Application of multi-factorial experimental designs for optimization of biotin Production by a *Rhizopus nigricans* strain

Heba A. El-Refai[1], Ehab R.El-Helow[2], Magdy A. Amin[3], Lotfy A. Sallam[1], Hebat-Allah A. Salem[1]

[1] Chemistry of Natural and Microbial Products Department, National Research Center, Dokki, Cairo, Egyp [2] Department of Botany and Microbiology, Faculty of Science, University of Alexandria, Alexandria, Egypt; [3] Department of Microbiology, Faculty of Pharmacy, University of Cairo, Egypt. dr_mona_zaki@yahoo.co.uk

Abstract: The main objective of the present work is to demonstrate the efficiency of multi-factorial experimental designs to elucidate factors affecting the microbial production of biotin and to predict their optimum settings. A local *Rhizopus nigricans* strain was selected as a remarkable wild type biotin (vitamin H) producer. A preliminary medium formulation experiment suggested sucrose and peptone as appropriate donors of carbon, nitrogen and sulphur. An incomplete two level factorial experiment showed that concentrations of sucrose and peptone, as well as fungal growth stage are the most effective independent variables. A three level response surface methodology was then applied to accomplish a polynomial model which correlates the three key variables to biotin accumulation. When compared to the basal culture, the optimum condition predicted according to this model achieved about 10.4, 13.9, 5.7, 7.6 and 4.2-fold increases in production, product yield coefficient, specific product yield coefficient, productivity and specific productivity, respectively. [Journal of American Science 2010;6(6):179-187]. (ISSN: 1545-1003).

Keywords: Biotin, vitamin H, *Rhizopus nigricans*, experimental designs, response surface methodology

1. Introduction

Biotin ($C_{10}H_{16}N_2O_3S$), also known as vitamin H or B7, is a coenzyme essential for many cellular carboxylation and decarboxylation reactions, fatty acid biosynthesis, gluconeogenesis, and amino acid metabolism (Streit and Entcheva, 2003). It also affects gene expression through a diverse array of cell signaling pathways (Rodriguez-Melendez and Zempleni, 2009). Deficiency of biotin may cause, for example, seborrhea, dermatitis, loss of appetite and lassitude (Birch *et al*., 2000). Since its bioavailability is rather low, it is commonly added to many food, feed and cosmetic products.

While humans and animals require several hundred micrograms per day, most microorganisms and plants have the ability to synthesize biotin (Streit and Entcheva, 2003). Some wild isolates of *Saccharomyces cerevisiae* are biotin prototrophs and others are not (Hall and Dietrich, 2007). Research has led to understanding the complete biotin biosynthesis pathways in many different microorganisms. Among the most important microorganisms reported as biotin producers are *Rhizopus delmar* (Shchelkovo and Vorob'ev, 1982; Maksimov *et al.*, 1983), *Rhizobium sp.* (Sierra *et al.*, 1999) and *Kurthia sp* (Hoshino *et al*., 1999). Biotin over-production by fermentations of recombinant microorganisms such as *Sphingomonas sp.* (Saito *et al.*, 2000) and *Candida utilis* (Hong *et al*., 2006) has been reported.

Since its bioavailability is rather low, biotin is commonly added to many food, feed and cosmetic products. However, the majority of vitamin H in the market is synthesized chemically. To overcome the disadvantages faced by the chemical biotin industry, including high energy input and the production of considerable amounts of chemical waste, (Streit and Entcheva 2003), efficient fermentation processes to produce biotin are in great demand. Thus, the main goal of the present study is to contribute a model that can be applied for optimization of biotin production fermentations. Traditional optimization of fermentation factors (one at a time) is generally a time consuming and labor-intensive process. On the contrary, statistically designed multi-factorial experiments proved to be valuable tools for optimizing microbial culture conditions (Hooijkaas *et al.*, 1998; Lotfy *et al.*, 2007). One of the advantages of applying experimental designs is that, they consider the interaction between the non-linear natures of the response in short experiments (Gresham and Inamine, 1986).

2. Material and Methods

Microorganism:

The fungus used in this study is *Rhizopus nigricans* NRC strain FR105 which is a local isolate obtained from the cultures collection of the National Research Centre, Dokki, Cairo, Egypt. This strain had been

selected as a promising biotin producing fungus (Salem, 2009).

Growth media:

Maintenance medium: Potato-dextrose agar (PDA) medium was supplied as a dry powder preparation which has the following composition expressed as a percentage (g/100 ml): potato infusion 0.4 (infusion from 200 g potatoes); glucose, 2; agar 1.5. The pH was adjusted to 5.6. Prepared PDA slants were inoculated by the pure fungal cultures and incubated at 28°C. Slant cultures were transferred every month.

Preliminary biotin production medium: The medium chosen for investigating biotin production by the experimental *Rhizopus* strain contained (%): glucose, 5; peptone, 1; KH_2PO_4, 0.5 and KCl, 0.25. The pH value was adjusted to 5.5 before autoclaving (Mahato *et al.*, 1988).

Fungal Cultivation:

The inoculum was prepared by adding 3ml of sterile distilled water to each PDA slant followed by scratching with a sterile needle. This suspension was used to inoculate 50 ml of sterile biotin fermentation medium dispensed in a 250 ml Erlenmeyer flask. Flasks were then incubated on a rotary shaker with 150 rpm at 30°C for different periods of incubation. Thereafter, cultures were centrifuged at 5000 rpm for 10 min to separate the fungal mycelia from the culture filtrate. Final pH, fungal biomass (mass after being dried at 60°C till constant weights) and biotin content were then determined.

Isolation and determination of biotin:

Biotin was separated, purified, chemically characterized and compared to an authentic biotin sample based on the TLC technique described by Birch *et al.* (2000). Biotin concentration was estimated calorimetrically as described by Shimada *et al.* (1969).

Experimental designs:

The Plackett-Burman design: The Plackett-Burman experimental design, a fractional factorial design (Plackett and Burman, 1946; Yu *et al.*, 1997), was used to reflect the relative importance of various environmental factors on biotin production as well as dry weight and final pH in liquid cultures. Seven independent variables were screened in nine combinations organized according to the Plackett-Burman design matrix described in the Results section. For each variable, a high level (+) and low level (-) was tested. All trials were performed in duplicates and the averages of biotin production results were treated as the responses. The main effect of each variable was determined with the following equation:

$$E_{xi} = (\sum_{i+} M - \sum_{i-} M) / N$$

Where E_{xi} is the variable main effect, M_{i+} and M_{i-} are biotin production in trials where the independent variable (xi) was present in high and in low settings, respectively, and N is the number of trials divided by 2. For determination of variable significance, statistical t-values for equal unpaired samples were calculated with respect to observations.

Box-Behnken design: In the second phase of medium formulation for optimum biotin production, the box-behnken experimental design, which is a response surface methodology (Box and Behnken, 1960), was applied. In this model, the most significant independent variables, named (X_1), (X_2) and (X_3) are included and each factor can be examined at the three different levels, low (-), high (+) and central or basal (0).

Here, these factors included concentrations of sucrose (X_1) and peptone (X_2) and KH2PO4 (X_3) were treated as independent variables. Thirteen combinations and their observations (shown in the Results section) were fitted to the following second order polynomial mode:

$$Y=b_o+b_1X_1+b_2X_2+b_3X3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+$$

$$b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2$$

Where, Y is the dependent variable (biotin production); X_1, X_2 and X_3 are the independent variables; b_o is the regression coefficient at center point; b_1, b_2 and b_3 are linear coefficients; b_{12} b_{13} and b_{23} are second-order interaction coefficients; and b_{11} and b_{33} are quadratic coefficients.

The values of the coefficients were calculated and the optimum concentrations were predicted using Statistica software. The quality of the fit of the polynomial model equation was expressed by R^2, the coefficient of determination.

3. Results and Discussion

Screening for biotin production substrates

Biotin has the chemical formula $C_{10}H_{16}N_2O_3S$. Accordingly, a quick identification of carbon, nitrogen and sulphur sources suitable for biotin production by *R. nigricans* NRC, FR105 was aimed. This was performed by two simple screening experiments. In the former, substitution of the glucose content in the preliminary biotin production medium by equal amounts of fructose, sorbose,

sucrose, starch, lactose, maltose and glycerol was examined. Biotin content was determined after four days of incubation. As shown in Table (1), variation of carbon sources in the basal medium dramatically modulated the efficiency of the fungus to produce biotin as well as biomass.

Among the different tested carbon sources, sucrose afforded the highest biotin production record (2.81 mg/l) with a 1.8-fold increase when compared to glucose. The obtained results and calculations suggest that sucrose obviously enhanced biotin synthesis rather than mycelial production and consequently, attained the highest specific product yield (0.205 mg product/g fungal biomass). Considerable enhancements in biotin production were also recorded by fructose, sorbose and glycerol cultures. On the contrary, the experimental fungus failed to produce biotin in the presence of starch or lactose.

Sierra *et al.* (1999) demonstrated that the nature of the carbon source as well as the age of the fungus culture strongly influence the pattern of vitamins released by *Rhizopial* cultures. It has been also reported that glycerol (6%) is suitable as a carbon source for biotin formation by *Kurthia sp* (Furuichi *et al.*, 2000). Similar to our results, sucrose proved to be appropriate for growth and finally converted to biotin by *E. coli* (Brich *et al*., 2000).

For the evaluation of alternative donors of nitrogen and sulphur, representative organic sources (including yeast extract, baker's yeast, corn steep liquor and urea) and an inorganic source (ammonium sulphate) were examined as peptone substiuents in the fermentation medium. Each was employed at a nitrogen concentration equivalent to 1% peptone in the presence of the most favorable carbon source, sucrose. However, none of the examined nitrogenous compounds exceeded peptone with respect to biotin production.

Considerable biotin production results were also recorded by the cultures contained yeast extract and corn steep liquor (2.3 and 1.87 mg/l). However, corn steep liquor dramatically stimulated the experimental *R. nigricans* strain for mycelial growth rather than biotin production. It recorded a 2.2–fold increase in biomass production when compared to peptone (data not shown).

In the case of urea, no detectable biotin was accumulated in the medium. The inability of the fungus to synthesize biotin under such a condition is simply a consequence of the absence of sulphur, a component of the biotin molecule. This observation demonstrates the sensitivity of the applied biotin estimation method. On the other hand, the results showed that ammonium sulphate did not support biotin biosynthesis or even mycelial growth. Accordingly, it can be suggested that ammonium sulphate cannot serve as a nitrogen or sulfur contributor in cultures of *R. nigricans* NRC strain FR105. Similarly, Maksimov *et al*. (1983) has found that *R. delmar* does not efficiently assimilate ammonium sulphate and urea which offered negligible quantities of biotin.

Peptone, a peptic digest of fresh meat, has relatively high contents of nitrogen and sulphur (Atlas, 2004) which are elementary for building biotin molecules. Therefore, among the nitrogenous compounds examined in this work, peptone has been chosen as a component of the fermentation medium.

Optimization of biotin production by multi-factorial designs:

The medium formula containing sucrose and peptone as substrates for biotin synthesis was chosen as the basal medium for an optimization strategy that involved a two-phase experimental design. The first step was to evaluate the relative importance of various fermentation factors by applying a fractional factorial design. In the second phase, levels of the variables, which have significant influences on biotin formation, were further investigated.

Elucidation of fermentation factors affecting biotin production:

The relative importance of various environmental factors involved in the process of biotin production was explored using the Placket-Burman design (Plackett and Burman, 1946) described in the Material and Methods section. Examined levels of 7 culture variables are presented in Table (2). The design was applied with 9 different fermentation conditions as shown in Table (3). All experiments were performed in duplicates and the averages of the observations are presented in the table. Fungal growth, final pH and biotin production were determined after 3, 4 or 5 days according to the proposed design. The main effects of each variable upon biotin production, as well as fungal growth, were estimated and expressed graphically in Figure (1). The results showed clearly that the dry weights of the mycelia are positively affected by the presence of high levels of sucrose, peptone and inoculum size. On the other hand, high concentrations of sucrose and KH_2PO_4 showed negative effects on biotin production.

The calculated main effects of this experiment suggested also that increasing the level of peptone in the culture medium, decreasing sucrose

concentration and extending the incubation period stimulate biotin production. On agreement with our results, it has been previously reported that the rate of biotin synthesis by a *R. delemar* strain increased drastically when the major portion of sugar was consumed and the fungal growth was terminated (Shchelkovo and Vorob'ev, 1982). All these observations suggest that the genes responsible for expressing biotin synthesizing enzymes in *Rhizopus* are growth-phase dependent. It is likely that they are triggered post-exponentially or under limiting growth conditions. Moreover, biotin production by other microbial cultures including *Sphingomonas sp.* (Shaw *et al.*, 1999) and *Agrobacterium* (Saito *et al.*, 2000) were relatively high under limiting growth conditions.

Based on the results obtained from the Placket-Burman experiment, a formula of the following composition (%) is predicted to be near optimum for biotin production: sucrose, 4; peptone, 1.5; KH_2PO_4, 0.4; KCl, 0.15 and inoculum size, 3 with an initial pH of 4.5 and an incubation period of 5 days. In order to determine the accuracy of this experiment, a verification test was carried out in a triplicate. The predicted near optimum levels of independent variables were examined against the basal condition settings. The average of the achieved biotin content was 20.1 mg/l which represent about 7-fold increase when compared to the basal condition.

The results of this experiment suggested also that the most effective variables, concerning biotin production are the concentrations of peptone and sucrose in addition to the incubation period. Among those, statistical analyses of the data (t-test) demonstrated the significance of peptone and sucrose Table (4).

Optimization of biotin production by Box-Behnken design:

In order to approach the optimum response region of biotin production, the effective independent variables including sucrose concentration (X_1), peptone concentration (X_2) and incubation time (X_3) were further investigated, each at three levels according to the Box and Behnken design (Box and Behnken, 1960). However, KCl, initial pH, inoculum size and KH_2PO_4 were treated as constant factors: KCl (0.15%), KH_2PO_4 (0.4%), inoculum size (3%) and initial pH (4.5). Table (5) represents the design matrix of the coded variables together with the experimental results of final pH, growth and biotin production.

As shown in Table (5), the highest biotin accumulation records (28.2, 23.3 and 26.2 mg/l) were achieved by the trials number 3, 4 and 10, respectively, which contained peptone at its examined high level (3%). On the other hand, it is clear that the lowest biotin production records (18.1, 19.1 and 18.4 mg/l) were achieved by the trials number 2, 6 and 8, respectively, that contained sucrose at its examined high level (3%).

Expressing the experimental results in the form of surface plots reflects the interactive effects of examined variables. Figure (2) shows the influences of variations in sucrose concentration and incubation time on the experimental fungus with respect to biotin production. From this figure, it can be suggested that, up to approximately 5.5 hours, the more the incubation time the more the biotin accumulation in the medium. It seams likely that a longer incubation time would not allow more biotin synthesis. However, an optimum level of sucrose appears to be close to the mean of the examined concentrations.

For a precise prediction of the optimal point, a second order polynomial function was fitted to the biotin production results of the applied Box-Behnken experiment. According to the obtained statistical analysis results, the correlation between the response and the three independent variables can be described by the following model.

$$Z = 4.93 + 2.44\,X_1 + 1.07\,X_2 + 5X_3 - 0.22X_1X_2 - 0.15X_1X_3 - 0.37X_2X_3 - 0.56\,X_1^2 + 0.74\,X_2^2 - 0.37\,X_3^2.$$

Where Z is the product of biotin (dependent variable); X_1, X_2 and X_3 are levels of sucrose, peptone and incubation time, respectively. The degree of fitting of the model is relatively high as the calculated coefficient of determination (R^2) was 0.99. The closer the R^2 value to 1, the stronger the model is and the better the response predicted.

Solving the model according to the data obtained from Table (5) revealed an optimum response at $X_1 = 1.5$, $X_2 = 3$ and $X_3 = 5.5$ with a predicted Z (response) of 29.5 mg/l. Thus, according to the results of the two optimization experiments, an optimum response (biotin production) is predicted under the following fermentation condition: sucrose, 1.5%; peptone, 3%; KCl 0.15%; KH_2PO_4, 0.4% initial pH, 4.5; inoculum size 3% and an incubation period of 5.5 days.

In order to evaluate this proposition, a verification experiment was performed in which the predicted optimal condition was practically compared with the basal fermentation settings in triplicates. The optimized culture condition attained a biotin accumulation average of 28.2 mg/l which is relatively

close to the theoretically predicted value. When compared to the basal culture condition this achievement resulted in about 10.4, 13.9, 5.7, 7.6 and 4.2-fold increases in production, product yield coefficient, specific product yield coefficient, productivity and specific productivity, respectively Table (6).

Table 1: Effect of different carbon sources on biomass and biotin production by *Rhizopus nigricans* NRC, FR105

Carbon source	Final pH	Mycelial dry weight (g/l)	Biotin content (mg/l)	Specific product yield (mg product/g biomass)
Glucose	4.7	12.6	1.50	0.119
Fructose	5.0	16.4	2.30	0.140
Sorbose	5.1	14.8	2.21	0.149
Sucrose	4.9	13.2	2.70	0.205
Starch	5.0	18.0	0.00	0.000
Lactose	7.5	16.6	0.00	0.000
Maltose	4.3	10.6	0.08	0.008
Glycerol	5.6	17.2	2.10	0.122

Table 2: Factors examined as independent variables affecting biotin production by *R. nigricans* NRC, FR105 and their levels in the Plackett-Burman experiment

Factor	Symbol	Level		
		-1	0	1
Time (days)	T	3	4	5
Sucrose (%)	S	4	5.5	7
Peptone (%)	P	0.5	1	1.5
$KH_2 PO_4$ (%)	KH_2	0.4	0.5	0.6
Inoculum size (%)	1S	1	2	3
Initial pH	pH	4.5	5.5	6.5
KCl (%)	KCl	0.15	0.25	0.35

Table 3: The Plackett-Burman experimental design for 7 variables and its results

Trial	Independent Variables [1]							Response		
	T	S	P	KH_2	IS	pH	KCl	Final pH	Dry weight (g/l)	Biotin (mg/l)
1	-	-	-	+	+	+	-	3.50	09.1	5.7
2	+	-	-	-	-	+	+	3.51	08.2	10.7
3	-	+	-	-	+	-	+	3.37	16.2	2.6
4	+	+	-	+	-	-	-	3.59	12.1	2.9
5	-	-	+	+	-	-	+	3.78	13.3	15.6
6	+	-	+	-	+	-	-	4.10	16.4	20.1

7	-	+	+	-	-	+	-	3.84	21.2	10.1
8	+	+	+	+	+	+	+	4.16	16.1	11.3
9	0	0	0	0	0	0	0	3.89	11.2	16.5
10	0	0	0	0	0	0	0	3.87	11.0	16.3

[1] Factor symbols are shown in Table 5

Table 4: Statistical analysis of the Plackett-Burman experimental results

Variable	Growth		Biotin	
	Main effect	*t*-value	Main effect	*t*-value
Time	-0.09	-0.92	2.75	0.67
Sucrose	0.23	**2.47**	-6.30	**-1.53**
Peptone	0.27	**2.83**	8.80	**2.13**
KH_2PO_4	-0.14	**-1.51**	-2.00	-0.48
Inoculum size	0.04	0.39	0.10	0.02
pH	-0.04	-0.46	-0.85	-0.21
KCl	-0.06	-0.67	0.35	0.08

Critical t-values at α= 0.05 and 0.1 are 2.015 and 1.467, respectively.

Table 5: Examined concentrations of the key variables and results of the Box-Behnken experiment

Trial	Independent variable			Response		
	Sucrose % X_1	Peptone % X_2	Days X_3	Final pH	Dry weight (g/l)	Biotin (mg/l)
1	1 (-)	1 (-)	5.5(0)	3.5	07.2	20.4
2	3 (+)	1 (-)	5.5(0)	3.4	15.5	18.1
3	1 (-)	3 (+)	5.5(0)	4.2	24.0	28.2
4	3 (+)	3 (+)	5.5(0)	3.7	20.3	23.3
5	1 (-)	2 (0)	4(-)	3.7	09.4	20.5
6	3 (+)	2 (0)	4(-)	3.6	08.0	19.1
7	1 (-)	2 (0)	7(-)	3.8	12.6	22.5
8	3 (+)	2 (0)	7(+)	3.7	14.4	18.4
9	2 (0)	1 (-)	4(-)	3.5	13.8	20.1
10	2 (0)	3 (+)	4(-)	4.56	09.2	26.2
11	2 (0)	1 (-)	7(+)	3.5	15.6	21.3
12	2 (0)	3 (+)	7(+)	3.8	12.0	20.7
13	2 (0)	2 (0)	5.5(0)	3.64	12.2	22.2

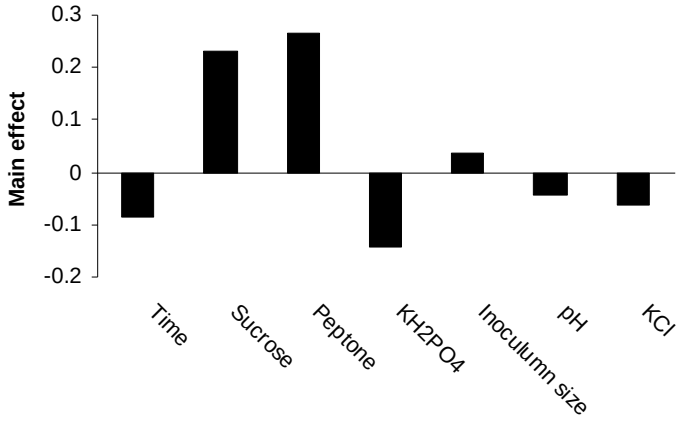
13	2 (0)	2 (0)	5.5(0)	3.63	12.0	22.3
13	2 (0)	2 (0)	5.5(0)	3.64	12.4	22.2

Table 6: Kinetic parameters and coefficients of biotin fermentation by FR105 under basal and optimized conditions

Parameter	Unit	Condition		Fold increase
		Basal	Optimum	
Production	(mg product/l)	2.700	28.20	10.4
Product yield coefficient	(mg product/g substrate[1])	0.045	0.63	13.9
Specific product yield coefficient	(mg product/g cells)	0.205	1.18	5.7
Productivity	(mg product/l/h)	0.675	5.13	7.6
Specific productivity	(mg product/g cells/h)	0.051	0.21	4.2

[1] Substrate = g sucrose + g peptone

(a)



(b)

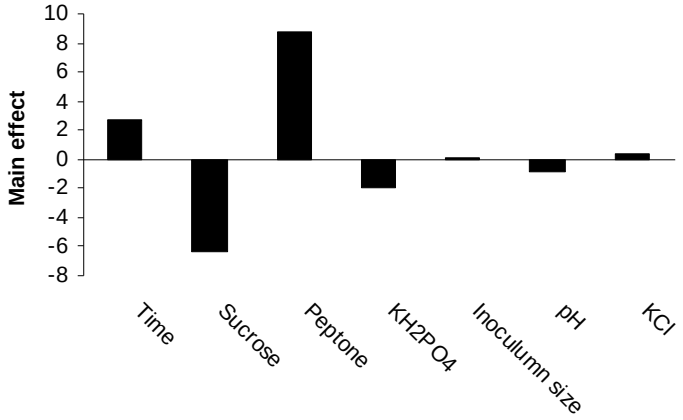


Figure 1: Main effects of independent variables upon growth (a) and biotin production (b) by *R. nigricans* NRC, FR105 according to the results of the Plackett-Burman experiment

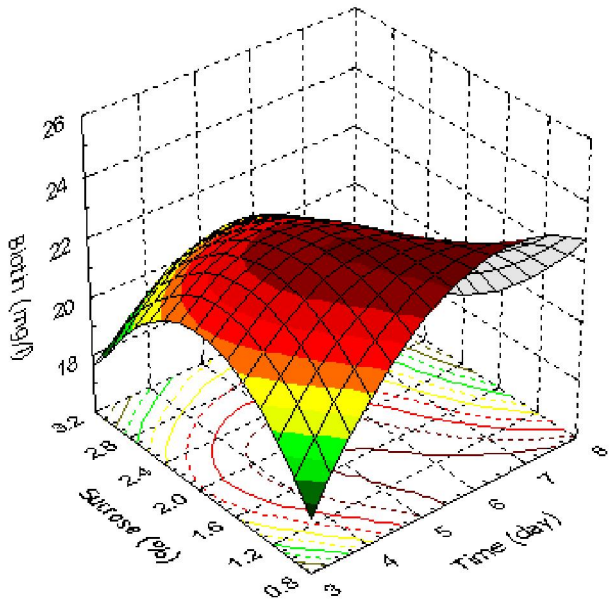


Figure 2: Sucrose and time as independent variables that affect the production of biotin by *R. nigricans* NRC, FR105 based on the results of the Box-Behnken experiment

Conclusion

It has been proposed that any fermentation process has to be able to produce more than 1 g biotin per liter in order to be cost-effective (Streit and Entcheva 2003). A recombinant strain of *Serratia marcescens* was able to produce biotin at the 500-mg/l level (Sakurai *et al.*, 1994). In addition, more than 1 g biotin per liter of culture medium was achieved by a *B. subtilis* strain that over-expresses the genes responsible for biotin synthesis (Bower *et al.*, 2001). For more cost-effective results, we suggest that the fermentation of such recombinant biotin over-producer strains could be considerably improved by a sequential economization and optimization approach which covers each of the following investigations: (1) screening for carbon, nitrogen and sulphur containing agro-industrial wastes that efficiently support the recombinant strain for growth, as well as biotin production, (2) elucidation of the fermentation factors the significantly regulate the synthesis of biotin and (3) application of a suitable multi-factorial experimental design for optimization of biotin production. In each optimization phase, a special attention should be given to other important biotechnological parameters including: product yield coefficient (g product/g substrate), specific product yield coefficient (g product/g cells) and productivity (g product/l/h).

References

1. Atlas R.M., 2004. Handbook of Microbiological Media, 3rd edn. Boca Raton, FL, USA: CRC Press.
2. Birch, O., Brass, J.J., Fuhrmann, M., Shaw, N., 2000. Biotechnological method of producing biotin. U.S. Patent 6083712.
3. Bower. S., Perkins, J.B., Yocum, R.R., Pero, J.G., 2001. Biotin biosynthesis in Bacillus subtilis. US Patent 6303377.
4. Box, G. E. P. Behnken, D. W., 1960. Some new three-level designs for the study of quantitative variables. Technometrics 2, 455-75.
5. Furuichi Y., Hoshino T., Kimura H., Kiyasu T., Nagahashi Y., 2000. Biotin biosynthetic genes. United States Patent 6117669.
6. Gresham, R., Inamine, E., 1986. Nutritional improvement of processes. In: Manual of Industrial Microbiology and Biotechnology, ed. Demain, A.L. and Solomon, N.A. Washington, ASM, pp. 41-8.
7. Hall, C., Dietrich, F.S., 2007. The reacquisition of biotin prototrophy in Saccharomyces cerevisiae involved horizontal gene transfer, gene

duplication and gene clustering. Genetics, 177, 2293-307.
8. Hong, Y., Chen, Y., Farh, L., Yang, W., Liao, C., Shiuan, D., 2006. Recombinant Candida utilis for the production of biotin. Appl. Microbiol. Biotechnol. 71, 211-21.
9. Hooijkaas, L.P., Wilkinson, E.C., Tramper, J., Buitelaar, 1998. Medium optimization for spore production of Conithyrium minitans using statistically-Based expereimental designs. Biotechnol. Bioeng. 64, 92-100.
10. Hoshino, T., Noro, A., Tazoe, M., 1999. Process for the production of d-biotin. U.S. Patent 5922581.
11. Lotfy, W.A., Ghanem, K.M., El-Helow, E.R., 2007. Citric acid production by a novel Aspergillus niger isolate: II. Optimization of process parameters through statistical experimental designs. Biores. Technol., 98, 3464-9.
12. Mahato, S.B., Banerjee, S., Podder, S., 1988. Oxidative side-chain and ring fission of pregnenes by Arthrobacter simplex. Biochem. J. 255, 769-774.
13. Maksimov, V.N., Shchelokova E.V., Vorob'eva L.I., 1983. Optimization of medium for biotin biosynthesis by Rhizopus delmar culture. Prikl. Biokhim. Mikrobiol. 19, 353-5.
14. Plackett, R.L., Burman, J.P., 1946. The design of optimum multi-factorial experiments. Biometrika. 33, 305–25.
15. Rodriguez-Melendez, R., Zempleni, J., 2009. Nitric Oxide Signaling Depends on Biotin in Jurkat Human Lymphoma Cells. J. Nutr. 139, 429-433.
16. Saito, I.I., Honda, H., Kawabe, T., Mukumoto F., Shimizu, M., Kobayashi ,T., 2000. Comparison of biotin production by recombinant Sphingomonas sp. under various agitation conditions. Biochem. Eng. J. 5, 129-36.
17. Sakurai, N., Imai, Y., Matsuda, M., Komatsubara, S., Sota, T., 1994. Improvement of a d-biotin-hyperproducing recombinant strain of Serratia marcescens. J. Biotechnol. 36, 63–73.
18. Salem, H.A. 2009. Phisiological and biochemical studies on microbiological production of biotin (Vitamin H). Thesis submitted for the fulfillment of the degree of master of pharmaceutical sciences, Cairo University.
19. Shaw, N.M., Lehner, B., Fuhrmann, M., Kulla, H.G., Brass, J.M., Birch, O.M., Tinschert, A., Venetz, D., Venetz, V., Sanchez, J-C., onella, L.T., Hochstrasser, D.F. 1999. Biotin production under limiting growth conditions by Agrobacterium/Rhizobium HK4 transformed with a modified Escherichia coli bio operon. J. Indus. Microbiol. Biotechnol. 22, 590-9.
20. Shchelkovo, E.V., Vorob'ev, L.I., 1982. Biotin formation by the fungus Rhizopus delemar. Prikl. Biokhim. Mikrobiol. 18, 630-5.
21. Shimada, K., Naganese, Y., Matsumoto, U. 1969. Colorimetric determination of biotin and analogs. Yakugaku Zasshi 89, 436-41.
22. Sierra, S., Rodelas, B., Martínez-Toledo, M.V., Pozo, C. González-López, J. 1999. Production of B-group vitamins by two Rhizobium strains in chemically modified media. J. Applied. Microbiol. 86, 850-8.
23. Streit, W.R., Entcheva, P., 2003. Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production. Appl. Microbiol. Biotechnol. 61, 21-31.
24. Yu, X., Hallett, S.G., Sheppard, J., Watson, A.K., 1997. Application of the Plackett-Burman experimental design to evaluate nutritional requirements for the production of Colleterichum coccodes spores. Appl. Microbial. Biotechnol. 47, 301-305.

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