4. RESULTS

4.1. Estimation of pH and moisture contents of soil

The pH and moisture contents of soil sample collected different areas ranges from 5.2 to 6.3 and 27.17% to 62.23% respectively (Table 4.1).

<table>
<thead>
<tr>
<th>Place of collection</th>
<th>pH</th>
<th>Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Explosive contaminated soil (army firing range, Bahadurgarh, Patiala Punjab)</td>
<td>5.2</td>
<td>27.17</td>
</tr>
<tr>
<td>Organophosphate pesticides contaminated dry soil sample (field crop area near Chandigarh)</td>
<td>5.8</td>
<td>28.41</td>
</tr>
<tr>
<td>Organophosphate pesticides contaminated moist soil sample (field crop area near Chandigarh)</td>
<td>6.3</td>
<td>62.23</td>
</tr>
</tbody>
</table>

Table 4.1 pH and moisture content of different soil sample

4.2. Isolation of TNP degrading bacteria

TNP degrading bacteria has isolated from explosive contaminated soil. Enrichment and sub-culturing yielded five isolates able to grow on M1 medium containing 1.32 mM TNP as the sole carbon and nitrogen source. Out of 5 strains, only one pure isolate (isolate no. 3, designated strain PU) had higher CFU/mL on medium supplemented with TNP (Fig 4.1). Strain PU was selected for further study and was streaked on M1 medium supplemented with 1.32 mM TNP and visible colonies were obtained (Fig 4.2).

![Fig 4.1: Growth (CFU/mL) of five isolated strains in M1 medium supplemented with 1.32 mM TNP after 4 days of incubation at 30 °C.](image-url)
4.3. Isolation of malathion degrading bacteria

Malathion contaminated soil sample was used to isolate malathion degrading bacteria. Growth and enrichment techniques resulted in isolation of two distinct bacterial isolates one each from moist soil sample (designated KB1) and dry soil sample (designated KB2) capable of growth in MS1 medium containing 0.15% malathion (Fig 4.3). Isolated strains showed visible growth on MS1 medium supplemented 0.15% (Fig 4.4). These strains were selected for further studies.

Fig 4.2: Colonies of strain PU in M1 medium supplemented with 1.32 mM TNP.

Fig 4.3: The growth (CFU/mL) of strains KB1 and KB2, in MS1 medium containing 0.15% malathion after 7 days of incubation at 30 °C.
Fig 4.4: Colonies of isolated strains (A) strain KB1 and (B) strain KB2 in minimal salt medium (MS1) containing 0.15% malathion after 7 days of incubation.

4.4. Isolation of *Rhizobium* from fenugreek roots

Washed and surface sterilized root nodules of fenugreek were macerated and streaked on YEM plates. Colonies were obtained on YEM medium after incubation at 29.4°C on day 2 (Fig 4.5). Pure isolate showed maximum growth (5.7×10^{11} CFU/mL) in YEM medium after 48 h of incubation.

Fig 4.5: Colonies of isolated strain on YEMA medium after 2 days of incubation.
The pH of the medium and broth during growth of isolates was changed from 7.0 to 6.0, thus showing the production of acid which is the characteristic of *Rhizobium* to produce acid during growth (DeVries et al. 1980; Baoling et al. 2007).

### 4.4.1. Tolerance of *Rhizobium* to TNP and malathion

*Rhizobium* showed no signs of growth in both M1 medium containing TNP at concentrations between 0.44 mM and 1 mM and MS1 medium containing malathion at concentrations between 0.05% and 0.1%. Therefore, *Rhizobium* strain was used as a standard control in the degradation studied of TNP and organophosphate pesticides along with other isolated cultures.

### 4.5. Morphological and Biochemical analysis

Table 4.2 describes the visible colony characteristics studied after growth for preliminary grouping of the bacteria.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Growth medium</th>
<th>Color</th>
<th>Morphology</th>
<th>Colony size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain PU</td>
<td>LB</td>
<td>White</td>
<td>Smooth</td>
<td>1.3-2 (after 12 h)</td>
</tr>
<tr>
<td>Strain KB1</td>
<td>LB</td>
<td>White</td>
<td>Smooth</td>
<td>1.3-2 (after 12 h)</td>
</tr>
<tr>
<td>Strain KB2</td>
<td>LB</td>
<td>Light yellow</td>
<td>Smooth</td>
<td>0.8-1.7 (after 12 h)</td>
</tr>
<tr>
<td><em>Rhizobium</em></td>
<td>YEM</td>
<td>White till 3-4 days of growth and turning yellowish in color after 4 days</td>
<td>Sticky appearance</td>
<td>5-7 (after 2 days)</td>
</tr>
</tbody>
</table>

Table 4.2 Morphological analysis of four isolated strains

To further characterize, various biochemical tests were carried out on isolated bacterial strains. The cells of isolated three strains, PU, KB1 and KB2 were gram-positive, aerobic, motile, rod shaped. Colonies of *Rhizobium* sp. were gram negative. They showed positive results for the starch hydrolysis.
assay, glucose peptone agar, and triple sugar iron agar and were not producing gelatinase (Table 4.3).

Rhizobial cells were able to grow on the glucose peptone media showing the utilization of glucose as the carbon source by the *Rhizobium*. It is a confirmatory test for *Rhizobium* and these are able to utilize glucose as carbon source (Kucuk et al. 2006). It was observed that rhizobial cells do not produce gelatinase enzymes as medium containing gelatin solidified when kept at 4°C for 30 as well as 60 min. Negative gelatinase activity is also a feature of *Rhizobium* (Hunter et al. 2007).

The maximum amylase titre of 1409, 1215 and 1321 U/mL were observed for strains, PU, KB1 and KB2, respectively at 28 h of incubation. The maximum amylase titre of 328 U/mL was observed for *Rhizobium* sp. at 48 h of incubation.

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th><em>Rhizobium</em></th>
<th>Strain PU</th>
<th>Strain KB1</th>
<th>Strain KB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Gelatin test</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Amylase</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Glucose peptone agar</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Triple sugar iron</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Catalase test</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Indole test</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

Table 4.3: Biochemical characteristic of four isolated strains

4.5.1. Salinity (NaCl), pH and Temperature Variation Assay

Salinity, pH and temperature are an important parameter for the growth of the organism. Slight variations in salinity, pH and temperature of medium might have enormous effects on the growth of organism.

Colonies of strains PU, KB1 and KB2 did not appear when salinity (NaCl) exceeded 5% and maximum growth occurred at 0.1% salinity. The cells grew well at pH range of 5 to 8 and showing maximum growth at pH-7. Strains were able to grow at temperatures range between 20-50 °C and maximum growth occurred at 30 °C ± 2 °C. *Rhizobium* sp. was able to grow on 1% NaCl containing medium but was unable to grow on higher
concentrations, thus showing that the isolate was sensitive to the salt. *Rhizobium* was found to grow at pH-7 and kept at 29.4°C. No growth was observed in medium with pH 4.0 and 9.0. Also cells were unable to grow when incubated at 37°C even in pH 7.0. Thus medium with pH 7.0 and temperature 29.4°C are the optimum parameters for growth of the organism.

4.6. Molecular taxonomic analysis

16S rRNA sequences are highly conserved and are commonly used to determine phylogenetic relatedness. Molecular analysis of 16S rRNA gene was performed for species identification of isolated strains.

4.6.1. Sequencing of 16S rRNA and phylogenetic analysis of strain PU

For amplification, cloning and sequencing of 16S rRNA sequence analysis of strain PU, commercial services provided by Microbial type culture collection (MTCC) centre, Institute of Microbial Technology (IMTECH), Chandigarh, India, were availed. A 939 bp long sequence (Fig 4.6) of 16S rRNA was amplified, sequenced and submitted to NCBI GenBank database (accession no. FJ387129).

![Partial 16S rRNA sequence (939 bp) of strain PU](image)

**Fig 4.6:** Partial 16S rRNA sequence (939 bp) of strain PU
Results

BLASTN analysis indicated strain PU shows nearly 99% similarity with *Bacillus cereus* and *B. thuringiensis*. Phylogenetic tree was constructed using for strain PU with different bacterial strains based on 16S rRNA similarity (Fig 4.7) Based on the morphology, cultural characteristics, physiological, biochemical properties, 16S rRNA gene sequence and phylogenetic tree, strain PU was identified as species closely related to *B. cereus*.

![Fig 4.7: Phylogenetic tree showing closeness of strain PU with different bacterial strains based on 16S rRNA similarity. Phylogenetic tree was constructed by neighbour-joining method using ClustalW and the Tree Explorer program. Strain PU, isolated in this study is underlined. Numbers in parentheses represented the sequence accession numbers in GenBank. Numbers at branch points represents bootstrap values.](image-url)
4.6.2. Cloning of 16S rRNA fragment from strains KB1 and KB2

4.6.2.1. Genomic DNA isolation

Genomic DNA was isolated from strain KB1 and strain KB2 (Fig 4.8). The ratio of absorbance between KB2 and KB1 was between 1.7-1.9.

![Genomic DNA isolated from strain KB1 and KB2. M represents 500 bp marker.](image)

Fig 4.8: Genomic DNA isolated from strain KB1 and KB2. M represents 500 bp marker.

4.6.2.2. PCR amplification

PCR amplification using 16S rRNA specific primers, 27 F and 1492 R, yielded amplicon of around 1.5 Kb (Fig 4.9).

![PCR amplification of partial 16S rRNA fragment from strains, KB1 and KB2. M represents 100 bp marker.](image)

Fig 4.9: PCR amplification of partial 16S rRNA fragment from strains, KB1 and KB2. M represents 100 bp marker.
4.6.2.3. Colony PCR and plasmid isolation

The amplified fragments from both the strains, KB1 and KB2 were separately cloned to pGEM®-T easy cloning vector (Promega, USA) and transformed into *E. coli* DH5α competent cells. Colony PCR confirmed the desired sized insert into vector (Fig 4.10).

![Colony PCR](image)

**Fig 4.10**: Colony PCR to confirm the insert of desired size into the vector. M represents 100 bp marker.

The results of colony PCR and plasmid isolation were shown in Fig 4.11.

![Plasmid Isolation](image)

**Fig 4.11**: Plasmid isolation from the bacterial cells containing insert of desired size.
4.6.2.4 16S rRNA sequencing and phylogenetic analysis of strain KB1

Sequencing of 16S rRNA gene of strain KB1 produced 1516 bp (Fig 4.12) and submitted to Genbank database vide on accession number HQ156458. BLASTN, ClustalW and phylogenetic analysis (Fig 4.13) indicated that sequence of strain KB1 showed nearly 99% similarities with 16S rRNA sequence of Lysinibacillus sp. Based on the morphology, cultural characteristics, physiological, biochemical properties, 16S RNA sequence and phylogenetic analysis, strain KB1 was identified as species closely related to Lysinibacillus sp.

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGAAAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTACCCATATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACCGAATAATCTCTTTTACTTCATGGTGAAAGACTGAAAGACGGTTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACCGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCGCAAGCCCGCGGTAATACGTAGTGGGCAAGCCTGTAAGGCTCCTGATTTAGCTGCGGCTACACACGTGCTACAATGGACGATACAAACGGTTGCAACTCGCGAGAGGGAGCTAATCCGATAAAGTCGTTCTCAGTTCGGATTGTAGGCTGCACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAGAGTTTGTAAC

Fig 4.12: Partial 16S rRNA sequence (1516 bp) of strain KB1.
Fig 4.13: Phylogenetic tree showing closeness of strain KB1 with different bacterial strains based on 16S rRNA similarity. Phylogenetic tree was constructed by neighbour-joining method using ClustalW and the Tree Explorer program. Strain KB1, isolated in this study is underlined. Numbers in parentheses represented the sequence accession numbers in GenBank. 0.05 denotes the genetic distance. Numbers at nodes represents branch length.

4.6.2.5. 16S rRNA sequencing and phylogenetic analysis of strain KB2

Sequencing of 16S rRNA gene of strain KB2 produced 1501 bp fragment (4.14). The sequence was submitted to NCBI GenBank database wide on accession number HQ156457. BLASTN, clustalW and phylogenetic tree analysis (Fig 4.15) indicated that sequence of strain KB2 showed nearly 99% similarities with 16S rRNA sequence of Brevibacillus spp. Based on the morphology, cultural characteristics, physiological, biochemical properties, 16S RNA gene sequence and phylogenetic analysis, strain KB2 was identified as species closely related to Brevibacillus sp.
Results

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGTCCCTTCGGGGGCTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCCTGCCGCATTACGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGTCCCTTCGGGGGCTAGCGGCGGACGGGTGAGTAACACGTAGGCAACC
TCGCCCTAGCATGCATAATAGGGAATTACATTACCAATCTTGGTTAAGCTGGCGGCGGACGGGTGAGTAACACGTAGGCAACC
TACGTACCGGCAAGGCGCCGCTGCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCC

Fig 4.14: Partial 16S rRNA sequence (1501 bp) of strain KB2.
Fig 4.15: Phylogenetic tree showing closeness of strain KB2 with different bacterial strains based on 16S rRNA similarity. Phylogenetic tree was constructed by neighbour-joining method using ClustalW and the Tree Explorer program. Strain KB2, isolated in this study is underlined. Numbers in parentheses represented the sequence accession numbers in GenBank. 0.05 denotes the genetic distance. Numbers at nodes represents branch length.

4.7. Analysis of TNP degradation by strain PU

4.7.1. Determination of nitrite concentration

Nitrite concentrations within the incubation samples were measured to determine whether strain produce nitrite from the breakdown of TNP or not. Nitrite is a product of TNP degradation and may be used by the bacteria as a source of nitrogen (Behrend & Heesche-Wagner, 1999). Degradation of TNP in M1 medium supplemented with 1.32 mM resulted in stoichiometric release of three moles of nitrite per mole of substrate (Fig. 4.16). Degradation rate
(μmol/h/g dry cell weight) was found to be 539. The growth rate and yield (dry cell weight in g/g of TNP) was found to be 0.025±0.005/h and 0.16±0.04 g/g, respectively. The stoichiometric release of mol of nitrite per mol of picric acid provided strong evidence that complete mineralization of picric acid occurred.

**Results**

![Graph showing release of nitrite by strain PU](image)

*Fig 4.16: Release of nitrite by strain PU in mineral salt (M1) medium supplemented with 1.32 mM TNP. Value are presented as mean ± standard error of three independent observations.*

**4.7.2. Medium Standardization**

Efforts were made to found a simple, reliable and low cost medium for growth of bacteria with high degree of TNP degradation. Growth of strain PU was monitored in KB medium and submediums KB1 to KB20 by varying different components of medium but containing 1.32 mM TNP (Fig 4.17). KB3 medium (glucose 0.045%, yeast extract 0.05%, K₂HPO₄ 0.02%, MgSO₄·7H₂O 0.006%, and TNP 1.32mM) was found to be an efficient medium for growth of bacteria. The results of medium standardization showed that maximum growth of strain PU was found in KB3 medium. Therefore KB3 medium was selected further to study the degradation analysis of TNP. Fig 4.18 depicts change in color from yellow to orange red during growth of strain PU in KB3 medium supplemented with 1.32 mM TNP.
4.7.3. Chemical analysis of TNP degradation by strain PU in KB3 medium

A simple and highly sensitive HPLC method was used for the determination of TNP and its metabolite. The metabolite exhibited a characteristic UV-visible light absorption at 420 and 490 nm. The peak of hydride meisenheimer complex was eluted at 3.66 min and of TNP was observed after retention time of 4.63 min (Fig. 4.19).
Fig 4.19: HPLC analysis of TNP and degradation in KB3 medium (A) in control without inoculums (B) with strain PU after 12 h of incubation at 30 °C.

TLC analysis was done on a total of 7 solvent systems, out of which chloroform and methanol (4:1) was chosen for its better separation results when visualized under iodine fumes. Both TNP and its metabolite from control (R. erythropolis) and test (B. cereus strain PU) appeared as yellowish and yellowish orange spots, respectively on a white background. Value of $R_f$
[(retention factor) distance travelled by the sample divided by the distance traveled by the solvent] was found to be 0.56 (Fig 4.23).

Fig 4.23: TLC analysis of TNP and its metabolite (A) control, TNP (B) TNP degradation with *R. erythropolis* (C) TNP degradation by strain PU.

### 4.7.3.1. Analysis of TNP degradation product

Bacterial growth in KB3 medium containing TNP resulted in formation of yellow to orange red colored metabolite. This orange red colored metabolite was due to formation of hydride meisenheimer complex (Lenke and Knackmuss 1992; Behrend and Heesche-Wagner 1999; Rieger et al. 1999). To confirm that orange-red metabolite was hydride meisenheimer complex, synthetic hydride meisenheimer complex was prepared and UV spectra and HPLC analysis of culture supernatants were compared with the synthetic complex. HPLC analysis showed that the chemically obtained product had the same retention time (3.66 min) (Fig 4.20) and UV-visible light spectrum (λ-max, 420 and 490 nm) as the orange-red metabolite of TNP (3.66 min., 420 and 490 nm) (Fig 4.19). Hydride meisenheimer complex exhibited a characteristic UV-visible light absorption at 420 and 490 nm which was correlated with the observed orange red metabolite (hydride meisenheimer complex) (Lenke and Knackmuss 1992; Behrend and Heesche-Wagner 1999; Rieger et al. 1999).
4.7.4. Biodegradation of TNP by strain PU

The growth of strain PU in KB3 medium containing 1.32 mM TNP and its ability to degrade TNP is shown in Fig. 4.21. The OD measurements at 600 nm showed a steady increase in bacterial mass. Simultaneously, the HPLC analysis showed a substantial reduction in the levels of TNP. After incubation for 12 h, about 67.95% of 1.32 mM TNP initially added to the KB3 medium was degraded by strain PU. No significant change in TNP concentration was observed in cultures that were not inoculated. TNP concentration of 1.10, 1.32 and 1.54 mM was taken (Fig. 4.22) to study the effect of various concentrations on TNP degradation in KB3 medium and maximum degradation was observed at concentration of 1.32 mM.

Fig 4.21: Degradation of TNP by *Bacillus cereus* strain PU in KB3 medium supplemented with 1.32 mM. Value is presented as mean ± standard error of three independent observations.

Fig 4.20: HPLC analysis of synthetic hydride Meisenheimer complex.
**Results**

**Fig. 4.22**: Effect of concentration on TNP degradation in KB3 medium.

4.7.6. Crude enzyme assay

Time-dependent UV-visible light spectrum related to the conversion of TNP after addition of total cell protein extract is depicted in Fig 4.24. After 30 min of incubation, increase in absorbance between 420 and 490 nm indicated the formation of H⁻-TNP as described previously for *R. erythropolis* HL PM-1 (Lenke and Knackmuss, 1992). No activity was detected in control at 0 time without any total cell protein extract and very little change was observed after 5 min of incubation with protein extract.

**Fig. 4.24**: TNP degradation by total cell extract of strain PU.
4.7.7. Malathion tolerance testing strain PU

Strain PU showed visible growth on MS1 medium containing 0.1% malathion (Fig 4.25). Therefore further malathion degradation studies was carried out by using strain PU also along with strains, KB1 and KB2.

Fig 4.25: Growth of strain PU in MS1 containing 0.1% malathion

4.7.8. TNP tolerance testing strain KB1 and KB2

Both strains showed no signs of growth at TNP concentration between 0.44 Mm and 1 mM. Therefore both strains were not selected for TNP degradation studies.

4.8. Analysis of malathion degradation by strains, PU, KB1 and KB2

4.8.1. HPLC analysis of malathion degradation

The HPLC analysis of standard MS1 medium showed a two peaks one at 215 nm of malaoxon and second at 230 nm of malathion (Fig 4.26) (FAO specifications). Malaoxon peak was observed after retention time 7.05 min and malathion was observed after retention time of 5.20 min. HPLC chromatograms of control and test reactions are shown in Fig 4.25. Percentage degradation of malathion and malaoxon was analyzed by measuring the peak area of chromatogram.
Results

4.8.2. Degradation of malathion by strains, PU, KB1 and KB2

The growth of strains PU, KB1 and KB2 on malathion and their ability to degrade malathion is shown in Fig 4.27. The OD measurements at 600 nm showed a steady increase in bacterial mass. Simultaneously, HPLC analysis showed substantial reduction in the levels of malaoxon and malathion. After incubation for 7 days, 54.67, 46.75 and 40.92% malaoxon of total 0.15% malathion initially added to the MS1 medium were degraded by strains PU, KB1 and KB2 respectively, when malathion was used as the sole carbon source. No significant change in malaoxon concentration was observed in cultures that were not inoculated.

Fig 4.26: HPLC-UV chromatogram of malathion degradation in MS1 medium after 7 days of incubation at 30 °C with (A) in control without inoculum (B) strain PU (C) strain KB1 (D) strain KB2.
Results

Fig 4.27: Contd ...

Concentration of malaoxon (mg L\(^{-1}\))

A

B

Cell density (OD at 600 nm)

Concentration of malaoxon (mg L\(^{-1}\))

Degradation of malathion as sole carbon source

Uninoculated medium

Growth on M1 medium

Time (days)

0 2 4 6 8 10 12
Fig 4.27: Degradation of malathion by (A) *Bacillus cereus* strain PU. Results shows % degradation of malaoxon where initial concentration of malaoxon was taken equal to 0.15% malathion (72.5 mg L$^{-1}$). Data is presented as mean and standard error of three independent observations. (B) *Lysinibacillus* sp. strain KB-1 (C) *Brevibacillus* sp. strain KB-2.

To test the effect of malathion concentration on degradation, initial malathion concentrations varied from 0.1% to 0.2% (Fig 4.28). Maximum malathion and malaoxon degradation was observed at concentration 0.15% malathion for all three strains.
Results

Fig 4.28: Effect of concentration on malathion degradation by (A) *Bacillus cereus* strain PU. Results shows % degradation of malaoxon where initial concentration of malaoxon was taken equal to (0.1 to 0.2%) malathion. Error bars represent the standard error of three replicates.
4.8.3. Effects of co-substrate on growth

To optimize the culture conditions required to achieve complete degradation of malathion, co-substrates yeast extract, glucose, succinate and sodium pyruvate were added as an additional energy source to MS1 medium. After 7 days, maximum growth and malathion degradation was observed in MS2 medium (Table 4.6). Result indicated 87.40% of malaoxon, 49.31% of malathion and 80.82% of malaoxon and 44.74% of malathion and 72.20% of malaoxon, 36.22% of malathion was degraded by strain PU, KB1 and KB2, respectively after 7 days of incubation. In the presence of either yeast extract (MS3) or glucose (MS4) in culture media, supplied with malathion, an increase in bacterial growth by $10^5$ fold as compared to malathion alone was observed after 7 days incubation. The growth rate and percent malathion and malaoxon degradation declined in the order MS2>MS3>MS5>MS4 >MS6>MS1.

The results of effect of co-substrates on the growth of strains (appendices 4.7.2) showed that maximum growth and malathion degradation of strains were found in MS2 medium. Therefore MS2 medium was selected further to study a malathion degradation time-course inoculated with all three strains, PU, KB1 and KB2 (Table 4.7).

Fig 4.28: Effect of concentration on malathion degradation by B) *Lysinibacillus* sp. strain KB-1 (C) *Brevibacillus* sp. strain KB2.

Results
### Results

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Bacterial count (CFU mL⁻¹)</th>
<th>% degradation of Malathion</th>
<th>% degradation of Malaoxon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PU</td>
<td>KB1</td>
</tr>
<tr>
<td>MS1</td>
<td>5.2×10¹⁰ ± 0.04</td>
<td>9×10⁸ ± 0.04</td>
<td>7×10⁸ ± 0.04</td>
</tr>
<tr>
<td>MS2</td>
<td>8.6×10¹⁵ ± 0.02</td>
<td>8×10¹⁵ ± 0.02</td>
<td>5.9×10¹⁴ ± 0.02</td>
</tr>
<tr>
<td>MS3</td>
<td>6.2×10¹³ ± 0.025</td>
<td>6.3×10¹³ ± 0.025</td>
<td>5.2×10¹³ ± 0.025</td>
</tr>
<tr>
<td>MS4</td>
<td>5.7×10¹³ ± 0.04</td>
<td>5.6×10¹³ ± 0.03</td>
<td>4×10¹² ± 0.03</td>
</tr>
<tr>
<td>MS5</td>
<td>5.8×10¹³ ± 0.03</td>
<td>6×10¹³ ± 0.03</td>
<td>5×10¹³ ± 0.03</td>
</tr>
<tr>
<td>MS6</td>
<td>4.2×10¹³ ± 0.03</td>
<td>4×10¹³ ± 0.03</td>
<td>3.4×10¹³ ± 0.04</td>
</tr>
</tbody>
</table>

Table 4.6: Growth and malathion degradation of strain KB1, PU and KB2 in MS1 to MS6 medium supplemented with 0.15% malathion after 7 days of incubation at 30 °C.

<table>
<thead>
<tr>
<th>Strain PU</th>
<th>Days</th>
<th>% degradation of Malathion</th>
<th>% degradation of Malaoxon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>10.25</td>
<td>32.78</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>40.24</td>
<td>76.73</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>49.31</td>
<td>87.40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain KB1</th>
<th>Days</th>
<th>% degradation of Malathion</th>
<th>% degradation of Malaoxon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>8.24</td>
<td>24.64</td>
</tr>
<tr>
<td></td>
<td>5</td>
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<th>% degradation of Malaoxon</th>
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<td>19.87</td>
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<tr>
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<tr>
<td></td>
<td>7</td>
<td>36.22</td>
<td>72.20</td>
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</tbody>
</table>

Table 4.7: The disappearance of malathion and malaoxon by strains, PU, KB1 and KB2 in MS2 medium containing 0.15% malathion incubated at 30 °C.
4.8.4. GC-MS analysis

The malathion degradation products were further characterized by GC-MS analysis. Two types of product were detected in MS2 medium, probably, malathion dicarboxylic acid and malathion monocarboxylic acid (Fig 4.29). The GC-MS spectrum pattern of standard (without inoculum) Fig 4.29 (A) show a parent ion peak at m/e 330 which was consistent with the molecular formula of malathion and was found to be very weak. The parent ion undergoes cleavage of S-C bond to form two highly abundant fragments, which appeared at m/e 173 and 125. The mass spectrum shows two additional peaks at m/e 258 and 184, which correspond to loss of one and two -COOC2H5 fragments, respectively from parent ion. Other peak was observed with the addition of H at m/e 285 due to evolution of CO2 from the parent ion.

Mass spectrum of Fig 4.29 (B) shows common features of malathion dicarboxylic acid (MDC) C6H13O6PS2, in which peak at m/e 230 due to evolution of CO2 from the parent ion. The parent ion undergoes cleavage of P-S bond to form highly abundant fragment which appeared at m/e 148.

Mass spectrum of Fig 4.29 (C) shows common features of malathion monocarboxylic acid (MMC) C8H15O6PS2, in which peak at m/e 258 due to evolution of CO2 from the parent ion. The parent ion undergoes cleavage of S-CH bond to form highly abundant fragment which appeared at m/e 145. The mass spectrum shows peak at m/e 118, which correspond to loss of one - S-CH-COOC2H5 fragment.
4.8.5. Crude enzyme assay

Degradation of malathion by all three strains, PU, KB1 and KB2 to malathion mono-acid and malathion diacid may occur through the action of carboxylesterase. Subsequently, the assay was carried out to determine the degree of carboxylesterase by all three strains. The result revealed that esterase activity increases with increasing incubation time and maximum esterase activity 270, 240 and 210 U mL\(^{-1}\) were observed by strain PU, KB1 and KB2 respectively, after 7 days of incubation as shown in Fig 4.30.
4.8.6. PCR amplification and sequence analysis of *carboxylesterase* gene

Based on the observation that *Bacillus cereus* strain PU had a high carboxylesterase activity, we decided to clone the *carboxylesterase* encoding gene involved in malathion degradation. The primers BRCE-f and BRCE-r amplified 744 bp open reading frame of *carboxylesterase* gene of *Bacillus cereus* strain PU (accession number-HQ288420), 747 bp of *Lysinibacillus* sp. KB1 (accession number-JF817352) and 756 bp of *Brevibacillus* sp. KB2 (accession number-HQ218054) (Fig 4.31).

![Fig 4.30: Esterase activity (U/ml) in culture filtrates of three strains in MS2 medium.](image)

![Fig 4.31: Agarose gel electrophoresis of the fully amplified BRCE gene of strains PU, KB1 and KB2. M represents 100 bp DNA marker.](image)
Results

BLASTX analysis indicated that sequence Bacillus cereus strain PU had highest degree of similarity (99%) with carboxylesterase of Bacillus cereus, B. thuringiensis, and B. anthracis. Also, it showed similarity (91%) to carboxylesterase of B. pseudomycoides, B. weihenstephanensis, 81% to B. megaterium, and 78% to B. pumilus.

Lysinibacillus sp. KB1 had highest degree of similarity (84%) with carboxylesterase of Bacillus cereus, 73% to Bacillus sp. NK13 and B. pumilus.

Brevibacillus sp. KB2 had highest degree of similarity (90%) with carboxylesterase of Bacillus cereus, B. thuringiensis, B. anthracis. Also, it showed similarity (76%) to carboxylesterase of Geobacillus sp., B. megaterium, 74% to B. pumilus and 73% to B. coagulans.

The translated amino acid sequence of carboxylesterase from all three strains (Fig 4.32) subjected to a search against the NCBI protein database. It was found that the sequence had the highest degree of identity to a carboxylesterase (EC 3. 1. 1. 1) of Bacillus sp.
Fig 4.32: (A) Nucleotide and deduced amino acid sequence of the carboxylesterase gene from Bacillus cereus strain PU.
Results

4.32: (B) Nucleotide and deduced amino acid sequence of the carboxylesterase gene from Lysinibacillus sp. strain KB1.

![Sequence Alignment]

Fig 4.32: (B) Nucleotide and deduced amino acid sequence of the carboxylesterase gene from Lysinibacillus sp. strain KB1.
Fig 4.32: (C) Nucleotide and deduced amino acid sequence of the carboxylesterase gene from Brevibacillus sp. strain KB2.

Multiple sequence alignment of deduced amino acid sequence of carboxylesterase of from all three strains and four closest relative strains was shown in Fig 4.33. Deduced amino acid sequence of all three strains showed 99% similarities with amino acid sequence of carboxylesterase of Bcillus s
Results

Lysinibacillus

```
MMKLASPKPFTFEGGDRAVLLLHGFTGNSADVRMLGRFLEKKGYTCHAPI 50
```

ZP-04153739

```
MMKLASPKPFTFEGGDRAVLLLHGFTGNSADVRMLGRFLEKKGYTCHAPI 50
```

ZP-04242075

```
MMKLASPKPFTFEGGDRAVLLLHGFTGNSADVRMLGRFLEKKGYTCHAPI 50
```

ZP-04093139

```
MMKLASPKPFTFEGGDRAVLLLHGFTGNSADVRMLGRFLEKKGYTCHAPI 50
```

B.cereus

```
MMKLASPKPFTFEGGDRAVLLLHGFTGNSADVRMLGRFLEKKGYTCHAPI 50
```

Brevibacillus

```
MMKLASPKPFTFEGGDRAVLLLHGFTGNSADVRMLGRFLEKKGYTCHAPI 50
```

AD016159

```
MMKLASPKPFTFEGGDRAVLLLHGFTGNSADVRMLGRFLEKKGYTCHAPI 50
```

Lysinibacillus

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ZP-04153739

```
YKGHGVPPEELVHTGPTDWWQDVTEAYQLLKDKGFEKIALVGLSLAGVFS 100
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ZP-04242075

```
YKGHGVPPEELVHTGPTDWWQDVTEAYQLLKDKGFEKIALVGLSLAGVFS 100
```

ZP-04093139

```
YKGHGVPPEELVHTGPTDWWQDVTEAYQLLKDKGFEKIALVGLSLAGVFS 100
```

B.cereus

```
YKGHGVPPEELVHTGPTDWWQDVTEAYQLLKDKGFEKIALVGLSLAGVFS 100
```

Brevibacillus

```
YKGHGVPPEELVHTGPTDWWQDVTEAYQLLKDKGFEKIALVGLSLAGVFS 100
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AD016159

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Lysinibacillus

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ZP-04153739

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B.cereus

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Brevibacillus

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AD016159

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Lysinibacillus

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ZP-04153739

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B.cereus

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Brevibacillus

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AD016159

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Lysinibacillus

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ZP-04153739

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B.cereus

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Brevibacillus

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AD016159

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Fig 4.33: Multiple sequence alignment of deduced amino acid sequence of carboxylesterase of from all three strains, PU,KB1 and KB2 and four closest relative strains: carboxylesterase from Bacillus pseudomycoides DSM 12442 (ZP-04153739), carboxylesterase from Bacillus cereus Rock1-15 ZP-04242075., ZP-04093139, carboxylesterase from Bacillus thuringiensis. Red color, identical amino acids present in all sequences, Blue, green and pink identical amino acid in most of aligned sequences.

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The prediction of secondary structure by SOPMA (Combet et al. 2000) indicated that the deduced amino acid sequence of carboxylesterase of strain PU contained 114 a-helices, 10 b-turns, 38 extended strands, and 85 random coils (Fig. 4.34), strain KB1 contained 118 a-helices, 9 b-turns, 39 extended strands, and 82 random coils (Fig. 4.34) and similarly, strain KB2 contained 120 a-helices, 16 b-turns, 40 extended strands, and 75 random coils (Fig. 4.34).

![Secondary Structure Diagram](image)

**Fig. 4.34:** Predicted secondary structure and comparison of deduced amino acid sequence of carboxylesterase from strains, PU, KB1 and KB2 by SOPMA. Helices, sheets, turns, and coils are indicated by the longest, second longest, second shortest, and shortest vertical lines, respectively.

### 4.9. Tolerance of bacterial isolates to chlorpyrifos and methyl parathion

The ability of these bacteria(s) to tolerate chlorpyrifos and methyl parathion is of interest as all three organophosphate pesticides are often used and therefore commonly co-contaminate the lands. The ability of the bacteria to tolerate chlorpyrifos and methyl parathion was assessed by their growth on MS (mineral salt media) plates containing both organophosphate pesticides at concentrations between 0.05% and 0.1%. All three strains showed no signs of growth at 0.05% and 0.1% methyl parathion.
4.10. Bioremediation of malathion

4.10.1. Characteristics of soil

The pH and moisture content of soil sample collected from botanical garden in the campus of Panjab University was found to be 5.8 and 30.45% (Table 4.8), respectively. Calcium carbonate: inorganic carbonate, either as calcium (calcite) or magnesium (dolomite) carbonate or mixtures of both, occurs in soil as results of weathering, or is inherited from parent material. A given weight of soil is reacted with an excess of acid and in this reaction CO₂ gas is released and the acid not used in the dissolution of carbonates is back-titrated with sodium hydroxide solution. In the titrimetric method, two equivalent of acid is assumed to be equivalent to one half mole of CaCO₃. Calcium carbonate in the soil sample was found to be 18%.

Organic matter

Soil organic matter represents the remains of roots, plant material, soil organisms in various stages of decomposition and synthesis, and is variable in composition. Though occurring in relatively small amounts in soils, organic matter has a major influence on soil aggregation, nutrient reserve and its availability, moisture retention and biological activity. Organic matter in the soil sample was found to be 5.3%.

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<td>Moisture</td>
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<tr>
<td>Calcium carbonate</td>
<td>18%</td>
</tr>
<tr>
<td>Organic matter</td>
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</table>

Table 4.8: Characteristics of soil used in this study

4.10.2. Bioremediation of malathion contaminated soil

Only 7.11% of the initially added 0.15% of malathion was degraded in uninoculated sterilized soil after 7 days (Fig 4.35). However, after inoculation
the sterilized soil with strain PU, the degradation of malathion increased to 70% during the same period. In fresh soil samples with and without inoculation, the degradation of malaoxon was 77.7% and 17.8% after 7 days respectively.

After inoculation the sterilized soil with strain KB1, the degradation of malathion increased to 66% after 7 days of incubation (Fig. 4.35 B). In fresh soil samples, the degradation of malaoxon was increased to 74%.

After inoculation the sterilized soil with strain KB2, the degradation of malathion increased to 54% after 7 days of incubation (Fig. 4.35 C). In fresh soil samples, the degradation of malaoxon was increased to 61%.

Similarly, after inoculation the sterilized soil with all three strains, the degradation of malathion increased to 80% after 7 days of incubation (Fig. 4.35 D). In fresh soil samples, the degradation of malaoxon was increased to 87%.

![Graph showing degradation of malaoxon](image)

Fig 4.35: Degradation of malaoxon in varied soil by (A) strain PU (B) strain KB1 (C) strain KB2 (D) by mixed culture. Results showed % malaoxon degradation where initial malaoxon was equal to malathion concentration of 1.5% kg⁻¹ soil. Data is presented as mean and standard error of three independent observations.

Fig 4.35: Contd....
Results

Fig 4.35: Contd...

- inoculated nonsterile soil
- inoculated sterile soil

% Malathion degradation

Time (days)
4.10.2. Bioremediation of TNP contaminated soil

Only 21% of the initially added TNP Swas degraded in inoculated sterilized soil after 12 h. No significant degradation of TNP was observed in uninoculated sterilized soil after 12 h. In fresh soil samples with and without inoculation, the degradation of TNP was 28% and 5% after 12h respectively.