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Optimization of Physical Parameters for Biodegradation of Caffeine by *Pseudomonas* sp.: A Statistical Approach

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Abstract: *Pseudomonas* sp. NCIM 5235 capable of degrading high concentrations of caffeine has been previously isolated from the soil of coffee plantation area. The isolate was capable of degrading 6.4 g L^{-1} initial concentration of caffeine at a rate of 0.1 g L^{-1} h^{-1}. In this study, the physical parameters viz., pH, temperature and shaking speed have been optimized using central composite design. The optimum values of pH, temperature and shaking speed were found to be 7.8, 28°C and 190 rpm, respectively. Under optimized condition of pH, temperature and shaking speed, the rate of degradation of caffeine has been enhanced from 0.18 to 0.29 g L^{-1} h^{-1} which is 1.6 fold higher than the normal rate. This is the first report on degradation of high concentration of caffeine at higher rates. Under optimal conditions, the strain has also been found to degrade caffeine at 15 g L^{-1} initial concentration efficiently within 48 h. This makes *Pseudomonas* sp. NCIM 5235 an attractive candidate for development of biodecaffeination strategies.

Key words: Caffeine degradation, *Pseudomonas* sp., statistical optimization, physical parameters

Introduction

Caffeine (1, 3, 7-trimethylxanthine), a purine alkaloid naturally occurring in more than 60 plant species (Steffen, 2000), forms an essential component of a variety of beverages like tea coffee and caffeinated soft drinks and numerous food products like chocolates and desserts as it increases alertness and concentration by overcoming fatigue (Nehlig, 1999). Apart from that caffeine is widely used in pharmaceutical preparations as it enhances the effect of certain analgesics and antipyretic drugs. Caffeine is also used as a cardiac, neurological and respiratory stimulant and as a diuretic (Mazzafera, 2002). This makes caffeine one of the most widely consumed psychoactive compounds with the global average consumption ranging from 80 to 400 mg caffeine per person per day (Gokulakrishnan *et al* ., 2005). Consequently, caffeine is also one of the major agro-industrial wastes generated from coffee and tea processing plants. Caffeine containing wastes are often released to the surrounding water bodies and it is detected in surface water, ground water and waste water effluents often at a high concentration (~ 10 g L^{-1}) (Buerge *et al*., 2003; Weigel *et al*., 2004; Glassmeyer *et al*., 2005).

In order to free the natural waters from this xenobiotic, as the ingestion of caffeine and its chrorinated byproducts (derived during chlorination of water) has severe adverse effect on the physiological system (Gould and Hay, 1982; White and Rasmussen, 1998), decaffeination of the byproducts becomes a very necessary step in treatment of coffee and tea wastes. Decaffeination of other food products and beverages is also being recommended, keeping in view the adverse effects

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of chronic caffeine consumption particularly on the cardiovascular system and health of women (Jenner *et al*., 1988; Bergman *et al*., 1990; Jee *et al*., 1999; Kalmar and Cafarelli, 1999). In this aspect, microbial and enzymatic techniques for decaffeination have been found advantageous than other conventional methods such as solvent extraction or super critical carbon dioxide oxidation (Gokulakrishnan *et al*., 2005).

Several microbial strains belonging to *Pseudomonas*, *Serratia*, *Aspergillus*, *Penicillium* and *Stemphylium* have been reported to be able to degrade caffeine (Schwimmer *et al*., 1971; Woolfolk, 1975; Bletcher and Lingens, 1977; Mazzaferra *et al*., 1994; Asano *et al*., 1994; Hakil *et al*., 1999; Yamaoka-Yana and Mazzaferra, 1999). However, these strains were not capable of degrading at higher concentrations of caffeine, except for *Pseudomonas* sp. capable of growing at 5 g L^{-1} of caffeine with a degradation rate of 0.09 g L^{-1} h^{-1}. Previously we have isolated a strain of *Pseudomonas* from the soil of coffee plantation area that is capable of utilizing caffeine as sole source of carbon and nitrogen. This strain closely resembles *Pseudomonas putida* based on 16S rRNA analysis (Dash and Gummadi, 2006). Kinetic studies on this bacteria shows that it follows substrate inhibition kinetics for caffeine degradation and caffeine concentration of 20 g L^{-1} was found to be inhibitory for growth (Gokulakrishnan and Gummadi, 2006). The degrading ability of this strain was also found to be better as it could bring about complete degradation of caffeine at an initial concentration of 5 g L^{-1} at the rate of 0.1 g L^{-1} h^{-1}. Optimization of medium components resulted in further increase in the rate of degradation to 0.18 g L^{-1} h^{-1} (unpublished data).

In the present study, physical parameters like pH, temperature and shaking speed were optimized using response surface methodology for studying the interaction effects among the variables and further increasing the rate of degradation of caffeine by this strain.

Materials and Methods

Chemicals

Pure caffeine was procured from Merck, India. All other reagents were of analytical grade procured in India.

Microorganism

Pseudomonas sp. NCIM 5235 was maintained on nutrient agar medium which had the following composition (g L^{-1}): beef extract 1; yeast extract 2; peptone, 5; NaCl, 5 and agar, 25 and was subcultured every two weeks.

Media

The composition of CAS medium was as follows (g L^{-1}): Na_2HPO_4, 0.12; KH_2PO_4, 1.3; $CaCl_2$, 0.3; $MgSO_4$. 7 H_2O, 0.3. sucrose, 5 caffeine, 1.2 and agar, 25. The initial pH of the medium was 6. For flask culture experiments optimized CAS medium with the following composition was used (g L^{-1}): Na_2HPO_4, 0.352; KH_2PO_4, 3.4; $CaCl_2$, 0.3; $MgSO_4$. 7 H_2O, 0.3. sucrose, 5 caffeine, 6.4 and 0.075% (w/v) of Fe^{2+}. The initial pH of the medium was adjusted as per experimental design (Table 2).

Flask Culture Experiments

Three loopfull of 36 h grown culture from CAS plate was transferred aseptically to 25 ml nutrient broth medium and incubated to mid-log phase (OD_{600nm}~1.4) for 2.5 h on a rotary shaker operating at 180 rpm at 30°C. Twenty five milliliter of sterile optimized CAS medium was inoculated with 6% (v/v) of the culture and incubated for four days at conditions specified by the experimental design (Table 2). At regular time intervals 1 mL of culture was taken and centrifuged at 10000 rpm for 10 min. The supernatant was used for analysis of caffeine. The cell pellet was washed twice with MilliQ water and OD_{600nm} was measured for cell dry weight.

Caffeine Estimation

Caffeine was estimated by HPLC (Agilent 1100 series) equipment using a ZORBAX C-18 column with 10 mM ammonium phosphate buffer (pH 2.5)/acetonitrile (4:1, v/v) as mobile phase. Pure caffeine (Merck) at 2 g L^{-1} was used as standard. The retention time of caffeine was found to be 4.9 min at a flow rate of 1 mL min^{-1} and at 28°C. Detection of caffeine was done at 254 nm using UV detector.

Optimization of Physical Parameters Using a Central Composite Design

The physical parameters viz. pH, temperature and shaking speed were optimized using central composite design (Box and Wilson, 1951; Box and Hunter, 1957). According to this design, the total number of treatment combinations is $2^k + 2k + n_0$ where k is the number of independent variables and n_0 the number of repetitions of the experiments at the center point. For statistical calculation, the variables X_i have been coded as x_i according to the following transformation:

$$xi = \frac{Xi - X_0}{\delta X} \quad (1)$$

where x_i is dimensionless coded value of the variable X_i, X_0 the value of the X_i at the center point and δX the step change. A 2^k-factorial design with six axial points and six replicates at the center point with a total number of 20 experiments was employed for optimizing the medium components.

The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \quad (2)$$

where Y is the predicted response, β_0 is the intercept term, β_i is the linear effect, β_{ii} is the squared effect and β_{ij} is the interaction effect. The regression equation (Eq. 2) was optimized for maximum value to obtain the optimum conditions using MATLAB® version 7.0 (Mathworks Inc., Natick, Massachusetts, USA).

Results and Discussion

The interaction between the physical parameters viz., pH, temperature and shaking speed in relation to caffeine degradation was studied by response surface methodology. A central composite design was used for studying the interaction of these variables within a range of -1.68 to +1.68 in relation to caffeine degradation (Table 1). The experimental plan is given in Table 2. The results obtained from the central composite design experiments were fitted to a second order polynomial equation to explain the dependence of caffeine degradation on the medium components.

Experiments were performed with various combinations of pH, temperature and shaking speed as given in Table 2. The rate of degradation of caffeine was highest (0.26 g L^{-1} h^{-1}) when pH,

Table 1: Coded and Uncoded values of physical parameters used for central composite design

	Coded values				
Variables	-1.68	-1	0	+1	+1.68
pH	3.47	4.5	6	7.5	8.52
Temperature (°C)	21.59	25.0	30	35.0	39.41
Shaking speed (rpm)	129.54	150.0	180	210.0	230.46

Table 2: Central composite design for optimizing physical parameters for caffeine degradation by *Pseudomonas* sp. NCIM 5235[a]

					Caffeine degradation rate (g L^{-1} h^{-1})	
Run No.	Block	pH	Temperature (°C)	Shaking speed (RPM)	Y (Experimental)	Y (Model predicted)
1	1	-1	-1	-1	0.00	0.04
2	1	+1	-1	+1	0.26	0.26
3	1	-1	+1	+1	0.00	0.01
4	1	+1	+1	-1	0.21	0.17
5	1	0	0	0	0.23	0.22
6	1	0	0	0	0.24	0.22
7	2	-1	-1	+1	0.00	0.03
8	2	+1	-1	-1	0.24	0.23
9	2	-1	+1	-1	0.00	-0.01
10	2	+1	+1	+1	0.22	0.18
11	2	0	0	0	0.21	0.22
12	2	0	0	0	0.22	0.22
13	3	-1.68	0	0	0.00	-0.05
14	3	+1.68	0	0	0.24	0.28
15	3	0	-1.68	0	0.17	0.13
16	3	0	+1.68	0	0.00	0.04
17	3	0	0	-1.68	0.18	0.18
18	3	0	0	+1.68	0.20	0.19
19	3	0	0	0	0.22	0.22
20	3	0	0	0	0.22	0.22

[a]R^2 = 0. 9376; R = 0.968. Experimental values are average of triplicates within±5% standard error

temperature and shaking speed were at +1, -1 and +1 levels, respectively (Run # 2). Caffeine degradation was completely inhibited in all cases where the pH was at -1 or -1.68 level i.e., at 4.5 or 3.4 indicating inhibition of bacterial growth at lower pH. The rate of degradation was however not much different in cases of where temperature and shaking speed were at -1 levels indicating that the negative effect of one of the variable was compensated by the other. When all the three variables were at +1 level (Run # 10), the rate of degradation was not much different from that of center points (Run # 5, 6, 11, 12, 19, 20).

The rate of degradation was lowest when the temperature -1.68 level and the other parameters at 0 levels (Run# 15). This shows that the bacteria can survive at a temperature lower but the enzymes responsible for bringing out degradation were not optimally active at that temperature. Similar results were also obtained when the shaking speed was at -1.68 level and the other parameters at 0 levels (Run# 17). In conditions of slower shaking speed, the oxygen availability is considerably lower and this in turn affects the degradation process which is supposed to be oxidative.

Interesting results were obtained in case of the shaking speed at + 1.68 level and the other two parameters at 0 levels (Run# 18). It was noted that at high shaking speed, caffeine degradation proceeded up to 80% within 24 h and then remained constant. Growth was also noted to be inhibited at that time point. Accumulation of metabolites, particularly xanthine, was also noted at this condition. pH was also observed to increase up to 9 which was probably due to the accumulation of metabolites. These findings indicate the possibility of feed back inhibition mechanism occurring with the caffeine degradation phenomena. At increased aeration with high caffeine concentration, the rate of conversion of caffeine to its metabolites is very rapid as compared to the rate of assimilation by the cells. This results in the accumulation of metabolites in the culture medium, which in turn inhibit the caffeine degrading enzymes and bring about a change in pH to alkaline resulting in growth inhibition. Inhibition of growth and caffeine degradation by *Pseudomonas* sp. NCIM 5235 at higher pH has been noted in

Table 3: ANOVA table for caffeine degradation: effect of pH, temperature and shaking speed

Source	Sum of squares	df	Mean square	F	P>F
Blocks	0.000216	2			
Model	0.195012	9	0.021668	13.34	0.0006
Error	0.012992	8	0.001624		
Total	0.208220	19			

earlier experiments with the strain (data not shown). So also inhibition of caffeine degradation at higher initial concentrations of caffeine has been reported earlier for *Pseudomonas putida* (Woolfolk, 1975). More studies on this feed back inhibition mechanism will prove useful in developing strategies of obtaining higher concentrations of metabolites of caffeine degradation pathway that have many applications.

The second order regression equation (Eq. 3) shows the dependence of caffeine degradation on pH, temperature and shaking speed. Multiple regression analysis of the experimental data generated the parameters of the equation which is represented as follows:

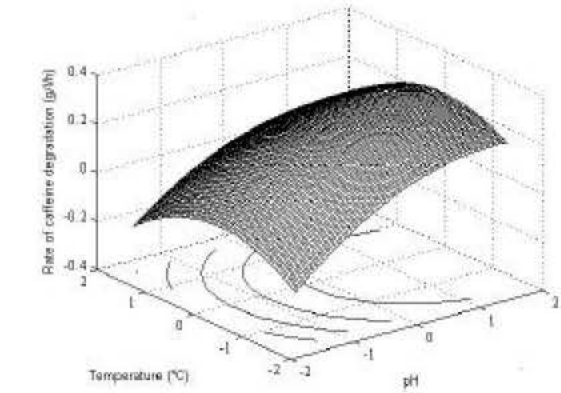
$$Y = 0.223468 + 0.097646x_1 - 0.026060x_2 + 0.004659x_3 - 0.038698x_1^2 - 0.051069x_2^2 - 0.013955x_3^2 - 0.008750x_1x_2 + 0.003750\ x_1x_3 - 0.001250x_2x_3 \quad (3)$$

where Y is the predicted response, x_1 is the coded value of X_1 (pH), x_2 is the coded value of X_2 (temperature) and, x_3 is the coded value of X_3 (shaking speed). Statistical testing of the model was performed by the Fisher's statistical test for ANOVA (Table 3). The F-value, which is the ratio of mean square due to regression to the mean square due to error, was calculated to be 13.34. The analysis of variance of the quadratic regression model also suggests that the model is very significant as indicated by a low probability value [$(P_{model}>F) = 0.0006$]. So also the values of R^2 (0.8518) and R (0.9376) also indicated that the model predicted values are in perfect agreement with the experimental values as shown in Table 2. Equation 3 also predicts the interaction of variables affecting caffeine degradation. Coefficients of interaction terms were significant as compared to linear terms indicating that the interaction between the variables cannot be neglected.

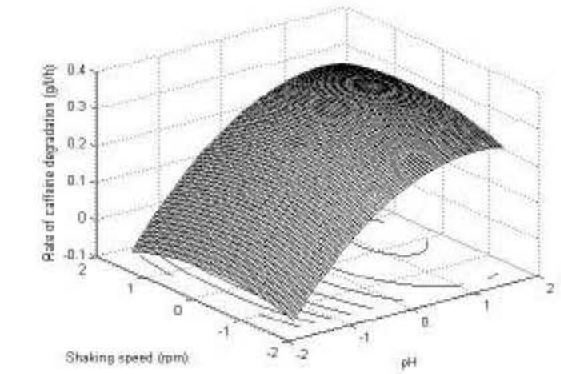
The model equation was solved using MATLAB 7.0 software to generate the optimal values for the above three variables. The optimum values of pH, temperature and shaking speed were found to be 7.8, 28°C and 190 rpm, respectively. The model predicted value of rate of degradation of caffeine was determined to be 0.29 g L^{-1} h^{-1} which is 1.6 fold higher than the rate of degradation under unoptimized conditions. Surface plots of the response using Eq. (3) when one of the variables is fixed at optimum value and the other two are allowed to vary are shown in Fig. 1 a-c. It was seen that with there is an increase in the rate of caffeine degradation with the increase in pH within the design region (Fig. 1a and b). In all the three cases the optimum rate of degradation predicted by the plots matched with the experimentally predicted value for optimum rate of caffeine degradation.

In order to verify the optimal conditions obtained using central composite design, experiments were performed under the optimal conditions and compared with the center points (Fig. 2). As seen from Fig. 2 the experimental results match with the model predicted one, proving that the optimal values of the physical parameters obtained were ideal. Such a condition may prove useful in studying the metabolism of caffeine by the strain of *Pseudomonas* in order to develop methods for biological decaffeination.

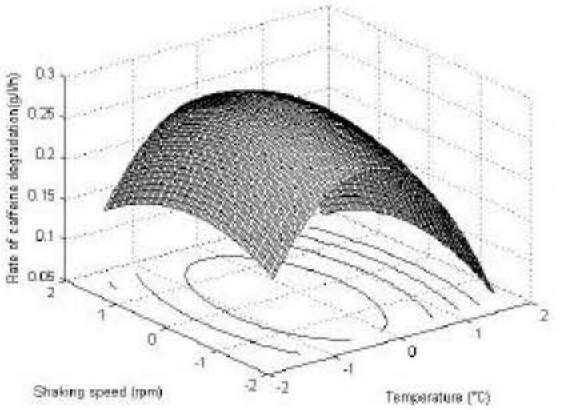
The bottleneck in biodecaffeination of caffeine containing effluents is the lack of a microorganism capable of tolerating and degrading caffeine at high concentrations, as the concentrations of caffeine in effluents can be as high as 10 g L^{-1}. The next objective, therefore, was to study the degradation of



(a)



(b)



(c)

Fig. 1: Response surface plots showing effect of pH, temperature and shaking speed on rate of caffeine degradation by *Pseudomonas* sp. NCIM 5235: (a) effect of temperature and pH at optimal shaking speed on rate of caffeine degradation; (b) effect of pH and shaking speed at optimal temperature on rate of caffeine degradation; (c) effect of shaking speed and temperature at optimal pH on rate of caffeine degradation

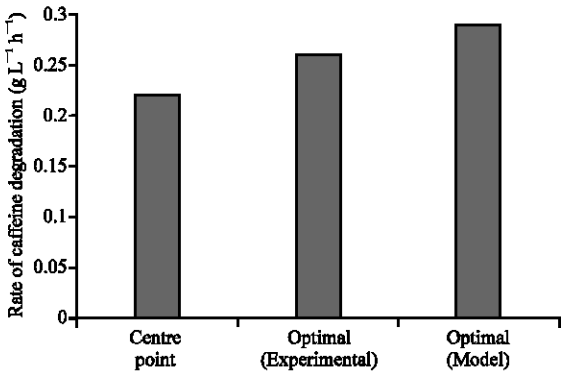


Fig. 2: Comparison of caffeine degradation by *Pseudomonas* sp. at optimized and unoptimized conditions of physical parameters. Experiments were performed at center point conditions as specified by CCD table (Table 2) and at optimal conditions obtained after solving Eq. 3. Samples were drawn at 6 h interval and caffeine was estimated by RP-HPLC on a C-18 column with 10 mM ammonium phosphate buffer (pH 2.5)/acetonitrile (4:1, v/v) as mobile phase

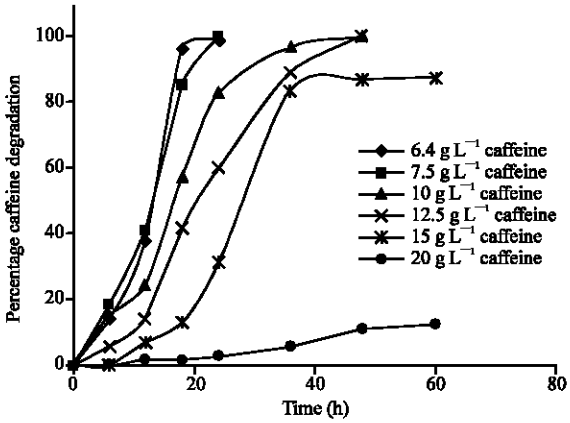


Fig. 3: Degradation of high initial concentrations of caffeine at optimal conditions of pH, temperature and shaking speed by *Pseudomonas* sp. NCIM 5235. The initial caffeine concentration was varied from 6.4 to 20 g L^{-1} and fermentation was carried out at optimal conditions of pH, temperature and shaking speed as predicted from Eq. 3. Samples were drawn at 6 h interval and caffeine was estimated by RP-HPLC on a C-18 column with 10 mM ammonium phosphate buffer (pH 2.5)/acetonitrile (4:1, v/v) as mobile phase

caffeine at still higher concentrations under the optimized conditions obtained. The strain was grown at initial caffeine concentrations up to 20 g L^{-1} and it could efficiently degrade about 80% of caffeine at an initial concentration of 15 g L^{-1}. Caffeine at initial concentrations of 7.5, 10 and 12.5 g L^{-1} could be metabolized completely within 24, 36 and 48 h, respectively (Fig. 3). Under optimized conditions the inhibition at high concentrations as depicted by kinetic studies on the strain (Gokulakrishnan and Gummadi, 2006) could be overcome and it could also degrade caffeine at initial concentrations of 20 g L^{-1} but at lower rates. This makes *Pseudomonas* sp. NCIM 5235 an excellent candidate for developing methods for effluent treatment as the need for dilution of effluent to bring caffeine to lower concentrations can be done away with, thereby reducing the cost of operation.

Conclusion

Optimization results show that with some modification in physical parameters viz. pH, temperature and shaking speed the degradation of a higher concentration of caffeine could be achieved and so also the rate of degradation could be enhanced. The degradation of caffeine under optimized conditions can prove useful in generation of important metabolites like theobromine and other methylxanthines or can be used to recover enzymes for development of biological methods of decaffeination. Response surface methodology can be successfully used to optimize the physical parameters and study the interaction effect among the same on caffeine degradation rate.

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