Medium optimization for the production of lipstatin by *Streptomyces toxytricini* using full factorial design of experiment

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Abstract: Full factorial design of experiment for medium optimization was employed for lipstatin production by *Streptomyces toxytricini* in shake flask study. The full factorial DOE was very much effective in screening of nutritional parameters within the stipulated time frame in a limited number of experiments. A maximum lipstatin production was achieved 3.290 g/l with the following optimized factors: soya flour 35g/l and soya oil 25g/l. Validation experiments were also carried out to verify the adequacy and the accuracy of the model. The results also give a scope for large scale fermentation of lipstatin production.

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1. Introduction

Lipstatin, a very potent inhibitor of pancreatic lipase is produced by *Streptomyces toxytricini*. Lipstatin contains a beta-lactone structure that probably accounts for the irreversible lipase inhibition. Lipstatin is of considerable importance as key intermediate for the preparation of tetrahydrolipstatin (THL, Orlistat), which is useful in the prophylaxis and treatment of diseases associated with obesity. The lipophilic β-lactone irreversibly inactivates lipase by covalent modification of the serine residue of its catalytic triad. The applications of lipstatin have promoted the commercial production of these compounds. Hence, the investigations on the improvement of the productions of these products are of commercial importance (Weibel *et al.,* 1987).

As medium composition can significantly affect the formation, concentration and yield of a fermentation end product, designing an appropriate fermentation medium is of crucial importance**.** Thus affecting the overall process economics therefore it is important to consider the optimization of fermentation medium in order to maximize the profits from fermentation process. There are many challenges associated with optimization of fermentation process; it is laborious, expensive, open ended and time consuming process involving many experiments (Stanbury *et al*., 1997; Panda *et al.,* 2007). However, there are conventional methods for optimizing medium composition *via* sequential manipulation of single parameter; but these often fail to identify the optimal conditions for the bioprocess, because interactions between different factors are neglected. So, effective problem solving methods are preferred (Xiong et al., 2008; Hong *et al.,* 2009). A factorial design has eliminated the drawbacks of classical methods and has proved to be powerful and useful for the optimization of the target metabolite production. It can also be used to evaluate the relative significance of several variables simultaneously (Li *et al., 2008;* Hong *et al.,* 2009).

The aim of this work was to apply the full factorial design, followed by the path of steepest ascent to optimize the culture medium composition for lipstatin production by *S. toxytricini*. The major variables affecting the performance of the culture in terms of lipstatin production were also investigated.

2. Material and Methods

Microorganism

The mutated strain (combined mutation of U.V and nitrosoguanidine) of *S. toxytricini* used in the study was stored in 20% glycerol at – 80 ^{O}C. The culture was maintained at 4 ^{O}C on yeast extract-malt extract-glucose (YMG) agar slants (Luthra *et al.,* 2013).

Medium

Lab inoculum medium adjusted to pH 6.8 was composed of (g/l): yeast extract 4; malt extract 10; dextrose 4. Seed medium adjusted to pH 6.8 was composed of (g/l): soyaflour 10; glycerol 10; yeast extract 5. The components of non-optimized fermentation medium were: glycerol, soyaflour, soyalecithin, soyaoil, polypropylene glycol. The pH of the medium was adjusted to 6.8 and the concentrations of components were adjusted according to the experimental design.

Lab and seed media were sterilized by autoclaving for 30 min and fermentation media for 45 min respectively at 121^{O}C. After sterilization, the pH of lab, seed and fermentation medium was 6.6.

Culture conditions

Fermentation was performed in three stages: lab growth, seed growth and lipstatin production. For the lab growth stage, mycelium from a slant culture was inoculated into 250 ml conical flask containing 35 ml of lab medium and grown at 28 ^{O}C with 220 rpm on a shaker incubator for 24 h. Then, 3% (v/v) lab culture was inoculated into seed medium. The strain was incubated at 28 ^{O}C with 220 rpm for 36 h. Then, 10% (v/v) seed cultures were inoculated into the fermentation medium. The strain was incubated at 28 ^{O}C with 220 rpm for 12 days (Luthra *et al.,* 2013).

Analytical methods

Lipstatin activity in the culture broth was determined by HPLC. The culture broth of 5.0 gm was taken in 50 ml volumetric flask with 30 ml acetone and sonicated for 10 minutes and the volume was made up with acetonitrile. The resulting extracted solution was injected into the HPLC (Waters 2496) having C-18 column (Hypersil ODS, 5u C18 (150mm X 4.6 mm) for the estimation of lipstatin. Concentrations of lipstatin were calculated by comparison of peak areas with those standard lipstatin and subsequently lipstatin activity was calculated. Biomass was measured in terms of percentage mycelial volume (%) (Ferreira *et al.,* 2005).

Experimental design and data analysis

Various treatments in a series of experiments were developed or generated according to several standard texts and software on designing experiments (Box *et al.,* 1987; Montgomery, 1997; Long-Shan *et al.,* 2002). In preliminary experiments, various carbon and nitrogen sources were evaluated for their suitability to sustain good lipstatin production by *S. toxytricini*. On the basis of literature study, glycerol and sorbitol were selected as carbon source; soyaflour and corn steep liquor along with cotton seed meal as a nitrogen source. Oil source chosen for the study were soya oil and groundnut oil and brij 35 and soyalecithin as an emulsifier.

The categorical factorial design of experiment was employed to select the best carbon, nitrogen, emulsifier and oil source.

Table 1: The categorical full factorial design for screening variables in lipstatin production

Factors	Code	High level (+1)	Low level (-1)	Effect
Carbon	**X1**	Glycerol	Sorbitol	0.45
Oil	**X2**	Groundnut Oil	SoyaOil	-0.23
Nitrogen	**X3**	Soyaflour	CSL + CSM	0.76
Emulsifier	**X4**	Brij 35	Soyalecithin	0.43

Abbreviation: CSL: Corn steep liquor; CSM: Cotton seed meal

The results revealed that glycerol, soyaflour, soyaoil, soyalecithin were best carbon, nitrogen, oil and emulsifier source respectively for the production of lipstatin. These components were chosen for further optimization.

Table 2: The full factorial design for screening variables in lipstatin production

Factors (g/l)	Code	Low level(-1)	High level (+1)	Effect	Coef
Glycerol	A	22.5	35	-0.01	-0.01
Soyaoil	B	10	20	0.06	0.032
Soyaflour	C	15	35	0.08	0.038
Soyalecithin	D	15	25	-0.06	-0.028

***Abbreviation*: Coef: coefficient**

Table 3: The full factorial design variables (in coded levels) with lipstatin as response.

Run	Variable levels				Lipstatin (mg/ml)
	A	B	C	D	
1	-1	-1	-1	-1	2.371
2	1	-1	-1	-1	2.406
3	-1	1	-1	-1	2.614
4	1	1	-1	-1	2.571
5	-1	-1	1	-1	2.527
6	1	-1	1	-1	2.573
7	-1	1	1	-1	2.561
8	1	1	1	-1	2.537
9	-1	-1	-1	1	2.526
10	1	-1	-1	1	2.298
11	-1	1	-1	1	2.422
12	1	1	-1	1	2.423
13	-1	-1	1	1	2.449
14	1	-1	1	1	2.528
15	-1	1	1	1	2.512
16	1	1	1	1	2.551

Full factorial design

Full factorial design was employed for screening the most significant parameters affecting lipstatin production by *S. toxytricini*. Each independent variable was tested at two levels, high and low, which are denoted by (+) and (-), respectively. The experimental design with the name, symbol code, and actual level of the variables is shown in Table 1, whereas Table 2 shows the detail of the design. Lipstatin production was carried out in duplication and the average value was taken as the response.

Path of the steepest ascent experiment

To get the optimum response, we used the technique of steepest ascent. The experiments were adopted to determine a suitable direction by increasing or decreasing the concentrations of variables according to the results of factorial experiment (Gheshlaghi *et al.,* 2005; Hong *et al.,* 2009).

Statistical analysis

Design expert 8.0 software was used for the experimental designs and subsequent regressional analysis of the experimental data. Stastistical analysis

of the model was performed to evaluate the analysis of the variance (ANOVA). Optimization procedure is facilitated by construction of an equation that describes the experimental results as a function of the factor level. A polynomial equation can be constructed in the case of a factorial design where the co-efficient in the equation are related to the effects and interactions of the factors. Statistical analysis of the model was performed to evaluate the analysis of the variance (ANOVA).

Result and discussion

Optimization by full factorial design

The importance of the four components, namely, glycerol, soyaoil, soyaflour, soyalecithin for lipstatin production was investigated by full factorial design. Table 1 shows the effect of these components on the response and significant levels.

Based on the statistical analysis, the effects of soyaflour were (+) 0.08 had confidence level above 90%. So, it was identified as influencing lipstatin production component significantly. Next to soyaflour, soyaoil has an effect of (+) 0.06. Others had less effect, so they were considered as insignificant.

In the Pareto chart (Fig. 1), the maximal effect was presented in the upper portion and then progress down to the minimal effect. In addition, it directly shows that the most important factor determining lipstatin production was soya flour.

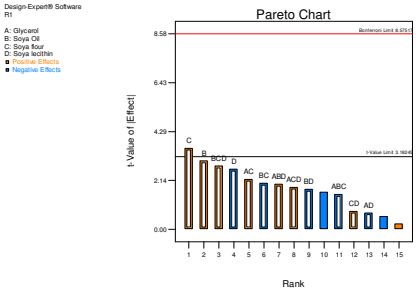

Fig. 1: Pareto chart of four factors and their interaction standard effects on lipstatin production. The most important term was soya flour

A normal plot is a plot of the absolute value of the effect estimates against their normal % probabilities. It is useful to distinguish between positive (upper right) and negative (lower left) effects of the variables and their interactions. The normal plot (Fig.2) shows that the variables D (Soya lecithin) and A (Glycerol) has negative effect while B (Soya oil) and C (Soya flour) has the positive effect on lipstatin production. Also, the interactive effect of DCB, AC, ABD, ACD, CD and ABCD variables has the positive effect whereas the variables CD, ACD., ABD, AC, and BCD has negative effect on lipstatin.

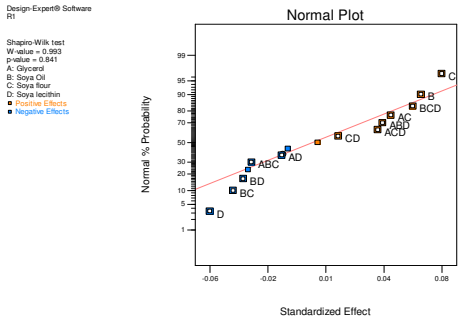

Fig. 2: Normal Plot for distinguishing between positive and negative effects of variables.

Analysis through ANOVA

Data shown in Table 3 were analyzed using Design experiment 8.0 software. The P-value was used to identify the effect of each factor on lipstatin production. P-value less than 0.05 indicate that the model terms are significant. The Model F-value of 2.81 implies there is a 8.57% chance that a "Model F-Value" could occur due to noise. In this case C *i.e.* Soya flour is significant model terms. Values greater than 0.1000 indicate the model terms are not significant. A negative "Pred R-Squared" implies that the overall mean is a better predictor of the response than the current model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 4.509 indicates an adequate signal. This model can be used to navigate the design space.

Table 4: Analysis of variance table

Factors	SS	DF	MS	F	P
A – Glycerol	0.001	1	0.001		
B – Soyaoil	0.016	1	0.016	4.02	0.08
C – Soyaflour	0.023	1	0.023	5.62	0.0451
D – Soyalecithin	0.013	1	0.013	3.1	0.1161
BC	0.007	1	0.007	0.21	0.1161
BD	0.006	1	0.006	0.27	
CD	0.001	1	0.001	0.61	
BCD	0.014	1	0.014	3.44	0.1006
Model	0.08	7	0.011	2.81	0.0857

Abbreviation: DF: Degree of freedom; SS: sum of squares, MS: mean square

R-Squared 0.7107
Adj R-Squared 0.4575
Pred R-Squared -0.1573
Adeq Precision 4.509

Optimization by the path of steepest ascent experiment

Based on the factorial design, the Soya flour and Soya oil effects were positive. Thus, increasing their concentration should result in a higher production of lipstatin. The center point of the

factorial design has been considered as the origin of the path. The titer of lipstatin production was obtained. These experiments (Table 4) showed the maximum production of lipstatin. This was obtained when the parameters were 35 g/l Soya flour and 25 g/l Soya oil. It suggested that this point is the nearest region of the maximum lipstatin response.

Table 5: Design and results of Steepest ascent experiment.

Run	Factors		Response(g/l)
	X1 (g/l)	X2 (g/l)	
1	25	15	2.513
2	30	20	2.652
3	35	25	3.290
4	40	30	2.720
5	45	35	2.510

X1: Soyaflour; X2: Soyaoil

Validation of final concentration

To verify the final concentration, three sets of experiments were carried out with the final composition of soyaflour (35 g/l) and soyaoil (25 g/l). The mean of lipstatin production was 3.275 g/l.

Conclusion

Statistical optimization of fermentation medium components for the production of lipstatin by mutated *Streptomyces toxytricini* had been proved to be a valuable tool for lipstatin production. A maximum lipstatin production of 3.290 g/l was achieved with the following optimized factors: 35g/l soyaflour and 25g/l soyaoil. Validation experiments were also carried out to verify the adequacy and the accuracy of the model. The results also give a basis for further study with large scale fermentation for production of lipstatin. From all the studies carried out, the final medium composition to get the higher titer value of lipstatin-

S.No.	Component	Concentration (g/l)
1	Glycerol	22.5
2	Soyaoil	25
3	Soyaflour	35
4	Soyalecithin	15
5	Polypropylene glycol	0.5

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