medium were favorable carbon source for both production of EPS and cell growth (Liu et al., 2010). EPS produced during different phases of growth have

specific properties and functions. For example, capsular form of EPS is generally produced during log phase that forms a tight envelope around the cell, promoting cell attachment to substratum (Costerton, 1984). Capsules may also provide better protection against predation and help bacteria survive in low pH (Decho & Lopez, 1993). Bacterial capsules also act as a good metal adsorbent (Brown & Lester, 1980) and may act as a buffer against metal toxicity. There are reports on EPS production by the genus *Klebsiella* (Cheng et al., 2004; Ramírez-Castillo & Uribelarrea, 2004). Thus the production of EPS in the exponential phase has ecological significance and may play an important role during the growth of specific bacteria.