Optimization of riboflavin production by *Rhodotorula glutinis* using statistical design

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Background and objective
Riboflavin is considered a significant nutritional and growth factor in humans, animals, plants, and microorganisms. This water-soluble vitamin is an intrinsic component of the basic metabolic processes and is a precursor of the oxidation-reduction coenzymes, flavin mononucleotide and flavin adenine dinucleotide. Riboflavin was synthesized by different microorganisms such as *Ashbya gossypii*, *Eremothecium ashbyii*, *Candida famata*, and *Bacillus subtilis*.
The factors controlling riboflavin production, such as environmental and nutritional factors, were implemented to increase the yield of riboflavin. A statistical experimental design, such as the Plackett–Burman, enables the finding out of the most effective factors that increase the yield of riboflavin.
Materials and methods
One-factor-at-a-time approach was used to evaluate the effect of different nitrogen and carbon sources for riboflavin production by a local yeast isolate *Rhodotorula glutinis*. To optimize the riboflavin production by *R. glutinis*, the combined effects of seven variables (lactose, yeast extract, KH_2PO_4, $MgSO_4$, time, pH, and rpm) were assessed using the Plackett–Burman design.
Results and conclusion
A preliminary medium formulation trial suggested that yeast extract and lactose were the appropriate nitrogen and carbon sources, respectively. The two-level Plackett–Burman design was implemented to study the nutritional and environmental factors for riboflavin production. Among the seven variables tested, lactose, KH_2PO_4, and rpm were identified as the most significant factors. The optimal levels of the three variables were determined by response surface methodology based on the Box–Behnken design. The validity of the model developed was verified, and the maximum riboflavin concentration was 88.25 μg/ml, representing 1.27 folds higher in the improved medium.

Keywords:
Box–Behnken design, Plackett–Burman design, *Rhodotorula glutinis*, riboflavin

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Introduction

Many valuable biological molecules such as nucleic acids, proteins, polysaccharides, and vitamins are useful in human and animal health. Vitamins are classified as chemical substances that control the physiological processes as well, as they are essential for the metabolism and the growth of animals, especially mammals. A variety of plants and microorganisms can synthesize vitamins, whereas the mammalian cells have lost the capacity to synthesize these vitamins [1].

Riboflavin can be produced commercially by chemical or biological synthesis, and the latter is preferred because it decreases the cost, reduces the waste and energy requirements, and makes use of renewable resources [2]. Riboflavin was synthesized by many microorganisms such as bacteria, yeast, and fungi [3]. *Clostridium acetobutylicum* was the first microorganism reported to produce riboflavin and subsequently two other microorganisms, ascomycetes, namely *Eremothecium ashbyii* and *Ashbya gossypii* [4–7]. Some microorganisms of the genus *Candida* were used as riboflavin producers [1]. In yeast, inhibition owing to iron should be overcome [8].

The medium composition in optimum ratio can influence both the efficiency and productivity of bioactive microbial metabolites. The conventional methods of optimizing medium composition are carried out via sequential variation of a single parameter (one-factor-at-a-time technique), whereas all other factors are fixed at a specific level [9]. These

often fail to identify the optimal conditions for the bioprocess because the effects of interactions between various factors are ignored [10].

Moreover, the conventional 'one-factor-at-a-time' approach needs a considerable amount of work and time and sometimes leads to unreliable results [11]. Hence, statistically based experimental designs have proved to be the most popular method for optimization of the microbial fermentation process.

The designing of an appropriate fermentation medium is of great importance to improve the riboflavin yield, concentration, volumetric production, and the ease of downstream product separation [12].

Response surface methodology (RSM) is a collection of statistical and mathematical techniques employed to design experiments, build models, and evaluate the effects of factors and has proved to be powerful and useful for the optimization of the target metabolites production [13,14]. A statistical experimental design decreases the error in determining the effect of parameters and showed the coinciding, systematic, and effective variation of all parameters [15]. RSM is an efficient tool for optimizing the process condition that used quantitative data from an appropriate experimental design at the same time to solve multivariate equations [16]. An equation is employed to explain and describe the combined effect of all the tested variables within the response.

For this reason, the present study was concerned with the optimization of riboflavin production using RSM.

Materials and methods

Microorganism and culture conditions

Rhodotorula glutinis used throughout the present work was kindly obtained from the microbiology Department, Faculty of Science, Ain Shams University.

The cultures were maintained on potato-dextrose agar slopes with transfer at monthly intervals.

Culture medium

An inoculum containing 8.4×10^7 CFU/ml of yeast cells was inoculated into 50 ml of yeast extract–peptone–dextrose enhancement medium containing 2% dextrose, 2% peptone, and 1% yeast extract and was aerobically incubated in a shaking incubator at 28°C and 170 rpm for 48 h [17,18].

Riboflavin production medium

When the culture reached a high cell density of OD_{600nm}=1.5, 0.2 ml of the yeast culture was inoculated into 50 ml of the production medium, which contained 2% of sucrose, 0.5% of $(NH_4)_2SO_4$, 0.1% of KH_2PO_4, 0.05% of $MgSO_4.7H_2O$, 0.01% of $CaCl_2.2H_2O$, 0.01% of NaCl, and 0.2% of yeast extract [17,18]. The flask was incubated in a shaking incubator at 28°C and 170 rpm for different time intervals [19].

Erlenmeyer flasks of cells were centrifuged at 4000 rpm for 15 min. The supernatant was used as the crude riboflavin solution for determination of riboflavin.

Qualitative assessment of riboflavin

The riboflavin was identified by the preparative thin-layer chromatography (TLC) technique, whereby samples of the tested material as well as the authentic riboflavin from Sigma-Aldrich (Steinheim, Germany) were introduced onto silica gel plates and then developed by a solvent system of n-butanol: acetic acid : distilled water (4 : 1 : 5 v/v). Riboflavin was detected by applying 2 µl of methylene chloride on the TLC plate before applying the tested sample and authentic riboflavin standard on TLC plate, and then the plate was examined in ultraviolet light at 365 nm. The developed spots on silica gel giving a yellow fluorescence were identified as riboflavin as compared with a standard sample [20].

Quantitative assessment of riboflavin

The amount of riboflavin in the supernatant was measured quantitatively at a wavelength of 440 nm using a spectrophotometer, and riboflavin from Sigma-Aldrich was used as a standard [19,21]. Moreover, the supernatant obtained at the end of incubation was filtered using a 0.22-µ membrane filter before the analysis with high-performance liquid chromatography (HPLC). For this method, C18 column with a mobile phase comprised of methanol : water (30 : 70) at a constant flow rate of 1 ml/min was used. A ultraviolet detector that set on a wavelength of 254 nm was employed for the detection of peaks [22].

Experimental designs

The Plackett–Burman design

The effects of levels of seven variables (lactose, yeast extract, KH_2PO_4, $MgSO_4$, time, pH, and rpm) were studied for the optimization of riboflavin production using the Plackett–Burman design [23]. The most critical parameters by *R. glutinis* were studied with JMP 8 software (SAS Institute Inc., Cary, North Carolina, USA) using Plackett–Burman design. For

each variable, high (+), low (−), and medium (0) levels were tested. Each trial represents the average value of riboflavin yield (μg/ml) as an independent response.

Box–Behnken design

RSM using Box–Behnken design was used for further optimization of riboflavin yield, and the significant factors were studied using Box–Behnken design [24]. The experimental design comprised 13 runs at three levels. Each run had three replicates, and the average value of riboflavin yield was represented as a dependent response. The significance of the model was determined by the analysis of variance. The regression equation was obtained. A P value less than 0.05 indicates that the model is significant. The fit of the model (R^2) was also determined. The values of the coefficient were calculated, and the optimum concentrations were predicted using JMP 8 Software (SAS Institute Inc., Cary, North Carolina, USA).

Validation of the experimental model

It was carried out under the conditions predicted by the experimental model. The experiment was examined in triplicates.

Results and discussion

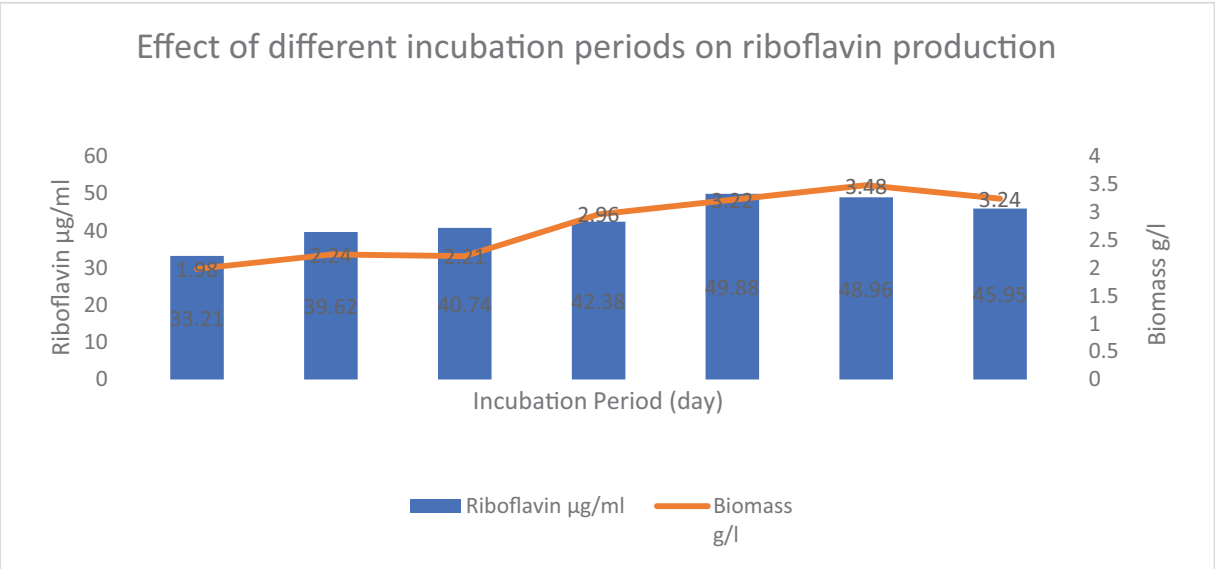
Effect of fermentation time on riboflavin biosynthesis

The growth of *R. glutinis* and the riboflavin production was estimated during a fermentation period extended for 7 days, as shown in Fig. 1. The results showed that the growth and maximal riboflavin production were attained after 6 and 5 days, respectively, which was the stationary phase of the microorganism. However, the yeast growth and vitamin content decreased with further extension of the fermentation period over 6 days. Similar results have been reported by Ertrk *et al.* [3], and Stahmann *et al.* [5]. This showed a two-phasic process of flavin synthesis: biomass production followed by the flavin formation. Similar results were obtained using *Mycobacterium* [25], yeasts [26], and fungi [20].

Effect of various nitrogen sources on riboflavin biosynthesis

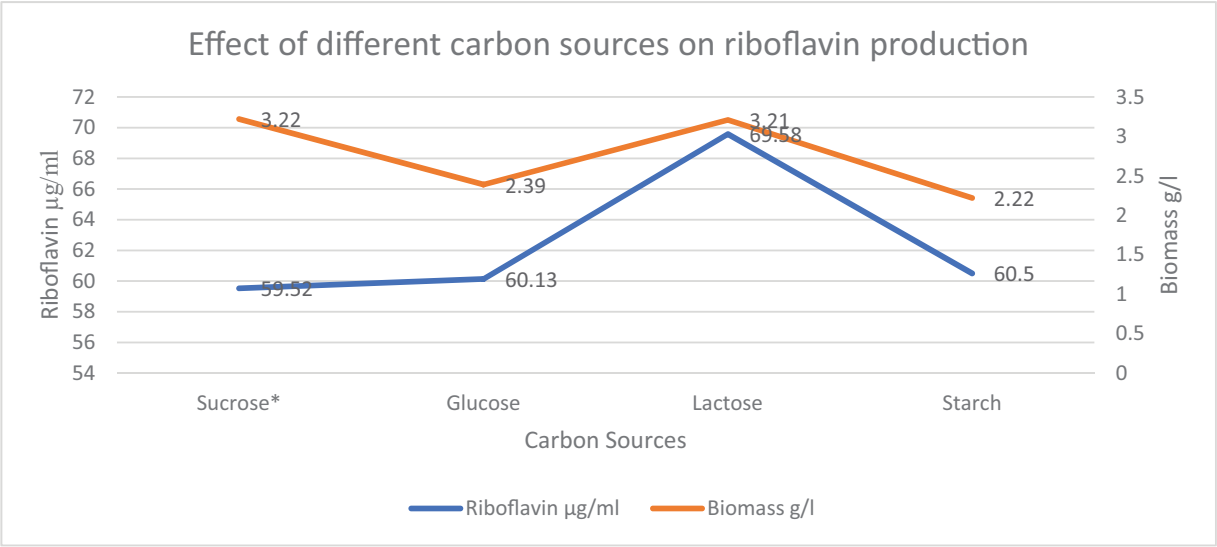
The influence of different types of nitrogen sources (yeast extract, peptone, ammonium sulfate, beef extract, and corn steep liquor) was investigated. Each nitrogen source was added on equivalent nitrogen base (0.2% yeast extract and 0.5% ammonium sulfate) of the basal fermentation medium using sucrose as a carbon source. The final riboflavin production was listed in Fig. 2. From the results, it is shown that yeast extract had a significant influence on riboflavin biosynthesis, in which the riboflavin production was 53.44 μg/ml. This may be owing to the fact that yeast extract is a complex raw material and rich in amino acids, peptides, growth factors, vitamins, and trace elements commonly used in fermentation processes [27]. In addition to its low cost, the presence of vitamin B in yeast extract may be important factor for riboflavin production [28]. Nishio and Kamikobu had reported that yeast extract and casamino acid stimulated riboflavin biosynthesis by *Pichia guilliermondii*, whereas other nutrients had no

Figure 1



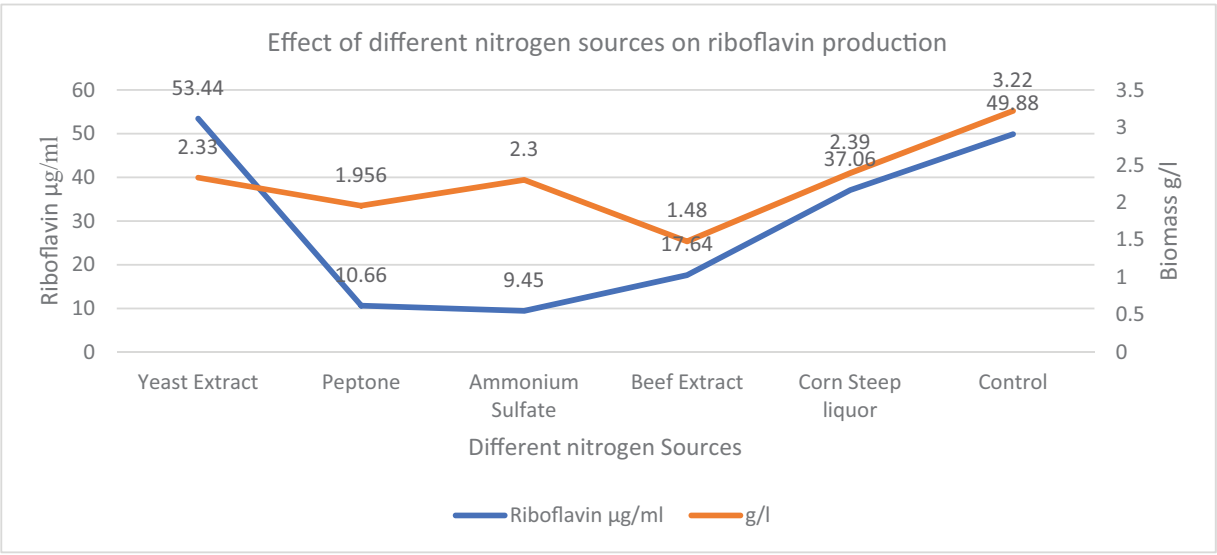
Effect of fermentation time on riboflavin production by *Rhodotorula glutinis*.

Figure 2



Effect of different nitrogen sources on riboflavin production by *Rhodotorula glutinis*.

Figure 3



Effect of different carbon sources on riboflavin production by *Rhodotorula glutinis*.

effect [29]. Suzuki *et al*. [28] showed that 0.5% yeast extract gave the highest yield of riboflavin. Wu *et al*. [21] reported that yeast extract was superior to other organic sources. Nonetheless, each organism has its own regulatory mechanism [18].

Effect of various carbon sources on riboflavin biosynthesis

For the investigation of the effect of different carbon sources on riboflavin production by *R. glutinis*, different carbon sources (glucose, lactose, and starch) were separately used in equimolar amounts to the basal carbon source (2% sucrose). After 5 days of shake flask cultivation, the final riboflavin production is shown in Fig. 3. It could be concluded that lactose was the optimal carbon source for riboflavin fermentation (69.58 µg/ml).

Carbon sources are important as they help in cell growth, maintenance of the cell, and riboflavin production. Different carbon sources were used for riboflavin biosynthesis where Suzuki *et al*. [28] showed that riboflavin was produced in the presence of sucrose, whereas Lim *et al*. [1] used glucose as a carbon source. Özbas and Kutsal [30] found that maximum riboflavin production was obtained in the presence of glucose. Vanetti and Aquarone [31] stated that maximum riboflavin production was obtained in

the presence D-xylose. However, each microorganism has its own regulatory mechanism, starting its substrate preferences regardless of the medium balanced [28].

Detection of riboflavin with high-performance liquid chromatography

HPLC analysis of riboflavin had been carried out with a Waters HPLC system (Waters Corporation, Milford, Massachusetts, United States) equipped with a quaternary pump, an auto sampler injector supplied with 100 µl fixed loop, injector thermostat compartment for the column, and ultraviolet detector. The chromatographic column was C18 kromasil (250 mm×4.6 mm, 5 µm). The column was kept at room temperature at a flow rate of 1 ml/min. Separation of riboflavin was carried out by elution with methanol : water (30 : 70). Detection wavelength for detection of riboflavin was set at 254 nm. The retention times of riboflavin test and standard were the same.

Statistical analysis for optimization of riboflavin production

The Plackett–Burman design

The medium formula containing lactose substrate and yeast extract as nitrogen sources for riboflavin synthesis was chosen as the basal medium for an optimization strategy that involved a two-phase experimental design. Several statistical experimental designs were applied to optimize all the parameters collectively to eliminate the limitations of one-factor-at-a-time process [32,33]. The first step was to evaluate the relative importance of various fermentation factors by applying a Plackett–Burman design. In the second phase, levels of the variables, which have significant influences on riboflavin formation, were further investigated.

The relative importance of various environmental factors involved in the process of riboflavin production was explored using the Placket–Burman design (1946). Examined levels of seven culture variables are presented in Table 1. The design was applied with nine trials with three levels of each variable and the corresponding riboflavin production, as shown in Table 2. The results in Table 2 showed a variation in vitamin production yield from 70.42 to 77.68 µg/ml. Thus, the optimization of the cultural conditions and medium is necessary for high riboflavin production [12]. Maximum production of riboflavin was observed in run number 5 where the yield was 77.68%. The main effects of each variable upon riboflavin production were estimated and expressed graphi graphically (Fig. 4). The results showed clearly that the riboflavin production was positively affected by KH_2PO_4 and rpm. On the contrary, lactose, yeast extract, time, pH, and $MgSO_4$ showed negative effect on riboflavin production. In terms of KH_2PO_4, the requirement of phosphorus for vitamin formation depends on the microorganism under investigation. A deficiency of phosphate stimulates riboflavin production by *Mycobacterium lacticolumn*

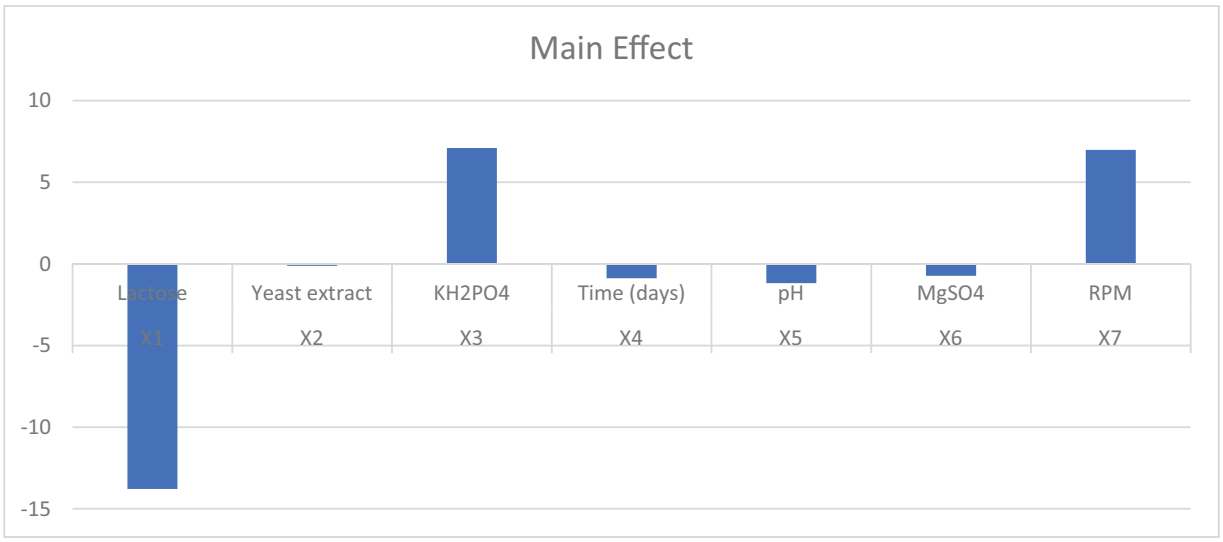
Table 1 Screened variables in the Plackett–Burman design including real values for the three levels of the variables

		Levels		
Independent variables	Symbol	−1	0	+1
Lactose %	X1	1	2	3
Yeast %	X2	0.05	0.1	1.5
KH_2PO_4%	X3	0.05	0.1	1.5
Time (days)	X4	4	5	6
pH	X5	5	6	7
$MgSO_4$%	X6	0.03	0.05	0.07
rpm	X7	100	150	200

Table 2 Plackett–Burman design with seven variables (in coded levels) with riboflavin production as a response

Trial	X1	X2	X3	X4	X5	X6	X7	Yield (µg/ml)
1	−	−	−	+	+	+	−	76.48
2	+	−	−	−	−	+	−	70.76
3	−	+	−	−	−	−	+	77.38
4	+	+	−	+	+	−	−	70.42
5	−	−	+	+	−	−	+	77.68
6	+	−	+	−	+	−	+	77.38
7	−	+	+	−	−	+	−	77.49
8	+	+	+	+	+	+	+	76.68
9	0	0	0	0	0	0	0	70.45

Figure 4



Main effects of independent variables upon riboflavin production by *Rhodotorula glutinis* according to the results of the Plackett–Burman experiment.

but not in *P. guilliermondii* [34]. Similarly, Abdulla *et al*. [35] reported the importance of the presence of KH_2PO_4 at 2.5 g/l for riboflavin production using *Bacillus subtilis* and *Bacillus tequilensis* by RSM design.

The presence of lactose is important for growth and vitamin production but the excessive carbon source may cause metabolic overflow and byproduct accumulation, which is a disadvantage to riboflavin production [21]. On the contrary, fructose was a superior carbon source in comparison with glucose, arabinose, and maltose for production of riboflavin using *B. subtilis* ATCC 6051 [36]. Different carbon sources were used such as oils [37], whey [3], and hydrocarbons [20]. However, each organism has its own substrate preferences [28].

In our study, maximum riboflavin production was at the highest rpm; similar results were reported by Lim *et al*. [38], who investigated that it is important to optimize the rate of agitation in case of using fungi, as they were sensitive to shear stress caused by mechanical agitation.

Box–Behnken design

To approach the optimum response region of riboflavin production, the effective independent variables including lactose concentration (X1), KH_2PO_4 concentration (X2), and rpm (X3) were further investigated, as shown in Table 3, each at three levels according to the Box and Behnken design (1960). However, yeast extract, initial pH, incubation time, and $MgSO_4$ were kept in their zero values.

Table 3 Screened variables in Box–Behnken design including real values for the three levels of the variables

		Levels		
Independent variables	Symbol	−1	0	+1
Lactose %	X1	1	2%	3
KH_2PO_4%	X2	0.05	0.1	1.5
rpm	X3	100	150	200

As shown in Table 4, the highest riboflavin production values (88.25, 78.24, and 77.25 µg/ml) were achieved by the trials number 4, 8, and 12, respectively. On the contrary, it is clear that the lowest riboflavin production records (65.26, 65.36, and 65.89 µg/ml) were achieved by the trials number 5, 11, and 7, respectively.

The three-dimensional response surface curves were then plotted to understand the interactions of the medium components for maximum riboflavin concentration. Figure 5 is the response surface for variation in riboflavin concentration, as a function of two variables with the other nutrients being at constant level.

The results in Fig. 5a–c showed that increasing concentrations of components has a positive influence on the maximum riboflavin production until an optimum value was obtained.

For a precise prediction of the optimal point, a second order polynomial function was fitted to the results of riboflavin production. The correlation between the response and the three independent variables can be

described by the following model..

$$\begin{aligned} Y \text{ yield} = & -11.9468(X_1) - 8.9353(X_2) \\ & - 1.0237(X_3)0.0146(X_1)(X_2)0.0241(X_1)(X_3) \\ & + 0.0281(X_2)(X_3) + 0.0987(X_1)^2 \\ & - 0.0931(X_2)^2 + 0.0051(X_3)^2 \end{aligned}$$

Table 4 Box–Behnken matrix for 3 variables with experimental values of riboflavin production

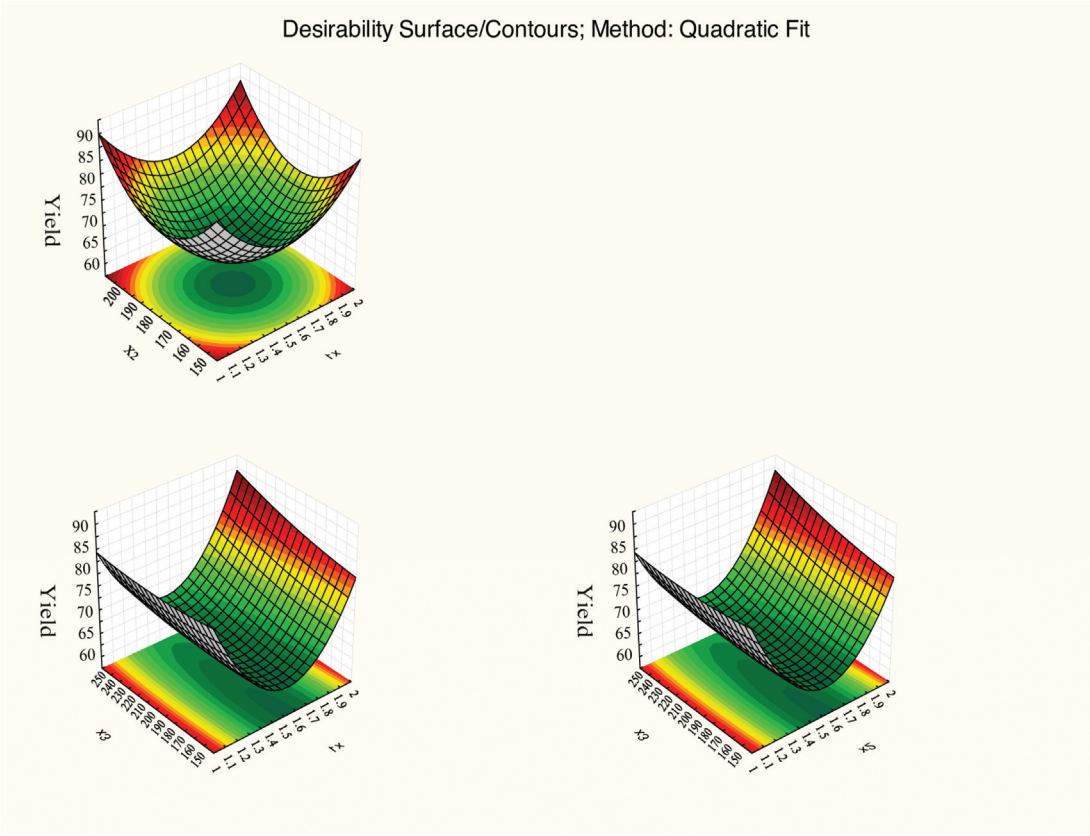
Trial	X1 lactose	X2 KH_2PO_4	X3 rpm	Yield (μg/ml)
1	–	–	0	67.25
2	+	–	0	74.36
3	–	+	0	77.24
4	+	+	0	88.25
5	–	0	–	65.26
6	+	0	–	74.98
7	–	0	+	65.89
8	+	0	+	78.24
9	0	–	–	67.26
10	0	+	–	74.32
11	0	–	+	65.36
12	0	+	+	77.25
13	0	0	0	70.26

Where Y activity is the response (riboflavin production) and X_1, X_2, and X_3 are the coded values of the test variables (lactose, KH_2PO_4, and rpm, respectively).

The plot of the predicated values versus experimental values (Fig. 6) also showed that all the predicted values of Box–Behnken model are located in close proximity to the experimental values. This supports the hypothesis that the model equation is sufficient to describe the response of the experimental observations pertaining to riboflavin production.

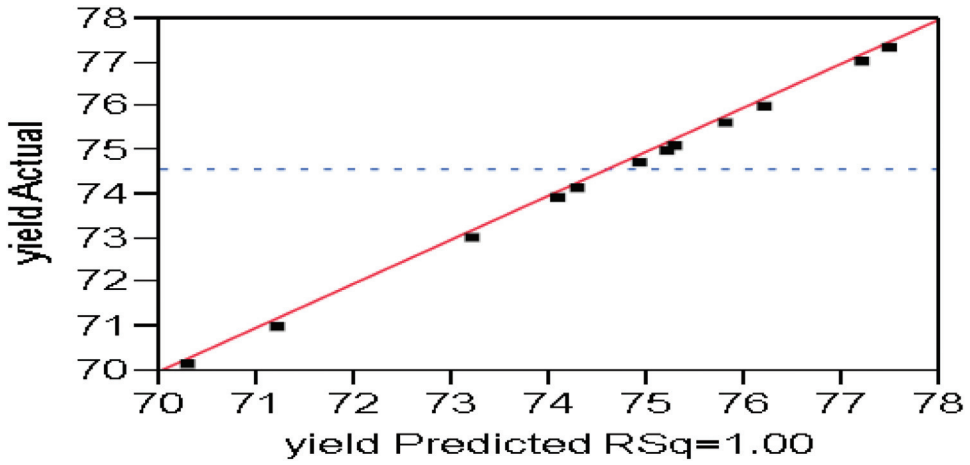
Table 5 shows the regression results from the data of Box–Behnken designed experiments. The results obtained by analysis of variance analysis showed a significant F value (20.77272), which implied the model to be significant. Model terms having values of $P>F$ (0.0001) less than 0.05 were considered significant. The determination of coefficient (R^2) was calculated as 0.9764 for riboflavin production (a value of $R^2>0.75$ indicated the aptness of the model), which indicates the statistical model can explain 97.64% of variability in the response. The goodness

Figure 5



Response surface and contour plots of riboflavin production by the yeast isolate showing the effect of two variables (other variable was kept at zero in coded unit). (a) Interaction between lactose and KH_2PO_4 (X1×2); (b) interaction between lactose and rpm (X1×3); (c) interaction between KH_2PO_4 and rpm (X2×3).

Figure 6



Comparison between the experimental values and the predicted values of RSM. RSM, response surface methodology.

Table 5 Results of regression analysis of Box–Behnken

	Coefficient	SE	*t*	*P*
Intercept	70.2600	1.789853	39.25462	0.000003
X1	−11.9468	2.192238	−5.44961	0.005508
$X1^2$	0.0987	0.012470	7.91294	0.001380
X2	−8.9353	2.192291	−4.07578	0.015151
$X2^2$	0.0931	0.016098	5.78450	0.004438
X3	−1.0237	2.192291	−0.46695	0.664838
$X3^2$	0.0051	0.016098	0.31504	0.768479
X1×2	0.0146	0.020362	0.71549	0.513850
X1×3	0.0241	0.020362	1.18463	0.301742
X2×3	0.0281	0.020620	2.18463	0.201762

Multiple R=0.988178, multiple R^2=0.976496, adjusted R^2=0.929487, SS model=352.3756, DF model=8, F=20.777272.

Table 6 Verification of Box–Behnken-predicted optimal conditions

Trial	Riboflavin	Theoretical yield
Basal	69.58	1
Preoptimized	88.25	1.27

of the model can be checked by the determination of coefficient (R^2) and correlation coefficient (R). The R^2 value is always between zero and one.

The confirmatory experiments were performed in three replicates to validate the optimal point. The riboflavin yield was 88.25 μg/ml, which was 1.27 folds of the improved medium, as shown in Table 6. This indicates that there is a good agreement between the predicted and the experimental results and verified the validity of the model.

Conclusion

In the present study, it was evident that the statistical experimental design offers a practicable approach to the implementation of medium optimization. Our optimized medium is principally composed of common and low-cost components that probably are used for industrial production of this vitamin. The results of verification experiments indicated that the use of the optimized medium led to an evident improvement in riboflavin production for industrial application. The validity of developed model was confirmed by comparing the observed and predicted values at the optimum conditions. The results of our study demonstrated the powerful advantage of Plackett–Burman design for the optimization of variables to achieve high riboflavin production.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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