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How induced cells of *Pseudomonas* sp. increase the degradation of caffeine

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Abstract: Decaffeination is an important process for the removal of caffeine from wastes generated by coffee and tea industries. Microbial degradation of caffeine is more useful than conventional chemical treatment because of its low cost and because it does not involve the use of toxic solvents. However, biodegradation of caffeine remains a problem because of the difficulty of finding a strain that can resist high concentration of caffeine in addition to be able to degrade caffeine at higher rates. In this study, we used the induced cells of *Pseudomonas* sp. for the degradation of caffeine. The induced cells (8 mg/ml) showed complete degradation of a initial concentration of caffeine of 1.2 g/l in 6 hours. The optimum pH was 7.0, the agitation rate was 180 rpm and the optimum temperature for degradation was 35 °C. Under these conditions and in the presence of magnesium, complete degradation of 1.2 g/l of caffeine was accomplished in 4 hours. Additional trials determined that induced cells completely degraded an initial concentration of caffeine of 10 g/l in 26 hours. This is the first report on a strain that can degrade high concentrations of caffeine (e.g., 10 g/l) at the maximum rate of 0.385 g/l per hour. These results suggest that the strain can be used to successfully in developing a biological process for the degradation of caffeine.

Keywords: Caffeine, biodegradation, induced cells, Pseudomonas sp., magnesium

1 Introduction

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6 dione) is a naturally occurring alkaloid found chiefly in tea, coffee, cola nuts, mate, chocolates and pharmaceutical products. Prolonged consumption of caffeine results in headache, fatigue, apathy, adrenal stimulation, irregular muscular activity, cardiac arrhythmias, osteoporosis, and also has

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deleterious effect on cardiac patients [1–5]. Consumption of caffeine during pregnancy causes malformation of fetus and reduces fertility rates [6]. Therefore, it is important to develop decaffeinated beverages and food products. In addition to concerns about health, degradation of caffeine is necessary from an environmental perspective. Caffeine is one of the major toxic compounds generated by solid wastes in the coffee and tea industries; i.e., coffee pulp, husk, and tea waste. In spite of the fact that these wastes are enriched with carbohydrates and proteins, they cannot be used as animal feed because of the presence of caffeine and other toxic compounds [7–9]. The liquid effluents in coffee and tea products have high concentrations of caffeine, thus they cannot be allowed to be fed into lakes and rivers [10]. Because of health as well as general environmental considerations, caffeine degradation is critical. Conventional methods of caffeine removal involve the use of toxic solvents, and such expensive methods as solvent extraction and supercritical fluid extraction. In addition, these methods are not specific to caffeine, therefore other flavor and aroma compounds are removed, resulting in poor quality coffee and tea. Thus, microbiological and enzymatic methods of caffeine removal are highly preferred [11].

Several bacterial species belonging to genera, *Pseudomonas* and *Serratia*, are capable of degrading caffeine, and fungal species belonging to *Aspergillus* have been cited in the degradation of caffeine [12–18]. The rates of degradation of caffeine reported for the organisms mentioned varied from 0.0053 g/l per hour to 0.1 g/l per hour. The significant organisms were *Pseudomonas* sp., with a degradation rate of 0.1 g/l per hour at a initial caffeine concentration of 5 g/l [12], *Serratia* sp. with a degradation rate of 0.008 g/l per hour at initial caffeine concentration of 0.6 g/l [15], and a mixed culture of *Klebsiella* and *Rhodococcus* with a degradation rate of 0.05 g/l per hour at a initial caffeine concentration of 0.5 g/l [18]. But the rates of degradation and resistance to high concentrations of caffeine do not match the demand, since the concentration of caffeine in effluents is often as high as 10 g/l [19]. Reports concerning the growth of *Pseudomonas* strains at approximately 50 g/l caffeine, lack data showing condition and degradation rate at this high concentration [17]. So far, only one strain belonging to *Pseudomonas* is capable of growing at high concentrations of caffeine [12]. Therefore, a strain that can tolerate high concentrations of caffeine and degrades at higher rates is very much needed to develop a successful bioprocess for degradation of caffeine.

In this context, we previously isolated a *Pseudomonas* strain from soils of coffee and tea cultivation area capable of utilizing caffeine as sole source of carbon and nitrogen. Based on 16-S rDNA analysis, the strain was identified as *Pseudomonas putida* [20]. Kinetic studies revealed that minimum inhibitory concentration of caffeine for growth was found to be 20 g/l [21]. The initial rate of degradation of caffeine for this strain was found to be 0.18 g/l per hour. However, after optimizing the nutrients and physical parameters (pH, agitation and temperature), the rate of caffeine degradation was increased to 0.29 g/l per hour. Under optimal conditions, the strain has been found to bring about the complete degradation of 12.5 g/l of caffeine and ∼90% degradation of 15 g/l in a 48 hour period. It has also been found that the enzymes required to degrade caffeine can be induced by growing the cells in a medium containing caffeine. In this study, we used the

induced cells to degrade caffeine so that the cells could be utilized as biocatalysts which then could be reused. Several factors influencing the degradation of caffeine by induced cells, such as amount of cells, pH, temperature, agitation and metal ions was studied and optimized.

2 Experimental procedures

2.1 Bacterial strain

Pseudomonas sp. previously isolated in our laboratory was identified as *P. putida*, based on 16-S rDNA analysis [20]. The culture was maintained on caffeine agar plates which consisted of the following concentrations (in g/l): caffeine, 1.2; KH_2PO_4, 1.3; Na_2HO_4, 0.12; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; Sucrose, 5 and agar, 25. The pH was adjusted to 6.0 before sterilization.

2.2 Preparation of caffeine induced cells

Three loops of actively grown cells from caffeine agar plates were transferred to 25 ml of nutrient medium containing 1 g/l of beef extract, 2 g/l of yeast extract, 5g/l of peptone and 5 g/l of NaCl, in 100 ml Erlenmeyer flasks. The culture was incubated on a rotary shaker at 180 rpm for 30°C till the OD_{600} reached 1.4-1.5. After achieving desired OD, 6 % (v/v) of inoculum was transferred to CAS medium containing 6.4 g/l of caffeine, 3.4 g/l of KH_2PO_4, 0.352 g/l of Na_2HO_4, 0.3 g/l of $CaCl_2$, 0.3 g/l of $MgSO_4.7H_2O$, 0.075% (w/v) of $FeSO_4.7H_20$, and 5g/l of sucrose. The culture was grown on rotary shaker for 30-36 hours – 90-95% of caffeine was degraded – at 190 rpm and 28°C. The initial pH of the CAS medium was adjusted to 7.8 before inoculation. The cells were harvested under aseptic conditions by centrifugation (Eppendorf centrifuge, 5810 R) at 10,000 x g for 10 min at 4°C. These cells were washed three times by 10 mM potassium phosphate buffer (at pH 7.0). The washed induced cells were re-suspended in 10 mM potassium phosphate buffer (at pH 7.0) such that the initial cell concentration was $\sim$ 550 g/l.

2.3 Caffeine degradation experiments with induced cells

In order to study the effects of different cell concentrations on caffeine degradation, different amount of induced cells were added to reaction medium containing 1.2 g/l of caffeine in 10 mM potassium phosphate buffer at pH 7.0. The concentration of cells in the reaction medium varied between 2 and 22 g/l. At different time intervals, samples were collected and analyzed for caffeine and growth. Once the optimum amount of cells was determined, the effect of pH was calculated by varying the pH of the reaction medium between 5.0 and 9.0. During this study, the optimal amounts of cells were added to the reaction medium. Similarly, the effect of temperature and agitation was calculated by varying the temperature of incubation between 25 and 40°C and agitation between 120

and 250 rpm respectively. The effect of metal ions was established by maintaining the initial concentration of different metal ions at 1mM in the reaction medium. Finally, the effect of initial concentration of caffeine was obtained by varying the initial concentration of caffeine in the reaction medium between 0.5 and 10 g/l.

2.4 Analytical determination of caffeine

The amount of caffeine was estimated by HPLC (Agilent 1100 series) using a ZORBAX C-18 column with 10 mM ammonium phosphate (pH 2.5)/ acetonitrile (4:1, v/v) as mobile phase at a flow rate of 1 ml/min at 28°C. Caffeine and its metabolites (theobromine, 7-methylxanthine and xanthine), monitored at 254 nm using UV detector. Percentage of caffeine degradation was calculated as follows:

Initial caffeine concentration (g/l) = a
Residual caffeine concentration (g/l) = b
Caffeine degradation (%) = ((a)-(b) × 100)/ (a))

3 Results and discussion

The bacterial strain *Pseudomonas sp.* was grown on CAS medium containing an initial concentration of caffeine of 6.4 g/l. Maximum growth was achieved in 25-30 hours and after 30 hours, a decrease in cell growth was observed. The complete degradation of 6.4 g/l of caffeine was achieved in 36 hours, and the cells were harvested before complete degradation of caffeine was attained (i.e. $\sim$ 30 hours) to get active induced cells. These induced cells were used for resting cell experiments and conditions required for maximum degradation of caffeine were determined in this study. Although sucrose was used in the growth medium, previous studies concerning this strain have shown that *Pseudomonas* sp. used in this study did not utilize sucrose. However, the presence of sucrose in the medium enhances only caffeine degradation and not cell growth. These results suggest that this strain utilizes caffeine as the sole source of carbon for growth.

3.1 Effect of cell concentration on degradation of caffeine

The effect of cell concentration was studied by varying the induced cell concentration between 2 and 22 g/l in the reaction medium (Fig. 1).

Caffeine degradation rate was very low when the cell concentration was 2 g/l, whereas maximum rates were obtained when the cell concentration was $\geq$ 8 g/l. Optimum cell concentrations of greater than 8 g/l was not considered for further examination since there is not much of an increase in caffeine degradation rates when the cell concentration was increased beyond that concentration. At 8 g/l cell concentration, complete degradation of 1.2 g/l of caffeine was achieved in 6 hours at a degradation rate of 0.2 g/l per hour. In all the experiments, it has been found that there is no increase in biomass, suggesting that induced cells are not growing but are catalyzing the degradation of caffeine. It has

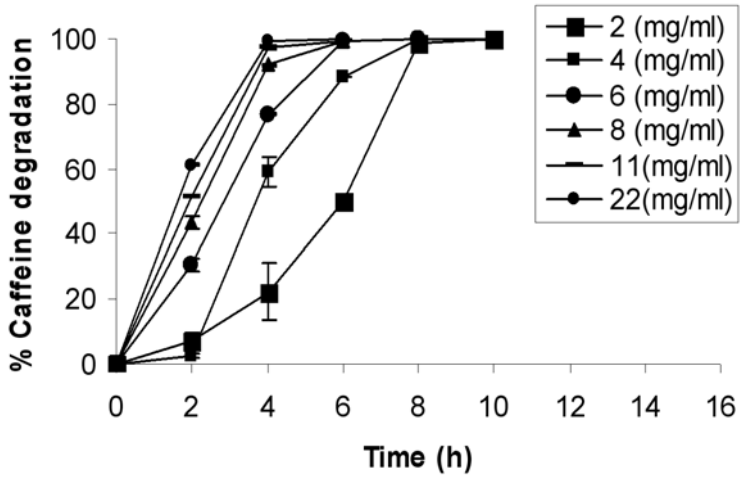

Fig. 1 Effect of cell concentration on caffeine degradation by induced cells of *Pseudomonas sp*. All experiments were performed in triplicate. The initial concentration of caffeine was at 1.2 g/l. The initial pH, temperature and agitation rate was maintained at 7.0, 30°C and 180 rpm respectively.

been reported that the isolate *Pseudomonas* sp. used in this study degraded caffeine by sequential demethylation leading to the formation of theobromine, 7-methylxanthine and xanthine during the growth in CAS medium [20]. It has also been observed that similar metabolites (theobromine, 7-methylxanthine and xanthine) were formed during the degradation of caffeine by induced cells of *Pseudomonas* sp. Similarly several *Pseudomonas* sp. are known to degrade caffeine by demethylation pathway [12, 13, 22]. The enzymes involved in this process, identified as demethylase, has been reported to be labile [23]. Caffeine is metabolized through a specific biphasic pathway driven by oxygen-demanding enzymes in *P. putida* [24]. However, it is not clear whether single enzyme or multi enzymes are involved in the conversion of caffeine to xanthine, something that requires further exploration. However, no caffeine demethylases have been purified and characterized so far.

3.2 Effect of pH on degradation of caffeine by induced cells

The effect of initial pH of reaction medium on caffeine degradation by induced cells of *Pseudomonas* sp. was studied. All the degradation experiments were performed with 8 mg/ml of initial cell concentration. Degradation rates were low at acidic (pH 5.0-6.0) and alkaline (pH 9.0) range (Fig. 2). The caffeine degradation rate was maximum (0.2 g/l per hour) in the pH range of 7.0 - 8.0 (Fig. 2). Therefore, in further studies, degradation was performed at pH 7.0. In a similar study, it has been found that optimum pH range for caffeine degradation by induced cells was found to be 7.0-8.0 pH for *P. alcaligenes* [25].

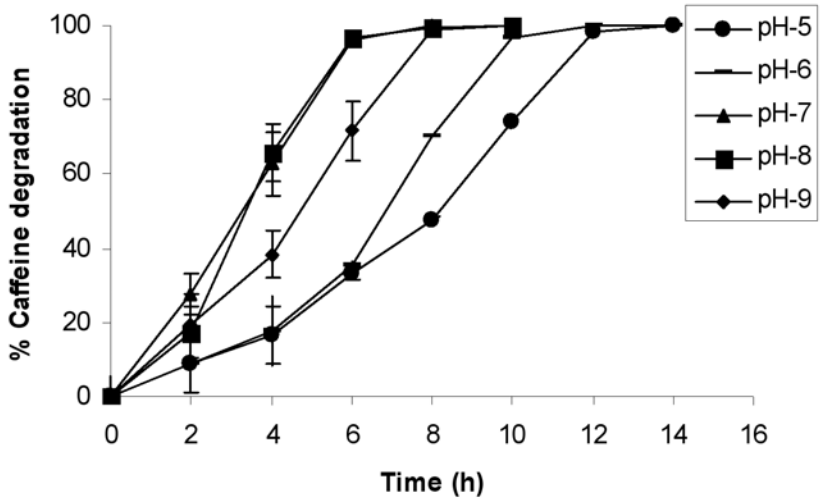

Fig. 2 Effect of pH on caffeine degradation by induced cells of *Pseudomonas sp.* All experiments were performed in triplicate. The initial concentration of caffeine and induced cells was at 1.2 g/l and 8 g/l, respectively. The temperature and agitation rate was maintained at 30°C and 180 rpm respectively.

3.3 Effect of temperature on degradation of induced cells

Temperature is also one of the important factors influencing the degradation of caffeine by the induced cells because degradation in turn is governed by intracellular enzymes. To determine the proper temperature, degradation was performed at different temperatures, ranging from 25 to 40°C. Maximum degradation of 1.2 g/l of caffeine was achieved at a temperature range of 30-35°C in 6 hours (Fig. 3). Temperatures above and below this range showed lower degradation rates. Even though at 30° and 35°C, the same 100% degradation in 6 hours was obtained, 35°C was chosen because the initial degradation rates were higher at 35°C than 30°C.

3.4 Effect of agitation on caffeine degradation by induced cells

The effect of different agitation rates on caffeine degradation by induced cells of *Pseudomonas* sp. was studied. The degradation rate was at a minimum at static conditions and maximum degradation was observed at 180 rpm (Fig. 4). Caffeine degradation was reduced when experiments were performed at 120 rpm and 250 rpm. Lower degradation at 120 rpm and at static condition was probably due to improper mixing. However, at higher speed (250 rpm) the degradation was lower because of vortex formation and or shear stress to cells.

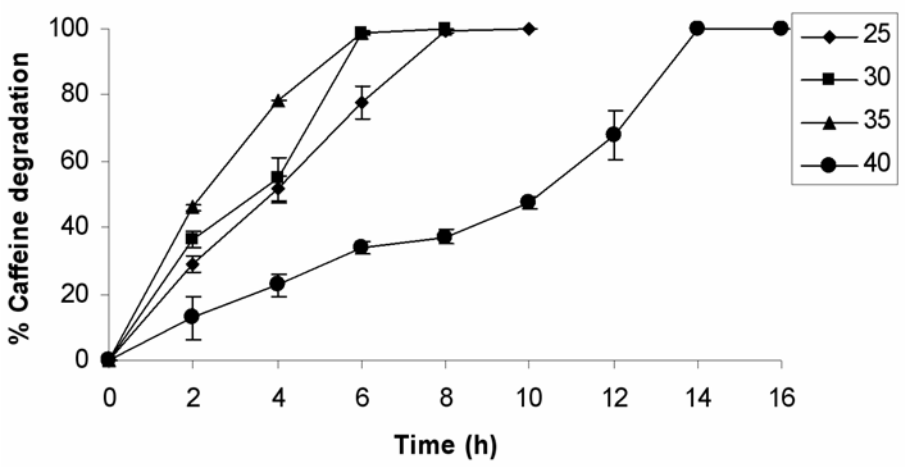

Fig. 3 Effect of temperature on caffeine degradation by induced cells of *Pseudomonas sp.* All experiments were performed in triplicate. The initial concentration of caffeine and induced cells was at 1.2 g/l and 8 g/l, respectively. The initial pH and agitation was maintained at 7.0 and 180 rpm.

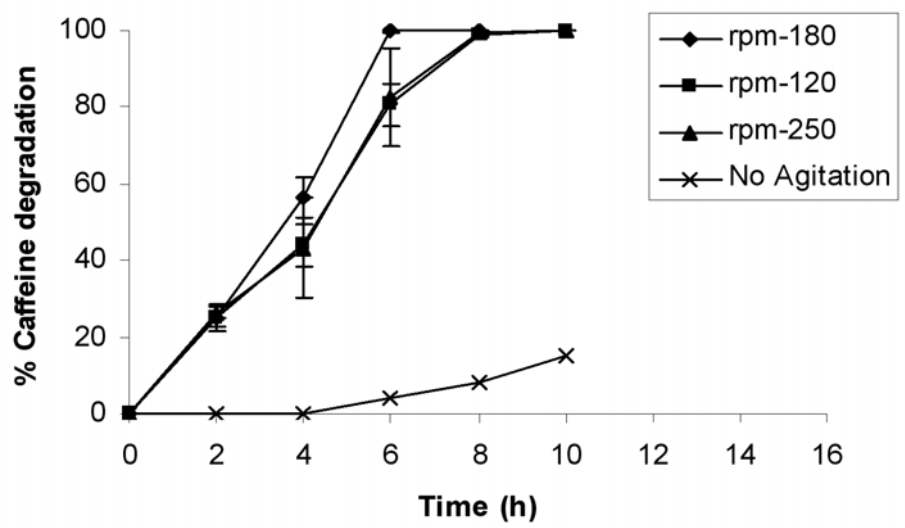

Fig. 4 Effect of agitation on caffeine degradation by induced cells of *Pseudomonas sp.* All experiments were performed in triplicate. The initial concentration of caffeine and induced cells was at 1.2 g/l and 8 g/l, respectively. The initial pH was maintained at 7.0 and the temperature was maintained at 35°C.

3.5 Effect of metal ions on caffeine degradation by induced cells

Metal ions play a crucial role in the degradation of caffeine by induced cell because certain metal ions act as cofactors for enzymes involved in the degradation. Various metal ions such as Zn^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, and Ni^{2+}were added to the reaction medium at a concentration of 1 mM. The maximum degradation of caffeine was observed

within 4 hours when metal ions such as Zn^{2+}, Mg^{2+} and Mn^{2+} were present in the reaction medium (Fig. 5).

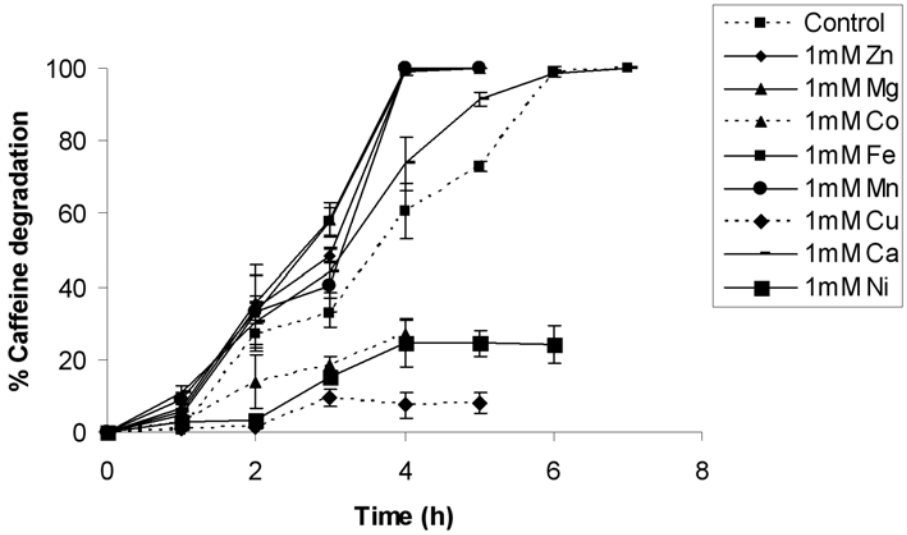

Fig. 5 Effect of metal ions on caffeine degradation by induced cells of *Pseudomonas sp.* All experiments were performed in triplicate. The initial concentration of caffeine and induced cells was at 1.2 g/l and 8 g/l, respectively. The initial pH, temperature and agitation rate was maintained at 7.0 35°C and 180 rpm respectively.

Among all the three, initial rates of degradation were maximum when Mg^{2+} was used. But the presence of metal ions such as Co^{2+}, Cu^{2+}, Ni^{2+} in the reaction medium showed less degradation of caffeine (∼ 20% in 4 hours) (Fig. 5). Experiments were also performed to check if metal ions have any synergistic effect on caffeine degradation by adding three metal ions–Zn^{2+}, Mg^{2+} and Mn^{2+}showed maximum degradation when added individually–togetherin the reaction medium. It has been found that the addition of three metal ions did not improve the rate of caffeine degradation. Thus, in additional studies, Mg^{2+} was added to reaction medium. In a study by Asano and coworkers, Zn^{2+} was seen to inhibit the conversion of theobromine obtained from the degradation of caffeine to subsequent metabolites [14, 26]. Thus in the presence of Zn^{+2} an accumulation of theobromine in the culture media occurred. In contrast, the addition of Zn^{+2} enhances the degradation of caffeine by induced cells of *Pseudomonas* sp. used in this study.

3.6 Effect of initial caffeine concentration

So far, it has been reported in literature that maximum concentration of caffeine that a bacterial strain can tolerate was 5 g/l by *Pseudomonas* sp. [12]. It has also been reported that strain used in this study could tolerate high concentration such as 10 g/l and maximum inhibitory concentration for the strain was 20 g/l [20, 21]. Hence, it is important to know the concentration of caffeine that the strain could degrade in resting cell experiments in order to test its efficiency to be used as biocatalyst. For this purpose,

degradation experiments were performed at various concentrations of caffeine ranging between 0.5 g/l to 10 g/l at a cell concentration of 8 g/l, pH 7.0, agitation rate of 180 rpm, temperature of 35°C and in the presence of 1 mM Mg^{2+} (Fig. 6). Interestingly it was found that induced cells were capable of bringing about the complete degradation of very high initial caffeine concentration of 10 g/l in 26 h with a degradation rate of 0.384 g/l per hour. The degradation rate was almost similar for 8, 6, 4 and 3 g/l of caffeine.

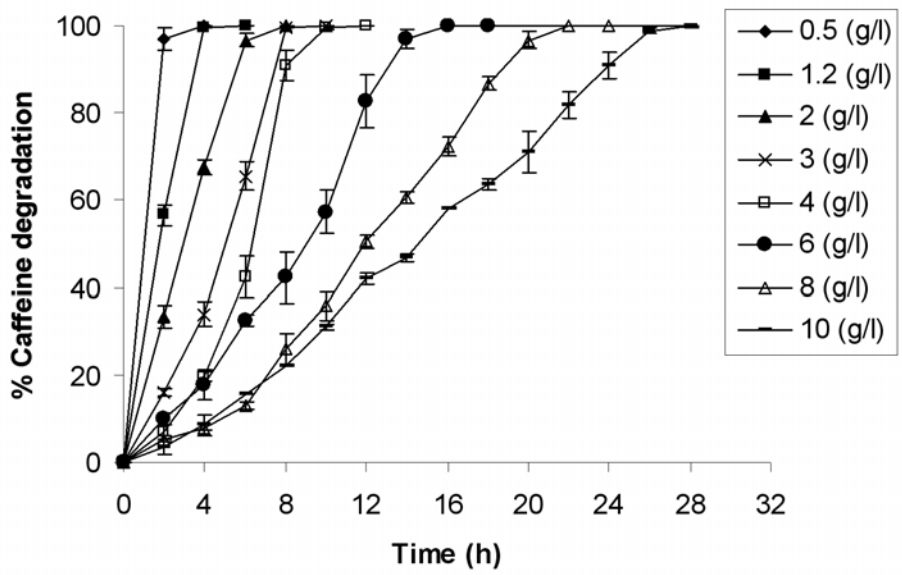

Fig. 6 Effect of initial caffeine concentration on caffeine degradation by induced cells of *Pseudomonas* sp. All experiments were performed in triplicate. The initial concentration of caffeine, induced cells and Mg^{2+} was at 1.2 g/l, 8 g/l and 1 mM, respectively. The initial pH, temperature and agitation rate was maintained at 7.0, 35°C and 180 rpm.

The degradation rate (∼0.3 g/l per hour) was slightly lower at low concentrations of caffeine (0.5 to 2 g/l). In a similar study, *P. alcaligenes* at a cell concentration of 100 g/l was used to degrade 1-5 g/l of caffeine [25]. It has been found that complete degradation of 1 g/l of caffeine by induced cells was attained in 4 hours. However, the isolate could degrade only 52, 45 and 30% of 2, 4 and 5 g/l caffeine respectively and caffeine concentration higher than 5 g/l was found to be inhibitory. In contrast, the isolate *Pseudomonas* sp. used in this study could degrade caffeine as high as 10 g/l at low cell concentration (8 g/l). This was the first report on high levels of caffeine degradation by induced cells at higher rates. Therefore, induced cells of *Pseudomonas* sp. used in this study showed greater potential for use as biocatalysts and can be successfully used to develop methods for biological degradation of caffeine.

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