International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 4, Issue 7, 2012, pp.-266-269.
Available online at http://www.bioinfo.in/contents.php?id=27

MEDIUM OPTIMIZATION OF LIPSTATIN FROM *Streptomyces toxytricini ATCC* 19813 BY SHAKE FLASK STUDY

UMESH LUTHRA* AND DUBEY R.C.

Department of Botany & Microbiology, Gurukul Kangri University, Haridwar-249 404, Uttarakhand, India.
*Corresponding Author: Email- umeshluthra@gmail.com

Received: March 30, 2012; Accepted: August 01, 2012

Abstract- Effect of medium composition on lipstatin production by *Streptomyces toxytricini ATCC* 19813 was investigated in shake flasks. The nutritional components of the medium was optimized by using Plackett-Burman, three factorial and one variable-at-a-time approach. Among the five factors studied, soya oil, soya lecithin and soya bean flour had significant effects on lipstatin production. The optimum levels of these key variables were further determined using a three factorial design and one variable-at-a-time. The highest lipstatin production was obtained in the medium consisting of glycerol 22.5 g/l, Soya flour 35 g/l, soya oil 15 g/l, soya lecithin 25 g/l and PPG 0.5 g/l at pH 7.20, 28°C and 220 rpm. The whole optimization strategy enhanced the lipstatin production from 0.097 g/l to 0.885 g/l. Here we report 8 fold enhancements in lipstatin production following Plackett-Burman, three factorial and one variable-at-a-time approach from *Streptomyces toxytricini ATCC* 19813.

Keywords- Medium optimization, Soya oil, Lipstatin, *Streptomyces toxytricini ATCC* 19813, Anti-obesity.

Citation: Umesh Luthra and Dubey R.C. (2012) Medium Optimization of Lipstatin from *Streptomyces toxytricini ATCC* 19813 by Shake Flask Study. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 4, Issue 7, pp.-266-269.

Introduction

Obesity is a leading preventable cause of death worldwide with increasing prevalence in adults and children, and as one of the most serious public health problems of the 21st century [1]. Obesity is stigmatized in much of the modern world (particularly in the Western world), though it was widely perceived as a symbol of wealth and fertility at other times in history (still is in some parts of the world) [4,8]. Lipstatin is one of the most important anti obesity drug. Lipstatin is a potent and irreversible inhibitor of pancreatic lipase activity. It is a natural product and was first isolated from the Actinomycetes *Streptomyces toxytricini*. The lipophilic β lactone irreversibly inactivates lipase by covalent modification of the serine residues of its catalytic triad [5]. The structure is characterized by, a β lactam ring carrying two aliphatic residues with chain lengths of 6 and 13 carbon atoms [5]. Orlistat is the saturated derivative of lipstatin, which is a potent natural inhibitor of pancreatic lipases isolated from *Streptomyces toxytricini* [7]. Orlistat (Xenical) is the most commonly used medication to treat obesity. Lipstatin fermentation to improve the level of production mainly depends on strain selection and fermentation processes (culture medium and culture conditions) *i.e.* the optimization of these two complementary aspects. The cost of lipstatin is one of the factors determining the economics of a drug production process and can be reduced by finding optimum conditions for their production, by the isolation of hyper producing mutants and medium optimization. Looking into the depth of technology, there is always a chance of improving better titer which is suitable for commercial exploitation. Thus, realizing the immense utility of microbial lipstatin, the present investigation was carried out. Till date *Streptomyces* stand out as the source of most commercially available lipstatin production. Here, we report medium optimization for maximum lipstatin production from *Streptomyces toxytricini ATCC* 19813 by Placket-Burman, three vaiable and one variable - at - a - time approach.

Materials and Methods

Streptomyces Strain, medium and Culture Characterization

The actinomycetes *Streptomyces toxytricini* (*ATCC* 19813) was used in the studies. The cells were maintained on yeast malt extract (YM), *Streptomyces toxytricini* (ST) isolation agar and actinomycetes isolation medium. The pH was adjusted to around 7.2

before autoclaving the medium at 121°C for 15 min. The cells were grown in 250 ml flask containing 30 ml of medium and incubated in orbital shaking incubator at 200 rpm and 28°C for 48±24 h. For inoculum preparation, the culture was grown in YMB medium (Yeast extract 4.0g/l, Malt extract 10.0g/l, D-glucose 4.0g/l) at 28°C and 200 rpm for 24 h. 2 % of lab inoculum at the age of 32 h was transferred into the seed media (Soya flour 10.0g/l, Glycerol 10.0g/l, Yeast extract 5.0g/l) and flasks were incubated at 28°C at 180 rpm up to 48 ± 24 h or till growth appears. The production medium was inoculated with 5% (v/v) inoculum. For each experimental condition, three replicates were used, and the standard deviation was calculated. Thereafter, the culture broth was used for lipstatin HPLC quantification.

Lipstatin Assay

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 sonicates it for 10 minutes and make up the volume 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 (%) [2].

Medium Optimization for Maximum Lipstatin Production

M1 media were selected from the available literature and tested for the production of lipstatin. M1 media was optimized by Placket-Burman, three factorial and by one-variable-at-a-time strategy for maximum lipstatin production.

Plackett-Burman Design

A Plackett-Burman design was used to screen the factors having significant effects on the lipstatin production. The variables to be evaluated are listed in Table 1.

Table 1- Variables to be Screened in Plackett-Burman Design

Components	Unit	High level (+)	Low level (-)
Soyalecithin	(g/l)	10	5
Polypropylene Glycol (PPG)	(g/l)	2	0.5
Glycerol	(g/l)	25	10
Soya Oil	(g/l)	20	10
Soya flour	(g/l)	30	15

Each independent variable was investigated at a high (+) and a low (-) level. The low levels of medium component were taken as their concentration in *ATCC* 19813. The design matrix and data analysis were similar as previously reported by Wen and Chen (2001). In summary, there were 8 runs of experiment, with 5 variables and 2 dummy variables (D1-D2) (Table 2). The effect of each variable on response was determined by subtracting the average response of the low level from the high level. The effects of dummy variables reflect the standard error of the experiments, which can be used to derive the significant level [10].

Three Factorial Design

On the basis of the data obtained from the Placket-Burman, the three variable factorial design was used to screen the effect of each media component as well as the effect of their interactions on the lipstatin production. The three variable used in the experiment were soya oil, soya lecithin and soya flour (listed in Table 3). The range (*i.e.* high and low level) was taken on the basis of above PB experiment [10].

Table 2- The Plackett-Burman Design of the Variables (Table 1) with Lipstatin yield as Response

Run	No of variables							Activity (g/l)
	Soya Lecithin (g/l)	PPG (g/l)	Glycerol (g/l)	Soya Oil (g/l)	Soya flour (g/l)	D1	D2	
1	+	+	+	-	+	-	-	0.775
2	-	+	+	+	-	+	-	0.623
3	-	-	+	+	+	-	+	0.801
4	+	-	-	+	+	+	-	0.846
5	-	+	-	-	+	+	+	0.523
6	+	-	+	-	-	+	+	0.565
7	+	+	-	+	-	-	+	0.796
8	-	-	-	-	-	-	-	0.365
	Σ(+) 2.982	Σ(+) 2.717	Σ(+) 2.764	Σ(+) 3.066	Σ(+) 2.945	Σ(+) 2.557	Σ(+) 2.685	
	Σ(-) 2.312	Σ(-) 2.577	Σ(-) 2.53	Σ(-) 2.228	Σ(-) 2.349	Σ(-) 2.737	Σ(-) 2.609	
FORMULA =	[I(Σ+) - (Σ-)I] / (NO OF VARIABLES / 2)							
(Σ+) - (Σ-)	0.67	0.14	0.234	0.838	0.596	-0.18	0.076	
(Σ+) - (Σ-)/4	0.168	0.035	0.059	0.21	0.149	-0.045	0.019	

Table 3- Variables to be Screened in Three Variable Factorial Design

Components	Unit	High level (+)	Low level (-)
Soya Oil	(g/l)	40	20
Soyalecithin	(g/l)	30	10
Soyaflour	(g/l)	50	30

Based on the above results, further medium optimization studies were carried out to study the interaction of three variables namely, soya flour, soya oil and soya lecithin. Each factor was studied at two levels (-1, +1).

Table 4- Three Factorial Design of the Variables (Table 3) with Lipstatin Yield as Response

Run	No of Variables							
	Soya Oil (A)(g/l)	Soya Lecithin (B)(g/l)	Soya Flour (C)(g/l)	AB	BC	AC	Activity I (g/l)	Activity II (g/l)
1	-1	-1	-1	1	1	1	0.563	
2	1	-1	-1	-1	1	-1	0.812	0.796
3	-1	1	-1	-1	-1	1	0.745	
4	1	1	-1	1	-1	-1	0.679	
5	-1	-1	1	1	-1	-1	0.669	
6	1	-1	1	-1	-1	1	0.712	0.723
7	-1	1	1	-1	1	-1	0.563	
8	1	1	1	1	1	1	0.823	
∑(+) = X	3.026	2.81	2.799	2.734	2.761	2.843		
∑(-) = Y	2.54	2.756	2.767	2.832	2.805	2.723		
X-Y	0.486	0.054	0.032	-0.098	-0.044	0.12		
X-Y/ (no.run/2)	0.1215	0.0135	0.008	-0.0245	-0.011	0.03		

One Variable at a Time (OVAT)

OVAT was carried out to screen the concentration of the single media component on the lipstatin production. OVAT experiment was carried out for Soya oil, Soya lecithin and soya flour.

Table 5- Optimization of Soya Oil Concentration for Lipstatin Production in Shake Flask

Sr. No.	Soya Oil (g/l)	Yield (g/l)
1	20	0.784
2	25	0.81
3	30	0.785
4	35	0.713
5	40	0.683

Table 6- Optimization of Soyalecithin Concentration for Lipstatin Production in Shake Flask

Sr. No.	Soya lecithin (g/l)	Yield (g/l)
1	10	0.734
2	15	0.825
3	20	0.701
4	25	0.653
5	30	0.666

Table 7- Optimization of Soyaflour Concentration for Lipstatin Production in Shake Flask

Sr. No.	Soyaflour (g/l)	Yield (g/l)
1	30	0.651
2	35	0.838
3	40	0.725
4	45	0.721
5	50	0.669

All tests were performed in triplicate and the data represents a mean of three. It was validated within and beyond the design space by selecting ten random experiments according to the conditions predicted by the model.

Results and Discussion

S. toxytricini colonies were elevated and covered with white aerial mycelia and spores. Diffused melanoid pigments were sometimes observed. On YM plates colonies were irregular, flat, covered with white aerial mycelia, on ST plates colonies were elevated, and covered with white aerial mycelia and spores and on AI plates colonies were elevated and covered with grayish aerial mycelia as described by Kmpfer (2006).

The organism was then quantitatively tested on the basis of HPLC assays. For this the organism was grown in minimal medium and then incubated at 28°C, 200 rpm and pH 7.0 for specified incubation period. The results of HPLC assays shows that the organism produced 0.094 mg/g lipstatin titers on minimal medium. Therefore, this strain was selected for further studies on lipstatin production.

Medium formulation is necessary for each fermentation process. It is necessary to optimize each and every component of fermentation media by varying the concentration of constituents in the medium in order to achieve the maximum production. The purpose of medium optimization is to support the efficient growth of microorganisms.

The medium comprised of a suitable carbon source and a suitable nitrogen source providing carbon- and nitrogen containing compounds to the microorganism in such a way that the microorganism may use/convert these compounds for growth/development/ reproduction and production of secondary metabolites, representing compounds of metabolism that are not essential for normal growth, development or reproduction of said microorganism (EP 1 860 194 A1).

Media components were optimized by Placket-Burman, three factorial and by one-variable-at-a-time strategy for maximum lipstatin production.

Plackett-Burman Design

The five independent factors were screened by using Plackett-Burman (Table 1). The results obtained by Plackett-Burman (Table 2) were analyzed by calculating the experimental error and their significant level.

Experimental Error $= \sqrt{[\{(-0.045)^2 + (0.019)^2\}/2]}$

$= \sqrt{[\{0.0020 + 0.00036\}/2]}$

$= 0.034$

Significant level = (exp error * 2) = (0.034* 2) = 0.0687

Sr. No.	Components	Effect
1	Soya lecithin	0.168
2	PPG	0.035
3	Glycerol	0.059
4	Soya oil	0.21
5	Soya flour	0.149

The values of PPG and glycerol are below the significant level (*i.e* 0.0687) while the values of Soya oil, Soya lecithin and Soyaflour are above the significant level. The above result indicates that Soya oil, Soya lecithin and Soyaflour has maximum effect on the lipstatin production from *S. toxytricini* while PPG and Glycerol has minimum effect on it.

Three Factorial Design

The three factors namely soya flour, soyalecithin and soya oil were screened by Three factorial design (Table 3). The results obtained by three factorial (Table 4) were analyzed by calculating the experimental error and their significant level.

Experimental Error $= \sqrt{[\{(D1)^2 + (D2)^2\}/2]}$

$= \sqrt{[\{(\text{Run } 2)^2 + (\text{Run } 4)^2\} / 2]}$

$= \sqrt{[\{0.000256 + 0.000121\}/2]} = 0.014$

Significant level = (0.014 * 2) = 0.028

Sr. No.	Components	Effect
1	Soya oil (A)	0.1215
2	Soya lecithin (B)	0.0135
3	Soya flour (C)	0.008
4	AB	0.0245
5	BC	0.011
6	AC	0.03

The values of Soya oil is above the significant level (i.e 0.028) while that of Soya lecithin, soya flour is below the significant level. Also the interactive effect of Soya oil and soya flour (AC) is significant than the other (AB and BC). The above result indicates that Soya oil has maximum effect on the lipstatin production from *S. toxytricini.*

One Variable At a Time (OVAT)

The three independent factors namely soya flour, soyalecithin and soya oil were screened by Three factorial design. The results obtained by Three factorial (Table 5,6,7) were analyzed. The above results shows that Soya oil, Soya lecithin, Soya flour at concentration 25, 15, 35 g/l respectively shows maximum activity of lipstatin by *S. toxytricini*.

The data obtained from all the above experiments indicates that Soya oil is the component which has maximum effect on Lipstatin production. By clubbing the above data, the final / optimized production media for lipstatin production from *S. toxytricini* is as follow:

Sr. No.	Components	Concentration (g/l)
1	Glycerol	22.5
2	Soya flour	35
3	Soya oil	15
4	Soya lecithin	25
5	PPG	0.5

By using the above composition, the maximum yield of lipstatin obtained was 0.885 g/l. Thus it may be concluded that Lipstatin production from *Streptomyyces toxytricini* has been reported in high titer. Maximum lipstatin production in *Streptomyyces toxytricini* was a function of close interaction between inducer, soya flour and soya lecithin. The statistical approach proved to be instrumental in predicting the optimal nutritional culture conditions and understanding the interactions among medium variables for maximum lipstatin production by *Streptomyyces toxytricini*.

Acknowledgements

The author thanks to IPCA Laboratories Ltd for the financial support and provided lab to do complete research work.

Reference

[1] Barnes A., Goodrick G.K. and Pavlik V. (2007) *J. Gen. Intern. Med*.

[2] Ferreira A.P., Lu´ıs M. Vieira, Joaquim P. Cardoso, Jos´e C. Menezes (2005) *J. Biotechnol*., 116, 403-409.

[3] Gasparic A., Svagelj N.B., Raspor P. Fujs S., Sladic G., Pelko M. and Petkovic H. (2007) *Eur. Pat*.

[4] Haslam D.W. and James W.P. (2005) *Lancet.,* 366 (9492), 1197-209.

[5] Hochuli E. Kupper E., Maurer R., Meister W., Mercadal Y. and Schmidt K. (1987) *J. Antibiotics,* 8, 1086-1091.

[6] Kmpfer P. (2006) *Prokaryotes,* 3, 538-604.

[7] Weibel E.K., Hadvary P., Hochuli E., Kupfer E. and Lengsfeld H. (1987) *J. Antibiotics,* 8, 1081-1085.

[8] Wen Z.Y. and Chen F. (2001) *Biotech. Bioeng.,* 75, 159-169.

[9] Woodhouse R. (2008) *Front Horm. Res*., 36, 271-286.

[10] Zhanyou C., Denver P., Zhiyou W., Craig F. and Shulin C. (2007) *Proc. Biochem*., 42, 1537-1545.