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MEDIUM COMPOSITION INFLUENCE ON BIOTIN AND RIBOFLAVIN PRODUCTION BY NEWLY ISOLATED *CANDIDA* SP.

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ABSTRACT

Complex B vitamins as Biotin and Riboflavin are required by living organisms, not only for growth but also for metabolite production, and the feed market classifies them as growth promoters. Since Brazil will soon be one of the world's biggest animal protein producers, feed production is a large consumer of vitamins and micronutrients. The industry requires 10 mg riboflavin/0.2 mg biotin per kilogram of feed; a ratio of 40 ~ 50:1. Although few studies have been conducted specifically on riboflavin production using factorial design and surface response method as an optimization strategy, it is a common practice in biotechnology with many research reports available. However, there are no reports on the use of statistical design for biotin production. This study set out to evaluate medium composition influence on biotin and riboflavin production using a statistical design. There are no studies relating biotin and riboflavin production by *Candida* sp LEB 130. In this preliminary study to improve the simultaneous production of biotin and riboflavin, the maximum riboflavin/biotin ratio of 8.3 µg/mL was achieved with medium component concentrations of: sucrose 30 g/L, KH_2PO_4 2 g/L, $MgSO_4$ 1 g/L and $ZnSO_4$ 0.5mL/L.

Key words: *Candida* sp., riboflavin, biotin, media development, vitamin production

INTRODUCTION

Although work reported during recent years has led to a nearly complete understanding of the metabolic role of biotin, comparatively little is known about the biosynthesis of this B-group vitamin largely because living cells usually synthesize extremely small amounts of it or of compounds with biotin-like activity (24).

Biotin is required by a variety of yeasts, fungi and bacteria, not only for growth but also for metabolite production. *Ashbya gossypii*, for instance, needs many nutrients for riboflavin production, biotin among them (2). In a preliminary study, most of the isolate strains produced biotin and riboflavin simultaneously with a maximum ratio of 15 µg riboflavin/µg biotin. In the study of Özbas and Kutsal (19), maximum riboflavin production was achieved by *Ashbya gossypii* with a D-biotin concentration of 0.4 µg/L, yielding a riboflavin concentration of 0.6 kg/g initial carbon concentration. Kojima et al, 1972 (12) also used a specific medium containing glucose with salts and biotin added to study

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riboflavin production by *Eremothecium ashbyii*. Kalingan and Krishnan (9) showed that the concentration of yeast hydrolysate (which contains biotin and thiamin) that induced the highest vitamin production by *Eremothecium ashbyii* was 30 kg/m^3, and the specific production rate increased by 48% when compared to control. However, contrary effects were also confirmed in the literature when riboflavin production by *C. guilliermondii* was studied. While vitamins such as thiamine, nicotinic acid, inositol, and *p*-aminobenzoic acid are said to stimulate riboflavin production by *C. guilliermondii*, vitamin B_6, biotin, pantothenic acid and folic acid are said to be ineffective (17).

Although few studies have been conducted specifically applying them to riboflavin production (20, 21, 22, 28), factorial design and surface response methodology as an optimization strategy is a common practice in biotechnology with many research reports of it (1, 5, 10, 13, 15, 23, 27). However, there are no reports on the use of statistical design for biotin production. Statistical design is a tool that uses planned testing to ensure the scientific rigor of the approach. Varying the factors simultaneously results in an improved analytical methodology reducing the number of experiments or repetitions needed (23), and that suggests that it might be a useful tool for optimizing metabolite production. Even so, there is no available statistical design for biotin production. For that reason, our initial study was to evaluate medium composition influence on biotin production using a statistical design. Furthermore, comparing biotin production with riboflavin production in *Candida* sp. LEB 130 yeast isolated in a previous study in our laboratory was an intriguing proposition since no data is available for this yeast as it has only recently been isolated.

MATERIALS AND METHODS

Microorganisms and maintenance

The strain *Candida* sp. LEB 130 wild type was isolated at the Laboratory of Food Biochemistry, Faculty of Food Engineering, UNICAMP, from a soil sample collected from a sugarcane plantation in São Paulo State It is maintained in cryotubes containing 10% glycerol at -80^0 C. To prepare subcultures, yeast was inoculated onto Yeast Malt agar (2% glucose, 1% yeast extract, 2% peptone, 3% agar) and incubated at 30^0 C for 24 h before fermentation tests. The Brazilian Collection of Environmental and Industrial Micro-organisms (CBMAI) made the taxonomical identification of the microorganism, and it is has been deposited with them for future investigation.

Medium and vitamin production

A liquid inoculation technique, adding 1 mL of sterile distilled water to the agar slant, was used to inoculate shaken flasks. The tube was gently shaken to get the cells from the solid medium and obtain a homogeneous suspension containing 8.2 10^7 CFU/mL.

The standard medium used in this study was composed of 25 g/l sucrose, KH_2PO_4, $MgSO_4.7H_2O$, $ZnSO_4$ (0.2% solution) and 10% sodium phosphate buffer 0.1M, pH 7.0. The fermentation was carried out in 50 mL Erlenmeyers flasks containing 15 mL of culture medium autoclaved for 20 min at 121^{0}C and 1 atm. The fermentation experiment was carried out for 48h at 30^0 C and 100 rpm in a TECNAL TE 421 rotary shaker, in aerobic conditions. Riboflavin and Biotin production was assayed in the culture broth after 48h, as described below, after centrifugation at 1,250 x g for 15 min at 5^0 C (Beckman J2-21). Every single experiment was carried out in the dark to avoid riboflavin oxidation (26).

Elucidation of the significant media components for vitamin production using a fractional factorial design (FFD)

A 2^{6-2} fractional factorial design was employed to determine the key ingredients that significantly affected vitamin production. There were six nutrient factors in the medium: Sucrose (g/L) (x_1), Ammonium tartrate (g/L) (x_2), KH_2PO_4 (g/L) (x_3), $MgSO_4$ (g/L) (x_4), Citrate (mL/L) (x_5),

$ZnSO_4$ (mL/L) (x_6). Each factor was examined at a high level (coded +1) and a low level (coded -1), which corresponded to the basal level ±50%, respectively. The center points were the trials under the basal level conditions (coded 0). Table 1 shows the variables and levels in detail. A ¼ fraction of the full factorial design was adopted and consequently the experiment included 16 (2^{6-2}) combinations plus four replicates at the center point, as shown in Table 1. Samples were taken after 48 h of fermentation and centrifuged, and the supernatant was separated for biotin and riboflavin analysis.

Table 1. Independent variables and the concentration levels studied in the 2^{6-2} factorial optimization design for both riboflavin and biotin production.

Variable	Components	Level of variables		
		-1	**0**	**+1**
X_1	Sucrose (g/L)	10	20	30
X_2	Ammonium tartrate(g/L)	0	0.5	1
X_3	KH_2PO_4 (g/L)	0	1	2
X_4	$MgSO_4$ .7 H_2O (g/L)	0	0.5	1
X_5	Ferric Citrate (mL/L)	0	0.5	1
X_6	$ZnSO_4.7H_2O$ (mL/L)	0	0.5	1

Data analysis

The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2, and its statistical significance was checked by *F*-test. The significance of the regression coefficient was tested by a *t*-test. The significance level was given as values of Prob > *F* less than 0.1. A differential calculation was then employed for predicting the optimum point.

Vitamin Assay

An assay based on the binding of the dye HABA to avidin and the ability of biotin to displace the dye in stoichiometric proportions was applied. The HABA-Avidin complex is at the core of this displacement assay that can estimate the extent of protein biotinylation. HABA dye binds to avidin to form a complex that absorbs strongly at 500 nm with extinction at that wavelength of 35,000 $M^{-1}cm^{-1}$ (6). The assay is based on the decrease in absorbance of the ($(HABA)_4$:Avidin) complex when HABA is displaced from the complex by biotin, and that was measured in a Beckman DU-640 Spectrophotometer at 500nm using an HABA-Avidin Sigma vial (Sigma-Aldrich, Saint Louis, Missouri, USA).

Riboflavin was determined in the supernatant of the culture medium after centrifugation of the broth at 1,250 x g for 15 min, at 5^0 C (Beckman J2-21), according to Ming et al. (16). Analysis occurred by measurement of the optical density in a Beckman DU-640 Spectrophotometer at 440nm. A standard curve was constructed using pure riboflavin standard (Sigma).

RESULTS AND DISCUSSION

Elucidation of the significant media components for biotin production using a fractional factorial design (FFD)

There are no published studies on the use of statistical design techniques for biotin production. Moreover, very few studies on carbon source and nitrogen source roles in enhancing biotin biosynthesis have been conducted in the past.

The parameters sucrose (x_1), ammonium tartrate (x_2), KH_2PO_4 (x_3), $MgSO_4$ (x_4), ferric citrate (x_5) and $ZnSO_4$ (x_6) were selected for fractional factorial design of the experiment. Table 2 summarizes the fractional factorial design matrix along with the experimental response for each individual experiment. Twenty experiments were performed using different

combinations of the variables designing a fractional factorial 2^{6-2} with four replicates at the centre point ($n_0 = 4$). Not only does the factorial design permit the identification of the components that are significantly relevant to biotin production, it also delineates the ranges of concentration within which they make a difference.

Table 2. A 2^{6-2} factorial design matrix of six variables with experimental riboflavin and biotin production values by *Candida* sp. LEB 130.

Run	Sucrose x_1 [a]	Ammonium x_2 [a]	KH_2PO_4 x_3 [a]	$MgSO_4$ x_4 [a]	Citrate x_5 [a]	$ZnSO_4$ x_6 [a]	Riboflavin Production (μg/mL)	Biotin Production (μg/mL)	Riboflavin / Biotin	
									Experimental [b]	Predicted
1	-1 (10)	-1 (0.0)	-1 (0.0)	-1 (0.0)	-1 (0.0)	-1 (0.0)	1.27	3.70	0.34	0.80
2	1 (30)	-1 (0.0)	-1 (0.0)	-1 (0.0)	1 (1.0)	-1 (0.0)	2.85	2.21	1.29	1.49
3	-1 (10)	1 (1.0)	-1 (0.0)	-1 (0.0)	1 (1.0)	1 (1.0)	2.09	5.61	0.37	-1.78
4	1 (30)	1 (1.0)	-1 (0.0)	-1 (0.0)	-1 (0.0)	1 (1.0)	0.00	2.38	0.00	0.54
5	-1 (10)	-1 (0.0)	1 (2.0)	-1 (0.0)	1 (1.0)	1 (1.0)	3.35	3.72	0.90	0.39
6	1 (30)	-1 (0.0)	1 (2.0)	-1 (0.0)	-1 (0.0)	1 (1.0)	2.50	1.80	1.39	2.71
7	-1 (10)	1 (1.0)	1 (2.0)	-1 (0.0)	-1 (0.0)	-1 (0.0)	5.40	4.76	1.14	1.36
8	1 (30)	1 (1.0)	1 (2.0)	-1 (0.0)	1 (1.0)	-1 (0.0)	5.83	2.76	2.11	2.06
9	-1 (10)	-1 (0.0)	-1 (0.0)	1 (1.0)	-1 (0.0)	1 (1.0)	3.32	3.50	0.95	0.79
10	1 (30)	-1 (0.0)	-1 (0.0)	1 (1.0)	1 (1.0)	1 (1.0)	2.49	2.15	1.16	1.49
11	-1 (10)	1 (1.0)	-1 (0.0)	1 (1.0)	1 (1.0)	-1 (0.0)	1.04	3.25	0.32	0.14
12	1 (30)	1 (1.0)	-1 (0.0)	1 (1.0)	-1 (0.0)	-1 (0.0)	3.13	2.14	1.46	2.46
13	-1 (10)	-1 (0.0)	1 (2.0)	1 (1.0)	1 (1.0)	-1 (0.0)	0.78	2.83	0.27	2.31
14	1 (30)	-1 (0.0)	1 (2.0)	1 (1.0)	-1 (0.0)	-1 (0.5)	12.52	1.51	8.29	4.63
15	-1 (10)	1 (1.0)	1 (2.0)	1 (1.0)	-1 (0.0)	1 (1.0)	2.89	2.74	1.06	1.36
16	1 (30)	1 (1.0)	1 (2.0)	1 (1.0)	1 (1.0)	1 (1.0)	5.36	3.15	1.70	2.05
17	0 (20)	0 (0.5)	0 (1.0)	0 (0.5)	0 (0.5)	0 (0.5)	3.32	1.93	1.72	1.43
18	0 (20)	0 (0.5)	0 (1.0)	0 (0.5)	0 (0.5)	0 (0.5)	2.33	2.08	1.12	1.43
19	0 (20)	0 (0.5)	0 (1.0)	0 (0.5)	0 (0.5)	0 (0.5)	4.22	2.54	1.66	1.43
20	0 (20)	0 (0.5)	0 (1.0)	0 (0.5)	0 (0.5)	0 (0.5)	2.53	2.03	1.25	1.43

[a]: the coded variables x_i are defined in Table 1.
[b]: Observed biotin production stands for the experimental data.

Biotin production varied from 1.5 μg/mL to 5.6 μg/mL among the different levels of compound concentrations in the medium. The latter value was the highest biotin excretion achieved with the following concentrations: sucrose 10 g/L, ammonium tartrate 1 g/L, ferric citrate solution 1 mL/L and $ZnSO_4$ solution 1 mL/L. This value was 2.9-fold higher than the data obtained with the standard media composition described in the Materials and Methods section.

According to Table 2, it seems that increasing ammonium tartrate concentration also raises biotin production. The same effect is also noticed with ferric citrate; sucrose concentration, however, should not be higher than 15 g/L. Zinc and potassium salts did not influence biotin production significantly.

The significance of each coefficient was determined by Student's *t*-test and *P*-values, which are set out in Table 3. The smaller the *P*-value is and the larger the magnitude of the *t*-value, the higher the significance of the corresponding coefficient. Consequently, the concentration of sucrose markedly influenced biotin biosynthesis, with *P*-values of 0.001, despite its negative effect. On the other hand, KH_2PO_4 and $ZnSO_4$ did not significantly affect the vitamin excretion within the limit of significance of 90%.

Table 3. Estimated effects, *t*-statistics and significance probability of the model for biotin production by *Candida* sp. LEB 130.

	Effects	**Std. Err.**	***t*-Value**	***p*-Value**
Interception*	2.84	0.06	46.92	0.0000
(1) Sucrose *	-1.50	0.07	-11.10	0.002
(2) Ammonium tartrate *	0.67	0.07	4.96	0.02
(3) KH_2PO_4	-0.21	0.07	-1.54	0.22
(4) $MgSO_4$ *	-0.71	0.07	-5.24	0.01
(5) Ferric Citrate *	0.39	0.07	2.91	0.06
(6) $ZnSO_4$	0.24	0.07	1.75	0.18

* Significant factors ($p \leq 0.10$).

Biotin production varied from 1.5 μg/mL to 5.6 μg/mL among the different levels of compound concentrations in the medium. The latter value was the highest biotin excretion achieved with the following concentrations: sucrose 10 g/L, ammonium tartrate 1 g/L, ferric citrate solution 1 mL/L and $ZnSO_4$ solution 1 mL/L. This value was 2.9-fold higher than the data obtained with the standard media composition described in the Materials and Methods section.

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In their study, Ohsugi et al. (18) reported carbohydrates like sucrose, mannose and galactose as being better carbon sources for biotin production by *Enterobacteriaceae* than Polypepton, peptone or casamino acids, after five days of fermentation.

Sulfur is important in the biotin biosynthetic pathway (8), so studying the influence of complex sulfur compounds, such as certain amino acids, would be useful. For instance, Rose et al. (24) found that amino acids as nitrogen sources were much more stimulating than NH_4Cl in biotin production by *Pseudomonas aeruginosa*.

Evaluation of media composition influence on riboflavin production by *Candida* sp LEB 130 using a 2^{6-2} factorial design

Riboflavin production by *Candida* sp. LEB 130 was also tested in the same factorial design experiment described for biotin production to identify the medium components that play the most notable role in riboflavin production as well as the ranges of concentration within which they make a difference. Table 2 summarizes the fractional factorial design plan along with the experimental response for each individual experiment.

Riboflavin production varied from zero to 12.5 μg/mL among the different levels of the compounds of the medium, with the latter being the highest riboflavin excretion, achieved at concentrations of: sucrose 30 g/L, KH_2PO_4 2 g/L, $MgSO_4$ 1 g/L and $ZnSO_4$ 0.5 mL/L.

According to Table 2, increasing the concentration of sucrose seems to improve riboflavin biosynthesis. The same effect is noticed when KH_2PO_4 concentration increases. Still analyzing the same results (Table 2), it is clear that zinc did not have a stimulatory effect on riboflavin production by *Candida* sp. LEB 130 in this study, contrary to the findings of Demain (2).

The influence of iron in riboflavin production by *Candida* sp. is controversial (2, 3, 4, 17). Ghanem *et al*. (4), for example, found out that $FeSO_4.7H_2O$ at 50 μg/L levels had an inhibitory effect on riboflavin production. On the other hand, Nishio and Kamikubo, (17) showed the positive effect of ferrous ion not only on growth but also on vitamin B_2 biosynthesis. In this factorial design study, however, the presence of iron ions did not significantly affect vitamin B_2 production (Table 4).

The significance of each coefficient was determined by Student's *t*-test and *P*-values, which are set out in Table 4. The concentration of KH_2PO_4 distinctly influenced riboflavin biosynthesis, with *P*-values of 0.01. On the other hand, ammonium tartrate and ferric citrate did not significantly affect the vitamin excretion.

Table 4. Estimated effects, *t*-statistics and significance probability of the model for riboflavin production.

	Effects	**Std. Err.**	***t*-Value**	***p*-Value**
Interception*	3.36	0.19	17.45	0.0004
(1) Sucrose *	1.82	0.22	4.22	0.02
(2) Ammonium tartrate	-0.41	0.22	-0.96	0.41
(3) KH_2PO_4 *	2.81	0.22	6.51	0.01
(4) $MgSO_4$ *	1.03	0.22	2.39	0.097
(5) Ferric Citrate	-0.91	0.22	-2.11	0.13
(6) $ZnSO_4$ *	-1.35	0.22	-3.15	0.05

* Significant factors ($p \leq 0.10$).

Evaluation of media composition influence on both simultaneous vitamin production by *Candida* sp LEB 130 using a 2^{6-2} factorial design

There are no studies relating biotin and riboflavin production by *Candida* sp., hence our main objective here was to compare production of both vitamins in order to apply them in the feed industry as a suitable mix product. The feed industry requires 10 mg of riboflavin and 0.2 mg of biotin in each 1 kg of feed, a ratio of 40 ~ 50:1 (11). In order to compare that figure to the experimental ones obtained in this study, it was established as one of the answers for the fractional factorial design described in Table 2.

Vitamin production ratios varied from zero to 8.3 μg/mL among the different levels of the medium compounds. The latter value was the highest obtained and achieved at concentrations of: sucrose 30 g/L, KH_2PO_4 2 g/L, $MgSO_4$ 1 g/L and $ZnSO_4$ 0.5mL/L. This was still 12 times lower than the ratio used by the feed industry. However, the improvement of the vitamin production observed during this study demonstrated potential and provided relevant data on these preliminary tests for further investigation of the strain and the culture media composition.

The significance of each coefficient was determined by Student's *t*-test and *P*-values, which are in Table 5. Only ammonium tartrate and ferric citrate did not affect the vitamin production relation to a level of significance of 99%.

Biotin is a stimulator of riboflavin biosynthesis, and many authors report its use in the medium (7, 14, 17). Hickey (7), for example, added 0.34 μg/L of biotin in his study of riboflavin production. Nishio and Kamikubo (17) reported that 1 μg/mL of biotin was not so effective in stimulating riboflavin production and potassium hydrophosphate resulted in maximal riboflavin production when added at a concentration of 5 g/L. When Levine et al. (14) changed biotin concentration in the medium from 1 μg/mL to 2 μg/mL, riboflavin production actually decreased, while Sabry et al. (25) found that biotin did not affect riboflavin production by *Candida guilliermondii* at all.

Table 5. Estimated effects, *t*-statistics and significance probability of the model for both vitamin production ratio produced by *Candida* sp. LEB 130.

	Effects	**Std. Err.**	***t*-Value**	***p*-Value**
Interception*	1.43	0.07	21.36	0.000
(1) Sucrose *	1.51	0.07	10.10	0.002
(2) Ammonium tartrate	-0.80	0.07	-5.39	0.013
(3) KH_2PO_4 *	1.37	0.07	9.19	0.003
(4) $MgSO_4$*	0.96	0.07	6.42	0.008
(5) Ferric Citrate	-0.81	0.07	-5.45	0.012
(6) $ZnSO_4$*	-0.96	0.07	-6.46	0.008

* Significant factors ($p \leq 0.01$).

CONCLUSION

A preliminary study of biotin and riboflavin production was conducted. The 2^{6-2} factorial design was used to analyze medium composition for biotin and riboflavin production by *Candida* sp. LEB 130. The highest biotin production occurred at a sucrose concentration of 10 g/L, ammonium tartrate 1 g/L, ferric citrate 1 mL/L and $ZnSO_4$ 1mL/L. This value was 2.9-fold higher than the data obtained with the standard media of the screening study. Sucrose was the most significant factor affecting biotin production, but ammonium tartrate, magnesium salt and citrate were also important with significance levels of 90%.

As for riboflavin production, sucrose and potassium salt were the most significant factors. The highest riboflavin production, 12.5µg/mL, was achieved at a sucrose concentration of 30 g/L, KH_2PO_4 2 g/L, $MgSO_4$ 1g/L and $ZnSO_4$ 0.5mL/L. That was at least six-fold higher than in the standard media composition. Lastly, in the study to evaluate the riboflavin/biotin ratio obtained from *Candida* sp. LEB130 with the tested media, sucrose and potassium salt should be considered for further studies since they were the most significant factors, according to this study. In short, the improvement of vitamin production observed during this study demonstrated the potential of the strain and provided relevant data on these preliminary tests for further investigation of the strain and the culture medium composition. The statistical designs proved to be important tools for evaluating medium performance and improving vitamin production. Also, this new medium compound formulation could be applied to improve vitamin production by other strains. Furthermore, even though this wild-type *Candida* sp. produces lower vitamin concentrations then commercial strains, it may be an important candidate for genetic modification to improve that production.

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