4.1. Collection of water and soil samples from hot water spring of Manikaran and Palampur tea garden.

Manikaran is one of the volcanic regions present at the bank of Parvati valley, between Beas and Parvati rivers in Kullu district of Himachal Pradesh, India. It is situated in the snowy mountains of Himalayas at an altitude of 1760 m (32.0268°N, 77.3511°E) and is located about 45 km from Kullu.

Figure 4.1: Sample collection sites. Map of India (A) showing Himachal Pradesh and location of geothermal springs in India, B) Map of Himachal Pradesh showing Kullu and Palampur, (C) Palampur tea garden, (D) Thermal hot spring, Manikaran, Kullu H.P, India, and (E) Thermal hot spring, Vashisht, Kullu, H.P, India.
Palampur is a tea capital of north India, located in district Kangara, H.P., India. Soil sample was collected from Bundla tea estate, 2 kilometer from Palampur city. It is located at 32.12°N 76.53°E, at a height of 1,470 m above sea level (Fig. 4.1 A-E). Water and soil sediment samples were collected in the month of January, year 2012.

4.1.1. Physical parameters (temperature and pH) of the sample collection sites

At the time of sample collection at Manikaran hot spring (site I), the pH of the soil sample was 6 and temperature was around 70°C at noon time. This site also showed the presence of orange precipitates on the surface of water and at the sides of water canal. Manikaran hot spring site II, had pH 7.5 and the temperature was 95°C at the time of sample collection. Palampur tea garden soil sample had pH 5 and temperature of 10°C. Water sample was collected from Vashisht hot spring, which had pH 6 and temperature 60°C (Table 4.1).

4.2. Isolation of caffeine degrading bacterial isolates from water and soil samples by enrichment technique.

4.2.1. Enrichment of caffeine degrading bacterial isolates

After 5 days of enrichment in M9 liquid medium containing 0.25 % (w/v) caffeine, samples were spread on nutrient agar medium or M9 agar medium containing 0.25 % (w/v) caffeine. Colony forming units (CFU) on nutrient agar medium plated with soil sample from site I and site II was 2 x 10⁴ and 3 x 10³ respectively (Fig 4.2 D and E respectively). On the other hand, NA agar medium plated with water sample from Vashisht showed CFU of 1 x 10³ (Fig 4.2 F). However, CFU on M9 agar containing 0.25 % caffeine plated with soil sample from site I and site II was 0.32 x 10² and 3 x 10² respectively (Fig 4.2 A and B respectively) as compared to water sample, which showed only five colonies (Fig 4.2 C). CFU count on NA medium plated with soil sample from Palampur tea garden was 5 x 10², as compared to 0.91 x 10² CFU of soil sample plated on M9 agar medium containing 0.25 % (w/v) caffeine (Fig. 4.2 G-H). All the isolated colonies observed on M9 medium containing caffeine as sole source of carbon and nitrogen were picked and purified by
restreaking on M9 agar medium containing 0.25% caffeine (w/v). Ten morphologically distinct (shape, size and texture) bacterial isolates were further tested for growth on M9 medium containing caffeine (0.25%) as a sole source of carbon and nitrogen. Based on growth characteristics, four bacterial isolates named as MS1-3, RT4, RT6 and RTPM4 were selected for further study. The source of isolation of bacterial isolates MS1-3, RT4, RT6 and RTPM4 was Manikaran hot spring site II, Manikaran hot spring site I, Vashisht hot spring and Palampur tea garden respectively (Table 4.1).

4.2.2. Biochemical characteristics of caffeine degrading bacterial isolates.

The three caffeine degrading bacterial isolates, RT4, RT6, RTPM4 were Gram negative and MS1-3 was Gram positive. All the four caffeine degrading bacterial isolates were whitish to creamish in color, and rod shaped ranging from small to medium and long rods.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Caffeine degrading bacterial isolates</th>
<th>Source of isolation</th>
<th>Place of isolation in H.P, India</th>
<th>Temperature (°C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RTPM4</td>
<td>Soil</td>
<td>Palampur tea garden, Palampur,</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>RT4</td>
<td>Soil from bed of the thermal spring site I</td>
<td>Manikaran thermal hot spring, Kullu</td>
<td>75</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>MS1-3</td>
<td>Soil from bed of the spring site II</td>
<td></td>
<td>95</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>RT6</td>
<td>Water</td>
<td>Vashisht thermal spring, Kullu</td>
<td>65</td>
<td>6</td>
</tr>
</tbody>
</table>

Further, bacterial isolates RT4 and RT6 showed fluorescence under UV light (Fig 4.3), whereas RTPM4 and MS1-3 were non fluorescent. This data suggested that RT4 and RT6 might be *Pseudomonas* species. All the bacterial isolates showed positive reaction for catalase, oxidase and glucose test, whereas negative reaction for lactose and urease test. Bacterial isolate RT4, RT6 and RTPM4 showed negative reaction for mannitol and methyl red, whereas MS1-3 showed positive reaction. Isolate RT4, RT6 and RTPM4 showed
positive reaction for nitrate and indole test and MS1-3 showed negative reaction for the same (Table 4.2).

**Figure 4.2:** Primary screening after enrichment of 5 days in M9 liquid medium containing 0.25 % (w/v) caffeine. Plating of soil sample from MS1 site (A), MSII (B), Palampur tea garden soil (H) and water sample from Vashisht (C), on M9 agar medium containing 0.25 % (w/v) caffeine. Growth was observed after 42 h of incubation at 30°C. Plating of soil sample from sites MS1 (D), MSII (E), Palampur tea garden soil (G) and of water sample from Vashisht (F), on nutrient agar plates. Growth was observed after 12 h of incubation at 30°C.

**Figure 4.3:** UV-Fluorescence of caffeine degrading bacterial isolates. Bacterial isolates as indicated were streaked on M9 medium containing caffeine, incubated at 30°C for 42 h and observed under UV-transilluminator. E.coli strain DH5α served as control.
Table 4.2: Biochemical characteristics of caffeine degrading bacterial isolates

<table>
<thead>
<tr>
<th>S.No</th>
<th>Biochemical characteristics</th>
<th>RT4</th>
<th>RT6</th>
<th>RTPM4</th>
<th>MS1-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Color of the isolate</td>
<td>Creamish</td>
<td>Creamish</td>
<td>Creamish</td>
<td>Creamish</td>
</tr>
<tr>
<td>1</td>
<td>Morphology</td>
<td>Rod shaped</td>
<td>Rod shaped</td>
<td>Rod shaped</td>
<td>Rod shaped</td>
</tr>
<tr>
<td>2</td>
<td>Gram reaction</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>Fluorescent</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Oxidase test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Catalase test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Mannitol test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Lactose test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Nitrate test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Urease test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Indole test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Methyl red test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Glucose test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

‘+’ sign indicates positive reaction and ‘–’ sign indicates negative reaction

4.2.3. Utilization of caffeine as a sole source of carbon and nitrogen by bacterial isolates.

Bacterial isolates MS1-3, RT4, RT6 and RTPM4 were individually plated on M9 agar medium containing 0.25 % (w/v) caffeine. Creamish color colonies were observed after incubation for 36 h at 30°C (Fig 4.4 A and B). All the bacterial isolates showed growth at 30°C. *E.coli* DH5α did not show detectable growth in caffeine containing medium as expected. To quantitatively test the caffeine degradation, cell free spent medium and whole cell extracts (WCE) were subjected to NBT reduction assay. Cell free spent medium did not show any caffeine degrading enzyme activity (data not shown), whereas WCE of MS1-3, RTPM4, RT4 and RT6 showed caffeine degradation activity (U/mg/min) of 1125, 666.67, 885.42 and 739.58 respectively. As expected protein extracts of DH5α grown in nutrient medium did not show any caffeine degrading activity as shown in figure 4.4. This data clearly confirm that the bacterial isolates MS1-3, RTPM4, RT4 and RT6 showed induction of caffeine inducible caffeine degrading enzyme activity. Bacterial isolates grown in absence of caffeine did not show caffeine degradation activity.
Figure 4.4: Growth comparison and caffeine degradation by bacterial isolates. Individual and isolated colonies of four bacterial isolates as indicated were streaked on M9 agar medium containing 0.25% (w/v) caffeine. Petriplates were incubated at 30°C for 42 h and observed for growth. In both the plates, E.coli strain DH5α was streaked as control (A and B). C. Caffeine degradation enzyme assay was performed using whole cell extracts and cell free spent medium by measuring NBT reduction at 566 nm in UV transilluminator.


4.3.1. Determination of optimum concentration of caffeine for growth of bacterial isolates.

Caffeine degrading bacterial isolates, RT4, MS1-3, RT6 and RTPM4 were grown in medium containing different concentrations of caffeine ranging from 0.1 to 1 % (w/v) (Fig 4.5). At lower concentration of caffeine (0.1 %), all the four bacterial isolates showed growth. Bacterial isolates RTPM4 and MS1-3 showed optimum growth at 0.3 % caffeine.
(w/v), whereas RT6 and RT4 showed optimum growth at 0.4 % and 0.5 % of caffeine (w/v) respectively. Caffeine at 0.5 % (w/v) inhibited the growth of RTPM4 whereas, growth of MS1-3 was inhibited at caffeine concentration of 0.6-0.7 % (w/v). Bacterial growth was completely inhibited at 0.8 % caffeine for RT4 and RT6, whereas complete growth arrest was observed at 0.7 % for RTPM4 and MS1-3 respectively. None of the caffeine degrading bacterial isolate could tolerate 1 % (w/v) caffeine in the growth medium (Fig 4.5).

![Graph](image_url)

**Figure 4.5: Determination of optimum concentration of caffeine for growth of bacterial isolates.** Bacterial isolates were grown in M9 liquid medium containing 0.1 - 1 % caffeine (w/v) for 72 h at 30°C and absorbance was measured at 600 nm.

### 4.3.2. Determination of rate of utilization of caffeine and increase in microbial growth

To study the rate of utilization of caffeine and its role in building the cell mass, depletion of caffeine from the growth medium was observed at 273 nm, whereas growth was observed at 600 nm for different time intervals. Cell density corresponding to 0.1 OD at 600 nm of each bacterial isolate was inoculated into the M9 medium supplemented with optimum concentration of caffeine (0.3 % for MS1-3 and RTPM4, 0.4 % and 0.5 % for RT6 and RT4 respectively). The inoculated flasks were incubated at 30 °C in a shaking incubator (200 rpm). Samples were withdrawn at regular intervals of 12 h. During the
exponential phase of growth, there was a concomitant utilization of caffeine by RTPM4, MS1-3, RT6 and RT4 isolates and ~90-95% caffeine was utilized by 102 h of incubation (Fig 4.6). The rate of disappearance of caffeine was dependent on the growth of bacterial isolates. Among the four bacterial isolates, RT4, MS1-3 and RT6 showed almost complete caffeine degradation at 102, 90 and 90 h of incubation respectively, as compared to that of RTPM4, which showed complete caffeine degradation at 114 h of incubation. All the four caffeine degrading bacterial isolates displayed a lag phase, among which MS1-3, RT4 and RT6 showed lag phase of less than 36 h, as compared to the 36 h of lag phase of RTPM4 isolate. Bacterial isolates RT4 and RT6 showed steep rise in growth after 24 h of incubation. RT6 consumed 50% of caffeine at 54 h of incubation with OD of 1.06. RT4 and RTPM4 showed 50% consumption of caffeine at 54 h with absorbance of 2.61 and 1.12 at 600 nm, whereas MS1-3 showed 50% caffeine consumption at 66 h of incubation (Fig 4.6).

4.3.3. MS1-3 could utilize xanthine and its derivatives as a sole source of carbon and nitrogen for growth.

Bacterial isolates RT4, RT6, MS1-3 and RTPM4 were grown on theophylline theobromine, xanthine and caffeine as a sole source of carbon and nitrogen. RT4, RT6 and RTPM4 did not show detectable growth on medium supplemented with xanthine, theophylline and theobromine, except MS1-3, which showed growth on medium supplemented with theobromine, theophylline and xanthine (Fig 4.6 A). However, growth was comparatively less in comparison to medium supplemented with caffeine alone. Whole cell extracts of bacterial isolates MS1-3, RT4, RT6 and RTPM4 when grown in presence of theobromine, theophylline and xanthine did not show caffeine degradation activity (Fig 4.7 B). This data further substantiate the fact that bacterial isolates MS1-3, RT4, RT6 and RTPM4 possess caffeine inducible, caffeine degrading enzyme activity.
4.4. Effect of physical parameters on growth of caffeine degrading bacterial isolates

4.4.1. Determination of effect of temperature on growth and caffeine degrading enzyme activity

Growth of the caffeine degrading bacterial isolates in M9 liquid medium containing 0.25% (w/v) caffeine was determined at different temperatures ranging from 25 to 50°C. Bacterial isolate RT6, RT4 and RTPM4 showed optimum growth at 30°C, but growth of MS1-3 was not affected even up to 50°C (Fig. 4.8 A). This data suggest that bacterial isolate MS1-3, is a thermophile, which was isolated from thermal hot spring site II where the temperature was ~95°C at the time of sample collection. This is the first report of thermophilic bacterial isolate capable of utilizing caffeine as sole source of carbon and nitrogen.

![Graph showing growth and utilization of caffeine](image-url)

**Figure 4.6: Growth of bacterial isolates and utilization of caffeine.** Bacterial isolates were grown in M9 medium supplemented with optimum concentration of caffeine (w/v) and were incubated at 30°C. Cell density was measured at 600 nm and caffeine consumption at 273 nm. Absorbance at each wavelength was monitored at 12 h interval. Data of two independent experiments was plotted with standard deviation.
Optimum caffeine degradation activity was observed at 55°C for MS1-3 (1875 U/mg/min), 50°C for RT4 and RTPM4 (859.37 and 484.3 U/mg/min respectively), and 45°C for RT6 (835.9 U/mg/min) as shown in (Fig. 4.8 B).

Figure 4.7: Utilization of xanthine and its derivatives as a source of carbon and nitrogen for growth of caffeine degrading bacterial isolates. MS1-3, RT4, RT6 and RTPM4 were grown in M9 liquid medium containing xanthine and its derivatives as indicated for 72 h at 30°C. Growth was measured at 600 nm (A). B. Caffeine degrading enzyme activity was measured in WCE (prepared from bacterial isolates grown in medium supplemented with theobromine theophylline, xanthine and caffeine) using NBT assay.
Caffeine degrading activity of MS1-3 isolate was decreased to 50% at 60°C (1101 U/mg/min) and was completely inhibited at 75°C (23.625 U/mg/min). Caffeine degrading activity was decreased to 41% at 30°C. Caffeine degrading activity of RTPM4 was decreased to 41% at 60°C (218 U/mg/min) and retained 45% activity at 30°C (218.75 U/mg/min). Caffeine degrading activity of RT4 was reduced to 20% at 30°C and to 61% at 60°C. Caffeine degrading activity of RT6 was reduced to 20% at 60°C (218.75 U/mg). This data suggest that MS1-3 is a thermophile and could degrade caffeine efficiently even at 65°C. Bacterial isolates RT6, RT4 and RTPM4 did not show growth at temperature higher than 40°C, but caffeine degradation activity was optimum at 50°C and 55°C for RT4 and MS1-3 respectively (Fig. 4.8 B).

4.4.2. Effect of pH on growth and caffeine degrading enzyme activity

Caffeine degrading enzyme activity was performed in a sodium phosphate buffer set at pH 8 and 9 and sodium acetate buffer set at pH 3, 4, 5 and 6. Bacterial isolates RTPM4, RT6, RT4 showed optimum growth and caffeine degradation at pH 7, whereas bacterial isolate MS1-3 showed optimum growth and caffeine degradation at pH 6 (Fig. 4.9A and B). RTPM4, RT6 and RT4 could not tolerate acidic pH ≤ 5 and alkaline pH > 8. On the other hand, bacterial isolate MS1-3 could not tolerate acidic pH ≤ 3 and alkaline pH ≥ 8. MS1-3 showed highest caffeine degrading enzyme activity at pH 6 (2634 U/mg). RT6, RT4 and RTPM4 showed highest caffeine degrading enzyme activity at pH 7 with 2508, 2442 and 2810 U/mg/min respectively, whereas no activity was detected at pH 5. Caffeine degrading enzyme activity was retained at pH 8 in RT6, RT4 and RTPM4 with 1020, 1248, 1530 U/mg respectively. Further, growth of microbial cultures and caffeine degradation assays were performed at optimum temperature and pH.
Figure 4.8: Determination of effect of temperature on growth and caffeine degrading enzyme activity. The bacterial isolates were inoculated in M9 liquid medium supplemented with caffeine (0.25%) and were incubated at indicated temperatures for 72 h. Cell density was measured at 600 nm (A). Caffeine degrading enzyme activity in cell free extracts of MS1-3, RTPM4, RT6 and RT4 was measured at different temperature (30-75°C) using NBT assay (B). Data of two independent experiments was plotted with standard deviation.
Figure: 4.9: Determination of effect of pH on growth of caffeine degrading bacterial isolates. The bacterial isolates were inoculated in M9 liquid medium supplemented with caffeine (0.25% (w/v)), and adjusted to different pH as indicated and incubated at 30°C for 72 h. Cell density was measured at 600 nm and data of two independent experiments was plotted with standard deviation (A). Whole cell extracts of bacterial culture grown at optimum pH were tested for caffeine degradation using NBT assay with assay buffer set at different pH (B).
4.5. Effect of chemical parameters on growth and caffeine degrading enzyme activity

4.5.1. Effect of different carbon sources on the growth and caffeine degrading enzyme activity

In general, all the four bacterial isolates were capable of utilizing wide range of carbon sources (glucose, lactose, sucrose, starch, maltose, trehalose, caffeine and sorbitol), except galactose (Fig. 4.10). Specifically, bacterial isolate RT4 and RTPM4 showed trace growth in medium supplemented with sorbitol. Optimum growth was observed in medium supplemented with glucose (RT6, RT4, RTPM4), followed by caffeine (MS1-3), and trehalose (RT6, RTPM4 and MS1-3) as shown in Fig. 4.11 A. Comparable growth was observed in medium supplemented with lactose, sucrose, starch, and maltose. All the four bacterial isolates grown in presence of caffeine, bacterial isolate MS1-3 grown in presence of lactose and sucrose, and bacterial isolate RT4 grown in presence of sucrose showed comparable caffeine degradation enzyme activity. Caffeine degradation activity was inhibited, when bacterial isolates were grown in presence of trehalose, maltose, and lactose (except RT6). Though bacterial isolates RT4, RT6, RTPM4 and MS1-3 showed growth in presence of starch, but caffeine degradation activity was completely inhibited (Fig. 4.11 B). Similarly, cultures were grown in presence of lactose (RT4 and RTPM4) and trehalose (MS1-3) did not show caffeine degradation enzyme activity. Growth of RT4 and RTPM4 was inhibited when M9 medium containing 0.25 % (w/v) caffeine was supplemented with sorbitol as carbon source. In conclusion, glucose and lactose for RT6 and sucrose for RT6 and RT4 showed induction of caffeine degradation very much similar to that of caffeine (Fig 4.11 B).

4.5.2. Effect of different nitrogen sources on growth and caffeine degrading activity

Comparatively, supplementation of yeast extract, peptone, and urea inhibited the growth of all the bacterial isolates, whereas ammonium chloride specifically inhibited the growth of RT6 and RT4 (Fig 4.13 A). Caffeine degradation enzyme activity was induced in the presence of casein acid hydrolysate (CAH) for RT4 with specific activity of
2550 U/mg/min, ammonium chloride and urea for RTPM4 with specific activity of 2800 and 3550 U/mg respectively.

**Figure 4.10: Effect of carbon sources on growth of bacterial isolates.** Bacterial isolates were streaked on M9 agar medium supplemented with caffeine as a nitrogen source and sorbitol (a), glucose (b-c), trehalose (d), maltose (e), sucrose (f,g), lactose (h,i), as a carbon source. Caffeine, both as a nitrogen and carbon source served as control (J and K). The plates were incubated at 30° C for 72 h.
Figure 4.11: Determination of effect of different carbon sources on growth of caffeine degrading bacterial isolates. The bacterial isolates were inoculated in M9 liquid medium supplemented with caffeine (0.25%) as nitrogen source and different carbon sources as indicated. The cultures were incubated at 30°C and cell density was measured at 600 nm after 72 h of incubation (A). (B) Caffeine degradation enzyme assay were performed using whole cell extracts prepared from culture grown in presence of different sugars as indicated. Extracts of caffeine grown culture were considered as reference.
Figure 4.12: Effect of different nitrogen sources on growth of caffeine degrading bacteria isolates. The different bacterial isolates as indicated were streaked on M9 agar medium supplemented with caffeine as a carbon source and ammonium chloride (a-b), peptone (c-d), yeast extract (e-f), urea (g-h) as a nitrogen source. Caffeine as a carbon and nitrogen source served as a control (i-j). Plates were incubated at 30°C for 72 h.
Figure 4.13: Effect of different nitrogen sources on growth and caffeine degrading enzyme activity of bacterial isolates. (A) MS1-3, RTPM4, RT6 and RT4 were grown in M9 liquid medium containing 0.25 % (w/v) caffeine (carbon source) and different nitrogen sources such as CAH, ammonium chloride, urea, peptone or yeast extract and growth was determined at 600 nm (A). WCE were prepared from culture grown in A and were subjected to caffeine degrading enzyme activity using NBT assay.
Peptone and yeast extract did not show much effect on bacterial growth and caffeine degrading enzyme activity of all the four bacterial isolates. Though, urea as a nitrogen source inhibited the bacterial growth, but caffeine degradation enzyme activity of MS1-3 was induced in comparison to caffeine grown cultures (Fig 4.13 B).

### 4.5.3. Effect of metal ions on bacterial growth and caffeine degrading enzyme activity of bacterial isolates

To study the effect of metal ions on growth and caffeine degrading enzyme activity, salts of metal ions were supplemented in the growth medium or in the caffeine degradation assay. Supplementation of zinc (1 mM) increased the growth of MS1-3 and iron (1 mM) and manganese ions increased the growth of RT6 (Fig: 4.14 C and E). Other metal ions, such as copper, cobalt, iron, calcium, nickel and magnesium inhibited the growth of MS1-3 (Fig: 4.14 C). Caffeine degrading activity of MS1-3 was increased by two fold, when reaction mixture was supplemented with 1 and 5 mM of zinc with relative activity of 197 and 187 % respectively. However, 1 and 5 mM of manganese had less additive effect on caffeine degrading enzyme activity with relative activity of 113 and 103 %. Nickel also had additive effect on caffeine degrading enzyme activity, whereas supplementation of calcium showed comparable caffeine degrading enzyme activity with or without metal ions (96 and 99 % activity at 1 mM and 5 mM respectively). Supplementation of copper, manganese, iron and calcium ions at 5 mM enhanced caffeine degrading activity of RT6 with relative activity of 108, 180, 224 and 278 % respectively (Fig 4.14 F). Supplementation of 5 mM or 10 mM zinc and manganese respectively enhanced growth of RTPM4 (Fig 4.14 A). Addition of 1 mM zinc and 10 mM of zinc and nickel ions had additive effect on caffeine degrading enzyme activity of RTPM4 with relative activity of 142, 141 and 131 % respectively (Fig 4.14 A). Calcium (1 mM) and magnesium (5 mM) also had additive effect on growth of RT4 bacterial isolate (Fig 4.14 G). All the three concentrations of calcium and magnesium enhanced the caffeine degrading enzyme activity of RT4 with relative activity of more than 110 % (Fig 4.14 H). Other metal ions did not have much effect on growth of RT4 and RTPM4 (Fig 4.14 G).
Figure 4.14: Effect of metal ions on growth and caffeine degradation by bacterial isolates. Bacterial isolates were grown in presence of metal ions at 30°C, for 72 h. Growth was measured at 600 nm (A,C, E,G) and whole cell extracts were used for performing NBT assay (B,D,F,H). (A-B) RTPM4, (C-D) MS1-3, (E-F) RT6, and (G-H) RT4, represents effect of metal ions as indicated on growth and caffeine degradation respectively.
4.5.4. Effect of chaotropic agents on the growth of caffeine degrading bacterial isolates

The caffeine degrading bacteria isolates were grown in M9 media containing caffeine (0.25%, w/v) supplemented with phenol, detergents, such as sodium dodecyl sulfate (SDS), Triton X-100 and protease inhibitor (5 mM) such as phenyl methyl sulfonyl fluoride (PMSF) and ethylene diamine tetraacetic acid (EDTA). M9 medium with caffeine served as control. All the four bacteria isolates did not show detectable growth in medium supplemented with SDS, phenol, Triton X-100, PMSF and EDTA after 72 h of incubation. Likewise, no caffeine degrading enzyme activity was observed. As expected, all the bacterial isolates when grown in M9 medium supplemented with caffeine showed detectable growth (Fig 4.15).

![Graph showing effect of chaotropic agents on bacterial growth](image)

**Figure 4.15: Effect of different chaotropic agents on growth of bacterial isolates.** The bacterial isolates were inoculated in M9 liquid medium supplemented with caffeine (0.25%) as a sole source of carbon and nitrogen and chaotropic agents as indicated (5 mM). Cell density was measured at 600 nm and data of two independent experiments was plotted with standard deviation.
4.6. Kinetic parameters ($K_m$ and $V_{max}$) of caffeine degrading enzyme activity of bacterial isolates

Kinetic parameters like $V_{max}$ and $K_m$ were determined for all the caffeine degrading bacterial isolates by performing caffeine degradation enzyme assay using different concentration of caffeine (0.1-1 mM) at 50$^\circ$C and Michaelis Menten and Lineweaver-Burk plots were plotted.

![Diagrams showing Lineweaver-Burk plots for different bacterial isolates](image)

Figure 4.16: Determination of kinetic constant of caffeine degrading enzyme activity of bacterial isolates. Caffeine degrading enzyme activity was measured using 10 $\mu$g of protein extracts and increasing amount of caffeine (0.1-1 mM). The $K_m$ and $V_{max}$ was calculated using Lineweaver burk plot and Michaelis Menten graph (A-D and E respectively). Data of two independent experiments was plotted with standard deviation.
Chapter 4

Results

RTPM4, RT6 and MS1-3 showed $V_{\text{max}}$ of 1321.8, 617.8, 687.3 and 1007.8 U/mg respectively. RTPM4, MS1-3, RT6 and RT4 had $K_m$ of 0.25 mM, 6 mM, 0.23 mM and 0.22 mM for caffeine respectively (Fig: 4.16 A-E).

4.7. Identification of caffeine degrading bacterial isolates by 16S rDNA sequencing and phylogenetic analysis.

To identify the bacterial isolates, total genomic DNA of bacterial isolates was isolated as shown in figure 4.17A. 16S rDNA gene was PCR amplified and PCR product of approximately 1.5 kb was detected in all the four bacterial isolates (Fig 4.17 B).

4.7.1. Caffeine degrading *Pseudomonas* spp (RT6, RT4 and RTPM4) and *Bacillus licheniformis* (MS1-3) were identified from thermal hot spring and soil of tea garden

PCR-amplified 16S rDNAs were sequenced on both the strands using 27F and 1492R primers (Tanner et al., 2000; Appendix A1 and A2). A complete nucleotide sequence of 1328, 1324, 1447 bp was generated for RT4, RT6, RTPM4 and MS1-3 respectively. Nucleotide sequences were subjected to BLAST analysis. Isolate RT6, RTPM4 and RT4 showed 99\% homology with *Pseudomonas putida*, whereas MS1-3 showed 99\% homology to *Bacillus licheniformis*. All the 16S rDNA sequences were submitted in the Gen bank database with accession number KM012010.1 (RT4), KJ907483.1 (RTPM4), KM012011.1 (RT6) as different strains of *Pseudomonas putida* and KF885931.1 as *Bacillus licheniformis* (MS1-3) (Appendix; AII). Multiple sequence alignment shown in Figure 4.18 reveals the differences in the 16S rDNA gene sequence of the four isolated strains.

*Bacillus licheniformis* MS1-3 isolated from Manikaran hot spring showed close relationship with *Pseudomonas putida* RT6 isolated from Vashisht hot water spring, which is situated at a distance of 102 km, from Manikaran hot spring. *Pseudomonas putida* RT4 showed phylogenetic evolution with *Pseudomonas putida* RTPM4, isolated from Palampur tea garden soil.
4.7.2. Phylogenetic relationship of *Psuedomonas putida* strains (RT6, RT4 and RTPM4) and *Bacillus licheniformis* (MS1-3) isolated

*Psuedomonas putida* RT4 was closely related to *Pseudomonas sp*, a root endophyte isolated from native plant of republic of Korea (KM252967.1) and *Pseudomonas putida* JQ581 capable of degrading nicotine and nicotinic acid (KT726936). *Pseudomonas putida* RTPM4 was located with the phylogenetic branch of hydrogen-oxidizing bacteria, isolated from rhizosphere soil of *Medicago sativa* (EU807444.1). *Pseudomonas putida* RT6 share a phylogenetic clad with cholesterol- and deoxycholate-degrading bacteria isolated from soil samples (Merino *et al.*, 2016) and *Pseudomonas* sp. CR 4-06, which is a root endophyte isolated from Korea.

![Identification of caffeine degrading bacterial isolates by 16S rDNA](image)

*Figure 4.17: Identification of caffeine degrading bacterial isolates by 16S rDNA.* Total genomic DNA was isolated from MS1-3, RTPM4, RT6 and RT4 (A) and subjected to 16S rDNA amplification (B). ‘M’ represents molecular size marker in kb.
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Figure 4.18: Multiple sequence alignment of 16S rDNA of *Pseudomonas* sp (RT4, RTPM4, RT6) and *Bacillus licheniformis* (MS1-3). 16S rDNA sequences of RT4, RT6, RTPM4 (*P. putida*) and MS1-3 (*Bacillus licheniformis*) were aligned using CLUSTAL W. Sequences marked with * are conserved.

Figure 4.19: Phylogenetic evolution of caffeine degrading *Pseudomonas* spp (RT4, RTPM4, RT6) and *Bacillus licheniformis* (MS1-3). 16s rDNA sequences of *Pseudomonas* sp and *Bacillus licheniformis* were used to construct phylogenetic tree using MEGA 7.
**Figure 4.20:** Phylogenetic evolution of *Pseudomonas putida* (RT6, RT4 and RTPM4). Phylogenetic tree was constructed using 16s rDNA sequences obtained after BLAST search showing more than 99% homology with 16s rDNA of *Pseudomonas putida* (RT6, RT4 and RTPM4) using MEGA 7.
The results of phylogenetic analysis of three *Pseudomonas* species (RT4, RTPM4 and RT6) were consistent with the difference in location of isolation of the strains, as all the three *Pseudomonas* spp. were distantly related. This data clearly distinguish the thermal hot spring *Pseudomonas* (RT6 and RT4) from that of *Pseudomonas* (RTPM4) of a tea garden soil isolate (Fig: 4.20). *Bacillus licheniformis* (MS1-3) isolate was unique and located in a phylogenetic branch of *Bacillus* sp (LT161882.1, isolated from water sample of Sambhar lake, India), *Bacillus sonorensis* strain CM2H3L, isolated from fish gut (KF623291.1), and *Bacillus* sp. B-1-29B, isolated form *Alcyonium digitatum* from the Baltic Sea (Merino et al., 2016) (Fig. 4.21). *Bacillus licheniformis* (MS1-3) showed a unique divergence from known *Bacillus licheniformis* strains may be due to fact that *Bacillus licheniformis* (MS1-3) is a thermophile capable of caffeine degradation (Fig. 4.21). It was observed that *Bacillus licheniformis* (MS1-3) evolved with *Bacillus* sp SMB5, *Bacillus sonorensis* and *Bacillus* sp B1-29B, but by an independent branch (Fig: 4.21).

4.8. *Bacillus licheniformis* (MS1-3) showed demethylation as a mechanism of caffeine degradation

For further study, *Bacillus licheniformis* (MS1-3) was selected as there are very few reports of its caffeine degradation property by thermophilic bacteria. It was isolated from Manikaran hot spring with unique property of degrading caffeine at high temperatures viz. 65°C, which may find industrial application in the future. Moreover, this bacterial isolate showed growth on xanthine and its derivatives, we hypothesize demethylation as one of the mechanism for caffeine degradation.

4.8.1. *Bacillus licheniformis* (MS1-3) metabolizes caffeine and produce xanthine as intermediate product of metabolism.

Caffeine degradation assay measured using NBT suggested a role of oxidase in caffeine degradation. Bacterial isolate *Bacillus licheniformis* MS1-3 retained caffeine degradation activity at 55°C, we analyzed the caffeine degradation and appearance of its degradation products through HPLC analysis. At 24 h of incubation, cell free spent medium showed the presence of caffeine, theophylline, theobromine, and xanthine with retention time of 6.015, 4.528, 3.490, and 2.577 min respectively (Fig 4.22 C).
Figure 4.21: Phylogenetic evolution of *Bacillus licheniformis* (MS1-3). Phylogenetic tree was constructed using 16s rDNA sequences obtained after BLAST search showing more than 99% homology with 16s rDNA of *Bacillus licheniformis* MS1-3 using MEGA 7.
Figure 4.22: Caffeine degradation analysis of *Bacillus licheniformis* (MS1-3) using RP-HPLC: HPLC chromatograms of M9 medium without caffeine supplementation (A), medium containing 0.25% (w/v) caffeine before inoculation of *Bacillus licheniformis* MS1-3 (B), cell free spent medium of *Bacillus licheniformis* (MS1-3) grown in M9 medium containing caffeine at 42 h of incubation (C), and spent medium of *Bacillus licheniformis* (MS1-3) grown in M9 medium containing caffeine at 102 h of incubation (D).
A caffeine peak at 6.015 min was also detected at 0 h of incubation of *Bacillus licheniformis* (MS1-3) isolate in M9 medium supplemented with caffeine (Fig. 4.22 B). Very surprisingly, at 102 h of incubation of *Bacillus licheniformis* (MS1-3) in caffeine containing medium, caffeine, theophylline, and theobromine were undetectable and xanthine peak was observed in contrast to 0 h (Fig. 4.22 D). Since, NBT assay suggested the role of oxidase enzyme and HPLC analysis showed the xanthine as one of the intermediate in caffeine metabolism, we propose that caffeine undergo demethylation and produce xanthine, which is further processed by oxidation to produce uric acid. Based on caffeine degradation assay by measuring NBT reduction and appearance of xanthine as a intermediate in caffeine metabolism. We propose demethylation followed by oxidation of caffeine in *Bacillus licheniformis* (MS1-3) isolate (Fig 4.24).

### 4.8.2. Analysis of caffeine inducible caffeine degrading protein by SDS PAGE

We observed that *Bacillus licheniformis* (MS1-3) showed caffeine inducible caffeine degradation activity even at 55°C. We speculated that *Bacillus licheniformis* (MS1-3) grown in absence or presence of caffeine should have difference in protein profile. *Bacillus licheniformis* (MS1-3) was grown in M9 liquid medium containing 0.25 % (w/v) caffeine as a sole source of carbon or nitrogen or M9 medium containing glucose as a carbon source and ammonium chloride as a nitrogen source. Culture was withdrawn at 72 h, 92 h, 102 h and 114 h to analyze the difference in protein expression. WCE representing 15 µg of proteins were separated through SDS-PAGE and was subjected to Coomassie staining. Total proteins were separated through 12 % SDS PAGE. Specific protein of ~45kDa was observed in caffeine grown medium at 72 h of incubation. The caffeine inducible protein was reduced at 92 h of incubation and finally disappeared by 102 h (Fig 4.23). Caffeine induced protein production was reduced with time, which indicated utilization of caffeine present in the medium. It appears that the unique protein of ~45 kDa was induced specifically at 72 h of incubation, during which cellular growth is at the exponential phase (Fig 4.6). Caffeine induced protein decreased at 92 h of incubation, followed by further drop at 92 h of incubation was completely undetectable at 102 h of incubation.
Figure 4.23: SDS-PAGE profile of protein extracts of *Bacillus licheniformis* (MS1-3) caffeine degrading bacterial isolate. *Bacillus licheniformis* (MS1-3) was grown in presence and absence of caffeine. Samples were withdrawn at 72, 92, 102 and 114 h of growth. Whole cell extracts (WCE) containing 15 µg of protein were separated through 12 % SDS PAGE and stained with Comassie brilliant blue. Lane M represents molecular weight marker (kDa) as indicated M, whereas lane no 2, 3, 4 and 5 represents WCE of *Bacillus licheniformis* grown in presence of caffeine for 72, 92, 102 and 114 h respectively. In lane 1, extracts were prepared from bacterial culture grown in absence of caffeine.
Figure 4.24: Proposed mechanism for caffeine degradation in *Bacillus licheniformis* (MS1-3).

Since, NBT assay suggested the role of oxidase enzyme and HPLC analysis showed the xanthine as one of the intermediate in caffeine degradation, we propose that caffeine undergo demethylation and produce xanthine, which is further processed by oxidation to produce uric acid (Fig.4.24). Protein profile through SDS PAGE analysis showed a ~45 kDa caffeine inducible caffeine degrading enzyme in *Bacillus licheniformis* (MS1-3)
HPLC analysis of spent medium of 72 h showed presence of theobromine, theophylline and xanthine, which suggest concomitant demethylation of caffeine to theobromine or theophylline by demethylase. Di-methylxanthine are further converted to methylxanthine. Methylxanthine is converted to xanthine and xanthine is acted upon by xanthine oxidase and was measured using NBT assay. NBT get reduced by the oxygen to purple color formazan, and its production was detected at 566 nm. Formation of formazan suggest oxidation of xanthine to uric acid by xanthine oxidase in *B. licheniformis* MS1-3 (Fig: 4.24). This is the first report, where we showed that a thermophilic *Bacillus licheniformis* (MS1-3) possess thermaly stable cafffeine degradation by demethylation, followed by xanthine oxidation (Fig 4.24).

### 4.9. *In silico* studies of caffeine degrading enzymes in bacteria

Caffeine is known to be metabolized by various enzymatic pathways such as demethylase, dehydrogenase, and oxidase. The main objective of *in silico* study is to decipher the binding of caffeine and the intermediate products such as theophylline, theobromine, and xanthine to demethylase, dehydrogenase, and oxidase from bacterial system. We performed *In silico* analysis to study the evolutionary relationship, secondary structure prediction, and docking to understand the molecular interaction of caffeine degradation enzymes with its substrates such as caffeine, theophylline, theobromine and xanthine.

#### 4.9.1. Amino acid conservation of N demethylases in bacteria

Very few N demethylases are reported in the literature and most of them are from bacteria. To understand the molecular evolution of N-demethylase in bacteria, methylxanthine N-demethylase of *Pseudomonas putida* (electron transfer protein from *Pseudomonas putida*) was used as template and subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Very surprisingly, N-demethylase showed homology with oxidoreductases and flavodoxin proteins. A total of 13 N demethylase homologs showing more than 50 % homology were subjected to multiple sequence alignment (MSA) using Praline program (http://www.ibi.vu.nl/programs/praline). The amino acid sequences were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) and conserved residues were predicted. MSA showed high conversation among the
homolog’s starting from amino acid 300 and up to the end of protein at C-terminus (Figure 4.25A).

The amino acid residues from F301 – L598 represent a flavin mononucleotide (FMN), nicotinamide adenine dinucleotide (NAD), and iron binding sites which is more conserved than N-terminus, which belongs to Rieske super family (Fig 4.25B). The highly conserved C-terminus might have role in binding its substrates. To understand the evolutionary relationship between N-demethylase and oxidoreductases, all the thirteen Methylxanthine N-demethylase homologs were subjected to phylogenetic analysis. N-demethylase of Pseudomonas putida shared a root with oxidoreductases of Rhizobium clan, which involves transfer of electrons from electron donor to the electron acceptor (Fig. 4.26). The small branches in the horizontal dimension indicated that evolutionary lineages have not much changed over the time. Further, it showed that there is one nucleotide substitution per site (number of changes divided by the length of the sequence). Less homology was observed in reiske superfamily domain in comparison to the iron binding site of the methylxanthine demethylase (Fig 4.25).

Another enzymes involved in caffeine metabolism is xanthine oxidase and dehydrogenase, which convert xanthine to uric acid by oxidation. Both enzymes are fundamentally similar in regards of prosthetic group content and molecular properties, but differ in utilization of electron acceptors, which depend on the economy of cells from which they are obtained (Woolfolk, 1977). Xanthine dehydrogenases uses NAD\(^+\) as electron acceptor, whereas xanthine oxidase transfer reducing electron to molecular oxygen. Xanthine dehydrogenase is a dimer which can be converted to xanthine oxidase by irreversible proteolytic modification or reversible sulphhydryl reduction (Woolfolk, 2007). To study the conservation among xanthine oxidases and dehydrogenases, amino acid sequence of xanthine oxidase of Pseudomonas mendocina was retrieved from NCBI database and was subjected to BLAST search. Amino acid sequences having homology > 50% were subjected to multiple sequence alignment using Praline and we observed conservation of sequences. However xanthine oxidases showed homology with xanthine dehydrogenase (Fig 4.27).
Figure 4.25: Multiple sequence alignment of methylxanthine demethylase in bacteria. BLAST of amino acid sequence of methylxanthine N-demethylase was performed and proteins showing greater than 50% homology were selected and multiple sequence alignment was done using PRALINE (A). B. Conserved domains of methylxanthine demethylase were determined using CDD tool of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/).

Figure 4.26: Phylogenetic evolution of methylxanthine N-demethylase. BLAST was performed using amino acid sequence of Methylxanthine demethylase of *P. putida* and phylogenetic tree was constructed with sequences greater than 50% homology.
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Figure 4.27: Multiple sequence alignment and phylogenetic evolution of xanthine oxidase and dehydrogenases among bacteria. Xanthine oxidase amino acid sequence of *P. mendocina* NK-01 was retrieved from NCBI and BLAST search was performed. Proteins sequences showing homology greater than 50% were subjected to MSA with PRALINE software (A). Xanthine dehydrogenase and xanthine dehydrogenase from different species were subjected to MSA and phylogenetic tree construction using MEGA 7 (B).
Function of any protein is directly linked to its 3-dimensional conformation. Since, understanding of 3-dimensional structure of a protein is a very exhaustive and time-consuming process, bioinformatics tools to predict the tertiary structure of a protein and even interaction with ligand/substrates has narrowed the bridge.

We, therefore predict the tertiary structure of proteins involved in caffeine degradation (N-demethylase, oxidase, and dehydrogenase) in bacterial system. A homology-based tertiary structure of Methylxanthine demethylase of *P. putida* and xanthine oxidase of *Pseudomonas mendocina* was predicted using PSIPred and Swiss model software. Tertiary structure of Methylxanthine demethylase (Pro272-Leu578) was generated based on the phthalate dioxygenase reductase as a template with protein coverage of 94% and 35.26% identity. Methylxanthine demethylase showed 27 α-helix and 13 β-strands. Ribbon diagram of Methylxanthine demethylase and surface view are shown in Fig. 4.28-IA and B. Tertiary structure of xanthine oxidase (Val25-Gln779) was generated based on xanthine dehydrogenase (chain B) of *Pseudomonas mendocina* as a template, with protein coverage of 94% and 49.67% similarity. Ribbon diagram of xanthine oxidase and surface view of xanthine oxidase are shown in Fig. 4.28-IIA and B. Xanthine oxidase showed 19 α-helix and 29 β-strands. Further, xanthine dehydrogenase tertiary structure was generated (Val17 and Gln743) based on xanthine dehydrogenase 2 of *Rhodobacter capsulatus* as a template (Fig 4.28). Model had sequence similarity of 49.67% and coverage was 95%. The structure had 19 α-helix and 28 β-strands. The tertiary structures were further studied for interaction with its substrates.

4.9.2. *In silico* docking of N-demethylase with caffeine and its degradation products

In order to study the binding of substrates such as caffeine, theophylline, theobromine, and xanthine with N-demethylase or xanthine oxidase, we constructed homology-based tertiary structures of Methylxanthine demethylase of *P. putida* and xanthine oxidase of *Pseudomonas mendocina* using Swiss model software.
Figure 4.28: Tertiary structure of methylxanthine demethylase, xanthine and dehydrogenase. Tertiary structure was constructed using Swiss model. Chain structure (A), surface view (B) of methylxanthine demethylase (I), xanthine oxidase (II) and xanthine dehydrogenase (III).
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It is cumbersome to get insights into each protein/enzyme interaction with corresponding substrates and ligands. *In silico* studies provide in depth prediction of such interactions, which could be further tested by *in vitro* study. In order to study the binding of substrates with N-demethylase or xanthine oxidase, structures of theophylline, theobromine, caffeine and xanthine were retrieved from Chemspider database in .mol format and mol files were converted into .pdb files by open label software.

**Caffeine binds deeply in methylxanthine demethylase.**

*Caffeine* The binding of caffeine with methylxanthine demethylase showed that basic nitrogen in the pyrrolidine ring of caffeine formed hydrogen bond with Pro 438, and Met 467 with a bond length of 2.85 and 3.35 Å (alignment score of both amino acids was 6 and 5 in MSA; Fig: 4.25 A). All the interacting residues are present in FMN binding domain. High alignment score of amino acid involved in the bonding suggest that microbial oxidoreductases and methylxanthine demethylase are highly conserved. Hydrophobic interactions were also observed between the ligand and amino acid Thr 441, Arg 442, and Phe 410 (Figure 4.29 a, b and c).

**Theophylline, theobromine and xanthine.** These three substrates had interaction with methylxanthine N demethylase, but affinity was less in comparison to caffeine. Theobromine, theophylline and xanthine formed single hydrogen bond with Thr 313 with bond length of 3.43, 2.18 and 5.20 Å respectively (Fig 4.29 d-k). Thr 313 position had low alignment score of 3 as shown in MSA (Figure 4.25A). This suggests that caffeine, which is trimethyl xanthine possess a specific binding site on N-demethylase. Dimethyl xanthine (theophylline and theobromine) thus formed becomes a substrate for the next enzymatic reaction. Similarly, xanthine also showed interaction with Thr 313. This data suggest that theobromine, theophylline and xanthine may not be the true substrate for methylxanthine N demethylase. Likewise, the consistency of conservation was 4 out of 7 (Fig 4.29 d-k). All the three substrates also showed hydrophobic interaction with Leu 361, Pro 315 and Thr 359. Bond length >3.9 is not considered as effective, so such bonds have been considered as putative.

Conserved domains analysis using CDD of NCBI showed FMN binding pocket, FNR like domain and Rieske super family as conserved domains (Fig. 4.32) FMN binding pocket.
was between 320 and 490 amino acids, which showed conservation in multiple sequence alignment (Fig 4.25). FMN binding moieties are mainly acidic. Rieske super family domain is present towards C-terminal of the protein from amino acid 10-120. Multiple sequence alignment showed conserved residues such as His 107, His 89 and Cys25 etc.

Rieske super family domain accepts protein from ferridoxin and donates it to iron and it mainly contains cisteine and histidine residues. FAD binding domain is at 317aa-322aa, which binds to FAD (Fig.4.25). Docking was performed using Hex software (http://hex.loria.fr/). Docking analysis predicts that methylxanthine N-demethylase electron transfer protein from Pseudomonas putida showed high binding affinity for caffeine with the E total of -155.9, followed by theophylline (-148.41), theobromine (-146.76) and xanthine (-140.5).

### 4.9.3. In silico docking of xanthine oxidase and xanthine dehydrogenase with caffeine and its degradation products

Xanthine oxidase is involved in degradation of xanthine to uric acid (Woolfolk, 1975). We propose that after demethylation of caffeine to xanthine via dimethylxanthine, xanthine is converted to uric acid by xanthine oxidase in Bacillus licheniformis. In silico study was performed using tertiary structure of xanthine oxidase and binding mode of substrates and reaction products was determined.

**Xanthine oxidase from Pseudomonas mendocina.** Xanthine oxidase from Pseudomonas mendocina showed high affinity to xanthine with the formation of two hydrogen bonds with Gln 470 (H...O 3.57Å) and Thr 531 (H...O 2.36Å). There was complete homology throughout the sequences selected for the multiple sequence alignment with the score of 9 (Fig 4.28). Pro 530, Leu 539, Gly 489, 488, 536, Ala 471, Thr 528 and 531, Asn 540 formed hydrophobic interactions with xanthine (Fig 4.30 j-l). As expected xanthine oxidase did not formed bond with caffeine (Fig, 4.30 a-c). Xanthine oxidase also showed interaction with calcium ion. All the conserved residues including Glu 193, Phe 195, Gln 192 and 676, Val 673, Pro 703 were involved in hydrophobic interactions. As expected,
theobromine and theophylline did not interact with xanthine oxidase (Fig. 4.30 d-i). Conserved domain analysis of xanthine oxidase showed xanthine interact in the molybedoprotein binding domain of aldehyde dehydrogenase (Fig 4.32). These enzymes requires FAD molybdenum as a cofactor. Binding energies and number of bond formed by each enzyme with the substrates are listed in table 4.3.

Amino acid sequences involved in the bonding were conserved, which suggest simultaneous evolution of xanthine oxidases and dehydrogenases. Xanthine dehydrogenase has strong affinity for xanthine, as it showed high affinity to xanthine with the formation of two hydrogen bonds with Gln 470 (H....O 3.57Å) and Thr 531 (H...O 2.36Å). Gly538, Ala 471, Leu 539, and Thr 528 were involved in hydrophobic interactions. In docking studies, xanthine was not seen on surface of xanthine dehydrogenases, and binding pocket was embedded in the enzyme. Xanthine dehydrogenase, like xanthine oxidase did not form any bonds with theophylline and theobromine.

All the amino acids which are involved in binding with ligands, are also conserved throughout the sequences of xanthine dehydrogenases studied using MSA. All the binding sites were present in molybdopterin binding domain and all amino acid residues were reserved. Xanthine dehyderogenase did not formed bond with caffeine. Gln 192, 674, Tyr 363, Val 673, Pro 703, Phe 195, Ile 702 were involved in hydrophobic interactions. Xanthine dehydrogenases have property of interacting non-covalently with molybdenum ions through molybdopterin binding domain.

Summary of binding energies and number of bonds formed between receptor and ligands is listed in Table 4.3. Outcome of modeling supports strong affinity of demethylase for caffeine and xanthine oxidase and xanthine dehydrogenase for xanthine.
Figure 4.29: Binding of xanthine and its derivatives to methylxanthine N demethylase. Docking of caffeine (a, b, c), theobromine (d, e and f), theophylline (g, h and i) and xanthine (j, k and l) with methylxanthine N demethylase. Panel a, d, g, and j represent surface view, whereas panel b, e, h, and k represent enlarged view showing binding of caffeine, theobromine, theophylline, and xanthine respectively in the cavity of methylxanthine N demethylase. Panel c, f, i, and l depicts specific amino acid residue involved in interaction with caffeine, theobromine, theophylline, and xanthine respectively. Docking was performed using Hex docking tool.
Figure 4.30: Binding of xanthine oxidase with xanthine and its derivatives. Docking of xanthine oxidase was performed with caffeine (a, b, c), theophylline (d, e, f), theobromine (g, h, i) and xanthine (j, k, l). Panel a, d, g, and j represent surface view, whereas panel b, e, h, and k represent enlarged view showing binding of caffeine, theophylline, theobromine, and xanthine respectively in the cavity of xanthine oxidase. Panel c, f, i, and l depicts specific amino acid residue involved in interaction with caffeine, theophylline, theobromine, and xanthine respectively. Docking was performed using Hex docking tool.
Figure 4.31: Binding of xanthine dehydrogenase with xanthine and its derivatives. Docking was performed with caffeine (a-c), theophylline (d-f), theobromine (g-i) and xanthine (j-l) using Hex online tool. Panel a, d, g and j represents surface view, whereas panel b, e and h represent enlarged view showing binding of caffeine, theophylline, theobromine, and xanthine respectively in the cavity of xanthine dehydrogenase. Panel c, f, i, and k depicts specific amino acid residue involved in interaction with caffeine, theophylline, theobromine, and xanthine respectively. Docking was performed using Hex docking tool.
Figure 4.32: Conserved domains of xanthine oxidase and xanthine dehydrogenase. Conserved domains determined using CDD tool of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/).

Table 4.3: E-total and number of bonds formed by dimethylxanthine demethylase, xanthine oxidase and xanthine dehydrogenase

<table>
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<th>Enzyme</th>
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<th>Substrate</th>
<th>E-Total</th>
<th>Number of hydrogen bonds with the receptor</th>
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<td></td>
<td></td>
<td>Theobromine</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Theophylline</td>
<td>-148.41</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthine</td>
<td>-140.5</td>
<td>1</td>
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<tr>
<td>Xanthine oxidase</td>
<td><em>Pseudomonas mendocina</em></td>
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