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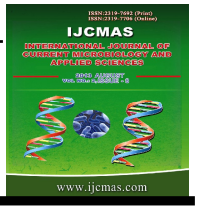

Original Research Article

Isolation and characterization of caffeine degrading bacteria from West Karnataka, India

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A B S T R A C T

Isolation of bacteria from soil and leaves of coffee plants and coffee cherries collected in coffee-growing areas of West Karnataka. Thirty two bacterium strains were isolated and exhibited high caffeine tolerance. Pure cultures were maintained on standard conventional nutrient agar medium.According to the physiological-biochemical characteristics the strains were identified as *Pseudomonas* and *Brevibacterium*. The bacterium was characterized with conventional tests and used to study the tolerance to different concentration of caffeine in both solid and liquid media. *Brevibacterium* sp. was grown in a liquid minimal medium containing 1-10g/L caffeine with glucose and sucrose separately. The bacterium was able to tolerate up to 8g/L of caffeine in solid medium and 3g/L in liquid medium. From the bacterium, a plasmid of about 2000bp MW was isolated. *Pseudomonas* sp. tolerated up to 10g/L of caffeine in solid medium and 5g/L in liquid medium.

K e y w o r d s

Bacteria; caffeine; coffee pulp; *Brevibacterium;* plasmid.

Introduction

Extremophiles are unique organisms that habitat in harsh environments including the extreme temperature, pH, pressure and salinity. Exploring microbial diversity under extreme environmental conditions and understanding adaptation mechanisms of extremophiles to survive in such extreme conditions will lead to much new knowledge of biology and also contribute to the future development of biotechnology.

Caffeine (1, 3, 7-trimethylxanthine) is a commercially important purine alkaloid synthesized by plants. It is an active psychostimulant, which increases alertness and sustains concentration by overcoming fatigue. Environmentally, caffeine has been suggested as a chemical indicator of ecosystem since it is difficultly metabolized (Ogunseitan, 2002). When the exposure dosage of caffeine in water was higher than 300 mg L-1,no zebrafish

embryos could survive, and caffeine-treated embryos exhibited significantly reduced tactile sensitivity frequencies of touch-induced movement even when exposure dosages were very low (Chen *et al*., 2008).

Decaffeination is a necessary step in coffee processing to reduce the caffeine content in food products and also for the treatment of caffeine containing effluents that are toxic to environment or for rendering coffee pulp and husk for other uses. To date, four major approaches for reducing caffeine content from caffeine-containing products are conventional breeding, physicochemical methods, Genetic engineering and microbial degradation whilst approach for wastes decaffeination is rarely concerned.

In this aspect microbial degradation of caffeine (1,3,7-trimethylxanthine) and related methylxanthines has been the focus of research in the recent past owing to major advantages that it has over conventional techniques of decaffeination. The present study aims at Isolation of bacteria from soil, leaves of coffee plants and coffee cherries collected in coffee-growing areas of West Karnataka, Screening and Amplification for CT (Caffeine Tolerance) bacteria and isolation of plasmid for further transformation studies.

Materials and Methods

Sample Collection and Isolation of Bacteria

Coffee pulp was collected from coffee cherry, leaves, soil processing site of the coffee estate in a sterilized container. Isolation of bacteria was carried out by spread plate method and pure cultures were obtained by streak plate method.

Amplification of the caffeine tolerant bacteria

Solid screening medium (SSM) for isolating the caffeine-tolerant bacteria was prepared by mixing the mineral solution with caffeine (2.5 g/ L) and agar (1.5%) and autoclaved at 121°C for 10 min. Solid purifying medium (SPM) was also prepared as SSM except different concentrations of caffeine (1 to 10 g/L) was supplemented. Liquid amplifying medium (LAM) was obtained after addition of caffeine (0.5 g /L) and sucrose/glucose (5.0 g /L) in the mineral solution and disinfection.

Soil (100 g) and the other samples were soaked in 1 L distilled water for 30 min. Solution containing microorganisms obtained by filtration and diluted 10^3-10^6 times, and spread over the SSM surface in Petri dishes (0.5 ml solution for each dish). After incubation at 30°C for 2-3 d, the fast-growing single colony was picked up with tooth tip and inoculated on SPM surface for further purification. Colonies, which could grow normally on the SPM, were separately picked up again and transferred to the tubes containing 1.0 ml LAM medium (each colony for one tube), then incubated at 150 r min-1 and 30°C in a shaking incubator for amplification. Three days later, 0.3 ml of bacterial solution was mixed with 0.1 ml dimethyl sulfoxide and stored at -70°C and the remaining solution was used for further tests.

Bacterial Identification

Pure cultures were maintained on nutrient agar medium at 4ºC and were sub-cultured at an interval of every 2 week. Various morphological, physiological and biochemical tests were performed to identify the bacteria and identification.

Preparation of Minimal Medium

The minimal medium was prepared by dissolving the following ingredients in distilled water (g L-1): KH2PO4, 3; Na2HPO4, 6; NaCl, 5; NH4Cl, 2; glucose or sucrose, 8; in the end, a solution of MgSO4 (0.1 g/L distilled water) was added and *p*H of the medium was adjusted to 6.7. After inoculation of the bacterium into the minimal medium, the temperature for culture condition was maintained at 37^{o}C.

Flask Culture Experiment for Growth Curve

The fresh (24 h) grown culture (10 µL) of nutrient broth was inoculated into 100 mL of the minimal media containing 1-10 g/L caffeine and the control was without caffeine. A blank was also maintained for the purpose of taking optical density (OD) and growth of the bacteria was measured by taking the OD at 600 nm by using Spectronic 20 spectrophotometer at every 3 h interval from the time of inoculation.

Isolation of Plasmid from Bacterium

Birnboim and Doly alkaline hydrolysis method was adopted for the isolation of plasmid from 24 hours culture of the bacterium. 20 µL of sample containing 0.2 mg/mL concentration of plasmid DNA suspended in Tris-EDTA buffer was loaded per well of 2% agarose gel.

Results and Discussion

After screening, a total of 89 colonies were obtained based on rapid proliferation on the SSM medium containing 2.5 g L-1 caffeine. These colonies exhibited a similar appearance, circular in shape and ivory-white in colour. Thirty two strains of them could grow normally but somewhat slowly on the SPM medium supplemented with 1to10.0 g L-1 caffeine after further purification, and were recorded as CT03, CT05, CT09, CT10, CT12, CT18,CT21, CT23, CT25, CT26, CT28, CT33, CT38, CT39, CT42, CT45, CT47, CT52, CT55, CT57, CT59, CT62, CT64, CT66, CT70, CT75, CT78, CT79, CT80, CT84, CT87 and CT89 respectively

From the morphology and conventional biochemical tests as given in Tables 1, it was confirmed that the bacterium (CT 10) isolated from coffee pulp was *Brevibacterium* sp. and *Pseudomonas* sp. 9 (CT 28) A number of workers have shown that selective removal of caffeine in aqueous medium by *P. putida* is possible 20,21. In the present study, the bacterium isolated from the coffee pulp was able to grow in the solid nutrient medium containing caffeine up to 8 g/L.

Growth curve of the *Brevibacterium* sp. at different concentration of caffeine in minimal media containing glucose is shown in Figure 1. It is evident from the growth curve that there was hardly any growth with the increasing concentration of caffeine beyond 8 g/L, whereas at 1-2 g/L concentration, the lag phase was prolonged to 24-40 h.

Growth curve of the bacterium at different concentration of caffeine in minimal media containing sucrose is shown in Figure 2. It is evident from the growth curve that there was only growth in 1 and 2 g/L concentration of caffeine with sucrose, whereas no growth was observed at 4 and 8 g/L of caffeine with sucrose. Moreover, the lag phase at 1 and 2 g/L caffeine concentration was prolonged up to 72 h. In the control treatment, growth started at about 48 h after incubation and

Table.1 Physiological and biochemical characteristics of CT bacteria

Characteristic	CT10	CT28
Glucose fermentation	+	+
Lactose fermentation	+	+
VP & MR	-	-
Citrate Utilisation	-	+
Starch hydrolysis	-	+
oxidase	+	-
Indole production	-	-
Hydrogen Sulfide production	-	-
Gelatin hydrolysis	-	-
Urease	+	+
Nitrate reduction	+	-

Figure.1 CT 10 and 28 growth in the media with addition of caffeine @ different concentration-with glucose

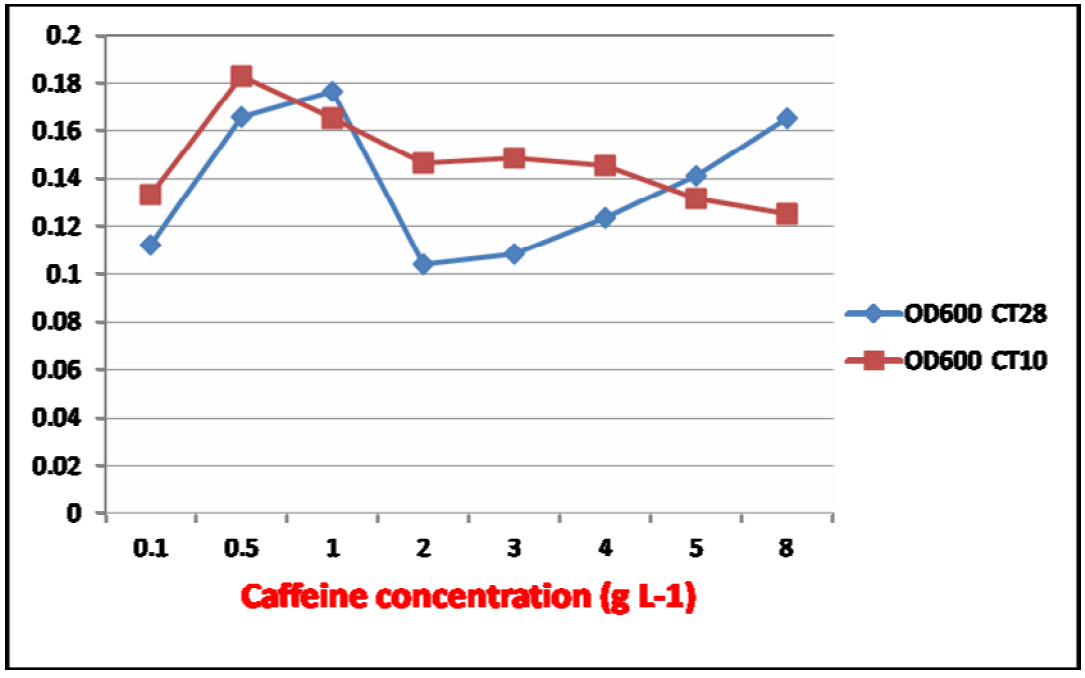


Figure.2 CT 10 and 28 growth in the media with addition of caffeine @ different concentration-with sucrose

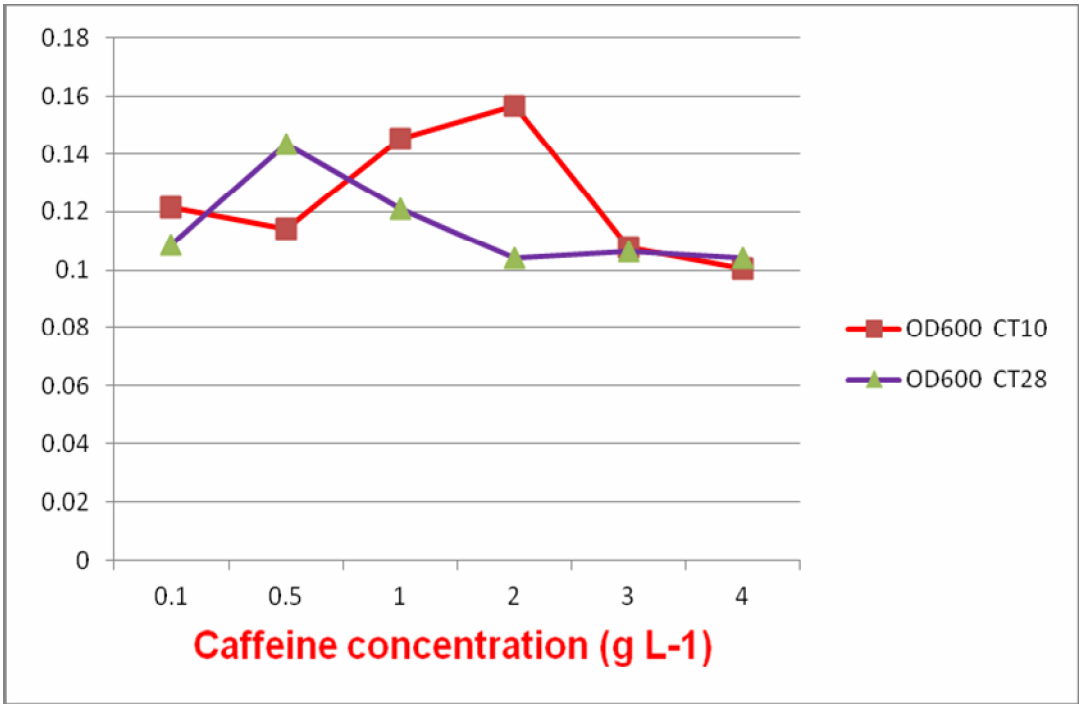


Table.2 CT growth in the media with addition of different concentration of caffeine (With glucose)

Caffeine concentration (g L-1)	OD600 CT10	OD600 CT28
0.1	0.1329 ± 0.0021d	0.1124± 0.0026d
0.5	0.1828± 0.0076e	0.1656± 0.0054e
1.0	0.1654± 0.0049e	0.1765± 0.0062e
2.0	0.1468± 0.0085d	0.1043± 0.0078d
3.0	0.1489± 0.0058c	0.1087± 0.0046c
4.0	0.1456± 0.0037b	0.1236± 0.0032b
5.0	0.1313± 0.0025a	0.1413± 0.0021d
8.0	0. 1249± 0.0013a	0.1654± 0.0010a

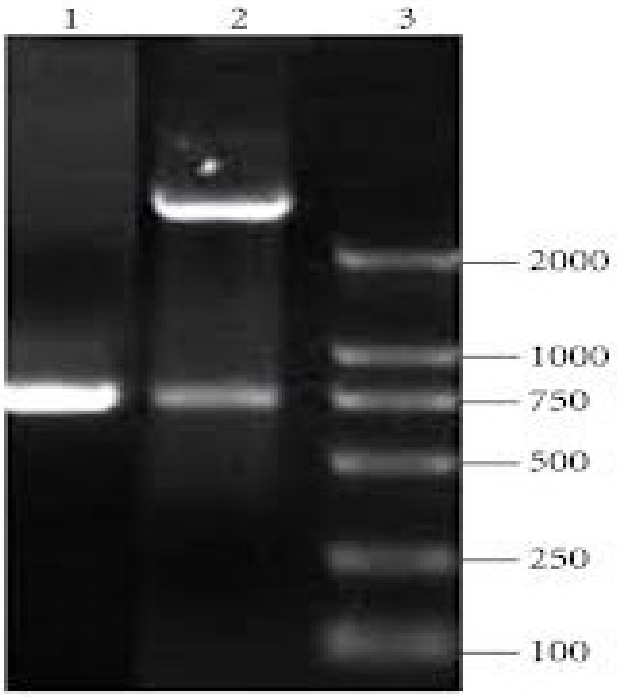
Incubation time was 30 h, and the different letters in column indicated significant difference at $p<0.05$.

Table.3 CT growth in the media with different caffeine concentration of caffeine (With sucrose)

Caffeine concentration (g L-1)	OD600 CT10	OD600 CT28
0.1	0.1214± 0.0014d	0.1089± 0.0012d
0.5	0.1143± 0.0021d	0.1432± 0.0018d
1.0	0.1453± 0.0024d	0.1212± 0.0021d
2.0	0.1564± 0.0034a	0.1043± 0.0028a
3.0	0.1076± 0.0042c	0.1064± 0.0034c
4.0	0.1007± 0.0067a	0.1042± 0.0029bd

Incubation time was 70 h, and the different letters in column indicated significant difference at $p<0.05$.

Figure.3 Agarose gel profile of the plasmid 1 CT10 (approx 2000bp),2 CT28 and 3 DNA ladder



there was a steep rise in the population without a lag phase, which stabilized and entered into stationary phase after reaching 65 h.

Carbohydrate sources like glucose and sucrose in the minimal media showed minimal effect on growth pattern of bacteria at low concentration of caffeine, i.e., 1 and 2 g/L, except that the lag phase was much more prolonged in the sucrose containing media (72 h) as compared to glucose (24-40 h) at the same concentration of caffeine (Table 2, 3) . Dash and Gummadi (2005) isolated the bacterium, *Pseudomonas* sp. NCIM 5235, from the soil of coffee estate, which was capable of degrading highest concentration of caffeine (10 g/L) at a maximum rate of 0.3 g/L/h as a whole cell biocatalyst without any growth. The strain was also known to tolerate high concentration of caffeine (~20 g/L). In the bacterium, a plasmid was found, which had the ability to degrade the caffeine when *E. coli* DH5a cells were transformed by this plasmid (Dash and Gummadi (2005). In the present study also, a plasmid of approximately 2000kb was isolated from the *Brevibacterium* sp. (Figure. 3). This plasmid is to be used for bringing about transformation in *E. coli* DH5α bacterium.

From the comparative study of growth of *Brevibacterium* sp. in minimal medium with glucose or sucrose as carbohydrate source and different concentrations of caffeine (1-10g/L), it is evident that the growth was almost the same for 1 g/L concentration of caffeine in both the medium, whereas the growth decreased in sucrose containing minimal medium at 4g/L caffeine concentration. Moreover, when the concentration of caffeine exceeded beyond 4g/L in sucrose containing minimal medium, there was hardly any growth (Table 2, 3).

Pseudomonas sp. isolated by Gokulakrishnan *et al*1from the soil of a coffee estate was able to tolerate caffeine up to 20 g/L. Further, Yamaoka-Yano and Mazzafera(Yamoka-Yano and Mazzafera, 1999) reported that *Pseudomonas putida* strain isolated by them was capable of degrading caffeine upto 25 g/L in liquid medium and up to 50 g/L in solid medium. Compared to this, *Brevibacterium* sp. Isolated from the coffee pulp and used in the present study was able to tolerate caffeine only up to8 g/L in solid medium and 6 g/L in liquid medium. From the environmental perspective, it is important to explore new species of bacteria and fungi that are naturally capable of degrading caffeine so that these organisms could be exploited by genetic engineering to enhance the biodegradation of caffeine in the environment. Solid wastes, such as, coffee pulp and husk, are the major contributors of environmental pollution from the coffee estates (Bressani, 1979; Adams and Dougan, 1981). The presence of caffeine in soil can also affect soil fertility as it inhibits seed germination and growth of seedlings (Friedman and Waller, 1983). Coffee pulp containing waste water is often discharged to the surrounding water bodies resulting in contamination of freshwater (Buerge *et al*., 2003; Glassmeyer *et al*., 2005) . The ingestion of caffeine and its chlorinated byproducts (derived during chlorination of water) have severe adverse effects on the physiological system (Mohapatra et al., 2006) [12]. However, bio-decaffeination can be used effectively for the solid wastes like coffee husk and pulp, which can then be used as animal feed ((Mohapatra *et al*., 2006; Mazzafera, 2002). This can be achieved only through exploitation of

naturally occurring organisms in the caffeine containing waste and by genetically engineering them to enhance their capacity of caffeine degradation.

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