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Potential of Microbial-decaffeination Process: A Review

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ABSTRACT: Caffeine is described as an essential naturally occurring, viable, and marketable purine alkaloids which can be degraded by bacteria; the competence of bacteria to use caffeine as its sole source of carbon and nitrogen has been elucidated more than four decades ago. This paper presents a review of the potential of microbial decaffeination process using standard techniques of harvesting recent and appropriate information and data from Online and Library sources focusing on to bacterial caffeine degradation processes: *N*-demethylation and C-8 oxidation. These two processes were observed to be more efficient, safe, specific, and economically crucial to caffeine degradation. Various organisms have been isolated across the globe that are capable to degrading caffeine such as *Klebsiella, Rhodococcus, Alcaligenes*, *Serratia*, *Phanerochaete,* and *Bacillus* sp. Furthermore, innumerable biotechnological application of the bacterial caffeine degradation has been identified such as bioremediation of caffeine-polluted environment, bio-decaffeination, chemical production, and diagnostic tools.

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Caffeine (1, 3, 7-trimethylxanthine or 3, 7-dihydro-1, 3, 7-trimethyl-1H-2, 6-dione) is a member of the purine alkaloids. It is a white crystalline alkaloid of xanthine, which is odourless, bitter, and amorphous in its pure form and used as a drug activator with the empirical formula of $C_8H_{10}N_4O_2$, a molecular weight of 184.2 g/mol, and half-life of 5hours. The parent chain of the compound is hydrophilic while its methyl groups are hydrophobic (Kudema *et al.,* 2023). The consumption of caffeine as a source of food and beverages had been in practice for decades before its isolation in a pure state by Friedrich Ferdinand in 1891 (Heishman and Henningfield, 2020). Caffeine mainly is a defence chemical against pests, herbivores, and other organisms that affects plants with a profound effect on the metabolism of these organisms. Coffee is one of the major plantation crops and the world's second most tradable commodities after oil. Coffee processing produces a huge amount of waste that is hazardous in terms of its removal and has a negative impact on the health of humans, animals, as well as the environment (Nanjundaiah *et al.,* 2017). The caffeine-containing agro-industrial waste is discarded into the environment which contaminates the surrounding soil and water as caffeine cannot be easily hydrolyzed and degraded in nature (Carmen *et al.,* 2020). Reuter et al. (2021) reported that 3% of ingested caffeine is found in the urine, representing its potential to pollute water and soil. Sousa et al. (2018) reported that caffeine is one of the frequent compounds with a high

concentration in groundwater samples in Europe during their evaluation of the presence of chosen polar organic persistent pollutants in groundwater. The caffeine in the liquid effluents of coffee and tea industries cannot be allowed to be fed into lakes and rivers as it would affect the aquatic ecosystem (Ibrahim *et al.,* 2016a, b). Caffeine present in the soil also affects soil fertility, as the compound inhibits the growth of seedlings and seed germination (Batish *et al.,* 2008; Lakshmi and Nilanjana, 2013). Therefore, there is a strong need for caffeine degradation from products and waste streams by routes other than conventional extraction techniques (Gokulakrishnan *et al.,* 2005; Mohanty, 2013). Hence, caffeine degradation is important in view of environmental concerns. Besides its environmental implications, caffeine has the ability to cross the blood-brain barrier and placental barrier have a high chance of resulting in fetal malformation by inhibiting the expression of genes that are crucial to development, this is due to the inability of the fetus to degrade caffeine as the enzymes have not been produced (Doepker *et al.,* 2016). Caffeine has also been associated with the leading cause of heart disease, cancer, and complications in aged and elderly people. Therefore, caffeine degradation is a crucial process that is required based on its environmental and health influence. Historically, the decaffeination process has been carried out using conventional methods such as supercritical fluid extraction and solvent extraction processes. Ibrahim et al. (2014) reported that the conventional approaches to caffeine elimination such as supercritical fluid and solvent extraction are expensive and involve hazards. These methods involve the use of high-tech equipment that are expensive, toxic solvents, and are not specific for extraction of caffeine as other compounds such as flavonoids and cannabinoids use the same process and principle. This lack of specificity would result in reducing the palatability and quality of food substances (Zabot, 2019). Hence, there is a need for a more specific, economical, and time-effective method for the decaffeination process. One such process is the microbial and enzymatic methods of decaffeination that are economic, eco-friendly and specific to caffeine (Abdulrasheed *et al.,* 2020; Jin *et al.,* 2014).

Therefore, microbial degradation of caffeine and related methylxanthines has been the focus of research in the recent past, owing to the major advantages it has over conventional techniques of decaffeination. Ibrahim et al. (2016c) reported that the biodegradation of caffeine by microorganisms and enzymes is more specific, eco-friendly, sustainable, and relatively a low-cost strategy to overcome the problems associated with conventional methods.

However, before discussing the degradation of caffeine by microorganisms, it is crucial to discuss the industrial process of degrading caffeine.

Degradation of caffeine using industrial methods: Decaffeination is the act of removing caffeine from coffee beans and tea leaves. Most decaffeination processes are performed on unroasted (green) coffee beans, but the methods vary somewhat. It generally starts with the steaming of the beans (Vuong and Roach, 2014). They are then dipped into solvent for several hours (Pietsch, 2017). The process is repeated for 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 (Zabot, 2019). According to the study by Pietsch, (2017), there are three different methods of decaffeination, widely used, are; water decaffeination, solvent decaffeination and carbon dioxide decaffeination. Although caffeine is water soluble above 175^{o} F, water alone is generally not used to decaffeinate coffee because it strips away too many of the essential flavor and aroma elements (Iswanto *et al.,* 2023). Decaffeination by solvents can be through two methods: direct and indirect contact. In the first the beans come directly in contact with the decaffeinating agents, after being softened by steam. Ethyl acetate and methylene chloride are the most frequent solvents used. However, methylene chloride is often to be used in the industries. This substance is more suitable to eliminate caffeine without removing the aroma and taste of the coffee. According to the United States Food and Drug Administration (FDA), most decaffeinated coffee has only below than 0.1 ppm methylene chloride residual (Iswanto *et al.,* 2023). Solvent decaffeination process involves steaming, pre-wetting, caffeine extraction, steam stripping, and drying (Figure 1). Green coffee beans were moved into an extractor, steamed in order to make the surface more porous so that it can easily extracted when the solvent comes in contact with the caffeine. The beans were then steeped in wet to increase their moisture content by 40% in weight. Pre-wetting water and solvent (ethyl acetate or methylene chloride) were included together in the step. The ratio of beans to solvent is 1:4. Caffeine content in the beans was then extracted after the solvent was being heated in a temperature of 70.8 °C. It takes about 10 h for the caffeine to be completely extracted (Pintauro, 1975). Steam and solvent stripping subsequently take place on the green coffee beans, so as to get rid of any residual solvent or methylene chloride is the major reason of this step. It is subsequently dried and stored. In recent years, the use of solvent decaffeination has significantly reduced due to health effect. The use of water as the solvent to decaffeinate coffee is currently

commercially used under the trade-mark "Swiss Water Process" by The Swiss Water Decaffeinated Coffee Company of Canada, British, Burnaby. The water decaffeination is possibly the most widely conventional technique used for coffee decaffeination (Vegesna, 2007). This technique is based on the ordinary capability of water to make caffeine to become soluble. Though, in this method the water acts non-selectively on the untreated coffee, thereby removing all the soluble components, such as the flavor and aromas.

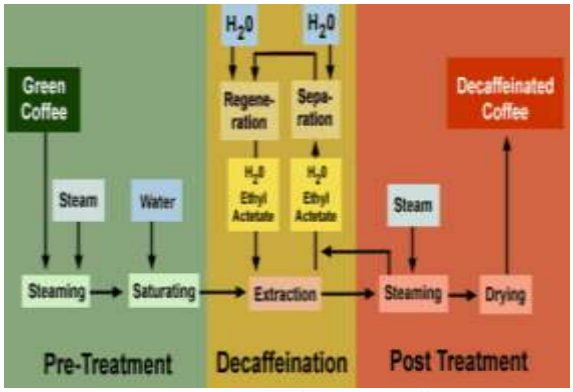

Fig 1: Schematic representation of solvent decaffeination process

In order to avoid the extraction of all soluble water apparatus of coffee beans, water have basically a symmetrical amount of the non-caffeine soluble solids. The beans were kept for about 8h in the extractor in order to remove 98% of the pure caffeine. The extract water together with caffeine, flavor, coffee aroma and coffee solids were subjected to caffeine extraction by solvents (Katz, 1987). The crude dissolved as well extracts flavor and aroma of the caffeine. The organic solvent must be isolated, because the extract water has been recycled. The coffee extract returned to the beans to reabsorb the components flavour, after removing the solvent. The decaffeinated beans are washed down afterwards, desiccated and stored (Vegesna, 2007). Decaffeination by swiss water is quite an easy method. The beans are extracted in hot water, to remove the flavor parts and the caffeine from the beans into the water (Figure 2). Caffeine is isolated by passing the water via carbon filters, after the water has been saturated. The carbon filters adsorbed the caffeine and the beans reabsorbed the extracted free caffeine, which are dried and roasted. Another method of industrial caffeine degradation is supercritical carbon dioxide which is believed to be a safer method than the solvent decaffeination. By using only water and carbon dioxide as a natural method of decaffeination, this method is most acceptable by many researchers. It uses condensed CO_2 and subject to a high temperature. Combination of high pressure and temperature enables carbon dioxide to turn into a solvent. The process starts with the beans pre-wetting with vapor, putting the pre-wetted beans into an extract or at the same time solid permeable is put into a container (Vegesna, 2007). In the container, damp CO_2 is also included that have solid absorbent and coffee beans. Supercritical CO_2 is then distributed between the solid accumulator (adsorber) container and the extractor. As the CO_2 bypass through the extractor, caffeine is isolated and the CO_2 rich in caffeine passes through the accumulator where the caffeine is accumulated. Then, free-caffeine CO_2 again moves for another cycle. This procedure is repeated continuously until the required decaffeination level is attained. The beans are then dehydrated and stored (Figure 3).

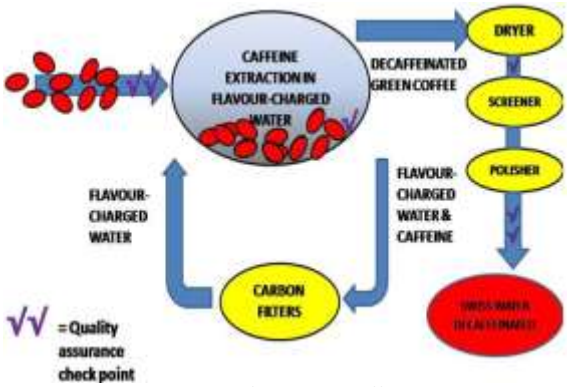

Fig 2: Schematic process for water decaffeination. (Vegesna, 2007)

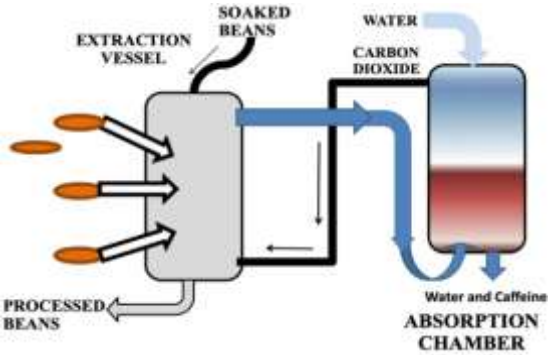

Fig 3: Schematic process for carbon dioxide decaffeination (Vegeena, 2007)

Degradation of caffeine using biological methods: Bio-decaffeination is the removal of caffeine from coffee, tea and other caffeine containing materials by the action of externally added microbial cells or enzymes. The concept of bio-decaffeination is a relatively new area of decaffeination and there is a growing interest in this area of biotechnology due the advantages it offers like being environmentally safe, economical and in preserving the quality of the beverages (Ibrahim *et al.,* 2016a). Development of

biological or enzymatic methods of decaffeination demands a deep understanding of the caffeine metabolism in microbial, plant and animal systems (Dash and Gummadi, 2012). A thorough knowledge of the caffeine metabolism, the enzymes involved and various factors involved in the caffeine degradation in different living systems will give deep insights into the development of efficient bio-decaffeination processes (Baker *et al.,* 2012). Detailed information on different enzymes involved in the degradation of caffeine in different organisms could help in developing an enzymatic process for caffeine removal. In this study we focus on the decaffeination process in prokaryotes whole cell, with specificity on bacteria.

Degradation of caffeine by microorganisms: Caffeine was once thought to be toxic to microorganisms, and research showing that it can be degraded by microbes weren't published until 1970 (Zhipeng *et al.,* 2023). Even while microbial sovereignty research on the enzymology and biochemistry of caffeine degradation in bacterial cells is well studied (Gopishetty *et al.,* 2011; Summers *et al.,* 2012, 2011; Yu *et al.,* 2008, 2009). It has been discovered that at low concentration, caffeine inhibits bacterial strains in the growth medium. Addition of anti-microbial substances like chloramphenicol has been shown to have some synergistic effects (Summers *et al.,* 2014). Caffeine degradation by microbes was first reported in the early 1970's (Kurtzman and Schwimmer, 1971). Since then, advancements have been made in the use of caffeine as a source for microbial growth (Schwimmer *et al.,* 1971; Vogels and Drift, 1976; Woolfolk, 1975).

In bacterial systems, particularly those of the genus Serratia and others like *Rhodococcus*, *Leifsonia Klebsiella* and *Pseudomonas* species, caffeine degradation has been well studied. Contrary to higher species, bacteria may use caffeine as their only source of energy, nitrogen, and carbon for growth (Madyastha *et al.,* 1999; Mazzafera *et al.,* 1996; Mohanty *et al.,* 2012; Mohanty, 2013; Yu *et al.,* 2008, 2009; Ibrahim *et al.,* 2018). At high concentrations, caffeine is toxic to bacteria which are essential for bactericide action (Bogo and Mantle, 2000). According to Raj and Dhala, (1965), some microorganisms can grow in the presence of caffeine, and their survival would depend on their ability to degrade the alkaloid. Actually, it's rare to come across bacterial strains that are caffeine-resistant (Ibrahim *et al.,* 2014; Woolfolk, 1975). Purines can be used as a source of nitrogen or carbon by some microorganism, such as *Klebsiella pneumoniae*. Genus *Serratia* and *Pseudomonas* are the bacterial strains capable of degrading caffeine. Caffeine at high concentration greater than 2500 mg/L in the growth medium has been found to inhibit the growth of many bacterial species. Efforts were made with the help of inhibitors for the bio-production catabolic intermediates of caffeine. *Pseudomonas* strain was used to produce theobromine (Iswanto *et al.,* 2023). Theobromine was accumulated at various concentrations ranging from 5000 mg/L and above, using 1000 µM Zn^{2+}. Tryptone and fructose were found to be the best nitrogen and carbon (Asano *et al.,* 1993; Dash and Gummadi, 2012). Theobromine production increased in the medium by 10 times in the presence of 0.04% Fe^{2+}, indicating that Fe^{2+} may function as a co-factor or enhance the production of demethylating enzymes. Presently, about 35 different strains of bacteria with the potential to degrade caffeine have been isolated and are summarized in Table 1 below. Although there exist differences in the types of isolated bacteria, a large proportion are mainly *Pseudomonas*, specifically *Pseudomonas putida.* Caffeine-degrading bacteria are geographically dispersed, and have been found in coffee fields (Ibrahim *et al.,* 2015), wastewater streams (Arimurti *et al.,* 2018) and garden soil (Summers *et al.,* 2013). Metabolic studies with these caffeine-degrading bacterial isolates have revealed only two catabolic pathways: *N*-demethylation and C-8 oxidation (Arimurti *et al.,* 2018). The *N*-demethylation pathway appears to be the most common, as it has been observed in over 80% of reported isolates where metabolism has been characterized.

Table 1: Characteristics of previously isolated caffeine-degrading bacteria.

Species	Place of isolation	Catabolic pathway	References
Pseudomonas sp. CES	Iowa, USA	*N*-demethylation	(Chi *et al.,* 2008)
Pseudomonas putida CBB5	Iowa, USA	*N*-demethylation	(Chi *et al.,* 2009)
Acetobacter sp. T3	India	N.R.	Babu et al., 2005
Alcaligenes fecalis T1	India	N.R.	Babu et al., 2005
Pseudomonas sp. CBB1	Iowa, USA	C-8 Oxidation	Chi *et al.,* 2008)
Alcaligenes sp.	Canada	C-8 Oxidation	(Mohapatra *et al.,* 2006)
Moraxella sp.	Brazil	N.R.	(Yamaoka-Yano *et al.,* 1999)
Pseudomonas putida IF-3	Japan	N-demethylation	(Koide *et al.,* 1996)
Serratia marcescens	Brazil	N-demethylation	(Mazzafera *et al.,* 1996)
Klebsiella and *Rhodococcus*	India	C-8 Oxidation	(Madyastha and Sridhar 1998)
Pseudomonas putida WS	Germany	N-methylation	(Springer-Verlag *et al.,* 1987)
Leifsonia sp. SIU	Malaysia	N-methylation	(Ibrahim *et al.,* 2016c)
Pseudomonas stutzeri Gr 21 ZF	Lebanon	C-8 Oxidation	(El-Mched *et al.,* 2013)
Brevibacterium sp.	India	N-methylation	(Nayak *et al.,* 2012)

In both pathways, bacteria break caffeine down to carbon dioxide and ammonia to harvest energy and cellular building blocks (Summers *et al.,* 2013). In the process of *N*-demethylation of caffeine, the caffeine molecule is sequentially *N*-demethylated to form xanthine. Each of the three methyl groups is removed with the incorporation of molecular oxygen to produce one formaldehyde and one water molecule per reaction. Theobromine (3,7-dimethylxanthine) is the major metabolite formed from the first step in the pathway, with small amounts of paraxanthine (1,7-demethylxanthine) also reported in some strains (Chi *et al.,* 2008). The second step of the pathway is the N_3-demethylation of theobromine or the N_1-demethylation of paraxanthine to form 7-methylxanthine. 7-Methylxathine is further N_7-demethylated to form xanthine. Finally, xanthine is converted to uric acid, which enters the normal purine catabolic pathway (Purwoko *et al.,* 2023).

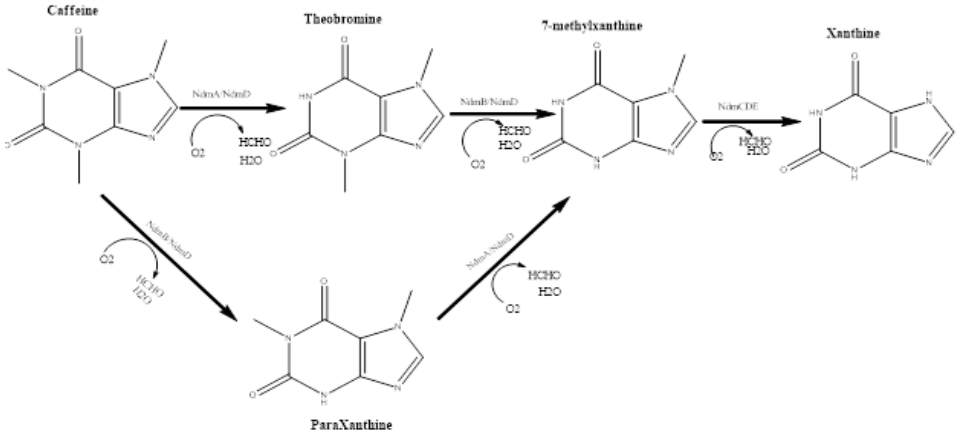

Fig 4: N-methylation of Caffeine

The second metabolic pathway of caffeine involves C-8 oxidation where caffeine is oxidized to form 1,3,7-trimethyluric acid (TMU), which is further degraded by a pathway homologous to the uric acid metabolic pathway. This pathway has been observed in both mixed and pure culture (Mohanty, 2013a; Mohanty *et al.,* 2012). 1,3,7-trimethyluric acid is further metabolized to sequentially form 1,3,7-trimethyl-5-hydroxyisouric acid (TM-HIU), 3,6,8-trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (TM-OHCU) and 3,6,8-trimethylallantoin (TMA) (Mohanty, 2013). Further degradation of TMA has not yet been fully characterized. However, it is believed that only S-(+)-TMA is formed enzymatically and its degradation proceeds through trimethylallantoic acid (TMAA) before being mineralized to glyoxylic acid, dimethylurea and monomethylurea as elucidated in the study by Mohanty (2013). These latter compounds are then assumed to enter the central metabolic cycles of the bacterial cell, figure 2 below summarizes the oxidation pathway of caffeine degradation.

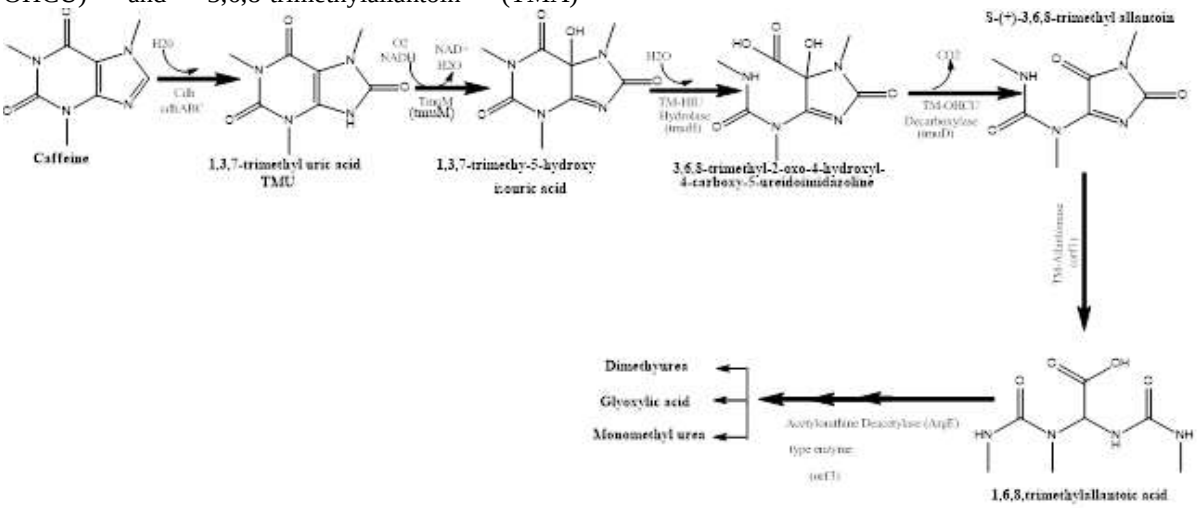

Fig 5: C-8 Oxidation of Caffeine by bacteria

Microbial Enzymes: Demethylases and oxidases are the enzymes responsible for microbial degradation of caffeine (Asano *et al.,* 1993; Dash and Gummadi, 2012; Yamaoka-yano and Mazzafera, 1999; Yamoka-Yano and Mazzafera, 1998). Attempts were made with the help of some inhibitors to purify demethylase enzyme but found that the purified enzyme was not stable and swiftly lost its activity (Yamoka-Yano and Mazzafera, 1998). Sideso et al. (2001) reported that to improve the stability of the enzyme, it has been observed that cryo-protectants and freeze-drying were used to low the moisture contents. Generally, caffeine-degrading enzymes are unstable and more research needs to be done in order to enhance the stability of the enzymes. In a mixed culture of *Rhodococcus* sp. and *Klebsiella* sp; caffeine oxidase at the C-8 position directly oxidised caffeine to 1,3,7-trimethyluric acid and this route does not have demethylation steps (Figure 5). Only partial characterisation of this enzyme is done (Zhipeng *et al.,* 2023). The oxidative caffeine degradation to 1,3,7-trimethyluric acid (single step) emerge to be proficient for development of enzymatic caffeine degradation. Studies on enzymes stability, cloning and over expression of the enzyme in appropriate hosts will lead to development of biotechnological process for efficient caffeine degradation. In yeast and fungi, caffeine is being degraded by cytochrome P450, signifying that yeast metabolic pathway might be related to mammals. Though, application of cytochrome P450s for microbial decaffeination process is not spontaneous as the caffeine degradation rates are too slow and their co-factor requirement is very high (Szlapinski *et al.,* 2023). Furthermore, these enzymes are expressed at extremely little amount and are microsome bound requiring the separation of these enzymes in the form of microsome which will increase the costs of a commercial process to inhibitory levels. It is known that caffeine degradation by bacterial enzymes is more efficient, and their requirements for co-factors are lower. In addition, the isolation and purification of these enzymes is quite simple and crude cells also have high enzymes activities.

Variables that influence degradation of caffeine by microorganisms: Caffeine degradation by microorganisms (bacteria) is affected by many cellular and physiological factors such as temperature, pH, high caffeine concentration, additional carbon and nitrogen source. Caffeine degradation plays a vital role in controlling the rate of enzymes production, degradation of caffeine, which involved in proliferation of caffeine-degrading microbial cells population and caffeine metabolism in caffeine-rich environment (Gummadi *et al.,* 2012). In addition to that, there are some factors that should be taken into consideration in developing a caffeine degradation method. Among them is temperature, which is an important factor in the decaffeination of hot beverages using biological method (Iswanto *et al.,* 2023).

Application of microbial caffeination degradation process: An understanding of the genes involved in bacterial caffeine degradation may open up several new biotechnological applications. Some of these include biological decaffeination of coffee, tea and caffeinated plant matter, environmental remediation of soils and waters with high caffeine concentrations, synthesis of alkylxanthines and alkyl uric acids for use as chemicals or pharmaceuticals and development of a rapid diagnostic test to detect caffeine and related methylxanthines (Ibrahim *et al.,* 2014). These applications can be observed in the following areas.

Bio-decaffeination: Bio-decaffeination of coffee and tea using whole microbial cells or enzymes has been discussed for a number of years (Gopishetty *et al.,* 2011). *Pseudomonas putida* CBB5 can completely decaffeinate coffee and tea extracts, while *Pseudomonas* sp. CBB1 has also been used to decaffeinate tea extracts (Gopishetty *et al.,* 2011). In terms of relative efficacy, strain CBB5 used the ***N***-demethylation pathway to degrade a higher amount of caffeine in a shorter amount of time than strain CBB1 through the C-8 oxidation pathway. An immobilized mixed culture of *Klebsiella* sp. and *Rhodococcus* sp. was also used to decaffeinate tea extract *via* C-8 oxidation under both batch and continuous processes (Summers *et al.,* 2014). Overall, the *N*-demethylation pathway appears to be more efficient than C-8 oxidation for use in the microbial decaffeination of coffee. However, the use of bacterial cells for the bio-decaffeination of beverages may not be feasible due to the potential for the release of endotoxins. Alternately, the use of purified caffeine-degrading enzymes (either soluble or immobilized) may provide a viable alternative to eliminate endotoxin problems (Gopishetty *et al.,* 2011). The *ndmABCDE* genes could be cloned into *Escherichia coli* for large-scale recombinant enzyme production in order to carry out the bio-decaffeination of beverages. Another approach is to clone caffeine-degrading genes into *Saccharomyces cerevisiae* or another generally regarded as safe (GRAS) organism for whole-cell bio-decaffeination (Gopishetty *et al.,* 2011), thus circumventing the endotoxin problem. Through this method, optimized genetic cassettes could be transformed into the GRAS organism, creating an enhanced caffeine-degrading strain.

Bioremediation: Bioremediation is the process of using enzymes, fungi, or bacteria the transformation of

toxic compounds into a more harmless and non-toxic compounds in the environment (Ibrahim, *et al.,* 2016b). Bioremediation of coffee waste is an enhanced approach that comprises the use of bacteria to convert and degrades toxic coffee wastes in the environment into an eco-friendly substance. Bioremediation is targeted at reducing the level of toxic wastes in an environment through the use of bacteria, plants, fungi, and animals to breakdown the toxic substances (Summers *et al.,* 2015).

There are many routes by which caffeine enters the environment, where it can exhibit toxic effects on the surrounding plants, insects and microbes (Szlapinski et al., 2023). In coffee and tea fields, fallen leaves, stems and seeds decompose, releasing caffeine into the soil. Solid and liquid wastes from coffee and tea processing plants also contain high levels of caffeine, which enter soil and groundwater. The widespread use of caffeine in foods, beverages and pharmaceuticals leads to high levels of caffeine in human wastewater streams, as well. In fact, caffeine can be used as an anthropogenic marker for wastewater contamination in the environment (Gracia-Lor *et al.,* 2017). In all of these cases, either wild-type or recombinant caffeine-degrading microorganisms may be of use in removing caffeine from contaminated environments.

Diagnostic tool: The adulteration of food and beverages with caffeine has resulted in growing concerns of government food agencies and also caffeine-sensitive consumers culminating in a requirement for a reliable in-home test process to detect caffeine in foods. Currently, a Cdh enzyme-based colourimetric test has been developed. This test was rapid and sensitive enough to detect caffeine in beverages, including coffee, soft drinks and nursing mother's milk, within minutes (Yu *et al.,* 2014); Mohanty, 2013). Based on the type of dye (electron acceptor) used, the test developed a bright colour upon exposure to caffeine even at 1–5 ppm level. The test could successfully detect caffeine in samples with a wide range of pH and variations, with milk and sugar, or with other active pharmaceutical ingredients. Thus, this test is now deemed to be highly suitable for further development into an 'in-home' type strip-based test (Yu *et al.,* 2014).

Chemical production: While caffeine is a relatively inexpensive molecule, many of the metabolites formed by both *N*-demethylation and C-8 oxidation of caffeine and their analogues are high value chemicals. Many of these chemicals have great potential in the pharmaceutical and cosmetic industries. Uric acid and methyluric acids are antioxidants (Summers *et al.,* 2014), and 8-oxomethylxanthines may be used in treatments for obesity, skin cosmetics and anti-dandruff products (Arimurti *et al.,* 2018). Methylxanthines have been used as diuretics, bronchodilators, antioxidants and asthma control (Gracia-Lor *et al.,* 2017). Most methylxanthines and methyluric acids are difficult to synthesize chemically because selective alkylation of each nitrogen atom is difficult to achieve (Szlapinski et al., 2023). The recent discovery of genes encoding bacterial caffeine degrading enzymes may help to facilitate synthesis of these high-value chemicals. The *ndmABCDE* genes catalyse specific *N*-demethylation of alkylxanthines, which leave a specific methyl group open for chemical derivatization. Caffeine dehydrogenase from *Pseudomonas* sp. CBB1 can oxidize caffeine to TMU, and displays some activity towards other methylxanthines, as well. Currently, there is only one report of methylxanthine production from caffeine using engineered cells (Kudema *et al.,* 2023). The genes *ndmA* and *ndmD* were cloned into *E. coli*, resulting in a bacterial strain that was able to effectively convert caffeine to theobromine. A second *E. coli* strain was constructed to convert theobromine to 7-methylxanthine using *ndmB* and *ndmD* genes. This preliminary report demonstrated the feasibility of specific methylxanthine production from caffeine.

Conclusion: Caffeine is a purine alkaloids which can be degraded by microorganisms. The recent discovery of bacterial genes responsible for metabolism of caffeine, by both *N*-demethylation and C-8 oxidation routes, opens numerous potential biotechnological applications. These novel genes and enzymes may be of great use in home diagnostic tests, remediation of caffeine-contaminated environments and production of chemicals, pharmaceuticals, animal feed and biofuels.

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