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Biodegradation of Caffeine by *Pseudomonas* sp. NCIM 5235

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Abstract: A bacterial strain capable of utilizing caffeine as the sole source of carbon and nitrogen has been isolated from soil of coffee plantation area and deposited at National Collection of Industrial Microorganisms as *Pseudomonas* sp. based on biochemical studies. In this study, the isolate showed close resemblance to *Pseudomonas putida* strain T-57 based on 16S rRNA analysis. The ability of *Pseudomonas* sp. to degrade caffeine was studied by growing the isolate in caffeine medium with 1.2, 5, 7.5 and 10 g L^{-1} of initial concentration of caffeine. It was observed that the isolate could efficiently degrade caffeine at an initial concentration of 5 g L^{-1} within 48 h. It could also bring about 59.9 and 21.5% degradation of caffeine in 96 h when the initial concentration of caffeine in the medium was 7.5 and 10 g L^{-1}, respectively. Analysis of the metabolites formed during the course of time showed that degradation followed a demethylation pathway with 3, 7-dimethylxanthine as the first major product of degradation. Furthermore, a 12 kb plasmid was isolated and the involvement of this plasmid in caffeine degradation was indicated by growth of transformed *E. coli* DH5α strain. This strain can therefore prove to be a very good candidate for the biotreatment of residues of coffee and tea processing plants and also a potential strain for the development of improved methods of decaffeination.

Key words: *Pseudomonas*, caffeine, biodegradation, demethylation, 16S rRNA analysis, plasmid

Introduction

Caffeine (1, 3, 7-trimethylxanthine) is an important naturally occurring purine alkaloid present in the leaves and fruits of certain plant species like *Coffea, Camellia* and *Theobroma*. Due to its stimulatory effect, caffeine has been the key ingredient in many popular beverages like tea, coffee and various soft drinks. It also finds application in pharmaceutical preparations as an adjuvant to drugs like paracetamol. Commercial caffeine is obtained from coffee cherries which generate nearly two million tons of residues in the form of pulp, husk, spent-grounds and residual water annually depending upon the type of extraction procedure followed. The pulp and husk though rich in carbohydrates, proteins and minerals cannot be used as animal feed due to presence of appreciable amount of antinutrional factors like tannins, polyphenols and caffeine which render it unpalatable to ruminants (Bressani, 1987; Elías, 1987). As the pulp, husk and other residues of coffee processing industries do not find any commercial use, these are often dumped into water bodies and soil around the coffee processing regions posing hazard to the environment (Adams and Dougan, 1981; Bressani, 1987). The tannins and polyphenols and other organic solids are toxic in nature and cannot be used beneficially. The sugars, proteins and minerals in the coffee residues promote the growth of microorganisms, which, if not

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treated readily, causes environmental pollution. As an example, in 1990 in Columbia, it was estimated that one metric ton of green coffee generated waste water which was equivalent to the domestic sewage of 2000-2500 people (Gathuo *et al*., 1991).

Caffeine in higher concentration is toxic to saprophytic organisms that are involved in essential biotransformation in the environment, which causes a disturbance in ecological balance. The disposal of coffee waste represents an enormous pollution problem in the producer countries. Therefore, from the environmental and economic point of view attempts have been made to utilize the coffee pulp as a source of animal feed. For this purpose the removal of the antinutritional factors, particularly caffeine, become necessary. The use of microorganisms for decaffeination purposes have been advocated to be more beneficial than other chemical and physical methods currently employed (Gokulakrishnan *et al* ., 2005). Several bacteria and fungi capable of utilizing caffeine as the sole source of carbon and nitrogen have been isolated (Schwimmer *et al*., 1971; Hakil *et al*., 1999; Ashihara and Crozier, 2001; Mazzafera, 2002) and these can serve as potential targets for biological decaffeination. Bacterial species would be more favoured for this purpose keeping in mind the ease of manipulating these microorganisms as compared to others.

The intermediates of caffeine degradation pathway are as useful as caffeine and find various applications. So bacterial degradation of caffeine not only becomes essential overcoming environmental issues but also can serve as a method of recovery of other commercially important products. In this aspect, the isolation of an efficient caffeine degrading strain becomes crucial. Previously we have isolated a bacterial strain from the soil of coffee plantation area which is capable of utilizing caffeine as the sole source of carbon and nitrogen. Based on biochemical tests the isolate was identified as *Pseudomonas* sp. and has been deposited at the National Chemical laboratory, Pune, India (accession number NCIM 5235). In the present study the taxonomic position of the isolated strain was evaluated by 16S rRNA analysis. The degradation profile at higher initial concentrations of caffeine and the various metabolites formed during the course of degradation were also studied. In addition, we also report for the first time the involvement of a plasmid in this degradation process. Therefore, this strain offers new perspectives in the degradation of environment polluting caffeine wastes and can also serve as a model for development of microbial methods of decaffeination.

Materials and Methods

Chemicals

Pure caffeine was obtained from Merck. 3, 7-dimethylxanthine, 7-methylxanthine and xanthine were obtained from Sigma. All other reagents were of analytical grade.

Bacterial Strain

Pseudomonas sp. NCIM 5235 was previously isolated in our laboratory from soil of coffee plantation area of Ooty, India during August 2003. The isolate was maintained on Nutrient Agar and was subcultured every two weeks.

Media

The composition of basal medium (BM) was as follows (g L^{-1}): Na_2HPO_4, 0.12; KH_2PO_4, 1.3; $CaCl_2$, 0.3; $MgSO_4$. 7 H_2O, 0.3. CAS medium consisted of BM supplemented with 5 g L^{-1} sucrose and caffeine at various concentrations mentioned elsewhere in the manuscript. 0.02% of Fe^{2+} was added to CAS medium after autoclaving. The final pH of the medium before sterilization was 6. For solid medium, agar (25 g L^{-1}) was added to CAS medium.

Analysis of 16S rRNA and Taxonomic Position of Pseudomonas sp. NCIM 5235

Genomic DNA was isolated as described earlier (Sambrook *et al*., 1989). The gene encoding the small ribosomal subunit (16S rRNA) was entirely amplified by PCR with the primers MM3 (5'-GCAGCAGTGGGGAATTTTGG-3') and P13 (5'-AGGCCCGGGAACGTATTCAC-3') (*Escherichia coli* 16S rRNA positions 371 to 1370; accession number JO695) (Ferroni *et al*., 2002) and determined by sequencing. Forward and reverse sequences thus obtained were aligned to produce a high quality consensus sequence. Related sequences were retrieved from Ribosomal Database Project (RDP II Release 9) using BLAST search program. A phylogenetic tree was constructed with CLUSTAL X software based on the 16S rRNA sequences of 10 strains close to strain NCIM 5235.

Flask Culture Experiments

A single colony of the strain from nutrient-agar plates was transferred to CAS medium plate containing 1.2 g L^{-1} caffeine and cultured at 30°C for 36 h. After sufficient growth on plates, three loopfuls of culture was transferred to 25 mL sterile nutrient medium and incubated in a rotary shaker at 30°C and at 180 rpm. After the seed OD_{600} reached ~1.3, 6% (v/v) of the culture was transferred to 25 mL of caffeine medium in 100 mL Erlenmeyer flasks and incubated on a rotary shaker at 180 rpm and at 30°C. At regular intervals, samples were collected and analyzed for cell growth and caffeine degradation. T he ability of the isolate to degrade caffeine was studied by varying the initial concentration from 1.2 to 10 g L^{-1}. All experiments were performed in triplicates u nder identical conditions and all results had a standard deviation of 5 to 8% about the mean.

Analytical Determinations

Cell density in the medium was monitored by measuring the optical density at 600 nm (OD_{600} of 0.5 corresponds to 0.379 g dry weight L^{-1}. Caffeine and the metabolites were estimated by HPLC (Agilent 1100 series) equipment using a ZORBAX C-18 column with 10 mM ammonium phosphate(pH 2.5)/ acetonitrile (4:1, v/v) as mobile phase. Pure caffeine (Merck) at 2 mg mL^{-1} and 3, 7-dimethylxanthine, 7-methylxanthine and xanthine (Sigma) at 0.5 mg mL^{-1} were used as standards. The retention times of caffeine, 3, 7-trimethylxanthine, 7-methylxanthine and xanthine were found to be 4.9, 3.5, 3.0 and 2.8 min, respectively at a flow rate of 1 mL min^{-1} and at 28°C. Detection of caffeine and its metabolites was done at 254 nm.

Isolation of Plasmid DNA

Plasmid DNA from *Pseudomonas* sp. NCIM5235 was isolated using the MiniPrep plasmid isolation kit (Qiagen). Isolated plasmid was analyzed on 0.8% agarose gel with a 1 kb DNA ladder (Biolabs Inc.) as marker.

Transformation of E. coli DH5α with Plasmid from Pseudomonas sp. *NCIM 5235*

Transformation of *E. coli* DH5α was carried out by electroporation in a Biorad Gene Pulser apparatus as described earlier (Sambrook *et al*., 1989). Transformed *E. coli* colonies were screened by spread plate technique on CAS medium containing 1.2 g L^{-1} caffeine and non-transformed cells were plated on CAS medium as control. The plates were incubated at 37°C and growth was observed after 48 h of incubation.

Results and Discussion

Identification of Bacterial Strain

Although the biological removal of caffeine from coffee and tea wastes offers an excellent alternative to the present existing methods of decaffeination, it is still in the research stage more so because of the lack of an efficient microbial strain, which would serve the purpose. In this aspect, we have isolated a bacterial strain capable of utilizing caffeine (1.2 g L^{-1}) as the sole source of carbon and nitrogen. This strain was grossly identified to be *Pseudomonas* sp. based on colony morphology, Gram staining and routine biochemical tests. In this study the partial 16S rRNA sequence of *Pseudomonas* sp. NCIM 5235 (comprising of 1399 nucleotides) was determined and a phylogenetic tree was constructed based on 16S rRNA sequences (Fig. 1). The isolate was related to the *Pseudomonas* sp. lineage and closely clustered with one strain *P. putida* T -57 (DDBJ database accession AB188094.1). Therefore, the isolate was tentatively identified to be *Pseudomonas putida*. Most of the bacterial species involved in caffeine degradation have been identified to belong to the *Pseudomonas* group (Burr and Caesar, 1985).

Growth of Pseudomonas sp. *NCIM 5235 at Higher Caffeine Concentration*

The ability of *Pseudomonas* sp. NCIM 5235 to degrade caffeine was studied by growing the isolate in CAS medium with 1.2, 5, 7.5 and 10 g L^{-1} of initial concentration of caffeine. The complete degradation of caffeine in medium containing 1.2 g L^{-1} of caffeine was achieved in 15 h and it required 48 h to degrade 100% of caffeine when the initial concentration was 5 g L^{-1} (Table 1). The degradation rate was found to be 0.105 g L^{-1} h^{-1}. The degradation of caffeine at this concentration (5 g L^{-1}) has been previously reported with a *Pseudomonas* strain isolated from soil with a rate of 0.095 g L^{-1} h^{-1} and the isolate was acclimatized for several years to grow utilizing caffeine as the sole carbon and nitrogen source (Woolfolk, 1975). Perhaps this isolate, being isolated from an environment rich in caffeine has been naturally acclimatized to utilize higher concentration of caffeine and the need for further acclimatization is therefore eliminated. The isolate reported in this study showed degradation rate higher than reported in literature (Woolfolk, 1975). In addition, the caffeine degrading ability by *Pseudomonas* sp. NCIM5235 was investigated further at higher concentrations. It was found that isolate could bring about 59.9 and 21.5% degradation of caffeine in 96 h when the initial concentration of caffeine in the medium was 7.5 and 10 g L^{-1}, respectively (Table 1). The corresponding cell dry weight was also observed to be highest at the point where degradation is maximum indicating that the isolate utilized caffeine for growth (Table 1). Although tolerance for caffeine has been reported at concentrations as high as 20 to 50 g L^{-1} (Middelhoven and Bakker, 1982; Yamaoka-Yano and Mazzafera, 1998), the degradation of caffeine by any microorganisms has not been reported for concentrations more than 5 g L^{-1}. This makes *Pseudomonas* sp. NCIM 5235 an efficient strain which can be developed for devising strategies for biological decaffeination. It is anticipated that the isolate could degrade caffeine more efficiently at higher concentrations if the microbiological parameters and the nutrients are optimized.

Metabolites of Caffeine Degradation and Pathway of Degradation

The major catabolic route of caffeine degradation by bacteria is through demethylation pathway. The subsequent products of caffeine degradation are formed by sequential removal of methyl groups from the basic 1, 3, 7-trimethylxanthine structure to 3, 7-dimethyxanthine (theobromine) as the first major metabolite (Woolfolk, 1975; Blecher and Lingens, 1977; Gluck and Lingens, 1987).

Table 1: Percentage degradation of caffeine and corresponding cell dry weight of *Pseudomonas* sp. NCIM 5235 at various initial concentrations of caffeine

	% degradation at various initial concentrations (g L^{-1})				Cell dry weight at various initial concentrations (g L^{-1})			
Time	1.2	5	7.5	10	1.2	5	7.5	10
0	0	0	0	0	0.12	0.09	0.13	0.07
9	65.42	-	-	-	0.1	-	-	-
12	72.09	-	-	-	0.13	-	-	-
15	99.9	-	-	-	0.13	-	-	-
18	100	-	-	-	0.2	-	-	-
24	100	18.11	4.6	4.67	0.22	0.32	0.19	0.13
36	-	70.94	8.47	7.42	0.15	0.86	0.19	0.13
48	-	94.33	12.7	5.43	-	0.84	0.24	0.11
60	-	100	40	16.1	-	0.48	0.24	0.13
72	-	100	45.03	25.7	-	-	0.44	0.17
84	-	100	54.49	27.3	-	-	0.94	0.2
96	-	100	59.98	27.54	-	-	0.92	0.36

The experiment was repeated thrice and the values represent the mean of the different experiments with a standard deviation of ±5% to ± 8%. The maximum inhibitory concentration of caffeine at which the cell growth ceases was found to be 20 g L^{-1}.

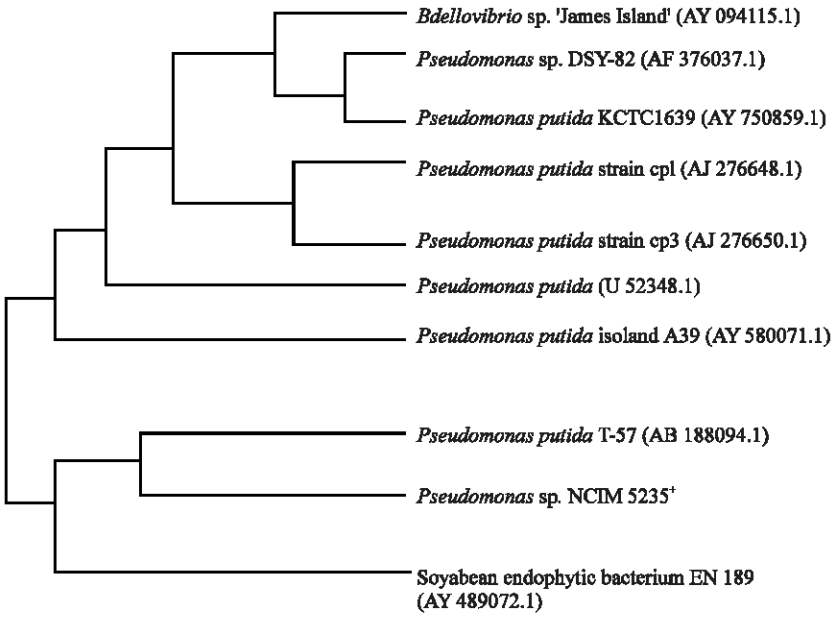

Fig. 1: Phylogenetic tree diagram based on 16S rRNA analysis using CLUSTALX software. Numbers in parentheses indicate Genbank /EMBL /DDBJ database accession numbers.* indicates the isolated strain in this study.

1, 3-dimethylxanthine (theophylline) is formed in lesser amount. Hence degradation through theophylline route can be considered as the minor pathway of degradation (Blecher and Lingens, 1977). In order to investigate the route of caffeine degradation by *Pseudomonas* sp. NCIM 5235 the metabolite profiles were obtained at various time points during the course of degradation and compared with the profiles of the standards (Fig. 2). It was observed that degradation of caffeine by

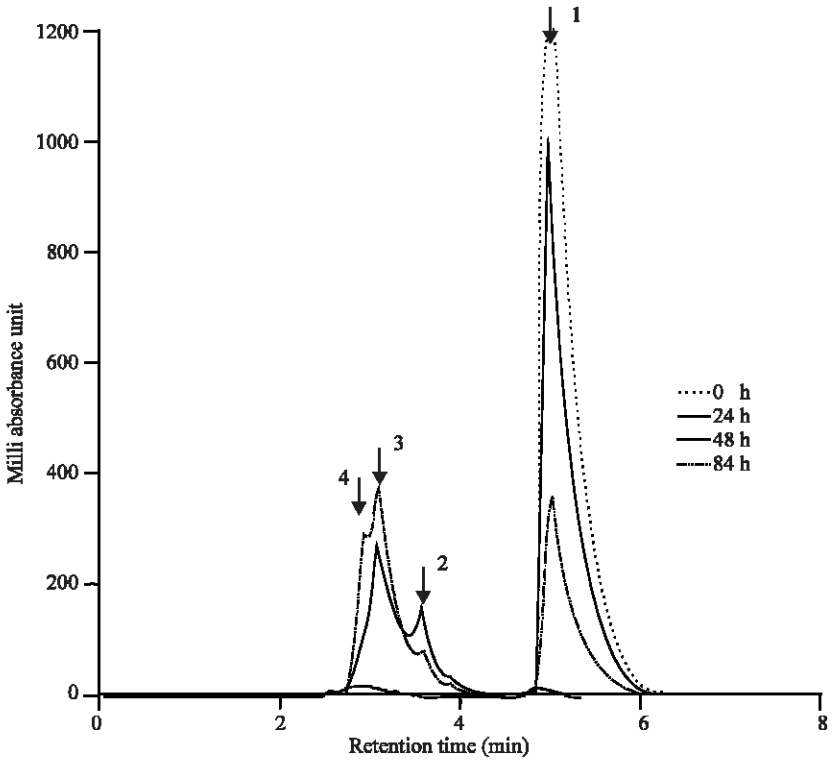

Fig. 2: Metabolites formed at various time points during degradation of caffeine by *Pseudomonas* sp. NCIM5235 when the initial concentration is 5 g L^{-1}. The analysis was performed by RP-HPLC on Zorbax C18 column with 10 mM ammonium phosphate: acetonitrile 1:4 as mobile phase. Peak 1 corresponds to caffeine at 4.9 min. Peak 2 corresponds to 3, 7-dimethylxanthine at 3.5 min. Peak 3 corresponds to 7-methylxanthine at 3.0 min. Peak 4 corresponds to xanthine at 2.8 min

Pseudomonas sp. NCIM 5235 follows the demethylation pathway with the formation of 3, 7-dimethylxanthine and then subsequent derivatives (Fig. 2). At 24 h of degradation both dimethylxanthine (3, 7-dimethylxanthine) at the concentration of 0.28 g L^{-1} and monomethylxanthine (7-methylxanthine) at the concentration of 0.37 g L^{-1} are formed. At 48 h of degradation, the amount of dimethylxanthine (3, 7-dimethylxanthine) decreases appreciably to 0.15 g L^{-1} with the increase in amount of monomethylxanthine (7-methylxanthine) to a concentration of 0.78 g L^{-1}. Formation of xanthine at a concentration of 0.32 g L^{-1} is also noted at that time. These results clearly suggested that the degradation of caffeine by the isolate occurs by demethylation pathway. This is in agreement with the previously reported findings regarding the degradation pathway in *Pseudomonas* sp. (Blecher and Lingens, 1977; Gluck and Lingens, 1987; Yamaoka-Yano and Mazzafera, 1999). At 84 h, all metabolites were completely utilized for cell growth. The knowledge of the metabolites formed and the pathway of degradation can provide an insight for the manipulation of conditions in order to derive metabolites of choice. This will provide an excellent opportunity of recycling the coffee/tea waste generated by processing plants for more useful products (such as theobromine) and the simultaneous removal of caffeine toxicities.

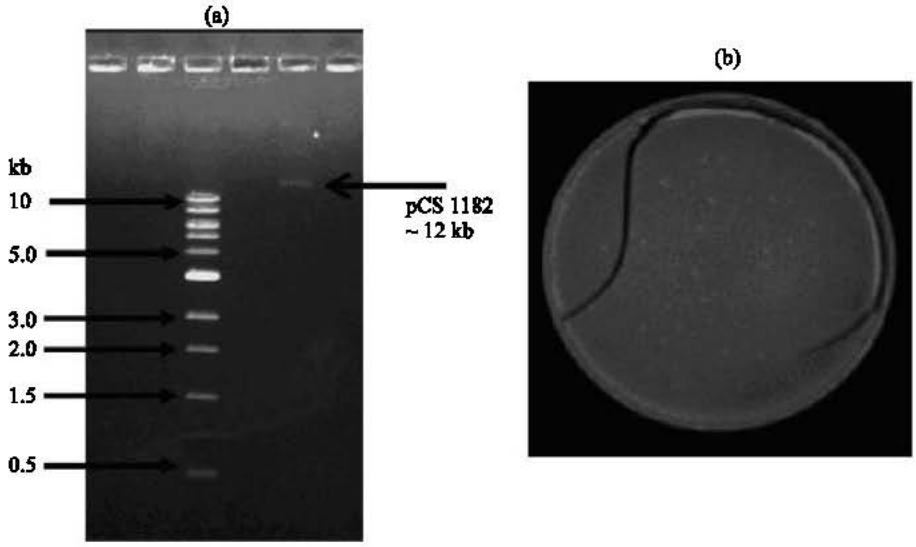

Fig. 3: Isolation of plasmid from *Pseudomonas* sp. NCIM 5235. (a) Agarose gel profile of plasmid pCS1182. (b) Growth of *E. coli* DH5α transformed with plasmid pCS1182 on CAS agar medium.

Isolation of Plasmid and Transformation Experiments

Microorganisms have been known to breakdown and mineralize organic pollutants. In fact, the best-studied bacterial genera capable of degrading organic compounds are *Pseudomonads* and their close relatives (Dennis and Zylstra, 2004). It has been shown repeatedly that many of these bacteria extend their nutritional diversity by harboring catabolic pathways for the degradation of simple or complex aromatic compounds on large low copy number plasmids. Some of the best-known catabolic plasmids include CAM, NIC, OCT and TOL, for the degradation of camphor, nicotine, octane and toluene respectively (Williams and Sayers, 1994). In order to investigate the possible role of plasmid in degradation of caffeine by *Pseudomonas* sp. NCIM 5235, plasmid isolation was performed and a 12 kb plasmid, named as pCS1182, was isolated from the strain (Fig. 3a). pCS1182 was then transformed into *E. coli* DH5α strain that was normally incapable of growing on CAS medium containing 1.2 g L^{-1} of caffeine. The growth of transformed *E.coli* DH5α colonies on CAS medium (Fig. 3b) is a clear indication that the caffeine degrading ability of *Pseudomonas* sp. NCIM 5235 is plasmid borne. The transformed *E.coli* strain was also able to bring about the complete degradation of caffeine at an initial concentration of 1.2 g L^{-1}. This is the first report of the involvement of plasmid in caffeine degradation.

Finally, we note that *Pseudomonas* sp. NCIM 5235 is the first bacterial strain able to efficiently degrade caffeine at concentrations never reported before and this property can be attributed to the plasmid that it harbours. This capability make this strain a very good candidate for the biotreatment of residues of coffee processing plants and also a potential strain for the development of improved methods of decaffeination.

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References

Adams, M.R. and J. Dougan, 1981. Biological management of coffee processing. Trop. Sci., 123: 178-196.

Ashihara, H. and A. Crozier, 2001. Caffeine: A well known but little mentioned compound in plant science. Trends in Plant Sci., 6: 407-413.

Blecher, R. and F. Lingens, 1977. The metabolism of caffeine by a *Pseudomonas putida* strain. Hoppe-Seyler's Zeitschrift fur Physiologische Chemie, 358: 807-817.

Bressani, R., 1987. Antiphysiological Factors in Coffee Pulp. In: Brahan, J.E. and R. Bressani (Ed.), Coffee pulp: Composition, technology and utilization. Guatemala City: Institute of Nutrition of Central America and Panama, pp: 83-88.

Burr, T.J. and A. Caesar, 1985. Beneficial plant bacteria. CRC Cri. Rev. Plant Sci., 2: 1-20.

Dennis, J.J. and G.J. Zylstra, 2004. Complete sequence and genetic organization of pDTG1, the 83 kilobase naphthalene degradation plasmid from *Pseudomonas putida* strain NCIB 9816-4. J. Mol. Biol., 341: 753-768.

Elías, L.G., 1987. Chemical Composition of Coffee-berry By-products. In: Brahan, J.E. and R. Bressani (Ed.), Coffee pulp: Composition, Technology and Utilization. Guatemala City: Institute of Nutrition of Central America and Panama, pp: 11-16.

Ferroni, A., I. Sermet-Gaudelus, E. Abachin, G. Quesne, G. Lenoir, P. Berche and J. Gaillard, 2002. Use of 16S rRNA gene sequencing for identification of nonfermenting gram-negative *Bacilli* recovered from patients attending a single cystic fibrosis center. J. Clin. Microbiol., 40: 3793-3797.

Gathuo, B., P. Rantala and R. Määttä, 1991. Coffee industry waste. Water Sci. Technol., 24: 53-60.

Gluck, M. and F. Lingens, 1987. Studies on the microbial production of theobromine and heteroxanthine from caffeine. Applied Microbiol. Biotechnol., 25: 334-340.

Gokulakrishnan S., K. Chandraraj and S.N. Gummadi, 2005. Microbial and enzymatic methods for the removal of caffeine. Enzyme and Microbial. Technol., 37: 225-232.

Hakil, M., F. Voisinet, GV. Gonzαlez and C. Augur, 1999. Caffeine degradation in solid-state fermentation by *Aspergillus tamarii*: Effects of additional nitrogen sources. Process Biochem., 35: 103-109.

Mazzafera, P., 2002. Degradation of caffeine by microorganisms and potential use of decaffeinated coffee husk and pulp in animal feeding. Sci. Agric., 59: 815-821.

Middelhoven, W.J. and C.M. Bakker, 1982. Degradation of caffeine by immobilized cells of *Pseudomonas putida* strain C3024. Eur. J. Applied Microbiol. Biotechnol., 15: 214-217.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schwimmer, S., R.H. Kurtzmann and E. Heftmann, 1971. Caffeine metabolism by *Penicillium roqueforti*. Arch. Biochem. Biophys., 147: 109-113.

Williams, P.A. and J.R. Sayers, 1994. The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. Biodegradation, 5: 195-217.

Woolfolk, C.A. 1975. Metabolism of N-methylpurines by a *Pseudomonas putida* strain isolated by enrichment on caffeine as the sole source of carbon and nitrogen. J. Bacteriol., 123: 1088-1106.

Yamaoka-Yano, D.M. and P. Mazzafera, 1998. Degradation of caffeine by *Pseudomonas putida* isolated from soil. Allelopathy J., 5: 23-34.

Yamaoka-Yano, D.M. and P. Mazzafera, 1999. Catabolic pathway of caffeine and purification of a xanthine oxidase responsible for methyluric acid production in *Pseudomonas* L. Revista de Microbiologia, 30: 60-72.