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Research Article

A PRELIMINARY STUDY AND FIRST REPORT ON CAFFEINE DEGRADING BACTERIA ISOLATED FROM THE SOILS OF CHITTOOR AND VELLORE

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ABSTRACT
An attempt on basic study of the caffeine degrading organism and screening of potential 'caffeinase' producing bacteria has been studied and reported. Caffeine is present in soft drinks, coffee plants, tea leaves, and kola nuts and is used extensively in human consumption. Various health and environmental demerits makes it significant to reduce the levels of caffeine into a much less harmful compound, which can be done biologically using specific microorganisms. The enzyme responsible for caffeine degradation plays a major role and hence needs to be studied for caffeinase isolation and improvement of available caffeine products. Isolation of such microorganisms and their study of extent of caffeine degradation would prove to be helpful in generating an economic and safer method of caffeine removal in food products and coffee left over which could be less harmful to human health and the environment.
KEY WORDS: Caffeine, Degradation, Isolation, Microorganism, Caffeinase, Immobilization.

INTRODUCTION

Caffeine or 1, 3, 7-Trimethylxanthine is a plant product, present in beverages like coffee, tea, caffeinated soft drinks and cola[9, 12]. It is a potent stimulant that increases attentiveness, anxiety and cognitive performance[13]. A statistical study in United States has reported that average caffeine intake among adult consumers varies from 106 to 170 mg/day which is of major concern in the long run[5]. Caffeine acts as a psychotropic drug which is also consumed by significant population of children and women during pregnancy and childbirth[8]. Long term intake of caffeine is known to affect memory and performance of human brain, especially in due course of withdrawal which then leads to decreased attentiveness, increased cloudiness of the brain, physical and mental dependance[4, 8, 9, 19]. Also adverse effects of caffeine include increased frequencies of headaches and fatigue during caffeine withdrawal[11, 19].

Amounts of caffeine need to be reduced to lesser quantities in food to reduce side effects and the risk of dependence. Coffee pulp and husk can be utilized in the form of animal feed as they serve rich source of carbohydrates and proteins. Also these can be used for biogas production, fertilizers and other food sources, which can only be possible after sufficient detoxification by removal of caffeine[7, 14]. A majority of coffee waste products like pulp and husk are discarded into the environment, contributing to the pollution of water bodies. Decaffeination would assist in lowering down the levels of hydro-toxicity in rivers and lakes[10].

Earlier studies have reported the effects of pH, temperature, amount of inoculum and caffeine concentration on degradation of caffeine[1, 2]. These parameters were taken under consideration in making caffeine enriched media (CEM) for the screening and isolation of the specific organism. It is much more desirable to use enzymes for decaffeination process rather than using bacteria directly as this would affect sensory qualities of that food component[1,22]. Also another result showed that cellular extracts of bacteria could utilize caffeine upto 55 times, which serves advantageous in efficient removal of caffeine[3]. Hence it is crucial to purify and study the enzyme degrading caffeine. The enzyme of our study was termed 'caffeinase' as the exact nature and composition of the enzyme was unknown.

MATERIALS AND METHODS

Screening and Isolation

A caffeine enriched media (CEM) was prepared using Lauryl Sulphate HiVeg Broth (30.0g/l), anhydrous caffeine (0.3g/l), sodium chloride (0.5g/l) and coffee husk extract (0.5% w/v). Soils taken from near coffee shops and industry were serially diluted and then inoculated with incubation for 48 hours at 37°C. The colonies grew on the plate with four visible clear zones. These colonies were streaked in four different plates having CEM for further study.

Biochemical Test

The samples were subjected to a set biochemical tests using Himvic media for the characterization of the bacteria. The results were seen after 24 hrs of incubation and were studied with Bergey's Manual for Systematic Bacteriology[20] and Advanced Bacterial Identification Software (ABIS) to identify the bacteria.

UV-Visible Spectrophotometry

Earlier studies showed that caffeine gave its maximum absorbance at 275nm[16].The four isolated samples were grown in CEM at 37°C in an orbital shaker. The samples at two intervals, 24 hours and 48 hours were centrifuged at 12,000 rpm and the supernatant was checked for absorbance at 275nm using UV-Visible spectrophotometer, keeping CEM without caffeine component as blank.

Cell Immobilization

A mixture of 3% (w/v) of sodium alginate solution and 0.1% (w/v) sodium chloride was incubated for 4 hours to form an entrapment mix. 10% (w/v) calcium chloride solution was prepared and stored at 15°C. 3% of culture filtrate was added to entrapment mix and this mixture was divided into four parts. Extracts from carrot, turmeric powder, and beetroot for orange, yellow and red colours respectively were prepared by boiling them for 30 minutes. 1ml of each extract was added to the entrapment mix, leaving one as control. The coloured mixtures were separately added drop wise using a syringe to 20 ml of calcium chloride solution and were left for 30

minutes for hardening of beads. After solidification, they were washed twice stored using millipore water at 4°C.

Caffeinase Assay

0.1, 0.2, 0.5, 0.7 and 1mg of anhydrous caffeine was dissolved in a solution containing 1ml millipore water, 1ml phosphate buffer (pH 7.0), 5ml culture filtrate grown for 24 hours. After addition of 1ml glacial acetic acid, the mixture was incubated at 4°C for 30 minutes and checked for O.D at 540 nm using UV-Vis Spectrophotometer. Similar procedure was followed after 48 hours of culture.

KEGG Analysis

In the 'Kyoto Encyclopedia of Genes and Genomes' database, various pathways of caffeine degradation metabolism were checked. A potential end product, N-methyl urea was targeted for study. Earlier studies had showed the absorbance of N-methyl urea to be 265nm, which was qualitatively analyzed using double beam spectrophotometer, using a control broth devoid of caffeine component and the test having degraded caffeine(after 48 hours of incubation)[21].

RESULTS AND DISCUSSION

Screening and Isolation

The colonies, after incubation in CEM showed visible zones of degradation shown in figure 1.(a). These zones were hypothesized to be that of degraded caffeine and colonies from such four major zones were isolated. The isolated colonies were named Sample 1, 2, 3 and 4 according to the figure 1(b), (c), (d), (e), which all showed similar morphological characteristics on the CEM media, indicating the presence of the same organism.

Biochemical Test

Biochemical characteristics were studied for the samples and following results were obtained, shown in Table1. The results indicated the presence of gram positive rods. Strains of *Paenibacillus macerans* were deciphered and confirmed using Bergey's Manual[20] and the ABIS software.

UV-Visible Spectrophotometry

The UV-Visible spectrophotometry results gave different peaks at 280nm. The peaks dropped after 24 hours and 48 hours of incubation with minor shifts, indicating positive results for degradation of caffeine which are shown in fig. 2 (a), (b), (c), (d) for samples 1, 2, 3 and 4 respectively. Out of the four samples, sample 1 and 3 gave significant results for degraded caffeine, showing major drop in peaks, figure 2(a) and 2(c). These strains might produce caffeinase enzyme much more than the other strains and hence were taken for further study of the enzyme activity.

Table 1

Types of Test	Sample1	Sample2	Sample3	Sample4
Motility	+	+	+	+
Catalase	+	+	+	+
Indole Production	-	-	-	-
Methyl Red Test	+	+	+	+
Voges Proskauer Test	-	-	-	-
Citrate Utilization	-	-	-	-
Starch Hydolysis	+	+	+	+
Glucose Utilization	+	+	+	+
Adonitol Utilization	-	-	-	-
Arabinose Utilization	+	+	+	+
Lactose Utilization	-	-	-	-
Sorbitol Utilization	-	-	-	-
Mannitol Utilization	+	-	+	+
Rhamnose Utilization	-	-	-	-
Sucrose Utilization	+	+	+	+

Note: (+) Positive result and (-) Negative result

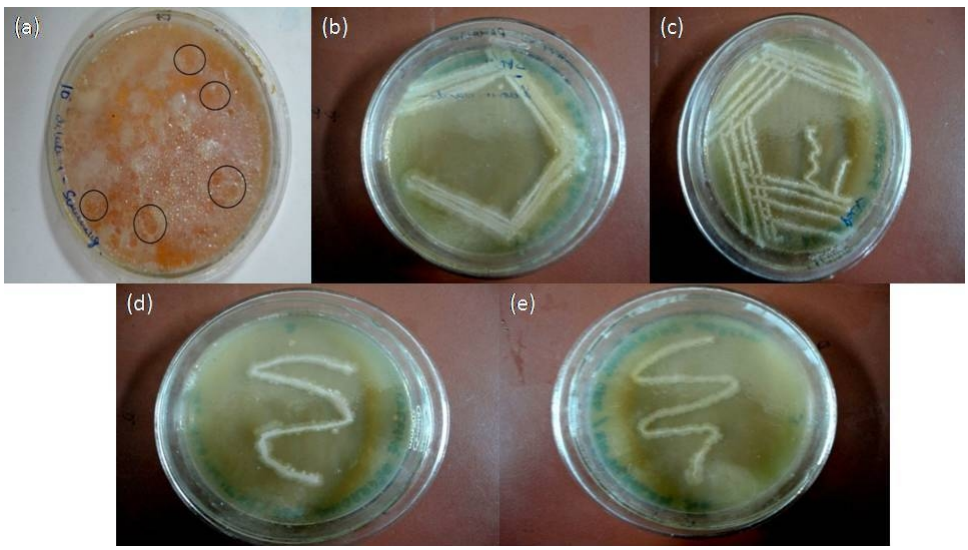


Fig.1 (a) Selective media showing zones of caffeine degradation from organisms present in sample soil; (b), (c), (d),(e). Pure and isolated samples of caffeine degrading organisms

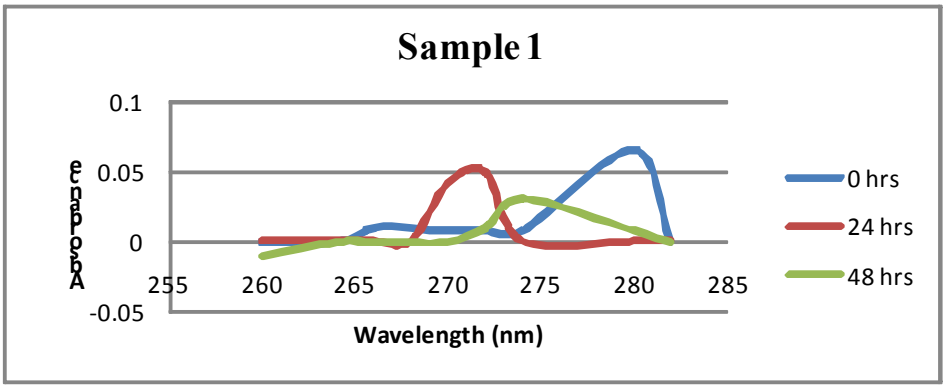


Fig.2(a).Sample 1 showing caffeine degradation in 48 hours

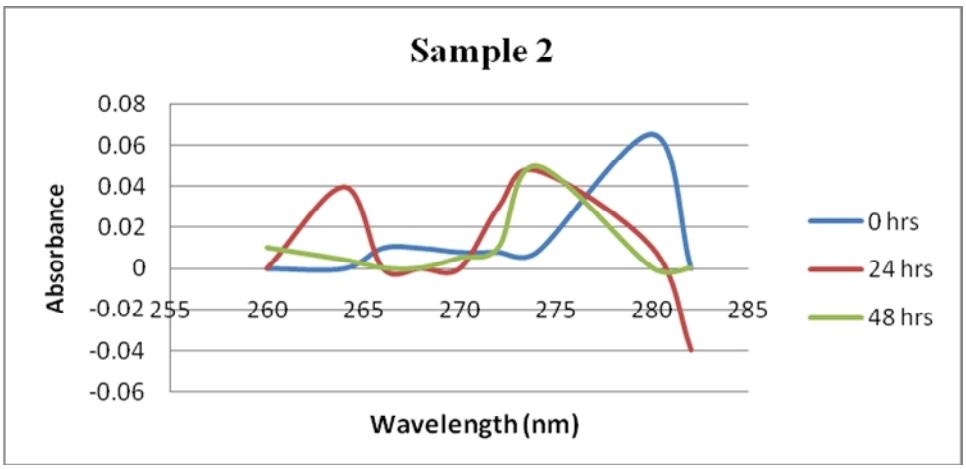


Fig.2(b).Sample 2 showing degradation in 24 hours and constant level of caffeine after 48 hours

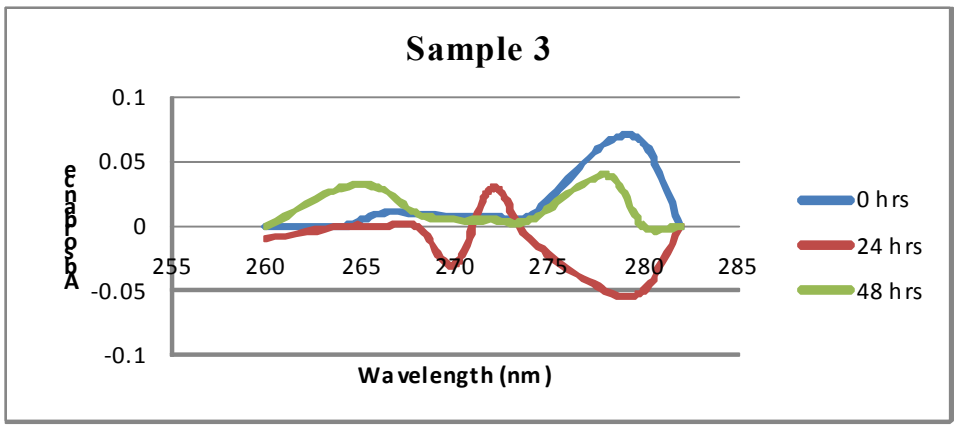


Fig.2(c).Sample 3 showing significant degradation in 24 hours and an increase in the peak after 48hours

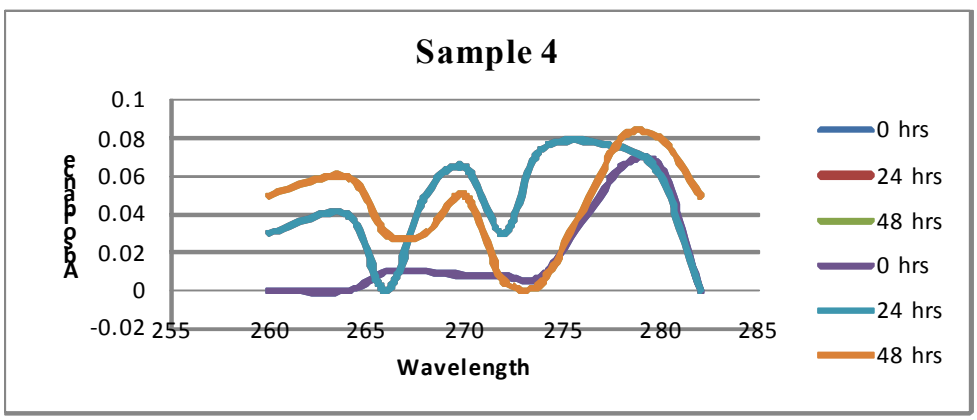


Fig.2(d).Sample 4 showing significant degradation in 24 hours and an increase in the peak after 48 hours

Cell Immobilization

The cells of *Paenibacillus macerans* were immobilised to form coloured beads illustrated by figure 3. The concentration of calcium chloride was varied to check the stability of the beads formed. Any concentration less than 10% (w/v) yielded unstable beads which dissolved in the solution and thus 10%(w/v) of calcium chloride was standardized. Beetroot, carrot and turmeric extracts yielded red, orange and yellow colored beads respectively, which were stored for further use.

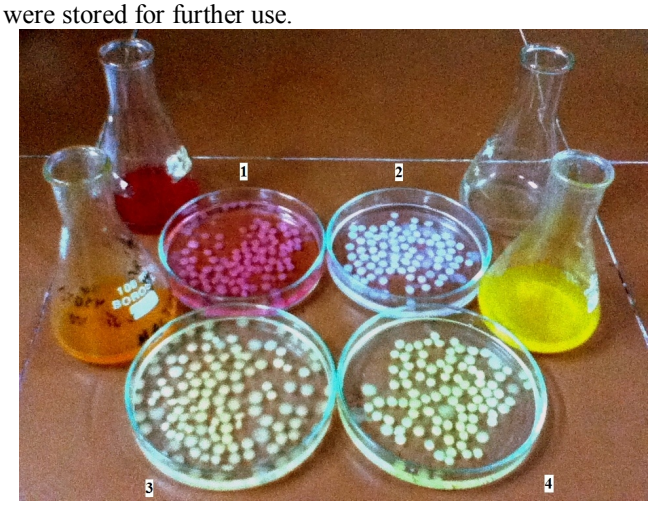


Fig.3. The Figure shows immobilized cells of *Paenibacillus marcens* that have been colored using beetroot extract(1), carrot extract(3), turmeric extract(4), and the control(2) to yield red, white, orange and yellow beads respectively

Caffeinase Assay

Previous studies have been done related to the effect of caffeine substrate on growth of microorganisms and the production of enzyme[1, 15]. Caffeine has been found to serve as a limiting component in the growth of cells[15].The assay result indicated the activity of the enzyme caffeinase formed by sample 3 culture, as a function of the substrate caffeine, which showed a gradual increase in the activity with a drop in peak at 0.7mg of caffeine, given in figure 4. This shows deviation from the conventional Michaelis–Menten curve, which may prevail due to the repressor activity of caffeine, which was absent on further increase in the concentration of caffeine.

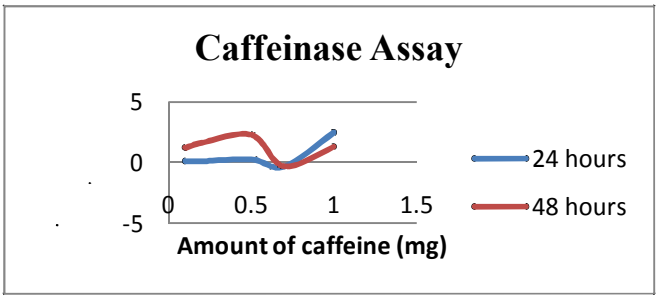


Fig.4.Caffeinase assay after 24 hours and 48 hours of incubation

KEGG Analysis

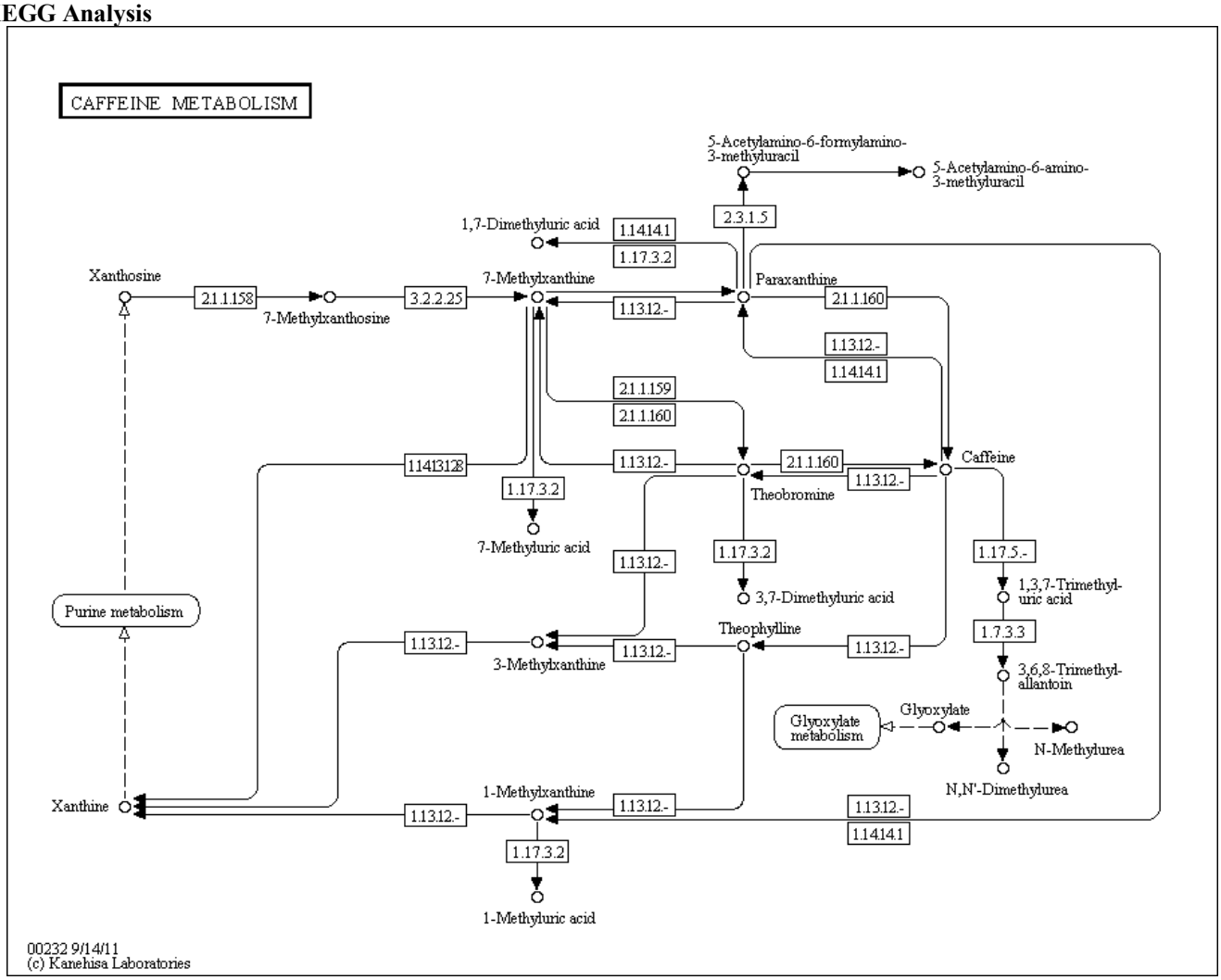


Fig.5 Caffeine metabolism reference pathway derived from KEGG Pathway database

Caffeine metabolism was found to be a part of xanthine pathway, shown in Figure 5, which generally yields N-methyl urea and N, N'- Dimethyl urea. Another pathway prevails where removal of three methyl groups results in the formation of xanthine which further gets degraded to yield CO_2 and NH_3 by acid hydrolysis[6]. However N-Methyl urea was formed instead of xanthine here. The UV/Vis spectrophotometer results, given in figure 6 gave a peak at 255nm, stating the presence of N-Methyl urea and confirming degradation of caffeine through this pathway[21]. Also a pathway study of *Paenibacillus macerans* using MetaCyc database was done to check for the existence of any caffeine metabolic pathway. Results showed three similar pathways where caffeine was utilized by demythylation or oxidation, further confirming that the enzyme caffeinase could either be caffeine demethylase or caffeine oxidase, which is accordance with the results reported by Gokulakrishnan et al[17]. Also the presence of caffeine metabolic pathway again confirms the ability of the origanism to degrade caffeine.

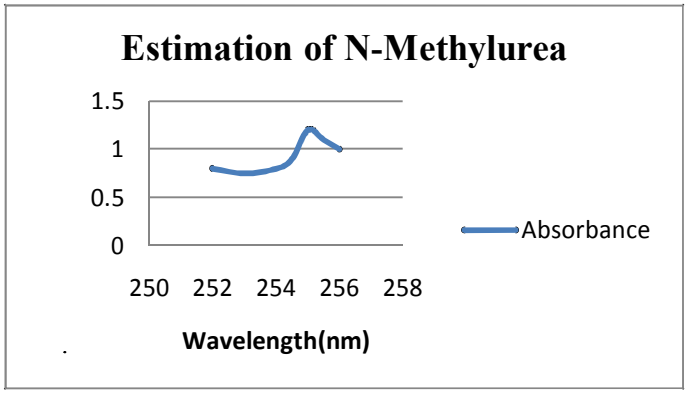


Fig.6. Gives the absorbance peak at 255nm, which shows the presence of the metabolite N-Methyurea

CONCLUSION

The isolate, *Paenibacillus macerans* was found to degrade caffeine, which may have potential applications in an environmental friendly and biological removal of caffeine in caffeinated food products and beverages. The study has formed a foundation and has created more scope for fabrication of more improved methods and techniques that would be considered in the future.

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REFERENCES

1. Sarath Babu VR, Patra S, Thankur MS, Karanth NG, Varadaraj MC. Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708. Enzyme And Microbial Technology, 2005, 37: 617-624.
2. Gokulakrishnan S, Chandraraj K, Gummadi SN. A preliminary study of caffeine degradation by Pseudomonas sp. GSC 1182. International Journal of Food Microbiology, 2007, 113: 346-350.
3. Juan GB, Richard LL, Wayne AB. Activity and stability of caffeine demethylases found in *Pseudomonas putida* IF-3. Biochemical Engineering Journal, 2006, 31: 8-13.
4. Peter JR, Claire D. Regular Caffeine Consumption: A Balance of Adverse and Beneficial Effects for Mood and Psychomotor Performance. Pharmacology Biochemistry and Behavior, 1998, 59: 1039-1045.
5. Knight CA, Knight I, Mitchell DC, Zepp JE. Beverage caffeine intake in US consumers and subpopulations of interest: estimates from the Share of Intake Panel survey. Food and Chemical Toxicology, 2004, 42: 1923-1930.
6. Hiroshi A, Hiroshi S, Alan C. Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering. Phytochemistry, 2008, 69: 841-856.
7. Walter P, Mario RM, Roberto GB, Ricardo B. Solid-State Fermentation: an Alternative to Improve the Nutritive Value of Coffee Pulp. Applied and Environmental Microbiology, 1985, 49: 388-393.
8. Gianluigi T, Steven RG. Alteration of the Behavioral Effects of Nicotine by Chronic Caffeine Exposure. Pharmacology Biochemistry and Behavior, 2000, 66: 47-64
9. Hiroshi A, Alan C. Caffeine: a well known but little mentioned compound in plant science. Trends in Plant Science, 2001,6: 407-413.
10. Hakil M, Denis S, Viniegra-Gonza´lez AC. Degradation and product analysis of caffeine and related dimethylxanthines by filamentous fungi. Enzyme Microbial Technology, 1998, 22: 355-359.
11. Roland RG, Phillip PW. Caffeine Physical Dependence: A review of human and laboratory animal studies. Psychopharmacology, 1988, 94: 437-451.
12. Anthony L, John RH, Jacob AG. Absorption and Subjective Effects of Caffeine from Coffee, Cola and Capsules. Pharmacology Biochemistry and Behavior, 1997, 58: 721-726.
13. Smith A. Effects of caffeine on human behavior. Food and Chemical Toxicology, 2002, 40: 1243-1255.
14. Dash SS, Sathyanarayana NG. Enhanced biodegradation of caffeine by *Pseudomonas* sp. using response surface methodology. Biochemical Engineering Journal, 2007, 36: 288-293.
15. Gokulakrishnan S, Sathyanarayana NG. Kinetics of cell growth and caffeine utilization by *Pseudomonas sp.* GSC 1182. Process Biochemistry, 2006, 41: 1417-1421.
16. Abebe B, Kassahun T, Mesfin R, Araya A. Measurement of caffeine in coffee beans with UV/vis spectrometer. Food Chemistry, 2008, 108: 310-315.
17. Gokulakrishnan S, Chandraraj K, Sathyanarayana NG. Microbial and enzymatic methods for the removal of caffeine. Enzyme and Microbial Technology, 2005, 37: 225-232.
18. Manish MP, Joseph I. Rapid determination of caffeine content in soft drinks using FTIR–ATR spectroscopy. Food Chemistry, 2002, 78: 261-266.
19. Paul TQ, Joan L, Karen LM, Jennifer A, Jane AR, Dawn CO. The Acute Physiological and Mood Effects of Tea and Coffee: The Role of Caffeine Level. Pharmacology Biochemistry and Behaviour, 2000, 66: 19-28.
20. Sneath PH, Mair NS, Sharpe ME, Holt JG, editors. Bergey's manual of systematic bacteriology. MD: Williams & Wilkins, Baltimore, 1986, 2: 965-1600.
21. Sidney MH, John WK. Mechanism of the base-induced decomposition of N-nitroso-N-methylurea. Journal of Organic Chemistry, 1973, 38: 1821-1824.
22. Avinash S, Anshul S, Suneetha V. Feather Waste biodegradation as a source of Amino acids. European Journal of Experimental Biology, 2011, 1,56-63.

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