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BIODEGRADATION OF CAFFEINE BY *Psuedomonas sp.* IN DIFFERENT COFFEE POWDERS

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ABSTRACT

Caffeine is an alkaloid naturally occurring in coffee,cocoa beans and tea leaves and it is described as the methylated xanthine alkaloid derivative.The presence of caffeine leads to severe health problems for which decaffeination process was carried out to degrade it. The main aim of the study was to degrade the caffeine in different coffee powders .The amount of coffee powder to be used for estimation of CF content was standardised to be 10 mg. The caffeine content for various coffee powders were estimated using methanol and water extraction using which the sample with high caffeine content was determined. Different stains of organisms were isolated from the soil sample , in which *Pseudomonas fluroscens* was used for caffeine degradation. *Pseudomonas fluroscens* was characterized using two tests Gram staining which found to be negative and Catalase test which found to be positive. Effect of different sources like carbon , nitrogen and physical parameters like pH , rpm were estimated and finally medium optimization using PBD was performed .The sample with high caffeine content was estimated to be S_2 – 0.243g , S_4 -0.289 ,S_9-0.226.Further these were degraded to the percentage of 35.95% , 54.35% , 42.22% respectively after 24 hrs. Then in 48 hrs the degradation increased to 49% , 64% , 51.5% respectively .The effect of the carbon source showed high degradation in 1% than 4%.Whereas nitrogen source showed decrease in caffeine degradation.The physical parameters like pH showed high degradation at pH 6.5 to be 70.4% and at around 190 rpm the degradation was around 77.52%.

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LIST OF ABBREVIATIONS

CF	Caffeine
SPI	Sodium Meta Periodate
MBTH	3-methyl 2-benzo thiazolinone hydrazone hydrochloride
HPLC	High performance liquid chromatography
DCM	Dichloromethane
CNS	Central Nervous System
IUPAC	International Units of Physics And applied Chemistry
NOS	Not Otherwise Specified
CLM	Caffeine Liquid Media
°C	Degree Centigrade
UV-Vis	Ultra Violet - Visible
PDA	Potato Dextrose Agar
SMF	Standard Measuring Flask
NB	Nutriet broth
NA	Nutrient agar
NaOH	Sodium hydroxide
HCl	Hydrochloric acid

Hr	Hour
Mg	Milligram
G	Gram
PBD	Plackett-Burman design
Ml	Milliliter
Nm	Nanometer
CS	Carbon sources
NS	Nitrogen sources

INTRODUCTION

1. INTRODUCTION

Caffeine is a colourless, odourless compound having a slight bitter taste. It's a crystalline methylxanthine that acts as a psychoactive stimulant drug and a mild diuretic (speeds up urine production) (Maughan *et al.,* 2003) in humans and other animals. It's IUPAC Name is 1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*) - dione. Other names can be given as trimethylxanthine, theine, methyltheobromine. Molecular formula can be given as $C_8H_{10}N_4O_2$. Caffeine was discovered by a German chemist, Friedrich Ferdinand Runge, in 1819. He coined the term "kaffein", a chemical compound in coffee, which in English became caffeine. The organoleptic characters of caffeine are, it sublimes at 180^0C and its melting point is 236.5^0C. It dissolves in boiling water but in room temperature it dissolves in organic solvents. In aqueous solution caffeine associates to form at least a dimer and probably polymer. Caffeine crystallizes from water as monohydrate. (Gilkey *et al.,* 1984).

Caffeine is biosynthesized from theobromine in coffee plant with the help of an bifunctional enzyme S-adenosyl-L-methionine:theobromine 1-N-methyltransferase (Mazzafera *et al.,* 1994). Caffeine is an achiral molecule, and therefore has no enantiomers; nor does it have other stereoisomers. The disparity in experience and effects between the various natural caffeine sources could be due to the fact that plant sources of caffeine also contain widely varying mixtures of other xanthine alkaloids, including the cardiac stimulants theophylline and theobromine and other substances such as polyphenols which can form insoluble complexes with caffeine (Balentine *et al.,* 1998).

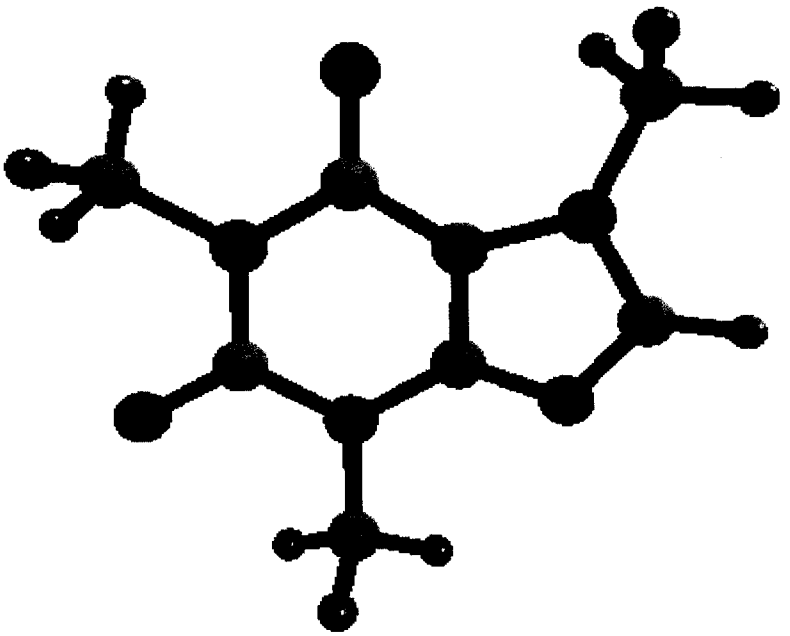

Fig.No.1.1: STRUCTURE OF CAFFEINE

Generally, caffeine is a central nervous system (CNS) stimulant, having the effect of temporarily warding off drowsiness and restoring alertness. Caffeine is the world's most widely consumed psychoactive substance, but unlike many other psychoactive substances it is legal and unregulated in nearly all jurisdictions. In the brain, its principal mode of action is as an antagonist of adenosine receptors. The caffeine molecule is structurally similar to adenosine, and binds to adenosine receptors on the surface of cells without activating them (an "antagonist" mechanism of action). Therefore, caffeine acts as a competitive inhibitor.

Because caffeine is primarily an antagonist of the central nervous system's receptors for the neurotransmitter adenosine, the bodies of individuals who regularly consume caffeine adapt to the continual presence of the drug by substantially increasing the number of adenosine receptors in the central nervous system. This increase in the number of the adenosine receptors makes the body much more sensitive to adenosine, with two primary consequences.

phenomenon known as a tolerance adaptation. Second, because these adaptive responses to caffeine make individuals much more sensitive to adenosine, a reduction in caffeine intake will effectively increase the normal physiological effects of adenosine, resulting in unwelcome withdrawal symptoms in tolerant users (Green *et al.,* 1986).

Caffeine from coffee or other beverages is absorbed by the stomach and small intestine within 45 minutes of ingestion and then distributed throughout all tissues of the body (Ted Wilson, Norman J. Temple 2004).The half-life of caffeine—the time required for the body to eliminate one-half of the total amount of caffeine—varies widely among individuals according to such factors as age, liver function, pregnancy, some concurrent medications, and the level of enzymes in the liver needed for caffeine metabolism. In healthy adults, caffeine's half-life is approximately 4.9 hours. In women taking oral contraceptives this is increased to 5–10 hours (Meyer *et al.,* 1991) and in pregnant women the half-life is roughly 9–11 hours (Ortweiler, *et al.,* 1985). Caffeine can accumulate in individuals with severe liver disease, increasing its half-life up to 96 hours. In infants and young children, the half-life may be longer than in adults; half-life in a newborn baby may be as long as 30 hours. Other factors such as smoking can shorten caffeine's half-life (Springhouse 2005). Fluvoxamine reduced the apparent oral clearance of caffeine by 91.3%, and prolonged its elimination half-life by 11.4-fold (from 4.9 hours to 56 hours).

In humans and other animals, caffeine is first degraded to either paraxanthine (1,7-dimethylxanthine), theobromine or theophylline. The demethylation process is carried out by cytochrome P450 (Elias, 1986; Berthou *et al.,* 1992). After a short period of caffeine ingestion, these dimethylxanthines can be detected in the urine. However, they can also be further demethylated to

methylxanthines produced during the catabolic pathway can be oxidized to the corresponding methyluric acids (Berthou *et al.*, 1992).

Caffeine tolerance develops very quickly, especially among heavy coffee and energy drink consumers. Complete tolerance to sleep disruption effects of caffeine develops after consuming 400 mg of caffeine 3 times a day for 7 days. Complete tolerance to subjective effects of caffeine was observed to develop after consuming 300 mg 3 times per day for 18 days, and possibly even earlier (Roland *et al.*, 2000). In another experiment, complete tolerance of caffeine was observed when the subject consumed 750–1200 mg per day while incomplete tolerance to caffeine has been observed in those that consume more average doses of caffeine (Griffiths *et al.*, 2003).

Caffeine is not stored in the body, but you may feel its effects for up to 6 hours. Many people feel that caffeine increases their mental alertness. Caffeine can also interfere with normal sleep. Most adults need seven to eight hours of sleep each night. But caffeine can interfere with this much-needed sleep (Cauli *et al.*, 2005). Chronically losing sleep whether it's from work, travel, stress or too much caffeine results in sleep deprivation. Sleep loss is cumulative, and even small nightly decreases can add up and disturb ones daytime alertness and performance.

Caffeine sensitivity (the amount of caffeine that will produce an effect in someone) varies from person to person. On average, the smaller the person, the less caffeine needed to produce side effects. Caffeine sensitivity is most affected by the amount of caffeine a person has daily. People who regularly take in a lot of caffeine soon develop less sensitivity to it. This means they may need more caffeine to achieve the same effects.

An acute overdose of caffeine, usually in excess of about 300 milligrams, dependent on body weight and level of caffeine tolerance, can result in a state of central nervous system over-stimulation called *caffeine intoxication*,

colloquially "caffeine jitters". The symptoms of caffeine intoxication are not unlike overdoses of other stimulants. It may include restlessness, nervousness, excitement, insomnia, flushing of the face, increased urination, gastrointestinal disturbance, muscle twitching, a rambling flow of thought and speech, irritability, irregular or rapid heartbeat, and psychomotor agitation. In cases of much larger overdoses mania, depression, lapses in judgment, disorientation, disinhibition, delusions, hallucinations and psychosis may occur, and rhabdomyolysis (breakdown of skeletal muscle tissue) can be provoked.

In large amounts, and especially over extended periods of time, caffeine can lead to a condition known as *caffeinism* (Mackay *et al.,* 1989). Caffeinism usually combines caffeine dependency with a wide range of unpleasant physical and mental conditions including nervousness, irritability, anxiety, tremulousness, muscle twitching (hyperreflexia), insomnia, headaches, respiratory alkalosis, and heart palpitations (Leson *et al.,* 1988). Furthermore, because caffeine increases the production of stomach acid, high usage over time can lead to peptic ulcers, erosive esophagitis, and gastroesophageal reflux disease.

Higher doses of caffeine can cause anxiety, dizziness, headaches, and the jitters. If someone is susceptible to the effects of caffeine, just small amounts, even one cup of coffee or tea may prompt unwanted effects, such as anxiety, restlessness, irritability and sleep problems (Retey *et al.,* 2007). How one react to caffeine may be determined in part by how much caffeine they used to drink. So, people who don't regularly consume caffeine tend to be more sensitive to its negative effects (Attwood *et al.,* 2007). Other factors may include body mass, age, smoking habits, drug or hormone use, stress and health conditions such as anxiety disorders. Sex may even play a role: Research suggests that men are more susceptible to caffeine than are women (Adan, 2008).

Caffeine may also cause the body to lose calcium, and that can lead to

bone loss over time. Drinking caffeine-containing soft drinks and coffee instead of milk can have an even greater impact on bone density and the risk of developing osteoporosis.

Caffeine can aggravate certain heart problems. It may also interact with some medications or supplements. If you are stressed or anxious, caffeine can make these feelings worse. Although caffeine is sometimes used to treat migraine headaches, it can make headaches worse for some people. Caffeine also effects the release of free fatty acids from adipose (fatty) tissues.

Excessive amounts of ingested caffeine may result in nervousness, dehydration, irregular heartbeat, trembling and/or insomnia. Intake of caffeine by pregnant women indicate risks for miscarriages or delays in fetus development when caffeine doses exceed 300 mg/ day (equivalent to three cups of coffee). Also, caffeine is mildly addictive and users may experience withdrawal symptoms such as headache, fatigue, depressed mood, difficulty concentrating, irritability, nausea/ vomiting and or muscle pain/ stiffness.

Certain medications and herbal supplements negatively interact with caffeine. Here are some examples.

- **Ciprofloxacin (Cipro) and norfloxacin (Noroxin)** — types of antibacterial medications — can interfere with the breakdown of caffeine. This may increase the length of time caffeine remains in the body and amplify its unwanted effects.
- **Theophylline (Theo-24, Uniphyl, others).** This medication — which opens up bronchial airways by relaxing the surrounding muscles (a bronchodilator) — tends to have some caffeine-like effects. Taking it along with caffeinated foods and beverages may increase the concentration of theophylline in the blood. This can cause ill effects, such as nausea, vomiting and heart palpitations.
- **Ephedra (ma-huang).** This herbal dietary supplement increases the risk

becomes especially risky. The Food and Drug Administration (FDA)has banned ephedra because of health concerns. The ban applies to dietary supplements but not herbal teas, which may still contain this herb.

The dry harvesting of coffee generates large amounts of coffee husks. Farmers use a small percentage of these husks as fertilizer, but discard most of the product. Another potential application of the husk material is to use it in animal feed as a cereal grain replacement. However, animals find the husks unappealing due to the caffeine content. This observation has led to an interest in using microbial enzymes to biodegrade the husk caffeine (Mazzafera, 2002).

1.2. OBJECTIVES

1. Estimation of caffeine in different coffee powders.
2. Isolation of Caffeine degrading organism (*Pseudomonas sp.*) from coffee plantation soil.
3. Comparative study on biodegradation of caffeine in different coffee powders.
4. Study of Caffeine degradation efficiency by the *Pseudomonas sp.* in different parameters.

LIRERATURE REVIEW

2. LITERATURE REVIEW

2.1. Decaffeination - An Overview

The adverse effects of caffeine have given rise to the development of several methods for the removal of caffeine from beverages. Decaffeination is the act of removing caffeine from coffee beans, cocoa, tea leaves and other caffeine-containing materials. (While caffeine-free soft drinks are occasionally referred to as "decaffeinated".

In the case of coffee processing, various methods have been used. The process is usually performed on unroasted (green) beans, and starts with steaming of the beans. They are then rinsed in solvent that contains as much of the chemical composition of coffee as possible without also containing the caffeine in a soluble form. The process is repeated from 8 to 12 times until it meets either the international standard of having removed 97% of the caffeine in the beans or the EU standard of having the beans 99.9% caffeine free by mass. Coffee contains over 400 chemicals important to the taste and aroma of the final drink; this effectively means that no physical process or chemical reaction will remove only caffeine while leaving the other chemicals at their original concentrations.

Coffea arabica normally contains about half the caffeine of Coffea robusta. A *Coffea arabica* bean containing little caffeine was discovered in Ethiopia in 2004. Also, genetic engineering may be eventually applied to create an inherently caffeine-free coffee.

2.2. Caffeine content of decaffeinated coffee

Almost all brands of decaffeinated coffee still contain some caffeine. Drinking five to ten cups of decaffeinated coffee could deliver as much caffeine as it would do with one or two cups of regular coffee, according to research at the University of Florida Maples Center for Forensic Medicine. In one

independent research on 10 popular decaffeinated coffees, researchers found that all but one contained caffeine. The 16-ounce cups of coffee samples contained caffeine in the range of 8.6 milligrams to 13.9 milligrams. In another similar study of popular brands of decaf coffees, the caffeine content was from 3 milligrams and up to 32 milligrams.

2.3. Different types of decaffeination processes

2.3.1. Roselius process

The first commercially successful decaffeination process was invented by Ludwig Roselius and Karl Wimmer in 1903. It involved steaming coffee beans with a brine (salt water) solution and then using benzene as a solvent to remove the caffeine. Coffee decaffeinated this way was sold as Coffee (Kaffee, Koffie etc.,) HAG (from Kaffee Handels Aktiengesellschaft or Coffee Trading Company) in most of Europe, as *Café Sanka* in France and later as Sanka brand coffee in US. Due to health concerns regarding benzene, this process is no longer used commercially and Coffee Hag and Sanka are produced using a different process.

2.3.2. Swiss water process

The Swiss water process is a method of decaffeinating coffee beans developed by the Swiss Water Decaffeinated Coffee Company. To decaffeinate the coffee bean by the Swiss water method, a batch of green (unroasted) beans was soaked in hot water, releasing caffeine. When all the caffeine and coffee solids were released into the water, the beans were discarded. The water then passes through a carbon filter that traps caffeine but lets the coffee solids also to pass through. The resulting solution, called "flavor-charged" water by the company, is then put in a similar filtration device, and new coffee beans were added. Since the flavor-charged water cannot remove any of the coffee solids from the new beans, only the caffeine was released. The process was repeated,

beans were removed and dried, and thus retained most if not all of their flavour and smell. Although the process originated in Switzerland in the 1930s, today the world's only swiss Water decaffeination facility is based near Vancouver, Canada.

2.3.3. Direct method

In the direct method the coffee beans were first steamed for 30 minutes and then repeatedly rinsed with either methylene chloride (Dichloromethane/DCM) or ethyl acetate for about 10 hours. The solvent was then drained away and the beans were steamed for an additional 10 hours to remove any residual solvent. Sometimes coffees which were decaffeinated using ethyl acetate were referred to as naturally process because ethyl acetate can be derived from various fruits or vegetables, but because of the impracticality of gathering natural ethyl acetate, the chemical used for decaffeination is synthetic.

2.3.4. Indirect method

In the indirect method, the beans were first soaked in hot water for several hours, essentially making a strong pot of coffee. Then the beans were removed and either methylene chloride or ethyl acetate was used to extract the caffeine from the water—as in other methods, the caffeine can then was separated from the organic solvent by simple evaporation. The same water was recycled through this two-step process with new batches of beans. Equilibrium was reached after several cycles, where the water and the beans had a similar composition except for the caffeine. After this point, the caffeine was the only material removed from the beans, so no coffee strength or other flavorings were lost. Because water has used in the initial phase of this process, sometimes indirect method decaffeination is referred to as "water processed" even though chemicals were used.

2.3.5. CO_2/O_2 process

This process is technically known as supercritical fluid extraction. Pre-

73 to 300 atmospheres. After a thorough soaking, the pressure was reduced allowing the CO_2 to evaporate, or the pressurized CO_2 was run through either water or charcoal filters to remove the caffeine. The carbon dioxide was then used on another batch of beans (Marleny *et al.*, 1999). This same process was also done with oxygen (O_2). These liquids worked better than water because they were kept in supercritical state near the transition from liquid to gas so that they had the high diffusion of gas and the high density of a liquid. This process had the advantage that it avoids the use of potentially toxic solvents.

2.3.6. Triglyceride process

Green coffee beans were soaked in a hot water/coffee solution to draw the caffeine to the surface of the beans. Next, the beans were transferred to another container and immersed in coffee oils that were obtained from spent coffee grounds. After several hours of high temperatures, the triglycerides in the oils remove the caffeine - but not the flavor elements - from the beans. The beans were separated from the oils and dried. The caffeine was removed from the oils, which were reused to decaffeinate another batch of beans. This was a direct contact method of decaffeination.

2.3.7. Conventional method

Conventional method of decaffeination usually involve the use of solution containing aqueous extract of coffee plus decaffeinating agents such as methylene chloride, ethyl acetate, charcoal or carbon, triglycerides and supercritical CO_2 (Katz,1987). The drawback of this process was that the flavor elements and oil were drawn off from the beans; presence of residual solvent, which was not safe for human health and the time taken for the whole process, was more than 24 hours.

However, these methods suffered from the ill effects of the decaffeinating agent being harmful or highly expensive. Use of microorganisms was being researched into in the recent years as a potential method for decaffeination

purpose had been advocated to be more benificial 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 had been isolated (Schwimmer *et al.,* 1971; Hakil *et al.,* 1999; Ashihara and Crozier, 2001; Mazzafera, 2002).

2.3.8. Enzymatic methods for caffeine degradation

Development of a process involving an enzymatic (specific) degradation of caffeine to non-toxic compound was necessary for solving the problems of chemical extraction of caffeine in food products as well as treating the caffeine containing waste products. The literature revealed that major caffeine degrading strains belong to *Pseudomonas* and *Aspergillus*. Though the enzymes involved in degradation of caffeine by microorganisms were known, in vitro enzymatic studies for caffeine degradation was not yet reported (Gokulakrishnan *et al.*, 2005).

2.3.9. Microbial methods for caffeine degradation

2.3.9.1. Isolated strains from soil for decaffeination

Some reports in the literature have described that the isolation of bacterial strains from the soil with an ability to degrade caffeine (Blecher *et al.,* 1997; Asano et al., 1993; Gluck *et al.,* 1988; Middlehoven *et al.,* 1984). *Psuedomonas sp.*, which used caffeine as a sole source of carbon and nitrogen, were reported to degrade caffeine at high concentrations 20-30 g/l (Asano *et al.,* 1994). Due to its high ability to degrade caffeine, *Pseudomonas* isolates was considered as a valuable tool for biotechnological application such as production of caffeine derivatives as well as for the studies on molecular biology towards a naturally decaffeinated coffee plant.

Caffeine degrading microorganisms utilizing caffeine as the sole source of carbon and nitrogen had been isolated and characterized, which had enzymes that bring about the actual degradation of the substrate. The intermediates of

caffeine degradation pathway were as useful as caffeine and found to have various applications. So bacterial degradation of caffeine not only became essential overcoming environmental issues but also served as a method of recovery of other commercially important products (Swati Sucharita Dash *et al.*, 2006).

2.3.9.2. Degradation by *Pseudomonas* sp.

The isolate identified as *Pseudomonas* sp. GSC 1182 showed 80% degradation of caffeine in 48 h when caffeine was used as the sole carbon and nitrogen source. In the presence of sucrose (5 g/l), 100% degradation of caffeine was achieved within 36–40 hrs. The degradation rate was also found to increase when fructose, lactose and galactose were used as carbon source. The isolate showed decreased level (< 10%) of caffeine degradation in the presence of glucose. At an initial pH of 6.0, the complete degradation of external nitrogen source decreased the caffeine degradation to 35% and 70% respectively(Gokulakrishnan *et al.*, 2007).

Pseudomonas is a genus of gamma proteobacteria, belonging to the larger family of pseudomonads. Recently, 16SrRNA sequence analysis had redefined the taxonomy of many bacterial species (Anzai *et al.*, 2000). As a result the genus *Pseudomonas* included strains formerly classified in the genera *Chryseomonas* and *Flavimonas*. Other strains previously classified in the genus *Pseudomonas* were now classified in the genera *Burkholderia* and *Ralstonia*.

Several studies were carried out to investigate the use of purines, including caffeine, as a source of energy for microorganism growth (Mazzafera *et al.*, 1994; Middelhoven and Bakker, 1982; Schwimmer *et al.*, 1971; Woolfolk, 1975; Woolfolk and Downard, 1977).A 'comprehensive review on purine utilization by microorganisms was published by Vogels and van der Drift (1976). Although fungi growing on caffeine had been isolated, most of the

the Pseudomonads group, with particular attention to *Pseudomonas putida* (Burr and Caesar, 1985).

2.3.9.3. *Pseudomonas putida*

Pseudomonas putida is a gram-negative rod-shaped saprotrophic soil bacterium. Based on 16S rRNA analysis, *P. putida* had been placed in the *P. putida* group, to which it lends its name (Anzai *et al.,* 2000). It demonstrates very diverse metabolism, including the ability to degrade organic solvents such as toluene (Marques *et al.,* 1993). This ability has been put to use in bioremediation, or the use of microorganisms to biodegrade oil.

Fig.No.2.1: *Pseudomonas putida*

Use of *P. putida* is preferable to some other *Pseudomonas* species capable of such degradation as it is a safe strain of bacteria, unlike *P.aeruginosa* for example, which is an opportunistic human pathogen.

The isolate was identified as close resemblance to *Pseudomonas putida* based on 16s r RNA .The ability of the *sp.* to degrade caffeine was studied by growing the isolate in caffeine medium with 1.2,5,7.5,10 g/l of initial concentration of caffeine.It was observed that the isolate could degrade caffeine at an initial concentration of 5 g/l within 48 hours. Metabolites formed during

with 3,7- dimethylxanthine as the first major product.(Swati *et al.*, 2006).

2.3.9.4. *Pseudomonas alcaligenes*

Pseudomonas alcaligenes is a Gram-negative aerobic bacterium used as a soil inoculants for bioremediation purposes, as it can degrade polycyclic aromatic hydrocarbons (O'Mahony *et al.*, 2006). It can be a human pathogen but occurrences are very rare (Valenstein *et al.*, 1983). Based on 16S rRNA analysis, *P. alcaligenes* has been placed in the *P.aeruginosa* group (Anzai, *et al.* 2000).

The isolate was identified to be the strain of *Pseudomonas alcaligenes* CFR 1708, isolated from coffee plantation soil. The enzyme responsible for caffeine degradation were found to be inducible. Pre induction of the microbial cells in medium containing caffeine as the sole source of carbon and nitrogen was carried out for 48 hrs. It gets completely degraded within 4-6 hrs in the pH range of 7-8. (Sarath babu *et al.*, 2005).

2.3.9.5. *Pseudomonas fluorescens*

Pseudomonas fluorescens is a common Gram-negative, rod-shaped bacterium (Palleroni, 1984). It belongs to the Pseudomonas genus; 16S rRNA analysis has placed *P. fluorescens* in the *P. fluorescens* group within the genus(Anzai *et al.*, 2000), to which it lends its name.

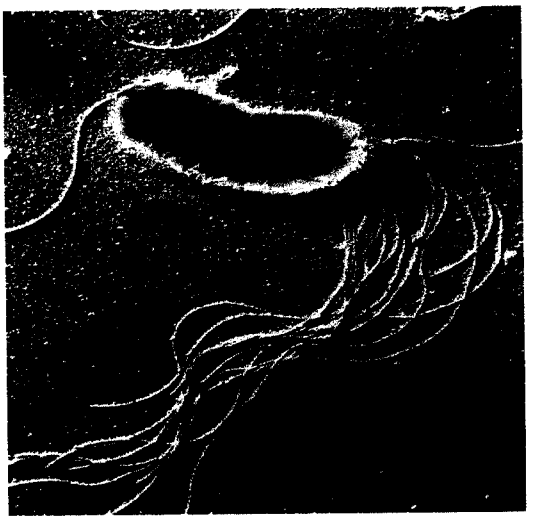

Fig.No.2.2: *Pseudomonas fluorescens*

P. fluorescens has multiple flagella. It has an extremely versatile metabolism, and can be found in the soil and in water.

Strictly aerobic, straight or slightly curved rods, but not helical, 0.5-1.0 μm x 1.5-5.0 μm in size. There is no resting stage or spores are known. Nearly all species are motile and many species are also denitrifiers. No unusual structures are formed, it is Catalase positive and also Oxidase positive or negative. Colonies are usually white to cream to yellow-pigmented (Environmental microbiology). Many species produce extracellular fluorescent pigments.

Heat stable lipases and proteases are produced by *Pseudomonas fluorescens* and other similar *pseudomonas* . These enzymes cause milk to spoil, by causing bitterness, casein breakdown, and ropiness due to production of slime and coagulation of proteins (Ray, 1996).

Dickstein *et al.,* (1957) and Bergmann *et al.* (1964) studied the degradation of 3 monomethylxanthine mediated by dehydrogenase activity in *Pseudomonas fluorescens.* They did not find activity with 1-

P.fluorescens strain with ability to grow on caffeine to demonstrate dehydrogenase activity against both monomethyl xanthines. A hydrolytic enzyme degraded caffeine, with the methyl groups being removed by sequential hydrolysis suggested. Methanol and xanthine were the final reaction products, and indications were that methanol was further oxidized to CO_2.

2.3.10. Effect of glucose

Caffeine degradation was inhibited when glucose was present in free form in the medium and at a concentration > 1 g/l. The glucose being the primary carbon source for all organisms, the addition of glucose will result in the utilization of glucose as the carbon source first rather than utilizing Caffeine. Thus the degradation of Caffeine was decreased due to the presence of glucose. Two stage culture experiments indicated that glucose probably interferes with the induction of enzymes involved in the caffeine degradation pathway. The addition of glucose at early stages of growth results in the complete inhibition of caffeine degradation whereas addition at latter stages of growth has no inhibitory effect .Disaccharides like sucrose and lactose enhanced the rate of caffeine degradation without being utilized as carbon source whereas glucose completely inhibits. (Sathyanarayana *et al.*, 2007).

2.3.11. Effect of physical parameter

Pseudomonas sp. capable of degrading high concentration of caffeine has been isolated from soil .It was capable of degrading 6.4 g/l initial concentration of caffeine at rate of 0.1 g/l/h. Here the physical parameter like pH, temperature and rpm were optimized. The optimum values of pH, temperature and rpm 7.8, 28°C, 190 rpm, respectively. Under optimized conditions the rate of degradation of caffeine has been enhanced 1.6 fold higher than the normal rate. Under optimal conditions the strain has also been found to degrade caffeine at 15 g/l within 48 hrs. (Swati *et al.*, 2007).

2.4. Medium optimization

Different combinations and sequences of process conditions need to be investigated to determine the growth conditions which produce the organism with the physiological state best constituted for Caffeine degradation. There may be a sequence of phases each with a specific set of optimal conditions.

Medium optimization by the classical method of changing one independent variable (nutrient, pH, temperature, etc.) while fixing all the others at the certain level can be extremely time consuming and expensive for a large number of variables. To make a full factorial search which would examine each possible combination of independent variable at appropriate levels could require a large number of experiments, x^n, where x is the number of levels and n is the number of variables. Industrially the aim is to perform the minimum number of experiments to determine the optimal conditions. Other alternative strategies must therefore be considered which allow more than one variable to be changed at the time (Mc Daniel *et al.*, 1976).

When more than five independent variables are to be investigated, the Plackett-Burman Design (PBD) may be used to find the most important variables in a system, which are then optimized in further studies (Plackett and Burman, 1946). This technique allows for the evaluation of X-1 variables by X experiments. X must be multiples of four e.g. 8, 12, 16, etc. Any factors not assigned to a variable can be designated as dummy variable. Alternatively, factors known do not have any effect may be included and designated as dummy variables. The incorporation of the dummy variables into an experiment makes it possible to estimate the variance of an effect (experimental error).

Table 2.3.12.1 shows a PBD for seven variables (A-G) at the high and low levels in which two factors, E and G, are designated as Dummy variables. These can be used in the design to obtain an estimate of error (Stowe and Mayer *et al.*, 1966). Each horizontal row represents a trial and each vertical column

Table No.2.4.1

Placket-Burman Design for Seven variables (Nelson, 1982).

Trial	Variables						
	A	**B**	**C**	**D**	**E**	**F**	**G**
1.	H	H	H	L	H	L	L
2.	L	H	H	H	L	H	L
3.	L	L	H	H	H	L	H
4.	H	L	L	H	H	H	L
5.	L	H	L	L	H	H	H
6.	H	L	H	L	L	H	H
7.	H	H	L	H	L	L	H
8.	L	L	L	L	L	L	L

H- High level

L- Low level

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1METHODS FOR THE ESTIMATION OF CAFFEINE

The methods reported for the determination of caffeine include High performance liquid chromatography (HPLC), Spectrophotometry, flourimetry, ion chromatography, and electrochromatography. Most of the reported spectrophotometric methods suffer from many disadvantage like time consuming, unstable product etc,.

The sensitive and rapid spectrophotometer method for the determination of caffeine, is based on the oxidation of caffeine with sodium meta periodate (SPI) in the presence of acetic acid and coupling with 3-methyl 2-benzo thiazolinone hydrazine hydrochloride (MBTH) reagent. This method offers sensitivity and stability.

Materials

1. Caffeine working standard (0.01 %)
2. SPI (0.01 M)
3. MBTH (0.01 M)
4. Acetic acid (0.1 M)
5. Distilled and double distilled water
6. Methanol
7. 25ml SMF
8. 1ml pipette

9. Cotton wool

10. A thermostatically controlled water bath

11. Beckman coulter DU ® 530 Life science UV/Vis Spectrophotometer with 1 cm matched cells.

Procedure

1. Aliquots of the working standard solutions of Caffeine (2.5 to 25 µg) were transferred into a series of 25 ml SMF.
2. Then 1.0 ml of SPI and .5 ml of acetic acid solution were added to each flask.
3. These solutions were diluted with distilled water (approximate 10 ml).
4. Then the solutions were kept in a boiling water bath for about 10 min.
5. After that, 2 ml of MBTH solution was added and further heated for about 2 min.
6. The solutions were cooled to room temperature.
7. The volume was made up to mark with distilled water.
8. The absorbance of the solutions were measured at 630 nm against a reagent blank prepared and treated similarly.
9. Calibration graph was constructed by plotting concentration (in µg) on X axis and absorbance (OD) values on Y axis.

3.2 ISOLATION OF CAFFEINE DEGRADING ORGANISM FROM COFFEE PLANTATION SOIL

Caffeine degrading microorganisms utilizing caffeine as a sole source of carbon and nitrogen have been isolated and characterized, which have enzymes that bring about the actual degradation of the substrate.

Materials

1. Soil Sample
2. Conical flasks
3. CLM
4. Orbital shaker
5. Test tubes
6. Sterilized Distilled water
7. 1ml μ pipette
8. Sterilized tips
9. Sterile petriplates
10. L rod
11. Incubator

Procedure

a. Soil samples from coffee gardens were collected from yercard.

b. Different growth media were used for isolation of caffeine degrading organisms.

c. A medium designated as M9 after modification with supplementaion of 0.3g/l caffeine was used for screening.

d. For enrichment of isolates capable of degrading caffeine as well as for induction of the organism CLM was used.

e. CLM was used as the growth medium with caffeine as the sole source of carbon and nitrogen.

f. Soil sample (1g) was taken and mixed with 100ml of CLM in a 250ml flask and kept for incubation for 72 h at 30°C on an orbital shaker at 120 rpm.

g. Caffeine agar medium was prepared by addition of 2.5% agar to CLM and poured into petri plates.

h. Serial dilutions (10^{-1} to 10^{-9}) of the above grown culture were then made.

i. 0.1 ml of the same was inoculated on to each petri plate and incubated at 37°C for a time period of 2 days.

j. Isolated single colonies growing on the above plates were purified further to obtain pure cultures.

k. These purified cultures were inoculated on to fresh slants of caffeine agar medium with subsequent subculturing.

3.3 Characterization of isolates

Soil samples was collected from yercaud .The samples were plated on nutrient agar and morphologically different colonies were isolated.These colonies underwent screening by gram staining and the catalase test.

3.3.1 Gram staining

Gram staining is an empirical method of differentiating bacteria into two large groups based on the differences in their cell walls. The cell walls for Gram-positive microorganisms have a higher peptidoglycan and lower lipid content than gram negative bacteria. Bacterial cell walls were stained by the crystal violet.Iodine was subsequently added as mordant to form the crystal violet-iodine complex so that the dye was not removed easily.However ,subsequent treatment with ethanol dissolved the lipid layer from the gram-negative cells enhanced the leaching of the primary stain from the cells. In contrast ,the solvent dehydrated the thicker gram-positive cell walls, closing the pores as the cell wall shrunk during dehydration. As a result, the diffusion of the violet – iodine complex was blocked and the bacteria remainded stained.Finally , a counterstain of safranin was applied to the smear to give decolorized gram-negative bacteria a pink color..

Materials

1. Microbial strains
2. Crystal violet
3. Gram's Iodine
4. Safranin

5. Ethanol (95%)

6. Glass slides

7. Inoculation loop

8. Bunsen burner

Procedure

1. A small drop of distilled water was placed on the surface of a clean glass slide.

2. Using a sterile loop , a thin smear of the culture was made on the slide

3. The smear was flooded with Crystal Violet for 1 min after which the stain was washed off under running water.

4. The dye was fixed with Gram's Iodine solution and was washed with water after one min.

5. The smear was decolorized using ethanol (95%) and washed under running water.

6. Finally, the smear was counter-stained with safranin for 30 sec and then washed with water.

7. The smear was air dried and visualized under 100x oil immersion microscopy.

3.3.2 Catalase test

The catalase test identifies organisms which produce the catalase enzyme,this enzyme converts hydrogen peroxide to water and oxygen gas. Catalase helps protect bacterial cells against hydrogen peroxide.Hydrogen peroxide is a highly-reactive compound which damages cell components . It is sometimes formed when the electron transport chain is used to produce energy.When a catalase – positive organism is exposed to hydrogen peroxide ,the hydrogen peroxide will bubble.

Materials

1. Hydrogen peroxide
2. Microbial strains
3. Glass slide
4. Inoculation loop

Procedure:

1. A small drop of water was placed on the glass slide.
2. Using a sterile inoculation loop, a bacterial colony was smeared on the slide.
3. Using a dropper , a few drops of hydrogen peroxide were added to the smear and observed for any signs of bubble evolution.

3.4. ESTIMATION OF CAFFEINE IN DIFFERENT COFFEE POWDERS

The amount of coffee powders to be taken was standardised under different concentrations of coffee powders. Two trails have been done using methonal and boiled water and finally caffeine content was estimated using spectophotometric method.

3.4.1. Extraction using methonal

Caffeine cannot be dissolved in water and hence organic solvents like methonal was used.Standardised amount of coffee powder was taken and extracted with methonal.

Materials

1. coffee powders – 10 samples
2. Caffeine working standard (0.01 %)
3. SPI (0.01 M)
4. MBTH (0.01 M)
5. Acetic acid (0.1 M)
6. Distilled and double distilled water
7. Methanol
8. 25ml SMF
9. 1ml pipette

10. Cotton wool

11. A thermostatically controlled water bath

12. Beckman coulter DU ® 530 Life science UV/Vis Spectrophotometer with 1 cm matched cells.

Procedure

1. 10 mg of coffee powders were taken and to that 2 ml of methonal was added.

2. Then the solution was stirred for 5 mins.

3. These were made upto 10 ml and finally 0.1 ml of the sample was used for estimation.

4. The standard caffeine estimation procedure was performed and finally the caffeine content was estimated.

3.4.2. Extraction using boiled water

The standardised amount of coffee powders were taken and water is added and boiled ,from these, samples were taken and estimated.

Materials

1. Coffee powders-10 samples

2. Caffeine working standard (0.01 %)

3. SPI (0.01 M)

4. MBTH (0.01 M)

6. Distilled and double distilled water

7. Methanol

8. 25ml SMF

9. 1ml pipette

10. Cotton wool

11. A thermostatically controlled water bath

12. Beckman coulter DU ® 530 Life science UV/Vis Spectrophotometer with 1 cm matched cells.

Procedure

1. 10 mg of coffee powders were taken and to that 2 ml of water was added.

2. Then the solution was boiled for 2 mins.

3. These were made upto 10 ml and finally 0.1 ml of sample were used for estimation.

4. The standard caffeine estimation procedure was performed and finally the caffeine content was estimated.

3.5. DEGRADATION OF CAFFEINE

Degradation process were done industrially using organic solvents and other chemical methods but these were found to be toxic and carcinogenic and even more costly and hence an alternative approach for the degradation was carried out using microorganisms.

Materials

1. Bacterial strain
2. Nutrient broth
3. coffee powders
4. Conical flasks
5. Inoculum loop
6. Orbital shaker
7. Sterilized distilled water
8. 1ml micro pipette
9. Sterilized tips

Procedure:

1. 3 conical flasks containing 50 ml Nutrient broth was prepared and to that 0.3(g/l)CF content of coffee powder was added.
2. These conical flasks were then sterilized.
3. To the sterilized flasks , a loop full of inoculum was added and incubated in orbital shaker at 120 rpm for 24 hrs.
4. After 24 hrs,the samples were collected and the caffeine estimation was performed using spectrophotometric method.

3.6. OPTIMIZATION OF PARAMETERS

3.6.1. Carbon source

Carbon sources like sucrose and lactose enhanced the rate of caffeine degradation by the organism without being utilised as the carbon source,where as glucose inhibited the rate of caffeine degradation since it interferes with the induction of the enzymes involved in the caffeine degradation pathway.

Materials

1. sucrose
2. lactose
3. Caffeine
4. Nutrient broth
5. Bacterial strain
6. Conical flasks
7. Orbital shaker
8. Sterlized tip
9. Distilled water
10. Inoculum loop

Procedure

1. 4 conical flasks containing 50 ml Nutrient broth were taken.To which 0.015g CF was added.
2. Concentration containing 0.5g of sucrose was added to one conical flask and 5g concentration sucrose to another .
3. Similarly concentration of 0.5g and 5g of lactose were added to another

set of conical flasks.

4. These were then kept for sterilization.

5. Then to this 200µl of 24 hrs pre- grown inoculums was added.

6. The conical flasks were then incubated at 37°C in orbital shaker at 120 rpm for 24 hrs.

7. Samples were collected after 24 hrs and caffeine content was e stimated using spectophotometric method.

3.6.2 Nitrogen source

Caffeine degrading organisms utilise carbon and nitrogen as their sole source.Nitrogen sources like ammonium sulphate,urea has the efficiency of degradading caffeine.Ammonium chloride has less effect on degradation.

Materials

1. Ammonium chloride
2. Caffeine
3. Nutrient broth
4. Bacterial strain
5. Conical flasks
6. Orbital shaker
7. Sterilized tip
8. Distilled water

Procedure

1. 2 conical flasks containing 50 ml Nutrient broth were taken.To that 0.015g CF was added.
2. Concentration containing 0.5g of ammonium chloride was added to one conical flask and 5g concentration ammonium chloride to another .
3. These were then kept for sterilization.
4. Then to this 200µl of 24 hrs pre- grown inoculums was added.
5. The conical flasks were then incubated at 37°C in orbital shaker at 120 rpm for 24 hrs.
6. Samples were collected after 24 hrs and caffeine content was estimated using spectophotometric method.

3.6.3. Inoculum size

Depending upon the variation in inoculum size ,the caffeine gets degraded.

Materials

1. Caffeine
2. Nutrient broth
3. Bacterial strain
4. Conical flasks
5. Orbital shaker

7. Distilled water

8. Inoculum loop

Procedure

1. 2 conical flasks containing 50 ml Nutrient broth were taken.To which 0.015g CF was added

2. Sterilization was performed.

3. In one conical flask 50µl of 24 hrs pre grown culture was inoculated and in another 1000µl of culture was inoculated.

4. The conical flasks were then incubated at 37°C in orbital shaker at 120 rpm for 24 hrs.

5. Samples were collected after 24 hrs and caffeine content was estimated using spectophotometric method.

3.6.4. pH

The physical parameters like pH,temperature enhances the rate of caffeine degradation.These have been optimized using central composite design.This enhanced by 1.6 fold higher than the normal rate.

Materials

1. NaOH

2. HCl

3. Caffeine

4. Nutrient broth

5. Bacterial strain

6. Conical flasks

7. Orbital shaker

8. Sterilized tip

9. Distilled water

10. Inoculum loop

Procedure

1. 4 conical flasks containing 50 ml Nutrient broth were taken. To which 0.015 g CF was added

2. pH from 6-8 was adjusted by using 0.1 NaOH and 0.1 HCl in the respective conical flasks.

3. Conical flask were kept for the sterilization.

4. Then to this 200 µl of 24 hrs pre- grown inoculums were added.

5. The conical flasks were then incubated at 37°C in orbital shaker at 120 rpm for a time period of 24 hrs.

6. Samples were collected after 24 hrs and caffeine content was estimated using spectophotometric method.

3.6.5.rpm

Rpm is the physical parameter which enhances the rate of caffeine degradation.Here the nutrients will be distributed equally and it enhances the growth of the organisms, whereas in standby equal distribution will not be there.

Materials

1. Caffeine
2. Nutrient broth
3. Bacterial strain
4. Conical flasks
5. Orbital shaker
6. Sterilized tip
7. Distilled water
8. Inoculum loop

Procedure

1. 4 conical flasks containing 50 ml Nutrient broth were taken. To which 0.015 g CF was added.
2. Conical flasks were kept for the sterilization.
3. Then to this 200 µl of 24 hrs pre- grown inoculums were added.
4. The conical flasks were then incubated at 37°C in orbital shaker at different rpm from 180-200 for a time period of 24 hrs.
5. Samples were collected after 24 hrs and caffeine content was estimated using spectophotometric method.

3.7 .MEDIUM OPTIMIZATION

Different combinations and sequences of process conditions need to be investigated to determine the growth conditions which produce the organism with the physiological state best constituted for Caffeine degradation. Industrially the aim is to perform the minimum number of experiments to determine the optimal conditions. Hence PBD was used.

Materials

1. Sucrose
2. Lactose
3. Ammonium chloride
4. Caffeine
5. Nutrient broth
6. Bacterial strain
7. Conical flasks
8. Orbital shaker
9. Sterlized tip
10. Distilled water
11. Inoculum loop

Procedure

1. 300 ml of NB was prepared containing 0.3% of caffeine 0.25 ml of broth were transferred into 12 conical flasks.

2. Add 1 g of sucrose, 0.25 g of lactose, 0.25 g of ammonium chloride and pH was adjusted to 6.0 with 0.1N NaOH.

3. Sterilization was performed.

4. To that 1000 µl of inoculum was added and incubated in orbital shaker at 200 rpm.

5. To the second flask, add 1 g of sucrose, 0.25 g of lactose, 0.25 g of ammonium chloride and pH was adjusted to 7.5 with 0.1N HCl. Similaraly, for the rest of the 10 flasks different concentration of sources were added and adjusted to the corresponding pH.

6. Then sterilization was performed.

7. Then corresponding innoculum was added and incubated in orbital shaker at different rpm.

8. Samples were collected at 24 hrs and 48 hrs time of interval.

9. Caffeine estimation was performed.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Caffeine is an alkaloid present in many food supplements like coffee bean, tea leaves, cocoa and beverages. It has the complex structure of trimethyl xanthine. Its presence stimulates the CNS by acting as antagonist for the adenosine receptor. The presence of excess amount of CF will result in continuous stimulation of the nerves and thus leads to many ill effects.

Caffeine is a major constituent of many common "nonprescription" drug preparations such as cold remedies, diet pills, diuretics, and other stimulants. Excess intake of this xanthine derivative causes many undesired side effects and gives rise to many symptoms. Therefore degradation of this alkaloid is necessary.

The industrial decaffeination process involves the usage of solvent which are carcinogenic and some other processes which are highly expensive. So microbiological approach for the degradation of CF was formulated. CF was found to have antimicrobial activity against some organisms. Though the presence of CF will eliminate some organism, the organism isolated from the coffee plantation garden has the ability to degrade CF. Those organisms were found to be of *Pseudomonas sp.* and some other fungal species.

With the help of these organisms the CF from coffee extracts were degraded. The effects of the presence of other nutrient sources (Carbon, Nitrogen) and other physical parameters for the CF degradation by the organisms were studied.

4.1. Caffeine Estimation

Caffeine estimation was usually done with High Performance Liquid Chromatography (HPLC), Spectrophotometry, flourimetry, ion chromatography,

nd electrochromatography. The new rapid and sensitive method of Caffeine stimation is done by oxidizing Caffeine with SPI in acidic condition, followed by the coupling of MBTH. This results in the formation of blue colored product which could be measured in spectrophotometer at 630 nm.

The first objective of our study was to determine the Caffeine content from various commercially available coffee powders. Ten different coffee powders were therefore purchased from the departmental stores for the caffeine estimation. In order to calculate the Caffeine concentration from the coffee powders, the standard calibration graph was constructed with the help of pure stock Caffeine solution (Fig.No.4.1.1) by using the spectrophotometric method. The standardization of Caffeine was carried out with the help of Spectrophotometric method and the absorbance values were taken at 630 nm. For the CF concentration of 0.5 µg the values obtained was 0.009, similarly for the CF concentration of 1.0, 1.5, 2.0, 2.5 µg the absorbance values obtained were found to be 0.039, 0.058, 0.079, 0.135 respectively (Table No.4.1.1).From the slope of the calibration graph the Caffeine concentration of the samples was calculated with the availability of absorbance value at 630 nm.

Since Caffeine being an alkaloid which does not dissolve in polar solvents, the Caffeine stock solution were prepared by dissolving 10 mg of pure CF to 20 ml methanol by shaking it for 10 mins. Then the solution was made up to 100 ml with double distilled water.

Table No.4.1.1

Standardisation of Caffeine

No.	Reagents	B	S1	S2	S3	S4	S5
	Volume of Stock (µl)	0.0	5.0	10.0	15.0	20.0	25.0
	Conc. of Stock (µg)	0.0	0.5	1.0	1.5	2.0	2.5
	Volume of SPI (ml)	0.2	0.2	0.2	0.2	0.2	0.2
	Volume of Acetic acid (ml)	0.1	0.1	0.1	0.1	0.1	0.1
Kept in boiling water bath for ten minutes							
	Volume of MBTH (ml)	0.4	0.4	0.4	0.4	0.4	0.4
The solution was heated for 2 minutes							
Cooled to room temperature and made up to 10 ml							
6.	Absorbance value at 630 nm.	0.000	0.009	0.039	0.058	0.079	0.135

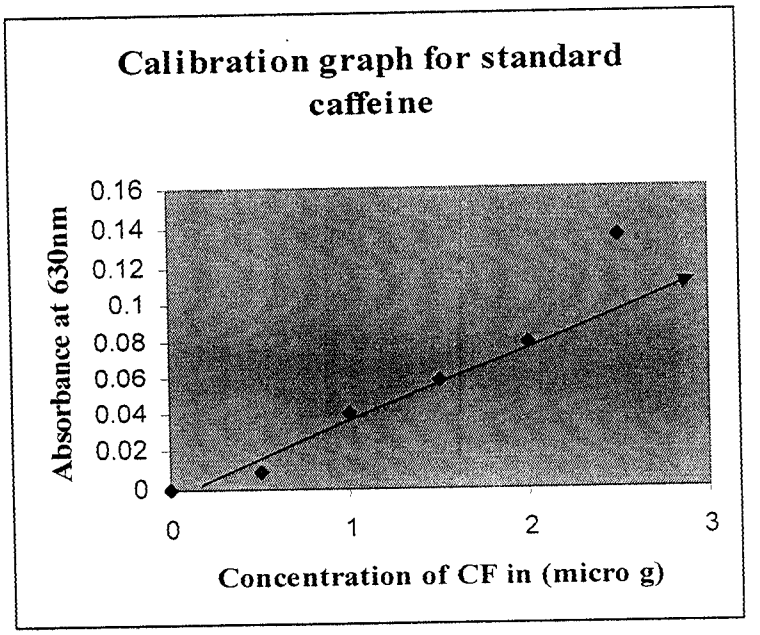

Fig.No.4.1.1: Calibration graph for standard caffeine

4.1.1. Estimation of caffeine in different coffee powders (Methanol Extraction)

To extract the Caffeine from coffee powders, organic solvent like Methanol was used. First, the amount of coffee powders to be taken to detect the CF content by spectrophotometric method. For that coffee powders of different concentrations were taken, 50 g of coffee powder was dissolved in 20 ml methanol and made up to 100 ml with distilled water, and the CF estimation procedure was carried out. Then 5 and 0.5 g of coffee powders were dissolved in 20 ml methanol and made up to 100 ml with distilled water and standard procedure was carried out (Table No.4.1.2), the resulting solution was found to be dark brown for 50 g coffee powder made up to 100 ml, brown color for the 5 g and light brown for the 0.5 g coffee powders (Fig.No.4.1.1.1).

So the further experiment was tried with lesser amount of coffee powders. 0.1, 0.2, 0.3, 0.4 g of coffee powders was taken and extracted with 20 ml methanol and these were diluted to 100 ml with the distilled water. Then CF estimation procedure was done for all these samples, where 0.1 ml of the

oupling with an MBTH reagent. The resulting blue colored solution indicates e presence of CF, which was measured at 630 nm (Table. No.4.1.2). The color otained for 0.1 g and 0.2 g of coffee powders was blue, the color obtained for e remaining samples was green and dark green based on the concentration ig.No.4.1.1.2).

able No. 4.1.1.1

tandardisation of amount of coffee powders for caffeine estimation

.No.	Amount of Coffee Powders (g)	Sample Numbers	Colour formed	Absorbance at 630 nm
.	50	S1	Dark Brown	3.00
.	5.0	S2	Brown	2.871
.	0.5	S3	Light Brown	1.025
.	0.4	S4	Dark Green	1.308
.	0.3	S5	Green	1.185
.	0.2	S6	Dark blue	0.907
.	**0.1**	**S7**	**Blue**	**0.874**

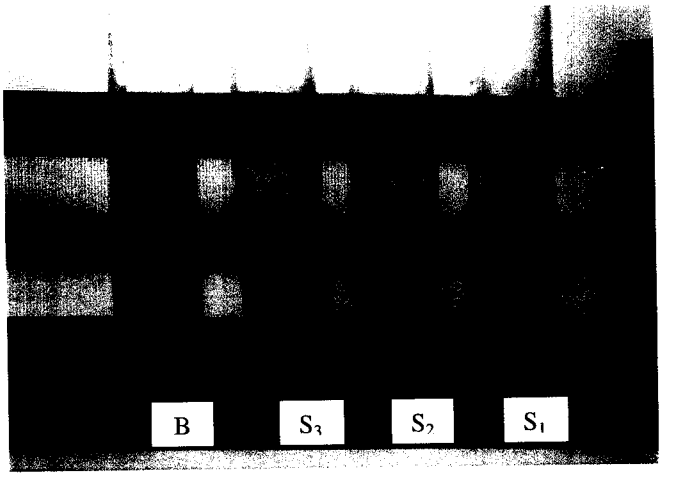

Fig.No.4.1.1.1: Standardisation of amount of coffee powders

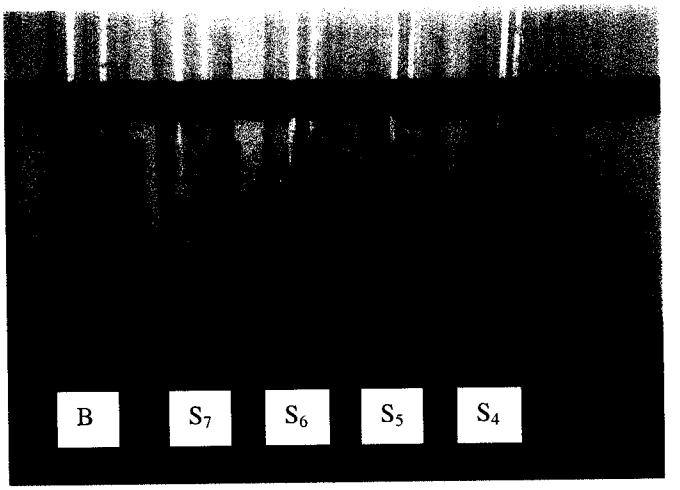

Fig.No.4.1.1.2: Standardisation of amount of coffee powders

Thus from the above experiment it was decided that 0.1 g of coffee powder should be dissolved in 20 ml methanol and diluted to 100 ml for the CF estimation.

The caffeine content from ten different commercially available coffee samples was estimated with the same procedure of oxidizing 0.1ml of sample with SPI in acidic medium, followed by coupling with an MBTH reagent. The obtained blue color solution was measured for its optical density at 630 nm in a spectrophotometer (Table No.4.1.2). The Caffeine concentration was calculated with the help of standard graph (Fig.No.4.1.1).

Table No.4.1.1.2

Caffeine Estimation in different coffee samples

Sample Number	Absorbance Value at 630nm	Concentration of Caffeine in g/g of Coffee Powder
1	0.507	0.13
2	**0.949**	**0.243**
3	0.714	0.183
4	**1.126**	**0.289**
5	0.746	0.192
6	0.595	0.154
7	0.622	0.16
8	0.630	0.162
9	**0.879**	**0.226**
10	0.597	0.154

4.1.2.Estimation of caffeine in different coffee powders (water extraction)

Since Caffeine being an alkaloid does not dissolve in polar solvents the extraction was done with methanol, but the traditional way of making coffee is

dissolving the coffee powders in boiling water with the addition of milk. So, the coffee powder was boiled in water for Caffeine estimation. The boiling time for Caffeine extraction was standardized by testing the amount of Caffeine dissolved in water at different boiling time. Samples were collected before boiling, before boiling, at boiling, one minute after boiling and the Caffeine content was estimated (Table No.4.1.2.1). At boiling condition, the Caffeine was found to be more so, it was chosen for further studies.

Table No.4.1.2.1

Standardization of boiling time for caffeine estimation

S.No.	Boiling Time	Absorbance value at 630nm
1.	Before Boiling	0.892
2.	During Boiling	0.855
3.	**At Boiling**	**0.950**
4.	One min of Boiling	0.730

Caffeine from coffee powders

Then Caffeine content from coffee powders at boiling condition was estimated, with the same procedure of oxidizing 0.1ml of sample with SPI in acidic condition and addition of MBTH after ten minutes of boiling the solution. Then the solutions were cooled to room temperature and made up to 25 ml with distilled water. The resulting blue colored solutions were measured at 630 nm for absorbance value (Table No.4.1.2.2). The Caffeine concentration was calculated with the reference of standard graph. The comparison of Caffeine content from the methanol and water extraction was made (Fig. No.4.1.2.1). Sample 2, 4, and 9 were chosen for further analysis, because of high Caffeine

Table No.4.1.2.2

Estimation of concentration of caffeine in different coffee powders (Water Extraction)

Sample Number	Absorbance Value at 630nm	Concentration of Caffeine g/g of Coffee Powders
1.	0.023	0.0059
2.	**0.378**	**0.0973**
3.	0.198	0.0506
4.	**0.874**	**0.225**
5.	0.206	0.053
6.	0.007	Not Defined
7.	0.095	0.0254
8.	0.112	0.0286
9.	**0.281**	**0.07325**
10.	0.053	0.01442

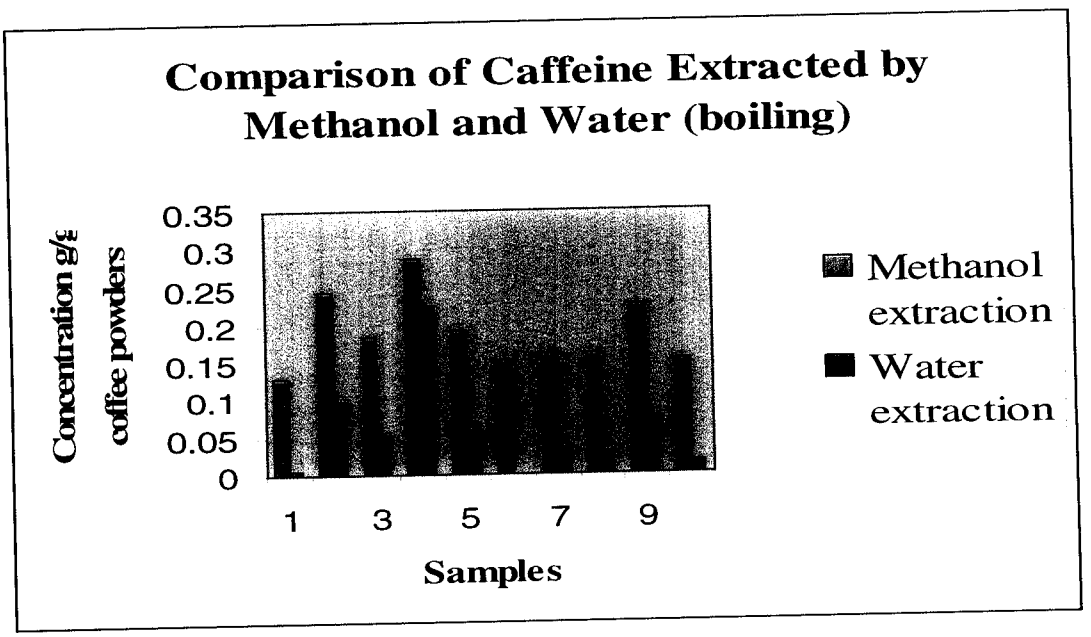

Fig.No.4.1.2.1: Comparison of Caffeine Extracted by Methanol and Water

4.2. Isolation of caffeine degrading organism from soil

The second objective of the study was to isolate the Caffeine degrading organism from coffee plantation soil (from yercaud). 1g of soil from coffee garden was added to CLM and incubated at 37°C for 24 hrs in rotary shaker with 120 rpm. Serial dilution (10^{-1} to 10^{-9}) of the above grown culture was then made and 0.1ml of the same was inoculated on to each Petri plate with CAM and incubated at 37°C for 48 hrs (Fig.No.4.2.1).

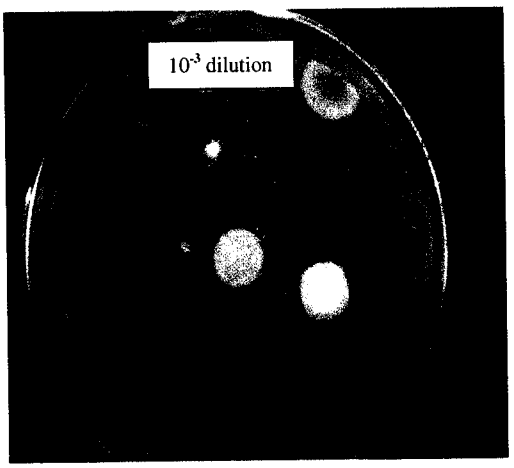

Fig.No.4.2.1: Isolated colonies from serial dilution

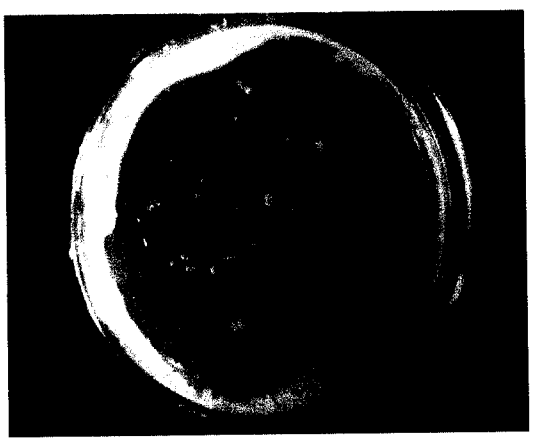

Fig.No.4.2.2: Sub-cultured isolated colony in new agar plate

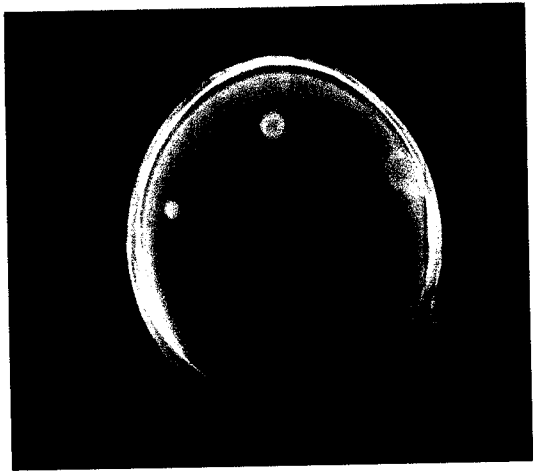

Fig.No.4.2.3: Pure culture isolated

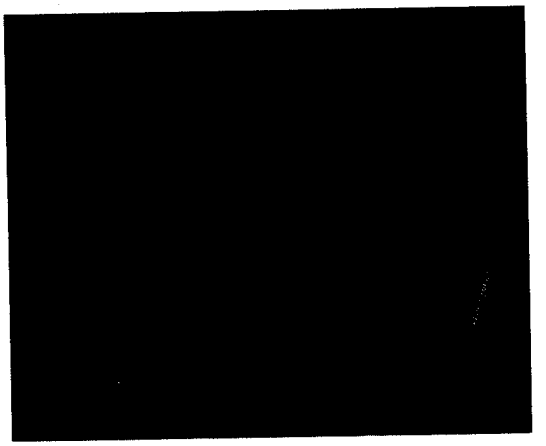

Fig.No.4.2.4: Single colony isolated

4.3. Characteristics of the isolated organism

4.3.1. Gram Staining

The isolated organism was subjected to gram staining with the help of gram staining kit.

The gram staining was done to identify the colony morphology. The isolated organism was found to be Gram negative, rod shaped bacterium.

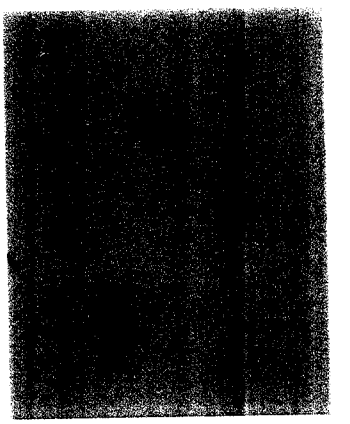

Fig.No.4.3.1: Gram Staining

4.3.2. Catalase Test

Then biochemical test was carried out for the organism, for their characterization. The organism was suspected to be *Pseudomonas fluorescence* because of the light reflected by the organism in agar plate (appearance of fluorescent colony) and confirmed by the catalase test (Fig.No.4.3.2) which was catalase positive.

Here the bubble formation was due to the release of oxygen molecule as a result of the catalase enzyme 's reaction with Hydrogen peroxide. When hydrogen peroxide was poured over the colonies on an agar plate with the help of dropper, the catalase enzyme secreted by the organism reacts with hydrogen peroxide and released oxygen and water.

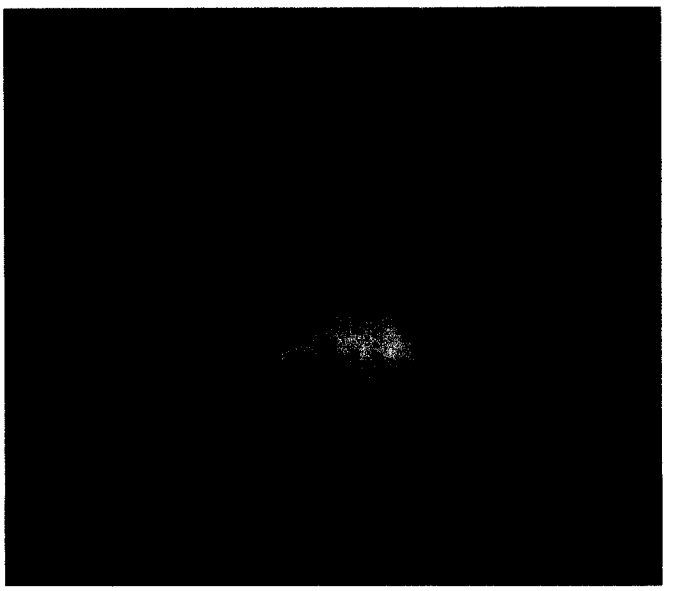

Fig.No.4.3.2: Catalase test

4.4. Caffeine degradation

The third objective of the work was to cultivate the isolated Caffeine degrading microorganism in coffee extracts. The nutrient broth was prepared to that coffee powders equivalent to 0.1% Caffeine was added. O.4% of 24 hr grown pre inoculum was added to the broth and incubated in rotary shaker for 24 hrs at 37°C at 180 rpm. The flasks without the organism were made as control. The samples were collected from the incubated medium for a period of every 24 hrs. The Caffeine content was estimated for every 24 hrs till a time period of 72 hrs and then the absorbance and concentration were calculated. Percentage degradation of Caffeine was achieved to >50% after 48 hrs of incubation. The

Table No.4.4.1

Caffeine degradation after 24 hours of incubation

S.No.	Samples	OD at 630 nm	Conc. of CF mg/g	% Degradation
1.	Control 1	0.554	7.091	-
2.	Sample 1	0.354	4.535	35.975
3.	Control 2	0.639	8.227	-
4.	Sample 2	0.292	3.755	54.35
5.	Control 3	0.707	9.103	-
6.	Sample 3	0.407	5.24	42.44

Table No.4.4.2

Caffeine degradation after 48 hrs of incubation

S.No.	Samples	OD at 630 nm	Conc. of CF mg/g	% Degradation
1.	Control 1	0.554	7.091	-
2.	Sample 1	0.281	3.615	49
3.	Control 2	0.639	8.227	-
4.	Sample 2	0.230	2.96	64
5.	Control 3	0.707	9.103	-
6.	Sample 3	0.343	4.41	51.5

Table No.4.4.3

Caffeine degradation after 72 hrs of incubation

S.No.	Sample	OD at 630 nm	Conc. of CF mg/g	% Degradation
1.	Control 1	0.554	7.091	-
2.	Sample 1	0.267	3.425	51.69
3.	Control 2	0.639	8.227	-
4.	Sample 2	0.226	2.905	64.6
5.	Control 3	0.707	9.103	-
6.	Sample 3	0.332	4.28	52.98

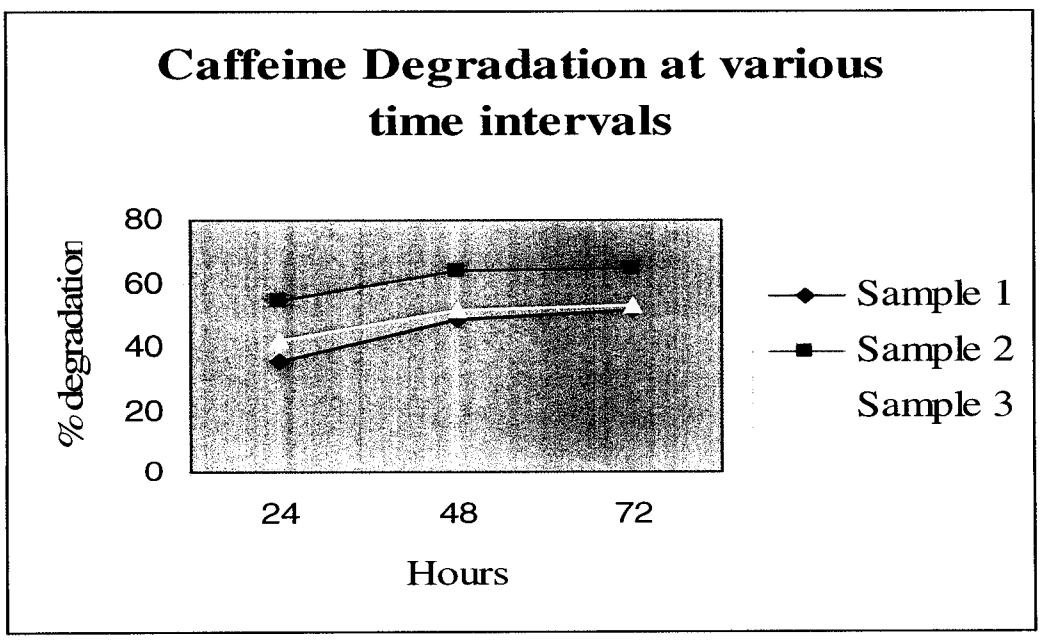

Fig.No.4.4.1: Caffeine Degradation at various time intervals

4.5. Caffeine degradation with different sources

Then the Caffeine degrading efficiency of the organisms were studied with different parameters like carbon source, nitrogen source, pH, rpm, and also by varying inoculum size.

4.5.1. Carbon source

The degradation efficiency decreased to <10% by the presence of glucose (Gokulakrishnan *et al.*, 2007). Carbon sources like disaccharides, fructoses were found to increase the degradation efficiency. So, the carbon sources like sucrose and lactose at high concentration of 4% (4 g/100 ml) and low concentration of 1% (1 g/100 ml) were carried out. The nutrient broth was prepared with the addition of 0.3% Caffeine and to that media sugar samples at different concentrations were added and kept for sterilization. The sterilized media were inoculated with the organisms (400 µl/100 ml) and incubated at 37°C in an orbital rotary shaker for 48 hrs at 180 rpm. We collected the samples and 0.1 ml of the collected samples was undergone Caffeine estimation to calculate the Caffeine degradation efficiency in percentage. High Caffeine degradation was observed in 1% of both the sugars to that of 4%.

The cell mass after 48 hrs of incubation was measured colorimetrically at 650 nm by setting the corresponding control (nutrient broth with 0.3% Caffeine and sugars at different concentration without organism) as blank.

Table No.4.5.1

Caffeine degradation after 48 hrs of incubation with carbon source

S.No.	Sugar samples	Concentration of carbon source (CS) (g/100 ml)	Colorimeter value at 650nm (48 hrs)	Caffeine estimation after 48 hrs of incubation	
				Absorbance at 630 nm	Percentage degradation
1.	Sucrose	1	1.43	0.112	90.4
2.		4	1.26	0.262	77.6
3.	Lactose	1	1.11	0.181	84.5
4.		4	1.02	0.201	82.82

The presence of 1% sucrose has increased the Caffeine degradation percentage to 90.4 and 1% lactose increased the degradation percentage to 84.5.

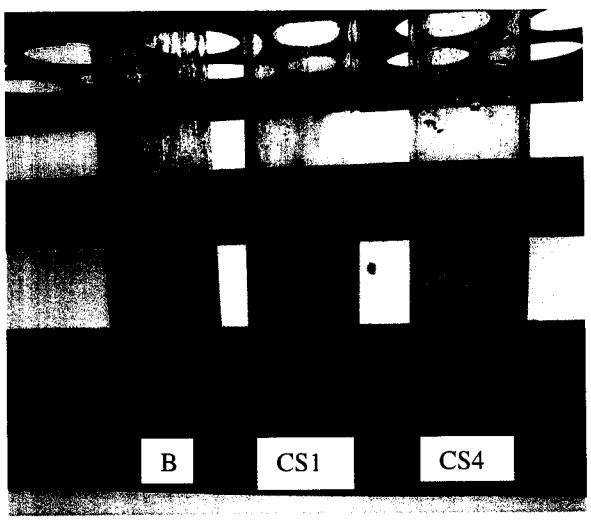

Fig.No.4.5.1.1: Sucrose

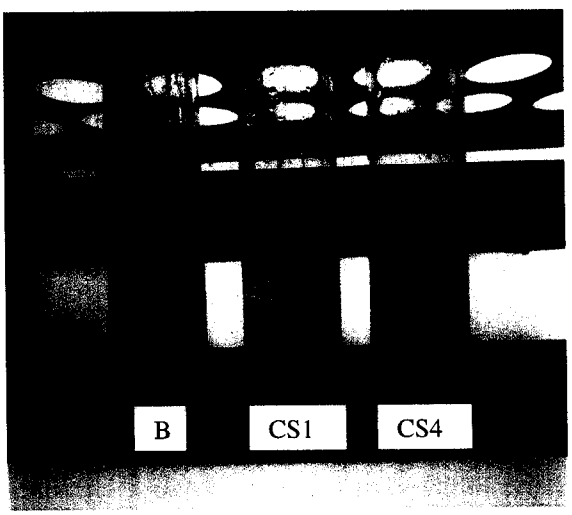

Fig.No.4.5.1.2: Lactose

4.5.2. Nitrogen source

Then the study of Caffeine degradation in the nitrogen source was carried with the addition of ammonium salts. The addition of nitrogen source like ammonium sulphate, urea was found to decrease the Caffeine degrading efficiency by 70% and 35% respectively (Gokulakrishnan *et al.*, 2007).

Ammonium chloride was chosen as the nitrogen and its effect on Caffeine degradation was observed at 1% and 4% of ammonium chloride. Nutrient broth was prepared with 0.3% Caffeine and ammonium chloride was added to get the final solution with 1 and 4% concentration. Then the media was sterilized and after sterilization the organisms were added to each flask. Then the inoculated flasks were placed in the incubator which maintained the same condition as it was followed for carbon source. After 48 hrs of incubation the samples were collected and Caffeine estimation was done to find the percentage degradation. The addition of nitrogen source decreased the degradation efficiency to 38% at a concentration of 1% ammonium chloride and 14% at a concentration of 4% ammonium chloride. The density of biomass grown after the incubation time was measured at 650 nm in the colorimeter.

Table No.4.5.2

Caffeine degradation rate after 48 hrs of incubation with nitrogen source

S.No.	Concentration of nitrogen source (NS) (g/100ml)	Colorimeter value at 650nm(48hrs)	Caffeine estimation after 48 hrs of incubation	
			Absorbance at 630 nm	Percentage degradation
1.	1	0.65	0.717	38.7
2.	4	0.04	0.995	14.9

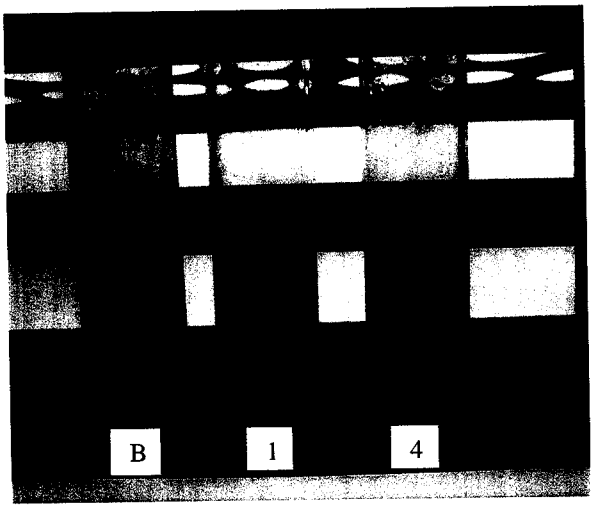

Fig.No.4.5.2: NH_4Cl_2

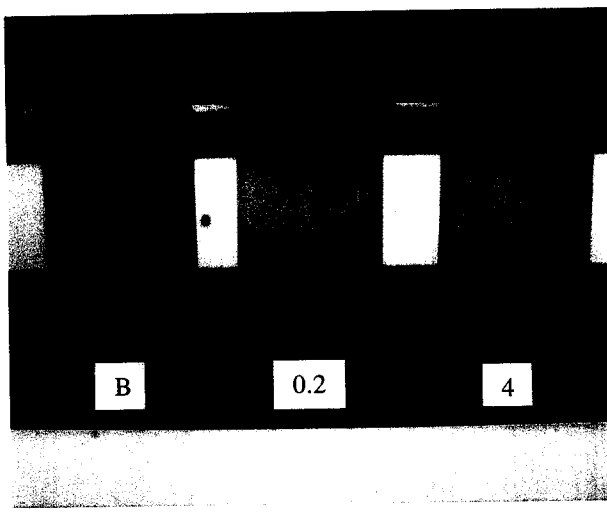

Fig.No.4.5.3: Inoculumsize

4.5.4. Physical parameter

The degradation of Caffeine undergone demethylase pathway (**Stephan M. Cameron**, University of Minnesota) and the enzyme involved is demethylase. All enzymes will have its own optimum reaction condition. The reaction condition involves several factors, mainly pH and temperature. Thus Caffeine degradation efficiency at different pH were studied by adjusting the pH of the medium, before inoculation.

4.5.4.1. pH

The degradation of CF by the organism was studied in the medium at different pH and the degradation rate is given in Table No.4.5.4.1

The presence of nitrogen sources reduced the degradation efficiency of the organism. 1% ammonium chloride resulted in a degradation of 38.7% and the 4% ammonium chloride resulted in a degradation of 14.9%.

4.5.3. Inoculum size

Similar studies were carried out by varying the inoculum size. By increasing the inoculum size, the lag phase of the organism for its accommodation with the surrounding medium got reduced. Thus the degradation started soon in the flask with high concentration of initial inoculum. Caffeine degradation was found after 48 hrs of incubation.

Table No.4.5.3

Caffeine degradation after 48 hours of incubation with different inoculum size

S.No.	Amount of inoculum (ml/100ml)	Colorimeter value at 650nm(48hrs)	Caffeine estimation after 48hrs	
			Absorbance at 630nm	Percentage Degradation
1.	0.2	1.03	0.045	96.15
2.	4	1.24	0.005	99.57

The size of the initial inoculums has the positive influence on the Caffeine degradation. 0.2% of the initial inoculum gave the degradation percentage of 96.15% and 4% of the initial inoculum gave 99.57%.

Table No.4.5.4.1

Caffeine degradation after 48 hours of incubation with different pH

S.No.	pH	Caffeine estimation after 48hrs	
		Absorbance at 630nm	Percentage degradation
1.	6.0	0.578	50.6
2.	6.5	0.346	70.4
3.	7.0	0.412	64.8
4.	7.5	0.428	64.78

The optimum pH for the degradation of Caffeine by this organism was found to be 6.5.

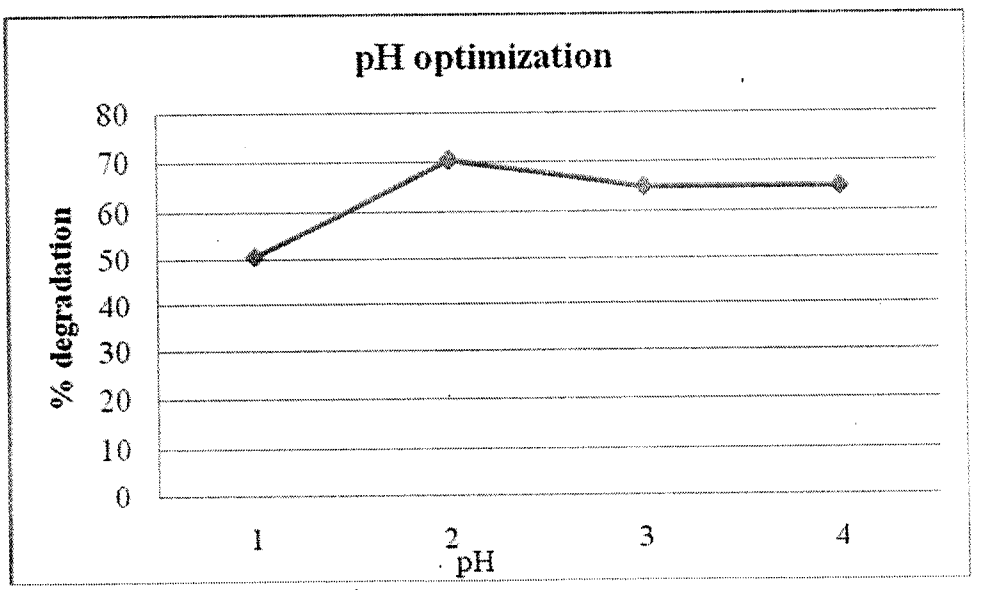

Fig.No.4.5.4.1: pH optimization

4.5.4.2. RPM

The degradation of CF was studied by keeping the media in different RPM in an orbital shaker for equal distribution of nutrition among the organisms to degrade the caffeine content.

Table No.4.5.4.2

Caffeine degradation after 48 hours of incubation maintained at different rpm

S.No.	RPM of the shaker	Caffeine estimation after 48hrs	
		Absorbance at 630nm	Percentage degradation
1.	180	0.321	72.56
2.	190	0.263	77.52
3.	200	0.317	72.9

Though the Caffeine degradation was not much influenced by the change in rpm, the maximum efficiency of Caffeine degradation was found to be at the 190rpm speed.

4.6. Medium Optimization

The organism's growth condition will tend to vary with the variance in the nutrient content as we saw already. Thus the optimization of those nutrients is required for the study of the effect of each parameter towards the product formation, here Caffeine degradation. The combinations of different sources will have different effect to the microbial growth. Since the parameters considered here exceeds three, we adopted Plackett-Burman Design to make our work easier. The nutrient sources were varied based on PBD and the Caffeine degradation was studied for each composition, the influence of each source

Table No.4.6.1

The parameters to be maintained for the identification of its influence towards Caffeine degradation

S.No.	Trial Number	Carbon source		Nitrogen source	Inoculum size (µl/25ml)	Growth conditions		
		Sucrose (g/25ml)	Lactose (g/25ml)	NH_4Cl_2 (g/25ml)		pH	RPM	Tempe rapture(°C)
1.	1	1	0.25	0.25	1000	6.0	200	37
2.	2	1	0.25	0.25	50	7.5	180	37
3.	3	0.25	0.25	1	1000	7.5	180	37
4.	4	0.25	0.25	1	1000	6.0	180	37
5.	5	0.25	1	0.25	1000	6.0	200	37
6.	6	1	1	0.25	1000	7.5	180	37
7.	7	1	1	1	1000	7.5	200	37
8.	8	1	0.25	1	50	6.0	200	37
9.	9	0.25	0.25	0.25	50	7.5	200	37
10.	10	0.25	1	1	50	7.5	200	37
11.	11	1	1	1	50	6.0	180	37
12.	12	0.25	1	0.25	50	6.0	180	37

4.6.1. Caffeine estimation

The CF estimation was carried out after 48 hrs of incubation at the required conditions.

Table No.4.6.2

Caffeine estimation after 48 hrs of incubation

S.No.	Trial No.	Absorbance at 630nm after 48 hrs	Percentage degradation
1.	1	1.02	12.72
2.	2	1.002	14.28
3.	3	0.666	43.09
4	4	0.223	80.96
5.	5	0.968	17.23
6.	6	1.052	10.06
7.	7	0.506	56.72
8.	8	0.442	62.18
9.	9	0.920	21.34
10.	10	0.344	70.59
11.	11	0.381	67.4
12.	12	1.04	11

The absorbance value at 630 nm was observed and the degradation efficiency was calculated for each trial. From those values the coefficient for each parameter was obtained with the help of PBD software. The positive coefficient refers the influence of that particular parameter to the degradation and the negative coefficient possessing parameter will decrease the degradation efficiency. The obtained coefficient for each parameter was,

Sucrose	=> 13.571
Lactose	=> 9.9711
Ammonium chloride	=> - 16.5178
Inoculum size	=> 0.0209
pH	=> 4.4344
rpm	=> - 0.83508

Thus sucrose has the high influence towards the Caffeine degradation since it has high positive value and the presence of ammonium chloride will decrease the degradation efficiency.

CONCLUSION

5. CONCLUSION

Since the presence of CF leads to severe health problems like, nervousness, sleep disorder, stress etc. The coffee powders with different brands were chosen to check the degradation percentage of Caffeine content. The amount of coffee powder for the Caffeine estimation was standardized to be 10 mg, from the blue colour obtained from the samples S6 and S7. This sample concentration was chosen for the further studies. Various trials were performed with different boiling time, for estimating the high caffeine content in different brand of coffee powders. It was concluded that, at boiling time, high amount of Caffeine was obtained which was used for the further analysis. Coffee powders with high Caffeine content was used for the further degradation studies.

Isolation of Caffeine degrading organisms from the coffee plantation soil was performed and *Pseudomonas fluorescens* was isolated and used for the degradation studies. Nearer to 50% degradation was observed after 48 hrs of incubation. Various parameters were taken up for checking its influence on the Caffeine degradation. The chosen parameters were, carbon source, nitrogen source, pH, rpm and Inoculum size. Finally using PBD software, the coefficients for the five parameters were obtained to be 13.5711 for sucrose; 9.9711 for lactose; -16.5178 for ammonium chloride; 0.02019 for inoculum size; 4.4344 for pH and -0.83508 for rpm. The positive coefficient parameter will show the influence and the negative coefficient parameter will not have any influence. Thus it was concluded that, sucrose has the high influence over the Caffeine degradation efficiency.

APPENDIX

APPENDIX

CAFFEINE LIQUID MEDIA (CLM)

Composition per litre

$Na_2HPO_4.7H_2O$	-6.4g
KH_2PO_4	-1.5g
NaCl	-0.25g
NH_4Cl	-0.5g
Caffeine	-0.3g
pH	- 7.2 with 0.1 N NaOH.

NUTRIENT AGAR

Composition per litre

Meat extract	– 3.og
Peptic digest of animal tissue	– 5.0g
Agar	– 15.0g
pH	– 6.8 ± 0.2 (at 25°c)

NUTRIENT BROTH

Composition per litre

Peptic digest of animal tissue	– 5.0g
Sodium chloride	– 5.0g
Beef extract	– 1.50g
Yeast extract	– 1.50g
pH	– 7.4 ± 0.2 (at 25°c)

SODIUM (META) PERIODATE

M.W – 213.89

CAFFEINE

Caffeine – 98.50%

M.W – 194.19

3-METHYL -2-BENZOTHIAZOLINADE HYDRAZONE HYDROCHLORIDE

Sulphated ash – 0.1%

pH – 3-4

M.W – 233.71

GRAM STAIN REAGENTS

Crystal violet stain (g/100 ml)

Solution A

Crystal violet – 2 g

Ethanol (95%) – 20 ml

Solution B

Ammonium oxalate – 0.8g

Distilled water – 80 ml

Mix solution A and B; store for 24 h before use.

Gram's Iodine solution (g/300 ml)

Iodine crystals – 1 g

Potassium iodide – 2 g

Distilled water – 300ml

Grind dry Iodine crystals and potassium iodide thoroughly in a mortar, adding water (few ml at a time) and rinse into a new bottle with the

Safranin solution (g/100 ml)

Stock Safranin solution

Safranine – 0.25 g

Ethanol (95%) – 100 ml

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REFERENCES

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