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Optimization of MRS media components using response surface methodology for the riboflavin production by *Lactobacillus fermentum* isolated from yoghurt sample

Sathyanarayanan, J., *Kunthala, J. and Gurumurthy, K.

School of Biosciences and Technology, Vellore Institute of Technology University, Vellore-632 014, India

Abstract: Riboflavin producing lactic acid bacteria were isolated from fermented milk samples. *Lactobacillus fermentum* MTCC 8711, which produced higher amount of riboflavin, was used for the fermentative production of riboflavin. Response surface methodology with central composite design was used for the optimization of the fermentation medium for riboflavin production. Peptone, beef extract, glucose and K_2HPO_4 of the MRS broth were selected as variables. Among the tested variables, beef extract, glucose and K_2HPO_4 showed significant effect on the riboflavin production. Higher concentration of K_2HPO_4 exhibited positive response on riboflavin production. The validation run based on the optimum production in response surface plot resulted in the maximum (9.43 mg L^{-1}) riboflavin yield, which was almost equivalent to the predicted value of 9.63 mg L^{-1}. The Lb. fermentum strain produced four folds higher riboflavin in the optimized medium when compared to the unoptimized medium (2.28 mg L^{-1}).

Keywords: *Lactobacillus, riboflavin*, MRS medium, di-potassium hydrogen phosphate, central composite design

Introduction

Lactic acid bacteria (LAB) are industrially important microorganisms employed in the production of fermented food products, such as yogurt, cheese, sauerkraut and sausage, and are generally regarded as safe (GRAS) organisms (Stiles, 2004). These 'probiotic' organisms produce a wide range of metabolites collectively termed as 'nutraceuticals', which include vitamins like riboflavin (B_2), folate (B_{11}) cyano-cobalamine (B_{12}); low calorie sugars like mannitol and sorbitol; exopolysaccharides and diacetyl and L-alanine (Hugenholtz et al., 2002).

One of the important nutraceuticals produced by LAB is the vitamin 'riboflavin'. Riboflavin is a water-soluble vitamin (B_2) produced by plants and many micro-organisms. It is a basic component of the cellular metabolism since it is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Vitreschak et al., 2002). Higher animals lack the riboflavin biosynthetic pathway and therefore they must obtain this essential nutrient mainly from their diet. Although riboflavin is found in a wide variety of foods, riboflavin deficiency is common in many parts of the world, particularly in developing countries (Boisvert et al., 1993). The deficiency can be treated with dietary supplement of riboflavin or by consuming fermented foods such as cheese, yogurt fortified with riboflavin in the daily diet. Riboflavin has traditionally been produced by chemical processes, but in recent times this has been replaced by microbial fermentation processes using organisms like Ashbya gossypii (Stahmann et al., 2000).

Identifying key components of a medium plays a major role in the commercialization of fermentation products. It has large effect on product concentration, yield and volumetric productivity (Kennedy and Krouse, 1999). LAB is heterotrophic and fastidious with complex nutritional requirements. The medium designed by de Man, Rogosa and Sharpe (MRS) (de Man et al., 1960) is widely used for cultivation of *Lactobacillus* spp. While several other media have been designed for commercial production of lactic acid (Hujanen et al., 2001), optimization for the production of probiotic microorganism (Liew et al., 2005), especially vitamin production has not been optimized with LAB. We report the optimization of the MRS medium components, peptone, beef extract, glucose and K_2HPO_4 for enhanced production of

*Corresponding author.
Email: *scientistjaya@gmail.com*
Tel: 91-416-220-2901, Fax: 91-416-224-3092

riboflavin by *Lb. fermentum* MTCC 8711 using a factorial response surface design.

Materials and Methods

Microorganism

A lactic acid bacterial strain producing riboflavin was isolated from yoghurt. It was identified as *Lactobacillus fermentum* MTCC 8711 and is deposited in Microbial Type Culture Collection and Gene Bank, Chandigarh, India (Accession number MTCC 8711). The 16S rDNA sequence of this strain is available in GenBank (NCBI) with the accession number GU213430.

Fermentation

Seed culture was prepared by growing a single colony of *Lb. fermentum* in 25 ml of MRS broth at 37ºC under static conditions for 24 h. Batch fermentation was carried out under static conditions at 37ºC in 250-ml Erlenmeyer flasks containing 50 ml of fermentation medium inoculated with 1% v⁄v seed culture. After 16 hours of fermentation, the samples were harvested and the quantity of riboflavin and the growth (A_{600}) were estimated.

Estimation of riboflavin

Riboflavin was estimated as described by Sauer et al. (1996). In brief, 0.8 ml of culture broth was mixed with 0.2 ml of 1 M NaOH. From this, 0.4 ml was neutralized with 1 ml of 0.1 M potassium phosphate buffer (pH 6.0). The absorbance was read at 444 nm in Shimadzu spectrophotometer, Shimadzu Corporation, Tokyo Model Pharmaspec UV-1700. Riboflavin concentration was calculated using an extinction coefficient of 1.04×10^{-2} $M^{-1}cm^{-1}$. Growth was estimated by measuring the absorbance at 600 nm against un-inoculated medium as blank.

Experimental design

A factorial central composite design (CCD) for four factors with replicates at the central point and star points was used for optimization of the fermentation medium. Four components of the MRS broth i.e. peptone, beef extract, glucose and K_2HPO_4, each at five coded levels namely, low star point, low level, central level, high level and high star point (indicated as -α, -1, 0, + α, +1 respectively as shown in Table 1) were designed as experimental runs using Design-Expert 7.1.6 (StatEase, Inc., Minneapolis, USA). The CCD experiments contained a total of 30 experimental trials that included 16 trials for factorial design, eight trials for axial points (two for each variable) and six trials for the replication of the central points (Box and Wilson, 1951) (Table 2). All experiments were carried out at 37ºC and at pH 6.5 for 16 h. The growth and riboflavin production was analyzed with the statistical software package, Design-Expert 7.1.6.

Results and Discussion

Riboflavin production by Lb. fermentum

Lb. fermentum MTCC 8711 produced 2.28 mg L^{-1} of riboflavin with a cell density of A600 value 1.7 after 16 h of fermentation in the unoptimized MRS broth. The same strain under optimal composition (run 27) produced a maximum riboflavin production of 9.43 mg L^{-1} with elevated K2HPO4 (7 g L^{-1}) and the normal concentrations of peptone, beef extract and glucose. The maximum growth with A600 value of 3.59 was also observed in the same run. Among the tested models, linear model was found to be the 'best fit model' for the riboflavin production with the highest F-value when compared to other models (Table 3). ANOVA for the linear model of riboflavin

Table 1. Variables and their levels for optimization of fermentation medium

Factor (g L^{-1})	Low level star point (-α)	Low level factorial (-1)	Central Point (0)	High level factorial (+1)	High level star point (+α)
A – Peptone	15	20	25	30	35
B –Beef extract	15	20	25	30	35
C – Glucose	30	40	50	60	70
D – K_2HPO_4	3	4	5	6	7

Table 2. Experimental design and results of central composite design for optimization of fermentation medium

Run	A	B	C	D	Riboflavin production (mg L^{-1})	Growth (A_{600})
1	0	0	0	-α	3.98	2.34
2	1	1	-1	-1	4.97	2.61
3	α	0	0	0	5.57	2.88
4	- α	0	0	0	7.69	2.98
5	0	- α	0	0	8.15	3.11
6	1	1	1	-1	4.97	2.58
7	-1	-1	1	1	8.01	3.00
8	0	0	0	0	7.76	2.92
9	-1	-1	-1	-1	7.62	2.74
10	0	0	0	0	7.62	2.90
11	0	0	0	0	7.62	3.01
12	0	0	0	0	7.62	2.90
13	1	-1	1	1	7.87	3.00
14	0	α	0	0	5.51	2.86
15	0	0	α	0	5.60	2.73
16	-1	1	-1	-1	4.97	2.65
17	1	-1	-1	-1	7.64	2.72
18	-1	-1	-1	1	8.94	3.48
19	0	0	- α	0	8.13	3.12
20	1	1	1	1	5.57	2.87
21	-1	-1	1	-1	4.98	2.66
22	1	-1	1	-1	4.78	2.56
23	-1	1	-1	1	8.12	3.08
24	-1	1	1	-1	4.89	2.63
25	-1	1	1	1	8.18	3.06
26	1	1	-1	1	9.34	3.34
27	0	0	0	α	9.43	3.59
28	0	0	0	0	7.78	2.93
29	1	-1	-1	1	9.25	3.34
30	0	0	0	0	7.79	2.87

Table 3. Model fit summary for riboflavin production

Model	Sum of Squares	Degrees of freedom	Mean Square	F Value	p-value Prob > F
Linear	61.27	4	15.32	28.56	< 0.0001
Two Factor Interaction (2FI)	2.78	6	0.46	0.83	0.5635
Quadratic	3.61	4	0.90	1.93	0.1582
Cubic	6.06	8	0.76	5.49	0.0184

Table 4. Regression analysis (ANOVA) for riboflavin production

Source	Sum of squares	Degrees of freedom	Mean Square	F-Value	p-Value (Prob > F)
Model	61.27	4	15.32	28.56	< 0.0001
A	1.29	1	1.29	2.40	0.1337
B	7.44	1	7.44	13.87	0.0010
C	11.56	1	11.56	21.57	< 0.0001
D	40.98	1	40.98	76.42	< 0.0001
Residual	13.41	25	0.54		
Lack of fit	13.37	20	0.67	89.64	< 0.0001
Pure error	0.037	5	0.007457		
Total	74.67	29			

Table 5. Regression analysis (ANOVA) for the growth

Source	Sum of squares	Degrees of freedom	Mean Square	F-Value	p-Value (Prob > F)
Model	2.07	4	0.52	62.51	< 0.0001
A	0.0096	1	0.0096	1.16	0.2924
B	0.058	1	0.058	6.99	0.0139
C	0.24	1	0.24	28.44	< 0.0001
D	1.77	1	1.77	213.46	< 0.0001
Residual	0.21	25	0.008298		
Lack of fit	0.20	20	0.009798	4.27	0.0572
Pure error	0.011	5	0.002297		
Total	2.28	29			

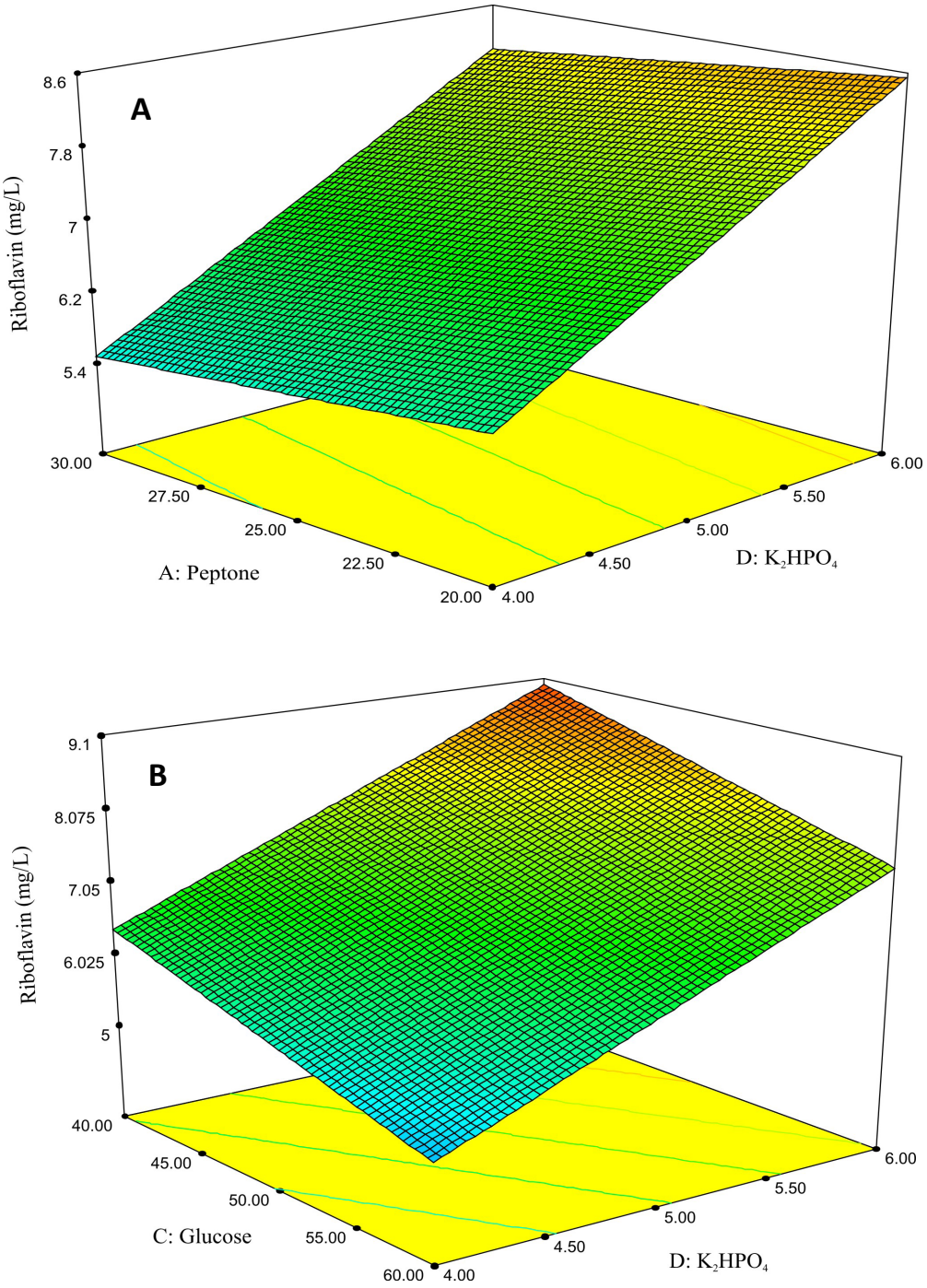

Figure 1. Response surface plots of riboflavin production by *Lb. fermentum* MTCC 8711.

A- Effect of peptone and K2HPO4 upon riboflavin production
B- Effect of glucose and K2HPO4 upon riboflavin production

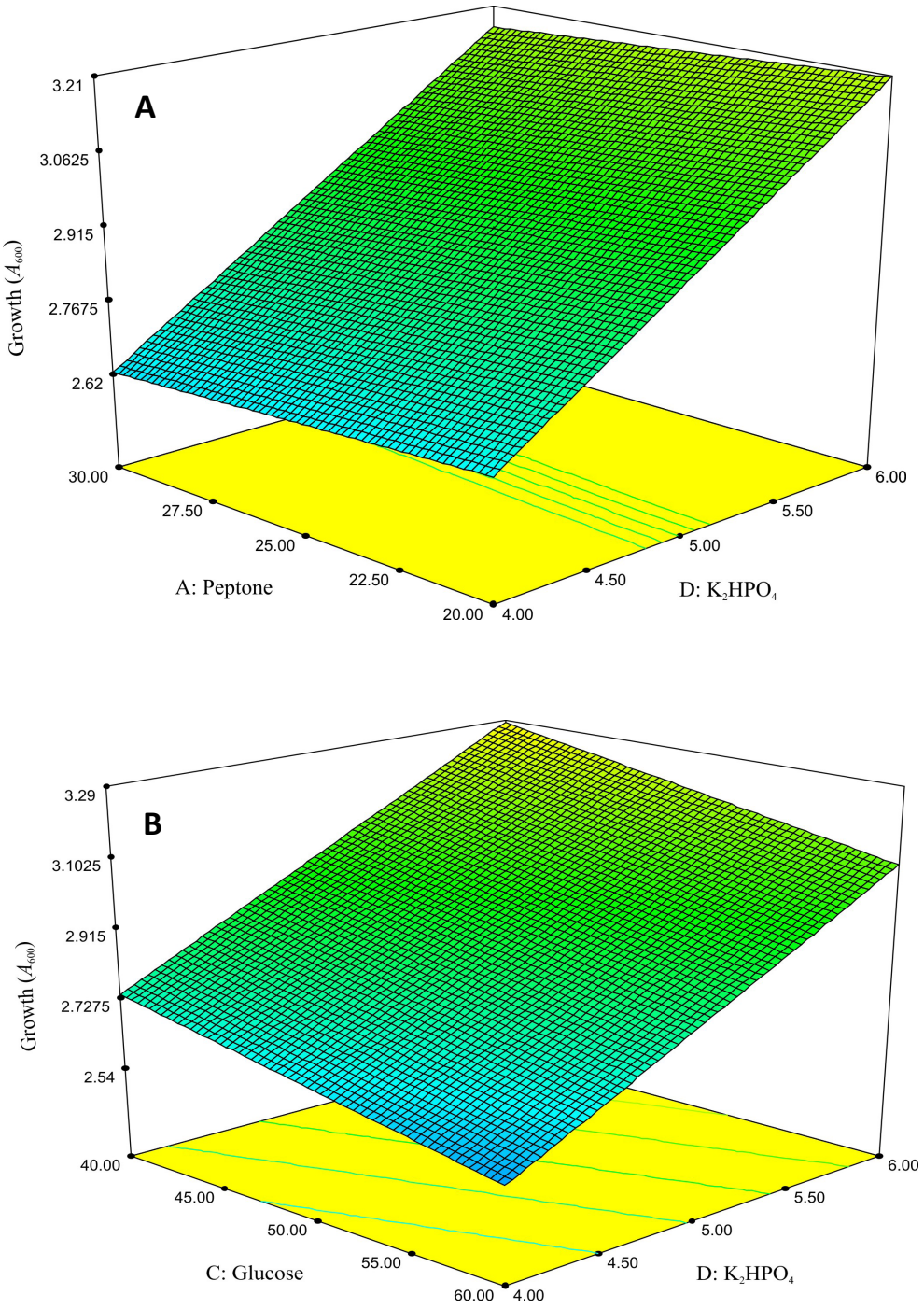

Figure 2. Response surface plots of growth by *Lb. fermentum* MTCC 8711

A- Effect of peptone and K2HPO4 upon growth
B- Effect of glucose and K2HPO4 upon growth

production showed that the confidence levels were greater than 99.99% (Table 4). The F-test with very low error probability (P_{model}>F=0.001) demonstrated a very high significance for the regression model (Khuri and Cornell, 1987).

The goodness of fit of the model was verified by the determination coefficient (R^2). In this study, the value for the riboflavin production was 0.8205. The value of the adjusted determination coefficient (Adj R^2 =0.7917) advocates a high significance of the model (Akhnazarova and Kafarov, 1982). The higher correlation coefficient (r) value of 0.945 indicated good correlation between the observed and the expected values (Box et al., 1978). A lower value of the coefficient of variation (CV=10.44%) confirmed the reliability and precision of the experiment (Box and Wilson, 1951).

Beef extract (B), glucose (C) and K_2HPO_4 (D) were found to be the significant model terms for riboflavin production. The 'lack of fit tests' which compares the residual error to the 'pure error' from replicated design points indicated a 'lack of fit F-value' of 89.64%, which significantly implies that there is only a 0.01% chance that a 'lack of fit F-value' could occur because of noise. The adequate precision measures the signal (response) to noise ratio (deviation). An adequate precision ratio greater than 4 is desirable. The ratio of 18.66 obtained in this study indicates an adequate signal of the model to be significant for the process of riboflavin production.

The observed values are the experimentally obtained values and the predicted values were calculated based on the respective model equation for each experimental run. There was a good coordination between the observed and the predicted values in all four models. Regression equation of the linear model was analyzed using 3D response surface plots, which help to understand the effect of medium components and their range of optimum concentrations required for higher riboflavin production. 3D response surface plots were obtained by plotting the response (riboflavin production) on the Z-axis against any two variables while keeping other two variable at their '0' level. (Figure 1). K_2HPO_4 exhibited positive response on riboflavin production. Increasing the concentrations of K_2HPO_4 showed higher riboflavin production in all combinations of other variables.

The final equation for riboflavin production in terms of the actual factors is as below

$$Y_1=+7.89-0.046A-0.11B-0.069C+1.3D \quad (1)$$

where, Y_1 is the riboflavin production in mg L^{-1}; A is Peptone (g L^{-1}); B is beef extract (g L^{-1}); C is glucose (g L^{-1}) and D is the concentration of K_2HPO_4 (g L^{-1}).

Similarly, the ANOVA for linear model for growth showed a model F-value of 62.51, which implies that the model is significant and there is only a 0.01 % chance that a model F-value could occur because of noise (Table 5). Beef extract (B), glucose (C), K_2HPO_4 (D) were found to be the significant model terms for the growh also. The "adequate precision" ratio of 29.22 obtained in this study indicates an adequate signal of the model to be significant for the process of growth. The R^2 value for growth in the model was 0.909. In order to check the effect of factors on the response as well as to determine the optimal level of the K_2HPO_4 on the growth, three dimensional response surface plots were constructed (Figure 2). Similar to the riboflavin production, K_2HPO_4 played a key role in the increase of growth also.

The final equation for growth in terms of the actual factors is given as

$$Y_2 =2.39-0.004A-0.0098B-0.0099C+0.27D \quad (2)$$

where, Y_2 is the growth in terms of A600. A is peptone (g L^{-1}); B is beef extract (g L^{-1}); C is glucose (g L^{-1}) and D is the concentration of K_2HPO_4 (g L^{-1}).

The statistical analysis of the data suggested that except for the individual factors, none of the interactions had significant effect on the riboflavin production as well as the growth. Riboflavin production by *Lb. fermentum* MTCC 8711 was improved by the higher concentration of K_2HPO_4. It acts as a good source of both potassium and phosphorous moieties. Similarly, Wu et al (2007) have reported that K_2HPO_4 acted as one of the most significant variables that influenced the riboflavin production by Bacillus subtilis. They have shown that increasing the concentration of K_2HPO_4 resulted in the riboflavin overproduction suggesting the positive effect of K_2HPO_4 by offering phosphorous as the source of energy, which is vital for the growth of the cells and the riboflavin synthesis . Riboflavin is synthesized from GTP as the precursor molecules. The purine biosyntheic pathway for formation of purines is related to riboflavin production since the pyrimidine moiety of the riboflavin is offered by the GTP molecules, that are basically guanine derivatives (Baugh and Krumdiek, 1969). The purine biosynthesis pathway is positively influenced by potassium molecules too (Friedman and Fox, 1954). In our study also, K_2HPO_4 was found to influence riboflavin production significantly.

The process of fermentation increases the riboflavin content. Also riboflavin production

by biological methods involving fermentation is preferred at the commercial scale. This is because the process is economical involving less energy and reduces the amount of waste also (Lim et al., 2003). The media components used in this study are the constituents of the MRS broth and they are commercially available standard ingredients, which could be used at a commercial scale. Based on the results obtained, optimized fermentation medium designed with the components (g L^{-1}): peptone 25; beef extract 25; glucose 50; K_2HPO_4 7; yeast extract, 5; tween 80, 1; sodium acetate, 5; ammonium citrate, 2; magnesium sulphate, 0.2; manganese sulphate, 0.05. *Lb. fermentum* MTCC 8711 grown in this medium produced 9.43 mg L^{-1} of riboflavin with the growth (A600) of 3.43. The validation run based on the optimum production in run 27 resulted in the maximum (9.43 mg L^{-1}) riboflavin yield as compared with the predicted value of 9.63 mg L^{-1}. The riboflavin produced in the optimized medium was four folds higher than that of the unoptimized medium (2.28 mg L^{-1}). Since the *Lb. fermentum* produces high-level of riboflavin in the optimized media, the organism can be considered as a potential candidate for the food industry.

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