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Biodecaffeination of coffee husk using bacterial enzyme from *Enterobacter aerogenes* isolated from coffee husk dumped soil

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Abstract
Bioremediation being a problem solver for many different environmental and other major hazards, they play an important and cost effective role in the biodecaffeination process. Twenty strains were isolated from coffee husk dumped soil, among that one strain could grow on the medium supplemented with 15g/L caffeine and could effectively degrade up to 2.5 g/L of caffeine in the liquid media as a sole source of carbon and nitrogen. Morphological and biochemical characteristics identified the organism as *Enterobacter aerogenes*. The degradation of caffeine was authenticated by growth curve and UV- Spectrometry analysis. The enzyme responsible for decaffeination was partially purified and identified using SDS- PAGE.

Keywords: Bioremediation, biochemical characterization, decaffeination, UV- Spectrometry.

1. Introduction

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6 dione), a purine alkaloid, which acts as a stimulant of central nervous system and also has negative withdrawal effects and is present in different varieties of plants such as coffee plant, tea leaves, colanut, cocoa beans and other plant. It is also present in soft drinks and is being used extensively in human consumption and has in addition some therapeutic uses but in minimal amount. Evidence has proved the harmful effects of caffeine thus opening a path in the field of caffeine biodegradation. Biodegradation by bacteria is considered to be the most efficient technique in degrading caffeine within the environment. Even though there are available methods for the removal of caffeine using conventional methods such as water, supercritical and solvent decaffeination but they are lack of accuracy/specificity for the removal of caffeine and in addition to the existing caffeine which sometime remains [1]. Caffeine content in regular and instant coffee ranges from 0.43 to 0.85 and from 0.61 to 0.82 mg/ml, respectively [22]. It stimulates the central nervous system, shows toxicity when fed in excess and is even mutagenic in vitro [4-8]. Excessive consumption of caffeine through beverages is associated with a number of health problems like adrenal stimulation, irregular muscular activity [3-14], cardiac arrhythmias [7] and increased heart output. Excess caffeine is reported to cause mutation [3], inhibition of DNA repairs and inhibition of adenosine mono phosphodiesterase [1] and during pregnancy causes malformation of fetus and may reduce fertility rates [16].

Studies on caffeine degradation by microorganisms were not reported till 1970 probably because caffeine was regarded as toxic to bacteria [23-24]. Caffeine concentration greater than 2.5 mg/ml in the growth medium has been found to inhibit the growth of many bacterial species. Moreover, caffeine is also one of the major agroindustrial wastes generated from the coffee and tea processing plants and these wastes are often released into the water bodies [17-18]. Therefore, decaffeination of waste is very necessary from the point of view of environmental conservation [9-10]. At higher concentration, caffeine is toxic to saprophytic microorganisms that are concerned in the important biotransformation within environment, which causes disorderliness in environmental stability. Coffee waste disposal signifies vast pollution problem in the producer countries. The attempt to make use of the coffee pulp as an animal feed source has been made from the economic to the environmental view point. For this reason, the removal of anti-nutritional parts such as caffeine becomes essential [15].

As microorganisms have gained the capability to adapt itself to grow in harsh environments through evolutionary processes, isolation of organisms from such environmental condition can yield interesting organisms with special characteristics. Generally caffeine hinders the growth of many organisms so, in the present work the source of bacterial isolation was selected based on were the organism would have best adapted to uptake caffeine as a sole source of carbon and nitrogen, after processing coffee by dry method the coffee husk is dumped in soil or burnt to ash. Hence from that soil the organism capable of degrading caffeine can be isolated efficiently. The present work deals with the isolation of caffeine degrading organisms from coffee hush dumped soil and investigating its ability to degrade caffeine by using different concentrations of caffeine in the medium provided for the growth of the organism and identification of the enzyme responsible for the degradation process.

2. Materials and Methods

2.1. Isolation of caffeine degrading bacteria

2.1.1. Collection of sample

Coffee husk dumped soil from AVT Coffee Company, Vellakinar Village, Coimbatore, Tamil Nadu. Initially before

serial dilution 1g of soil sample was inoculated into enrichment media (g/l) containing Disodium hydrogen phosphate (0.12), Potassium dihydrogen phosphate (1.3), Calcium chloride (0.3), Magnesium sulphate (0.3), Ferrous sulphate (0.02) [2]. From that medium 1ml was taken and transferred to serial dilution tubes and organisms were isolated by spread plate technique.

2.1.2. Screening of caffeine degrading bacteria

Enrichment media supplemented with caffeine (2.5 g/L) and agar was used as primary screening media. Organisms selected from the primary media was then inoculated into secondary screening media in which different concentrations of caffeine (0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 g/100ml) was used. The biochemical characterization of the isolates were done by performing IMViC, Oxidase test, Triple sugar iron test, Carbohydrate fermentation test for glucose, sucrose, maltose and lactose, Gelatin hydrolysis, Caesin hydrolysis, Starch hydrolysis, Catalase and Urease production.

The strain capable of growing in high concentration of caffeine was selected and transferred to the enrichment broth with caffeine (2.5g/L) and incubated at 37˚C in shaking incubator 120 rpm, for 48 hrs. The organism with high caffeine degradative capacity was identified using MALDI-TOF analysis (MALDI- Biotyper)

2.2. Analytical methods

2.2.1. UV- Spectrometry Analysis

Samples from the enrichment broth were retrieved at different intervals (24 and 48 hrs) and checked for absorbance at 275nm in UV- Spectrometry [6], were caffeine was used as control. The ability of the organism to degrade caffeine was determined by comparing the spectrum of caffeine with that of the sample.

2.2.2. Growth curve experiment

The growth curve experiment was carried out to understand the effect of caffeine on the growth kinetics of the organism. In the control flask the organism was inoculated into enrichment media without caffeine so that the normal growth of the organism can be analyzed and the test flask media was supplemented with Caffeine (2.5g/L) [13].

2.3. Partial purification of enzyme

2.3.1. Ethanol precipitation

To 1 volume of crude enzyme 3 volumes of pre-chilled ethanol was added and incubated overnight at 4 ˚C. The precipitated enzymes were pelleted by centrifuging at 10000 rpm for 20 min at 4˚C. Then the pellets were dissolved in 0.1 M potassium phosphate buffer (pH 7.4) and stored at -20 ˚C for further analysis of enzyme activity.

2.3.2. Acetone precipitation

To 1 volume of crude enzyme 9 volumes of pre-chilled acetone was added and incubated over night at 4 ˚C. The precipitated enzymes were pelleted by centrifuging at 10000 rpm for 20 min at 4 ˚C. Then the pellets were dissolved in 0.1 M potassium phosphate buffer (pH 7.4) and stored at -20 ˚C for further analysis of enzyme activity

2.3.3. SDS-PAGE Analysis

Estimation of protein was done by Lowry's method [26]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) [27] using 12% cross-linked polyacrylamide gel. Comassie Brilliant blue staining was carried out to visualize protein bands on the gel [25]. From these protein bands the molecular weight of this protein is compared with the help of standard molecular marker (220-14 KDa) (Bio-rad-USA). The partially purified enzyme from acetone precipitation and crude extract was loaded in SDS PAGE for the identification of the enzyme.

3. Results

For the isolation of caffeine degrading bacteria the coffee husk dumped soil (1g) was primarily inoculated in the enrichment media, from which it was further taken for serial dilution which yielded about 20 different bacterial strains.

The 20 strains were then subjected to different levels of screening process to find the potent caffeine degrading bacterial strain. The primary screening results showed that about five isolates (1C, 2C, 3C, 4C, 5C) were capable of degrading caffeine at a level of 2.5g/L. The Table 1 shows the biochemical and physiological characterization of the isolates.

Table 1: Biochemical characterization of all the isolates.

TEST	1C	2C	3C	4C	5C
Indole	+	-	-	+	-
Methyl red	+	+	+	+	-
Vp	-	-	-	-	-
Citrate	+	-	+	+	+
Alkaline slant	+	+	+	+	+
Alkaline butt	-	-	-	-	-
Gas production	+	+	-	+	-
Casein	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-
Catalase	+	+	+	+	+
Urease	-	-	-	-	-
Gelatin	-	-	-	+	-
Carbohydrate fermentation:					
Lactose	+/NG *	+/ NG	+/ NG	+/G	+/ NG
Sucrose	+/ G *	+/ G	+/ NG	+/ G	+/ NG
Maltose	+/ G	+/ G	+/ NG	+/ G	+/ NG
Dextrose	+/ G	+/ G	+/ NG	+/ G	+/ NG
Oxidase	+	-	+	-	-

*NG- No gas production, G- Gas production.

From the 5 isolates taken for secondary screening 3C was able to tolerate up to 15 g/L of caffeine and 5C was capable of tolerating 20g/L of caffeine supplemented with other media components. The two isolates (3C and 5C) were inoculated into enrichment broth with different concentration of caffeine to analyze how much amount of caffeine was degraded by the organism within 24hrs of growth in UV- Spectrometry.

5C was able to degrade up to 1.5g/L of caffeine and taken for further analysis. 5C was identified as *Enterobacter aerogenes* by physiological, biochemical and MALDI- TOF analysis were it was authenticated as *Enterobacter aerogenes* with a score value of 1.985.

Analyte ID	Organism (Best Match)	Score value
5C	*Enterobacter aerogenes*	1.985

The UV spectrometry analysis revealed the best organism which could degrade caffeine better which was shown in the Fig1.

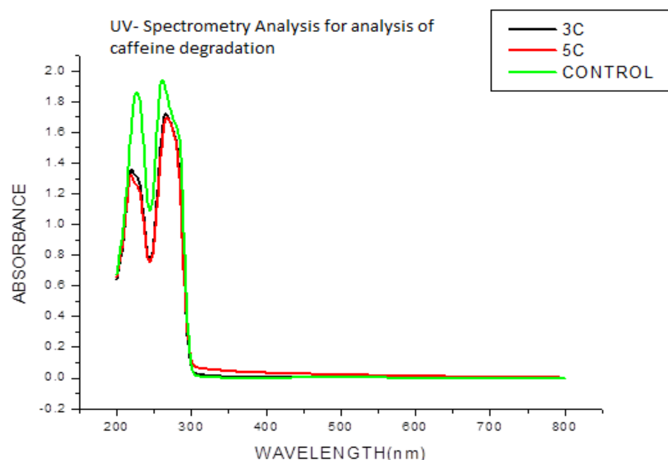


Fig 1: The UV spectrometry analysis of 3C, 5C along with control to analyze the degradative capacity of caffeine.

To study the effect of caffeine on the growth pattern of the organism (5C) the organism was inoculated into medium containing caffeine and a control medium without caffeine being inoculated with the same organism, the results were shown in Fig: 2

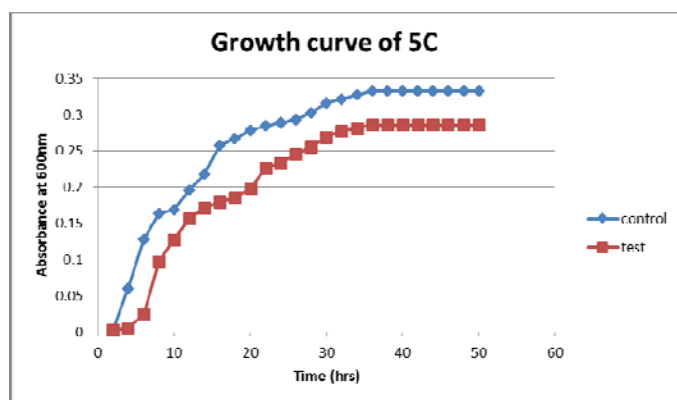
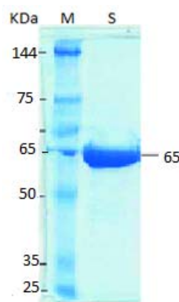


Fig 2: Growth curve of 5C showing difference in growth pattern with (Test) and without caffeine (Control).

The enzyme produced by *Enterobacter aerogenes* was analysed by SDS- PAGE analysis. 8600µg/100 ml protein was observed from acetone precipitation and 8000µg/100 ml protein from ethanol precipitation.



The SDS gels exhibited a clear band at the position were the marker showed about 65 KDa of protein.

4. Discussion

The enrichment media helps the growth of bacterial colonies at lesser population in the soil, hence the soil sample was first inoculated into enrichment broth and after incubation for 24-48 hrs and 1ml from the broth was taken and serially diluted. About 20 different bacterial colonies were isolated. 5 isolates were able to grow in the primary screening plates supplemented with 2.5g/L of caffeine which actually clearly indicates that how some species of organism get adapted to grow utilizing different substrates for their growth.

5C having high caffeine degradation ability which was revealed by the UV spec peak differences when comparing with that of standard caffeine. The UV spec analysis revealed that the intensity of the peak produced by the organism was lesser than that of the standard peak of caffeine. In the flasks with caffeine concentration more than 1.5g/L hindered the growth of the bacteria and there was no degradation of caffeine. Since that *Enterobacter aerogenes* was able to degrade 1.5g/L of caffeine.

As the organism was able to uptake caffeine its effect on the growth of the organism was monitored by growth curve experiment. The control flask showed a normal growth of the bacterium and there were changes in the growth pattern of organism on the test flask. This may be due to the fact that caffeine disturbs the cellular metabolism of the bacteria so that the log phase was prolonged and delayed lag phase along with less cell density was observed.

It was known that caffeine was degraded by different type of enzymes like caffeine oxidase, caffeine demethylase and calferine dehydrogenase [19-20], the SDS- PAGE analysis of the enzyme produced by *Enterobacter aerogenes* was done using two types of precipitation procedures. Acetone precipitation was considered a better method for the partial purification of the protein responsible for caffeine degradation. The SDS-PAGE analysis showed a marked band near the 65 KDa marker. By referring the works of Madyastha [28] and Mohapatra [29] the enzyme produced by the organism may be caffeine oxidase which has a molecular weight of 65 KDa.

5. Conclusion

The present investigation focuses on the isolation of Caffeine degrading bacteria from the environment where caffeine would be the sole source of carbon and nitrogen. The partially purified enzyme from *Enterobacter aerogenes* isolated from coffee husk dumped soil might be caffeine oxidase capable of degrading caffeine up to 1.5 g/l efficiently with an incubation time of 24 hrs.

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