CHAPTER-VII

Biofilm formation during phosphate solubilization process
1. INTRODUCTION

A biofilm can be defined as an assemblage of surface associated microbial cells embedded in an extracellular organic polymeric matrix of microbial origin. For bacteria, there are several advantages of biofilm formations, such as protection from antibiotics (Costerton, 1999), disinfectants or dynamic environments and also to survive in nutrient deficient conditions (Danhorn et al., 2004). About 99% of the world populations of bacteria produce diverse biofilm structures at various stages of their growth (Dalton and March, 1998). Biofilm formation is governed by a number of physical, chemical and biological processes. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems (Donlan, 2002). Different types of noncellular materials like mineral crystals, corrosion particles, clay or silt particles, or blood components, may also be found in the biofilm matrix depending on the environment of biofilm development (Donlan, 2002). In soil environment, a community of bacteria may form a biofilm on surfaces to get nutrients and to protect themselves from a variety of unfavorable conditions. Biofilm in natural environments are commonly found on coating around various particles that occurs in soils, lake, river etc.

One of the important features of most of the biofilm forming bacteria is the production of extracellular polymeric substances (EPS) consist larger of polysaccharide and smaller amount of protein and DNA. The EPS molecules are considered as the major factor that influence biofilm formation by microorganisms (Czaczyk and Myszka, 2007). The extensive production of EPS occurs during the specific adhesion stage of biofilm formation. EPS are also responsible for the architecture and morphology of the biofilm matrix (Mattos- Guaraldia et al., 2000; Langille et al., 2000).

Competition for nutrients and other growth requirements is definitely an important driving force for the biofilm development. Increased cell density favors chemical signals to communicate with the responding cells for social interactions in biofilms. Furthermore, the expression of different adhesions, their cognate receptors, and exopolymeric components by individual cell types within a biofilm community can contribute to overall biofilm development (Watnick anb Kolter, 2000; Davey and O'Toole, 2000). In particular, many bacteria are capable of using a quorum sensing
mechanism to regulate biofilm formation and other social activities (Parsek, 2005).

Bacteria communicate with neighbors and monitor their population density by producing and sensing signaling molecules in a process called quorum sensing (QS) (Suárez-Moreno et al., 2010). The concentration of the signaling molecule increases alongside the bacterial population density and, when it reaches a significant level, bacteria respond and modulate target gene expression. In Gram negative bacteria QS system involves the production and response to a signal molecule known as acylated homoserine lactone (AHL). In the genus *Burkholderia* QS system has been extensively studied. The species of BCC complex share a conserved QS system known as CepI/R (Gotschlich et al., 2001) that produces and responds to *N*-octanoyl homoserine lactone (C8-AHL). CepI/R Qs system regulates virulence and several important phenotypes like biofilm formation and siderophore production (Eberl, 2006; Huber et al., 2001; Venturi et al., 2004). It consists of CepI which synthesizes mainly C8-AHL and the CepR, C8-AHL sensor response regulator. On the other hand Suárez-Moreno et al. (2008) reported BrI/R quorum sensing system which produces and respond mainly to C12-3-oxo-AHL and stringently regulated by a repressor RsaL. Later on they have also reported that BrI/R QS system is related to EPS production and biofilm formation by some plant associated *Burkholderia* spp. (Suárez-Moreno et al., 2010).

The present study focused on the biofilm formation by isolated bacterial strains during P solubilization process. The tendencies of the organisms to produce biofilms in relation to avialbe P in the medium were also observed in details. EPS production by isolated organisms were also quantified to find out their role in biofilm formation as well as P solubilization process. Lastly attempts were taken to detect the QS systems in the isolated bacterial strains which probably help in biofilm formation during solubilization of insoluble P by isolated bacterial strains.
2. MATERIALS AND METHODS

2.1. Study of biofilm formation by isolated bacterial strains on insoluble P granules

2.1.1. Culture conditions and development of bacterial biofilm

Biofilm formation by isolated bacterial strains were checked using NBRIY broth supplemented with TCP or four different rock phosphates viz. Jordan rock phosphate (JRP), Purulia rock phosphate (PRP), Udaypur rock phosphate (URP) and Mussoorie rock phosphate (MRP) at a concentration of 5 g/L. All the bacterial strains were grown in nutrient broth with mild shaking at 28 °C. 100 µl of starter cultures were added to 100 ml of NBRIY broth in 250 ml Erlenmeyer flasks. Sterilized plastic chips (1 cm × 1 cm) were paced within each flask and incubated at 28 °C without shaking. After 72 hours of incubation, plastic chips were removed carefully from all the flasks without any agitation, slowly washed with sterilized distilled water to remove the planktonic cells and then air dried.

2.1.2. Study of bacterial biofilms under scanning electron microscope

All the plastic chips containing bacterial biofilms on insoluble phosphate granules were prepared for scanning electron microscopic (SEM) observation following proper fixation techniques and gold coating (Mandal et al., 2013). The details of the preparations for scanning electron microscopic observations are mentioned in Chapter II. Before gold coating the plastic chips were cut into small pieces (4 mm × 4mm) and placed on metal stubs. Biofilm formations by all the three bacterial strains on TCP as well as four different rock phosphates were observed under SEM (HITACHI S-530, Japan) and their biofilm formation patterns were compared.

2.2. Study of biofilm formations by isolated bacterial strains in relation to available P

In order to find out the relations of biofilm forming properties of isolated bacterial strains with available P, different concentrations of dipotassium phosphate (K$_2$HPO$_4$) were used in NBRIY medium by replacing the insoluble P source. Biofilm formations by the bacterial strains were quantified as well as biofilm morphologies were studied in the presence of different concentrations of K$_2$HPO$_4$. Planktonic cell densities were also determined to correlate the biofilm forming tendencies of the isolates in relation to available P.
2.2.1. Quantification of biofilm formation at different concentrations of K₂HPO₄

i. Culture condition for biofilm development

Biofilm formations were quantified in the presence of different concentrations of K₂HPO₄ (0, 25, 50, 100, 200, 300, 400, 500 µg/ml) in NBRIY medium using 24 well polystyrene cell culture plates (Stepanovic et al., 2000). 2 ml of NBRIY broth (pH 7.0) supplemented with different concentrations of K₂HPO₄ were taken in separate wells of 24 well cell culture plates. Wells were inoculated with 20 µl of bacterial culture (OD 600 nm = 0.5). Uninoculated wells for each concentration were considered as control. All the plates were then kept undisturbed in a BOD incubator at 28 °C for 48 hours. After 48 hours of incubation broths were decanted from each wells without disturbing the biofilms and the wells were washed twice with sterilized distilled water carefully to remove the planktonic cells.

ii. Staining of biofilm

After air drying in front of laminar airflow, 2 ml of 0.1% crystal violet was added to each well of the 24 well culture plates (Djordjevic et al., 2002). After 10 min of incubation at room temperature crystal violet was removed from each well and washed with sterilized distilled water for 3-4 times and again air dried.

iii. Measurement of optical density

1 ml of 33% acetic acid was applied to each well for extracting the crystal violet from adhered cells (Adetunji and Isola, 2011). After addition of acetic acid plates were kept at room temperature for 30 minutes with mild agitation for extracting the crystal violet completely. Optical densities were then measured at 595 nm using UV-VIS spectrophotometer (Thermo Scientific, UV-2700) against respective controls.

2.2.2. Estimation of planktonic cell densities at different concentrations of K₂HPO₄

In addition to quantification of biofilm formations planktonic cell densities were also measured in the presence of different concentrations of K₂HPO₄ in NBRIY broth after 48 hours of incubation. Before decanting the broth from 24 wells plates 100 µl of bacterial culture was taken from each well and sprayed on nutrient agar (NA) plates after serial dilution with sterilized distilled water. Plates were incubated at 28 °C for 24 hours and numbers of CFUs were counted.
2.2.3. Study of biofilm morphologies at different concentrations of K$_2$HPO$_4$

To observe the variation in biofilm morphologies in relation with available P, sterilized plastic chips were placed in slanting position before bacterial inoculation in NBRIY broth (pH 7) containing different concentrations of K$_2$HPO$_4$ (0, 25, 50, 100, 200, 300, 400, 500 µg/ml) within the wells of 24 well cell culture plates. Each well was inoculated with 1% bacterial inoculants (OD$_{600}$ nm = 0.5) and incubated at 28 °C for 48 hours. After 48 hours the plastic chips were removed carefully, washed slowly with sterilized distilled water and air dried. All the plastic chips were then stained with 0.1% crystal violet and finally observed under light microscope (LEICA DM2500).

2.3. Study of extracellular polymeric substance (EPS) production by bacterial isolates

2.3.1. EPS production in the presence of different insoluble phosphates

EPS production by isolated bacterial strains were studies in the presence of tricalcium phosphate (TCP) as well as in the presence of four different rock phosphates viz. JRP, PRP, MRP and URP. Each bacterial strain was inoculated to separate 100 ml NBRIY broth (pH 7.0) containing different insoluble phosphates (5 g/L) in 250 ml Erlenmeyer flasks and incubated at 28 °C for 48 hours. EPS production by all the bacterial isolates were quantified in terms of dry weight as well as total carbohydrate content.

2.3.2. EPS production in the presence of different concentrations of K$_2$HPO$_4$

In order to determine the relation of EPS production by bacterial strains with available phosphate in the media EPS production were also quantified in the presence of different concentrations of soluble P i.e. K$_2$HPO$_4$. Like biofilm formation experiments, insoluble phosphate source of NBRIY broth (pH 7.0) was replaced by K$_2$HPO$_4$. Each bacterial strain was inoculated to separate 100 ml NBRIY broth (pH 7.0) containing different concentrations of K$_2$HPO$_4$ (0, 25, 50, 100, 200, 300, 400, 500 µg/ml) in 250 ml Erlenmeyer flasks and incubated at 28 °C with mild shaking (120 rpm). Dry weight and total carbohydrate contents of EPS were determined after 48 hours of incubation.
2.3.3. Extraction and quantification of EPS

Bacterial cultures were centrifuged at 1000 rpm for 20 minutes to get the cell free supernatants (CFS). EPS from CFS was precipitated by adding same volume of ice cold ethanol followed by incubation at 4 °C for overnight (Ohno et al., 2000). Precipitated EPSs were separated by centrifugation at 7000 rpm for 20 minutes. To measure the dry weight EPSs were taken in petriplates and dried at 60 °C. On the other hand for total carbohydrate measurement, precipitated EPSs were resuspended in 5 ml of sterilized distilled water. 1 ml of sample was taken to measure the total carbohydrate by phenol sulfuric acid method (Dubois et al. 1956) as described in Chapter II.

2.4. Detection of bacterial quorum sensing system

In the genus *Burkholderia* two major types of quorum sensing (QS) system have been reported: (i) CepI/R system found in the members of *Burkholderia cepacia* complex (BCC) which produce and respond to C8-AHL (Lewenza et al., 1999; Gotschlich et al, 2001) and (ii) BraI/R system found in many nitrogen fixing, plant associated *Burkholderia* species which produce and respond to C-12-3oxo AHL (Suárez-Moreno et al., 2008). In the present study QS systems in the isolated bacterial strains were detected by PCR amplification followed by sequencing of genes related with QS systems. Amplified DNA were resolved on 1.2% agarose gel and purified using Xcelgen DNA Gel/PCR Purification Miniprep kit as described in chapter II. Sequencing reactions were carried out on ABI 3730xl genetic analyzer from Xcelric Genomics, India. Sequence alignment program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to assemble the nucleotide sequences. Nucleotide sequences were used to carry out BLAST with the NCBI GeneBank database (http://www.ncbi.nlm.nih.gov/) and aligned by CLUSTAL O (1.2.1) multiple sequence alignment program.

2.4.1 Detection of BraI/R QS system in *B. tropica* P4 and *B. unamae* P9

To detect the presence of BraI/R QS system in *B. tropica* P4 and *B. unamae* P9 two genes viz. *braR* and *rsaL* were amplified using the primers as described by Suárez-Moreno et al. (2008). Genomic DNA was used as template and appropriate annealing temperatures during amplification were selected using PeqLab gradient PCR. Details of PCR mixture used for amplification are mentioned in chapter II.
i. Amplification of braR

braR gene of two bacterial strains (B. tropica P4 and B. unamae P9) were amplified at an annealing temperature of 62 °C. Following primer pairs were used for the amplification of braR gene for both the bacterial strains-

pQEbraRfw: 5’-GGGGATCCTCGCCGATACTGGCCGCATC-3’
pQEbraRrv : 5’-GGGAAGCTTTTCAGCCCCGGATCTTATAAGGCC -3’

ii. Amplification of rsaL

rasL gene of B. tropica P4 and B. unamae P9 were also amplified at an annealing temperature of 48 °C. Details of PCR mixture used for amplification are mentioned in chapter II. Following primer pairs were used for the amplification of rsaL gene for both the bacterial strains-

braLFw: 5’-TTGTGAAATAAAGTCCCAG-3’
braLRv: 5’-CTGGAAAAATCCTGGCA-3’

2.4.2. Detection of CepI/R QS system in B. cepacia P10

To detect the presence of CepI/R QS system in B. cepacia P10 cep loci was amplified using the primer pairs described by Gotschlich et al, (2001). Details of PCR mixture used for amplification are mentioned in chapter II. Genomic DNA was used as template during PCR amplification. Following primer were used to amplify the cep loci-

cepIR-BaIV : 5’-GGAAACGCGCGTTCCGGCTCAGGCGGCGATAGC-3’
cepIR-Ba2R : 5’-CGTGAGCTGGACCCGGAAGGAAGCGGAGCC-3’

In B. cepecia presence of BraI/R QS system was also checked by using the same primers which were used to amplify braR and rsaL genes of B. tropica P4 and B. unamae P9.
3. RESULTS

3.1. Biofilm formation on insoluble P granules

Biofilm formation on insoluble P granules during solubilization process by isolated bacterial strains were studied using scanning electron microscope (SEM) where TCP and four different rock phosphates were used as insoluble P sources. After 48 hours of incubation when the plastic chips were carefully removed from NBRIY broth and observed under SEM following suitable preparations, improved adhesions of bacterial cells on TCP (Fig 7.1) as well as rock phosphate granules (Fig 7.2 and 7.3) by all the three strains were observed. In addition to attachments with insoluble P granules prominent cell to cell adhesions were also noticed for all the three species of *Burkholderia*. Depending on the nature of insoluble P, production of extracellular polymeric matrix was also observed during biofilm formation by the isolates. Although formations of biofilm were found for all the insoluble P granules but the extent of biofilm formations varied when different insoluble P were used in the broth. Among the different rock phosphates maximum bacterial attachments and cell to cell adhesions were observed on MRP granules by all the isolates (Fig. 7.3). On MRP granules compact biofilm structures with dense extracellular polymeric matrix were found under higher magnification of SEM (Fig. 7.3). On the other hand comparatively reduced bacterial adherences with thin EPS matrix were noticed for JRP granules (Fig. 7.2).

![Figure 7.1](image)

**Figure 7.1.** Scanning electron micrographs of biofilm formation by isolated bacterial strains on tricalcium phosphate (TCP) granules: a- *B. tropica* P4; b- *B. unamae* P9 and c- *B. cepacia* P10.
Figure 7.2. Scanning electron micrographs of biofilm formation by isolated bacterial strains on Jordan rock phosphate (JRP) granules: a- B. tropica P4; b- B. unamae P9 and c- B. cepacia P10.

Figure 7.3. Scanning electron micrograph of biofilm formation by isolated bacterial strains on Mussoorie rock phosphate (MRP) granules: a- B. tropica P4; b- B. unamae P9 and c- B. cepacia P10.
3.2. Quantification of biofilm formation at different degrees of available phosphate

In order to find out the relationship of biofilm formation by *Burkholderia* spp. with available P in the media the degrees of biofilm formations were quantified using different concentrations of soluble P (K$_2$HPO$_4$). When the crystal violet stain from different wells of 24 wells culture plates were extracted in 33% acetic acid, visual differences in biofilm formations at different concentrations of K$_2$HPO$_4$ were noticed for all the three isolates (Fig. 7.4a, 7.5a and 7.6a). UV-VIS spectrophotometric measurements of optical densities (OD) of crystal violet solutions suggested maximum biofilm formation by all the three bacterial isolates in the presence of 25 µg/ml of K$_2$HPO$_4$ (Fig. 7.4b, 7.5b and 7.6b). Maximum optical densities of 2.135 ± 0.15 and 1.86 ± 0.17 were recorded for *B. tropica* P4 and *B. unamae* P9 respectively at 25 µg/ml of K$_2$HPO$_4$. Biofilm formations markedly decreased along with increased concentrations of K$_2$HPO$_4$ for *B. tropica* P4 (Fig. 7.4b) as well as *B. unamae* P9 (Fig. 7.5b) and OD of crystal violet solutions suggested almost no biofilm formation by these two isolates in the presence of K$_2$HPO$_4$ at a concentration of 500 µg/ml. On the other hand although *B. cepacia* P10 followed similar pattern of biofilm formation up to 200 µg/ml of K$_2$HPO$_4$ but variations were detected above that concentration. In case of *B. cepacia* P10 maximum absorbance (1.61 ± 0.16) of crystal violet solution was recorded in the presence of 25 µg/ml of K$_2$HPO$_4$, and it reduced to 0.298 ± 0.12 at 200 µg/ml of K$_2$HPO$_4$. Above 200 µg/ml of K$_2$HPO$_4$ further increased in biofilm formation was detected by crystal violet staining (Fig. 7.6b). In the complete absence of K$_2$HPO$_4$ in the growth medium biofilm formations were also negligible for all the three isolates.

![Figure 7.4](image_url)

*Figure 7.4.* Quantification of biofilm formation by *B. tropica* P4 in the presence of different concentrations of K$_2$HPO$_4$: a- crystal violet staining of biofilm, b- OD of crystal violet solution extracted from biofilm.
3.3. Planktonic cell densities at different degrees of available phosphate

Counting of CFUs for planktonic cells at different concentrations of K₂HPO₄ showed that unlike biofilm formations planktonic cell densities increase along with increased concentration of soluble P (K₂HPO₄) for both *B. tropica* P4 (Fig 7.7a) and *B. unamae* P9 (Fig. 7.7b). On the other hand for *B. cepacia* P10 numbers of planktonic cells increased up to 200 µg/ml of K₂HPO₄ but decreased for further higher concentrations of K₂HPO₄ (Fig 7.7c).
3.4. Biofilm morphologies at different degrees of available phosphate

To determine the structural variations during biofilm formations at different concentrations of available P, biofilms were developed on plastic chips and observed after crystal violet staining (Fig. 7.8, 7.10, 7.12). Under light microscope compact biofilm structures were observed for all the three strains at lower concentrations of K$_2$HPO$_4$ than higher concentrations. Thick biofilms with maximum substratum coverage were noticed in the presence of 25 µg/ml of K$_2$HPO$_4$. Both the thickness as well as substratum coverage decreased along with increased concentrations of K$_2$HPO$_4$ for B. tropica P4 (Fig. 7.9) and B. unamae P9 (Fig. 7.11). Almost no patchy microcolonies were observed for both the strains above 300 µg/ml of K$_2$HPO$_4$. On the other hand in B. cepacia P10 biofilm thickness decreased up to 200 µg/ml concentration of K$_2$HPO$_4$ but increased with further increased concentrations of K$_2$HPO$_4$ (Fig. 7.13). Almost no biofilm structures were noticed for all the strains in the absence of K$_2$HPO$_4$ in the medium.
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Figure 7.8. Crystal violet staining of biofilm formation on plastic chips by *B. tropica* P4 grown at different concentrations of K$_2$HPO$_4$ in NBRIY broth.

Figure 7.9. Biofilm morphologies (under light microscope) of *B. tropica* P4 grown in the presence of different concentrations of K$_2$HPO$_4$ in NBRIY broth.
**Figure 7.10.** Crystal violet staining of biofilm formation on plastic chips by *B. unamae* P9 grown at different concentrations of $K_2HPO_4$ in NBRIY broth.

**Figure 7.11.** Biofilm morphologies (under light microscope) of *B. unamae* P9 grown in the presence of different concentrations of $K_2HPO_4$ in NBRIY broth.
Figure 7.12. Crystal violet staining of biofilm formation on plastic chips by B. cepacia P10 grown at different concentrations of K$_2$HPO$_4$ in NBRIY broth.

Figure 7.13. Biofilm morphologies (under light microscope) of B. cepacia P10 grown in the presence of different concentrations of K$_2$HPO$_4$ in NBRIY broth.
3.5. EPS productions by bacterial isolates in the presence of different insoluble P

In addition to biofilm formation EPS production by all the three isolates were also determined in the presence of TCP as well as four different rock phosphates. EPS production by the isolates were quantified in terms of dry weights as well as total carbohydrate contents. For all the three bacterial strains maximum EPS production was observed in the presence of MRP followed by URP, where as in the presence of TCP minimum EPS production was recorded by all of them ((Table 7.1). In the presence of TCP, *B. tropica* P4, *B. unamae* P9 and *B. cepacia* P10 produced 2.75 ± 0.25, 2.68 ± 0.27 and 2.08 ± 0.22 g/L of EPS respectively after 48 hours of incubation. On the other hand during MRP solubilization they produced 5.01 ± 0.29, 4.86 ± 0.32 and 5.78 ± 0.16 g/L of EPS respectively. In terms of total carbohydrate also EPS production was almost two times higher in MRP than TCP for both *B. tropica* P4 as well as *B. unamae* P9 (Table 7.1). In *B. cepacia* P10 total carbohydrate content increased nearly three times in the presence of MRP than TCP (Table 7.1). EPS production in the presence of other rock phosphates were also mentioned in the Table 7.1.

Table 7.1. EPS production in terms of dry weight as well as total carbohydrate (TC) by isolated bacterial strains during solubilization of different insoluble P.

<table>
<thead>
<tr>
<th>Insoluble P used in NBRIY broth</th>
<th>EPS production by isolated bacterial strains after 48 hours of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>B. tropica</em> P4</td>
</tr>
<tr>
<td></td>
<td>Dry wt. (g/L)</td>
</tr>
<tr>
<td>TCP</td>
<td>2.75 ± 0.25</td>
</tr>
<tr>
<td>JRP</td>
<td>2.82 ± 0.27</td>
</tr>
<tr>
<td>PRP</td>
<td>3.22 ± 0.22</td>
</tr>
<tr>
<td>URP</td>
<td>4.69 ± 0.17</td>
</tr>
<tr>
<td>MRP</td>
<td>5.01 ± 0.29</td>
</tr>
</tbody>
</table>

3.6. EPS production in the presence of different concentrations of K$_2$HPO$_4$

EPS production by all the isolates in the presence of different concentrations of K$_2$HPO$_4$ also showed similar patterns like biofilm formations. Maximum EPS production were observed in the presence of K$_2$HPO$_4$ at a concentrations of 25 µg/ml which decreased gradually along with increased concentrations of K$_2$HPO$_4$ for *B. tropica* P4 (Fig 7.14a) as well as *B. unamae* P9 (Fig. 7.14c). In the presence of 25
µg/ml of K₂HPO₄ in NBRIY broth B. tropica P4 and B. unamae P9 produced 5.65 ± 0.28 g/L and 5.02 ± 0.34 g/L of EPS respectively, whereas it decreased to 0.75 ± 0.08 g/L and 0.78 ± 0.1 g/L respectively at 500 µg/ml of K₂HPO₄ in the medium. On the other hand in B. cepacia P10 5.32 ± 0.26 g/L EPS production were measured in the presence 25 µg/ml of K₂HPO₄ and decreased gradually up to 3.86 ± 0.19 g/L for 200 µg/ml of K₂HPO₄, but increased again along with increased concentrations of K₂HPO₄ (Fig. 7.14e). In terms of total carbohydrate contents also EPS productions by the isolates showed similar patterns (Fig 7.14b, 7.14d and 7.14f).

Figure 7.14. Production of EPS in terms dry weight (a, c, e) and total carbohydrate (b, d, f) by bacterial isolates at different concentrations of K₂HPO₄ in NBRIY broth: a & b- B. tropica P4; c & d-B. unamae P9; e & f- B. cepacia P10.
3.7. Presence of BraI/R QS sytem in B. tropica P4 and B. unamae P9

To detect the presence of BraI/R QS system two genes braR and rsaL were amplified using the primers described earlier (Suárez-Moreno et al., 2008). When the amplified DNA were resolved on 1.2% agarose gel single band of nearly 720 bp for braR genes were detected for both B. tropica P4 as well as B. unamae P9 (Fig. 7.15). On the other hand nearly 225 bp bands were observed for rsaL gene of the strains (Fig. 7.16). After sequencing nucleotide sequence of each gene were generated using forward and reverse sequence data (Fig. 7.17 and 7.18) and confirmed as braR and rsaL gene by BLAST analysis on NCBI GenBank database.

Analysis using CLUSTAL O (1.2.1) multiple sequence alignment program reveled that braR of B. tropica P4 showed 81.62% identity with B. kururiensis M130 and 95.58% identity with B. unamae MTI-641T where as braR of B. unamae P9 showed 80.77% identity with B. kururiensis M130 and 96.44% identity with B. unamae MTI-641T (Fig. 7.19). On the other hand 225 bp fragment of rsaL gene of B. tropica P9 showed 77.58% identity with B. kururiensis M130 and 82.59% identity with B. unamae MTI-641T where as 225 bp fragment of rsaL gene of B. unamae P9 showed 77.13% identity with B. kururiensis M130 and 82.14% identity with B. unamae MTI-641T (Fig. 7.20).

Figure 7.15. Amplified DNA for braR gene on 1.2% agarose gel: lane 1-100 bp DNA ladder; lane 2- braR of B. tropica P4; lane 3- braR of B. unamae P9.
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Figure 7.16. Amplified DNA for rsaL gene on 1.2% agarose gel: lane 1-100 bp DNA ladder; lane 2- rsaL of B. tropica P4; lane 3- rsaL of B. unamae P9.

Figure 7.17. Nucleotide sequence of braR (a) and rsaL (b) of B. tropica P4.
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**Figure 7.18.** Nucleotide sequence of *braR* (a) and *rsaL* (b) of *B. unamae* P9.

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**Figure 7.19.** CLUSTAL O (1.2.1) multiple sequence alignment of *braR* (702 bp). (1- *B. tropica* P4, 2- *B. unamae* P9, 3- *B. kururiesis* M130; 4- *B. unamae* MTI-641\(^2\)).
### 3.8. Quorum sensing system in *B. cepacia* P10

Although attempts were taken to detect the presence of CepI/R quorum sensing system in *B. cepacia* P10 but no amplifications were found on 1.2% agarose gel. Even after changing different PCR parameters using the same primers described by Gotschlich et al. (2001) the *cep* loci was not amplified. BraI/R quorum sensing system was also not detected in *B. cepacia* P10 using the primers used for amplification of *braR* and *rsaL* genes of *B. tropica* P4 and *B. unamae* P9.

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**Figure 7.20.** CLUSTAL O (1.2.1) multiple sequence alignment of *rsaL* (224 bp). (1- *B. tropica* P4, 2- *B. unamae* P9, 3- *B. kururiensis* M130; 4- *B. unamae* MTI-641³).
4. DISCUSSION

In environment several microorganisms concentrate at solid-liquid interfaces to form adherent multicellular structures which in generally known as biofilm. During biofilm formation bacterial cells stick to each other and often these cells adhere to a surface. Sometimes these adherent cells embedded within an extracellular matrix made up of polysaccharides and other macromolecules. Microorganisms produce biofilm to overcome several environmental stresses like nutrient limitation, desiccation and predatory grazing (Danhorn et al., 2004). Biofilm formation by pathogenic microorganisms serve as one of the important protecting mechanism against several antimicrobials (Stewart, 2002) and makes threat to medicinal treatments. In addition to various drawbacks of microbial biofilm formations there are several positive aspects also (Robertson and McLean, 2015). As for example biofilm formation by several bacterial strains on crop plant rhizosphere confers protection against various fungal diseases (Maddula et al., 2008).

In the present endeavor biofilm formation by isolated bacterial strains were studied during solubilization of insoluble P. After 48 hours of incubation prominent biofilm formations on insoluble P granules were observed by all the three strains of *Burkholderia* using scanning electron microscopic techniques (Eighmy et al., 1983). During solubilization of insoluble P, biofilm formation by the bacterial isolates probably create a close environment that help better solubilization of phosphate granules by organic acids secreted by the isolates. Mukhopadhyay et al. (2010) also observed biofilm formation by *B. cepacia* C11 on JRP granules during solubilization. Comparison between the extents of biofilm formation on different rock phosphate granules revealed maximum biofilm formation by all the isolates on MRP granules. It have been reported earlier that phosphate content in MRP was lowest in comparison to other rock phosphates (Pal, 2006). By assuming the role of available P on the extent of biofilm formation by the isolates, quantitative approaches were taken where biofilm formation by the isolates were quantified using crystal violet staining technique in the presence of different concentrations of soluble P (K$_2$HPO$_4$). Measurement of optical densities of crystal violet solutions indicated maximum biofilm formation by all the three isolates in P limitation conditions. These outcomes suggested that the P solubilizing bacterial isolates attached to the surfaces of insoluble P granules and release more soluble P to overcome the stress generated by P
limitation. Huang et al. (1998) also reported upregulation of alkaline phosphatase activity in phosphorous limited biofilm. It was also found that phosphorous limiting condition enhances the biofilm formation of plant pathogenic Agrobacterium tumefaciens (Danhorn et al., 2004). Increase of planktonic cell densities along with increased concentrations of K$_2$HPO$_4$ also supported the positive correlation between P limitation and increase of bacterial biofilm. Although maximum biofilm formation by the strains were observed in the presence of 25µg/ml of K$_2$HPO$_4$ but they were unable to form biofilm in the absence of soluble P due to inhibition of bacterial growth in the absence of essential macroelement phosphorous. In B. cepacia P10 although biofilm formation gradually decreased along with increased concentrations of K$_2$HPO$_4$ like two other bacterial strains but further increase in biofilm formation above 200 µg/ml was observed. Probably in the higher concentrations of soluble P, other stress conditions were generated that again influenced the bacterial cells to form biofilm structures. Further studies are required to find out the actual reason behind the increase of biofilm formation in case of B. cepacia P10 in the presence of higher concentrations (>200µg/ml) soluble P. Studies on biofilm morphologies showed compact and thick biofilm structures by the isolates in P limitation condition. Decrease in thickness and substratum coverage along with increased concentrations of K$_2$HPO$_4$ especially for B. tropica P4 and B. unamae P9 strongly suggested the influence of P limitation over biofilm development.

The biosynthesis of EPS serves many functions like promotion of bacterial attachments to solid surfaces and formation as well as maintenance of microcolony and mature biofilm structure (Czaczyk and Myszka, 2007). EPS enhances the resistance of biofilm to environmental stresses. In some cases EPS matrix also help the bacteria to accumulate the nutrients (Pontefract, 1991). Increase of EPS along with biofilm production by bacterial isolates strongly indicated their direct involvement in biofilm formation during P limitation condition. Maximum EPS production in the presence of MRP probably directs the bacterial organisms to produce more condensed biofilm structures to release sufficient soluble P from MRP. Due to high phosphate content in TCP and JRP, bacterial strains faced less difficulties to produce sufficient amount of soluble P, which ultimately suppressed the necessities of EPS production to create condense biofilm structures. Highest degrees of EPS production in the present of lowest concentration of K$_2$HPO$_4$ also suggested its strong correlation with available phosphate. On the other hand increase in EPS production at higher concentration of
K$_2$HPO$_4$ (>200 µg/ml) were observed in case *B. cepacia* P10 due to increased biofilm structures. This also indicated the positive role of EPS on biofilm development. Involvement of EPS production in biofilm formation have been reported by several workers for various bacterial species (Czaczyk and Myszka, 2007). Chunha et al. (2004) reported that although EPS was not required for biofilm initiation in *Burkholderia cepacia* complex but plays major role in the establishment of thick biofilm. Yi et al. (2008) found four bacterial strains under the genus *Enterobacter, Arthrobacter* and *Azotobacter* which were able to produce significant amount of EPS and showed strong abilities for TCP solubilization. In different members of *Burkholderia* a branched acetylated heptasaccharide known as cepaci an was detected as the major exopolysaccharide which play crucial role in the survival of these organisms in different environmental conditions like desiccation, metal ion stress etc. (Ferreira et al., 2010).

AHL dependent quorum sensing system plays significant role in biofilm formation in various Gram negative bacterial organisms. In the present study in *B. tropica* P4 and *B. unamae* P9 presence of BraI/R QS system was detected. As the primer sets were effective to amplify the braR and rsaL gene from 20 species of *Burkholderia* (Suarez and Moreno et al., 2008) therefore they were used to detect the presence of BraI/R QS system in isolated bacterial strains. Suarez-Moreno et al. (2010) also reported that biofilm formation and EPS productions were regulated by BraI/R QS system in some plant associated *Burkholderia* spp. In the present observation it was found that biofilm formation and EPS production by *B. tropica* P4 as well as *B. unamae* P9 was highly correlated with available P in the medium. So there may be some positive relation of this QS system which regulates the biofilm formation or EPS production during P solubilization by the isolates. Further studies are required to establish the relationship between BraI/R QS system with P solubilization by *B. tropica* P4 and *B. unamae* P9. On the other hand no amplification was detected for *cep* loci of *B. cepacia* 10 using the above mentioned primers. So, further studies are also required to detect the QS system in *B. cepacia* P10 and to find out its relation with biofilm formation during P solubilization. The difference in their QS system may also be the reason for variation in biofilm formation pattern as observed in the present experiment for *B. cepacia* P10 with two other *Burkholderia* strains.