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Optimization of Process Conditions for Biotransformation of Caffeine to Theobromine using Induced Whole Cells of *Pseudomonas sp*

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Abstract

The obromine is a metabolic intermediate produced in caffeine degradation pathway by many bacterial species, which has potential applications in food and pharmaceutical industries. Conventional methods of Theobromine production from xanthine involve harsh physical and chemical conditions which are harmful to the environment. To overcome this, we employed biotechnological route to convert caffeine to theobromine by single demethylation reaction using induced cells of *Pseudomonas sp.* Initially we screened various divalent metal ions for the production of Theobromine by *Pseudomonas sp*. from caffeine. Co^{2+} and Ni^{2+} accumulates 400 and 100 mg/l of theobromine under initial reaction conditions (2 g/l caffeine, 8 g/l cell loading, pH 7.0,30°C). Co^{2+} was chosen for further optimization of reaction conditions for Theobromine production using response surface methodology. Data were fitted into a quadratic model and the optimal condition for theobromine production was found to be 3.2 g/l caffeine, 11.3 g/l initial cell loading and pH 7.0. Quadratic regression models were validated at the optimized conditions and the experimental theobromine produced 689.7 mg/l corresponds to the model predicted theobromine 729.4 mg/l. Theobromine production was further improved to 1.08 ± 0.10 g/l by optimizing the reaction temperature. This study reports highest production of theobromine from caffeine using induced cells of *Pseudomonas sp.* Induced cells are better suited for metabolite production as it is metabolically very active and can be re-used several times. Optimization of reactor parameters will enable us to make microbial production of theobromine feasible in industries at reduced cost.

Keywords: Caffeine; Theobromine; Central composite design; Resting cells; *Pseudomonas sp*; Co^{2+}

Introduction

Theobromine (3, 7-dimethylxanthine) is a metabolite formed in the first step of caffeine metabolism by bacteria [1] and is naturally present in cocoa bean. It has potential applications in food and pharmaceutical industries. Even though theobromine is similar to caffeine in its structure, its effect on human physiology is milder and beneficial. While caffeine increases blood pressure, theobromine reduces blood pressure by dilating blood vessels. Theobromine sodium salicylate, a derivative of theobromine was found to act as a vasodilator early in 1935 [2]. According to the U.S. National Institutes of Health, an antitussive drug BC1036which contains theobromine is under phase III clinical trials. The antitussive action of this drug was found to be because of direct inhibition of cough reflex by theobromine [3]. It is also an intermediate used for the production of a vasotherapeutic agent, Trental and Hextol [4]. Conventionally theobromine has been produced by extraction from cocoa beans or by synthesis from 3-methyl urea. It is also produced by methylation of 3-methylxanthine using dimethyl sulfate as methylating agent in methanolic solution [4]. Exposure of dimethyl sulfate to human has toxic effects like genital and mucous membrane lesions, conjunctivitis and keratitis in eyes, ulceration of lips, etc. [5]. Hence, there is a need to develop alternative methods which are less toxic to the environment and human health. Biotechnological route has been used as an eco-friendly and cheap alternative to chemical methods to produce a number of industrially important molecules. Since theobromine is a metabolite formed in the caffeine metabolic pathway by certain bacterial species, it can be effectively produced from caffeine using such bacteria in an eco-friendly method by single demethylation reaction. Caffeine (1,3,7-trimethylxanthine) is a naturally occurring purine alkaloid abundantly present in tea leaves, coffee beans, cocoa beans and cola nuts. It has been used as a psycho-stimulant [6] and as food ingredients by humans. Acute intake of caffeine causes elevated blood pressure [7], osteoporosis [8], sterility [9], nervousness, mood change and many types of cancer [10,11]. Hence, there is a need to degrade caffeine in food products and as well as in effluents from tea and coffee industries. Instead of degrading completely, it will be advantageous to convert caffeine into less harmful and value added molecule such as theobromine.

Microbial production of theobromine can be used as a cheaper and ecofriendly alternative to the conventional chemical methods available. Very few studies are reported in literature on the production of theobromine from caffeine using microbes [12-14]. A new strain of *Pseudomonas sp* that is capable of growing on very high concentration of caffeine as the sole carbon and nitrogen source was isolated from the soil of coffee plantation area [15]. It was reported that the growth of *Pseudomonas sp.* gets inhibited by caffeine concentration greater than 2.5 g/l while it was able to grow on medium containing as high as 10 g/l of caffeine with low specific growth rate [16]. After optimization of all other media components, caffeine degradation rate was as high as 0.29 g/l h which is the highest reported so far [17,18]. It was also reported that the enzyme system required for caffeine degradation in *Pseudomonas sp.* is inducible [19] and certain divalent metal ions (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) strongly inhibit further degradation of theobromine accumulated from demethylation of caffeine [20]. Metal ions have been known to

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inhibit enzymes thereby resulting in the accumulation of metabolites. Certain heavy metals have been shown to accumulate metabolite in plants [21]. Asano et al. reported that the strain *Pseudomonas putida* No. 352 accumulates theobromine due to the inhibition of a monooxygenase specific for theobromine demethylation by Zn^{2+} [22]. Even at low concentration (8 g/l) of induced cells of *Pseudomonas sp.* as high as 10 g/l of caffeine was found to be degraded in 25 h [23]. Since *Pseudomonas sp.* was shown to be very effective in caffeine degradation, in the present study we attempted to exploit its ability to accumulate theobromine from caffeine in the presence of certain metal ions.

Materials and Methods

Chemicals

Pure caffeine was obtained from Merck India. 3, 7 dimethyl xanthine, 7 methyl xanthine and xanthine were obtained from Sigma India. Solvents used for HPLC analysis were of HPLC grade. All other reagents were of analytical grade.

Bacterial strain

Pseudomonas sp. was previously isolated in our laboratory from soil of coffee plantation area of Ooty, India during August 2003 [15]. The isolate was maintained on Caffeine Added Sucrose (CAS) agar with sub-culturing every 36 h. CAS agar has the following composition -Na_2HPO_4 (120 mg/l), KH_2PO_4 (1.3 g/l), $CaCl_2$(300 mg/l), $MgSO_4$ (300 mg/l), sucrose (5 g/l) and agar (2.5%). pH adjusted to 6.0.

Production of induced cells of *Pseudomonas sp.*

Pseudomonas sp. was maintained on CAS agar at 30°C. After 36 h, three loops full of cells were inoculated in 25 ml seed culture medium and incubated at 30°C and 180 rpm. The seed medium has the following composition -beef extract (1 g/l), yeast extract (2 g/l), peptone (5 g/l), NaCl (5 g/l). Once A_{600} reaches 1.6-1.8, production media was inoculated with 6% of seed culture and incubated at30°C and 180 rpm till 90-95% of initial caffeine gets degraded. The production medium has the following composition - KH_2PO_4 (3.4 g/l), Na_2HPO_4 (0.36 g/l), caffeine (6.4 g/l), sucrose (5 g/l), $CaCl_2$ (0.3 g/l), $MgSO_4.7H_2O$ (0.3 g/l), $FeSO_4$(10.05 g/l). Sucrose and trace elements were prepared and autoclaved separately. Production medium was prepared just before inoculation by mixing sterilized media components under aseptic conditions. Initial pH was adjusted to 7.8 with 3N NaOH. Once 90-95% of caffeine gets degraded, cells were harvested by spinning at 10,000 rpm, 4°C for 5 minutes under sterile conditions. At this point, the enzymes required for caffeine metabolism are induced and expressed at high levels and therefore these induced cells are metabolically very active in utilizing caffeine.

Screening of metal ions for theobromine accumulation by the induced cells of *Pseudomonas sp.*

Harvested induced cells were washed with 10 mM potassium phosphate buffer pH 8.0 and resuspended in 10 mM potassium phosphate buffer pH 8.0, so that the induced cell concentration in the buffer was 100 g/l (catalyst stock). Resuspended induced cells were inoculated in the reaction media containing 2 g/l caffeine such that initial concentration of induced cells in reaction media was 8 g/l. Metal ions were added at a concentration of 1 mM and incubated at 30°C, 180 rpm. Samples were collected every hour till caffeine was completely degraded in control flask, where no metal ion was added and the collected sample was centrifuged at 10,000 rpm for 10 min. The supernatant was analyzed by reverse phase High Performance Liquid Chromatography (HPLC) using JASCO HPLC system (Multi-wavelength detector MD-2010 plus) at 254 nm to determine the concentration of caffeine, theobromine and other methylxanthine derivatives remaining in the medium. C-18 column was used with 30% methanol in water as mobile phase. Retention time of caffeine and theobromine was 9.2 min and 4.5 min respectively under the above mentioned stationary and mobile phases.

Optimization of reaction media parameters for maximized theobromine production using Central Composite Design (CCD)

CCD was chosen to show the statistical significance of the effects of pH, initial cell loading and initial caffeine concentration on theobromine production by *Pseudomonas sp.* The software 'Design Expert 9', a trial version from Stat-Ease, Inc., was used to do a two-level factorial design in which three independent variables (X_1, X_2 and X_3) were represented as three dimensionless variables x_1, x_2 and x_3 with coded levels at -1, 0 and 1.Statistical relationship between coded and actual values is given below,

$$x_i = (X_i - X_0)/\delta X$$

Where X_0 is the corresponding center point value and δX is the step change. The coded values and its corresponding actual values are given in table 1. Total number of experiments that need to be performed according to this design is given by $2^k+2k+n_0$where 'k' is the number of independent variables and n_0 the number of repetitions of the experiments at the center point. For three independent variables (caffeine concentration, cell concentration and pH), 20 runs were performed in three blocks with two center points in each block. pH of the reaction media (X_3) was varied from 6.32 to 9.68. 10 mM potassium phosphate buffer was used for pH ranges 6.32 to 8.0 and 10 mM carbonate buffer was used for pH 9.68. Appropriate amount of caffeine (X_1) (ranges from 0.32 g/l to 3.68 g/l) was dissolved in appropriate buffers according to the experimental design in table 2. Induced cells (X_2) (ranges from 1.27 to 14.73 g/l) were resuspended in corresponding buffers to make a stock of 100 g/l and inoculated in reaction media according to the design. Theobromine produced after 4 h, molar yield of theobromine and productivity were measured as the responses.

The dependence of these independent variables (x_1, x_2, x_3) on the responses can be approximated by the quadratic equation (Equation 1) as given below,

$$Y = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \beta_3\chi_3 + \beta_{11}\chi_1^2 + \beta_{22}\chi_2^2 + \beta_{33}\chi_3^2 + \beta_{12}\chi_1\chi_2 + \beta_{13}\chi_1\chi_3 + \beta_{23}\chi_2\chi_3 \quad (1)$$

where Y is the predicted response, β_0 is the intercept term, β_1,β_2 and β_3 are the linear coefficients, β_{11}, β_{22} and β_{33} are the quadratic coefficients and β_{12}, β_{13} and β_{23} are the interaction coefficients.

Effect of reaction temperature

Medium components	Coded values				
	-1.68	-1	0	1	1.68
Caffeine concentration X_1 (g/l)	0.32	1	2	2	3.68
Initial cell loading X_2 (g/l)	1.27	4	8	12	14.73
pH X_3	6.32	7	8	9	9.68

Table 1: Coded and actual values of the medium components chosen for optimization using CCD.

The effect of temperature on conversion of caffeine to theobromine was studied by incubating reaction media with induced cell sat three different temperatures 25°C, 30°C and 35°C.The reaction conditions were 3.2 g/l caffeine in 10 mM potassium phosphate buffer, pH 7.0 and induced cell concentration 11.3 g/l. Samples were collected at regular intervals of time and the supernatants were analyzed for theobromine and caffeine concentration by HPLC.

Results

Screening of metal ions for theobromine accumulation from caffeine by *Pseudomonas sp.*

Divalent metal ions like Co^{2+}, Cu^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}were tested for their ability to accumulate theobromine from caffeine by *Pseudomonas sp.*Cu^{2+} completely inhibits caffeine degradation by *Pseudomonas sp.* whereas Ca^{2+}, Fe^{2+} and Mg^{2+} slightly enhanced caffeine degradation and Mn^{2+} has no effect. Out of all the metal ions screened, only $Ni^{2+}$and Co^{2+} accumulated significant amount of theobromine in reaction media (Figure 1A). After 4 h of adding induced cells to the reaction media, $Ni^{2+}$and Co^{2+}accumulated 106.67 ± 4.73 mg/l and 384.53 ± 54.01 mg/l with molar yields of 7.31 ± 1.10% and 29.89 ± 4.23% respectively. We also observed that when these metal ions were added, caffeine degradation by *Pseudomonas sp.* was less compared to control till fourth hour and completely stopped there after (Figure 1B). Experiments were carried out to check synergism between Ni^{2+} and Co^{2+} on theobromine accumulation and caffeine degradation (Figures 1C and 1D) by adding 1 mM each in the media and it was observed that they produce only around 182.89 ± 36.75 mg/l with a molar yield of 13.59 ± 3.04%; thus confirming that they do not act synergistically in accumulating theobromine from caffeine. Amount of theobromine accumulated by synergistic experiment was less than that accumulated by cobalt ions but more than what was produced in the presence of nickel ions. Hence, $Co^{2+}$was chosen for further studies.

Optimization of reaction parameters for maximum theobromine accumulation by CCD

From the preliminary experiments, the upper and lower values for each parameter were fixed and the experimental design for CCD is given in table 1. The design required 20 experimental runs which were performed in three blocks with two center point values in each block. The experiments were carried out in random run order so as to avoid systematic error. Theobromine production (mg/l), molar yield (%) and productivity (mg/l) were considered as the responses after 4 h reaction time. Analysis of the data from CCD experiments

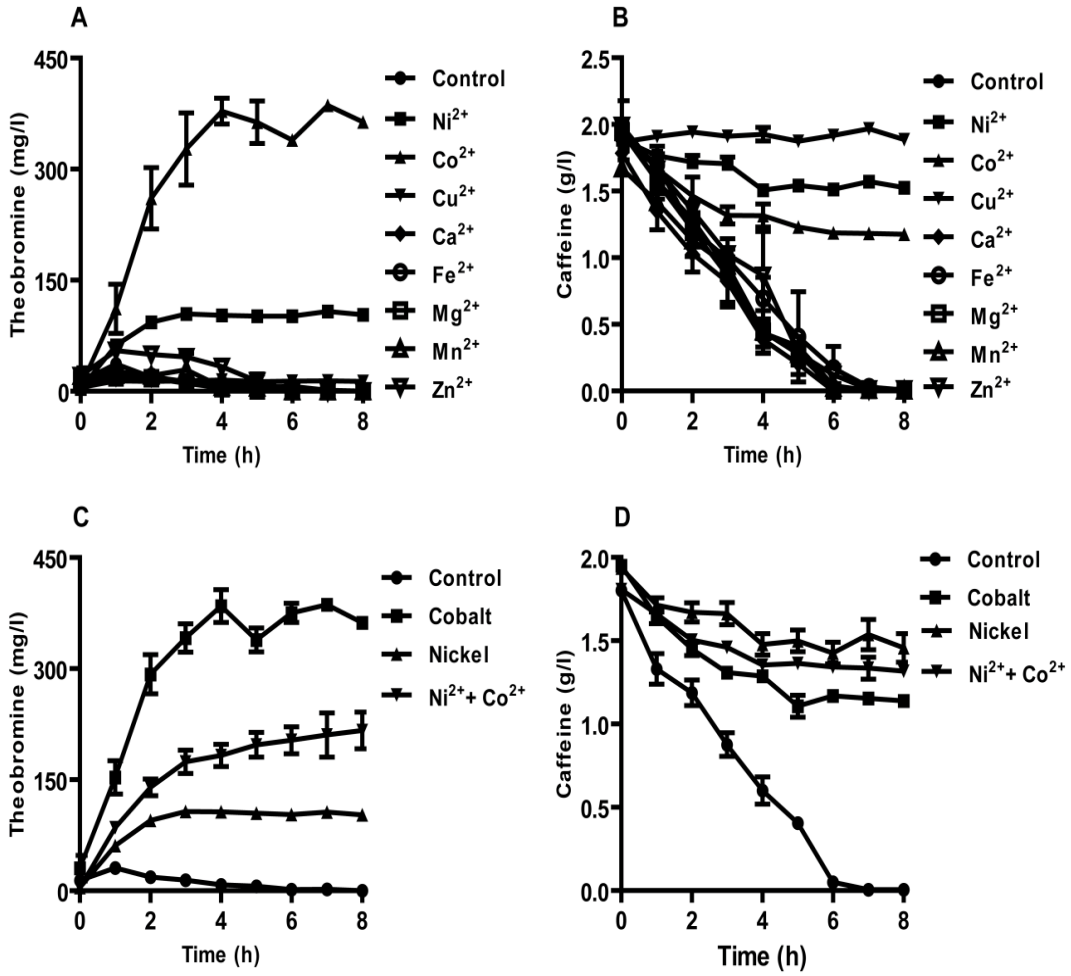

Figure 1: Screening of metal ions for theobromine production from caffeine using *Pseudomonas sp.* Effect of various divalent metal ions on (a) caffeine degradation, (b) theobromine accumulation. Synergistic effect of Ni^{2+} and Co^{2+} on (c) caffeine degradation, (d) theobromine accumulation.

showed that theobromine production was enhanced when pH was lower while caffeine and cell concentration were higher than that used in the preliminary experiments (center point values). When caffeine concentration and cell loading were maintained at +1 with pH at -1 level, maximum theobromine accumulation was observed (643.09 mg/l, run 6, table 2). Similarly, when we compared the runs where caffeine concentration and cell loading were maintained at +1 with varying pH (-1 and +1), it was found that lower levels of pH favored theobromine production [643.09 (run 6, table 2) and 256.90 (run 11, Table 2) mg/l respectively]. Reduction in theobromine production (487.05 mg/l, run 17) observed at -1.68 level of pH with other two parameters at 0 levels, shows that the dependence of theobromine production on pH is not linear and the optimal value lies somewhere between 0 and -1.68. Huge difference in theobromine accumulation was observed when only the caffeine concentration was changed from level +1 to -1 [643.09 (run 6) to 298.82 (run 9) mg/l] with cell loading and pH maintained at +1 and -1 respectively. This trend was also observed for cell loading and thus at higher concentrations of cell loading and caffeine, theobromine accumulation was favored (run 6, 13 and 14, Table 2). Marginal increase in theobromine production at +1.68 levels of cell loading (572.56 mg/l, run 14) and caffeine concentration (637.4 mg/l, run 13) when compared to center point values (556.46 mg/l, run 2) suggests that the optimal value of cell loading and caffeine concentration lies within the range of values (-1.68 to 1.68) selected for optimization. Productivity followed the same pattern as that of theobromine produced after 4 h, which can be explained from the relation that productivity is proportional to the amount of theobromine produced at a particular time. Molar yield was higher at higher levels of caffeine concentration, cell loading and pH (+1, +1 and +1 respectively) (run 11, Table 2). Yield was found to have reduced in experiments where the pH level was +1 and cell loading was maintained at -1 or less (run 1, 7,16). Otherwise not much variation in theobromine yield was observed between the CCD experiments.

Using 'Design Expert9' software, the response data were fitted into a quadratic equation to explain the effects of the three variables on theobromine production and yield. The quadratic regression models predicting theobromine accumulation, molar yield and productivity are given below

$$T = 546.85 + 118.56x_1 + 130.71x_2 - 126.27x_3 + 30.34x_1x_2 - 55.52x_1x_3 - 6.36x_2x_3 - 81.78x_1^2 \quad (2)$$

$$Y = 63.27 + 5.57\chi_1 + 15.26\chi_2 - 9.45\chi_3 + 3.32\chi_1\chi_2 + 2.13\chi_1\chi_3 + 11.58\chi_2\chi_3 - 5.06x_1^2 - 11.18x_2^2 \quad (3)$$

$$P = 136.71 + 29.64\chi_1 + 32.68x_2 - 31.37x_3 - 7.58x_1x_2 - 13.88x_1x_3 - 1.59x_2x_3 - 40.45\chi_1^2 - 25.9\chi_2^2 \quad (4)$$

Where, T is the predicted theobromine produced (mg/l), Y is the predicted molar yield, P is the predicted productivity (g/l) and x_1, x_2 and x_3 are the coded values of X_1, X_2 and X_3 respectively. The co-efficient for the interaction term x_1x_3 and x_2x_3 in equation (2) is negative indicating that the variable x_3 interacts negatively with the other variables x_1 and x_2. It shows that the effect of x_3 on theobromine production pattern is different from the effect of other two variables (x_2 and x_3). Similar pattern was observed in equation (4), which represents the model equation for theobromine productivity. Even the coefficient for the factor x_3 is negative in equation (1) and equation (3), which represents the model equation for molar yield of theobromine produced after 4 h. It was reflected in experimental data which showed increase in theobromine production and theobromine productivity as pH was lowered from the center point values (Table 2).

Low p-value (0.0001) for theobromine production (Equation 2) in ANOVA analysis indicates that the quadratic regression model is very significant (Table 3). R^2 value is coefficient of determination which examines how well the data fits into the model.R^2value closer to 1 indicates that the model significantly fits the experimental data. R^2 value of 0.9598 and adjusted R^2 value of 0.9146 obtained for the model equation (2) indicates that the quadratic regression model can predict theobromine production at any given point of x_1, x_2 and x_3 with good precision. Since productivity is directly related to theobromine produced at a particular time, quadratic model predicting theobromine

Run no	Caffeine concentration (g/l)	Cell loading (g/l)	pH	Theobromine produced (mg/l)		Molar yield %		Productivity (mg/l/h)	
				Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	1 (≡3)	-1 (≡4)	1 (≡9)	7.38	35.33	4.80	12.03	1.84	8.83
2	0 (≡2)	0 (≡8)	0 (≡8)	556.46	546.85	66.97	63.27	139.11	136.71
3	-1 (≡1)	1 (≡12)	1 (≡9)	149.79	157.95	48.40	50.31	37.44	39.49
4	0 (≡2)	0 (≡8)	0 (≡8)	556.46	546.85	66.97	63.27	139.11	136.71
5	-1 (≡1)	-1 (≡4)	-1 (≡7)	145.19	98.71	53.59	49.59	36.29	24.67
6	1 (≡3)	1 (≡12)	-1 (≡7)	643.09	721.01	61.60	63.83	160.77	180.25
7	-1 (≡1)	-1 (≡4)	1 (≡9)	6.55	-30.07	10.77	3.27	1.63	-7.53
8	0 (≡2)	0 (≡8)	0 (≡8)	546.26	546.85	60.06	63.27	136.56	136.71
9	-1 (≡1)	1 (≡12)	-1 (≡7)	298.82	312.17	62.85	50.31	74.70	78.05
10	1 (≡3)	-1 (≡4)	-1 (≡7)	353.05	386.19	57.05	49.83	88.26	96.55
11	1 (≡3)	1 (≡12)	1 (≡9)	256.90	344.71	73.66	72.35	64.22	86.17
12	0 (≡2)	0 (≡8)	0 (≡8)	539.56	546.85	62.25	63.27	134.89	136.71
13	1.68 (≡3.68)	0 (≡8)	0 (≡8)	637.40	515.21	60.19	58.34	159.35	128.78
14	0 (≡2)	1.68 (≡14.73)	0 (≡8)	572.56	474.01	52.85	57.35	143.14	118.51
15	-1.68 (≡0.32)	0 (≡8)	0 (≡8)	67.09	116.85	27.77	39.63	16.77	29.19
16	0 (≡2)	-1.68 (≡1.27)	0 (≡8)	8.45	34.82	0.47	6.07	2.11	8.70
17	0 (≡2)	0 (≡8)	-1.68 (≡6.32)	487.05	453.57	58.96	70.42	121.76	113.40
18	0 (≡2)	0 (≡8)	1.68 (9.68≡)	67.92	29.30	40.16	38.67	16.98	7.32
19	0 (≡2)	0 (≡8)	0 (≡8)	530.29	546.85	60.06	63.27	132.57	136.71
20	0 (≡2)	0 (≡8)	0 (≡8)	548.26	546.85	63.56	63.27	137.06	136.71

Experimental response values are the average responses from three independent experiments

Table 2: Central Composite Design for optimization of reaction conditions for theobromine production by Pseudomonas sp.

productivity (Equation 4) was found to be proportional to the model which predicts theobromine production (Equation 2). For the model which predicts theobromine yield (Equation 3), low p-value (0.0004) indicates that the model is significant. High value of R^2 (0.9431) and adjusted R^2 (0.8792) confirm the significance of the model to predict theobromine yield. Hence, the model developed using 'Design Expert 9' for the responses of theobromine production, theobromine yield and molar yield were found to be significant after statistical analysis (Table 3).

Response surface plots showing the two factor interaction effect on theobromine production (Figures 2A-2C), theobromine yield (Figures 2D-2F) and theobromine productivity (Figures 2G-2I) when the other factor was maintained at optimal value, are given in figure 2. The two dimensional response surface plots are the graphical representations of the quadratic regression model equation. Optimum values of the variables for maximum response can be predicted from contour plots. The contour lines represent the interaction of the two variables when the other is maintained at optimal value predicted by solving the model equation. Area enclosed by the smallest ellipse is the optimal response area and the optimal point is the intersection point of the major and minor axes of the ellipse. Figure 2A shows the effect of caffeine concentration (x_1) and cell loading (x_2) on theobromine production when pH is maintained at optimal value (x_3= -0.913). Elliptical contours in Figure 2A indicate that there is interaction between two variable x_1(caffeine concentration) and x_2 (initial cell loading) in determining the response (theobromine production). Similarly, perfect elliptical contours in Figure 2B, Figure 2D, Figure 2E, Figure 2G and Figure 2H indicates that the variables represented in the plots have interaction effects on the corresponding responses. Almost circular contour plots in Figure 2C and Figure 2I indicates that the interaction between the variables represented is less. Parallel contour lines in Figure 2F show that the interaction between pH (x_1) and initial cell loading (x_2) is not significant in determining the molar yield of theobromine. In all the contour plots in Figure 2, the intersection point between the major and minor axes of the smallest ellipse lies in the contour which indicates that the optimum point lies within the ranges of X_1, X_2 and X_3taken for optimization.

All three model equations were solved for the values of x_1, x_2 and x_3 at which the responses were maxima. Model predicted values of theobromine production and productivity at optimal values of X_1, X_2 and X_3 (3.2 g/l, 11.33 g/l and 7.0) are 729.43 mg/l and 182.35 mg/l, which correspond to the responses obtained from experimental values of 689.72 ± 22.86 mg/l and 172.46 mg/l respectively with <4% error. Predicted quadratic regression model for molar yield was completely different from that obtained for theobromine production and productivity, and thus the optimal values of X_1, X_2 and X_3were also different. The maximum experimental yield obtained at optimal conditions of X_1, X_2 and X_3 (2.28 g/l, 8.17 g/l and 6.64 respectively) was 64.46 ± 3.87% and it was comparable with model predicted yield of 70.4%. At points X_1, $X_2$and X_3, where we got maximum theobromine production (3.2 g/l caffeine, 11.3 g/l induced cell and pH 7.0), experimental molar yield was only 43 ± 3.9%which is 33% less than the molar yield obtained at optimized point for molar yield (2.28 g/l caffeine, 8.17 g/l induced cells and pH 6.64). Figure 3 shows the distribution of experimental and model predicted theobromine production (Figure 3A), molar yield (Figure 3B) and productivity (Figure 3C). Almost all the points fall within ± 1σ for theobromine production, productivity and theobromine yield.

Response variable	Source	DF	SS	MS	F-value	p-value
Theobromine accumulated[a]	Model	9	1027000	114100	21.23	0.0001
	Error	8	42973.56	5371.69		
	Lack of fit	5	42789.67	8557.93	139.62	0.0009
	Pure error	3	183.89	61.3		
	Total	19	1073000			
Molar yield[b]	Model	9	8086.74	898.53	14.74	0.0004
	Error	8	487.52	60.94		
	Lack of fit	5	479.01	95.80	33.76	0.0077
	Pure error	3	8.51	2.84		
	Total	19	8852.86			

[a]R^2 = 0.9598; adjusted R^2= 0.9146
[b]R^2 = 0.9431; adjusted R^2= 0.8792

Table 3: ANOVA for the quadratic models predicted for each response variable.

Effect of reaction temperature

All the preliminary experiments and the optimization of reaction media parameters were carried out at 30°C which is the optimal temperature for *Pseudomonas sp.* to grow on CAS medium. In an effort to understand the effect of reaction temperature on theobromine production, reactions were carried out at three different temperatures 25°C, 30°C and 35°C. It was observed that after 4 h, theobromine production was maximum (880.57 ± 18.46 mg/l) at 25°C (Figure 4b) which is 27% more than that observed at 30°C (689.72 ± 22.86 mg/l) with molar yield of 57.77 ± 9.8%. At 35°C, theobromine production was only around 525.93 ± 19.32 mg/l but the yield was higher (79.6 ± 5.18%). We also observed that at 30°C and 35°C, there is no significant conversion of caffeine to theobromine after 4 h whereas at 25°C, *Pseudomonas sp.* converted caffeine to theobromine (Figure 4A and Figure 4B) even after 4 hours with maximum theobromine concentration at 6thh (1.08 ± 0.10 g/l) which is 57% more than that observed at 30°C with a molar yield of 57.92 ± 11.9%. Initial conditions used for converting caffeine to theobromine was 2 g/l caffeine, 8 g/l induced cells and pH 8.0 at 30°C which were optimized to 3.2 g/l caffeine, 11.33 g/l induced cells, pH 7.0 and at 25°C. The comparison in theobromine production between optimized and unoptimized conditions is given in Figure 4C. Theobromine production was increased from 0.384 ± 0.054 g/l to 1.08 ± 0.1 g/l thereby obtaining a 181% increase after optimization of reaction parameters. Molar yield was also increased from 62.70 ± 9.9 % to 68.23 ± 17.6 %.

Discussion

It was reported that a caffeine degrading strain, *Pseudomonas putida* No. 352 accumulated theobromine when zinc ions were added to the growth medium [13]. About 20 g/l of theobromine was produced from caffeine with a yield of 92%. Later they showed that zinc specifically inhibited a theobromine specific monooxygenase [22]. However, this study was performed under growing conditions of *Pseudomonas putida* No. 352 in the presence of zinc in a medium which contained complex components like tryptone and expensive micronutrients like vitamins and amino acids which increases the cost of production. In addition, downstream processing to recover theobromine will be tedious as the growth medium has several compounds. In another study, a mutant strain *Pseudomonas putida IF-3-9C-21* developed from a wild strain *Pseudomonas putida IF3* was found to degrade caffeine at a faster rate and accumulated high theobromine in the media. It was cultured at 30°C in a medium containing caffeine, glucose and sodium glutamate with 1 vv m aeration. Caffeine was continuously added into the medium after 9 h culture time so as to maintain caffeine concentration between 0.5 g/l to 2.5 g/l. It was observed that when 165 g of caffeine was introduced 133 g of theobromine was produced with final caffeine

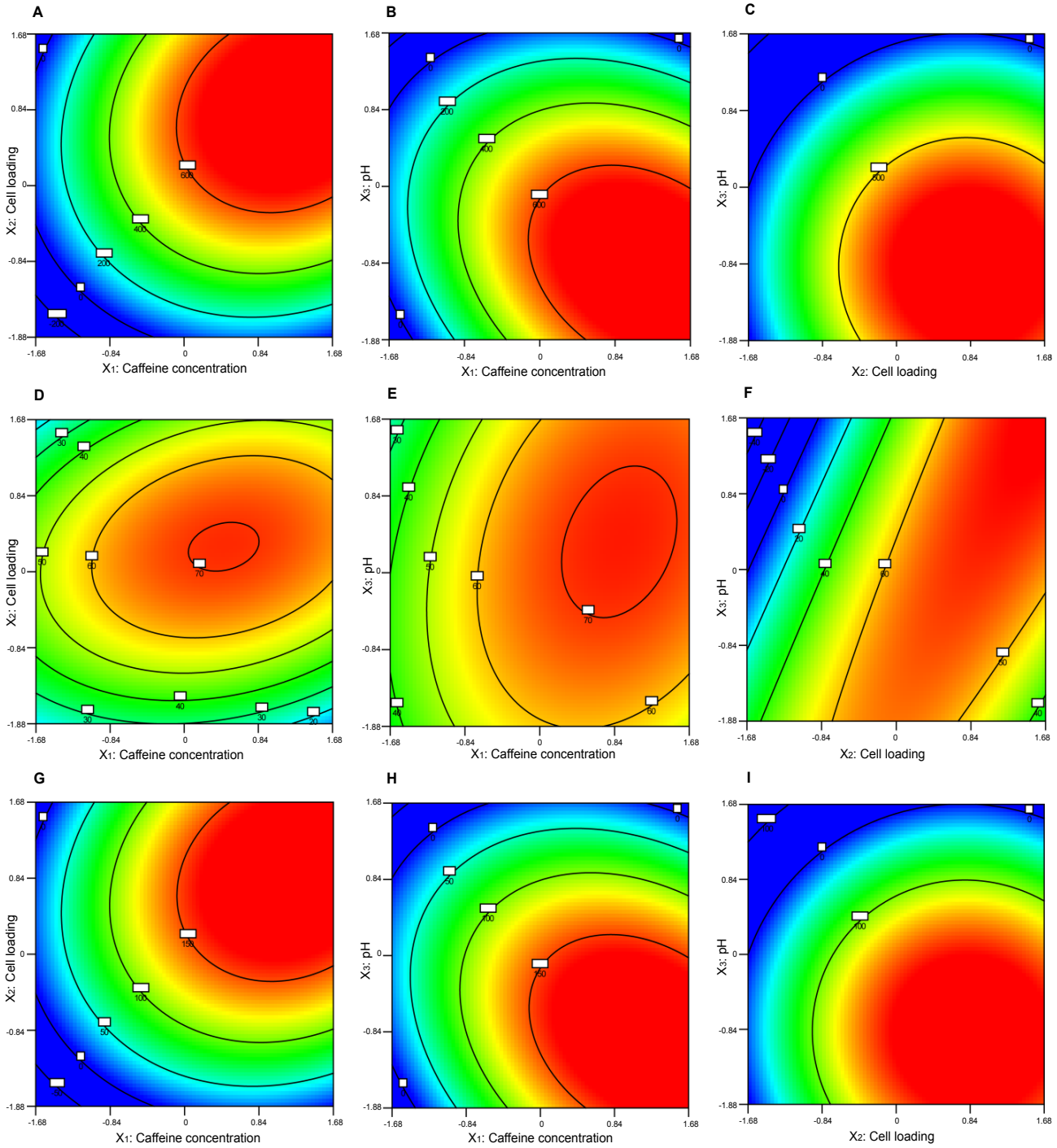

Figure 2: Contour plots showing the effect reaction parameters on responses. (a) theobromine production at optimal pH, (b) theobromine production at optimal cell loading, (c) theobromine production at optimal caffeine concentration, (d) theobromine yield at optimal pH, (e) theobromine yield at optimal cell loading, (f) theobromine yield at optimal caffeine concentration, (g) theobromine productivity at optimal pH, (h) theobromine productivity at optimal cell loading, (i) theobromine productivity at optimal caffeine concentration.

and theobromine concentration of 0.3 g/l and 39.8 g/l respectively [14]. Even though a process for theobromine production from caffeine using a *Pseudomonas* strain was developed with very good conversion (88%), it was done in the growing conditions in the presence of many other complex nutrients which need tedious downstream processing. As reported by Glück et al. [12], there is also a possibility of the mutant strain reverting to its wild type. All these studies were performed to produce theobromine in the growth medium with complex components.

However, we used induced cells of *Pseudomonas sp.* as the catalyst to convert pure caffeine in 10 mM potassium phosphate

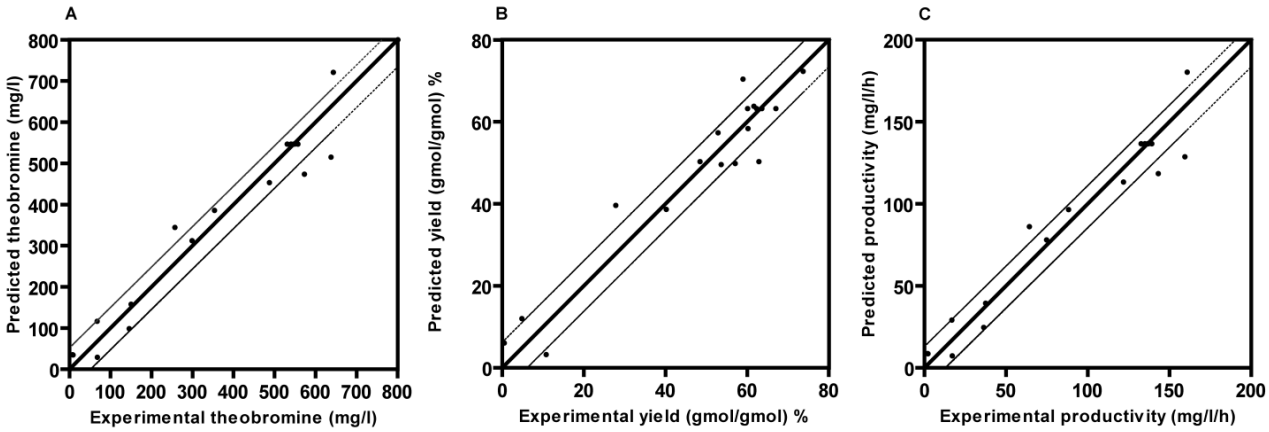

Figure 3: Parity plots for the quadratic models. (a) Parity plot showing the distribution of experimental and predicted theobromine production (σ = 51.48), (b) Parity plot showing the distribution of experimental and predicted theobromine yield (σ = 6.17), (C) Parity plot showing the distribution of experimental and predicted theobromine productivity (σ = 12.8).

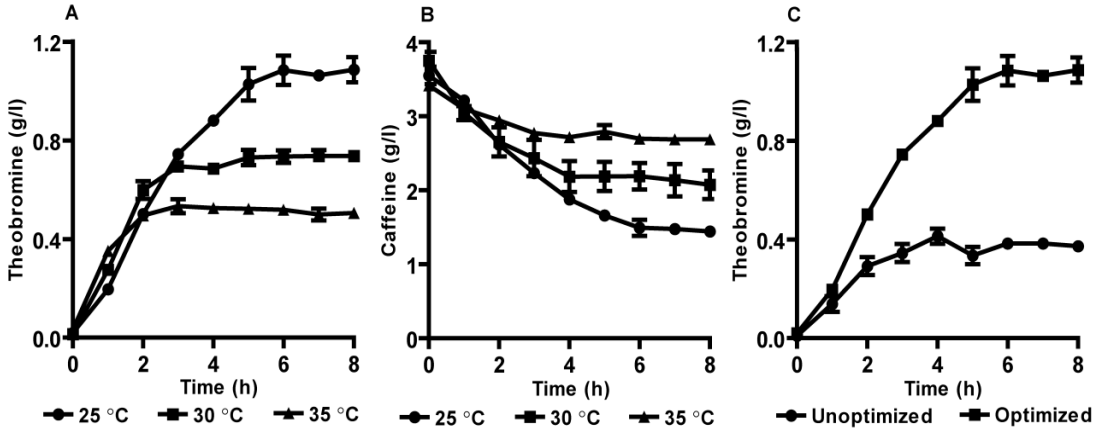

Figure 4: Optimization of reaction temperature at optimized caffeine concentration, cell loading and pH. Effect of temperature on (a) caffeine degradation, (b) theobromine production, (c) plot showing increased theobromine production under optimal conditions.

buffer to theobromine. Whole cell catalyst ensures reusability and low production cost. We report highest theobromine production (~1 g/l) from caffeine using resting cells of *Pseudomonas sp.* as compared to already reported 225 mg/l by Glück et al. They reported accumulation of theobromine and 7-methylxanthine, using resting cells of *Pseudomonas sp. H8* which is a mutant of *Pseudomonas putida WS* [12]. But very less amount of theobromine was accumulated (around 100 mg/l) and they shifted their focus to metabolite production during growth of *Pseudomonas H8* in a medium containing caffeine. Even during growth, only around 225 mg/l of theobromine was reported along with 425 mg/l of 7-methylxanthine which is very less for industrial production. Major advantages of using induced whole cell biocatalyst for theobromine production as demonstrated in this study are: 1. Induced cells are metabolically active as it has enough caffeine degrading enzymes synthesized already during growth in production media. Therefore, conversion of caffeine to theobromine is really fast. Productivity of theobromine after 6 h under optimized conditions is 220.14 ± 4.61 mg/l/h which can be increased by applying continuous or fed-batch strategy and 2. Reaction media contains only caffeine and theobromine which can be easily separated by preparative HPLC.

Conclusions

Theobromine is a commercially important molecule which is currently produced in industries using toxic chemicals and tedious procedures. Microbial production of theobromine is practically a safer and economical approach compared to the chemical methods available. We optimized the reaction parameters for theobromine production from caffeine by *Pseudomonas sp.* as 3.2 g/l caffeine; 11.3 g/l induced *Pseudomonas sp.*, pH 7.0 at 25°C. Under this condition, up to 1 g/l of theobromine was produced from caffeine in 6 h with a molar yield of 57%. More research is needed on increasing the yield and production of theobromine from caffeine by employing bioprocess strategies so that microbial production of theobromine in industry will become feasible in future.

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