CHAPTER 4

RESULTS

4.1. Isolation of endophytic bacteria from different plant samples

4.1.1. Sampling of plant parts from different medicinal plants
We had collected plant parts from 6 different plants according to their medicinal properties from Shoolini University Botanical Garden, Bajhol, Solan District, Himachal Pradesh. For convenience, the samples were abbreviated as EL (leaf part of *Echinacea purpurea*), EF (flower part of *E. purpurea*), ER (root part of *E. purpurea*), MO-L (leaf part of *Mentha officinalis*), MS-L (leaf part of *M. spicatal*), CA (leaf part of *Centella asiatica*), CS (leaf part of *Cannabis sativa*), WL (leaf part of *Withania somnifera*), WR (root part of *W. somnifera*), WS (stem part of *W. somnifera*), LS (stem part of *Lonicera japonica*) and LF (flower part of *L. japonica*).

Table 4.1. Different plant samples with their plant parts.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plant</th>
<th>Plant part</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Echinacea purpurea</em></td>
<td>Leaf (EL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flower (EF)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root (ER)</td>
</tr>
<tr>
<td>2.</td>
<td><em>Mentha officinalis</em></td>
<td>Leaf (MO-L)</td>
</tr>
<tr>
<td></td>
<td><em>Spicatal</em></td>
<td>Leaf (MS-L)</td>
</tr>
<tr>
<td>3.</td>
<td><em>Centella asiatica</em></td>
<td>Leaf (CA)</td>
</tr>
<tr>
<td>4.</td>
<td><em>Cannabis sativa</em></td>
<td>Leaf (CS)</td>
</tr>
<tr>
<td>5.</td>
<td><em>Withania somnifera</em></td>
<td>Leaf (WL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Root (WR)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stem (WS)</td>
</tr>
<tr>
<td>6.</td>
<td><em>Lonicera japonica</em></td>
<td>Stem (LS)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flower (LF)</td>
</tr>
</tbody>
</table>
4.1.2. Determination of total colony forming unit (CFU)

Plant part after crushing in pestle and mortar was analysed for CFU per gram of plant sample. As an example shown in Fig 4.1, EF sample contained $1535 \pm 36$ CFUs per g of flower part. Autoclaved flower plant was also processed as control which showed no colonies that concluded bacteria isolated have no environmental bacteria (Fig 4.1B). Similarly, last water washing was also tested (Fig 4.1C) and we found no colony, that shows that colonies in Fig 4.1A were indeed endophytes.

![Fig 4.1. Determination of CFUs in plant sample (*Echinacea purpurea* flower). (A) Plant sample Number of colonies = 179 (B) Autoclaved plant sample (C) Last water washing during surface sterilization.](image)

CFUs of all the plant samples were calculated and were summarized in Table 4.2. Maximum CFUs was observed in WR whereas minimum CFU was observed in MO-L and MS-L.

**Table 4.2.** Average CFUs of endophytic bacteria per g of plant sample. Abbreviations for plant parts are mentioned in Table 4.1.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plant</th>
<th>Plant part</th>
<th>CFUs per gram of plant part</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Echinacea purpurea</em></td>
<td>EL</td>
<td>$970 \pm 28$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EF</td>
<td>$1535 \pm 37$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ER</td>
<td>$165 \pm 8$</td>
</tr>
<tr>
<td>2.</td>
<td><em>Mentha officinalis</em></td>
<td>MO-L</td>
<td>$28 \pm 4$</td>
</tr>
</tbody>
</table>
4.1.3. Purification of colonies

We further went on to purify endophytic microorganism(s). For that, approximately 10 colonies which differ in color, size and morphology were randomly selected from each plant part. Colonies were streaked on NAM plates for colony purification (shown in Fig 4.2) and later their plant growth promoting activities were checked.

![Fig 4.2. Purification of isolated endophytic strains on NAM plate to obtain single purified colony.](image)

### Table 4.1: Plant growth promoting activities

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Strain</th>
<th>Activity</th>
<th>Value ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mentha spicata</em></td>
<td>CA</td>
<td>MS-L</td>
<td>28 ± 4</td>
</tr>
<tr>
<td><em>Centella asiatica</em></td>
<td>CA</td>
<td>MS-L</td>
<td>286 ± 8</td>
</tr>
<tr>
<td><em>Cannabis sativa</em></td>
<td>CS</td>
<td>MS-L</td>
<td>1240 ± 32</td>
</tr>
<tr>
<td><em>Withania somnifera</em></td>
<td>WL</td>
<td>MS-L</td>
<td>305 ± 7</td>
</tr>
<tr>
<td></td>
<td>WR</td>
<td>MS-L</td>
<td>7855 ± 16</td>
</tr>
<tr>
<td></td>
<td>WS</td>
<td>MS-L</td>
<td>3055 ± 9</td>
</tr>
<tr>
<td><em>Lonicera japonica</em></td>
<td>LS</td>
<td>MS-L</td>
<td>1428 ± 31</td>
</tr>
<tr>
<td></td>
<td>LF</td>
<td>MS-L</td>
<td>225 ± 16</td>
</tr>
</tbody>
</table>

4.2. Screening of strains for phosphate solubilisation and siderophore activity

4.2.1. Phosphate solubilisation

Bacterial ability to solubilize mineral phosphates has been of interest to agricultural microbiologists as it can enhance the availability of Pi for microbial and plant growth. Most of the soils contain insoluble phosphates. Out of 120 isolates, 6 isolates (5%), named
EF.B2, EF.B3, LS.B11, WS.B8, WS.B9 and CS.B10, showed phosphate solubilization activity on Pikovskaya’s agar medium by inducing halo zones around the colonies (Fig 4.3). Out of these six strains, EF.B2, EF.B3 and LS.B11 exhibited a higher phosphate solubilization activity (8-15 mm) than WS.B8, WS.B9 and CS.B10 (2-3 mm) (Table 4.3). *M. officinalis, M. spicatal* and *C. asiatica* are the plants whose endophytic bacteria did not show any phosphate solubilisation activity.

![Image of bacterial colonies with halo zones](image)

**Fig 4.3.** Formation of clear halo zone around bacterial colony on Pikovskaya media showed solubilisation of inorganic phosphate.

**Table 4.3.** Strain showed phosphate solubilization index and formation of halo zone around bacterial colony.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Strain</th>
<th>Total diameter (mm)</th>
<th>Colony diameter (mm)</th>
<th>Halo zone diameter (mm)</th>
<th>Phosphate Solubilization Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>EF.B2</td>
<td>18</td>
<td>10</td>
<td>8</td>
<td>1.8</td>
</tr>
<tr>
<td>2.</td>
<td>EF.B3</td>
<td>31</td>
<td>16</td>
<td>15</td>
<td>1.94</td>
</tr>
<tr>
<td>3.</td>
<td>LS.B11</td>
<td>25</td>
<td>16</td>
<td>9</td>
<td>1.56</td>
</tr>
<tr>
<td>4.</td>
<td>WS.B8</td>
<td>12</td>
<td>10</td>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td>5.</td>
<td>WS.B9</td>
<td>14</td>
<td>12</td>
<td>2</td>
<td>1.17</td>
</tr>
<tr>
<td>6.</td>
<td>CS.B10</td>
<td>13</td>
<td>10</td>
<td>3</td>
<td>1.18</td>
</tr>
</tbody>
</table>
4.2.2. Siderophore activity

The production of siderophores by plant-associated bacteria has received major attention because of their role in both biological control of diseases and in virulence of plant-pathogens (Neilands and Leong, 1986; Loper and Buyer, 1991). 25 isolates (20.8%) out of 120 strains were found to confer siderophore production. They changed colour of the CAS medium from blue to orange as shown in Fig 4.4. When strains were incubated on CAS agar plates, the following three responses were observed: no growth, growth but no halos surrounding colonies, and growth and small to large orange halos surrounding the colonies as illustrated in Fig 4.4. Out of these twenty five strains, EF.B3, LS.B8, LS.B11, WR.B1, WR.B2 and WL.B2 showed high siderophore activity (8-11 mm); LS.B12, LF.B1, WR.B6, WS.B1 and WS.B6 showed moderate siderophore activity (5-7 mm); whereas EF.B2, EF.B5, EF.B7, EL.B11, LF.B5, WR.B3, WR.B5, WS.B2, WS.B3, WS.B4, WS.B5, WS.B7, WL.B6 and CA.B10 showed low siderophore activity (2-4 mm) (shown in Table 4.4). Since the assay is based on the competitive exchange of iron (III), potential chelators are detectable corresponding to their affinity for the metal i.e., strong chelators like siderophores chelates the metal iron more strongly than the weaker ones.

Fig 4.4. Siderophore production on CAS agar media. WR.B1, WR.B2, WR.B3, WR.B5, WR.B6, WL.B2, WL.B4, EL.B11, WS.B4, LS.B11, EF.B3 and EF.B2 strains showed orange zone around bacterial colony. But few strains, WL.B5, ER.B1, ER.B2, ER.B5 and CS.B10 does not show any growth on CAS media plates.
Table 4.4. Strains showed siderophore activity with their halo zone diameter.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Strain</th>
<th>Total diameter (mm)</th>
<th>Colony diameter (mm)</th>
<th>Halo zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>EF.B2</td>
<td>12</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td>EF.B3</td>
<td>15</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>EF.B5</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>4.</td>
<td>EF.B7</td>
<td>15</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>5.</td>
<td>EL.B11</td>
<td>15</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>6.</td>
<td>LS.B8</td>
<td>17</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>7.</td>
<td>LS.B11</td>
<td>27</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>8.</td>
<td>LS.B12</td>
<td>14</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>9.</td>
<td>LF.B1</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>10.</td>
<td>LF.B5</td>
<td>14</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>11.</td>
<td>WR.B1</td>
<td>21</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>12.</td>
<td>WR.B2</td>
<td>20</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>13.</td>
<td>WR.B3</td>
<td>12</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>14.</td>
<td>WR.B5</td>
<td>15</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>15.</td>
<td>WR.B6</td>
<td>16</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>16.</td>
<td>WS.B1</td>
<td>11</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>17.</td>
<td>WS.B2</td>
<td>11</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>18.</td>
<td>WS.B3</td>
<td>11</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>19.</td>
<td>WS.B4</td>
<td>13</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>
A large number of strains like CA.B1, CA.B2, CA.B6, EF.B6, EF.B8, EL.B3, EL.B7, ER.B2, ER.B5, LS.B13, LF.B2, CS.B10, WL.B1, WL.B7, WR.B4, WS.B3 and WS.B2 showed no growth on CAS agar medium. It shows that it may be difficult to grow fastidious microorganisms on the CAS agar plate and some ingredients of the CAS agar may have innate antibacterial activity (Verma et al., 2012). The detergent used in the preparation of CAS medium may be toxic to some bacteria. On the other hand, CA.B8, CA.B2, EF.B4, EL.B4, EL.B6, EL.B13, ER.B1, LS.B10, WL.B5 strains showed growth on CAS medium but did not show any halos surrounding colonies. Rest of the isolates did not show any appreciable activity.

For further analysis, we took LS.B11 and EF.B3 for detailed study. We also took CS.B10 which conferred moderate phosphate solubilisation activity and no siderophore activity. Both the strains LS.B11 and EF.B3 were different based on their colour and colony morphology.
4.3. Biofertilizer activity of LS.B11 and EF.B3

4.3.1. Detail analysis of phosphate solubilization activity

4.3.1.1. Inorganic phosphate solubilization

Quantitative estimation

Phosphate solubilization activity was quantified by chlorostannous reduced molybdophosphoric acid blue method as described in materials and methods (Ahmad et al., 2008). We found that 1 OD$_{600}$ of LS.B11, EF.B3 and CS.B10 cells produced 569.75±43 µg ml$^{-1}$, 685.5±34.7 µg ml$^{-1}$ and 182.25 µg ml$^{-1}$ of soluble phosphate from tricalcium phosphate, respectively.

4.3.1.2. Organic phosphate solubilization

Organic phosphate solubilization activity was checked on calcium phytate agar plate and sodium phytate agar plate as described in material and methods. EF.B3 and LS.B11 are the two strains that formed clear peripheral zones on turbid agar plate whereas CS.B10 did not show any halo around bacterial strain (shown in Fig 4.5).

![Fig 4.5. Plates showing organic phosphate solubilization by forming clear halo zone around bacterial colony on (A) calcium phytate agar media; (B) sodium phytate agar media. EF.B3, LS.B11 and Burkholderia sp. strain AU4i as positive control showed clear zone around bacterial colony whereas CS.B10 showed no growth on media plate.](image-url)
Organic phosphate solubilization activity on calcium phytate media plate was significantly higher for EF.B3 than LS.B11 that showed halo zone formation of 16 ± 0.8 mm and 11 ± 0.4 mm, respectively whereas Burkholderia sp. strain AU4i as positive control (accession number: KF114029), which was isolated from pea rhizosphere in our lab, showed 13 ± 0.6 mm halo zone formation (Fig 4.5A). But in sodium phytate agar plate, halo zone formation around bacterial colony was observed as 7 ± 0.4 mm and 14 ± 0.5 mm in EF.B3 and LS.B11, respectively (Fig 4.5B). It shows that two strains LS.B11 and EF.B3, both have organic phosphate activity but give different zone on different substrates. They might be producing two different types of phytase enzymes.

4.3.2. Nitrogen Fixation
The nitrogen fixing ability of bacterial endophytes was screened by their ability to grow on N₂-free BAz media. As compared to zero time (Fig 4.6 A-E), EF.B3 and LS.B11 showed growth on BAz media plate but CS.B10 did not show growth (shown in Fig 4.6 F-J). This suggests that both the isolates EF.B3 and LS.B11 has the capability to grow in nitrogen free media or use atmospheric nitrogen as nitrogen source for their growth but CS.B10 is unable to do that. Burkholderia sp. strain AU4i as positive control also showed growth whereas E. coli as negative control did not grow on BAz media.

![Fig 4.6. Nitrogen fixation. A-E shows the patched strains at 0 h and F-J shows the growth after 3 days on BAz media.](image-url)
4.3.3. Ammonia production

Ammonia production was analyzed qualitatively by two methods. In the first method, ammonia production was checked in peptone water after addition of Nesseler’s reagent. We observed that all the three strains, LS.B11, EF.B3 and CS.B10, transformed their colour to yellow after addition of Nesseler reagent as compared to negative control which is uninoculated peptone water or positive control *Burkholderia* sp. strain AU4i (Fig 4.7 A-E). It indicates the production of ammonia in LS.B11, EF.B3 and CS.B10.

In the second method, we tested ammonia production via urease test. LS.B11, EF.B3 and CS.B10 were capable of changing the urea agar to dark pink indicates production of ammonia (Fig 4.7 F-J). *Klebsiella pneumoniae* acted as positive control (Fig 4.7J) whereas *E. coli* acted as negative control (Fig 4.7I) for the test. These three strains are capable of producing urease enzyme which break down urea to ammonia which is an attribute of plant growth promoting bacteria.

### Fig 4.7. Ammonia production. (A-E) represents ammonia production by Nesseler’s reagent. (F-J) represents ammonia production on urea agar media.

4.3.4. Indole-3-acetic acid (IAA) production

IAA production by endophytic bacteria is correlated with their ability to induce root elongation and root hair formation (Lambrecht et al., 2000). The ability to produce IAA in the presence and absence of L-tryptophan was analyzed. Tryptophan, is a precursor in the biosynthesis of IAA. As shown in Fig 4.8, all endophytic isolates produced significant amount of IAA as observed with development of pink colour. We found that in the absence...
of L-tryptophan, LS.B11 (6.5±0.3 μg ml⁻¹) synthesized more IAA as compared to EF.B3 (2.8±0.1 μg ml⁻¹) and CS.B10 (1.4±0.05 μg ml⁻¹). Similarly, in the presence of L-tryptophan, the biosynthesis of IAA was highest in LS.B11 (5.1±0.5 μg ml⁻¹) followed by EF.B3 (3.5±0.2 μg ml⁻¹) and CS.B10 (1.8±0.04 μg ml⁻¹). Uninoculated with any strain was acted as negative control (Fig 4.8 A&F) and E. coli DH5α was taken as positive control (Fig 4.8 E&J).

The results suggested that LS.B11 produce more IAA both in presence or absence of tryptophan than EF.B3 and CS.B10. These results indicated that these endophytes could produce IAA in tryptophan independent pathway or dependent pathway.
4.4. Biocontrol activity

4.4.1. Siderophore activity
As we noted earlier that LS.B11 and EF.B3 showed siderophore production on CAS media (Fig 4.9A). We further compared their siderophore production. Siderophore production was quantified in Chrome azurol S liquid medium. We found that 1 OD$_{600}$ cells of LS.B11 strain produced ~9 fold and EF.B3 produced ~6 fold higher amount of siderophore as compared to *E. coli* DH5α (Fig 4.9B).

![Fig 4.9. (A) Siderophore production by LS.B11, EF.B3 and *Burkholderia* sp. strain AU4i (taken as positive control) (B) Graph showing siderophore production of LS.B11 and EF.B3 as compared to *E. coli.*](image)

4.4.2. Hydrogen cyanide production
Hydrogen cyanide production by bacteria was analyzed by sodium picrate assay as described in material and methods.

![Fig 4.10. Hydrogen cyanide production by bacterial endophytes (A-E). LS.B11, EF.B3 and CS.B10 produced HCN (A-C). Uncultured plate with any strain was taken as negative control (D) and *Burkholderia* sp. strain AU4i was taken as positive control (E).](image)
HCN produced by bacteria that reacts with sodium picrate present on whatmann filter paper which result in release of isopurpuric acid that gives yellow to red colour on whatmann filter paper (shown in Fig 4.10). LS.B11 showed red colour on whatmann filter paper that showed high production of hydrogen cyanide (shown in Fig 4.10C). EF.B3 showed orange colour (shown in Fig 4.10D) whereas CS.B10 showed light orange (shown in Fig 4.10E). This concluded that LS.B11 showed highest production of HCN followed by EF.B3 and CS.B10. Unstreaked modified nutrient agar plate with glycine acted as negative control that showed yellow colour (shown in Fig 4.10B) and *Burkholderia* sp. strain AU4i acted as positive control that showed orange colour (shown in Fig 4.10A).

### 4.4.3. Antifungal activity against plant pathogens

As these two strains, LS.B11 and EF.B3, showed ability for siderophore and HCN production. So, we further tested them against plant pathogenic fungi. The anti-fungal activity was evaluated by the inhibition of hyphal growth of the plant pathogens adjacent to the bacterial strains. Selected endophytic bacteria were tested for their *in vitro* inhibitory activity against *Rhizoctonia* sp., *Fusarium* sp., *Alternaria* sp. and *Pythium* sp.

Bacterial strain LS.B11 inhibited the growth of *Rhizoctonia* fungus (70.9%) (Fig 4.11 A vs E), *Fusarium* sp. (62.2%) (Fig 4.11 B vs F), *Alternaria* sp. (53.75%) (Fig 4.11 C vs G) and *Pythium* sp. (54.2%) (Fig 4.11 D vs H) (Table 4.5).

EF.B3 strongly inhibited the growth of *Rhizoctonia* sp. (65.48%) (Fig 4.11 I vs M), *Fusarium* sp. (68.4%) (Fig 4.11 J vs N), *Alternaria* sp. (71.2 %) (Fig 4.11 K vs O) and *Pythium* sp. (61.1 %) (Fig 4.11 L vs P) (Table 4.5). These results suggests that LS.B11 showed highest activity against *Rhizoctonia* sp. and EF.B3 showed highest activity against *Alternaria* sp. and *Fusarium* sp.

CS.B10 had weak or negligible antifungal activity against phytopathogenic fungi (Fig 4.11 Q-X).
Fig 4.11. *In vitro* antifungal activity of bacterial strain LS.B11, EF.B3 and CS.B10 against plant pathogenic fungi. Fungal disc of pathogenic fungi *Rhizoctonia* sp., *Fusarium* sp., *Alternaria* sp. and *Pythium* sp. was placed in the centre of potato dextrose agar plate. Bacterial strain were streaked at 2 cm away from the disc in second row.
Table 4.5. Percentage growth inhibition of phytopathogenic fungus by endophytic strains.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Fungal strain</th>
<th>LS.B11</th>
<th>EF.B3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Rhizoctonia</em> sp.</td>
<td>70.9%</td>
<td>65.48%</td>
</tr>
<tr>
<td>2.</td>
<td><em>Fusarium</em> sp.</td>
<td>62.2%</td>
<td>68.42%</td>
</tr>
<tr>
<td>3.</td>
<td><em>Alternaria</em> sp.</td>
<td>53.75%</td>
<td>71.25%</td>
</tr>
<tr>
<td>4.</td>
<td><em>Pythium</em> sp.</td>
<td>54.2%</td>
<td>61.1%</td>
</tr>
</tbody>
</table>

Table 4.5 concludes that LS.B11 shows more inhibition activity against *Rhizoctonia* sp. than EF.B3. It could be due to high amount of siderophore production and HCN production by LS.B11 as compared to EF.B3. On the contrary, EF.B3 shows more inhibition activity against *Fusarium* sp., *Alternaria* sp. and *Pythium* sp. than LS.B11 strain. It may be secretion of some bioactive compounds (secondary metabolites) that has additional effect with siderophore production and HCN production. Endophytes from the *Echinacea purpurea* and *Lonicera japonica* plants appeared to have higher antifungal activity than those from the *Cannabis sativa* plant.
4.5. *In plantae* effects of LS.B11 and EF.B3

4.5.1. Growth promotion

The presence of various biofertilizer activities like IAA production, nitrogen fixation and phosphate solubilization prompted us to hypothesize that two strains, LS.B11 and EF.B3, could stimulate plant growth. Therefore, LS.B11 and EF.B3 strains were further explored for their ability to promote seedlings growth. Surface sterilized pea seeds were treated with LS.B11 and EF.B3 as described in experimental procedures.

![Fig 4.12. Effect on growth by LS.B11 and EF.B3 strains using pea seedling. Both LS.B11 and EF.B3 strains stimulated roots and shoots formation. Surface sterilized pea seeds were grown in dark for 6 days. Seedlings were treated either with nutrient broth (A), bacterial culture of LS.B11 (B) or EF.B3 (C) with a density of $10^7$ cells ml$^{-1}$.](image)

Interestingly, as compared to the untreated seeds (shown in Fig 4.12, Table 4.6 lane 1), both LS.B11 and EF.B3 treated seeds showed enhanced root and shoot formation. We found that LS.B11 treatment increased the root length by 4.27-fold and shoot length by 1.13-fold as compared to untreated seeds (Fig 4.12 panel A vs B, Table 4.6 lane 2). On the other hand, EF.B3 increased root length by 2.95-fold and shoot length by ~2-fold (Fig 4.12 panel A vs C, Table 4.6 lane 3). Similarly, seeds treated with LS.B11 and EF.B3 showed ~1.5-fold increase in secondary roots as compared to untreated seeds.
4.5.2. Biocontrol activity

Pea seedlings are affected by plant pathogenic fungi. *Fusarium* sp., *Rhizoctonia* sp. and *Pythium* sp. infects the pea plant and cause Fusarium root and stem rot, Rhizoctonia seedling blight and Pythium root rot, respectively (Hagedorn, 1991). As these strains, LS.B11 and EF.B3, inhibited the growth of plant pathogens *in vitro* as shown in Fig 4.11. So, we designed an experiment to test that endophytic strains, LS.B11 and EF.B3 can protect the plants from fungal pathogens. We found that both LS.B11 and EF.B3 inhibits the growth of the fungus and promote the growth of the seedlings.

![Figure 4.13](image)

**Fig 4.13.** Antagonism of phytopathogenic *Fusarium* fungus by LS.B11 and EF.B3 strains using pea seedling. Both LS.B11 and EF.B3 strains conferred biocontrol activity. Surface sterilized pea seeds were grown in dark for 6 days. Seedlings were treated with 7 days old *Fusarium* sp. culture and later treated either with nutrient broth (A), bacterial culture of LS.B11 (B) or EF.B3 (C).

As expected, fungi (*Fusarium* sp., *Pythium* sp., *Rhizoctonia* sp. and *Alternaria* sp.) treatment inhibited the growth of seedling as compared to control (Fig 4.12A vs 4.13A, 4.14A, 4.15A, 4.16A). In case of *Fusarium* & LS.B11 treatment (Fig 4.13B, Table 4.6 lane 5) and *Fusarium* & EF.B3 treatment (Fig 4.13C, Table 4.6 lane 6), there is 1.2-fold and 1.53-fold increase in shoot length, 9.3-fold and 5.78-fold increase in root length and 6.46-fold and 5-fold increase in secondary roots, respectively as compared to *Fusarium* fungus alone (Fig 4.13A, Table 4.6 lane 4).

As compared to *Pythium* fungus alone (Fig 4.14A, Table 4.6 lane 7), root length increased by 5-fold and 5.88 fold and secondary roots by 26 fold and 25-fold in case of *Pythium* &
LS.B11 treatment (Fig 4.14B, Table 4.6 lane 8) and *Pythium* & EF.B3 treatment, respectively (Fig 4.14C, Table 4.6 lane 9).

**Fig 4.14.** Antagonism of *Pythium* fungus by LS.B11 and EF.B3 strains using pea seedling. Both LS.B11 and EF.B3 strains conferred biocontrol activity. Surface sterilized pea seeds were grown in dark for 6 days. Seedlings were treated with 4 days old *Pythium* sp. culture and later treated either with nutrient broth (A), bacterial culture of LS.B11 (B) or EF.B3 (C).

*Pythium* fungus deteriorates the pea seeds. But this effect was shielded by endophytic strains, LS.B11 or EF.B3.

**Fig 4.15.** Antagonism of phytopathogenic *Rhizoctonia* fungi by LS.B11 and EF.B3 strains using pea seedling. Both LS.B11 and EF.B3 strains conferred biocontrol activity. Surface sterilized pea seeds were grown in dark for 6 days. Seedlings were treated with 5 days old *Rhizoctonia* sp. culture and later treated either with nutrient broth (A), bacterial culture of LS.B11 (B) or EF.B3 (C).
In case of *Rhizoctonia* & LS.B11 treatment (Fig 4.15B, Table 4.6 lane 11) and *Rhizoctonia* & EF.B3 treatment (Fig 4.15C, Table 4.6 lane 12), there is 1.5-fold and 1.16-fold increase in shoot length, 7.3-fold and 6.6-fold increase in root length and 19.28-fold and 12.1-fold increase in secondary roots, respectively as compared to *Rhizoctonia* fungus alone (Fig 4.15A, Table 4.6 lane 10).

**Fig 4.16.** Antagonism of phytopathogenic *Alternaria* fungi by LS.B11 and EF.B3 strains using pea seedling. Both LS.B11 and EF.B3 strains conferred biocontrol activity. Surface sterilized pea seeds were grown in dark for 6 days. Seedlings were treated with 10 days old *Alternaria* sp. culture and later treated either with nutrient broth (A), bacterial culture of LS.B11 (B) or EF.B3 (C).

In last case, *Alternaria* & LS.B11 treatment (Fig 4.16B, Table 4.6 lane 14) and *Alternaria* & EF.B3 treatment (Fig 4.16C, Table 4.6 lane 15), there is 1.1-fold and 1.37-fold increase in shoot length, ~7-fold and 5.57-fold increase in root length and 2.28-fold and 1.7-fold increase in secondary roots, respectively as compared to *Alternaria* fungus alone (Fig 4.16A, Table 4.6 lane 13). Increase in shoot length, root length and number of secondary roots may be the result of secretion of some bioactive compounds.
Table 4.6. Effect of endophytes on pea seedlings under different fungus treatment.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatment given</th>
<th>Shoot length (cm)</th>
<th>Root length (cm)</th>
<th>Number of side roots</th>
<th>Fresh wt. of seedling (g)</th>
<th>Dry wt. of seedling (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Untreated</td>
<td>3.26 ± 0.829 b</td>
<td>3 ± 0.209 b</td>
<td>19.13 ± 2.9 a</td>
<td>0.737 ± 0.102 a</td>
<td>0.136 ± 0.023 a</td>
</tr>
<tr>
<td>2.</td>
<td>LS.B11</td>
<td>3.78 ± 0.92 a</td>
<td>12.8 ± 0.95 a</td>
<td>29 ± 3.74 a</td>
<td>0.842 ± 0.133 b</td>
<td>0.138 ± 0.010 a</td>
</tr>
<tr>
<td>3.</td>
<td>EF.B3</td>
<td>6.87 ± 0.83 a</td>
<td>8.8 ± 0.12 a</td>
<td>29.75 ± 3.59 a</td>
<td>0.916 ± 0.07 a</td>
<td>0.14 ± 0.013 a</td>
</tr>
<tr>
<td>4.</td>
<td>Fusarium sp.</td>
<td>3.3 ± 0.837 a</td>
<td>1.1 ± 0.33 a</td>
<td>4 ± 2.87 b</td>
<td>0.516 ± 0.062 a</td>
<td>0.113 ± 0.011 a</td>
</tr>
<tr>
<td>5.</td>
<td>Fusarium sp. + LS.B11</td>
<td>3.972 ± 0.56 a</td>
<td>10.33 ± 0.3 a</td>
<td>25.86 ± 3.97 a</td>
<td>0.746 ± 0.029 a</td>
<td>0.135 ± 0.017 a</td>
</tr>
<tr>
<td>6.</td>
<td>Fusarium sp. + EF.B3</td>
<td>5.08 ± 1.137 a</td>
<td>6.38 ± 0.084 a</td>
<td>20.11 ± 3.586 a</td>
<td>0.842 ± 0.091 a</td>
<td>0.136 ± 0.031 a</td>
</tr>
<tr>
<td>7.</td>
<td>Pythium sp.</td>
<td>4.4 ± 0.858 a</td>
<td>1.82 ± 0.540 b</td>
<td>1 ± 0.1 c</td>
<td>0.613 ± 0.045 a</td>
<td>0.115 ± 0.018 a</td>
</tr>
<tr>
<td>8.</td>
<td>Pythium sp. + LS.B11</td>
<td>4.9 ± 0.459 a</td>
<td>9.57 ± 0.531 a</td>
<td>26 ± 3.26 a</td>
<td>0.798 ± 0.071 a</td>
<td>0.129 ± 0.025 b</td>
</tr>
<tr>
<td>9.</td>
<td>Pythium sp. + EF.B3</td>
<td>2.4 ± 0.589 b</td>
<td>10.7 ± 0.56 a</td>
<td>26 ± 4.5 b</td>
<td>0.727 ± 0.006 a</td>
<td>0.129 ± 0.025 b</td>
</tr>
<tr>
<td>10.</td>
<td>Rhizoctonia sp.</td>
<td>2.875 ± 0.17 a</td>
<td>1.28 ± 0.19 a</td>
<td>1.6 ± 0.547 b</td>
<td>0.521 ± 0.049 b</td>
<td>0.103 ± 0.008 b</td>
</tr>
<tr>
<td>11.</td>
<td>Rhizoctonia sp. + LS.B11</td>
<td>4.4 ± 0.316 a</td>
<td>9.22 ± 0.35 a</td>
<td>32.8 ± 1.92 a</td>
<td>0.758 ± 0.018 a</td>
<td>0.125 ± 0.001 a</td>
</tr>
<tr>
<td>12.</td>
<td>Rhizoctonia sp. + EF.B3</td>
<td>3.35 ± 0.44 a</td>
<td>8.2 ± 0.616 a</td>
<td>20.2 ± 2.280 a</td>
<td>0.736 ± 0.122 a</td>
<td>0.119 ± 0.004 a</td>
</tr>
<tr>
<td>13.</td>
<td>Alternaria sp.</td>
<td>4.1 ± 0.55 a</td>
<td>0.982 ± 0.39 a</td>
<td>12 ± 1.64 a</td>
<td>0.661 ± 0.096 a</td>
<td>0.117 ± 0.017 a</td>
</tr>
<tr>
<td>14.</td>
<td>Alternaria sp. + LS.B11</td>
<td>4.51 ± 0.165 a</td>
<td>6.84 ± 0.27 a</td>
<td>27.2 ± 2.28 a</td>
<td>0.777 ± 0.061 a</td>
<td>0.140 ± 0.012 a</td>
</tr>
<tr>
<td>15.</td>
<td>Alternaria sp. + EF.B3</td>
<td>5.6 ± 1.134 b</td>
<td>5.40 ± 0.066 a</td>
<td>20.67 ± 3.07 a</td>
<td>0.864 ± 0.076 a</td>
<td>0.137 ± 0.006 a</td>
</tr>
</tbody>
</table>

Pea seedlings were treated as described in material and methods. Lane 1: untreated seeds (control); Lane 2: seeds treated with LS.B11; Lane 3: seeds treated with EF.B3; Lane 4 seeds treated with *Fusarium* sp.; Lane
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5: seeds treated with *Fusarium* sp. and LS.B11; Lane 6: seeds treated with *Fusarium* sp. and EF.B3; Lane 7: seeds treated with *Pythium* sp.; Lane 8: seeds treated with *Pythium* sp. and LS.B11; Lane 9: seeds treated with *Pythium* sp. and EF.B3; Lane 10: seeds treated with *Rhizoctonia* sp.; Lane 11: seeds treated with *Rhizoctonia* sp. and LS.B11; Lane 12: seeds treated with *Rhizoctonia* sp. and EF.B3; Lane 13: seeds treated with *Alternaria* sp.; Lane 14: seeds treated with *Alternaria* sp. and LS.B11, Lane 15: seeds treated with *Alternaria* sp. and EF.B3.

Values are mean ± Standard deviation of four replicates with 25 seedlings for each treatment. For each column, values in row 1 are compared with rows 2 or 3; row 4 is compared with 5 or 6; row 7 is compared with 8 or 9; row 10 is compared with 11 or 12 and; row 13 is compared with 14 or 15.

*a* *p*-value < 0.001 is extremely significant; *b* *p*-value lies between 0.001 to 0.01 is very significant; *c* *p*-value lies between 0.01 to 0.05 is significant.
4.6. Identification of biosynthetic genes

4.6.1. Isolation of genomic DNA

Endophytic bacterial genomic DNA was isolated and was observed by agarose gel electrophoresis (shown in Fig 4.17). The concentration of genomic DNA was estimated by nanodrop values. The concentration of genomic DNA was calculated as 119.1 ng µl\(^{-1}\), 94.2 ng µl\(^{-1}\) and 77.8 ng µl\(^{-1}\) in case of LS.B11, EF.B3 and CS.B10, respectively. Purity of DNA was estimated by TECAN Nanoquant plate (Infinite 200). The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as “pure” for DNA. Ratio of absorbance was calculated as 1.81, 1.84 and 1.8 in LS.B11, EF.B3 and CS.B10, respectively. Isolated genomic DNA was further used for analysing the presence of NRPS and PKS genes.

![Fig 4.17. Isolation of genomic DNA from bacterial endophytes. Lane 1 consists of CS.B10 genomic DNA; Lane 2 consists of EF.B3 genomic DNA; Lane 3 consists of LS.B11 genomic DNA; Lane 4 consists of 1 kb plus DNA marker.](image-url)
4.6.2. Identification of genes encoding enzymes involved in the synthesis of bioactive compounds

We further tested if LS.B11, EF.B3 and CS.B10, isolated from medicinal plants, has biosynthetic potential to produce bioactive compounds. Previously, presence of genes coding for NRPS and PKS, was considered as an indicative of the potential to produce bioactive compound (Miller et al., 2012). We tested this idea by amplifying region coding for the conserved domain of PKS and NRPS viz., KS domain and A domain, respectively. We found 1,000 bp amplicon was amplified using genomic DNA of LS.B11, and not by EF.B3, using the degenerate primers for A domain of NRPS (Fig 4.18 lane 4 vs 2&3). Similarly, an amplicon of 650 bp was amplified from genomic DNA of EF.B3 using degenerate primers for KS domain of PKS (Fig 4.18 lane 6 vs 5&7). CS.B10 showed no amplicon product of either NRPS or PKS.

![Fig 4.18. NRPS and PKS gene amplification. Lane 1 consists of 1 kb DNA marker; Gene amplification of ~1,000 bp of A domain in CS.B10 (Lane 2); EF.B3 (Lane 3); LS.B11 (Lane 4); Gene amplification of ~650 bp of KS domain in LS.B11 (Lane 5); EF.B3 (Lane 6); CS.B10 (Lane 7).]

The amplicon product of NRPS in LS.B11 (Fig 4.18, lane 4) and PKS in EF.B3 (Fig 4.18, lane 6) were eluted out with the help of gel elution kit. Both the eluted product were ligated with pGEM-T vector with the help of T4 DNA ligase. The ligated vector was electroporated into E. coli top 10’ cells. Electroporated sample gave blue-white colonies on luria agar plate supplemented with ampicillin, X-gal and IPTG (shown in Fig 4.19).
Fig 4.19. LB agar plate showing blue-white color selection of recombinant *E. coli* using X-gal.

Transformants which contained the insert, showed white colonies were randomly picked and inoculated in 5 ml luria broth (LB broth) and incubated at 37 °C for overnight. Plasmids pLS.B11-NRPS and pEF.B3-PKS were isolated from overnight culture. Both the plasmids pLS.B11-NRPS and pEF.B3-PKS were further digested with *EcoRI* to check the inserts. After the restriction digestion, insert was checked on the gel. pLS.B11-NRPS showed the insert of 1,000 bp (Fig 4.20, lane 1 vs 2). Similarly, pEF.B3-PKS showed ~650 bp of insert after digestion (Fig 4.20, lane 4 vs 5).

Fig 4.20. Restriction digestion of plasmid using *EcoRI* enzyme. Lane 1 & 2 contains digested and undigested pLS.B11-NRPS plasmid, respectively; Lane 4 & 5 contains digested and undigested pEF.B3-PKS, respectively; Lane 3 consists of 1kb DNA ladder.
Both plasmids, pLS.B11-NRPS and pEF.B3-PKS, were sent for sequencing with T7 forward primer (5’-TAATACGACTCACTATAGGG-3’).

After sequencing, we got sequence of 936 nucleotides in case of A domain of NRPS gene, amplified from LS.B11 strain (Fig 4.21).

$$\text{ATGCTCCCGCGCCATGCGCGCGCGAGGCATTTGCGCGGGAATTCGATTGCGCGGAATTCGCCGGTGGTGCGTAT}$$
$$\text{GTACCCCTGGATCCCGACTATCTCTCGCAGCGGTTCTCCTACATGCTGGAAGACAGCGGCGTA}$$
$$\text{CGGCTCTGCTACTACAGCGCCGAGCCGCGGTGCCTGTATCGTCATGCCTAAGGCTCAGCAGAGCAAGGATCCGCAG}$$
$$\text{GCGCTGCTGGCCCTCATCGAAGAGGCTGAGGTACGACATTATCCAGGCGACGCCGCGACTTG}$$
$$\text{GAGGATGCTGCTGAACGCGCTCCCGGAGCGCGCGGAGTTCTGCTGCGCGCAAGGCGCTCT}$$
$$\text{GTCTTCCGACCCGCTCTATGCTGAGACCGGAGAAGCGGATTCGCAGGCTCGACCGGCAGGCCGAAAGGTGT}$$
$$\text{CCAGATCAGAATCTCGTTGGCTGCTCAACTTCCTCGGCCAGCATGCGCGGAGCGCGGAGGTTGCAC}$$
$$\text{CTCTTCCGACCCGCTCTGCTGCAATTGACCTCTCTGTCTCTCGATATCGCTGGCGCTGTAGCTATAC}$$
$$\text{CTGGGCTCTGACCCGCGGTGCCTGTATCGTCATGCTGCAAGGCCTACAGGCAGAAGGATCCGCAAG}$$
$$\text{GCCTGCTGCTGGCCCTCATCGAAGAGGCTGAGGTACGACATTATCCAGGCGACGCCGCGACTTG}$$
$$\text{GAGGATGCTGCTGAACGCGCTCCCGGAGCGCGCGGAGTTCTGCTGCGCGCAAGGCGCTCT}$$
$$\text{GTCTTCCGACCCGCTCTATGCTGAGACCGGAGAAGCGGATTCGCAGGCTCGACCGGCAGGCCGAAAGGTGT}$$
$$\text{CCAGATCAGAATCTCGTTGGCTGCTCAACTTCCTCGGCCAGCATGCGCGGAGCGCGGAGGTTGCAC}$$
$$\text{CTCTTCCGACCCGCTCTGCTGCAATTGACCTCTCTGTCTCTCGATATCGCTGGCGCTGTAGCTATAC}$$
$$\text{CTGGGCTCTGACCCGCGGTGCCTGTATCGTCATGCTGCAAGGCCTACAGGCAGAAGGATCCGCAAG}$$
$$\text{GCCTGCTGCTGGCCCTCATCGAAGAGGCTGAGGTACGACATTATCCAGGCGACGCCGCGACTTG}$$
$$\text{GAGGATGCTGCTGAACGCGCTCCCGGAGCGCGCGGAGTTCTGCTGCGCGCAAGGCGCTCT}$$
$$\text{GTCTTCCGACCCGCTCTATGCTGAGACCGGAGAAGCGGATTCGCAGGCTCGACCGGCAGGCCGAAAGGTGT}$$
$$\text{CCAGATCAGAATCTCGTTGGCTGCTCAACTTCCTCGGCCAGCATGCGCGGAGCGCGGAGGTTGCAC}$$
$$\text{CTCTTCCGACCCGCTCTGCTGCAATTGACCTCTCTGTCTCTCGATATCGCTGGCGCTGTAGCTATAC}$$

**Fig 4.21.** Nucleotide sequence of A domain of NRPS gene amplified from LS.B11 strain.

BLAST N analysis revealed that DNA sequence was 99% similar to the A domain of *PvdD* gene of *Pseudomonas aeruginosa* LESB58 (Accession no – emb|FM209186.1|) (shown in Fig 4.22). *PvdD* gene of LESB58 has a role in siderophore production (Mossialos et al., 2002). The nucleotide sequences of A domain of NRPS gene in LS.B11 was submitted in GenBank with accession numbers KM225659.
Fig 4.22. BLAST result of A domain of NRPS gene, amplified from LS.B11 strain, compared with *pvdD* gene of *Pseudomonas aeruginosa* LESB58 strain.

Upon translation, Expasy translate tool gave protein sequence of A domain of NRPS gene amplified from LS.B11 strain which was 312 amino acids as shown below.
ClustalW alignment showed that amino acids sequence have maximum proximity with *pvdD* gene, of *Pseudomonas aeruginosa* LESB58 (accession number - YP_002440488) with 94.5% identity (Fig 4.23). The GenBank accession number for the protein sequence of A domain of NRPS gene in LS.B11 strain is AIT42266.1.

**Fig 4.23.** Amino acid sequence of A domain of NRPS, amplified from LS.B11 strain, compared with *pvdD* gene of *Pseudomonas aeruginosa* LESB58 by clustalW alignment.
Fig 4.24. Phylogenetic analysis of NRPS in bacterial endophyte. Relationship inferred by maximum likelihood analysis of bacterial NRPS, A domain. Branch length indicates inferred divergence of amino acids. The scale bar represents change in 0.2 amino acid.

We further investigated the homology of A domain. The deduced amino acid sequence of A domain of LS.B11 was used in the reconstruction of phylogenies by constructing the tree using previously reported bacterial A domains (Miller et al., 2012). A domain of LS.B11
is closely related to pyoverdine A synthetase of *Pseudomonas fluorescens* (Fig 4.24) which was shown to be involved in siderophore production (Mossialos et al., 2002).

**Fig 4.25.** Schematic representation of nucleotide (A) and amino acids (B) of A domain of NRPS found in LS.B11 strain as compared to *pvdD* gene of *Pseudomonas aeruginosa* LESB58.
On the other hand, pEF.B3-PKS plasmid isolated from amplified product of KS domain, which gave insert after digestion were sent for sequencing with T7 forward primer (5’-TAATACGACTCACTATAGGG-3’).

After sequencing the plasmid with T7 forward primer, we got the sequence of 657 bp nucleotides for KS domain of PKS gene amplified from EF.B3 (shown in Fig 4.26).

![Fig 4.26. Nucleotide sequence of KS domain of PKS gene amplified from EF.B3 strain.](image)

DNA sequence showed 71% homology with DNA coding for beta-ketoacyl synthase gene of *Burkholderia gladioli* BSR3 chromosome 1 (Sequence ID: gb|CP002599.1|) as illustrated in Fig 4.27. The nucleotide sequence of KS domain of PKS gene in EF.B3 was submitted in GenBank with accession number KM225660.
Fig 4.27. BLAST result of KS domain of PKS gene, amplified from EF.B3 strain, compared with beta-ketoacyl synthase gene of *Burkholderia gladioli* BSR3 strain.

Upon translation, Expasy translate tool gave protein sequence of KS domain of PKS gene amplified from EF.B3 strain which was 227 amino acids as shown below.
ClustalW alignment showed that amino acids sequence have maximum proximity with beta-ketoacyl synthase of *Burkholderia gladioli* BSR3 chromosome 1 with 65% identity (Fig 4.28). The GenBank accession number for the protein sequence of KS domain of PKS gene in EF.B3 strain is AIT42267.1.

**Fig 4.28.** Amino acid sequence of KS domain of PKS, amplified from EF.B3 strain, compared with beta-ketoacyl synthase gene of *Burkholderia gladioli* BSR3 by clustalW alignment.
Fig 4.29. Phylogeny of bacterial PKS. Evolutionary relationship determined by maximum likelihood of bacterial PKS ketosynthase domain. Branch length indicates divergence in amino acids. The scale bar represents 0.05 amino acid changes.
We further investigated the homology of KS domain. The deduced amino acid sequence of KS domain of EF.B3 was used in the reconstruction of phylogenies by constructing the tree using previously reported bacterial KS domains (Miller et al., 2012). The phylogenetic tree of KS domain of EF.B3 revealed that it is closely related to microcystin synthase of *Microcystis aeruginosa* (Fig 4.29).

![Fig 4.30. Schematic representation of nucleotide (A) and amino acids (B) of KS domain of PKS gene found in EF.B3 strain as compared to beta-ketoacyl synthase gene of *Burkholderia gladioli* BSR3.]

These results tempted us to hypothesize that LS.B11 and EF.B3 could be producing some bioactive compounds as suggested earlier (Miller et al., 2012).
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4.7. Biochemical characterization of endophytic strains

4.7.1. Gram staining
Gram staining was performed for distinguishing between Gram positive and Gram negative bacteria. The strains exhibited different cell shapes varying from rod, chains to oval. LS.B11 and EF.B3 were bacilli in shape whereas CS.B10 was cocci in shape. But all the three strains LS.B11, EF.B3 and CS.B10 showed gram negative on staining as shown in Fig 4.31. Their respective results are summarized in Table 4.7.

![Fig 4.31. Gram staining of bacterial strains. LS.B11 (A) and EF.B3 (B) showed pink colored gram negative with rods. CS.B10 (C) showed pink color but cocci in shape.](image)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Strain</th>
<th>Shape of bacteria</th>
<th>Gram Stain</th>
<th>Catalase</th>
<th>Oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>LS.B11</td>
<td>Small rods</td>
<td>Gram –ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>2.</td>
<td>EF.B3</td>
<td>Small Rods</td>
<td>Gram –ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>3.</td>
<td>CS.B10</td>
<td>Cocci</td>
<td>Gram –ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>4.</td>
<td>MTCC8528</td>
<td>Rods</td>
<td>Gram +ve</td>
<td>+ve</td>
<td>NA</td>
</tr>
</tbody>
</table>

4.7.2. Catalase test
Catalase test is used to check the presence of catalase enzyme. All the three strains, LS.B11, EF.B3 and CS.B10, showed bubble formation on addition of hydrogen peroxide on bacterial culture due to the release of oxygen as shown in Fig 4.32.

![Fig 4.32. catalase test](image)
Fig 4.32. Catalase test. Bubble formation on addition of H$_2$O$_2$ on bacterial culture resulted evolution of oxygen in case of LS.B11 (A), EF.B3 (B) and CS.B10 (C). Formation of bubble in positive control MTCC 8528 (D) and no bubble formation in negative control MTCC 9768 (E).

### 4.7.3. Oxidase test

We found that LS.B11 strain was oxidase positive as it can oxidise tetramethyl p-phenylene diamine dihydrochloride (Wurster’s reagent), acts as a substrate, within 10-20 sec. and gives purple color due to production of cytochrome oxidase c. But EF.B3 and CS.B10 was unable to oxidise tetramethyl p-phenylene diamine dihydrochloride (shown in Fig 4.33).

Fig 4.33. Oxidase test. Purple color production due to oxidation of tetramethyl p-phenylene diamine dihydrochloride in LS.B11 (A). But no purple color formation in EF.B3 (B) and CS.B10 (C). *Staphylococcus aureus* was taken as negative control (D) and *Pseudomonas aeruginosa* was taken as positive control (E).
4.7.4. Identification of strains by 16S rRNA gene sequencing

Amplification of 16S rRNA gene was performed with primers 27f (5’-GCGCGGAATTCCAGATTCCTGGGCTCAG-3’) and 1492rev (5’-GCGCGCAAGCTTGGTTACCTTGTTACGACTT-3’) according to the protocol described in materials and methods.

![Agarose gel electrophoresis](image)

**Fig 4.34.** Agarose gel electrophoresis of 16S rRNA gene amplified product. Lane 1 consists of 1 kb DNA ladder. Lanes 2-4 consist of 16S rRNA gene amplified product of LS.B11, EF.B3 and CS.B10, respectively.

16S rRNA PCR products were found to be approximately ~1500 bp as expected (shown in Fig 4.34).
4.7.4.1. Identification of LS.B11 strain by 16S rRNA gene

Amplification of 16S rRNA gene of EF.B3 with 27f and 1492rev primers gave amplified product of ~1500 bp. The 16S rRNA gene sequence of one of the strain, LS.B11, was a continuous stretch of 1,344 bp as described in Fig 4.35.

*Biochemical analysis revealed that LS.B11 strain, isolated from Lonicera japonica stem part, was matched with Pseudomonas sp. The 16S rRNA gene sequence of LS.B11 was deposited in GenBank with accession number KM203877.*

**Fig 4.35.** Partial nucleotide sequence of 16S rRNA gene of LS.B11.

BLAST result showed that 16S rRNA gene sequence of LS.B11 was found to be 100% similar with 16S rRNA gene of Pseudomonas aeruginosa strain hqd-01 (Accession number - KC959478) (Fig 4.36). Bioinformatic analysis revealed that LS.B11 strain, isolated from Lonicera japonica stem part, was matched with Pseudomonas sp. The 16S rRNA gene sequence of LS.B11 was deposited in GenBank with accession number KM203877.
| LS.E11 1 | CAGTCGACGGGATGAGGGAGCTTGCTTCGCCGAGGTCGATCTAAATGCTT | 60 |
| Pseudo 22 | CAGTCGACGGGATGAGGGAGCTTGCTTCGCCGAGGTCGATCTAAATGCTT | 81 |
| LS.E11 61 | AGGAATCTGGGCTGGTAAGGGGTGTAACGTCCAAGCTGACAGTCAAGGTTT | 120 |
| Pseudo 02 | AGGAATCTGGGCTGGTAAGGGGTGTAACGTCCAAGCTGACAGTCAAGGTTT | 141 |
| LS.E11 121 | CTCGAGGGAAAGTGACGCCGTTACCTGAGGATGAGCCTAGGGGTTT | 180 |
| Pseudo 142 | CTCGAGGGAAAGTGACGCCGTTACCTGAGGATGAGCCTAGGGGTTT | 201 |
| LS.E11 181 | AGCTAGTGTTGGTGTTAGAGGGATCTACCTAGGAGCCGACCCAGTTACCTGACGTTACCTGCGAT | 240 |
| Pseudo 202 | AGCTAGTGTTGGTGTTAGAGGGATCTACCTAGGAGCCGACCCAGTTACCTGACGTTACCTGCGAT | 261 |
| LS.E11 241 | ATCGTCAACCTGGAGCTAGTACGACAGGCTGCGTTACCTGAGGATGAGCCTAGGGGTTT | 300 |
| Pseudo 262 | ATCGTCAACCTGGAGCTAGTACGACAGGCTGCGTTACCTGAGGATGAGCCTAGGGGTTT | 321 |
| LS.E11 301 | ATGGGAACATGGGGAAGGGCGACCTGATCCGAGGCCATGCCCGTGTTGAGAAGGTTCCTCGGA | 360 |
| Pseudo 322 | ATGGGAACATGGGGAAGGGCGACCTGATCCGAGGCCATGCCCGTGTTGAGAAGGTTCCTCGGA | 381 |
| LS.E11 361 | TTGTAAGGCAATCTTTAAGTTGCGAGGAAAGGCGAGCTAGAATACTTGGCTGTTGAGCCTTGCCTTG | 420 |
| Pseudo 382 | TTGTAAGGCAATCTTTAAGTTGCGAGGAAAGGCGAGCTAGAATACTTGGCTGTTGAGCCTTGCCTTG | 441 |
| LS.E11 421 | TACCACAGGAATGGACCGGCTAATCCTGCGGAGGCATAGGACGATGAGGAGGTTATGGG | 480 |
| Pseudo 442 | TACCACAGGAATGGACCGGCTAATCCTGCGGAGGCATAGGACGATGAGGAGGTTATGGG | 501 |
| LS.E11 481 | AAGGCTTATCAATCTGGCAGTAAAGGGCAGATGGCTATGGTTTCAAGCAAAGTTGGGATGTTT | 540 |
| Pseudo 502 | AAGGCTTATCAATCTGGCAGTAAAGGGCAGATGGCTATGGTTTCAAGCAAAGTTGGGATGTTT | 561 |
| LS.E11 541 | GAATCCCCGGSCCTCAACTGGGACGTCCATCAAATCTGAGCTAGCTACGTAGATGA | 600 |
| Pseudo 562 | GAATCCCCGGSCCTCAACTGGGACGTCCATCAAATCTGAGCTAGCTACGTAGATGA | 621 |
| LS.E11 601 | GGCTGCGCGAGAAATTCCTGCGGCTGAGCTAGCTACGTAGATGA | 660 |
| Pseudo 622 | GGCTGCGCGAGAAATTCCTGCGGCTGAGCTAGCTACGTAGATGA | 681 |
**Fig 4.36.** BLAST result of 16S rRNA gene sequence of LS.B11, compared with *Pseudomonas aeruginosa* strain hqd-01.
Fig 4.37. Phylogenetic analysis based on 16S rRNA gene sequences showing the phylogenetic relationship between *Pseudomonas aeruginosa* (LS.B11) and closely related species of *Pseudomonas* by MEGA 6 software. Relationship inferred by Neighbor joining tree method. The percentage of replicate tree in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown.
4.7.4.2. Identification of EF.B3

Amplification of 16S rRNA gene of EF.B3 with 27f and 1492rev primers gave amplified product of ~1500 bp. After sequencing, we got partial sequence of 1,383 bp nucleotides of 16S rRNA gene of EF.B3 (Fig 4.38).

![Partial nucleotide sequence of 16S rRNA gene of EF.B3.](image)

BLAST result showed that 16S rRNA sequence of EF.B3 was 100% identical with *Burkholderia gladioli pv. gladioli* strain CFBP 2427 (Accession number – NR_117553) (Fig 4.39). Bioinformatic analysis indicated that EF.B3 strain isolated from *Echinacea purpurea* belongs to *Burkholderia* genera. The 16S rRNA gene sequence of EF.B3 was deposited in GenBank with accession number KM203878.
### Chapter 4

#### Results

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF.B3 1</td>
<td>GCA/CACGGGTGCTTTGCACCTGCTGCGAGTGGCAGAGGAAATATACGCTGAAACA</td>
<td>60</td>
</tr>
<tr>
<td>B.glad 42</td>
<td>GCCACGGGTGCTTTGCACCTGCTGCGAGTGGCAGAGGAAATATACGCTGAAACA</td>
<td>101</td>
</tr>
<tr>
<td>EF.B3 61</td>
<td>TGCTCTTAGGGGCAATACCCCATCAGAATGGCTGATTAGCTAGT</td>
<td>180</td>
</tr>
<tr>
<td>B.glad 102</td>
<td>TGCTCTTAGGGGCAATACCCCATCAGAATGGCTGATTAGCTAGT</td>
<td>221</td>
</tr>
<tr>
<td>EF.B3 121</td>
<td>TGAAAGCAGGGGACCTTCCGCGCTCAGCRTAGGGGTGCGCAATGGCTGATTAGCTAGT</td>
<td>240</td>
</tr>
<tr>
<td>B.glad 162</td>
<td>TGAAAGCAGGGGACCTTCCGCGCTCAGCRTAGGGGTGCGCAATGGCTGATTAGCTAGT</td>
<td>291</td>
</tr>
<tr>
<td>EF.B3 241</td>
<td>ACACCTGCGACTGAGACGCAGGCAGACACTCCTACGGGAGGGAGGGATGGGAATTTGAGAC</td>
<td>300</td>
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<tr>
<td>B.glad 282</td>
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<td>341</td>
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<td>EF.B3 301</td>
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<td>360</td>
</tr>
<tr>
<td>B.glad 342</td>
<td>AATGGGCAAGCGCTGAGATTGGCCGCTCAGGCTGAGGAGAAGGCTCCTCGGGGTGTAATAA</td>
<td>401</td>
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<tr>
<td>EF.B3 361</td>
<td>GCACTTTTGCTCGGAAAGAAATCCTGAGGGCTTAATCTCCTCGGGAATGGGCTGTTGGAAGA</td>
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<tr>
<td>B.glad 402</td>
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<td>461</td>
</tr>
<tr>
<td>EF.B3 421</td>
<td>AAGATTAAGCAACGGCTAATCTGGCCAGCGCGCGCAATACGCTAGGGGTGCGACGT</td>
<td>480</td>
</tr>
<tr>
<td>B.glad 462</td>
<td>AAGATTAAGCAACGGCTAATCTGGCCAGCGCGCGCAATACGCTAGGGGTGCGACGT</td>
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</tr>
<tr>
<td>EF.B3 481</td>
<td>AATCGGAAATTACCTGGCCGAAAGCGTGCCGCGCGGTGTTGTTAGAAGACCTATGTGAAATCC</td>
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</tr>
<tr>
<td>B.glad 522</td>
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<td>581</td>
</tr>
<tr>
<td>EF.B3 541</td>
<td>CGGGCTCAACCTGGGAACCTGCTGCTGAGTGGCAGAGGAAATATACGCTGAAACA</td>
<td>600</td>
</tr>
<tr>
<td>B.glad 582</td>
<td>CGGGCTCAACCTGGGAACCTGCTGCTGAGTGGCAGAGGAAATATACGCTGAAACA</td>
<td>641</td>
</tr>
<tr>
<td>EF.B3 601</td>
<td>AAGATTCCACGCTGACGTGGAATACCTGGTAGAGAAGGAGGGGGGT</td>
<td>660</td>
</tr>
<tr>
<td>B.glad 642</td>
<td>AAGATTCCACGCTGACGTGGAATACCTGGTAGAGAAGGAGGGGGGT</td>
<td>701</td>
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</tbody>
</table>

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Fig 4.39. BLAST result of 16S rRNA gene sequence of EF.B3 compared with *Burkholderia gladioli*.
Fig 4.40. Phylogenetic analysis based on 16S rRNA gene sequences showing the phylogenetic relationship between *Burkholderia* sp. (EF.B3) and closely related species of *Burkholderia* by MEGA 6 software. Relationship inferred by Neighbor joining tree method. The percentage of replicate tree in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown.
4.7.4.3. Identification of CS.B10 strain by 16S rRNA gene

The 16S rRNA gene sequence of CS.B10 was a continuous stretch of 1,416 bp as described in Fig 4.41.

```
GCAGTGCAGGCGAACAGATAAAGGAGTTGCTCTTTTGACGTTAGCGGCGGACGGGTGAGTAA
CACGTGGATAAACCCTACTATAAGACTGGGATAAATTGCCGGGAAACCAGAGCTAATACCAGGAT
AACATATTGAACCCGATGTTCAATAGTGAAGAGCGGCTTGTGCTGACTTATAGATGGATTC
CGGCGCTGATAATTGCTAGTGTGTAAGTAACCCGGACGCTTACCAAGCGGAGGAGCTCG
TAGAGGGGTGATCGGATCGTAAACTCTGGTATTACAGGGAAAGAAATGTGTAAGTAGTGCACATC
TTGACGTACCTGATCAAGAACGCGCTAATTACGTCCAGCAGCGCCGTAAATACGTAA
GTTGGCAAAGCTTATCAGGAAATTATGCGCCTGAAAAAGCGGCTGAGGGGTTTTTC
ATGTGAAAGCCACCGCTCAACCGTGAGGAGGTCATTTGGAAGACTGGAAAATCTGGAGTAGCAGA
AGAGGAGAAGGTGAATTCCATCTGTGAAAGCGGTGAATGGAATCTGGAGAAGACCCAG
TGGCGAAGGGCACTGTTCTGTTGTAACAGCGGTATGTCGAAAAGCGGATTGGGATCAAAC
AGGATTAGATACCCGTATGTCATCGCCGATTAGCGAGTGGATGCTAAGTGGTTAGGGGTTTTCC
GCCCTTTATGTCGCACTAAGCGATATACCGCTCCAGGGACGACCGAGGATGCTTCCTCC
CTTGCCGGGCAAAAGTTGACACGGATGCTGTTCTGGTGTCGCTGCTGCTGCTGCTGCTGCTGCTG
GGTAAGTCCCGCAACCGAGCGGCAACCCTAAAGCCCGCTGATTGCCCATTATTAGTGGGCACTCT
AGATTGACTGCGCAGCCGAAAAAGCGGATGAGTGGGATGATATTGGGCTACACGTGCTACAATGGACAATACAAAGGGCAGCTAACCCGAGGCTACACAT
TGATTTGGGCTACACACGTGCTACAATGGGACAATACAAAGGGCAGCTAACCAGCGAGGTCGAGGACG
CAAGCAAATCCCAATACCTGTGCTCTCTGGGATTGTTCTGTTTGAAAATCTGGAGGATACCATGCTCA
CTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGTTGAATACGTTCCCGGGTCTTGTACA
CACCCGCGTCACACCACAGAGTTTGTAAACCACCGAGCCCGGTGAGTAACCATTATGGAGCTAGC
```

Fig 4.41. Partial nucleotide sequence of 16S rRNA gene of CS.B10.

The 16S rRNA gene sequence of CS.B10 was found to be 100% identical with *Staphylococcus pasteuri* isolate HF2011, partial 16S rRNA gene (Accession no – FR839669) (Fig 4.42). Bioinformatic results showed that CS.B10 strain, isolated from *Cannabis sativa* stem part, belongs to *Staphylococcus* sp. The 16S rRNA gene sequence of CS.B10 were deposited in GenBank with accession number KM203879.
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| CS.B10 1 | GCAGTCGAGCGGAGACGATAGAGAGCTTGGCTCGCTCTTAACGGGACGGGATGCTA | 60 |
| Staph 13 | GCAGTCGAGCGGAGACGATAGAGAGCTTGGCTCGCTCTTAACGGGACGGGATGCTA | 72 |
| CS.B10 61 | ACACCTGGATACCTGATCTAGATGCTGGGAAACCCGAACAGTAAAATCGG | 120 |
| Staph 73 | ACACCTGGATACCTGATCTAGATGCTGGGAAACCCGAACAGTAAAATCGG | 132 |
| CS.B10 121 | ATTAATAGTTGAAACCCGATGGTCTAATAGGAAAGCGGGTCTTGGCTGTCACTTATTGGTG | 180 |
| Staph 133 | ATTAATAGTTGAAACCCGATGGTCTAATAGGAAAGCGGGTCTTGGCTGTCACTTATTGGTG | 192 |
| CS.B10 181 | GATCCGGCCGCTGATTTAGCTAGTGTAAGGTAACCGCTTACCGAAGCCACGATACCGTG | 240 |
| Staph 193 | GATCCGGCCGCTGATTTAGCTAGTGTAAGGTAACCGCTTACCGAAGCCACGATACCGTG | 252 |
| CS.B10 241 | GCAACCAGAGGGATAGCCGCTAGCTGAGGTCGAGGAGCTGGCATCTAGTCCGGGAGG | 300 |
| Staph 253 | GCAACCAGAGGGATAGCCGCTAGCTGAGGTCGAGGAGCTGGCATCTAGTCCGGGAGG | 312 |
| CS.B10 301 | GCACCGATAGGGATACCTGCCGAGGCGGAAACCCGATACCGGACGATAGCTTG | 360 |
| Staph 313 | GCACCGATAGGGATACCTGCCGAGGCGGAAACCCGATACCGGACGATAGCTTG | 372 |
| CS.B10 341 | ATGAAAGGTCTCGCGATCGTGAACAAAGCTTGGGAAGCGGAAACAAAGTGTAAGTACTG | 420 |
| Staph 373 | ATGAAAGGTCTCGCGATCGTGAACAAAGCTTGGGAAGCGGAAACAAAGTGTAAGTACTG | 432 |
| CS.B10 421 | TGGCCTGCTGGATACGAGAAAGCCACCGCTAACTGACGCAAGCGCCGGG | 480 |
| Staph 433 | TGGCCTGCTGGATACGAGAAAGCCACCGCTAACTGACGCAAGCGCCGGG | 492 |
| CS.B10 481 | TAAATATCGCTAGGATGGTGGCTGGTTAACCGGAGTAATGCTGGAGCTTTG | 540 |
| Staph 493 | TAAATATCGCTAGGATGGTGGCTGGTTAACCGGAGTAATGCTGGAGCTTTG | 552 |
| CS.B10 541 | TTTTAAAGCTGATGCTTGAAGCGGACGCTTCTACCGGAGTTTAAACCGGGCTTGAGGGG | 600 |
| Staph 553 | TTTTAAAGCTGATGCTTGAAGCGGACGCTTCTACCGGAGTTTAAACCGGGCTTGAGGGG | 612 |
| CS.B10 601 | CTGGAAGTGCAGAAGGAAAAGTGCCATCAAGGCTAACTGATAGGCAAGAGATG | 66 |
| Staph 613 | CTGGAAGTGCAGAAGGAAAAGTGCCATCAAGGCTAACTGATAGGCAAGAGATG | 672 |
| Query 661 | GAGGAACACCACTGCGGGAAAGCGCTTTCTGCTGTAAGCTGACCCGCTGATGCTGGGAAACG | 720 |
| Staph 673 | GAGGAACACCACTGCGGGAAAGCGCTTTCTGCTGTAAGCTGACCCGCTGATGCTGGGAAACG | 732 |
### Fig 4.42.
BLAST result of 16S rRNA gene sequence of CS.B10 compared with *Staphylococcus pasteuri* isolate HF2011.
Fig 4.43. Phylogenetic analysis based on 16S rRNA gene sequences showing the phylogenetic relationship between *Staphylococcus* sp. (CS.B10) and closely related species of *Staphylococcus* by MEGA 6 Software. Relationship inferred by Neighbor joining tree method. The percentage of replicate tree in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown.
4.8. Endophytic nature of bacterial strains

4.8.1. Endophytic nature of EF.B3 in plantae

In order to confirm the endophytic nature and to assess the colonization capacity of the selected bacterial strains in pea seedlings, sprouts of this plant species were treated with the EF.B3 strain and collected at different times (Fig 4.44).

The plant parts were surface sterilized and endophytes were extracted. CFU was enumerated and it was noticed that CFU was increased continuously as shown in Fig 4.45 and Table 4.8.

We also observed that there was mainly one type of colony when we macerated pea seedlings inoculated with bacterial strain. It was also found that root and stem part which were not treated with any bacterial strain showed few colonies on plate but their CFU was
approximately same as shown in Table 4.8. Plate having last water washing during surface sterilization showed no colonies which concluded that strain isolated were endophytic in nature.

**Table 4.8.** Enumeration of CFU of bacterial strain isolated from roots and stems of pea seedlings from Day 1 to Day 8.

<table>
<thead>
<tr>
<th>Day</th>
<th>CFU in Root</th>
<th>Average CFU in Root + EF.B3</th>
<th>CFU in Shoot</th>
<th>Average CFU in Shoot + EF.B3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>555</td>
<td>$0.2 \times 10^4 \pm 89$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Day 2</td>
<td>8750</td>
<td>$3.1 \times 10^4 \pm 452$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Day 3</td>
<td>6050</td>
<td>$3.6 \times 10^4 \pm 1096$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Day 4</td>
<td>100</td>
<td>$4 \times 10^4 \pm 1244$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Day 5</td>
<td>1310</td>
<td>$5 \times 10^4 \pm 1018$</td>
<td>620</td>
<td>$2.1 \times 10^4 \pm 3139$</td>
</tr>
<tr>
<td>Day 6</td>
<td>2040</td>
<td>$10 \times 10^4 \pm 1301$</td>
<td>2300</td>
<td>$3.5 \times 10^4 \pm 439$</td>
</tr>
<tr>
<td>Day 7</td>
<td>5200</td>
<td>$30 \times 10^4 \pm 980$</td>
<td>3300</td>
<td>$8.5 \times 10^4 \pm 2206$</td>
</tr>
<tr>
<td>Day 8</td>
<td>4360</td>
<td>$76 \times 10^4 \pm 7495$</td>
<td>4900</td>
<td>$15 \times 10^4 \pm 3501$</td>
</tr>
</tbody>
</table>
Fig 4.46. Graphs showed the increase in CFU of EF.B3 bacterial strain isolated from pea roots (A) and pea shoots (B).

Colonies appeared after spreading on NAM were randomly picked and further patched on NAM plate. Patched colonies were then compared with original inoculated strain on the basis of their morphology, colour and shape. It was observed that re-isolated colonies had same morphology as inoculated strain of EF.B3 (Fig 4.47).

Fig 4.47. Patching of re-isolated EF.B3 strain on nutrient agar plate.
Re-isolated strains were then also patched on Pikovskaya plate. It was concluded that all the re-isolated strains showed same halo zone compared with EF.B3 strain as described in Fig 4.48.

![Fig 4.48. Formation of halo zone around re-isolated EF.B3 strain when patched on pikovskaya plate.](image)

The re-isolated strains were randomly picked and streaked on NAM plate for colony purification as shown in Fig 4.49. All the streaked strains showed similar colony morphology as compared with EF.B3.

![Fig 4.49. Colony purification of re-isolated strain from pea sprouts.](image)

The purified colonies of re-isolated strains were further used to isolate their genomic DNA using DNA purification kit (Zymo Research) as shown in Fig 4.50. Genomic DNA was isolated from 3 root isolated strains, 2 stem isolated strains, inoculated strain EF.B3 as positive control and *Burkholderia* sp. AU4i as negative control. Isolated DNA were further used for random amplified polymorphic DNA (RAPD) to confirm the endophytic nature of the bacterial strains.
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Fig 4.50. Isolation of genomic DNA. Lane 1 consists of 1 kb DNA ladder. Lane 2-3 consists of genomic DNA isolated from root; Lane 4-6 consists of genomic DNA from endophytes isolated from stem; Lane 7 consists of EF.B3 and Lane 8 consists of *Burkholderia vietnamiensis* as negative control.

For further confirmation of re-isolated strain compared with introduced strain of EF.B3, RAPD was performed for 5 re-isolated strains and one introduced strain EF.B3.

Fig 4.51. RAPD analysis. Genomic DNA were isolated from strains, and were subjected to RAPD analysis using random primer (5’- CATTTTGCTGGGTTC-3’). The amplicons were resolved on 1.4% agarose gel. Lanes 1-2 depict amplicons of RAPD PCR of strains isolated from root; Lanes 3-5 from stem; Lane 6 consists of EF.B3; Lane 7 *Burkholderia vietnamiensis* as negative control and Lane 8 depicts 1 kb DNA ladder.
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It was observed that re-isolated strains showed identical band pattern with the inoculated strain and different band pattern with *Burkholderia* sp. AU4i that acts as negative control as shown in Fig 4.51.

4.8.2. Endophytic nature of LS.B11 *in planta*

Endophytic nature of LS.B11 was checked by extracting the bacteria aseptically from the sprouts. Endophytes were isolated by spreading macerated stem and root sample.

![Fig 4.52. Growth of pea seedlings after bacterial treatment at different time intervals of Day 0 (A), Day 4 (B) and Day 8 (C).](image)

We observed that population density of bacterial strain was increased continuously as shown in Fig 4.54 and Table 4.17. It was also observed there was mainly of one type colonies (Fig 4.53).

![Fig 4.53. Colonies isolated from pea roots, after LS.B11 bacterial treatment, on Day 1 (A), Day 5 (B) and Day 8 (C). Last water wash during surface sterilization showed no colonies (D).](image)
Table 4.9. Enumeration of CFU of bacterial strain isolated from roots and stems of pea seedlings.

<table>
<thead>
<tr>
<th>Day</th>
<th>CFU in Root</th>
<th>Average CFU in Root + LS.B11</th>
<th>CFU in Shoot</th>
<th>Average CFU in Shoot + LS.B11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>200</td>
<td>$0.12 \times 10^5 \pm 2715$</td>
<td>0</td>
<td>$0.16 \times 10^4 \pm 41$</td>
</tr>
<tr>
<td>Day 2</td>
<td>600</td>
<td>$0.21 \times 10^5 \pm 1272$</td>
<td>200</td>
<td>$0.7 \times 10^4 \pm 106$</td>
</tr>
<tr>
<td>Day 3</td>
<td>1100</td>
<td>$0.44 \times 10^5 \pm 707$</td>
<td>600</td>
<td>$2.1 \times 10^4 \pm 834$</td>
</tr>
<tr>
<td>Day 4</td>
<td>1600</td>
<td>$1.16 \times 10^5 \pm 13506$</td>
<td>890</td>
<td>$3.6 \times 10^4 \pm 1485$</td>
</tr>
<tr>
<td>Day 5</td>
<td>2500</td>
<td>$4.3 \times 10^5 \pm 4242$</td>
<td>1000</td>
<td>$7.1 \times 10^4 \pm 2121$</td>
</tr>
<tr>
<td>Day 6</td>
<td>3200</td>
<td>$8.4 \times 10^5 \pm 44548$</td>
<td>1100</td>
<td>$14.1 \times 10^4 \pm 18385$</td>
</tr>
<tr>
<td>Day 7</td>
<td>4000</td>
<td>$12.5 \times 10^5 \pm 1414$</td>
<td>2200</td>
<td>$36.3 \times 10^4 \pm 1414$</td>
</tr>
<tr>
<td>Day 8</td>
<td>8500</td>
<td>$15.6 \times 10^5 \pm 2828$</td>
<td>2100</td>
<td>$68.8 \times 10^4 \pm 12021$</td>
</tr>
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</table>

Fig 4.54. Graphs showed increase in CFU of LS.B11 bacterial strain isolated from pea roots (A) and pea shoots (B).
Colonies appeared on spreading plate after 48 hrs was further patched on NAM plate. It was observed that all the re-isolated patched colonies were similar to the inoculated strain of LS.B11 on the basis of shape, colour and colony morphology (Fig 4.55).

Fig 4.55. Patching of re-isolated LS.B11 strain on nutrient agar plate.

Re-isolated strains were also patched on pikovskaya plate to check their phosphate solubilisation ability as compared to the inoculated strain. It was concluded that there was formation of halo zone around bacterial colony of re-isolated strain similar to LS.B11 strain as found in Fig 4.56.

Fig 4.56. Formation of halo zone around re-isolated strain when patches on pikovskaya plate.

To re-confirm the similarity of re-isolated strain and inoculated strain, we performed RAPD. For that, re-isolated strain were streaked on NAM plate for colony purification as shown in Fig 4.57.
Purified strains were used to isolate genomic DNA by using DNA isolation kit (Zymo Research) as shown in Fig 4.58. Genomic DNA was isolated from 3 root re-inoculated strains, 3 stem re-inoculated strains, inoculated strain LS.B11 as positive control and *Pseudomonas fluorescens* MTCC9768 as negative control. Isolated DNA were further used for random amplified polymorphic DNA (RAPD) to confirm the endophytic nature of the bacterial strains.

![Fig 4.57. Colony purification of re-isolated strain from pea sprouts after treatment with LS.B11 strain.](image)

![Fig 4.58. Isolation of genomic DNA. Lane 1 consists of 1kb DNA marker; Lane 2 consists of genomic DNA from LS.B11 strain; Lane 3 & 4 consists of genomic DNA isolated from root; Lane 5-7 consists of genomic DNA isolated from stem; Lane 8 consists of *Pseudomonas fluorescens* MTCC9768 as negative control.](image)
For further re-confirmation of the similarity of re-isolated strain with the inoculated strain, RAPD was performed. RAPD analysis showed that band pattern of inoculated strain and re-isolated strain was identical (Fig 4.59).

![Fig 4.59. Confirmation of re-isolates by RAPD.](image)

The identities of re-isolated bacteria and the inoculated strains LS.B11 were confirmed by RAPD analysis with K10 primer (5’- CATTTTGCTGCCGGTC-3’). The amplicons were resolved on 1.4% agarose gel. DNA band pattern between inoculated strain and re-isolated strain was found to be same. Lanes 1-2 depict amplicons of RAPD PCR of strains isolated from root; Lanes 3-5 depict amplicons of RAPD PCR of strains isolated from stem; Lane 6 depicts LS.B11 and Lane 7 depicts *Pseudomonas fluorescens* MTCC9768 as negative control.

4.9. Antimicrobial activity against Human pathogenic bacteria and fungus

As we showed that LS.B11 and EF.B3 contain NRPS and PKS genes, respectively. We further went on to check their ability to control human pathogens. The results showed that endophytic bacteria included in this study exhibited antimicrobial activity against variety of test organisms. A clear zone of 14 mm, 19 mm and 18 mm was formed around the filtrate of LS.B11 against *S. aureus*, *Bacillus* sp. and *Trichophyton* sp. plate, respectively. On the other hand, culture filtrate of EF.B3 was highly specific and showed antimicrobial activity having 20 mm clear zone against *Aspergillus* sp. (Fig 4.60). But isolate CS.B10 from *Cannabis sativa* stem part showed no activity against any of these pathogenic strain. But no strain showed antimicrobial activity against *E. coli*. 

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Fig 4.60. Antimicrobial activities of the secretory metabolites of endophytes. Human pathogenic strains S. aureus (A); Bacillus sp. (B); Trichophyton sp. (C); Aspergillus sp. (D); E. coli (E) were plated homogenously on nutrient agar medium. 30 µl of culture supernatant of LS.B11 (1), EF.B3 (2), and CS.B10 (3) is added, and incubated for 24 h at 30 °C. Nutrient broth (5) or antibiotics (4) were taken as negative or positive control.

Isolate LS.B11 from Lonicera japonica stem part showed broad spectrum anti-microbial activity. Isolate EF.B3, however, showed no activity against S. aureus, Trichophyton sp., Bacillus sp. whereas, isolate LS.B11 showed appreciable activity against S. aureus, Trichophyton sp., Bacillus sp. but did not show activity against Aspergillus sp.