IJBPAS, August, 2016, 5(8): 1869-1888

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ISOLATION AND CHARACTERIZATION OF CAFFEINE DEGRADING *PSEUDOMONAS PUTIDA* SPECIES AND *BACILLUS LICHENIFORMIS* FROM THERMAL HOT SPRINGS AND TEA GARDEN OF HIMACHAL PRADESH, NORTH WEST HIMALAYAS

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ABSTRACT

Bacterial isolates RT4, RTPM4, RT6 and MS1-3 capable of growth on caffeine (3-5 g/L) as a sole source of carbon and nitrogen were isolated from hot water spring situated in 32.0268° N, 77.3511° E (hot springs) and 32.1109° N, 76.5363° E (tea garden) of North West Himalayas. Complete caffeine degradation was observed at 102 h of incubation. Unlike other bacterial isolates, MS1-3 also utilized xanthine (2.5 g/L) as sole source of carbon and nitrogen. Caffeine degradation was measured in cell free extracts using NBT assay. The temperature and pH optima for growth and caffeine degradation were 30°C and 7 respectively for RTPM4 218 U/mg, RT6 (781.25 U/mg), and RT4 (179.68 U/mg). Bacterial isolate MS1-3 showed optimal growth at pH 6 and temperature 30-50°C. Moreover, bacterial isolate MS1-3 could degrade caffeine even at 55°C 382.8 (U/mg)**.** Urea as a nitrogen source enhanced the caffeine degrading activity of RTPM4. HPLC analysis of cell free medium of MS1-3 showed degradation of caffeine to theobromine, theophylline and xanthine at 42 hrs and to xanthine at 102 hrs. 16s rDNA analysis identified RT4, RT4 and RTPM4 as *Pseudomonas putida* and MS1-3 as *Bacillus licheniformis*. All the three *P. putida* showed phylogenetic divergence and appear in a separate phylogenetic clad. *Bacillus licheniformis* MS1-3 isolate was unique and evolved in parallel to *Bacillus species*.

Keywords: NBT, *Bacillus licheniformis, Pseudomonas putida*, caffeine, phylogenetic, caffeine degradation

INTRODUCTION

Caffeine (1, 3, 7-trimethylxantheine) is a plant alkaloid and is a member of a class of drugs known as methylxanthines [1]. Caffeine is the most widely consumed psychoactive drug in the world [2]. It is present in leaves, seeds, and fruits of many plant species of which coffee, tea, guarana, cocoa and yerba mate are the most well known [3]. It is a major human dietary ingredient and is found in common beverages and food products such as coffee, tea, chocolates and cold drinks [4]. Caffeine is used in many pharmaceutical preparations like apectol, anacin, dristan, vivarin, darvon, excedrin, percodan, soma, synalogos, butabital, norgesic and prolamine [5]. Caffeine acts as a central nervous system stimulant by antagonising adenosine in binding to the adenosine receptors [6, 7]. Low doses produce stimulation, including increase in alertness and concentration, which is often perceived as desirable, whereas excessive ingestion (> 250 mg) leads to state of intoxication called caffeinism [8]. Despite the stimulatory actions and medicinal value of caffeine, a growing belief that ingestion of caffeine can have adverse effects on health has increased the demand for decaffeinated beverages. Microorganisms capable of degrading caffeine have been isolated from natural environments. These include *Rhodococcus, Pseudomonas putida, Leifsonia, Aspergillus niger, and Penicillium roquefortii* [9]. Extensive study has been carried out in exploring metabolism mechanism of caffeine in *Pseudomonas* as it is easily cultivated on caffeine. Thus *Pseudomonas* has a wide role in decaffeination, bioremediation of contaminated waste waters and soil containing caffeine as a pollutant. Such decaffeinating enzymes can also be used in detection of caffeine in human body in diagnostic procedures and in beverages and food items. Recently diagnostic test has been developed, which utilizes caffeine dehydrogenase in a diagnostic assay [10]. The aim of this study was to identify caffeine degrading microbes from Manikaran hot spring located in suburbs of Himachal Pradesh. Till date, there is no report on the isolation and characterization of caffeine degrading microorganisms form thermal hot spring and tea garden soil of this region.

MATERIALS AND METHODS

Isolation of caffeine degrading bacteria

For isolation of caffeine degrading bacteria, 5 g of soil sample and 5 mL water sample were collected from hot water spring of Manikaran and Vashisht respectively,

situated in Kullu district of Himachal Pradesh, India. Soil sample was collected from tea garden of Palampur, Kangra district of Himachal Pradesh, India. Soil and water samples were enriched in M9 liquid media (pH 7) containing 0.25% (w/v) caffeine as sole source of carbon and nitrogen for 7 days at 30°C. Samples were centrifuged at 2,000 rpm for 2 min and the resultant supernatant was re-centrifuged at 5000 rpm for 10 min to pellet the enriched bacterial cells. Serial dilutions of supernatant up to 10^{-4} were prepared and 500 µL sample of each dilution was spread on nutrient agar plates and M9 medium containing 0.25% caffeine (w/v). The petriplates were incubated at 37°C, 40°C, 50°C, 60°C, 70°C for 36-48 hours or until isolated colonies were formed. Individual colonies were successively streaked three times on M9 agar medium containing 0.25 % (w/v) caffeine to obtain pure bacterial cultures. The purity of bacterial isolates was further verified under the light microscope and Gram's staining [11]. The pure bacterial cultures capable of caffeine degradation (MS1-3, RT4, RTPM4 and RT6) were maintained on M9 medium containing 0.25% (w/v) caffeine (pH 7.0) at 4°C and as glycerol (25 % v/v) stocks at -80°C.

Growth of caffeine degrading bacterial isolates in caffeine and xanthine derivatives. To find out the optimum concentration of caffeine for bacterial growth, MS1-3, RT4, RTPM4, and RT6 were grown in different concentration of caffeine ranging from 0.1 to 1 % (w/v) for 48 h at 30°C with shaking at 200 rpm. Bacterial growth was measured at 600 nm and caffeine degradation at 273 nm for different time period. Bacterial isolate MS1-3, RT4, RT6 and RTPM4 were also tested for their ability to degrade different xanthine derivatives such as theobromine, theophylline and xanthine itself at 0.25 % (w/v).

Effect of physical parameters on growth and caffeine degradation

Effect of Temperature

For finding relation between growth and utilization of caffeine as a sole source of carbon and nitrogen at different temperatures, MS1-3, RT4, RT6 and RTPM4 were grown in M9 liquid medium containing 0.25 % caffeine (w/v), and were incubated at temperature ranging from 25 to 50°C for 42 h of incubation at 200 rpm. Growth was measured at 600 nm and caffeine degrading enzyme activity was determined using NBT assay [9]. Crude enzyme was prepared by disrupting cells of MS1-3, RTPM4, RT6 and RT4, each suspended in lysing buffer 50 mM

sodium phosphate buffer (pH 7.5), 10 mM β mercaptoethanol, 0.01% lysozyme for 2 h at 30°C. Supernatant obtained after centrifugation at 10,000 rpm was used as intracellular source of crude enzyme. NBT assay was performed using 10 µg of each crude enzyme containing 50 mM sodium phosphate buffer, 0.5 mM caffeine, 0.5 mM NBT. NBT assay reaction mixture of each isolate were incubated at different temperatures ranging from 30-75°C. Formation of formazan was measured at 566 nm.

Effect of pH Effect of pH on growth and caffeine degrading activity was determined by growing MS1-3, RT4, RT6 and RTPM4 at different pH ranging from 3 to 9. NBT assay was performed at different pH ranging from 3 to 9 and reaction mixture was incubated at 30°C.

Effect of chemical parameters on growth and caffeine degradation

Carbon sources

To study the relation between growth and caffeine utilization in presence of alternate carbon source, NBT assay was performed using crude enzyme extracts of MS1-3, RTPM4, RT6 and MS1-3 grown in M9 medium containing 0.25% caffeine (as a nitrogen source) supplemented with different sugars (2 % each of glucose, galactose, lactose, sucrose, starch, maltose or trehalose) as a carbon source. Growth was measured at 600 nm.

Nitrogen sources

To study relation between growth and caffeine utilization in presence of alternate nitrogen source, NBT assay was performed using crude enzyme extracts of MS1-3, RTPM4, RT6 and RT4 grown in M9 medium containing 0.25% (w/v) caffeine (as a carbon source) supplemented with different nitrogen source (2 % each of CAH, ammonium chloride, urea, peptone or yeast extract). Growth was measured at 600 nm.

Caffeine degradation analysis of MS1-3 using HPLC

MS1-3 was precultured in 10 mL of M9 liquid medium containing 0.25 % (w/v) caffeine (pH 7.0) and incubated at 30 °C in an orbital shaker at 200 rpm. Caffeine degradation by MS1-3 was measured by HPLC fitted with C^{18} column. The 500 µL of clear culture supernatant was analyzed at 0, 24 and 102 h of incubation by measuring absorbance at 273 nm. Standard solutions and samples were filtered through a 0.25 mm nylon membrane filter before being injected into the HPLC unit. Samples were separated on a Hypersil BDS C-18 column (4.6 by 50 mm). Methanol-water-acetic acid (25:75:0.5, vol/vol/vol) was used as the mobile phase

with a flow rate of 0.5 mL/min and UV-visible absorption spectra was recorded.

Identification and phylogenetic analysis of caffeine degrading bacteria

Genomic DNA was isolated from MS1-3, RTPM4, RT6 and RT4 [12].16s rDNA gene amplification was carried out using 16S rDNA specific primers 27F, 1492R in 25 µL reaction mixture [13]. Amplified PCR products were sequenced on both strand and nucleotide sequences were submitted to Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed with 16sr DNA gene of each MS1-3, RTPM4, RT6 and RT4 and Phylogenetic tree was constructed using MEGA 6 (http://en.bio-soft.net/tree/MEGA.html) and blast hits showing more than 99% homology.

RESULTS

Caffeine degrading bacteria isolated form thermal hot spring and soil of tea garden:

Caffeine degrading bacterial isolates RTPM4, RT6 and RT4 were Gram- negative, whereas MS1-3 was Gram-positive. All the four bacterial isolates were rod shaped when observed under the microscope and creamish in color. Optimum concentration of caffeine for the growth of MS1-3 and RTPM4 was 0.3% (w/v), whereas RT6 and RT4 had optimum concentration of caffeine at 0.4 % and 0.5 % (w/v) respectively. RT6 and RT4 could tolerate 0.7 % (w/v) of caffeine, whereas MS1-3 and RTPM4 could tolerate only 0.6 % and 0.5 % caffeine respectively (Fig. 1A). During the exponential phase of growth, there was a concomitant utilization of caffeine by RTPM4, MS1-3, RT6 and RT4 isolate and 90-95% caffeine was utilized by 102 h of incubation (Fig. 1C). Interestingly, MS1-3 also showed growth on theophylline, theobromine and xanthine, but comparatively reduced growth as compared to caffeine (Fig. 1C).

Bacterial isolated MS1-3 is thermophile and degrade caffeine optimally at 55°C:

Bacterial isolate RT6, RT4 and RTPM4 showed optimum growth at 30^{o}C, but growth of MS1-3 was not affected even up to 50 ^{o}C (Fig 2A). Optimum caffeine degradation activity was observed at 55°C for MS1-3 (1875 U/mg), 50^{o}C for (859.37 U/mg), and 45^{o}C for RT6 and RTPM4 (835.9 and 484.3 U/mg respectively) as shown in Fig. 2B. Caffeine degradation was indirectly measured by the reduction of NBT to form blue formazan (NBT^{2+}). Caffeine degrading activity of MS1-3 isolate was reduced to 50% at 60^{o}C. All the bacterial isolates showed optimum growth and caffeine degradation at pH 7, except bacterial isolate MS1-3, which

showed optimum growth and caffeine degradation at pH 6 (Fig. 2C-D). RTPM4, RT6 and RT4 could not tolerate acidic pH ≤ 5 and alkaline pH ≥ 8, whereas bacteria isolate MS1-3 could not tolerate acidic pH ≤ 3 and alkaline pH ≥ 8. Further, growth of microbial cultures and caffeine degradation assays were performed at optimum temperature and pH. This data suggest the role of oxidase enzyme in caffeine degradation.

Caffeine degradation enzyme activity is inducible by carbon and nitrogen sources: 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. 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. 2A. 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 caffeine, lactose and sucrose, and bacterial isolate RT4 grown in presence of sucrose showed comparable caffeine degradation 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. 2B). Similarly, cultures grown in presence of lactose (RT4 and RTPM4) and trehalose (MS1-3) did not show caffeine degradation enzyme activity. 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.

Comparatively, supplementation of yeast extract, peptone, and urea inhibited the growth of all the bacterial isolates, whereas ammonium chloride specifically inhibited the growth of MS1-3 and RT4 (Fig. 2C). Caffeine degradation enzyme activity was induced in presence of casein acid hydrolysate (CAH) for RT4, ammonium chloride for RTPM4, and urea and caffeine for RT6, RT4, and RTPM4 isolates (Fig. 2D).

Bacterial isolate MS1-3 metabolizes caffeine and produce xanthine: Caffeine degradation assay measured using NBT suggested a role of oxidase in caffeine

degradation. Since bacterial isoalte MS1-3 retained caffeine degradation activity at 55^{o}C, we analyzed the caffeine degradation through HPLC analysis. At 24 h of incubation, 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. 3C). A caffeine peak at 6.015 min was also detected at 0 h of incubation of MS1-3 isolate in M9 medium supplemented with caffeine (Fig. 3B). Very surprisingly, at 102 h of incubation of MS1-3 in caffeine containing medium, caffeine, theophylline, and theobromine were undetectable and xanthine peak was observed in contrast to 0 h (Fig. 3B). 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. 6).

Caffeine degrading *Pseudomonas spp*. and *Bacillus licheniformis* were identified from thermal hotspring and soil of tea garden:

To identify the bacterial isolates, 16S rDNA gene was PCR amplified using total genomic DNA (Figure 5A) and PCR product of approximately 1.5 kb was detected in all the four bacterial isolates (Fig 5B). PCR-amplified 16s rDNAs were sequenced on both the strands and 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). The blast hits showing more than 99% homology with *Pseudomonas putida* and 99% homology with B. *Bacillus licheniformis* were subjected to phylogenetic analysis All the three *Psudomonas spp*. were distantly related, whereas *Bacillus licheniformis* showed a unique divergence from known *Bacillus licheniformis* strains (Fig. 5C-D).

DISCUSSION

Four caffeine degrading bacterial strains (MS1-3, RT4, RT6, and RTPM4) have been isolated from water and soil sample of Manikaran and Vashisht hot springs and soil of tea garden. All the four bacterial isolate could degrade caffeine (3-5 g/L) as a sole source of carbon and nitrogen. MS1-3 isolate could also grow specifically on theophilline, theobromine, and xanthine as a sole carbon and nitrogen source, albeit reduced growth in comparison to caffeine. More than 90% caffeine degradation was observed at 90 h of incubation. *Pseudomonas* sp NCIM 5235, isolated from soil of coffee plantation area of

Ooty, was capable of utilizing 1.2 g/L caffeine as the sole source of carbon and nitrogen [14]. *Pseudomonas alcaligenes* CFR 1708, isolated from soil sample of coffee Board, Mysore, India showed maximum degradation of caffeine (1 g/L), which occurred in less than 4 h. *Pseudomonas alcaligenes* CFR 1708 caffeine-degrading capability was exhibited up to a concentration of 5 g/L and higher concentration of caffeine proved to be inhibitory for its growth [15]. All the 16S rDNA sequences of all the bacteria have been submitted to the NCBI GenBank databases under the accession nos KJ907483.1(1447 bp), KM012010.1 (1328 bp), KM012011.1 (1326 bp), KF885931.1 (1319 bp), which identified bacterial isolates RT4, RT6 and RTPM4 as *Pseudomonas putida*, and MS1-3 was identified as *Bacillus licheniformis*. As *Pseudomonas* is known to degrade toxic compounds, we could get *Pseudomonas* as a caffeine degrading bacteria from three different sites. We were successful in isolating rare caffeine degrading *Bacillus licheniformis*, as there are few reports of caffeine degradation by this genus.

The temperature optima for growth of RT4, RT6, and RTPM4 were 30^{o}C, but the growth of *Bacillus licheniformis* MS1-3 was not inhibited even at 50^{o}C. Crude proteins from *Bacillus licheniformis* MS1-3 isolate showed optimum caffeine degrading activity at 55^{o}C, which may be due to the reason that *Bacillus licheniformis* was isolated from hot springs, where temperature ranged from 90-100^{o}C. Similarly, *P. putida* isolated from the same location also showed caffeine degradation activity at temperature 45-50^{o}C, but four times less caffeine degradation activity as compared to *Bacillus licheniformis* MS1-3 isolate. The optimum growth and caffeine degradation was observed at pH 7 for all the three *P. putida*, whereas pH 6 was optimum for optimum growth and caffeine degradation activity of *Bacillus licheniformis*. Optimum temperature for the growth of *Pseudomonas putida* CBB5 was 29^{o}C and was grown in media supplemented with soytone [16]. *Pseudomonas* sp isolated from roots of *Coffea Arabica* showed caffeine degradation at alkaline pH of 8-8.5 and attained optimum growth after 30 h of incubation [17].

Though, most of the carbon and nitrogen source did not stimulate growth or caffeine degradation, supplementation of urea as nitrogen source (caffeine as carbon source) in the growth medium marginally induced the caffeine degradation activity. Supplementation of growth medium with galactose completely inhibited the growth of

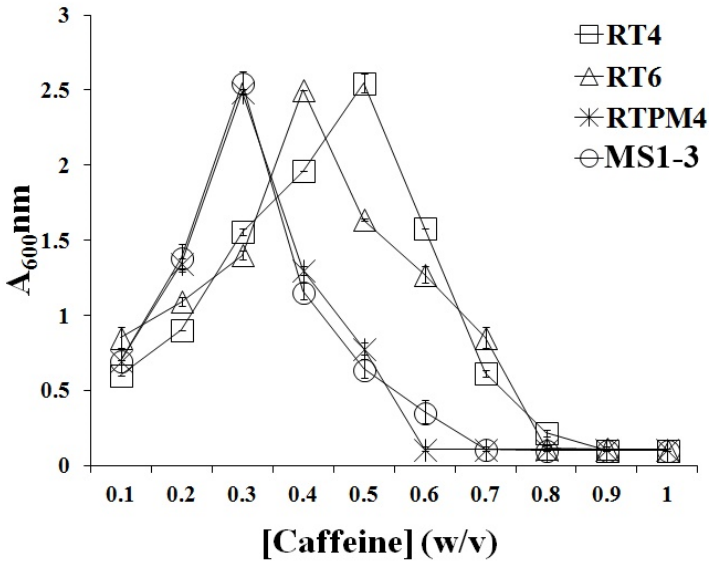
all the four isolates, whereas growth medium containing sorbitol drastically inhibited the growth of RT4 and RTPM4. Caesin acid hydrolysate (CAH) and ammonium chloride stimulated the growth of RTPM4 and MS1-3 in presence of caffeine, whereas growth of RT6 was enhanced in medium containing CAH and caffeine. Supplementation of urea, peptone and yeast extract inhibited the growth of all the four isolates. Fructose was the most suitable carbon source for the growth and tryptone was most suitable nitrogen source for growth of *P putida* No.352 [18]. Caffeine degradation by *Pseudomonas* sp. GSC 1182 was increased when medium containing caffeine was supplemented with carbon source such as galactose fructose and lactose, and was decreased when grown in medium supplemented with urea and ammonium sulphate [19]. Carbohydrate sources like glucose and sucrose in the minimal media showed minimal effect on growth pattern of bacteria at low concentration of caffeine, except that the lag phase was much more prolonged in the sucrose containing media (65 h) as compared to glucose (24-48 h) at the same concentration of caffeine [20]. Ammonium sulphate also enhanced growth of *Aspregillus tamarii* on caffeine [21]. There was significant decrease in caffeine content of leaves of *Camellia sinensis*, when *Bacillus licheniformis* was sprayed on leaves and showed significant decrease in caffeine content by 24 h [22].

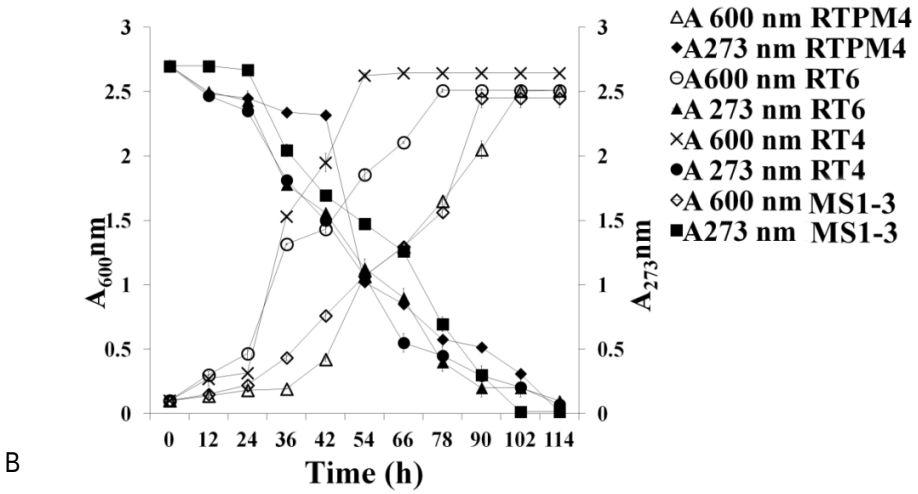
HPLC analysis of caffeine degradation products of *Bacillus licheniformis* MS1-3 at 24 h of incubation showed the presence of caffeine and its demethylated product as theobromine, theophylline and xanthine. Analysis of degradation products at 102 h of incubation showed the complete disappearance of caffeine, theophylline, and theobromine, but increase in appearance of xanthine. This data suggest the demethylation as first step of caffeine degradation in *Bacillus licheniformis* MS1-3. Degradation products of *Pseudomonas putida* isolated from waste water sample of Irvine Ranch water district (Irvine CA) included xanthine derivatives [23]. HPLC analysis of spent media removed at different times from CBB5 growing in soytone-supplemented M9-caffeine medium showed retention times identical to those of theobromine, 7-methylxanthine paraxanthine, xanthine, and uric acid [16].

Pseudomonas 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 [24]. *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 [25]. The phylogenic analysis clearly suggests that the *Bacillus licheniformis* MS1-3 is a novel bacterial isolate capable of caffeine degradation. Based on NBT assay and detection of xanthine as one the intermediate, demethylation and oxdative pathway of caffeine degradation has been proposed in *Bacillus licheniformis* MS1-3 isolate (Fig. 6).

A





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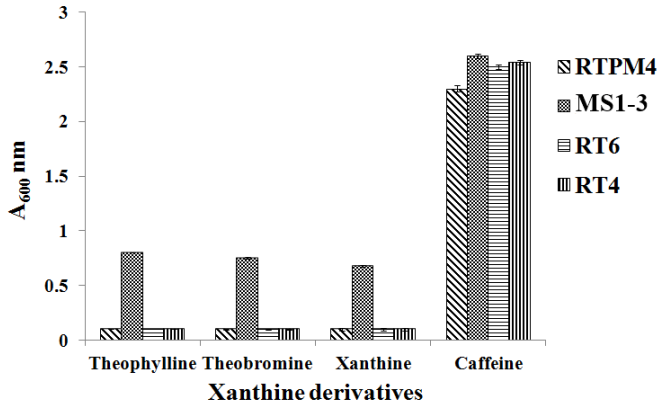

C

Figure 1. Growth of caffeine degrading bacterial isolates: A. Determination of optimum concentration of caffeine for the growth of MS1-3, RTPM4, RT6 and RT4. Bacterial isolates were grown in M9 liquid medium containing 0.1 - 1 % caffeine (w/v) for 42 h at 30°C and absorbance was measured at 600 nm. B. Bacterial isolates MS1-3, RTPM4, RT6 and RT4 were grown in presence of 0.6 %, 0.3 %, 0.3 % and 0.5 % (w/v) caffeine respectively. Growth was measured at 600 nm and caffeine degradation was measured at 273 nm, at 12 h interval, till 114 h of incubation at 30°C. C. Bacterial isolates MS1-3, RTPM4, RT4 and RT6 were grown in M9 liquid containing 0.25 % (w/v) xanthine and xanthine derivatives as indicated for 42 h at 30°C.

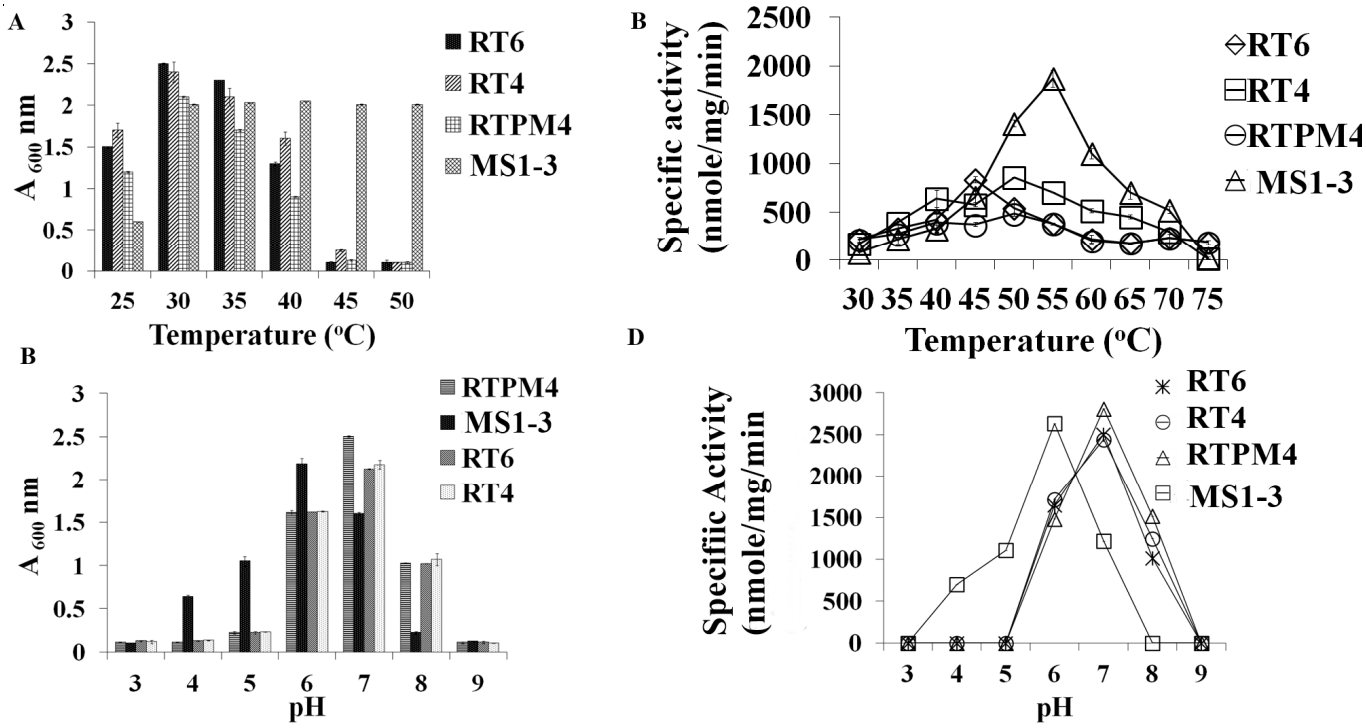

Figure 2. Effect of temperature and pH on growth and caffeine degrading enzyme activity: A. Bacterial isolates MS1-3, RTPM4, RT6 and RT4 were grown in M9 liquid medium containing 0.25 % (w/v) caffeine and were incubated at 25, 30, 35, 40, 45 and 50°C for 42 h and growth was determined at OD 600 nm. B. 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. Bacterial isolates MS1-3, RTPM4, RT6 and RT4 were grown in M9 liquid medium containing 0.25 % (w/v) caffeine at pH 3, 4, 5, 6, 7, 8, and 9 for 42 h at 30°C. Growth was determined at 600 nm (C) and caffeine degrading enzyme activity was measured at different pH (3-9) using NBT assay (D).

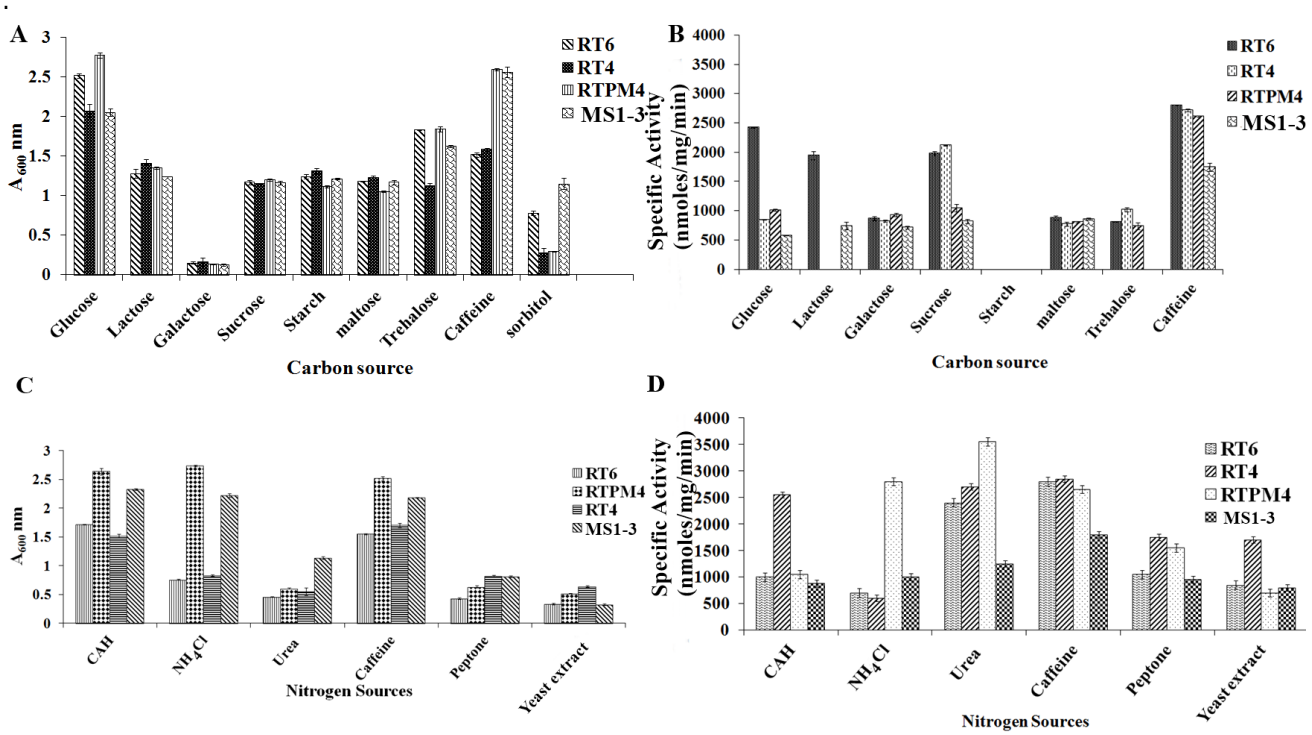

Figure 3. Effect of different carbon and nitrogen sources on the growth and caffeine degrading enzyme activity A. Bacterial isolate MS1-3, RTPM4, RT6 and RT4 were grown in M9 liquid medium containing 0.25 % (w/v) caffeine (nitrogen source) and different carbon sources such as glucose galactose, lactose, sucrose, starch, maltose, trehalose and growth was determined at OD 600 nm. B. Caffeine degrading enzyme activity was defined as nmoles/mg/min was detected with NBT assay. 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 (C). Caffeine degrading enzyme activity was defined as nmoles/mg/min was detected with NBT assay.

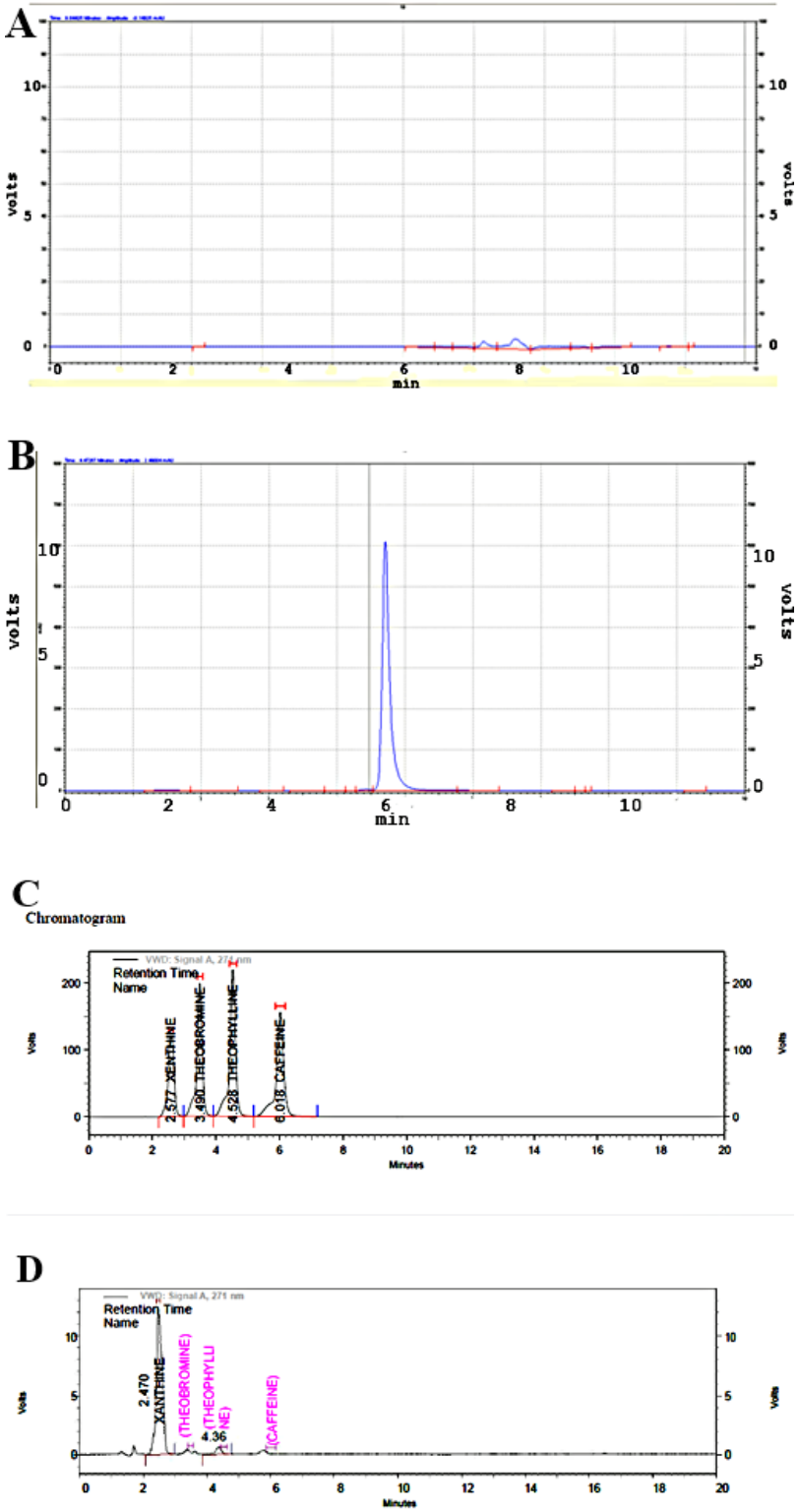

Figure 4. Caffeine degradation analysis of MS1-3 using RP-HPLC: HPLC chromatograms of M9 medium without caffeine supplementation (A), Medium containing 0.25% (w/v) caffeine (B), spent free medium of MS1-3grown in M9 medium containing caffeine at 24 h of incubation (C), and spent free medium of MS1-3grown in M9 medium containing caffeine at 102 h of incubation (D).

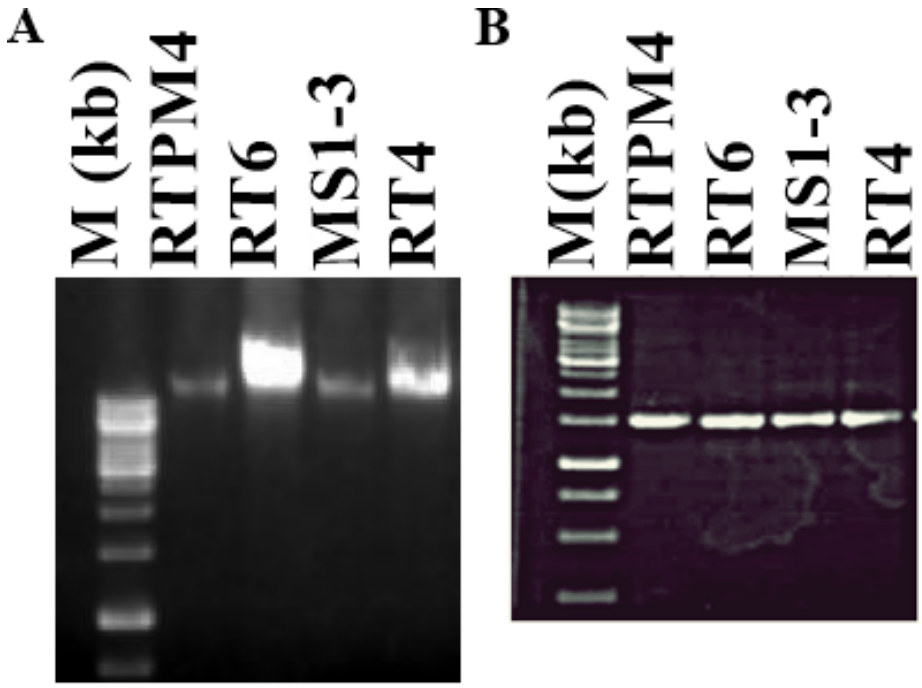

Figure 5. Identification of caffeine degrading bacterial isolates by 16s rDNA amplification and phylogenetic analysis Total genomic DNA was isolated from MS1-3, RTPM4, RT6 and RT4 (A) and subjected to 16s rDNA amplification

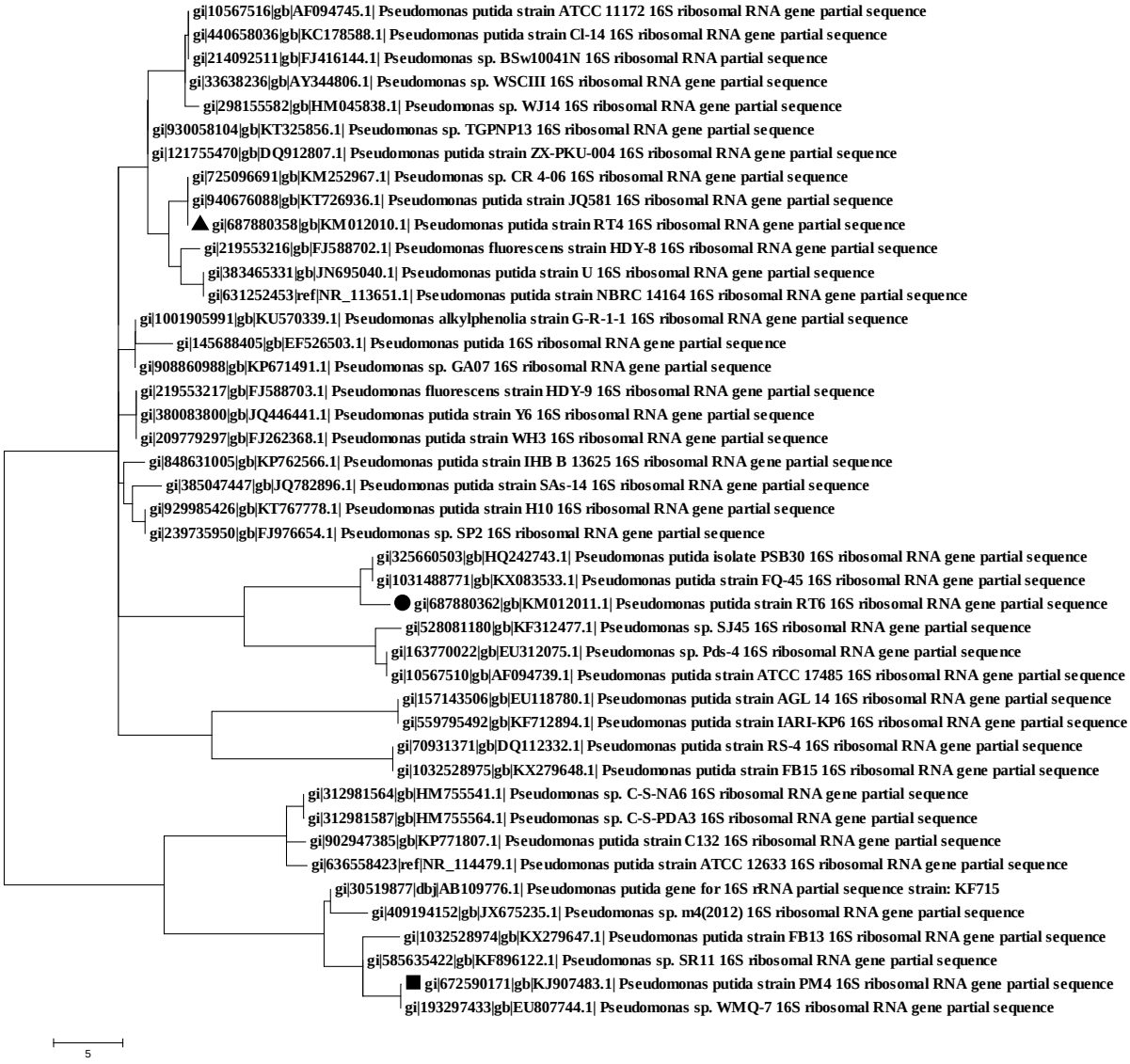

Figure 5 B: Phylogenetic tree was constructed using 16s rDNA of *Pseudomonas putida* (RT4, RT6, RTPM4) and BLAST hits showing more than 99% homology

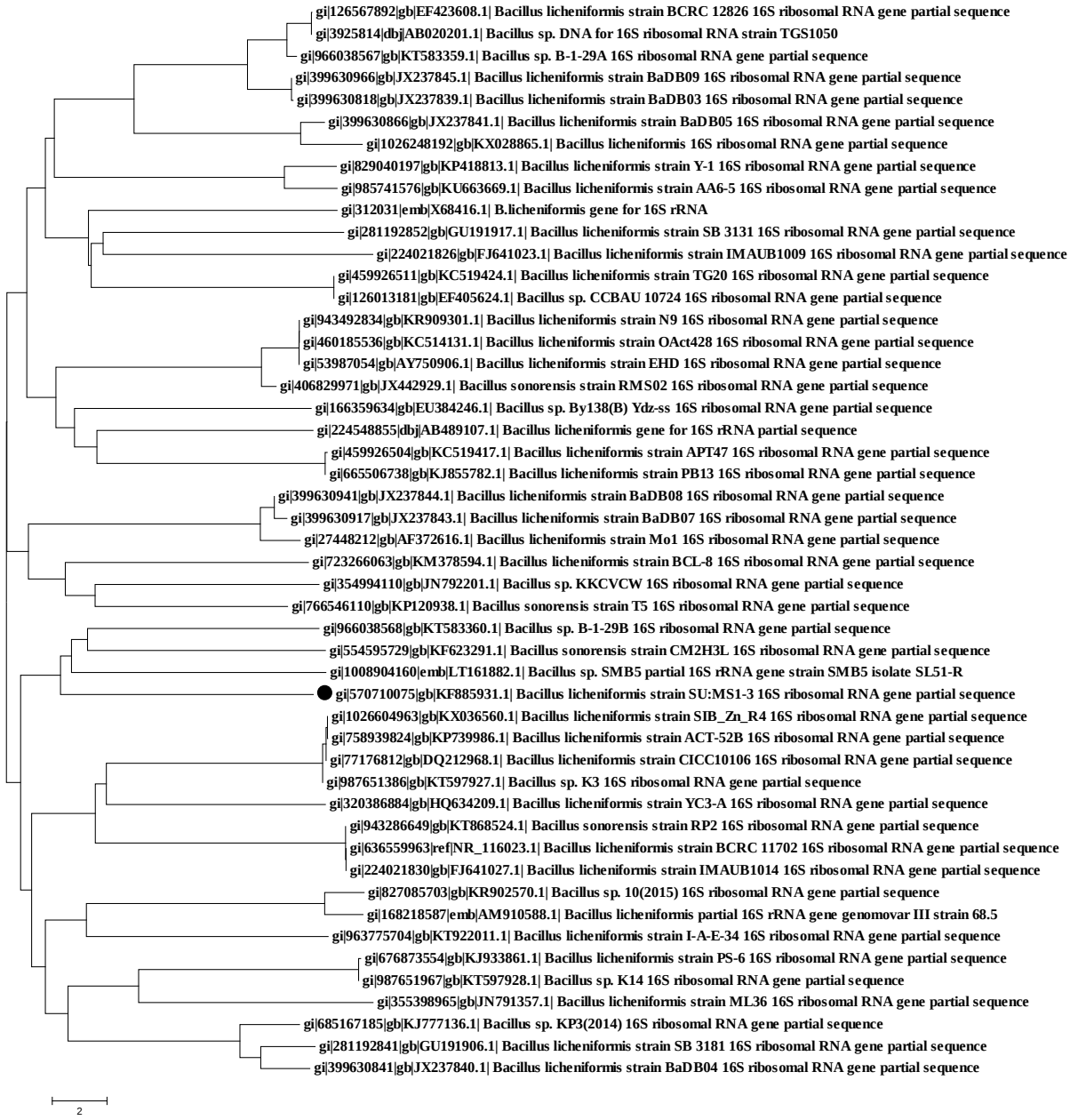

Figure 5 C: BLAST search showing more than 99% homology with 16s rDNA of *Bacillus licheniformis* MS1-3 were subjected to phylogenetci analysis using MEGA 7.

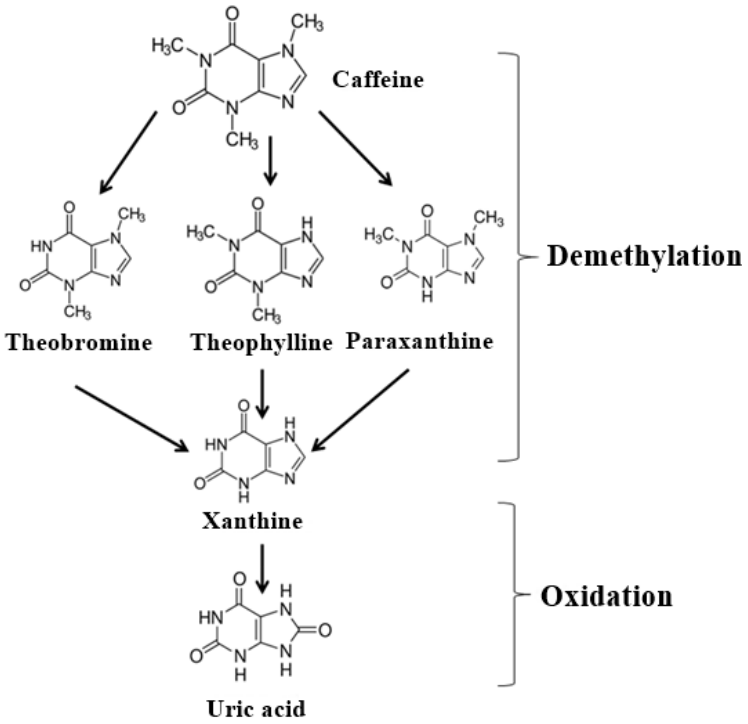

Figure 6: Proposed pathway of caffeine demethylation followed by xanthine oxidation in *Bacillus licheniformis* MS1-3. Caffeine undergoes demethylation and result in the production of theobromine, theophylline, or paraxanthine. Further demethylation leads to the generation of xanthine, which gets oxidized to produce uric acid.

CONCLUSION

In summary, we isolated highly diverged *P. putida* capable of caffeine degradation from different locations (hot water spring Manikaran and Vashist and tea garden soil, Palampur) situated in snowy mountains of North West Himalayas. *Bacillus licheniformis* MS1-3 is theromphilic bacteria capable of degrading caffeine via demethylation and xanthine oxidation at elevated temperature.

ACKNOWLEDGEMENT

Authors would like to acknowledge Shoolini University for infrastructural support and members of Yeast Biology laboratory for helpful discussions. RT acknowledges Dr Sonika Gupta for bringing soil sample of tea garden.

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