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Biodegradation of Caffeine

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Abstract: ***Caffeine is a purine alkaloid which is naturally present in different varieties of plants, tea leaves, cocoa beans etc. It act as stimulant to central nervous system and has various negative withdrawal effect on health. Caffeine can be treated chemically as well as biologically. Biodegradation by bacteria is considered to be the most efficient technique in degrading caffeine within environment. In the present study, four isolates capable of utilizing caffeine as a sole source of carbon were isolated from garden soil. The bacteria were characterized by conventional morphological and biochemical test. To identify the intermediates and degradation end products, thin layer chromatography was performed in which Isolate 1 and Isolate 3 degrades caffeine and coffee respectively to form theobromine as the end product. Isolate 2 and Isolate 4 degrades caffeine and coffee respectively to form theophylline as the end product. Infrared spectroscopy of medium containing caffeine and coffee treated by four isolates shows the presence of functional group such as alcohol, amines and amides***

Keywords: Caffeine, Biodegradation, Thin Layer Chromatography, Infrared Spectroscopy

1. Introduction

Caffeine is a methyl xanthine molecule which is the most widely consumed psychoactive substance in the world, most commonly from the beverages coffee, tea and soda. The English word caffeine comes from the French (Spanish and Portuguese) word for coffee: café. Because of its stimulatory nature, it was used as a cardiotonic till the end of 19th century (wijhe, 2002).In the first half of the 20th century; it was used as a stimulant of respiration and circulation in Dutch medicine. The Islamic physician was the first to exploit the medicinal use of coffee well before second millennium A.D, the first documented use as a beverage was by the Sufis of Yemen. With caffeine being increasingly used as a stimulant, it was prohibited from being used as it was thought that caffeine used was a cause for wices and is seditious. Coffee was introduced to England around 1650's and in Holland a decade later. The Dutch introduced the coffee plant to the island of Java in 1688. The Island's association with coffee production led to the use of "Java" as a nickname for high quality coffee.

Caffeine is found in about a hundred species of plants, but the most highly cultivated sources are the coffee beans. (*Coffea arabica*or , *Coffea canephora*, variety robusta), the leaves and leaf-buds of tea (*Thea sinensis* or *Camellia sinensis*), cola nuts (*Cola acuminata*) and cacao beans (*Theobroma cacao*) [1].

Coffee and tea plants are the major sources of natural caffeine and related compounds such as theophylline and theobromine are produced by a large number of plant species belonging to numerous genera, families, andorders. It is believed that methylxanthine producing plants accumulate these substances as part of a chemical defense system against pests and herbivores [1].

A very large proportion of the non-alcoholic beverages used in social settings contain caffeine. The most important beverages and foods containing caffeine are coffee, tea ,guarana, mate, cola nuts, cola drinks, cocoa, chocolate .The amount of caffeine found in these products varies ,but is generally high. Based on dry weight, the highest amounts are found in guarana (4-7%).Tea leaves contain approximately 3-5% caffeine, coffee beans 1.1-2.2%(Saldana et.al 2000),cola nuts 1.5% ,and cocoa beans 0.03% (Bogo and Mantle,2000;Kretschmar and Baumann,1999).Cocoa beans in addition contain about 1.8-2.5% theobromine .Caffeine also occurs in certain soft drinks, and so called "smart "drinks ,as well as in medicinal drugs. In these cases, however, purified or synthesized caffeine has often been added to the products. Caffeine is responsible for the stimulant action of coffee (Europaisches, 1978). It stimulates the central nervous system, increases the contraction power of the heart, widens the vessels of heart, kidney and the skin and exhibits broncholytical and diuretical action.(Europaisches,1978) [1]..

In mammals, ingested caffeine is rapidly absorbed, metabolized, and excreted in the urine as methyl xanthine derivative. Apart from being a stimulant to the central nervous system, if consumed in excess it causes mutation; it is teratogenic, causes inhibition of DNA repair, inhibition of cyclic AMP phosphodiesterase activity and inhibits seed germination (Friedman and Waller, 1983 a and b). It is the major cause of cancer ,heart diseases, and complications in pregnant woman and aging (Green and Suls,1996;Infante et. al.,1993;Srisuphan and Bracken,1986,Dlugosz et .al.,1996; Fenster et.al.,1991) [1].

The stimulant effect of caffeine is thought to be due to an increase in adrenaline release, which may stimulate the sympathetic nervous system, but the mechanism is not completely understood (Clarkson,1993).Caffeine enhances Acetylcholine release in the hippocampus in vivo by a selective interaction with adenosine A1 receptors. Carter, et al, 1995).

It is known that caffeine is responsible for many effects on the human body. Details of the mechanism of caffeine at cellular and organ level can give an insight into how this molecule affects different functions in the body and the reasons for adverse effects on the body.

Caffeine is 1,3,7-trimethylxanthine,meaning it is a xanthine molecule with methyl groups replacing all of the three hydrogen's bound to nitrogen's in the xanthine ring. The molecular weight of caffeine is 194.2 and structurally it is

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related to uric acid and contains Imidazole and a uracil ring[1].

Theobromine and paraxanthine are dimethylxanthines derived from the removal of methyl groups at 1 and 3 N position of xanthine ring of caffeine. Caffeine ,paraxanthine and theobromine have stimulatory effects on humans, but caffeine due to it is 3 methyl groups is associated with highest stimulatory activity and other ill effects on health.Dimethyl xanthines are known to possess anti asthmatic ,anti-cancer and anti-oxidant properties[1].

Recent studies show that excess consumption of caffeine has many health implications in humans. Therefore decaffeination technologies have been developed since 1920's through chemical routes, these chemical decaffeination methods have several disadvantages. Biodecaffeination as an alternative route in the budding stage and knowledge about this area of coffee and tea biotechnogy is scarce. Biodecaffeination is the process of removal of caffeine from coffee, tea and other caffeine containing materials by the action of externally added microbial cells or enzymes.

2. Materials and Methods

1) Collection of sample: [6]
Soil was collected from garden which contains coffee dust and tea dust.

2) Screening and Enrichment[6]
Caffeine liquid medium broth containing0.2gm % caffeine was inoculated with soil sample (0.1gm +10ml sterile distilled water) and was kept for lncubation at 37^0C for 48 hours.

3) Isolation:
After enrichment 0.1ml sample was spreaded on caffeine medium plate containing 0.2gm caffeine using spread plate technique. Plates were incubated at 37^0C for 48 hours.

4) Identification of bacteria: [6]
The bacterial isolates were characterized by biochemical tests according to Bergey's manual of determinative bacteriology. Catalase, oxidase, nitrate reduction, indole production ,methyl red test, Voges Proskauer test, starch hydrolysis ,citrate utilization, oxidation, fermentation, motility, gelatin hydrolysis, sugar production and sugar utilization tests were conducted for the identification of the bacterial isolates.

5) Extraction of caffeine from coffee: [4]
22.9 gm coffee powder added in 500ml distilled water along with 15 gm sodium carbonate. Mixture was boiled. After cooling down at room temperature, all glassware was rinsed with chloroform and the solution was transferred to separating funnel. 50 ml chloroform was added and swirled vigoursly. Mixture was allowed to stand and bottom chloroform layer was separated to a beaker (repeat it for five times). Calcium sulphate was added to the separated chloroform to remove water. The mixture was shaked well until fluffy, cloudy effect

Weight of the beaker which is going to hold the filtrate was recorded, excess calcium sulphate was removed. The beaker with filtrate was kept into the hot waterbath to evaporate chloroform (BP61.2^{0}C).Weight of the powder was recorded and the amount of powder extracted was calculated by taking final reading of burette. The above steps were repeated until two to three consistent results are obtained.

6) Thin Layer Chromatography: [11]
Solvent system used was 95% ethyl acetate : 5% Acetic acid . Spot developed were observed under U.V. Transluminator.

7) Infrared Spectroscopy: [6]
Caffeine liquid medium broth containing 0.2gm% caffeine was inoculated with respective isolates and was incubate at 37^0C for 48 hours. Broth was centrifuged at 3000rpm for 10min. Supernatant was used as a sample for Infrared Spectroscopy.

3. Result and Discussion

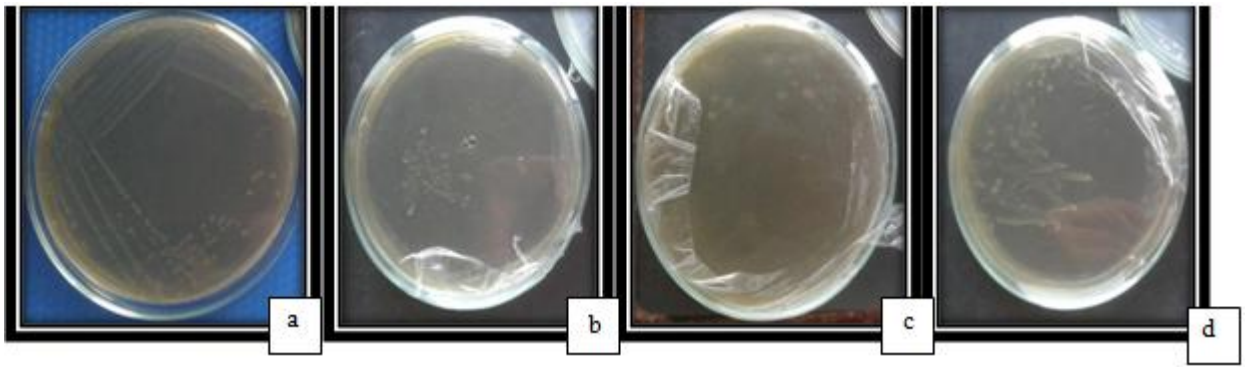

Figure 1: Four isolates showing growth on caffeine liquid medium. (0.2 gm% caffiene)

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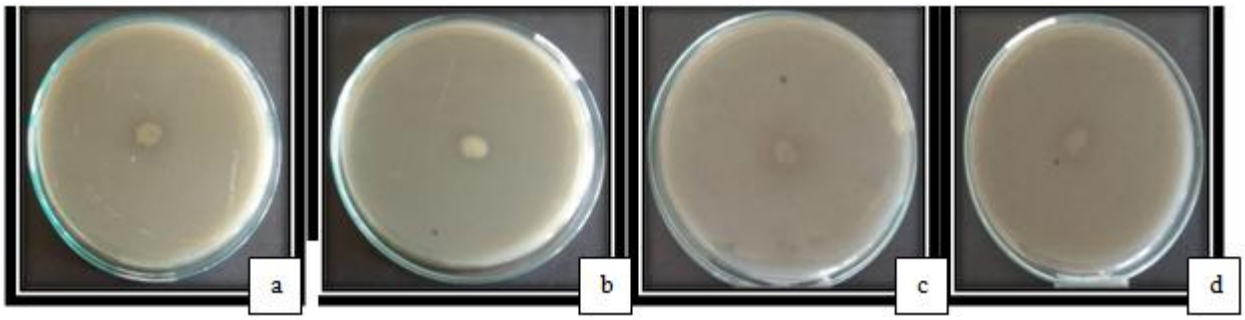

Figure 2: Zone of clearance observed around the colony of (a) Isolates 1, (b) Isolate 2, (c) Isolate 3, (d) Isolate 4 indicating extracellular degradation of caffeine. (0.2 gm% coffee powder)

Table I: Results for biochemical test of caffeine degrading isolates

Biochemical Test	Isolate-1	Isolate-2	Isolate-3	Isolate-4
Catalase	+	+	+	+
Oxidase	+	+	+	+
Nitrate reduction	-	-	+	-
Indole Production	-	-	-	-
Methyl red test	-	-	-	-
Voges Proskauer test	-	-	-	-
Starch hydrolysis	+	+	+	+
Citrate utilization	+	-	+	+
Gelatin hydrolysis	-	+	+	+
Casein hydrolysis	-	-	-	-
Urease	-	-	-	-
Triple Sugar Iron Agar	-	-	-	-

+=Positive Test, - = Negative Test

Table II: Results for sugar utilization and fermentation of caffeine degrading isolates

Sugar	Utilization				Fermentation			
	Isolate-1	Isolate-2	Isolate-3	Isolate-4	Isolate-1	Isolate-2	Isolate-3	Isolate-4
Inositol	+	-	+	+	+	-	-	-
Dextrose	+	-	-	-	+	-	+	-
Lactose	+	-	+	-	+	-	-	-
Sucrose	+	-	+	-	+	-	+	-
Xylose	-	+	-	-	+	-	+	-
Fructose	-	-	-	-	+	-	+	-
Maltose	+	+	+	+	+	-	+	-
Manitol	-	-	+	-	+	-	+	-

+=Positive Test, - = Negative Test

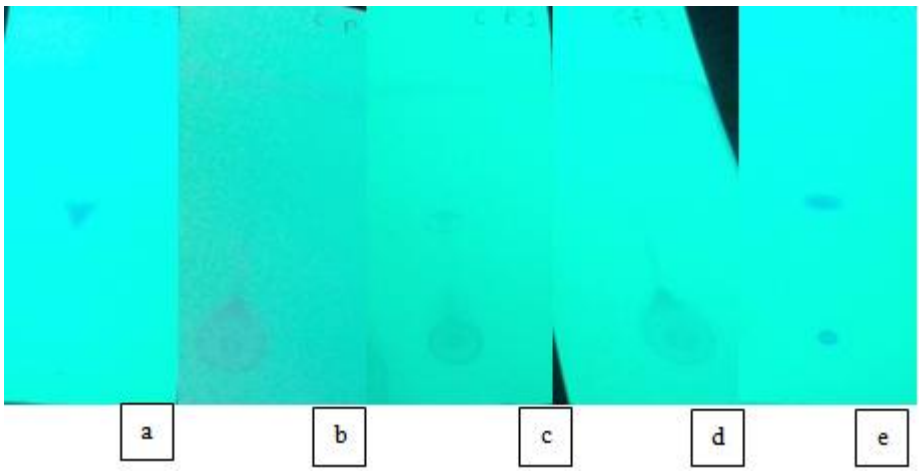

Figure 3: Thin layer chromatography showing degradation products of (a) Isolate 1, (b) Isolate 2, (c) Isolate 3,(d) Isolate 4 observed under UV (short wave length 200 nm). (e)- Control- uninoculated broth.

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Table III: R.F. Values of degradation products

Strain	R.F. value (coffee powder)	R.F. value (Caffeine)
Isolate 1	0.22	0.41
Isolate 2	0.33	0.32
Isolate 3	0.41	0.40
Isolate 4	0.36	0.36
Control (uninoculated)	0.38	0.30

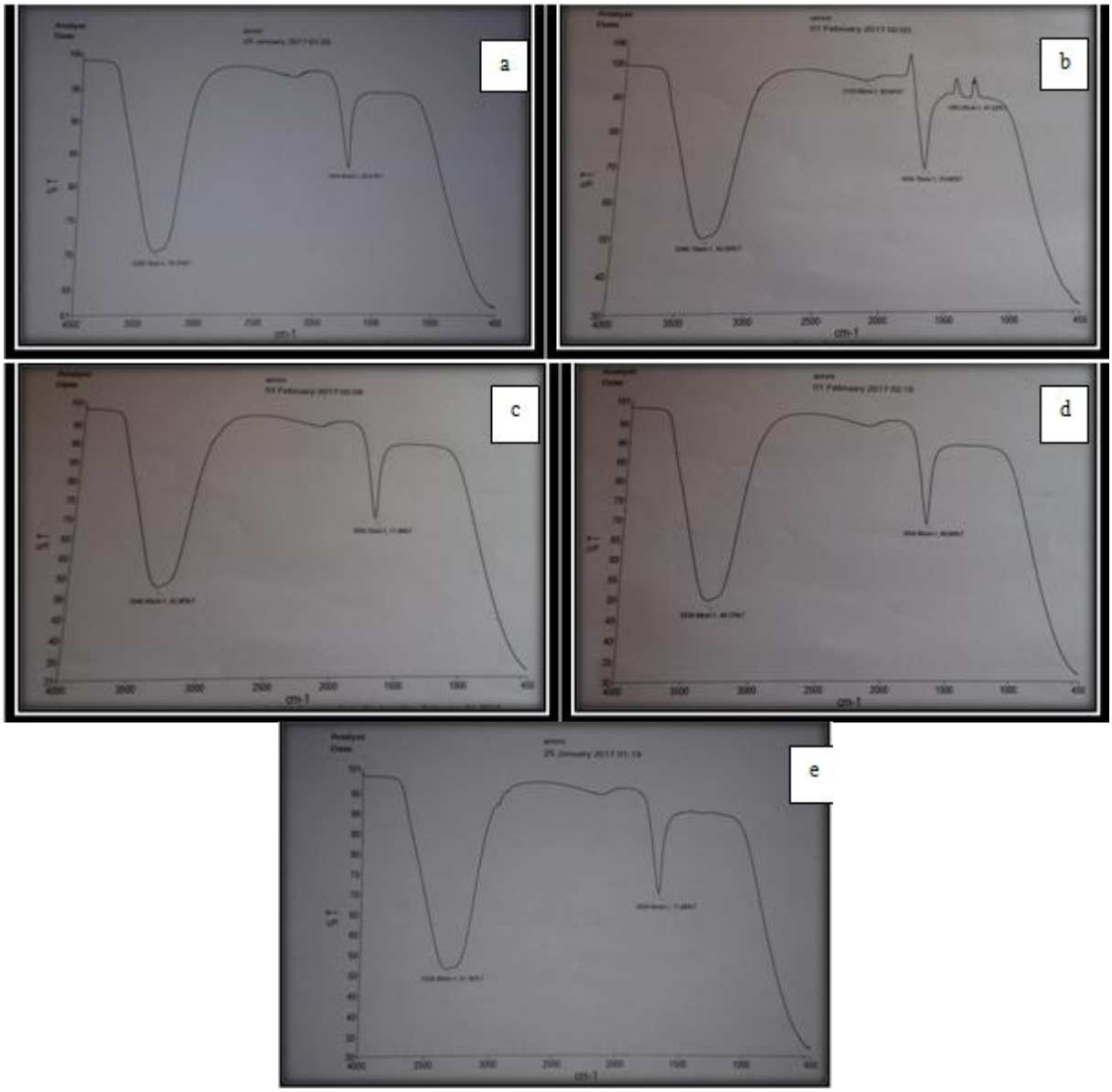

Figure 4: IR Spectra of (a) - Isolate-1,(b) - Isolate-2, (c)- Isolate-3,(d)- Isolate-4,(e)-control (uninoculated broth). Medium used CLM containing caffeine

Table IV: Functional groups of degradation product.Medium used CLM containing caffeine

Strain	Bond	Wave number	Functional Group
Isolate1	N-H	3339	Amines and Amides
	C=O	1634.40	Amides
Isolate 2	N-H	3340.19	Amines and Amides
	C=O	1634.76	Amides
Isolate 3	N-H	3340	Amines and Amides
	C=O	1634.72	Amides
Isolate4	N-H	3339.49	Amines and Amides
	C=O	1634.80	Amides

Four isolates were obtained from garden soil (fig1) on CLM medium. Four isolates were identified by morphological and conventional biochemical test as given in table no. I and II. The bacterial isolates 1 and 2 were identified using Bergey's manual of determinative bacteriology 9th edition as *Alcaligenes spp.* and *Acetobacte spp.* respectively. Isolate 3 and Isolate 4 are unknown.

From fig 2. Zone of clearance was observed around the colony of Isolate 1, Isolate 2, Isolate 3, Isolate 4 indicating extracellular degradation of caffeine.

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TLC performed for analysis of intermediates and end products of caffeine degradation by all isolates. Table 3 shows the RF value of intermediates & end product. isolates 1,2,3,4 can degrade caffeine up to uric acid, xanthine, therobronine, theophylline respectively as their RF value matches with standard RF values.[11]

Infrared Spectroscopy (FTIR) was performed to detect intermediate and end product after the treatment. Table IV shows the functional group with wave numbers. Comparing the functional group in table IV the intermediates or end product may be amines & amides [6]. The FTIR spectra support the degradation of caffeine by isolates.

From the result obtained it can be concluded that all four isolates can be used for biodecaffeination. All isolates shows extracellular enzymatic degradation of caffeine which should be studied to know the pathway and enzymes involved in the degradation. Biosensors using these isolates can be constructed to measure the caffeine content in the products such as coffee pulp or husk which contain large amount of caffeine.

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