



Microbial Degradation of Caffeine Using Himalayan Psychrotolerant *Pseudomonas* sp.GBPI_Hb5 (MCC 3295)

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Abstract

Caffeine, a xenobiotic compound, is continuously released into the environment. Fifteen psychrotolerant bacterial strains, isolated from the Indian Himalayan region, were screened for their caffeine degradation capacity. The medium for the growth of bacteria was optimized using Box-Behnken method. Among these bacteria, *Pseudomonas* sp. (GBPI_Hb5), showing the best response, was further used for caffeine degradation in batch mode. The culture medium, having caffeine as a sole source of carbon, was used for analyzing the effect of pH, agitation speed, temperature, inoculum volume, and caffeine concentration on bacterial growth and its caffeine degradation potential. The bacterium GBPI_Hb5 showed approx. 93% caffeine degradation up to 96 h under controlled conditions. The compounds produced during the degradation of caffeine were also studied. The study is likely to have implications in the bioremediation of caffeine from polluted environments.

Introduction

Fresh and clean water is the need of the day due to its scarcity throughout the world. Water can be made available to mankind through the sustainable use of available water and by treating the wastewater generated after different anthropogenic activities. Reuse of wastewater has been highlighted as playing an important role in water management. Recycled wastewater has a variety of possible uses, such as irrigation of agricultural areas and gardens, washing, cleaning, cooling water for industrial plants, and recharging groundwater bodies, etc. Wastewater contains various anthropogenic contaminants; caffeine is one of them [1, 2]. Caffeine follows several ways to come to the environment, and among these untreated sewage is one of the most important ones. It is believed to be an anthropogenic marker for domestic wastewater and had been detected in various water resources throughout the world [3]. It is one of the Pharmaceuticals and personal care products (PPCPs) compounds, which is commonly consumed by the human being in day-to-day

life in the form of beverages, food, or as medicines. It is a white crystalline alkaloid of xanthine with formula $C_8H_{10}N_4O_2$, with molecular weight 194.2 g/mol, and is bitter, odorless, and amorphous in its pure state. It also acts as a drug activator. It is estimated that a cup of coffee contains 0.43–0.82 mg/mL caffeine [4]. Due to its stimulatory nature, caffeine is also used as a stimulant or adjuvant in psychoactive medicine [5]. Its huge usages for domestic purposes, in food, medicines, and various production industries, causes the release of this compound in the environment through wastewater as effluents, if released untreated. The unintentional presence of caffeine in various compartments of the aquatic environment (e.g., water, sediments, and biota), at different concentrations, possess the ability to cause detrimental effects to the aquatic organisms as well as to human health [6]. Erukainure et al. 2017 [7] reported that caffeine harms liver cells by altering the substrate for P-glycoprotein and an inhibitor of CYP1A2 with the maximum tolerable dose for human beings of 0.536 log mg/kg/day. European Food Safety Authority (EFSA) 2014 shows several reports on the toxic effects of caffeine due to its higher intake than recommended (400 mg per day for adults). In animals, gestational caffeine exposure up to 500 μ M in zebrafish can cause hair cell damage and developmental toxicities at early developmental stages [8]. Fernandes et al. [9] reported that the presence of caffeine in wastewater can cause mutagenesis leading to DNA damage and toxicity to the aquatic organisms. Therefore, there is a need for ecofriendly treatment

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strategies for the removal of such types of compounds [10]. Several methods are available for the treatment of wastewater, such as solvent extraction, supercritical carbon dioxide extraction, ozonizes, biodegradation, and UV treatment [11, 12]. Among these methods, from an environmental viewpoint, the biodegradation of caffeine is considered to be an ecofriendly and cost-efficient approach. Different studies have been reported for potential caffeine-degrading microbes from bacterial and fungal genera and found that the microorganisms can use caffeine as their sole carbon and nitrogen source [13–15]. Microbiota of cold environments are increasingly receiving attention because of their ecological and biotechnological characteristics [16]. The bacteria can survive at different environmental conditions such as changing temperatures, pH, nutrition, etc. Due to such varying tolerance limits within the ecosystem, these are successful colonizers in all parts of the earth [17]. Due to the release of different types of enzymes, the bacteria are useful for various ecological and biotechnological applications and their physiological adaptations [18]. This property makes bacteria successful for the biodegradation of organic pollutants (e.g., PPCPs), found in wastewater/contaminated water, which is a severe problem throughout the world. The present study aims to optimize the media for the growth of bacteria using statistical techniques and to screen the psychrotolerant bacteria, isolated from Indian Himalaya, for analyzing their growth and caffeine degradation conditions.

Materials and Methods

Chemicals and Microbes

Caffeine (CAS.NO. C- 58-08-2 99% pure) was purchased from Merck Limited, Worli, Mumbai, India. Methanol (HPLC grade), Tryptone yeast (TY) (casein enzyme hydrolyase 5.0 g/L and yeast extract 3.0 g/L and agar), ammonium sulfate ((NH₄)₂SO₄), potassium dihydrogen phosphate (KH₂PO₄), sodium chloride (NaCl), and zinc chloride (ZnCl₂) were procured from Hi-media Limited, Mumbai, India and sodium dihydrogen phosphate (NaH₂PO₄), magnesium sulfate heptahydrate (MgSO₄·7H₂O), and calcium chloride dihydrate (CaCl₂·2H₂O) were purchased from-SRL Chemicals Limited, Mumbai, India. Bacterial strains, namely GBPI_Hb0, GBPI_Hb5, GBPI_Hb14, GBPI_Hb61, GBPI_CDB143, GBPI_CDB149, GBPI_507, GBPI_508, B0, GBPI_506, GBPI_CDB84, GBPI_CDB87, GBPI_Hb1, GBPI_Hb149, and GBPI_CDB94 (Table 1) were taken from the Microbiology Laboratory of the G. B. Pant National Institute of Himalayan Environment, Almora, Uttarakhand, India. These bacteria were originally isolated from high altitudes in the Indian Himalayan region [17]. The bacterial strains were then cultured in Tryptone yeast (TY) agar for 24 h at 25 °C. One loopful culture was taken in Mineral salt media (MSM) for further experiments. The composition of MSM is mentioned in TS 1 which was standardized for the optimum growth of bacteria before performing the actual

Table 1 Screening of bacteria responsible for caffeine degradation with its identification and percent degradation capacity ± SE (duration 48 h)

S No	Isolate (culture accession)	Response in petri plate with caffeine	Caffeine degradation ± SE (%) (N = 3)	16S rRNA identification (% similarity) (nucleotide accession)
1	GBPI_Hb0 (MCC 3300)	–	0	<i>P. granadensis</i> (99.79) (HG764746)
2	GBPI_Hb5 (MCC 3295)	++	93 ± 0.0029	<i>P. simiae</i> (99.79) (KT887192.1)
3	GBPI_Hb14 (MCC 3296)	+	15 ± 0.0011	<i>P. azotoformans</i> (99.79) (AF074384)
4	GBPI_Hb61 (MCC 3297)	+	23 ± 0.0055	<i>P. proteolytica</i> (99.30) (AJ537603)
5	GBPI_CDB143 (MCC3298)	+	27 ± 0.0084	<i>P. paralactis</i> (99.93) (D84009)
6	GBPI_CDB149 (MCC3294)	+	10 ± 0.0034	<i>P. marginalis</i> (99.77) (ND)
7	GBPI_507 (MCC2693)	+	5 ± 0.0075	<i>P. chlororaphis</i> subsp. <i>Aurantiaica</i> (100) (DQ682655)
8	GBPI_508 (MCC2692)	+	35 ± 0.0026	<i>P. palleroniana</i> (99.72) (AY091527)
9	<i>Pseudomonas putida</i> (B0) MTCC 6842	+	12 ± 0.0028	<i>Pseudomonas putida</i>
10	GBPI_506	+	22 ± 0.0033	<i>P. azotoformans</i> (99.86) (D84009)
11	GBPI_CDB84 (MCC3341)	+	15 ± 0.0025	<i>P. paralactis</i> (99.57) (AF074384)
12	GBPI_CDB87	+	25 ± 0.0079	<i>P. azotoformans</i> (99.66) (ND)
13	GBPI_Hb1	–	0 ± 0.0018	<i>Serratiaquinivorans</i> (99.87) (AJ233430)
14	GBPI_Hb149 (MCC3293)	+	30 ± 0.0036	<i>S. marcescens</i> subsp. <i>Sakuensis</i> (99.93) (JMPQ01000005)
15	GBPI_CDB94	+	18 ± 0.0041	<i>S. marcescens</i> subsp. <i>sakuensis</i> (100) (JPUX01000001)

– no growth, + = < 2 mm (colony size), ++ = 2–5 mm (colony size)

shaking experiments. Potassium dihydrogen phosphate and sodium dihydrogen phosphate were used to maintain the pH of the solution. PCR primers were procured from eurofins, India. Solvents used for chromatographic analysis were of HPLC grade.

Mineral Media Optimization

As the bacterial growth is affected by different types of minerals such as zinc, sodium, magnesium, ammonium, calcium, etc., required for their physiological activities, different combinations of respective salts were tested for optimization of MSM composition for all the selected bacterial strains. Organic compounds, except the target carbon compound (caffeine), were not added for allowing the bacteria to use only the target compound as a carbon and nitrogen source. TS 1 presents a different level of concentrations of five salts (i.e., ammonium sulfate (X_1), magnesium sulfate heptahydrate (X_2), sodium chloride (X_3), zinc chloride (X_4), and calcium chloride dihydrate (X_5)) and used optimization of media composition for GBPI_Hb5 bacterial strain. The experiments were planned using Box-Behnken design (BBD) with the help of Minitab-18 [19]. Each selected variable was studied at three levels—low, medium, and high, and coded as -1 , 0 , and $+1$ (TS 1). Overall a total of 46 experiments were designed (Table 1). All the experiments were carried out in triplicate at 25°C in a climate chamber (Jeio Tech) using 100 mL MSM in 250 mL flasks. The pH of MSM was maintained at 7 using potassium dihydrogen phosphate and sodium dihydrogen phosphate salts. The experimental variables were tested for their significant effect on the responses (bacterial growth). All three responses, corresponding to the combined effects of five variables, were studied in their specified ranges as shown in TS 1.

The role of each variable, their interactions, and statistical analyses in obtaining predicted yields are explained by applying the following second-order polynomial quadratic model, Eq. 1.

$$\begin{aligned}
 R = & \beta_0 + \sum \beta_1 X_1 + \sum \beta_2 X_2 + \sum \beta_3 X_3 \\
 & + \sum \beta_4 X_4 + \sum \beta_5 X_5 + \sum \beta_6 X_1 X_2 \\
 & + \sum \beta_7 X_1 X_3 + \sum \beta_8 X_1 X_4 + \sum \beta_9 X_1 X_5 \\
 & + \sum \beta_{10} X_2 X_3 + \sum \beta_{11} X_2 X_4 + \sum \beta_{12} X_2 X_5 \\
 & + \sum \beta_{13} X_3 X_4 + \sum \beta_{14} X_2 X_5 + \sum \beta_{15} X_4 X_5 \\
 & + \sum \beta_{16} X_1^2 + \sum \beta_{17} X_2^2 + \sum \beta_{18} X_3^2 \\
 & + \sum \beta_{19} X_4^2 + \sum \beta_{20} X_5^2
 \end{aligned} \quad (1)$$

where R is the response, β_0 is offset term (Intercept process effect), β_1 , β_2 , β_3 , β_4 , and β_5 are linear effects; β_6 , β_7 , β_8 , β_9 , β_{10} , β_{11} , β_{12} , β_{16} , β_{14} , and β_{15} are cross-product effects, and β_{16} , β_{17} , β_{18} , β_{19} , and β_{20} are squared effects. The interactions

are represented by $X_1 X_2$, $X_1 X_3$, $X_1 X_4$, $X_1 X_5$, $X_2 X_3$, $X_2 X_4$, $X_2 X_5$, $X_3 X_4$, $X_3 X_5$, and $X_4 X_5$. This equation represents the quadratic effect of X_1 , X_2 , X_3 , X_4 , and X_5 variables on bacterial growth. The equation was validated using ANOVA. Response surfaces were drawn to determine the individual and interactive effects of test variables on the growth of bacteria.

Screening of Caffeine-Degrading Bacteria

Fifteen psychrotolerant bacterial strains of *Pseudomonas* species were screened out for their ability to utilize caffeine as the sole carbon and nitrogen source in presence of MSM under controlled conditions of temperature (5, 15, 25, 30, 35, 40, 45, and 50°C), pH (4, 7, 9), and the caffeine concentration (10, 50, 100, 250, 500, 800 and 1000 mg/L) using plate-based assays. Different caffeine concentrations (10, 50, 100, 250, 500, 800 and 1000 mg/L) were maintained in MSM which was already impregnated with agar and then transferred into Petri plate for solidification. The concentration of caffeine was measured using a UV–Vis spectrophotometer (Shimadzu, UV-2600, Japan) before making the agar-based plate. When the plates were solidified, one loop full of the overnight culture was used to streak the plate containing MSM medium with different concentrations of caffeine. After streaking, the plate was kept in the incubator at 25°C for 48 h for the observation of bacterial growth, the strain which showed the highest growth in the MSM media containing caffeine was selected as a predominant caffeine-degrading strain. The plate without caffeine was set as a control. After the selection of bacterial strain, the shake flask experiments were performed for checking its biodegradation capacity for caffeine.

Shake Flasks Degradation Experiments

Shake flasks caffeine degradation study was conducted in an incubating shaker (Jeio Tech, ISS-3075R) using the screened bacterial strains. The experimental conditions included different combinations of pH (4, 7, 9), temperature (4, 15, 25, 30°C), caffeine concentration (10, 50, 100, 500, 1000 ppm), bacterial inoculum volume 2, 5, and 10% (v/v), and agitation speed (75, 110, 185, 200 rpm). Erlenmeyer flask (250 mL) containing 50 mL sterile MSM, containing a known amount of caffeine, was used to enrich the bacterial isolates. A cotton plug was used to close the Erlenmeyer flasks in order to avoid contamination. The experiments were conducted up to 96 h with the sample collection at every 24 h. The collected samples were tested for their bacterial growth and caffeine content remaining

after the degradation experiments. The percentage caffeine degradation was calculated using the Eq. (2)

$$\% \text{Degradation} = \left(\frac{C_0 - C_t}{C_0} \times 100 \right) \quad (2)$$

All the experiments were performed in triplicate. The control, taken under the study, was mineral media without the addition of caffeine. Cell growth was measured at 600 nm using a spectrophotometer (Shimadzu, UV-2600).

Analytical Methods

Chromatographic Analysis

The samples collected after degradation studies, at every 24 h, were analyzed using RP-HPLC (Shimadzu, Kyoto Japan) equipped with Reprosil-Pur Basic C18, 5 μ m 250 \times 4, 6 mm column, Germany. The methanol (HPLC grade) and MiliQ water were used as the mobile phase in the ratio of 55:45 with a flow rate of 1 mL/min at 25 °C temperature. Pure caffeine was used as a standard. Detection and quantification of caffeine were done at 273 nm using the PDA detector.

For GC–MS analysis, the samples were vacuum dried at 65 °C, dissolved in methanol, and then filtered using a 0.25 μ m syringe filter. The filtered samples were analyzed using GC–MS analysis equipped with QP2010 mass spectrophotometer (Shimadzu, Japan). The ionization voltage was 70 eV. Gas chromatography was conducted in the temperature programming mode using HP- 5 MS (0.25 mm \times 0.25 μ m \times 30 m) column. The initial column temperature was 60 °C for 4 min, then increased linearly at the rate of 10 °C per min up to 260 °C, and held for 4 min at this temperature. The temperature of the injection port was 260 °C. The GC–MS interface was maintained at 260 °C. Helium was used as carrier gas at a flow rate of 1 mL/min with 31 min run time. The compounds were identified based on mass spectra and using the NIST library (version 2.0) of the GC–MS.

Caffeine-Degrading Genes in *Pseudomonas* sp. (GBPI_Hb5) Proteome

The protein sequence database was searched to compare the caffeine-degrading genes in *Pseudomonas* sp. genome in comparison to the earlier reports on *Pseudomonas* simiae (genome no. ASM93456v1). The homology test was performed using the BLAST program available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST/>), with the default parameters.

PCR Screening of GBPI_Hb5 for *ndmA* Gene

Single colony were used as template for PCR amplification of methylxanthine N1-demethylase gene, *ndmA*. Primers used for *ndmA* were CBBcdmF (5'- TGG CAT CCC GTW TGT ACY GT-3') and CBBcdmR (5'- CTT GKA TAA CRA TTC GCA ACC-3') [20]. Twenty-five microliter (μ l) PCR reactions contained 3 μ l of diluted GBPI_Hb5 culture, 0.4 μ M of each primer, and 1X AmpliTaq PCR Master Mix (500 U AmpliTaq DNA polymerase, 2.5 mM MgCl₂, and 200 μ M of each dNTP from Hi-media India). PCR amplification was conducted in an Bio-Rad Mycycler thermocycler (S.No. 580BR6651). The amplification program consisted of an initial 5 min denaturation step at 95° C, followed by 35 cycles at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min and a final extension step at 72 °C for 10 min. Following amplification, PCR products were electrophoresed on 1% agarose gels containing 1X TAE and observed using UV transillumination to detect *ndmA* bands.

Statistical Analysis

Experimental designing for mineral media optimization and preparation of 3D surface plots was performed using Minitab-18 (trial version). ANOVA test for calculating significant variation for time-series experiments and significant errors calculations were carried out using Microsoft excel 2016.

Results and Discussion

RSM Optimization of Media

The results of 46-run BBD with five variables: ammonium sulfate (X₁), magnesium sulfate heptahydrate (X₂), calcium chloride dihydrate (X₃), zinc chloride (X₄), and sodium chloride (X₅) chosen for optimization of mineral composition for getting the best growth of bacterial strain (GBPI_Hb5) in the presence of caffeine at pH 7 and temperature 25°C. Multiple regression analysis of the experimental data yielded the regression Eq. (3) for the optimized media.

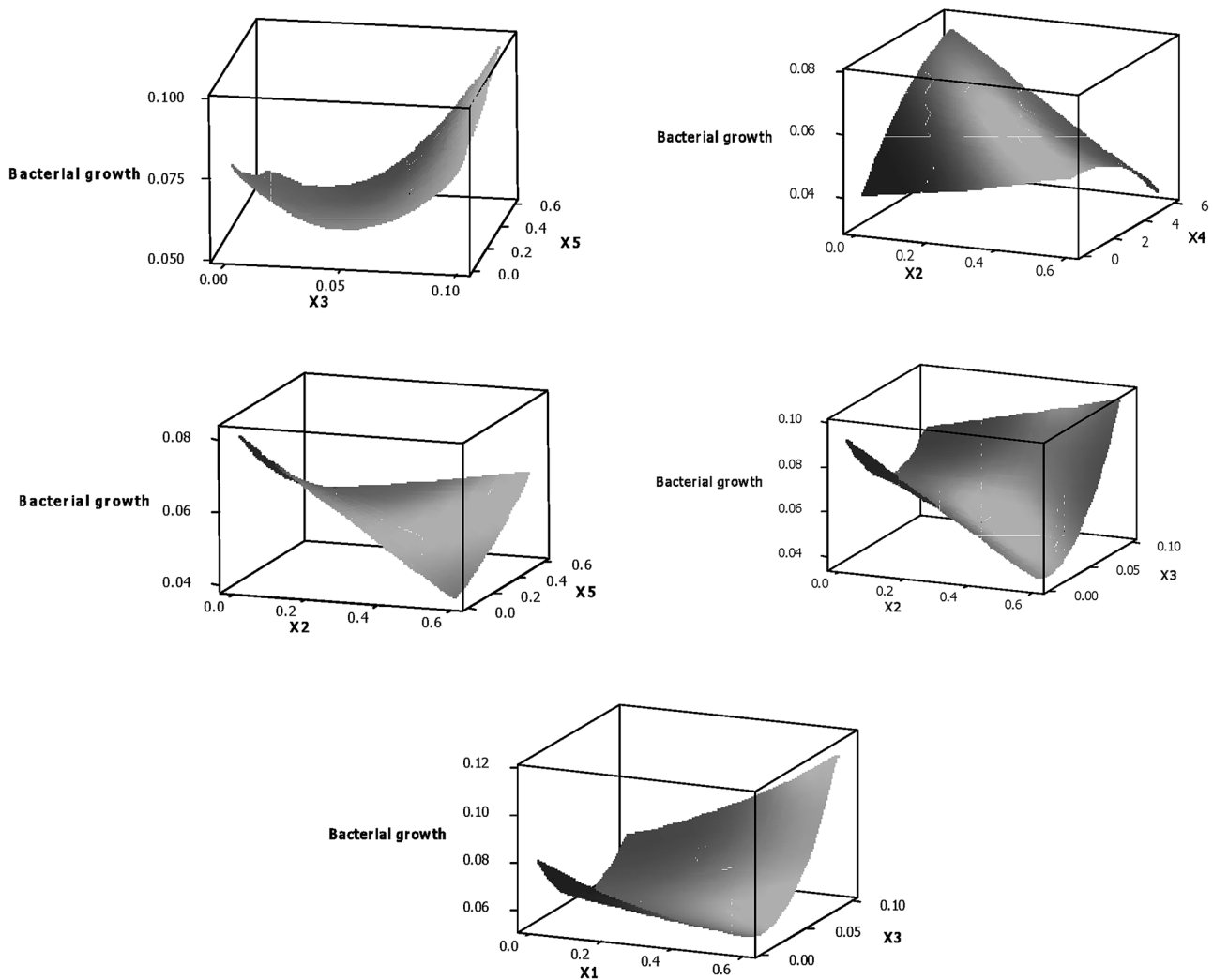


Fig. 1 3D surface plot of interactive effect of nutrient concentration significantly ($P < 0.05$) affecting bacterial growth using BBD

$$\begin{aligned}
 R = & 0.056167 + 0.00511X_1 - 0.008138X_2 \\
 & + 0.010500X_3 + 0.003688X_4 - 0.001438X_5 \\
 & - 0.006199X_1X_2 - 0.016500X_1X_3 - 0.005000X_1X_4 \\
 & + 0.000750X_1X_5 + 0.019250X_2X_3 - 0.014500X_2X_4 \\
 & + 0.012500X_2X_5 + 0.007500X_3X_4 + 0.010750X_3X_5 \\
 & + 0.001250X_4X_5 + 0.004200X_1^2 + 0.000534X_2^2 \\
 & + 0.019683X_3^2 - 0.006233X_4^2 + 0.002600X_5^2
 \end{aligned} \quad (3)$$

The value of the regression coefficient ($R^2 = 0.84$) was found closer to one which indicates that Eq. (2) can be used for the prediction of bacterial growth using any combination of five selected variables in similar experimental conditions.

Figure 1 shows the 3D surface plots which represent the changes in bacterial growth with the changes in MSM salt compositions [21]. Best combination (hold values) of MSM media for significant bacterial growth was found with the concentration of variables X_1 , X_2 , X_3 , X_4 , and X_5 as 0.6 g/L,

0.3 g/L, 0.05 g/L, 3 mg/L, and 0.3 g/L, respectively (TS 2), at pH7 and temperature 25 °C. This combination of selected minerals was used for further studies in Petri plate and shake flasks experiments for analyzing the caffeine degradation potential of the selected microbe.

TS 3 shows the ANOVA model for the optimized conditions required for the growth of bacteria. It is required to test the significance and adequacy of the model. The mean squares are obtained by dividing the sum of squares of each of the two sources of variations, the model, and the error variance, by the respective degrees of freedom. The T value is a standardized value that is calculated from sample data during the test hypothesis. It is the measure of variation in the data about the mean. Here the ANOVA of the regression model demonstrates that the model is significant in terms of some of the salts used for media preparation as evident from the calculated P value less than 0.05. In the case of individual parameters, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ salts significantly affect the bacterial growth, while in case of interactive effects, X_1X_3 , X_2X_3 , X_2X_4 , X_2X_5 , and X_3X_5 i.e., the combination of $(\text{NH}_4)_2\text{SO}_4/\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/\text{ZnCl}_2$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/\text{NaCl}$, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}/\text{NaCl}$, respectively, significantly affect bacterial growth (TS 2). TS 2 also indicates that the square effects of salt concentration ($P \leq 0.05$) had a significant influence on the growth of bacteria in the case of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

Mineral salt media optimization study has shown that on an individual level, calcium chloride and magnesium sulfate heptahydrate are playing a major role in the growth of target bacterium, while in case of interactive effects, the combinations such as $(\text{NH}_4)_2\text{SO}_4/\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/\text{ZnCl}_2$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/\text{NaCl}$, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}/\text{NaCl}$ significantly affected bacterial growth. This may be attributed to the fact that these salts may act as the cofactor for the growth

of bacteria by enhancing cell division and by helping them to carry out their physiological activities [22–24].

Screening and Selection of Potential Bacteria for Caffeine Degradation

All the bacterial strains except GBPI_Hb0 and GBPI_Hb1, taken for initial screening, are able to grow in presence of caffeine in plate assays. In the Petri plate screening experiments, the response of GBPI_Hb5 (MCC 3295) [17] with the nucleotide accession number KT887192.1 was found best. The bacterial growth was observed at every 24 h up to 96 h. There is very little change after the 48 h in the colony size. The growth of all the bacterial strains in presence of caffeine is measured in 48 h shown in Table 1. As the GBPI_Hb5 showed the maximum growth in the presence of caffeine, this strain was selected for shake flask experiments for the optimization of the best condition for the caffeine degradation.

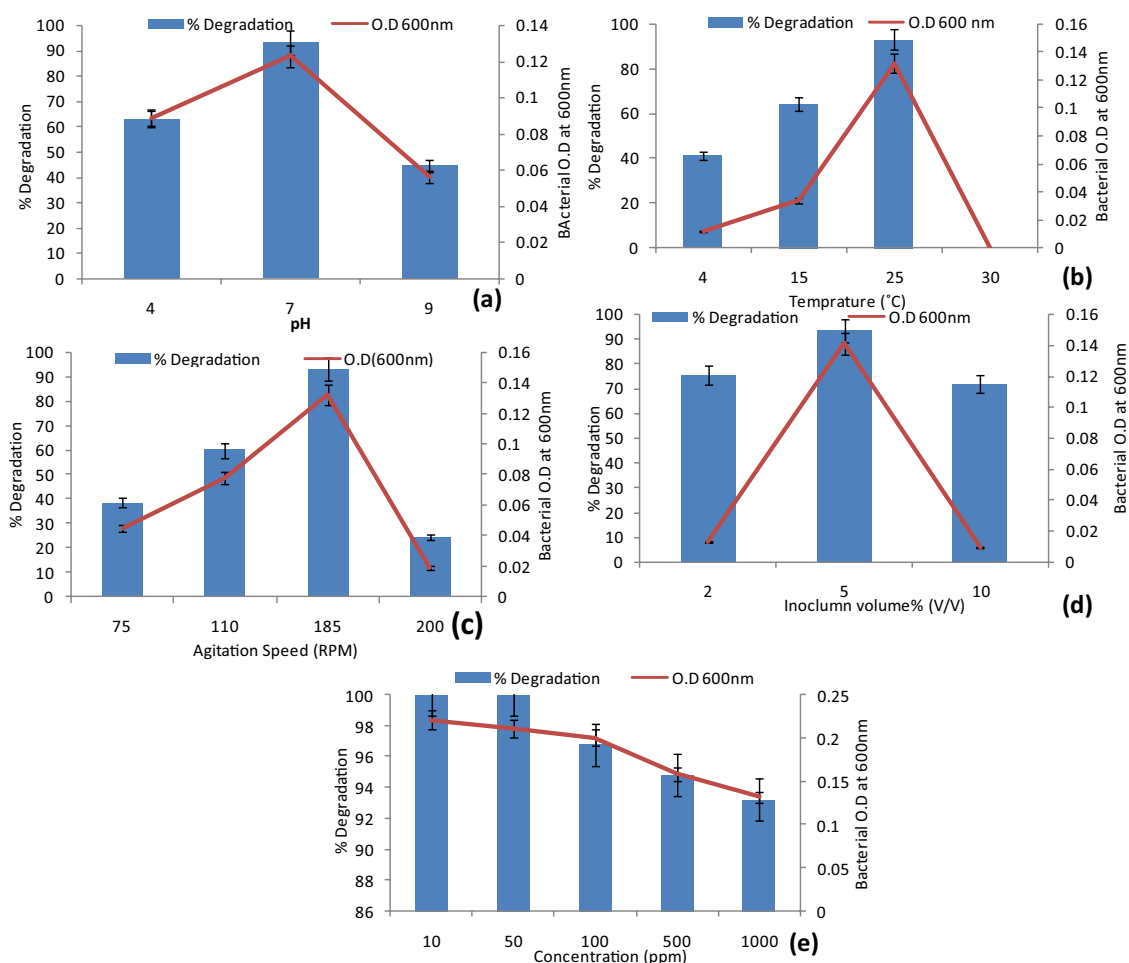


Fig. 2 Effect of **a** pH, **b** temperature, **c** agitation speed (RPM), **d** inoculum volume, and **e** concentration on caffeine degradation potential of GBPI_Hb5 bacterial strain at 96 h \pm SE ($N=3$)

Time Course of Growth and Caffeine Degradation by GBPI_Hb5

The time course of caffeine degradation is shown in FS 1 and the effect of experimental conditions on caffeine degradation potential of GBPI_Hb5 and its growth at 96 h is shown in Fig. 2, where the bacteria submerged in MSM was aerated by shaking in the presence of caffeine as the sole source of carbon at different media pH, temperature, inoculum volume, agitation speed, and caffeine concentration.

pH

The pH affects the growth and metabolism of all the microorganisms. It also plays a significant role in enzyme kinetics and bacterial growth. To study the effect of pH on caffeine degradation efficiency of GBPI_Hb5, pH of MSM was maintained at 4, 7, and 9, while the rest of the parameters remained constant (25 °C temperature, 5% (v/v) inoculum volume, 185RPM agitation speed, 1000 mg/L caffeine concentration). At pH 7.0, $93.2 \pm 0.0028\%$ caffeine was degraded up to 96 h and an increase in bacterial cell biomass was also observed. About $63 \pm 0.1358\%$ caffeine degradation was attained at pH 4 and $44.7 \pm 0.054\%$ degradation at pH 9 up to 96 h (Fig. 2a). The caffeine degradation potential was significantly affected ($P < 0.05$) by media pH along the time. Among all the selected media pH, 7 pH was found to be the best.

GBPI_Hb5 could grow at a pH range between 2 and 14 [17], with an optimal pH of 7, after which there is a decline in bacterial growth and caffeine degradation which is also depicted by the results where bacterial growth is lowest at 9 pH. These results indicate that the strain is a neutrophilic bacterium. High caffeine degradation has been reported using *Trichosporonasahii* at pH 6.5 [25]. Similarly, El-Mched et al. 2013 [26] reported maximum caffeine degradation at pH 7.0 using *Pseudomonas stutzeri* Gr 21 ZF, while maximum caffeine degradation was found at pH 7.5 for mixed culture of *Klebsiella* and *Rhodococcus* [25]. Karanth et al. [27] reported the highest caffeine degradation capacity at a pH range of 7.0–8.0 using *Pseudomonas alcaligenes* CFR 1708. Gokul Krishnan et al. [28] reported suitability of 6 pH for caffeine degradation with *Pseudomonas* sp. GSC 1182.

Temperature

The caffeine degradation capacity of GBPI_Hb5 was found to be affected by all the variables taken for the study. The temperature was observed as one of the major factors influencing caffeine degradation due to its effect on various biochemical reactions (Fig. 2b). Out of the four temperatures (5, 15, 25, and 30 °C), GBPI_Hb5 showed maximum degradation ($93.2 \pm 0.0028\%$) at 25 °C in 96 h (Table 2) in presence of 1000 ppm caffeine concentration, and pH of the media was maintained at 7, inoculum volume 5% (v/v), and agitation speed 185 RPM. Lakshmi and Das 2013 [25] reported that *Trichosporonasahii* degraded 73% of caffeine at 28 °C, while in a mixed culture of *Klebsiella* and *Rhodococcus* sp., 80% was observed at 40 °C. *Pseudomonas* sp. GSC 1182 had shown 80% caffeine degradation at 30 °C within 48 h [29], and *Pseudomonas alcaligenes* has reported 70% caffeine degradation at 35 °C [28].

The caffeine degradation, recorded at 4 °C and 15 °C, was found slow as compared to 25 °C. The maximum degradation observed at 4 °C, 15 °C, and 25 °C was $45 \pm 0.9143\%$, $63 \pm 0.089\%$, and $93.2 \pm 0.005\%$, respectively. The caffeine degradation potential was significantly affected ($P < 0.05$) by temperature along with the time. No detectable degradation was recorded at 30 °C. At 4 °C and 15 °C, bacterial growth and degradation efficiency were very low (45% and 64.45%, respectively), showing that the physiological activities of the bacterium are comparatively slow at these temperatures.

Agitation Speed

Agitation speed is one of the physical parameters which affects the growth of microorganisms. It is used for proper aeration and to maintain the homogeneity of the medium. The effect of agitation speed on cell growth and caffeine degradation capacity of GBPI_Hb5 was studied by varying the agitation speed at different levels such as 75, 155, 185, and 200 rpm while maintaining other conditions (7 pH, 25 °C temperature, 5% (v/v) inoculum volume, 1000 mg/L caffeine concentration) constant. 185 RPM agitation speed was found best with $93.2\% \pm 0.0028$ caffeine degradation up to 96 h with increased bacterial biomass (Fig. 2c). The

Table 2 Percent caffeine degradation with varying caffeine concentration at different durations using GBPI_Hb5 \pm SE ($N = 3$)

S. no	Caffeine concentration (ppm)	Percent degradation at different durations (%) \pm SE			
		24 h	48 h	72 h	96 h
1	10	100 ± 0.001			
2	50	100 ± 0.025			
3	100	95.2 ± 0.003	95.5 ± 0.006	96.6 ± 0.007	96.9 ± 0.0114
4	500	92.5 ± 0.001	92.4 ± 0.005	93.1 ± 0.0024	94.9 ± 0.005
5	1000	92.0 ± 0.001	92.4 ± 0.006	92.8 ± 0.006	93.3 ± 0.003

selected bacterium had shown the highest degradation at 185 RPM, while the further increase of agitation speed had shown a decrease in bacterial growth as well as caffeine degradation capacity (Fig. 2c). The caffeine degradation potential was significantly affected ($P < 0.05$) by agitation speed along the time. Similar findings were observed by Lakshmi and Das [25] where 72% caffeine degradation was observed at 120 rpm agitation speed. The agitation process is mainly responsible for mixing and shear of the degradation process, through which available oxygen, heat, and nutrients can be properly mixed and can be transferred efficiently in mineral media [30]. This process can help to disperse the air into small bubbles to improve the gas–liquid contact area. High agitation speed affects these processes negatively. It creates heterogeneous mixing and shear forces can damage the bacteria and their degradation capacities. In the case of low speed, the viscosity of media can slow down the growth and degradation process.

Inoculum Size

To get the best bacterial degradation of caffeine, the effect of inoculum size was tested. The best performance was observed with 5% (v/v) inoculum size (initial O.D 0.324) (Fig. 2d), where $93.2 \pm 0.0028\%$ caffeine degradation was observed up to 96 h, while the other variables were kept constant (i.e., 25 °C temperature, 7 pH, 185 rpm agitation speed, 1000 mg/L caffeine concentration). The caffeine degradation potential of the selected bacterial strain was significantly affected ($P < 0.05$) by inoculum volume along the time. Babu et al. [27] showed that 5% (v/v) inoculum size is best for the degradation of 1 g/L of caffeine. Inoculum less than 5% (v/v) was not performing well during the degradation of caffeine and had shown less growth. The increase of inoculum volume up to 5% (v/v) increased the bacterial growth along with the increased percent of caffeine degradation, which might be because the addition of cells in large inoculum, will not allow the marked decline in the total elimination of viable cells. As a result, caffeine is more consumed as a carbon and nitrogen source by the selected bacteria [31]. Less caffeine degradation and bacterial growth were observed with a higher inoculum size of 10% (v/v) ($71.6\% \pm 0.571$), which might be because too large inoculum size possibly destroys the ecological balance of the system leading to a decrease in caffeine degradation efficiency of the bacterium [32].

Caffeine Concentration

The effect of caffeine concentration on its bacterial degradation is shown in Fig. 2e and Table 2. 100% caffeine degradation was observed with MSM containing 10 and 50 mg/L caffeine even at 24 h. The degradation of caffeine decreased with increasing caffeine concentration.

Still, with 1000 mg/L caffeine concentration, GBPI_Hb5 bacterial strain was able to degrade up to $92.0 \pm 0.001\%$ within 24 h and $93.2 \pm 0.0028\%$ at 96 h while keeping the other variables constant (i.e., 25 °C temperature, 7 pH, 185 RPM agitation speed, 5% (v/v) inoculum volume). The decrease in caffeine concentration can also be observed through the HPLC chromatogram shown in FSI, where the caffeine was detected at 5.39 min at 273 nm. It was observed that the variation in caffeine concentration is significantly affecting the caffeine degradation potential of GBPI_Hb5 bacterial strain ($P < 0.05$) at definite duration, while time-series data show that there is no significant variation in caffeine degradation while comparing different sampling times i.e., 24, 48, 72, and 96 h. Caffeine at high concentrations is inhibitory to the growth of microorganisms. Nathanson [33] and Frischknecht et al. [34] observed degradation of 5 g/L caffeine using *Pseudomonas putida*, under ambient conditions, which have taken many years, and degradation of caffeine (1 g/L) was extremely slow, requiring about 1 week [35]. Numerous bacterial strains belonging to *Rhodococcus*, *Klebsiella*, *Acinetobacter*, *Brevibacterium* sp., *Serratia*, *Pseudomonas*, etc. have been reported for the capacity to degrade caffeine, but they are extremely sluggish [36, 37]. In contrast, GBPI_Hb5 was capable of degrading 1000 mg/L caffeine within 24 h of incubation under controlled conditions (Table 2 & FS2).

Observed Compounds During Caffeine Degradation

The samples collected at every 24 h time interval up to 96 h were estimated using GC–MS (FS1). The structure of observed compounds has been finalized based on the similarity index (SI) data obtained with GC/MS chromatogram (TS4 a and b). Main compounds observed during the caffeine degradation process at 24 h, 48 h, 72 h, and 96 h are 9-Octadecenoic acid methyl ester (E)-, 1,2-Benzenedicarboxylic acid, 9-Octadecenoic acid methyl ester, and (E)- and Bis (2-Ethylhexyl) Phthalate (Fig. 3). Caffeine degradation process shown by GBPI_Hb5 can be understood by observing the compounds produced at every 24 h up to 96 h (TS2 a and b). The majority of compounds were organic acids and their esters as identified through the GC–MS library (based on SI). Advance investigations on different aspects of microbial production of organic acids [38] are likely to be helpful to resolve the caffeine accumulation problem in affected areas in a more economical way. At 96 h, production of approx. 23.74 area percent of Bis-(2-ethyl hexyl) phthalate was also observed which is believed to be harmful to the environment. However, the degradation of caffeine up to 96 h can be used for the production of phthalate by the plastic industry. This intermediate was found missing up to 72 h, where 92.8%

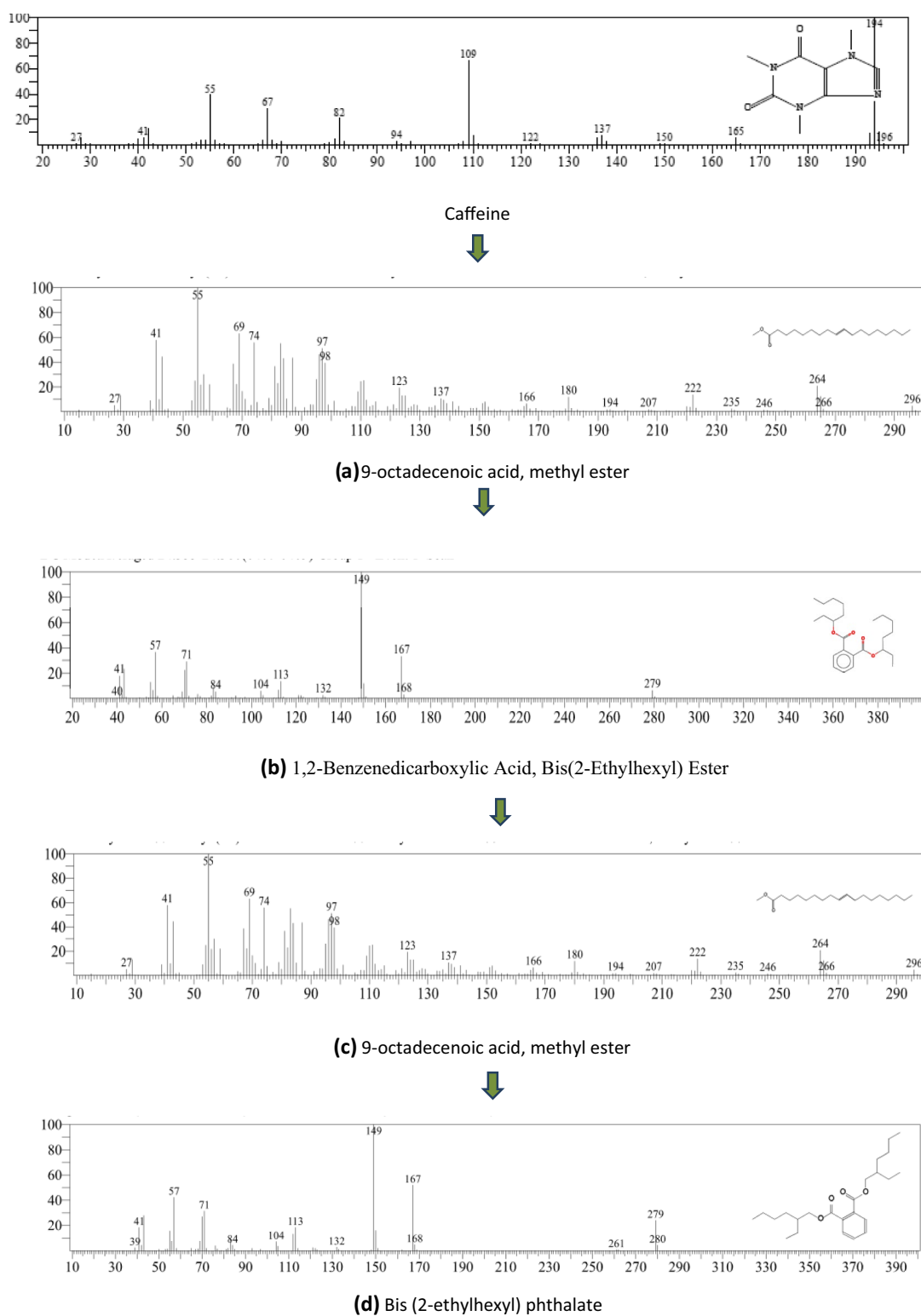


Fig. 3 GC–MS spectra of caffeine and major compounds detected at **a** 24 h, **b** 48, **c** 72 h, and **d** 96 h

caffeine was found degraded in comparison to 93.3% up to 96 h. Therefore, the termination of the process of caffeine degradation at 72 h is recommended.

Metabolic studies with these caffeine-degrading bacterial species revealed only two catabolic pathways: N-demethylation and C-8 oxidation. In the N-demethylation pathway, the caffeine molecule is sequentially demethylated to form xanthine and the C-8 oxidation pathway involves the oxidation of caffeine to form 1,3,7-trimethyluric acid (TMU), which is further degraded to the uric acid [39]. Yamaoka-Yano and Mazzafera [40] also reported the N-methylation pathway for the caffeine degradation using *Pseudomonas putida* where the responsible enzymes were reported as xanthine oxidase, caffeine demethylase, and dehydrogenase [11, 13, 15]. In the present study, the major compounds observed at different time intervals were 9-Octadecenoic acid methyl ester (E)-, 1,2-Benzenedicarboxylic acid (E)-, and Bis (2-Ethylhexyl) Phthalate.

Comparison of Possible Genes Responsible for Caffeine Degradation

Metabolic studies with the caffeine-degrading microorganisms normally follow two catabolic pathways: C-8 oxidation and N-demethylation [41, 42]. In both the pathways, bacteria break the caffeine molecule down to carbon dioxide and ammonia to harvest energy and cellular building blocks. Summers et al. 2015 [43, 44] had identified five novel genes, namely *ndmA* (AWV66915.1), *ndmB* (AWV66912.1), *ndmC* (PWB37176.1), *ndmD* (AWV66908.1), and *ndmE* (WP_116658531.1) responsible for the N-demethylation degradation of caffeine which was speculated encoding enzymes for sequential N-demethylation of caffeine in case of *Pseudomonas putida* CBB5 [39]. Yu et al. [45] had also found these genes in *Pseudomonas* sp. CES cells which are capable to degrade caffeine. In the present study, presence of homologs of *ndmA*, *ndmB*, *ndmC*, *ndmD*, and *ndmE* genes of *P. putida* were searched in the GBPI_Hb5 genome through the NCBI database. *ndmA* (Methylxanthine N1-demethylase), including one full length, and 3 for chain A, B, & C, has at least one significant (extremely low e-value) match in GBPI_Hb5 (TS 5); *ndmB* showed 3 entries, none matching with GBPI_Hb5 genome. *ndmC* showed 1 entry with no match with GBPI_Hb5, while *ndmD* (oxidoreductase) showed 1 entry with around 99 significant hits in the GBPI_Hb5 genome and *ndmE* with 1 entry with 12 good hits in GBPI_Hb5. The presence of these genes in the bacteria is indicating that these genes might be involved in caffeine degradation by *Pseudomonas* sp. GBPI_Hb5 (MCC 3295) [17]. Further, it reflected that the bacteria

might have followed the N-demethylation pathway for the degradation of the caffeine with some deviations which needs to be further investigated.

Identification ndmA Genes in GBPI_Hb5

Analysis of the sequence of a genomic DNA fragment of GBPI_Hb5 showed that the catabolic genes *ndmA* are involved in caffeine degradation (FS 3). The sizes of *ndmA* gene were similar found to the genome of *Pseudomonas putida* CBB5 [46]. The presence of *ndmA* genes in GBPI_Hb5 was expected, given that N-demethylation is the most commonly observed caffeine degradation mechanism. World-wide *Pseudomonas* genus is the most common caffeine-degrading genus. It is possible that GBPI_Hb5 bacterial strain might use *ndmA* gene for caffeine metabolism. The presence of *ndmA* a gene in the genus of GBPI_Hb5 shows that the caffeine degradation undergoes C-8 caffeine metabolic pathway. Further the metabolite pathway study needs to be done for the better understanding of caffeine degradation pathway for GBPI_Hb5 bacterial strain.

Conclusion

The present study emphasizes the bioprospection of bacterial diversity of colder regions under a mountain ecosystem. While the psychrotolerant bacteria possess mechanisms to protect their life, in which they secrete a range of metabolites responsible for various ecological processes such as biodegradation and mineralization. Based on the degradation study under controlled conditions and further analysis of intermediate compounds, *Pseudomonas* sp. (GBPI_Hb5) appears to be a promising species for the investigation of responsible enzymes and further demonstration of the study on a pilot scale.

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Declarations

Conflict of interest There is no conflict of interest.

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