The present study was undertaken to explore the bacterial isolates of Manikaran hotspring for their caffeine degrading ability. A total 4 bacterial isolates have been isolated from water, soil sediment of Manikaran hot spring and soil of tea garden Palampur. All the bacterial isolates were whitish to creamish in color, Gram’s negative and rod shaped except \(B.\) licheniformis (MS1-3), which was Gram positive. The bacterial isolates showed growth between 25°C- 40°C in M9 caffeine agar medium except \(B.\) licheniformis (MS1-3), which could grow even at 50°C. 16S rDNA analysis revealed that RT4, RT6, RTPM4 showed 99% similarity with \(Pseudomonas\) putida and MS1-3 was identified as \(B.\) licheniformis. The 16S rDNA sequences of all the bacteria have been submitted to the NCBI GenBank databases under the accession nos KJ907483.1, KM012010.1, KM012011.1, KF885931. \(Bacillus\) licheniformis (MS1-3) showed detectable growth even at 50°C. The optimum pH for growth of \(P.\) putida (RT4, RT6 and RTPM4) was observed at pH 7.5 but all the isolates showed trace growth even at pH 5 and 8. Different xanthine derivatives and xanthine was used as substrate for the growth of four bacterial isolates, \(Pseudoaononas\) putida (RT4), \(Pseudoaononas\) putida (RT6), \(Pseudoaononas\) putida (RTPM4) did not show growth on theobromine, theophylline and xanthine. \(Bacillus\) licheniformis (MS1-3) showed growth on xanthine, theobromine, and theophylline, but no caffeine degradation enzyme activity was observed. Further caffeine degradation enzyme activity was specifically observed in the intracellular fraction and no activity was observed in the cell free spent medium. We observed a caffeine degrading enzyme of ~45 kDa from \(Bacillus\) licheniformis (MS1-3) and detected theobromine, xanthine and theophylline in the spent medium by HPLC. We propose that \(Bacillus\) licheniformis possess demethylation as a mechanism of caffeine degradation. \(K_m\) of caffeine degrading enzyme from \(Bacillus\) licheniformis (MS1-3)was 6 mM, whereas it was 0.25 mM, 0.22 mM and 0.23 mM for RTPM4 (\(P.\) putida), RT4 (\(P.\) putida) and RT6 (\(P.\) putida) respectively.

Computational studies performed for studying interactions between substrates (caffeine, xanthine, theophylline, theobromine) with demethylase, xanthine oxidase and xanthine dehydrogenase was studied. We observed demethylase has higher affinity for caffeine than theobromine, xanthine and theophylline. Xanthine oxidase and dehydrogenase of \(P.\) mendocina showed higher affinity for xanthine and did not show interaction with theobromine, xanthine and theophylline. In methylxanthine demethylase,
bonding occurred in FMN binding domain, which showed many residues as conserved in multiple sequence alignment. Xanthine oxidase and xanthine dehydrogenase showed bonding with xanthine in molybedopterin binding domain, which was also conserved. We have isolated three different *P. putida* (RT4, RT6 and RTPM4) and rare *Bacillus licheniformis* (MS1-3), which are capable of caffeine degradation. *Bacillus licheniformis* (MS1-3) was a thermophile with inducible caffeine degrading protein of ~45 kDa, which carries caffeine degradation via demethylation and formation of xanthine and its derivatives was also supported by HPLC. Further we also performed *In Silico* studies to check xanthine and its derivatives interaction with reported caffeine degrading enzyme.