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Original Research Article

Statistical Optimization of Cellulase Production from Cassava Stem by Cellulomonas Fimi MTCC24 using Box-Behnken Design

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ABSTRACT

Effect of different media components such as carbon,nitrogen, and minerals on cellulase production from cassava stem production by *Cellulomonas fimi* MTCC24 inshake flask culture was investigated. Combination of Plackett – Burman design (PBD) and Box – Behnken design (BBD) were applied for optimization of different factors for cellulase production cassava stem by *Cellulomonas fimi* MTCC24. Among 11 factors, pretreated cassava stem, malt extract, Ammonium chloride and sodium chloride into the culture medium were selected due to significant positive effect on cellulase yield. Box - Behnken design, a response surface methodology, was used for further optimization of these selected factors for better cellulase activity. Data were analyzed step wise and a second order polynomial model was established to identify the relationship between the cellulase activity and the selected factors. The media formulations were optimized having the factors such as pretreated cassava stem 3.07g/L, malt extract 2.99 g/L, Ammonium chloride 2.81g/L and NaCl 2.49g/L.

Keyword: Response surface methodology; cellulose; cassava stem; Box-Behnken design

INTRODUCTION

Cassava (*Manihot esculenta Cranz*) is a very important crop grown for food and industrial purposes in several parts of the tropics. Nigeria, with a 2006 production of 49 million tonnes of cassava is the largest producer of the crop in the world [1]. Cassava is the third most important source of calories in the tropics, after rice and maize. Millions of people depend on cassava in Africa, Asia and Latin America. The broad agro-ecological adaptability of cassava

and its ability to produce reasonable yields where most crops cannot makes it the basis for food security at the household level and an important source of dietary energy.

Cassava stems are one source of agricultural residues that could be considered for bioconversion in tropical countries [2]. Potential applications of these materials include activated carbon production, energy generation and animal feed; however, the cassava stems are often left in the field, due to their low monetary value, or are burned, causing environmental problems. Cassava stems can be considered to be an alternative source for the production of bioethanol[3], and the effects associated with leftover cassava could be mitigated[4].Cassava stem rich in cellulose content which utilized for the cellulase enzyme production.

The response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models[5], evaluating the effects of several factors and obtaining optimum condition of factors for desirable responses [6], which provides the relationship between one or more measured dependent responses and a number of input factors[7]. It has some advantages that include a less number of experiments; suitability for multiple variables can reveals possible interactions between variable, search for relativity between multiple variables and finding of the most suitable correlation and forecast response. In many cases, a second-order model is easy to estimate the parameters due to its flexibility and it works well in solving real response problems. Therefore, the second-order model is widely used in RSM. The most common designs, that is central composite design[8] and Box-Behnken experimental design[10], of the principal response surface methodology have been widely used in various experiments. Box-Behnken design, a spherical and revolving design, has been applied inoptimization of media.

Statistical methodologies such as Plackett - Burman design and Box - Behnken design have shown to be efficient and effective approach to systematic investigation on the target factors. PBD is an effective screening design which considerably diminishes the number of experiment and gives information for the evaluation of the target factors as much as possible. Only the most effective factors with positive significance are selected for further optimization. The less significance or high negative effect on response value would be omitted for further experiments. PBD has been widely applied in many fields such as medium optimization, formulation of multi component and so on [5]. BBD can be used to optimize target parameters within the designed scopes. The number of trials is equal to the maximum number of the designed levels of the target factor and therefore presents the advantage.

In this present work medium components were optimized by one-factor-at-a-time method and combination of PBD and BBD was applied to the selection of medium components that significantly influenced the production of cellulase from cassava stem by *Cellulomonas fimi* MTCC24.

MATERIALS AND METHODS

Medium components

Magnesium sulphate, ferrous sulphate, manganese sulphate, copper sulphate, zinc sulphate, calcium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride, yeast extract, peptone, beef extract, malt extract, sodium molybdate, ammonium nitrate, ammonium chloride, ammonium phosphate and ammonium carbonate were purchased from Hi-Media Limited, Mumbai, India.

Microorganism and culture condition

Cellulomonas fimi MTCC24 was procured from MTCC, Chandigarh, India and maintained on Nutrient agar medium at 28^0C for overnight

.After the incubation the slants were kept at 4^0C and thereafter sub-cultured every 30 days.

Cellulase Assay

The culture medium was centrifuged at5000 rpm for 20 min at 4°C. The supernatant was used as crude enzyme source for cellulase assay[13].0.450ml of 1% CMC as the substrates in 0.2M citrate phosphate buffer (pH-5) and incubated at 55°C for 15min.The reaction was terminated by addition of 0.5ml of DNS reagent and tubes were kept at boiling water bath for 5min. After cooling the tubes at room temperature, 3ml of distilled water was added in each tube. The intensity of the color was read at 540nm.Standard curve was performed with glucose solution. One unit of enzyme activity was defined as the amount of enzyme required for release 1µ mol ofglucose per minute under assay condition. Enzyme activity was expressed in units. Cellulase activity was calculated using this formula:

IU/ml = concentration of glucose / 0.5×30×0.180

One micromole of glucose equals 0.180 mg

Optimization of process parameters

Screening of significant medium components using Plackett - Burman design

The Plackett – Burman experimental design identifies the critical physico-chemical parameters required for elevated prodigiosin production by screening n variables in n + 1 experiments [8]. The variables chosen for the present study were pretreated cassava stem (g/L), malt extract(g/L), ammonium chloride, ammonium nitrate, ammonium phosphate, $MgSO_4$ $CaCl_2$(g/L), NaCl (g/L) and trace salts (g/L) in the culture medium. The experimental design for the screening of the variables was presented in Table 1. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). Table 2 shows the PBD for 11 variables. From the regression analysis the variables, which were significant at or above 95% level ($P<0.05$), were considered to have greater impact on cellulase activity and were further optimised by Box-Benken design.

Table1: Nutrient screening using Placket-Burman design

	Factors	Low (-1)	High (+1)
A	Pretreated cassava stem	0.3	0.7
B	Malt extract	0.1	0.3
C	Ammonium chloride	0.1	0.3
D	Ammonium nitrate	0.1	0.3
E	Ammonium phosphate	0.1	0.3
F	$CaCl_2$	0.1	0.3
G	$MgSO_4.7H_2O$	0.1	0.3
H	NaCl	0.1	0.3
I	$MnSO_4$	0.005	0.015
J	$FeSO_4$	0.005	0.015
K	Na_2MoO_4	0.005	0.015

Table 2 Placket-burman design for screening of factors for cellulase production

	A	B	C	D	E	F	G	H	I	J	K	Cellulase activity (IU/ml)
1	+1	-1	+1	-1	+1	-1	+1	-1	+1	-1	+1	3.64±0.764
2	+1	+1	-1	+1	-1	+1	-1	+1	-1	+1	-1	10.31±0.63
3	-1	+1	+1	-1	+1	-1	+1	-1	+1	-1	+1	9.801±0.74
4	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	-1	5.064±0.458
5	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	7.9±0.37
6	+1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	5.792±0.201
7	-1	+1	-1	+1	-1	+1	+1	-1	+1	-1	+1	9.691±0.27
8	+1	-1	+1	-1	+1	-1	+1	+1	-1	+1	-1	3.49±0.179
9	-1	+1	-1	+1	-1	+1	-1	+1	+1	-1	+1	9.654±0.58
10	+1	-1	+1	-1	+1	-1	+1	-1	+1	+1	-1	4.189±0.87
11	-1	+1	-1	+1	-1	+1	-1	+1	-1	+1	+1	9.181±0.581
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	3.57±0.664

Response surface methodology

After determining the preliminary range of the production variables through one-factor-at-a-time method and PBD, a Box and Behnken experimental design, with three variables, was used to study the response pattern and to determine the optimum combination of variables. The effect of the variables X_1 (pretreated cassava stem), X_2(malt extract), X3(ammonium chloride) and X_4 (sodium chloride) at variation levels is shown in Table3.Four test variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta x} \quad i=1, 2, 3, 4$$

Where x_i is the coded value of an independent variable; X_i is the actual value of an independent variable; X_0 is the actual value of an independent variable at centre point; and X is the step change value of an independent variable. For predicting the optimal point, a second order polynomial model was fitted to correlate relationship between independent variables and response (cellulase yield).For the three factors, the equation is

$$Y_{activity} = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_{j=1}^{k} \beta_{jj} x_i x_j$$

Table 3: Ranges of variables used in RSM

Variables	Code	Levels(g/L)		
		-1	0	+1
Pretreated cassava stem	X_1	3	5	7
Malt extract	X_2	1	2	3
Ammonium chloride	X_3	1	2	3
Sodium chloride	X_4	1	2	3

Validation of the experimental model

Genetic algorithm (GA) (search heuristic), mimics the natural selection process, routinely used to generate solutions to optimization and search. To solve an optimization problem (regression equation), the GA randomly generates individual chromosomes which form the initial population, the chromosomes evolved in successive iterations had a better fitness value (optimal solution).The GA optimizations were implemented in MATLAB v7.12 (Math Works, Inc.).

RESULTS AND DISCUSSION

Screening of the Most Significant Medium Components by Plackett-Burman Design

Ali *et al.,* 2013 demonstrated, PBD showed huge variations in the cellulase activity which was an important consideration for optimization of medium to attain maximum productivity from the Pareto charts, among the 12 nutrients peptone, yeast extract, KH_2PO_4 and $CaCl_2.2H_2O$ were selected for the further optimization which has significant effects on cellulase production with 95% fo confidence level.

For the selection of significant medium for cellulase production, the components such as carbon, nitrogen (organic & inorganic) and mineral (macro &micro nutrients) sources were optimized by Plackett-Burman design using Statistical software MINITAB 17.A total of 11 parameters pretreated cassava stem, malt extract, ammonium chloride, ammonium nitrate, ammonium phosphate, $MgSO_4$ $CaCl_2$, NaCl, $MnSO_4$,$FeSO_4$and Na_2MoO_4were considered for the screening of each factors examined in two levels (-1 and +1). The cellulase activity of the cell free supernatant of *Cellulomonas fimi* MTCC24 culture at 16 h was maximum. The data in Table 2 indicated that there was a wide variation of cellulase activity from 3.49 IU/mL to 10.3 IU/mL in 12 trials.

The variation reflected the significance of factors on the enzyme activity. The variables with confidence levels greater than 95% were considered as significant. PCS and Malt extract were significant at 100% confidence levels for cellulase production, and Ammonium chloride and sodium chloride were found significant at 99.1 and 99.6% levels, respectively, for cellulase activity. By neglecting the insignificant factors, the model equation for cellulase activity can be written as

$Y_{activity} = 0.049+0.0239X_1+0.07742X_2+0.0474X_3-0.0036X_4$

PCS, Malt extract and Ammonium chloride were having significant effect on cellulase activity at high level and Sodium chloride having significant effect on cellulase activity at low level.

Response surface methodology

Ali *et al.,* 2013 optimized the selected nutrients using Central Composite Design and the optimal levels of components were obtained and the tapioca stem consisting the needed amount of carbon source for the effective cellulase production.At the end of the screening of the significant medium components from Plackett-Burman design four significant factors were selected and further optimized and effect of their interactions on cellulase production was determined by using Box-Benkhen design. In BBD, 30 experiments were performed at the various combinations of the factors shown in Table 4.The second-order polynomial model for cellulase production was correlate with an empirical relationship between cellulase production and the test variable in coded units, as given in the following equation:

$$Y_i = 9.598 - 0.2225X_1 + 0.2784X_2 - 4.055X_3 - 4.354X_4 - 0.191X_1X_2 - 0.1548X_1X_3 + 0.243X_1X_4 + 0.14574X_2X_3 - 0.14574X_2X_4 + 1.452X_3X_4 + 0.0302X_1^2 - 0.573X_2^2 + 0.4125X_3^2 + 0.2693X_4^2$$

Where Y_i is the cellulase activity (IU/ml) X_1, X_2, X_3 and X_4 are Pretreated cassava stem, Malt extract, Ammonium chloride and Sodium chloride respectively.ANOVA table for Box-Benhken design is shown in Table 5.The model F-value of 10.0652 for cellulase activity implies the model is significant and the values of p-value less than 0.05 shows that the model terms are significant.

The value of 1.283 for lack of fit implies that it is not significant comparing to the pure error 0.2904.P-values indicates the interaction effects of each variable and was used for checking of the significance of each coefficient.

Table 4: Box-Benken design of factors in coded levels with cellulase activities as responses

Run	X_1	X_2	X_3	X_4	Cellulase activity (IU/ml)	
					Experimental	Predicted
1	7	1	2	2	2.26	1.99
2	5	2	3	3	5.54	5.49
3	7	3	2	2	4.88	4.46
4	3	1	2	2	1.38	1.73
5	5	2	2	2	2.33	2.78
6	5	2	2	2	3.42	2.78
7	5	2	2	2	2.26	2.78
8	5	2	2	2	2.62	2.78
9	5	2	2	2	3.28	2.78
10	5	2	2	2	5.54	5.73
11	3	3	2	2	4.37	4.35
12	5	2	1	1	6.12	5.55
13	5	3	3	2	3.79	3.36
14	3	2	2	1	4.17	3.49
15	3	2	2	3	5.61	5.23
16	5	3	1	2	2.33	2.28
17	5	1	1	2	2.26	2.02
18	5	1	3	2	3.72	3.89
19	3	2	3	2	2.84	3.24
20	3	2	1	2	3.01	2.55
21	5	2	1	3	1.09	1.48
22	5	2	3	1	4.15	3.96
23	7	2	2	3	1.82	1.88
24	7	2	2	1	2.26	2.71
25	5	1	2	3	4.72	5.65
26	5	3	2	3	2.84	3.36
27	7	2	1	2	4.59	4.84
28	5	3	2	1	2.48	2.77
29	7	2	3	2	1.55	1.31

Table 5: Statistical analysis of Box-Benkhen design showing F-value and p-value

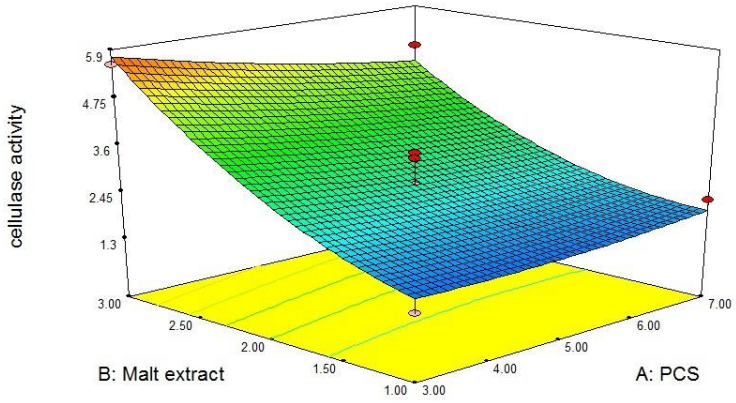
Source	Sum of squares	df	Mean square	F-value	p-value (Prob> F)
Model	49.22233	14	3.51588	10.0652	< 0.0001
A	0.753868	1	0.753868	2.15816	0.1639
B	31.41308	1	31.41308	89.92879	< 0.0001
C	0.003282	1	0.003282	0.009396	0.9242
D	3.664132	1	3.664132	10.48961	0.0059
AB	0.585395	1	0.585395	1.675857	0.2164
AC	0.383626	1	0.383626	1.098238	0.3124
AD	0.947972	1	0.947972	2.713838	0.1217
BC	0.084955	1	0.084955	0.243208	0.6295
BD	0.084956	1	0.084956	0.243211	0.6295
CD	8.436912	1	8.436912	24.15304	0.0002
A^2	0.095081	1	0.095081	0.272197	0.6100
B^2	2.136961	1	2.136961	6.117653	0.0268
C^2	1.10394	1	1.10394	3.160338	0.0972
D^2	0.470503	1	0.470503	1.346947	0.2652
Residual	4.890348	14	0.349311		
Lack of Fit	3.728585	10	0.372859	1.283768	0.4363
Pure Error	1.161763	4	0.290441		
Cor Total	54.11267	28			

Std.Dev.0.54, R^2 0.909, Mean 3.35, Adj R^2 0.8196, C.V.% 17.63, Pred R^2 0.569, PRESS 23.29, Adeq Precision 10.396

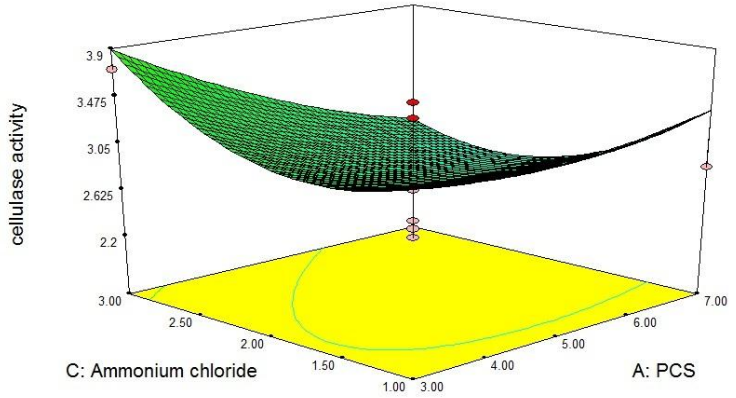
The regression of all the linear term(X_1, X_2, X_3 and X_4) and quadratic coefficients of X_1^2,$X_2^2X_3^2$and X_4^2were significant and interactive effects (X_1X_2, X_1X_3, X_1X_4,$X_2X_4$and X_2X_4) were also significant for cellulase production. Based on the determination of regression coefficient R^2, the model equation tested to fit. About the regression coefficient R^2 to 1, the model would explain the variance of the experimental to the predicted values. The regression coefficient (R^2) for cellulase activity was calculated as 90.9% of the independent factor responces. The interaction effects of variables on cellulase activity investigated using 3D response surface plots, ploted against two variables and kept an another variable at its central level. The 3D response surface plots of cellulase activity from the interactions between the variables shown in 1(a)-1(f).

Figures 1(a),1(d),1(e) shows the dependency of cellulase activity on malt extract, the cellulase activity increases about 5.9 IU/ml with the increasing level of malt extract. Likewise 1(a),1(b),1(c) shows the cellulase activity increases about 3.6 IU/ml with the increasing levels of PCS.1(e),1(f) shows that cellulase activity increases about 2.6 IU/ml by increasing level of Sodium chloride to 3g/L and thereafter cellulase activity decreases with the further increase in Sodium chloride. The dependency of cellulase activity on ammonium chloride showed from the figure 1(b), 1(d), 1(f).The cellulase actvity increases with the increasing amount of ammonium chloride to 2.89 g/L about 2.4 IU/ml and thereafter cellulase activity decreases. The optimum conditions of the production medium for maximum cellulase activity determined by sing response surface

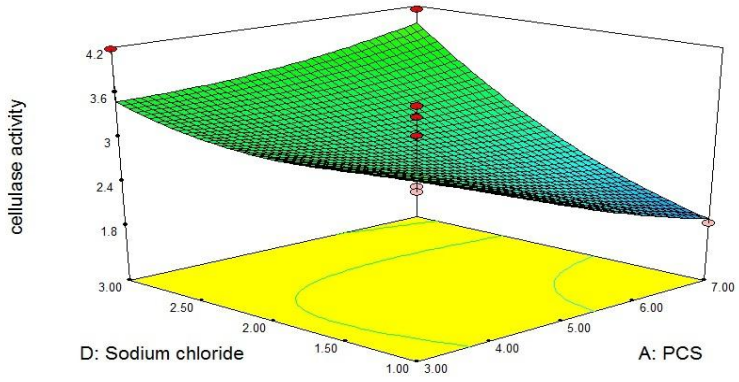
analysis and judge to be probable by statistical optimizer tool Design expert 7.0.0. The optimium conditions are pretreated cassava stem (3.07 g/L), Malt extract (2.99 g/L), Ammonium chloride (2.81 g/L) and sodium chloride (2.49 g/L).The predicted and experimental results were shown in Table 4.



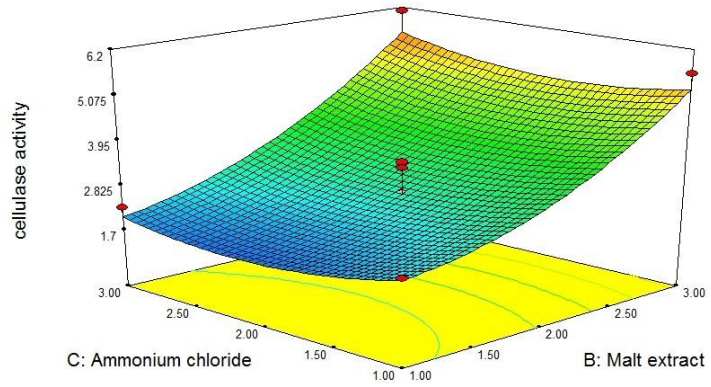
1(a):Three-dimentional response surface plot for cellulase production showing the effects of malt extrat and PCS



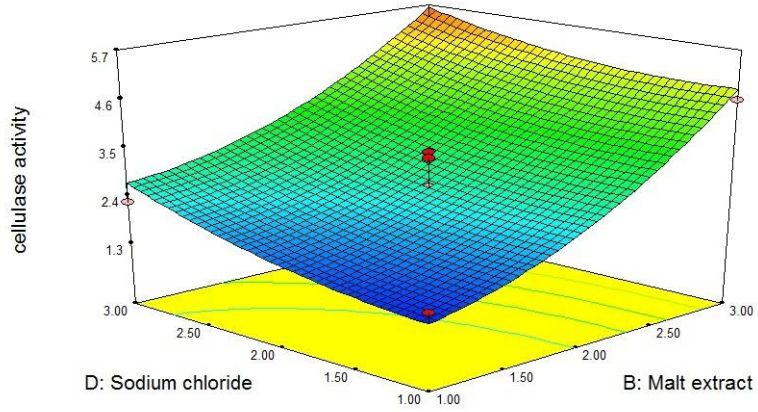
1(b): Three-dimentional response surface plot for cellulase production showing the effects of ammonium chloride and PCS



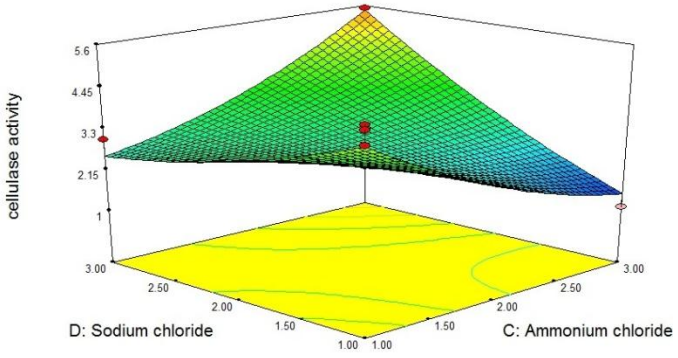
1(c): Three-dimentional response surface plot for cellulase production showing the effects of sodium chloride and PCS



1(d): Three-dimentional response surface plot for cellulase production showing the effects of ammonium chloride and malt extract



1(e): Three-dimentional response surface plot for cellulase production showing the effects of sodium chloride and malt extract



1(f): Three-dimentional response surface plot for cellulase production showing the effects of Sodium chloride and ammonium chloride

Fig. 1: Three-dimentional response surface plot for cellulase production

Validation of the experimental model

GA-predicted response (6.52 IU/ml) and the experimental response (5.96 IU/ml) showed a robust assent of validation experiments, a notable difference between RSM-predicted response (5.61 IU/ml) and observed response

(5.23 IU/ml)) was observed. The results showed the high adequacy of the GA based RSM strategy over RSM model, leading to a significant increase in cellulase production. Figure 2 shows the best fitness graph of GA for cellulase production generated by MATLAB v7.12 (Math Works, Inc.)

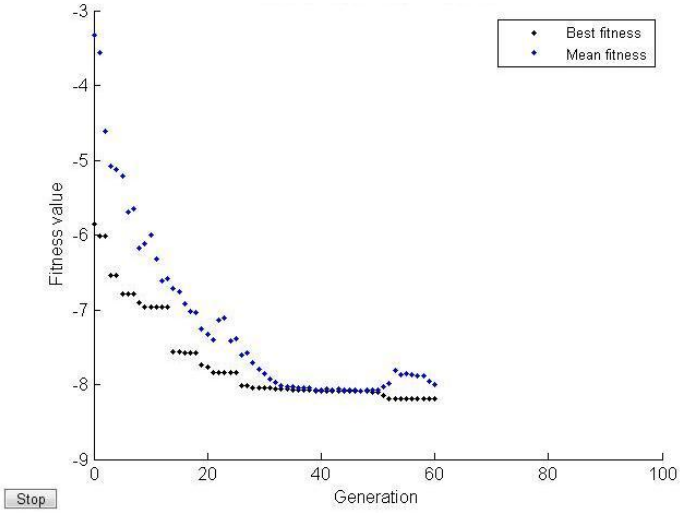


Fig. 2: Best fitness graph of GA for cellulase production generated by MATLAB v7.12 (Math Works, Inc.).

CONCLUSION

In order to optimise the process parameters of cellulase production from *Cellulomonas fimi* MTCC 24, in shake flask culture was investigated by the combination of Plackett – Burman design (PBD) and Box – Behnken design (BBD),an effective and reliable tool to select the significant factors and finding the optimal concentration of those factors in culture medium for cellulase production from Cassava stem var.YTP1. PCS, malt extract, ammonium chloride and sodium chloride were selected as the significant factors for cellulase production using Placket-Burman design. Box-Behnken design was applied to investigate four factors viz.PCS, malt extract, ammonium chloride and sodium chloride .The media formulations were optimized having the factors such as pretreated cassava stem 3.07g/L, malt extract 2.99 g/L, Ammonium chloride 2.81g/L and NaCl 2.49g/L.Data were analyzed step wise and a second order polynomial model was established to identify the relationship between the cellulase activity and the selected factors of maximum cellulase activity of 5.96 IU/ml.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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