JEB
Journal of Environmental Biology

Journal Home page : www.jeb.co.in
E-mail : editor@jeb.co.in

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Application of response surface methodology for optimising caffeine-degrading parameters by *Leifsonia* sp. strain SIU

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Publication Info

Paper received:
17 June 2014

Revised received:
12 March 2015

Accepted:
20 March 2015

Abstract

Caffeine is an important naturally occurring compound which can be degraded by bacteria. Previously, *Leifsonia* sp. strain SIU capable of degrading caffeine was isolated from agricultural soil. Plackett–Burman design was used to screen significant parameters that affect the rate of caffeine degradation. After the design was applied, response surface methodology (RSM) through Central Composite Design (CCD) was used to study significant parameters further, in order to get the most superior degradation conditions. The optimum concentrations of carbon source (sucrose), nitrogen source (NH_4Cl), pH and initial caffeine concentration was found to be 5.0 gl^{-1}, 0.4 gl^{-1}, 6.0 and 375 ppm respectively. Second order polynomial regression model accurately showed interpretation of experimental data with an R^2 value of 0.9989, Adjusted (Adj) R^2, Predicted (Pred) R^2 and F values of 0.9939, 0.9225 and 88.77 respectively.

Keywords

Caffeine degradation, Central composite design, *Leifsonia* sp., Plackett-Burman Design, Response surface methodology

Introduction

Caffeine or 1,3,7-trimethylxanthine is a purine alkaloid occurring naturally in beverages such as coffee, tea, cola nut, cocoa beans, tea leaves and soft drinks (Lakshmi and Das, 2013; Mohanpuria *et al.,* 2009). Excessive consumption of caffeine by individuals can actually lead to some adverse effects (Gokulakrishnan and Gummadi, 2006). Thus, demand for high, accurate and simple decaffeination process in beverages is at bottleneck nowadays. The side effects due to excessive caffeine intake through beverages are also associated to an increasing risk of miscarriage, and independent of pregnancy-related symptoms (Weng *et al.,* 2008). It can also lead to a number of health problems such as osteoporosis, adrenal simulation, irregular muscular activity, inhibition of DNA repair mechanism and adenosine mono phosphodiesterase inhibition (Lorist and Tops, 2003; Smith, 2002; Spriet *et al.,* 1992). Apart from the adverse effect, caffeine degradation is paramount from environmental point of view. Tea and coffee industries produce caffeine waste which are later channelled to the nearby lakes and rivers, making drinking water become polluted (Buerge *et al.,* 2003). The presence of caffeine in soil also affects soil fertility as it inhibits seed germination as well as growth of seedlings (Batish *et al.,* 2008). As a result, biodegradation of caffeine has been an interesting topic for many research groups (Gummadi *et al.,* 2007; Nayak *et al.,* 2012; Summers *et al.,* 2012; Yu *et al.,* 2008).

Microbial caffeine degradation is affected by numerous cellular and physiological factors. These factors may play a significant role in controlling caffeine degradation rate, and production of enzymes involved in caffeine degradation (Gummadi *et al.,*2012). Factors such as carbon source, external nitrogen source, pH, temperature, initial caffeine concentration, agitation speed and inoculums size are crucial parameters for caffeine degradation by microorganisms.

Optimisation of caffeine-degrading bacteria is done using RSM, via two stages, Plackett-Burman and central composite design (CCD). RSM has been significantly used for optimisation and study of interaction among various bioprocess parameters

using minimum number of experiments (Muntari *et al.,* 2012; Sivasubramanian and Namasivayam, 2014).

Plackett-Burman statistical method is a useful tool to screen out positive factors contributing to caffeine degradation. This could serve as a guide in developing efficient parameters to enhance caffeine degradation.

Response surface methodology is a collection of statistical methods for designing experiments, in order to determine optimal conditions, evaluate the effect of these factors and building models (Kalil *et al.,* 2000; Muntari *et al.,* 2012; Tripath and Srivastava, 2012). One of the advantage of using RSM is that we can decrease the number of experiments needed to optimise certain parameters as well as the way they interact with each other, as such leading to less time consuming (Chen *et al.,* 2005; Sharma *et al.,* 2009). Farliahati *et al.* (2010) reported that RSM is able to identify accurate optimum condition of a given parameter in addition to the capacity to reduce limitation of a single-factor optimisation process, such as finding an interaction between variables (Aktas, 2005; Zhou *et al.,* 2011). Besides this, optimisation using one-factor-at-a-time approach method is expensive and consume more time (Ray *et al.,* 2009). Example of such experimental designs are Box-Behnken, central composite design (CCD), 3-level factorial, distance based and one factor among others. CCD is an effective design and does not require large number of design points, thereby reducing the overall cost associated with the experiments (Korbhati *et al.,* 2007).

Previously, a bacterium capable of utilising caffeine as sole carbon and nitrogen source was isolated (Ibrahim *et al.*, 2015). According to this report, the strain was identified as *Leifsonia* sp. strain SIU. This present study aimed at improving caffeine degradation rate by optimising significant factors required for caffeine degradation by *Leifsonia* sp. strain SIU. The most significant factors for caffeine degradation are carbon source and nitrogen source, pH, temperature and effect of initial caffeine concentration. These factors were selected based on the previous preliminary study (Babu *et al.,* 2005; Gokulakrishnan *et al.,* 2007; Hakil *et al.,* 1999). Thus, optimisation of caffeine degradation by *Leifsonia* sp. strain SIU was done using Response surface methodology with central composite design.

Materials and Methods

Microorganism and media preparation: Previously isolated *Leifsonia* sp. strain SIU from agricultural soil, was cultured at 30 °C in sterilised caffeine liquid medium containing the following (gl^{-1}): 0.4 K_2HPO_4, 0.2 KH_2PO_4, 0.1 NaCl, 0.1 $MgSO_4$, 0.01 $MnSO_4.H_2O$, 0.01 $Fe_2(SO_4)_3$, 0.01 $NaMoO_4.2H_2O$ and 0.4 NH_4Cl. The media contained 0.3 gl^{-1} of caffeine in addition to the above composition. Any added carbon source to the medium was sterilised separately and then mixed with the medium under aseptic conditions. For solid medium, 25 gl^{-1} agar was added to caffeine medium. Isolates were maintained and sub-cultured every 2 weeks in caffeine agar medium.

Analytical determinations: Concentration of caffeine was estimated by HPLC (Agilent 1100 series from Agilent technologies, Waldbronn, Germany, Product No. G2170AA) using a ZORBAX® SB-C18 column (USA, Product No. 880975-902, Batch No. B03024) with 10 mM ammonium phosphate buffer (pH 2.5)/ acetonitrile (4:1, v/v) in a mobile phase. Pure caffeine (2 $mgml^{-1}$) was used as standard. Retention time of caffeine was found to be 2.1 min at a flow rate of 1 ml min^{-1} and at 30 °C. Detection of caffeine was done at 254 nm (detector sensitivity: 1×10^{-14} absorbance unit). The percentage of caffeine degradation was calculated using the formula below:

$$\text{Caffeine degradation (\%)} = (a-b)/a \times 100\%$$

where *a* is the initial concentration and *b* is the residual caffeine concentration.

Flask culture experiments: A single loop of fresh grown culture of caffeine agar plate was transferred to 10 ml nutrient broth medium and incubated on a rotary shaker at 150 rpm at room temperature for few hours till OD_{600nm} reached 1.3 - 1.4. About 4% (v/v) of the culture was incubated for 48 hr at room temperature and 150 rpm. After 48 hr, the samples were drawn and centrifuged for 10 min at 12,000 rpm. The supernatant was then filtered and used for caffeine analysis, or stored at 4 °C.

Data analysis with RSM and CCD: It consists of a group of statistical and mathematical methods that can be used to define relationship between response and independent variables. Plackett-Burman design consist of 12 experiments with five parameters to screen significant factors. Those significant factors were further optimised using central composite design with four parameters (sucrose concentration, nitrogen concentration, pH and initial caffeine concentration), which were considered to have a great impact towards caffeine degradation. A set of 30 experiments were run (= 2^k + 2k + 6), where k is number of parameters, with six replication of centre points. Design Expert Software (version 6.0.8, Stat-Ease, Inc. Minneapolis, USA) was used for regression analysis of experimental data and to plot response surface in order to obtain optimisation of significant parameters. All experiments were conducted in triplicates and the mean of degradation was taken as response. Correlation of response and independent variables was indicated using second order model that fitted with response variable. The general formula of the second degree polynomial model used is:

$$Y=\beta_0+\beta_1 X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{11}X_1+\beta_{22}X_2+\beta_{33}X3+\beta_{44}X_4+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{14}X_1X_4+\beta_{34}X_3X_4$$

where, Y is the predicted response parameter; and β_0, β_1, β_2, β_3, β_4, β_{11}, β_{22}, β_{33}, β_{44}, β_{12}, β_{13}, β_{23}, β_{24}, and β_{14} are constant regression coefficients of the model, in which β_0 is intercept term, β_1, β_2, β_3, and β_4 are linear coefficients, and β_{11}, β_{22}, β_{33}, and β_{44} are squared coefficients. On the other hand, X_1, X_2, X_3, and X_4 are independent parameters. Parameter combinations (X_1X_2) showed interaction between the variables (Suhaila *et al.,* 2013). The values/range of

five variables that affect caffeine degradation are as follows: Caffeine concentration 50 – 700 ppm, Carbon source 2 – 8 gl^{-1}; and Nitrogen source 0.1 – 0.7 gl^{-1}; temperature 25 – 40 °C, and pH 4 – 8.

Results and Discussion

In Plackett-Burman designs, major effects have a complex confusing relationship with two-parameter interactions. Hence, these designs should be used to study major effects when two-way interactions are negligible. In practical use, Plackett-Burman and fractional factorial designs are often used to screen important parameters that influence caffeine degradation. These designs are valuable for fitting first-order models (which detect linear effects) and can give insight on the existence of second-order effects (curvature) when the design includes centre points (Salihu *et al.,* 2013). To express the efficiency of Plackett-Burman design, a research was conducted to compare a Plackett-Burman design with a full factorial design. Full factorial design consisted of five parameters with two levels for each parameter. In this case, a total of about 32 different experiments were conducted and the characteristic of response, *Y*, was larger and better, while in Plackett-Burman design, a total of about 12 different experiments were conducted with five parameters. Thus, the Plackett-Burman saves time, chemical and energy usage due to less number of experiments to be conducted. More so, the responses are larger when compared to full factorial design.

Table 1: Experimental design and result of Placket-Burman on caffeine degradation

Run	A	B	C	D	E	Response (%)
1	50	2	0.1	40	8	69.11
2	50	8	0.7	25	8	98.20
3	50	8	0.7	40	4	82.40
4	700	8	0.1	40	8	81.50
5	700	2	0.1	25	8	76.10
6	50	8	0.1	25	4	67.64
7	700	8	0.1	40	4	46.34
8	50	2	0.1	25	4	65.67
9	700	2	0.7	25	4	36.56
10	50	2	0.7	40	8	56.76
11	700	8	0.7	25	8	89.80
12	700	2	0.7	40	4	42.45

A: Caffeine concentration; B: Carbon source (Sucrose); C: Nitrogen source (NH_4Cl); D: Temperature and E: pH

A total number of 12 experimental designs were made using Plackett–Burman design, with each row of the table consisting of five independent parameters namely carbon source (sucrose), nitrogen source (NH_4Cl), temperature, pH and initial caffeine concentration. High and low values of independent parameters were selected based on the literature. As shown in Table 1, the result of caffeine degradation for each experimental runs according to the design matrix. Based on the results obtained in this experiment, the lowest and highest caffeine degradation percent were found to be 36.56 and 98.20% as observed in runs 9 and 2, respectively. Analysis of variance in Table 2 shows the result of significant parameters which revealed that among all parameters used, temperature was not a

Table 2: Analysis of variance for caffeine degradation for Plackett-Burman

Source	Sum of squares	Degree of Freedom	Mean Square	F-Value	F- Value	
Model	4080.5	9	453.39	199.06	0.005	Significant
A	144.05	1	144.05	63.24	0.0154	
B	426.3	1	426.3	187.17	0.0053	
C	77.79	1	77.79	34.15	0.0281	
D	17.75	1	17.75	7.79	0.1079	
E	897.35	1	897.35	393.98	0.0025	
AD	120.92	1	120.92	53.09	0.0183	
AE	163.59	1	163.59	71.83	0.0136	
BC	313	1	313	137.42	0.0072	
CE	56.3	1	56.3	24.72	0.0382	
Residual	4.56	2	2.28			
Cor Total	4085.06	11				
Std. Dev.	1.51		R-Squared	0.9989		
Mean	67.71		Adj R-Squared	0.9939		
C.V.	2.23		Pred R-Squared	0.9225		
PRESS	316.62		Adeq Precision	44.122		

Table 3: Experimental design and result for optimisation of caffeine-degrading bacteria using CCD

Run	A: Conc.	B: Carbon source	C: Nitrogen source	D: pH	Experimental value	Predicted value
1	375	5	0.4	10	44.81	46.10
2	50	8	0.1	8	64.50	63.71
3	700	8	0.1	4	70.57	69.91
4	700	2	0.1	8	34.75	33.76
5	375	5	0.4	6	92.54	93.83
6	700	8	0.7	8	70.38	67.27
7	375	5	0.4	6	92.54	93.83
8	375	5	0.4	6	92.54	93.83
9	50	8	0.1	4	70.27	80.56
10	275	5	0.4	6	100.00	94.65
11	375	5	-0.2	6	34.00	35.07
12	50	8	0.7	8	84.67	86.21
13	50	2	0.7	8	75.00	70.70
14	700	2	0.1	4	40.00	35.78
15	375	5	0.4	6	92.54	93.83
16	700	8	0.7	4	70.38	71.55
17	50	8	0.7	4	71.88	72.17
18	50	2	0.7	4	44.68	43.00
19	375	11	0.4	6	80.00	80.41
20	50	2	0.1	8	44.00	44.43
21	700	2	0.7	4	41.08	41.17
22	375	5	0.4	6	92.54	93.83
23	375	5	0.4	2	32.00	34.08
24	375	0	0.4	6	45.56	49.84
25	375	5	1	6	60.66	62.97
26	50	2	0.1	4	33.92	34.37
27	700	2	0.7	8	65.00	61.04
28	1025	5	0.4	6	69.86	75.02
29	375	5	0.4	6	98.00	93.83
30	700	8	0.1	8	50.98	49.97

significant parameter for caffeine degradation by *Leifsonia* sp. strain SIU, as *p*-value was 0.05. The *p*-values of the remaining four parameters, *i.e.,* carbon source (sucrose), nitrogen source (NH_4Cl), pH, and initial caffeine concentration were observed to be 0.05, thus showing that these four parameters were significant. The determination coefficient (R^2) of the model was 0.9989. The Adj R^2 and the Pred R^2 values were found to be 0.9939 and 0.9225, indicating that there was a high correlation between the predicted and the experimental values. The percentage of caffeine degradation, obtained from Plackett-Burman design experiments, showed some variations which indicate the necessity for further optimisation using central composite design.

After the Plackett-Burman design was done to screen significant parameters which affect caffeine degradation, CCD further was used to study optimum combinations of significant parameters in order to get the most appropriate degradation activity. In this optimisation step, parameters namely initial caffeine concentration (A), carbon source (B), nitrogen source ©

Table 4: Analysis of variance for CCD

Source	Sum of square	DF	Mean square	F-value	Prob > F	
Model	13819.82	14	987.13	88.68	<0.0001	Significant
A	294.41	1	294.41	26.45	0.0001	
B	2875.97	1	2875.97	258.38	< 0.0001	
C	1167.61	1	1167.61	104.9	< 0.0001	
D	216.72	1	216.72	19.47	0.0005	
A2	128.45	1	128.45	11.54	0.004	
B2	1760.64	1	1760.64	158.17	< 0.0001	
C2	3523.24	1	3523.24	316.53	< 0.0001	
D2	5066.67	1	5066.67	455.19	< 0.0001	
AB	9.36	1	9.36	0.84	0.3736	
AC	10.56	1	10.56	0.95	0.3454	
AD	146.05	1	146.05	13.12	0.0025	
BC	64.4	1	64.4	5.79	0.0295	
BD	320.77	1	320.77	28.82	< 0.0001	
CD	479.17	1	479.17	43.05	< 0.0001	
Residual	166.96	15	11.13			
Lack of Fit	142.12	10	14.21	2.86	0.1288	Not Significant
Pure Error	24.84	5	4.97			
Cor Total	13986.79	29				
Std. Dev.	3.33	R-Squared	0.9881			
Mean	65.32	Adj R-Squared	0.9769			
C.V	5.1	R- Pred Squared	0.9362			
Press	892.69	Adeq Precis ion	25.825			

and pH (D) were further used. RSM-CCD also assisted in understanding how these parameters interact with each other. The results obtained from the central composite design experiments were fitted to second order polynomial equation to explain the dependence of caffeine degradation. By using RSM, the effects of independent factors and their interaction were represented and their response was predicted. Table 3 shows 30 experiments conducted according to CCD and the response (%) were experimentally and predicted. Table 3 shows the 30 experiments was conducted according to CCD and the response (%) were based on the experimental and predicted value. The minimum and maximum caffeine degradation concentrations was observed in run 23 (32%) and run 10 (100%), respectively.

As shown in Table 4, results of ANOVA based on the second-order polynomial response surface model was performed using Design-expert 6.08 software. In order to estimate the model, statistical analysis such as lack of fit, R^2-value and F-value were determined. In significant, lack of fit with a probability value 0.1 is required in order to show that data obtained from the experiment and predicted is almost similar. Aghaie *et al.* (2009) reported that if a model shows a significant lack of fit, it should not be used to predict the response. The *p*-value shows that this model for both responses was statistically significant with the probability F value for model parameter that is 0.05. The result obtained showed that A, B, C, D, A^2, B^2, C^2, D^2, AD, BC, BD and CD were significant model terms.

Coefficients determination,(R^2)of the model was found to be 0.9881 and the adjusted R^2 was found to be 0.9769, which indicates that there was high correlation between the experimental and the predicted values. Also, the predicted R^2 was found to be 0.9362 which is in conformity with the particular adjusted R^2. As such, it shows that the regression model provides an excellent explanation of the relationship between independent factors (variables) and response. The model showed lack of fit F value of 2.86 while lack of fit P value was 0.1288, which indicates that lack of fit was not significant. Suhaila *et al.* (2013) and Reddy *et al.* (2008) reported that lack of fit was not significant and explained that the model was a good fit.

Adequate precision measures signal to noise ratio and its value 4 is considered as advantageous (Ramanan *et al.,* 2010). The ratio of 25.825 signified that the models could be used to predict the response, while coefficient of variation at lower value, 5.10 % showed that the experiment was precise and reliable. Table 3 shows model coefficient and their significances estimated by multiples linear regression for caffeine degradation time.

Table 5: Predicted and experimental variable for optimal responses for caffeine degradation

Factor	Name	Level Pred	Level Exp.
A	Conc	375	375
B	C-source	5	5
C	N-source	0.4	0.4
D	pH	6	6
	Prediction	SE Mean	Experimental
Response 1	93.83	1.314425	91.47

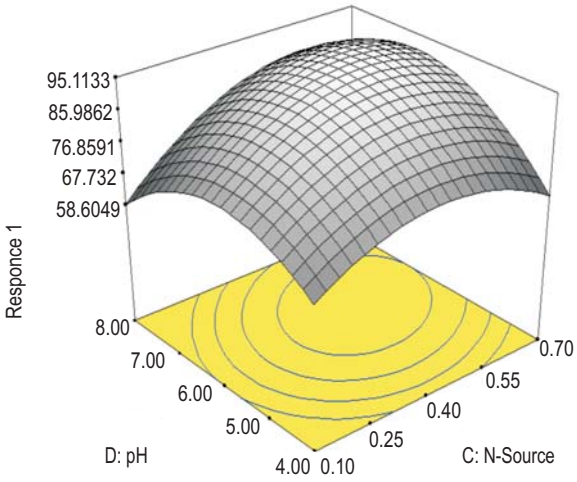

Fig 1.: Response surface plot showing the effect of pH and nitrogen source (NH_4Cl) concentrations on caffeine degradation. Value of variable caffeine concentration and carbon source (sucrose) was fixed at central point

Applying multiple regression analysis by neglecting the non significant values, the simplified quadratic model was in the form of the following equation.

$$Y = 93.83 - 3.89\,A + 11.35\,B + 6.98\,C + 3.01\,D - 2.76\,A^2 - 9.03\,B^2 - 11.20\,C^2 - 13.43\,D^2 - 3.02\,AD - 2.02\,BC - 4.48\,BD + 5.47\,CD.$$

where, Y is response value (in %); A, B C, and D are coded levels of caffeine concentration, carbon source, nitrogen source and pH, respectively.

The 3D response surface plot is a graphical representation of regression equation. It is plotted to understand the interaction of parameters and locate optimal level of each variable for maximal response (Lima *et al.,* 2010). Each response surface plotted for caffeine degradation signifies different combinations of two test parameters at one time, while maintaining the other parameter at constant level. The curved response surfaces indicate that there are well-defined optimal parameters. If the surfaces are rather flat and symmetrical near the optimum, the optimised values may not vary widely from single parameter conditions (Rao *et al.,* 2006). The graphical representation of response surface shown in Fig. 1 helps to see the effects of pH and nitrogen source (NH_4Cl) at fixed caffeine concentration and carbon source (sucrose). pH and nitrogen source are major components that control caffeine degradation. Thus, a strong interaction between them for degradation of caffeine is expected. Maximal caffeine degradation (92.34%) was obtained for pH values (6.00) and nitrogen source (0.4 gL^{-1}) in the central point region (Fig. 1). *Leifsonia* sp. strain SIU was noticed to withstand up to 700 ppm caffeine. The inhibitory effects became significant when caffeine concentration increased beyond 700 ppm. Increased or decreased NH_4Cl and pH leads to decrease in caffeine biodegradation. As such enhanced biodegradation of caffeine by bacterium.

Based on the response surface methodology, using central composite design result, optimum condition was found to be 5.0 gl^{-1} sucrose, 0.4 gl^{-1} ammonium chloride, pH 6.0 and 375 ppm caffeine concentration.

Degradation of caffeine by *Leifsonia* sp. strain SIU was done under optimal conditions as predicted by RSM for verification. Table 5 shows the verification result as predicted by RSM and verified using the experimental value. The results of one way ANOVA test showed that there was no significant difference in caffeine degradation percent between the predicted and the experimental value ($p > 0.05$).

This it can be concluded that the response surface methodology with central composite design can be used for optimising parameters, needed for caffeine degradation in order to enhance degradation by *Leifsonia* sp. strain SIU. The reliability of the model shows that the bacterium can be used for caffeine degradation in an optimised condition of industrialised scales.

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