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Optimization of the environmental and physiological factors affecting microbial caffeine degradation and its application in caffeinated products.

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Ten bacterial isolates were tested for their ability to utilize caffeine as the sole source of carbon and nitrogen source. The isolates identified as Pseudomonas stutzeri Gr 21 ZF showed 59% of 1.2 g/l caffeine degradation in 24 hrs when caffeine was used as the sole source of carbon and nitrogen source. In this study, the physical parameters viz, incubation time, temperature, culture volume, inoculums size and pH were carried out to select a suitable conditions that lead to maximum caffeine degradation. The optimum values were found to be 48hrs, 50 ml, 3ml and 7 respectively. In the presence of sucrose (5g/l), 80.1 % degradation of caffeine was achieved in 48 hrs. The addition of inorganic source NH₄Cl and KNO₃ increase the caffeine degradation to 80.2% and 86% respectively. The critical medium components viz Na₂HPO₄, KH₂PO₄, caffeine, sucrose, MgSO₄.7H₂O, CaCl₂, Fe²⁺ affecting the caffeine degradation were determined by Plackett- Burman design. The optimum concentration that lead to 99.07% of caffeine degradation with 10% of crude enzyme were found to be 0.2 g/l; 2.6 g/l; 0.3g/l; 0.6 g/l; 10 g/l; 5g/l and 0.02% respectively

Keywords: Caffeine degradation, microbial degradation, medium composition, Plackett-Burman.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine), a purine alkaloid occurs in more than 60 plant species including in the seeds of coffee, cacao, cola tree and in the leaves of tea (Ashihara and Crozier, 2001). Caffeine is also an important constituent of a variety of beverages such as coffee, tea, caffeinated cola and soda, and cocoa-derived food products such as chocolate, desserts and pastries (Weigl et al., 2004). Coffee, tea, cola and cacao contain about 100, 50, 40 and 10 mg of caffeine per serving, respectively (Clarke and Macrae, 1988).

An increase in the number of cardiovascular disorders as result of change in the lifestyle, decaffeination of other food products and beverages is being recommended, keeping in view the adverse effect of chronic caffeine consumption on the cardiovascular system (Greenberg et al., 2007). Caffeine consumption during pregnancy increase the risk of spontaneous abortion and affects fetal growth (Garattini, 1993). In addition, moderate to high caffeine intake greater than 150 mg caffeine /day, imbalances the bone mineral density leading to osteoporosis particularly in women (Cooper et al., 1992).

Hence there is a strong need for degradation caffeine from products and waste streams by alternative route other than conventional extraction techniques (Gokulakkrishanan et al., 2005). Decaffeination is being recommended for food, beverages and wastewaters because of potential chronic ingestion of caffeine can have adverse effects on the physiological systems.

Conventional decaffeination techniques (Dixon and Johnston, 1997) like solvent extraction or use of supercritical carbon dioxide can be expensive, toxic to the environment and non-specific. These methods often result in the removal of aroma and flavor imparting substances and their precursors resulting in an unpalatable product. To compensate for this, artificial flavours and color are often supplemented to the decaffeinated product. The application of genetically modified coffee plants produced by silencing the gene coding for enzymes responsible for synthesis of caffeine may be unfavourable because of the importance of caffeine such as a part of the plant defence mechanism against predators (Ogita et al., 2003). Hence there is a
strong need for caffeine degradation from products and waste streams by alternative routes other than conventional extraction techniques. The potential use of microorganisms and enzymes obtained from microbial system for developing biological decaffeination techniques offer a much attractive alternative to the present existing techniques since it’s cheaper, easier and faster (Mazzefera et al., 2002). In the present study an attempt was made to isolate and select a caffeine degraders and study the environmental and physiological factors of the most promising and selected isolate that lead to maximum caffeine degradation or complete caffeine degradation and its application for decaffeination of some caffeine products during processing.

MATERIALS AND METHODS

Chemicals

Caffeine anhydrous >99% was purchased from sigma, Aldrich USA. Other used chemicals are of analytical grade were obtained from recognized chemicals suppliers (Oxoid ltd and Fluka).

Media

The fermentation media used contained (g/l): Na₂HPO₄, 0.12; KH₂PO₄, 1.3; CaCl₂, 0.3; MgSO₄·7H₂O, 0.3; Fe²⁺, 0.02 % (w/v). Caffeine medium consisted of these salt supplemented with 1.2 g/l of caffeine. Carbone sources if any added to the medium were sterilized separately and then mixed to the medium under aseptic conditions. The pH of the medium after autoclaving was 6.

Soil samples

Soil samples were collected from different locations in Bekka Valley at 30 cm depth from soil surface under aseptic conditions were used to obtain microorganisms that degrade caffeine.

Isolation and purification of caffeine degrading bacteria

Serial dilutions of the soil sample (1/10, 1/10², 1/10³, 1/10⁴ and 1/10⁵) were prepared in sterile distilled water an plated on caffeine agar media plates and then incubated at 30°C ± 2 for 2 days.

Preparation of seed culture

Seed culture were prepared in 250 ml erlenmeyer flasks containing 50 ml nutrient broth medium with 3 ml of bacterial suspension prepared from 18 hrs old cultures and then incubated at 30°C on a rotary shaker (180 rpm) till reaches O.D₆₀₀ <1. These were used as standard inocula (1 ml/ 25 ml medium) unless otherwise stated.

Screening and identifying for caffeine degradation activities of the isolates under investigation

The cultivation of the isolated and purified bacterial strains was achieved in 250 ml Erlenmeyer flasks each containing 25 ml of fermentation media Na₂HPO₄, 0.12; KH₂PO₄, 1.3; CaCl₂0.3; MgSO₄·7H₂O, 0.3; sucrose, 5; caffeine, 1.2; Fe²⁺, 0.02% (w/v). The media were sterilized by autoclaving for 20 min then the filter sterilized caffeine was added and inoculated with 1 ml inoculums level unless otherwise stated and then incubated at 30°C ± 2 for 24 hrs under shaken conditions using incubator shaker (180 rpm). The isolates that utilized caffeine as sole carbon and nitrogen source were picked and sub-cultured for every two weeks. Further selection was made based on their ability to utilize caffeine in submerged fermentation.

Fermentation technique

A single colony from caffeine agar plates was transferred to 5 ml sterile caffeine medium. The tube with cotton plugs were aerated on a rotary shaker at 180 rpm and incubated for 24 h at 30°C ± 2. About 1 ml of the culture was transferred to the 25 ml of caffeine medium in 100 ml Erlenmeyer flasks with cotton plugs and aerated on a rotary shaker at 180 rpm and incubated for 48 h at 30°C ± 2.

Effect of incubation period

Samples were collected at different intervals of time (12, 24, 36, 48, 60 and 72 hrs) and measured for cell growth and caffeine degradation.

Effect of incubation temperature

In order to study the effect of incubation temperature on caffeine degradation, the caffeine degradation and the cell growth were estimated after 48 h incubation period at different incubation temperature (20, 30, 40, 50 and 60°C).

Effect of culture volume

In order to test the effect of culture volume on caffeine degradation, 250 ml of Erlenmeyer flasks were allowed to
receive different volumes 25, 50, 75 and 100 ml of fermentation media.

**Effect of inoculum levels**

Different inoculum levels 1, 3, 5, 7 and 9 ml of 18 hrs old culture were tested to study the effect of inoculum concentration on caffeine degradation.

**Effect of pH value**

The effect of pH was studied by varying the initial pH of the medium between 4 and 9.

**Effect of carbon source**

In order to study the effect of carbon sources different carbon sources viz., glucose, fructose, galactose and lactose were added to the caffeine medium at 5g/l and the time course of cell growth, carbon source utilization and caffeine degradation were measured.

**Effect of nitrogen source**

In order to study the effect of an additional nitrogen source on caffeine degradation, caffeine medium with 5 g/l sucrose was supplemented by 3g/l of KNO₃, NH₄Cl, casein, peptone, tryptone and yeast extract one at time to the media. All experiments were performed in triplicates under identical conditions and the data presented are mean of triplicate experiments.

**Biomass determination**

The cell pellets after centrifugation of the culture samples were washed twice with deionized water and O.D 600 nm was measured. For cell dry weight (O.D 600 nm of 0.5 corresponds to 0.379 g dry weight /100ml according to standard curve).

**Analytical determination of caffeine**

The residual caffeine in the culture was determined as follows: the culture was sampled and the cells were removed by centrifugation at 8000 r.p.m for 10 min at 4°C. The supernatant was used for analysis of caffeine immediately or stored at 4°C if immediate analysis not possible. Caffeine was estimated by HPLC equipment using (C-18-reserve phase column with polar mobile phase). Pure caffeine at 1.5 g/l was added as a standard. The retention time was found to be 6 min at a flow rate of 1 ml/min at 284-C (Dash and Gummadi, 2007).

**Experimental design nd data analysis**

**Screening of the medium components using a Plackett-Burman design.**

Plackett-Burman design (Plackett and Burman, 1946), well established statistical technique for medium component optimization (Xiong et al., 2005) was applied to screen the medium components were screened for seven variable at two levels, maximum (+) and minimum (-). According to the Plackett- Burman design, the number of positive signs (+) is equal to (N+1)/2 and the number of negative signs (-) is equal to (N-1)/2 in a row. A column should contain equal number of positive and negative signs. The first row contains (N+1)/2 positive signs and (N-1)/2 negative signs and the choice placing the signs is arbitrary. The next (N-1) rows are generated by shifting cyclically one place (N-1) times and the last row contains all the negative signs. Response is calculated as the rate of caffeine degradation expressed as percentage (%) or g/l.

The effect of each variable was calculated using the following equation:

$$E_{xi} = \frac{\sum M_{i+} - M_{i-}}{N}$$

where $E_{xi}$ is the effect of the tested variable, $M_{i+}$ and $M_{i-}$ the rates of caffeine degradation from trials in which the variables being measured were added to the medium at their maximum and minimum level respectively and $N$ is the number of experiments carried out, which is 8 in this case, as the number of variables to be tested is 7.

The standard error (S.E) of the variables was the square root of variance and the significance level (P value) of each variable is calculated by using the $t$- Test:

$$t = \frac{E_{xi}}{S.E}$$

where $E_{xi}$ is the effect of the tested variable. The variables with higher confidence levels were considered to influence the response or output variable.

**Effect of *Psudomonas stutzeri* Gr 21 ZF crude enzyme extract on caffeine degradation**

**Extracellular crude enzyme preparation**

Five hundred ml of *Pseudomonas stutzeri* Gr 21 ZF cultures grown in fermentation medium after 48 hrs incubation at 30°C under shaken condition (1800 r.p.m)
was collected and the supernatant fluid was separated by centrifugation at 5000 rpm for 10 min at 4°C, and acts as the crude enzyme (Olama et al., 1993).

**Entracellular and cell bound crude enzyme preparation**

The bacterial culture after centrifugation and supernatant separation the cells were washed twice with phosphate buffer pH 7 and resuspended in 50 ml of the same buffer. The cells were subjected to ultrasonic until the cells were broken, then the crude extract (filtrate) was collected by centrifugation at 8000 r.p.m for 10 min at 4°C.

**The effect of the crude *Pseudomonas stutzeri* Gr 21 ZF crude enzyme extract on caffeine degradation from different caffeine samples**

The crude enzyme extract was mixed with different caffeine samples collected from different sources in a ratio of 2, 5, and 10% (v/v) respectively. The mixtures were incubated at 30°C for 2 days. The same volume phosphate buffer pH 7 was mixed with caffeine samples as a negative control. The residual caffeine concentration was analyzed using HPLC (Olama et al., 1993).

**RESULTS AND DISCUSSION**

**Screening and identification of microorganisms degrading caffeine**

Ten strains were isolated based on their ability to utilize caffeine as the sole carbon and nitrogen source. Among the ten isolates, isolate T2 degraded caffeine efficiently (isolate T2 utilized 59% of 1.2g/l of caffeine in the medium in 24 h compared to other strains which are able to utilize less than 40% of caffeine in the same time). The Gram negative, non-spore forming, rod shaped isolate was identified as *Pseudomonas* sp. The isolate was positive for citrate utilization, catalase, oxidase, nitrate, citrate, urease and motility. The isolate was negative for indole, oxidation and fermentation test and triple sugar iron test (red slant). Morphological observations revealed that the colony was translucent and small with raised elevation. These data suggest that the isolate T2 is *Pseudomonas* sp.

**Genotypic characterization and phylogeny**

The most promising bacterial isolates T2 was identified by sequencing PCR amplified 16S rDNA. The obtained sequences were submitted to FASTA3 data base in order to find homologies with outer 16S rDNA. Table 1 shows the similarities percentage and accession numbers obtained after comparing the sequence of the tested strains (ZF) to the submitted sequences in gene bank. The tested strain was affiliated to the genus *Pseudomonas* with 95 % similarity to *Pseudomonas stutzeri* Gr 21. The phylogeny of the tested strain and closely relates species was analyzed using multi sequence alignment program. The strain named ZF showed 95% identity to *Pseudomonas stutzeri* Gr 21

**Effect of incubation period on caffeine degradation**

The time course of caffeine degradation by *Pseudomonas stutzeri* Gr 21 ZF was monitored during 72 h incubation period. Results showed that caffeine degradation was increased with the increase *pseudomonas stutzeri* Gr 21 ZF cell growth (Figure1). Maximum caffeine degradation (67.5%) was attained.

<table>
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<th>Isolate</th>
<th>Identify</th>
<th>Accession number</th>
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<tbody>
<tr>
<td>1</td>
<td><em>Pseudomonas</em> sp</td>
<td>AM887688</td>
</tr>
<tr>
<td>2</td>
<td><em>Bacterium</em> LZX26</td>
<td>DQ359948</td>
</tr>
<tr>
<td>3</td>
<td>Uncultured bacterium clone ncd2529d12c1</td>
<td>JF217466</td>
</tr>
<tr>
<td>4</td>
<td><em>Pseudomonas stutzeri</em> Gr21</td>
<td>FN813481</td>
</tr>
<tr>
<td>5</td>
<td><em>Pseudomonas stutzeri</em> Gr20</td>
<td>FN813480</td>
</tr>
<tr>
<td>6</td>
<td><em>Pseudomonas</em> sp. BC045</td>
<td>HQ105013</td>
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<tr>
<td>7</td>
<td>Uncultured bacterium clone nbt16f02</td>
<td>FJ893029</td>
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</tbody>
</table>

Table 1. 95 % Similarity percentage scores of 16S rDNA sequences for the selected isolate (T2) compared to those obtained from database.
after 48 h incubation followed by a decrease in the caffeine degradation to be 59% after 72 h. It was revealed that maximum caffeine degradation was achieved after 48 h at the end of logarithmic phase and the bacteria entered the stationary phase where the caffeine degradation started to decrease. *Pseudomonas alcaligenes* showed maximum caffeine degradation at 40 h (Mohapatra et al., 2006), on the contrary, 72 h was needed for the degradation of 60% of caffeine by *Trichosporon asahii* (Lakshmi and Nilanjana, 2008). The highest caffeine degradation by *Pseudomonas* GSC 1182 was obtained after 36 h of incubation (Gokulakrishnan and Gummadi, 2006).

**Effect of temperature on caffeine degradation by *Pseudomonas Stutzeri* Gr 21 ZF**

The optimum temperature that allowed the maximum caffeine degradation (67.5%) was at 30°C, then it starts to decrease with the temperature increase, it was noticed that 57.5% reduction was achieved at 40°C, 33.5% at 50°C and 14% at 60°C (Figure 2). The optimum temperature for caffeine degradation was found to be 35°C by *Pseudomonas alcaligenes* (Mohapatra et al., 2006) and 40°C by mixed cultures of *Klebsiella* and *Rhodococcus* sp (Madyastha et al., 1999). Dash and Gummadi (2007) reported that Pseudomonas sp NCIM
Effect of the culture volume on caffeine degradation

The best culture volume for caffeine degradation by *Pseudomonas stutzeri* Gr 21 ZF was found to be 50 ml/250 ml Erlenmeyer flask (Figure 3). The same culture medium was used for maximum caffeine degradation by *Pseudomonas alcaligenes*, *Pseudomonas* sp NCIM 5235 (Mohapatra et al., 2006 and Dash and Gummadi, 2007). Laksmi and Nilanjana (2008) found that 100% of caffeine degradation was achieved by 100 ml of culture medium by *Trichosporon asahii*.

Effect of inoculum size on caffeine degradation

The finite volume of culture medium means that it can only contain limited nutrients for the microorganism. Furthermore, the consumption of the nutrients is largely dependent on the population of bacteria. To ensure a high caffeine degradation in limited volume of medium, the bacterial inoculums size should therefore be controlled (Abusham et al., 2009). Inoculating the fermentation medium with various inoculum sizes of *Pseudomonas stutzeri* Gr 21 ZF could affect the caffeine degradation and bacterial growth. The maximum caffeine degradation and bacterial growth in the present work was achieved with an inoculum size of 3ml/50 ml of fermentation media (Figure 4). Similar data was reported by Dash and Gummadi (2007) for caffeine degradation by
Pseudomonas sp NCIM 5235. Maximum caffeine degradation by Pseudomonas alcaligenes CFR 1708 was achieved with an inoculum size of 5ml/50 ml fermentation media (Sarath Babu et al., 2005). Lakshmi and Nilanjana (2008) found that a higher inoculum size (9ml) reduce the caffeine degradation more than if the lower inoculum size (2 ml/50 ml of medium) was used. Therefore, high inoculums sizes do not necessarily give high caffeine degradation yield. The increase in the production of caffeine degradation using small inoculums sizes was suggested to be due to the higher surface area to volume ratio, which resulted in the increased caffeine degradation (Rahman et al., 2005). In addition, an improved distribution of dissolved oxygen and more effective uptake of nutrients also contributed to a higher caffeine degradation. If the inoculums sizes are too small, insufficient number of bacteria would then lead to a reduced amount of caffeine degradation (Shafee et al., 2005). However, higher inoculum sizes could lead to or cause a lack of oxygen and depletion of nutrients in the culture media.

**Effect of pH on caffeine degradation**

The caffeine degradation by bacterial strain is strongly controlled by the culture pH. Pseudomonas stutzeri Gr 21 ZF was active over a pH range from 6-9 with an optimum pH at 7(Figure 5). On the contrary with these findings, Dash and Gummaidi (2006) and Laksmi and Nilanjana (2008) reported that an optimum pH for Pseudomonas GSC 1182 and Trichosporon asahii respectively were in the acidic range pH (5.5-6.5). At pH 4 the caffeine degradation by Pseudomonas stutzeri Gr 21 was 0%, it can not grow at pH 4 (Lalucat, 2006). This is probably the reason why there is a negative reaction to the oxidation/fermentation test for the use of carbohydrates.

Many P. stutzeri Gr 21 strains give a neutral result, as the medium is not buffered and acidification inhibits further growth, even when the strain might be able to use the added sugar. P. stutzeri Gr 21 strains grow well under atmospheric oxygen. However, microaerophilic conditions have to be established when nitrogen-fixing strains are cultured as diazotrophs. All strains described to date are facultatively anaerobic with nitrate. Some strains are also anaerobic, with chlorate or perchlorate as terminal electron acceptors. Sarath Babu (2005) demonstrated that Pseudomonas alcaligenes CFR 1708 exhibited the maximum caffeine degradation ability at pH range from 7.0-8.0. The maximum caffeine degradation was found by mixed culture of Klebsiella and Rhodococcus at pH 7.5 (Madyastha et al., 1999).

**Effect of different carbon source on caffeine degradation**

Different carbon sources have different influences on caffeine degradation (Hakil et al., 1999 and Roussos et al., 1994). Different carbon sources were added to the sterile caffeine media; fructose, galactose, glucose, lactose one at a time instead of sucrose. Results indicated that glucose has an inhibitory effect on caffeine degradation (24%). The maximum caffeine degradation was achieved in the presence of sucrose as a carbon source (80.1%). All the other tested carbon sources have an inhibitory effect on caffeine degradation by Pseudomonas stutzeri Gr 21 ZF (Figure 6). Similar results were observed by Hakil et al., (1999) who showed that the addition of sucrose increase the caffeine degradation by Aspergillus and Penicillum sp. On the other hand, it has been reported that certain bacteria such as Serratia marcescens and Pseudomonas putida have the ability to degrade caffeine in the absence of...
Glucose or sucrose or any other carbohydrates in the medium (Mazzafera et al., 1994; Woolfolk, 1975). Gokulakrishnan and Gummadi (2006) revealed that caffeine degradation by Pseudomonas sp GSC 1182 was inhibited in the presence of glucose and enhanced by the presence of sucrose and lactose.

Effect of different nitrogen source on caffeine degradation

The bacterial process of denitrification is normally a facultative trait. It provides bacteria with a respiratory pathway for anaerobic life (Knowles, 1982 and Zumft, 1997). The distribution of denitrification capabilities among the prokaryotes does not follow a clear pattern (Philippot, 2002). The genus Pseudomonas is one of the largest taxonomic clusters of known denitrifying bacteria. This fact has largely favored the use of species of the genus Pseudomonas as model organisms for studying the denitrification process. Within the genus Pseudomonas, and probably also within the prokaryotes, much of the relevant work, advances in the biochemical characterization of denitrification, and essential genetics using highly interdisciplinary approaches have been achieved with P. stutzeri Gr 21. Denitrification is a stable trait for P. stutzeri Gr 21; it is one of the most active denitrifying, heterotrophic bacteria, and it has been considered a model system for the denitrification process (Zumft, 1997). The present investigation explored the effect of different nitrogen sources on caffeine degradation. Maximum caffeine degradation was reached 86% in presence of KNO$_3$ (Figure 7). In contradiction to our results, it was reported that the additional of external nitrogen sources (both organic and inorganic) inhibited the caffeine degradation and the inhibitory effect was stronger for urea and ammonium sulfate by Pseudomonas sp. GSC 1182 (Gokulakrishnan et al., 2005). Roussos et al. (1999) reported that the additional nitrogen sources in the medium inhibited the caffeine degradation. The external nitrogen sources inhibited the caffeine degrading capability of the yeast cells. The maximum degradation percentage attained was very low 45% (Lakshmi, 2009). Similar results were reported for
fungi 45 % Penicillium (Roussos et.al., 1995) and Aspergillus sp (Hakil et.al., 1999).

**Optimization of medium components using central composite design**

The Plackett-Burman design was applied to reflect the relative importance of various medium components involved in the caffeine degradation. Seven different factors (variables) were chosen to perform the optimization process (Srivians et al., 1994). The variables examined and their settings are shown in table 2. All experimental condition (trials) was performed in duplicate. The result (% of caffeine degradation) is given in table 3.

Caffeine degradation was shown wide variations throughout different trials of the experiment. It was revealed that optimized medium components having high efficiency for caffeine degradation. the analysis of the data from Plackett-Burman experiments involved a first order the main effect, was estimated as the difference between both average of measurements made at high level (+1) and at the low level (-1) of the factor (Lavilla et al., 1998; Abdel-Fattah et al., 2002). The main effect of the examined factors on caffeine degradation was calculated and represented graphically (Figure 8). on the analysis of the regression coefficients of the seven variables: disodium hydrogen phosphate, potassium hydrogen phosphate, sucrose and caffeine had shown a positive effect, whereas calcium chloride, iron and magnesium sulfate had shown a negative effect.

Statistical analysis of the data is demonstrated in as regression coefficients and t-value for the seven experimental variables. The significance levels were determined using the student’s test. The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance (Table 4).

if this probability is sufficiently small, the idea that the effect was caused by varying the level of the variable under test is accepted. confidence level is an expression of the P-value in percent. The factors which showed a high confidence percentage are sodium hydrogen phosphate, potassium dihydrogen phosphate and sucrose where the most significance variable affecting caffeine degradation is disodium hydrogen phosphate.

### Table 2. Variables and their levels employed in the Plackett-Burman design for screening the medium components affecting caffeine degradation.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Code</th>
<th>Low level (-1)</th>
<th>High level (+1)</th>
</tr>
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<tbody>
<tr>
<td>Disodium hydrogen</td>
<td>SHP</td>
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<td>0.24</td>
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<tr>
<td>phosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium dihydrogen</td>
<td>PDP</td>
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<td>2.6</td>
</tr>
<tr>
<td>Phosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>CC</td>
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<td>0.6</td>
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<tr>
<td>Magnesium sulfate</td>
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</tr>
<tr>
<td>Sucrose</td>
<td>SU</td>
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<td>10</td>
</tr>
<tr>
<td>Caffeine</td>
<td>CA</td>
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<td>2.4</td>
</tr>
<tr>
<td>Iron</td>
<td>Fe2+</td>
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<td>0.04</td>
</tr>
</tbody>
</table>

### Table 3. The experimental design using Plackett-Burman method for screening of medium components affecting caffeine degradation by Pseudomonas stutzeri Gr 21 ZF.
The effect of crude *Pseudomonas stutzeri* Gr 21 ZF enzyme on caffeine degradation

The coffee contained high caffeine concentration (1.3g/l) followed by black tea (0.8g/l) that was efficiently degraded after treatment with the crude enzyme extract (Extracellular, cell bound and intracellular enzyme) obtained from the bacterium under investigation. 99.07 and 84.60% of the caffeine in black tea leaves and coffee beans respectively were degraded using 10% crude enzyme. Black tea leaves sample ration indicating the high efficiency of the crude *Pseudomonas stutzeri* Gr 21 ZF enzyme in the degradation of caffeine (Table 5). Sarath Babu (2005) showed that the degradation of caffeine in tea sample using crude *Pseudomonas alcaligenes* CFR 1708 enzyme was found to be only 7.3% of the initial caffeine content (1.2g/l).

**CONCLUSION**

The isolate *Pseudomans stutzeri* Gr 21 ZF being reported is an efficient caffeine degrader, which may be useful in the development of an environmental friendly biodecaffeination process. Degradation efficiency was found to be greatly influenced by incubation time, temperature, pH, volume culture, inoculum sizes and the addition of carbon and nitrogen sources. Plackett-Burman experimental design can be successfully used to optimize the medium constituents affecting caffeine degradation.
REFERENCES


