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Research Article

Activation of Riboflavin Production by *Bacillus subtilis* (KU559874) and *Bacillus tequilensis* (KU559876)

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Abstract

The current study aimed to improve riboflavin production by native bacterial isolates and medium components. Bacterial species *Bacillus subtilis* ASU8 (KU559874) and *Bacillus tequilensis* ASU9 (KU559876) showed high potentiality for riboflavin production. The optimal growth and riboflavin production were achieved after 48 h. The impact of glycine addation to the basal medium was explored by applying different concentrations (0.1-3 g/l). The addition of glycine was effective and the influencing concentration was 1 g/l giving 144.7 and184.2 mg/l yields of riboflavin for *Bacillus subtilis* ASU8 and *Bacillus tequilensis* ASU9, respectively. Medium constituents were also optimized for high riboflavin yield using central composite design (CCD) of response surface methodology (RSM) to optimize the significant values and to study the mutual interactions between these constituents. The analysis revealed that optimal medium concentrations for maximum production of riboflavin by Bacillus subtilis (273.82 mg/l) and *Bacillus tequilensis* (288.33 mg/l) were (g/l): glucose 60, 40; $NaNO_3$ 3, 5; KH_2PO_4 2.5, 1.5; K_2HPO_4 1, 0.5 and $MgSO_4.7H_2$ 0.1, 0.5 for Bacillus subtilis and *Bacillus tequilensis*, respectively. Analysis of variance results showed that the model was significant and an R2 value of 98.06% (*Bacillus subtilis*), 99.34% (*Bacillus tequilensis*) demonstrated that the experimental results were fitted well with predicted values. Optimization of medium conditions using RSM experimental design is an efficient approach for the production of riboflavin through microbial fermentation by Bacillus subtilis and Bacillus tequilensis could be utilized in industrial application.

***Keywords**: Riboflavin; Bacillus subtilis; Bacillus tequilensis; glycine; RSM*

Introduction

Riboflavin (vitamin B2) is an essential nutritional component serving as a precursor of the coenzymes FMN and FAD that are involved mostly in the reactions of oxidative metabolism [1]. This vitamin also plays a significant role in energy metabolism of the cell, and in recent years riboflavin was shown to improve the efficiency of conventional therapies in different diseases such as *Staphylococcus aureus* infection and cisplatin-induced intestinal epithelial cell apoptosis [2,3]. Riboflavin deficiency can affect on mucocutaneous surfaces of the mouth, with the presence of inflammatory processes in lips (cheilitis) and tongue (glossitis) [4]. Riboflavin deficiency is also associated with impaired vision, reduced growth rate, increased levels of homocysteine with consequent cardiac risk [5], preeclampsia [6], and anemia [7].

Various *Bacillus* species have substantial increasing importance as host strains for industrial fermentation processes for, e.g., proteases, purine nucleotides or vitamins [8]. In particular, their ability to secrete large amounts of protein directly into the medium has rendered them very attractive for commercial applications. The nonpathogenic bacterium *Bacillus subtilis* has become a model organism, not only for the genus *Bacillus* but also for Gram positive bacteria in general [9].

Currently, riboflavin is produced industrially by chemical synthesis from ribose [10] but in recent decades, microbial riboflavin production has increased significantly and it may completely replace chemical synthesis [11,13]. *Bacillus subtilis*, a Gram-positive, rod-shaped

bacterium, is exploited in industry for this purpose [14]. Three microorganisms are exploited for riboflavin production: *Ashbya gossypii, Candida famata* and *Bacillus subtilis* with riboflavin production levels reaching 15 g l-1, 20 g l-1 and 14 g l-1, respectively [12,15].

Using recombinant strains of the Gram-positive bacterium *Bacillus subtilis* and filamentous fungus *Ashbya gossypii* were also successfully used in riboflavin production [11,16]. Currently recombinant *B. subtilis* use in large-scale fermentations and producing concentrations exceeding 15 g/L riboflavin [17]. Sauer et al. [9] reported that a recombinant, riboflavin-producing strain of *Bacillus subtilis* give productivities of 80 mg/L at 0.3 h-1 using a glucose-limited chemostat.

Riboflavin is synthesized by many other bacteria and its biosynthetic pathway has been studied extensively in *Bacillus subtilis* and *Escherichia coli*. This has been reviewed by Bacher et al. [18]. *Mycobacterium pheli* also produced small quantities of riboflavin from beet molasses [19]. Riboflavin production in *B. subtilis* was elevated by the increasing the copy numbers of the riboflavin operon and of the ribA gene, coding for GTP cyclohydro-lase II, the first enzyme of riboflavin biosynthesis [17,20].

Bacilli cells convert glucose to guanosine monophosphate (GMP), which is also a precursor of GTP and most of the metabolites concentrate upon GTP, which is the first precursor of riboflavin [11]. Much work showed that GTP (I) is a real immediate precursor of riboflavin. The most conclusive evidence was found in E. coli [21], B. *subtilis* [22], the yeasts *Pichia guilliermondii (Candida guilliermondii*) [23] and *Torulopsis candida (Candida flareri*) [24]. It has been shown that the 4 carbon, 5 carbon and 7 nitrogen atoms of the purine ring originate from glycine [25]. Therefore, glycine stimulates riboflavin overproduction as a precursor of purine and was not associated to cell growth, but only product formation [26]. The effect of glycine addition was studied by both *A. gossypii* [27] and *C. famata* [28] and the incorporation of 14C-glycine into riboflavin produced by *A. gossypii* was shown by Plaut [29] who reported that the effect of glycine supplement on productivity could mean that glycine is either only a limiting precursor or additionally an inducer.

Medium composition can significantly affect product production and designing an acceptable fermentation medium is of crucial importance. Response surface methodology (RSM) is a mathematical technique employed to design experiments, evaluates the effects of factors and describes the interactions between variables or factors with fewer experimental trials [30]. Also is an efficient approach for the production of metabolites through microbial fermentation [31]. The most common type of RSM models is the central composite design (CCD) to suit a second-order polynomial [32].

The purpose of the present study was to enhance simultaneous production of riboflavin by *Bacillus subtilis* (KU559874) and *Bacillus tequilensis* (KU559876) and the optimal medium constituent's concentration using response surface methodology.

Materials and methods

Isolation and identification of bacterial isolates

Different bacterial isolates were isolated from maize, Egyptian clover, lemon, zucchini, banana, and spinach plants grown in different localities in Assiut Governorate. The pure cultures of species were made and maintained on nutrient agar (NA) slants at 4ºC and subcultures monthly.

Screening for riboflavin production by bacteria

To test the ability of bacterial isolates to produce riboflavin, single colonies were transferred into 5 ml nutrient broth [35]. After 12 h aerobically incubation at 30 ± 1ºC and 200 rpm, 1 ml of these pre cultures were used to inoculate 50 ml modified basal medium contained g/l: 50 *glucose*, 5 $NaNO_3$, 0.5 $MgSO_4$, 1.5 KH_2PO_4, 0.5 K_2HPO_4, 0.0025 $ZnSO_4$ and 1000 ml distilled water, pH 6.5. Fermentation was carried out at 30 ± 1°C on a rotary shaker (200 rpm) for 72 h [36]. The culture was centrifuged in pre weighted tubes at 4,000 xg rpm for 15 min and the supernatant obtained sterilized by membrane filtration, using a membrane of pore size 0.22 mm and was used as the crude riboflavin solution for quantitative determination of extracellular riboflavin. The biomass was rewashed and recentrifuged using distilled water to remove any remaining sugars produced and dried at 40ºC overnight for cell dry mass (CDM) determination.

The highly riboflavin producers were identified firstly based on the morphological and biochemical characteristics according to Bergey's Manual [33]. Secondly, bacterial isolates were harvested from well-developed NA cultures for DNA extraction and molecular identi-

fication [34]. The bacterial isolates were grown on NA medium and incubate at 30 ± 1ºC for 24 h. The concentrated growth scraped with sterilized loop and suspended in 100 µl of sterilized distilled water and boiled at 100ºC for 15 min. for stopping the growth and stored at –80ºC. DNA extraction, amplification and sequencing were performed SolGent purification bed in SolGent Company (Daejeon, South Korea). The analysis of 16S rRNA genes was conducted using primer pairs 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) Sequences of the 16S rRNA of strains were first analyzed using the advanced BLAST search program at the NCBI website: http:// www.ncbi.nlm.nih.gov/BLAST/T in order to assess the degree of DNA similarity.

Bacteria growth with riboflavin production

To investigate the effect of different incubation times for biomass and riboflavin production of bacterial strains, the culture flasks inoculated with bacterial cells were kept for an extended period of 72 h and incubated at 30 ± 1°C. The riboflavin production and optical density at 600nm (OD600) were measured every 6 h.

Effect of different concentrations of glycine on riboflavin production

In the production medium, different concentrations of glycine 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3 g/l were tested. Each concentration was prepared with three replicates and incubated in the same fermentation conditions. Cell growth was determined by the measurement of optical density at 600nm (OD600) by a spectrophotometer and extracellular riboflavin was measured.

Optimization of medium constituents by response surface methodology

The RSM used in the present study is a central composite design (CCD) involving five different factors. Experiments were conducted in a randomized fashion. The CCD contains a total of 56 experiments, 54 factorial experimental runs with 2 additional runs at the center point level to check reproducibility was performed for five investigated parameters; *glucose* (A), $NaNO_3$ (B), KH_2PO_4 (C), K_2HPO_4 (D) and $MgSO_47H_2O$ (E), were varied at three coded levels [37]. The range and central point value of all the three process variables are shown in Table 1.

The variables X_i were coded as xi according to the following equation.

$$x_i = X_i - X_0/\delta X \quad (1)$$

Where x_i is the dimensionless value of the independent variable X_i; Xi is the real value of that independent variable; X_0 is the real value of that independent variable X_i at the center point; δX is the step change of real value of the variable *i* corresponding to a variation of a unit for the dimensionless value of the variable *i*.

An empirical relationship between the response and the screened variables was expressed by the following fitting quadratic polynomial equation:

$$Y = \beta_0 + \sum\beta_i x_i + \sum \beta_{ii} x_i^2 + \sum\beta_{ij}x_{ij} \quad (2)$$

Where Y is predicted response, β_0 is offset term, β_i is linear effect, β_{ii} is squared effect, β_{ij} j is interaction effect, x_{ij} the levels of the independent variables.

This regression equation was optimized for optimal values using Sigma XL (Version 6.12). The goodness of fit for the regression model equation was expressed by the coefficient determination as R2 and analysis of variance (ANOVA). The quadratic models were represented as response surface plots of which were generated to indicate an optimum condition using the fitted quadratic polynomial equations obtained by holding one of the independent variables at a constant value and changing the levels of the other four variables.

Analytical assessment

Fermentation broth samples were analyzed for cell dry weight, O. D. (600), glucose and riboflavin concentration: Cell growth was determined by the measurement of optical density at 600nm (OD600) by a spectrophotometer. Glucose was determined using anthrone-sulphuric method [38]. Standard curve of glucose with different known concentrations was prepared. The standard method of estimation

of riboflavin is spectrophotometric using T60 UV with a split beam UV visible spectrophotometer and fixed slit of 2 nm. The instrument covers a wavelength range of 190-1100 nm. A 0.8 ml of centrifuged culture broth was mixed with 0.2 ml of 1 M NaOH and neutralized with 1 ml of 0.1 M potassium phosphate buffer (pH 6.0) as modified by Tajima *et al*. [39]. The amount of riboflavin in the supernatant was measured quantitatively at 444 nm against substrate-free blank. The standard curve was prepared using pure riboflavin.

Results and discussion

Isolation and identification of bacterial isolates

Eighty five bacterial isolates were recovered from different parts of maize, Egyptian clover, lemon, zucchini, banana and spinach plants. The purified isolates were screened for their ability to produce riboflavin on fermented medium. Only two isolates were selected for further characters based on the highest riboflavin production. The selected bacterial isolates were identified firstly using morphological and biochemical characteristics as illustrated in Table 2. Then molecular identification using phylogenetic analysis of 16S rRNA gene sequences was performed. The sequence of approximately 1137 and 1151 base pairs of bacterial strains ASU 8 and ASU9, respectively have sequence with 99% similarity to *Bacillus subtilis* (GQ480495) and 99% *Bacillus tequilensis* (JF411314). So, the bacterial strains were identified as Bacillus subtilis ASU8 (KU559874) and Bacillus tequilensis ASU9 (KU559876). These strains produced the highest amounts of riboflavin on basal medium giving 81.58 ± 0.62, 74.43 ± 0.32 mg/l and dry mass 0.65 ± 0.012, 0.62 ± 0.05 g/l basal medium of *Bacillus subtilis* and *Bacillus tequilensis*, respectively and used for further experiments.

Bacteria growth and riboflavin production

Riboflavin production in the filtrate was detected over range of 0-72 h (Figure 1 A-B). The production not started until 18-24 h.; however growth started after 6h. The maximum optical density (OD 600) was 0.981, 0.947 and the maximum riboflavin production was 112.29, 108.95 mg/l by *Bacillus subtilis* ASU8 (KU559874) and *Bacillus tequilensis* ASU9 (KU559876), respectively. The riboflavin production of constructed strain RF18S was 1.8-fold higher as compared to that of recombinant strains of *B. subtilis* in flask batch culture, 387.6 mg L−1 riboflavin versus 220mg L−1 riboflavin [40] and higher than that of *A. gossypii* in flask batch culture was 228 mg L−1 riboflavin [41]. Lin *et al*. [42] found that, riboflavin accumulation and a titer of 225.1mg L−1 riboflavin was produced by strain *E. coli* RF01S. Riboflavin-producing *Escherichia coli* strain could produced 387.6 mg L−1 riboflavin with a yield of 44.8 mg riboflavin per gram glucose in shake-flask fermentations after over expressing rib B and engineering purine pathway [43]. Sauer *et al*. [9] reported that a recombinant, riboflavin-producing strain of Bacillus subtilis give productivities of 80 mg/l at 0.3 h-1 using a glucose-limited chemostat. Li *et al*. [44] found that when the specific growth rate is at 0.27/h, the theoretical specific riboflavin production rate in *B. subtilis* could reach 0.48 mmol/g CDW/h, *Clostridium acetobutylicum*, which had a productivity of about 100 mg/l, was one of the first organism used to produce riboflavin [11]. Engineered microbes are able to synthesize large quantities of riboflavin as well as catalyzing the other process. These microbes that incorporate all required functionalities into a single strain have proven inherently challenging. These challenges result from plasmid loss occurring during the fermentation process, fragile and instability of engineered cells. Two major reasons for plasmid loss include plasmid instability due to the segregational plasmid loss during cell division and depression of the growth rate of plasmid bearing cells [45, 46]. These drawbacks limit their use in real world applications like industrial bio processing. In sharp contrast to engineered microbes, natural producing isolates holds many appealing properties in a bio processing context, such as stability, functional robustness and the ability to perform complex tasks [47].

Effect of different concentrations of glycine on riboflavin production

To investigate the effect of the addition of glycine on riboflavin production in *Bacillus subtilis* ASU8 (KU559874) and *Bacillus tequilensis* ASU9 (KU559876), glycine added to the culture in the amounts of 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2 and 3 g/l, respectively (Figure 2 A-B). The addition of glycine support and increased the riboflavin production. With the addition of 1 g/l of the glycine, the increase was even 144.74, 184.21mg/l (1.77 %, 2.5%) higher than in control for *Bacillus subtilis* and *Bacillus tequilensis*, respectively. Riboflavin production decreased with the increase of the concentration of glycine over 1 g/l. Also it was found that there is a reverse relationship between growth, riboflavin production and glucose concentration on medium, with increasing growth glucose concentration decrease. Nanchen *et al*. [48] recorded that, varying from 0.11 to 0.38 mmol (glucose) $g^{-1}h^{-1}$, the substrate maintenance coefficient of *E. coli* is obviously lower than

that of wild-type *B. subtilis* (0.44 mmol (glucose) $g^{-1}h^{-1}$, [9], which might contribute to a higher riboflavin yield when a high cell density fermentation process is conducted. Glycine found to be stimulator for riboflavin overproduction as a precursor of purine and was not associated to cell growth, but only product formation [26]. The effect of glycine addition was studied by both *A. gossypii* [27] and *C. famata* [28] and the incorporation of 14C-glycine into riboflavin produced by *A. gossypii* was shown by Plaut [29] who reported that the effect of glycine supplement on productivity could mean that glycine is either only a limiting precursor or additionally an inducer.

Optimization of medium constituents by response surface methodology

By applying multiple regression analysis on the experimental data, the following fitting second-order polynomial equation was found to describe riboflavin production for Bacillus subtilis (Equation 3), Bacillus tequilensis (Equation 4):

Y1 = (174.26) + (-8.66) * A + (-25.2) * B + (7.3) * C + (13.22) * D + (-4.12) * E + (1.76) * AB + (13.78) * AC + (18.69) * AD + (19.82) * AE + (-4.91) * BC + (12.55) * BD + (0.04) * BE + (-3.94) * CD + (-15.91) * CE + (-32.52) * DE + (-37.3) * AA + (28.37) * BB + (-19.92) * CC + (-6.68) * DD + (37.0) * EE. (3)

Y1 = (195.03) + (-34.47) * A + (2.97) * B + (7.65) * C + (9.04) * D + (-3.78) * E + (1.74) * AB + (20.92) * AC + (-4.09) * AD + (1.75) * AE + (9.62) * BC + (1.83) * BD + (8.99) * BE + (-14.67) * CD + (-14.3) * CE + (-8.52) * DE + (4.35) * AA + (-29.19) * BB + (1.81) * CC + (-4.4) * DD + (7.18) * EE. (4)

Where Y is the predicted response of riboflavin production (mg/l), and A, B, C, D and E are the coded values of glucose, $NaNO_3$, KH_2PO_4, K_2HPO_4 and $MgSO_4 .7H_2O$, respectively.

The maximum experimental riboflavin production were 273.82, 288.33 mg/l, whereas the corresponding predicted value were 274.71, 281.4 mg/l for *Bacillus subtilis, Bacillus tequilensis*, respectively using the optimum levels of the medium constituents (Figure 3 A-B). All the predicted values of RSM model were located in close proximity to the experimental values as shown in Figure 3 A-B. These supported that the RSM model is sufficient to explain the data variations and to describe the actual relationships of variables to obtain the maximum riboflavin production. It was demonstrated the powerful advantage of RSM for the optimization of factors to achieve vitamin production from microorganisms [49].

The polynomial Eq. (3, 4) was further tested for adequacy by an analysis of variance (ANOVA) to estimate the significance and suitability of the model. The results of the parameter estimate and ANOVA were shown in Tables 3 and 4. The model F value of 88.63 (*Bacillus subtilis*), 62.77 (*Bacillus tequilensis*) implied that the models were significant. The lack of fit value of 1.67 (*Bacillus subtilis*), 5.93 (*Bacillus tequilensis*) implied that the lack of fit was no significant relative to the pure error (lack of fit $p<0.0001$). The R^2 value indicated that the 98.06 % (*Bacillus subtilis*), 99.34% (*Bacillus tequilensis*) of the entire variation was explained by the model equations and the adjusted R^2 value was 96.96 % *(Bacillus subtilis)*, 98.96% (*Bacillus tequilensis*). The ANOVA results showed that the variables AB (glucose / $NaNO_3$), BE ($NaNO_3$ / $MgSO_4 .7H_2O$), DD (K_2HPO_4 / K_2HPO_4) for Bacillus subtilis, AB (*glucose* / NaNO*3*), AE (glucose / $MgSO_4 .7H_2O$), BD ($NaNO_3$ / K_2HPO_4) for *Bacillus tequilensis* were found to be non-significant and the interactions were also non-significant.

Response surface plots can be used for the 3D visualization of the interaction between the pair-wise of the five factors selected, when the third factor and to determine the optimum concentration of each of them for maximum riboflavin production. Figure 4 (A (1-8), B (1-7)). The highest riboflavin production obtained using the optimal concentrations of the five components as Glucose 60, 40; $NaNO_3$ 3, 5; KH_2PO_4 2.5, 1.5; K_2HPO_4 1, 0.5 and $MgSO_4.7H_2$ 0.1, 0.5 for Bacillus subtilis, Bacillus tequilensis respectively.

Conclusion

In this work, the dynamic behavior of the riboflavin production under different glycine concentrations proved that the production of the strains was simulated. Using *Bacillus subtilis* ASU8 (KU559874) and *Bacillus tequilensis* ASU9 (KU559876) showed potentiality for riboflavin production reaching maximum production after 48h, addition of 1g/l glycine. Also the statistical method of RSM was demonstrated to be effective in selecting the significant factors of medium components and the optimal concentration of those factors. The

optimal conditions to achieve the maximum riboflavin yield were (g/l): *glucose* 60, 40; $NaNO_3$ 3, 5; KH_2PO_4 2.5, 1.5; K_2HPO_4 1, 0.5 and $MgSO_4.7H_2$ 0.1, 0.5 for *Bacillus subtilis*, *Bacillus tequilensis*, respectively. These results proved that the use of the optimized medium led to improvement riboflavin production. Hence, it was evident that the optimal condition for riboflavin production by *Bacillus subtilis* and *Bacillus tequilensis* could be utilized in biotechnological application.

Variable	Component	Level of variable (g/l)		
		-1	0	+1
A	Glucose	40	50	60
B	$NaNO_3$	3	5	7
C	KH_2PO_4	0.5	1.5	2.5
D	K_2HPO_4	0.1	0.5	1
E	$MgSO_4.7H_2O$	0.1	0.5	1

Table 1: *Independent variables (glucose, $NaNO_3$, KH_2PO_4, K_2HPO_4 and $MgSO_4.7H_2O$) and the concentration levels studied in the optimization design.*

Characteristics	ASU 8 *Bacillus subtilis* (KU559874)	ASU 9 *Bacillus tequilensis* (KU559876)
Shape	bacillus	bacillus
Spore formation	+	+
Motility test	+	+
Gram's staining	+	+
Ryu's method	watery	watery
Catalase test	+	+
Oxidation-Fermentation test	-	-
Nitrate reductase	+	+
Gelatin hydrolysis	+	+
Casein hydrolysis	+	-
Starch hydrolysis	+	+
Indole production	-	+
Arginine dihydrolase	-	+
Urease test	-	-
H_2S production	-	-
Voges-Proskauer	+	-
Carbon source utilization:		
L-Arabinose	-	-
D-Fructose	+	+
Citrate	+	-
D-Sorbitol	-	-
Sorbose	-	-
D-Galactose	+	-
Glycerol	+	+

Glucose	+	+
Sucrose	+	+
Lactose	+	-
Maltose	+	+
Mannitol	+	+
Growth at 4°C	-	-
Growth at 37°C	+	+
Growth at 41°C	+	+

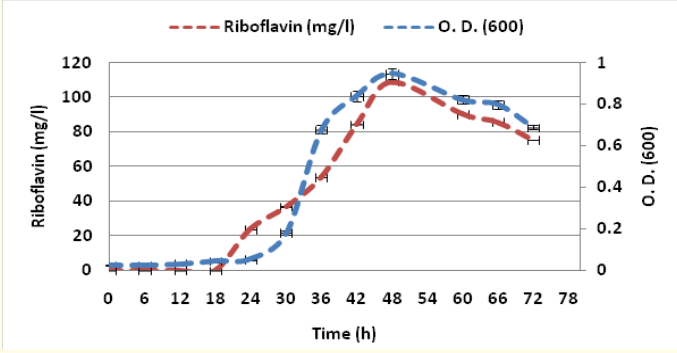
***Table 2**: Phenotypic characterization of riboflavin producing bacteria.*

Factor	Coefficient		t value		P value	
	B. subtilis	***B. tequilensis***	***B. subtilis***	***B. tequilensis***	***B. subtilis***	***B. tequilensis***
Constant	174.3	195.03	83.0	77.89	0.0000	0.0000
A: Glucose	-8.7	-34.5	-6.1	-44.6	0.0000	0.0000
B: $NaNO_3$	-25.2	-2.98	-17.8	-3.85	0.0000	0.0005
C: KH_2PO_4	7.3	7.67	5.1	9.92	0.0000	0.0000
D: K_2HPO_4	13.2	9.04	9.3	11.71	0.0000	0.0000
E: $MgSO_4.7H_2O$	-4.1	-3.78	-2.9	-4.89	0.0064	0.0000
A B	1.8	1.74	1.2	1.84	0.2494	0.0739
A C	13.8	20.93	9.2	22.1	0.0000	0.0000
A D	18.7	-4.09	12.4	-4.3	0.0000	0.0001
A E	19.8	1.75	13.2	1.85	0.0000	0.0726
B C	-4.9	9.62	-3.3	10.17	0.0025	0.0000
B D	12.5	-1.83	8.3	-1.94	0.0000	0.0611
B E	0.04	8.99	0.02	9.50	0.9800	0.0000
C D	-3.9	14.67	-2.6	15.51	0.0130	0.0000
C E	-15.9	-14.3	-10.6	-15.1	0.0000	0.0000
D E	-32.5	-8.531	-21.6	-9.01	0.0000	0.0000
AA	-37.3	4.36	-9.7	5.32	0.0000	0.0000
BB	28.4	-29.19	7.4	-35.62	0.0000	0.0000
CC	-19.9	1.8	-5.2	2.21	0.0000	0.0336
DD	-6.68	-4.4	-1.7	-5.37	0.0916	0.0000
EE	37.0	-7.18	-8.76	9.61	0.0000	0.0000
t – student's test, p – corresponding level of significance,* Significant at p ≤0.05, N, non-significant at p≥0.05						

Table 3: Estimated regression coefficient, t-test and P values for optimization of riboflavin using of the central-composite design.

Source	Degree of freedom	Sum of squares	Mean square	F-value	P-value
***Bacillus subtilis* (KU559874)**					
Model	20	128689	6434.4	88.631	0.0000
Error	35	2540.9	72.598		
Lack of Fit	6	652.96	108.83	1.672	0.0000
Pure Error	29	1888.0	65.102		
Total (Model + Error)	55	131230	2386.0		
***Bacillus tequilensis* (KU559876)**					
Model	20	150631	7531.5	62.77	0.0000
Error	35	1003.2	28.662		
Lack of Fit	6	552.86	92.143	5.934	0.0004
Pure Error	29	450.33	15.529		
Total (Model + Error)	55	151634	2757.0		

Table 4: *Analysis of variance (ANOVA) for the selected quadratic model.*



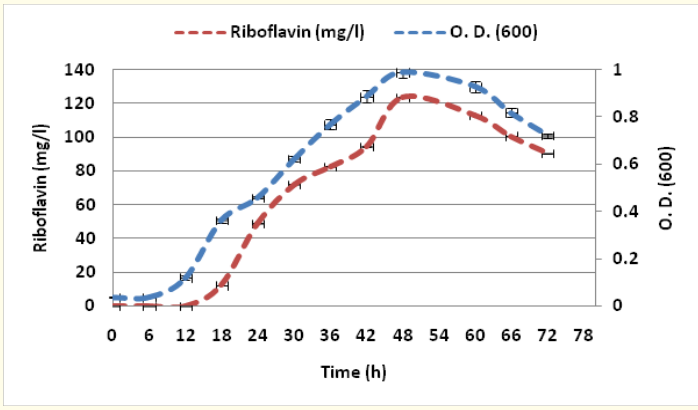
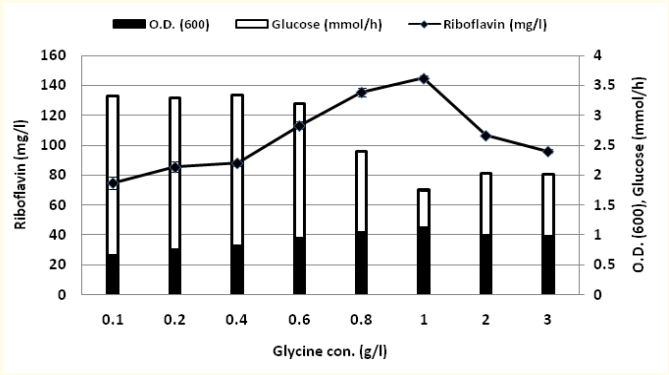


Figure 1: *Effect of incubation time on growth and riboflavin production by Bacillus subtilis ASU8 (A) and Bacillus tequilensis ASU9 (B).*



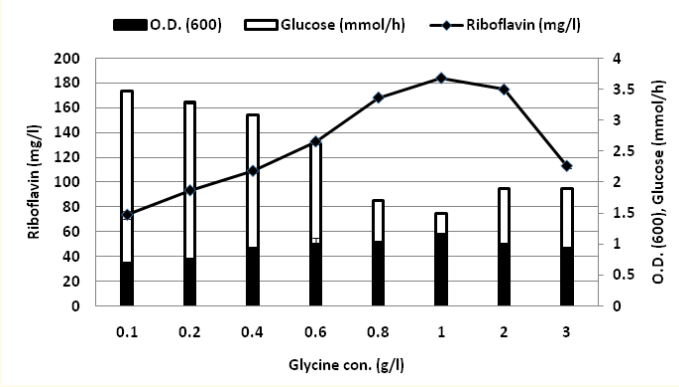
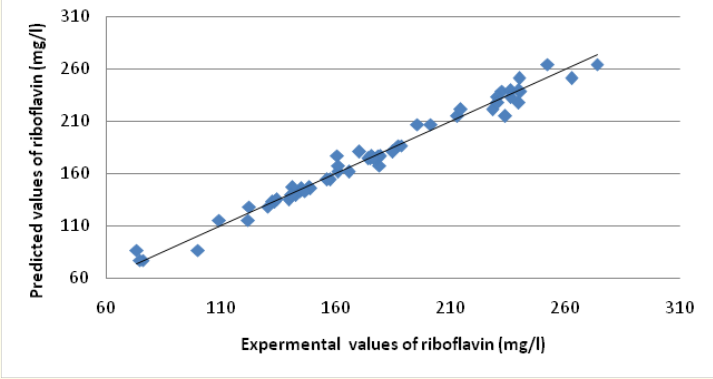


Figure 2*: Effect of adding glycine on growth and riboflavin production by Bacillus subtilis ASU8 (A) and Bacillus tequilensis ASU9 (B).*



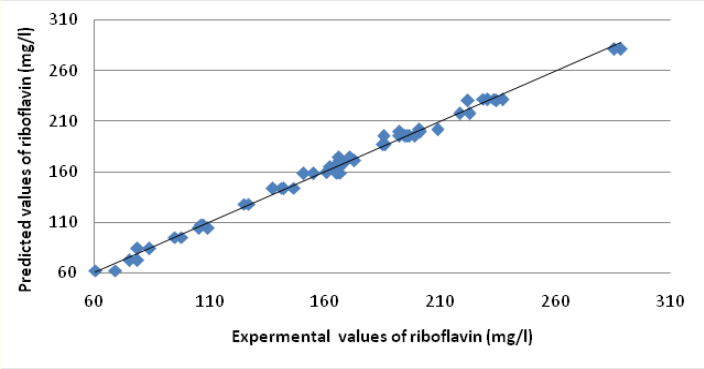


Figure 3: *Comparison between riboflavin production (mg/l) experimental and predicted values of the RSM model by Bacillus subtilis ASU8 (A) and Bacillus tequilensis ASU9 (B).*

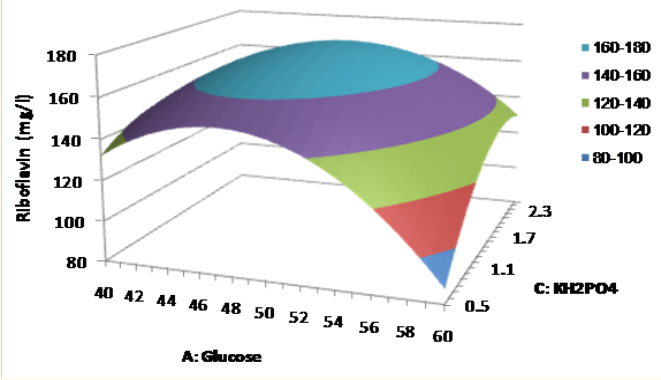


Figure 4A1*: Response surface plots of riboflavin production by Bacillus subtilis showing the effect of two variables (other variables were kept at zero in coded unit): significant interaction between*

A1: Glucose and KH_2PO_4.

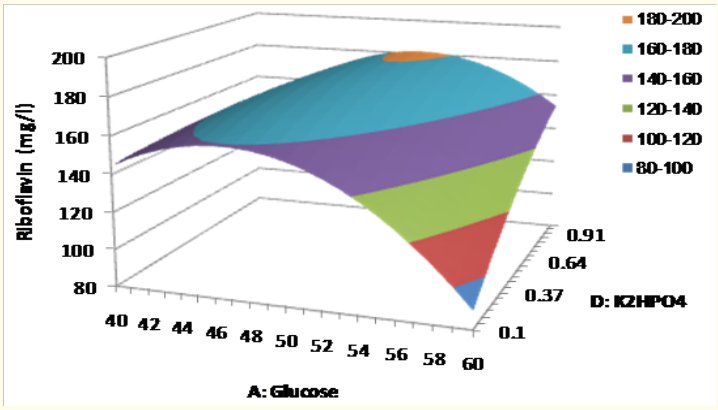


Figure 4A2*: Glucose and* K_2HPO_4.

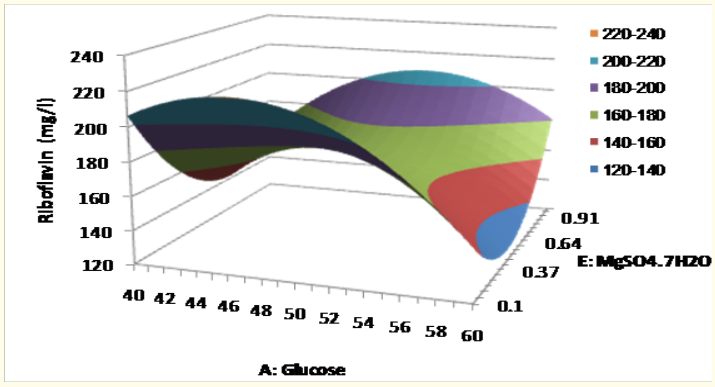


***Figure 4A3**: Glucose and $MgSO_4.7H_2O$.*

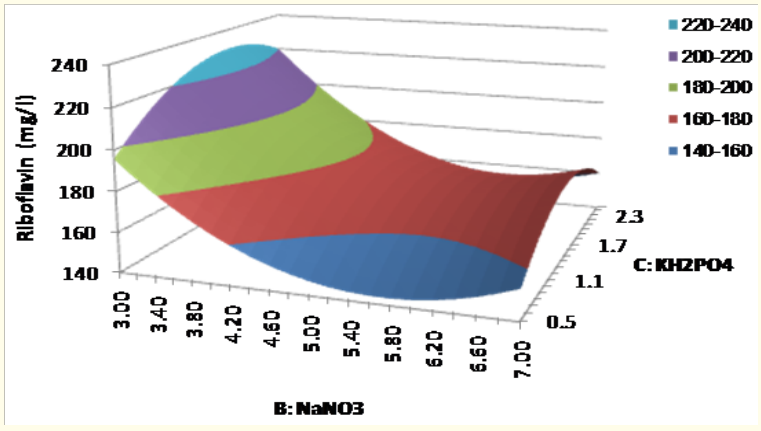


***Figure 4A4**: $NaNO_3$ and KH_2PO_4.*

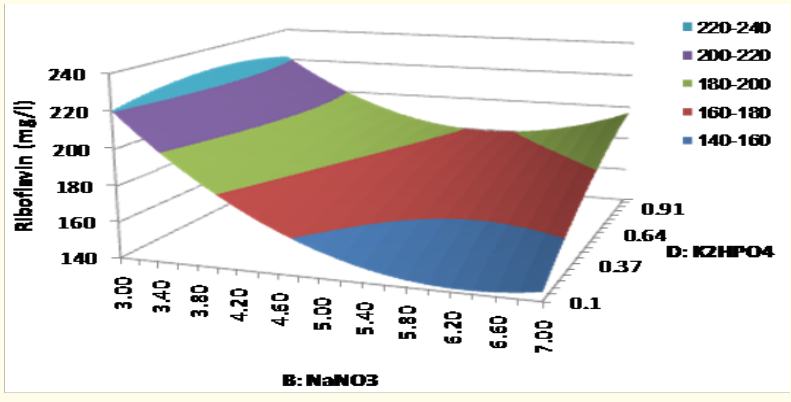


***Figure 4A5**: $NaNO_3$ and K_2HPO_4.*

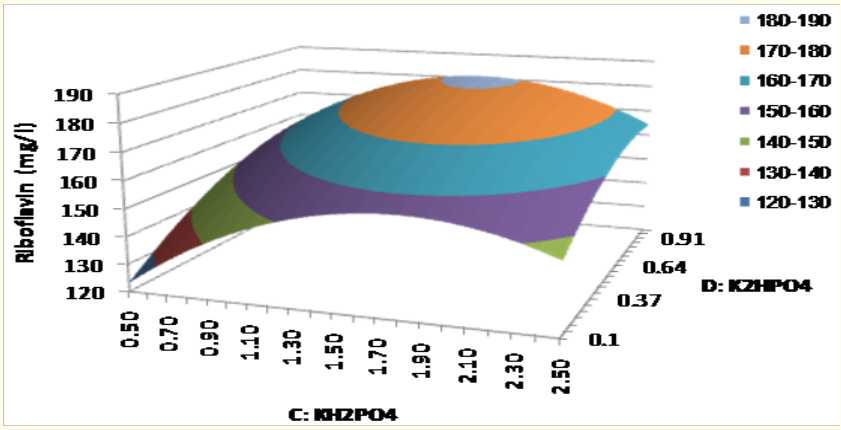


***Figure 4A6**: KH_2PO_4 and K_2HPO_4.*

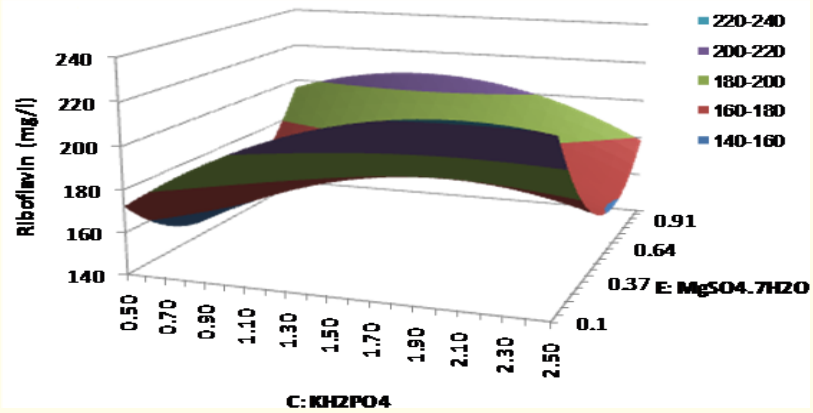


***Figure 4A7**: KH_2PO_4 and $MgSO_4.7H_2O$.*

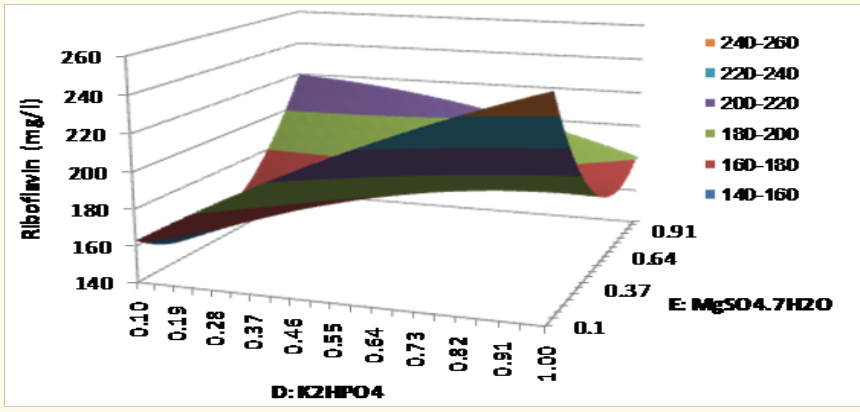


***Figure 4A8**: K_2HPO_4 and $MgSO_4.7H_2O$ for Bacillus subtilis ASU_8*

Response surface plots of riboflavin production by Bacillus subtilis & Bacillus tequilensis showing the effect of two variables (other variables were kept at zero in coded unit): significant interaction between

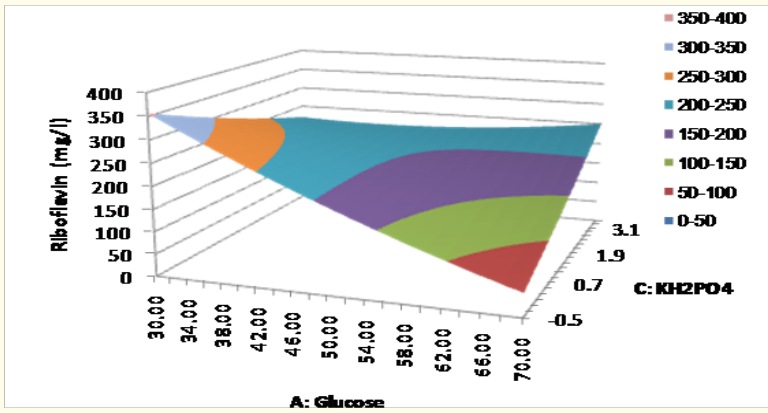


***Figure 4B1**: Response surface plots of riboflavin production by Bacillus tequilensis showing the effect of two variables (other variables were kept at zero in coded unit): significant interaction between*
B1: Glucose and KH_2PO_4.

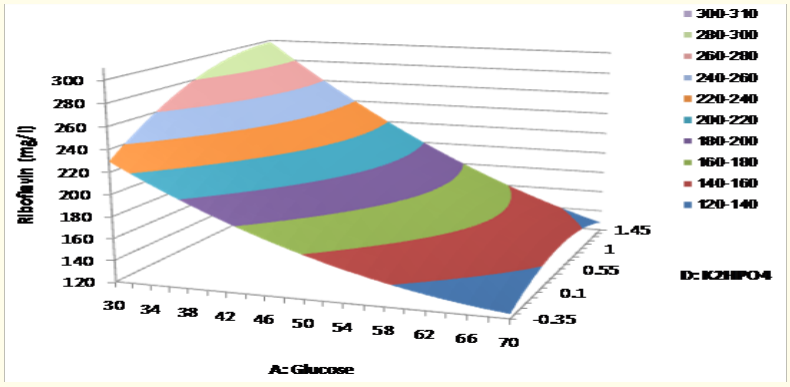


***Figure 4B2**: glucose and K_2HPO_4.*

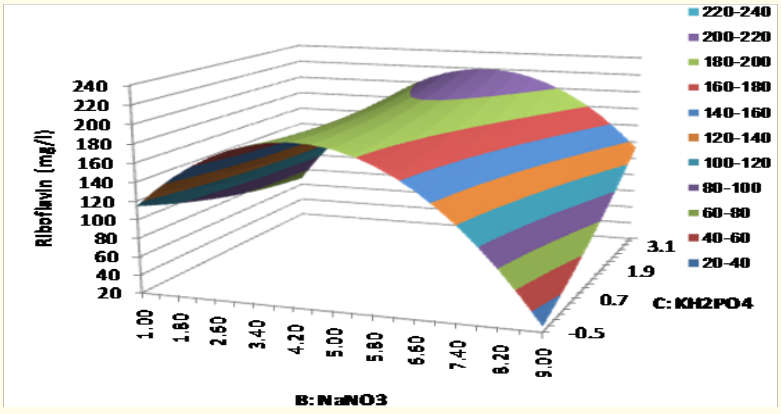


***Figure 4B3**: $NaNO_3$ and KH_2PO_4.*

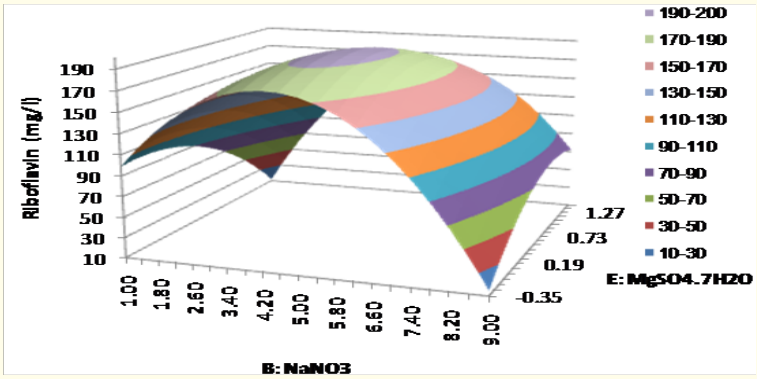


***Figure 4B4**: $NaNO_3$ and $MgSO_4.7H_2O$.*

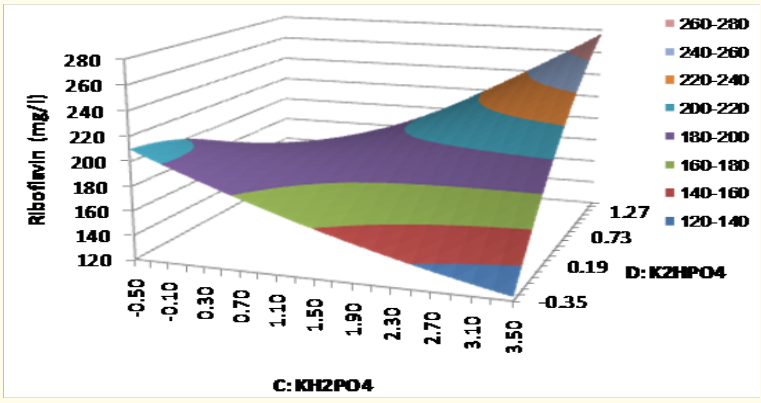


***Figure 4B5**: KH_2PO_4 and K_2HPO_4.*

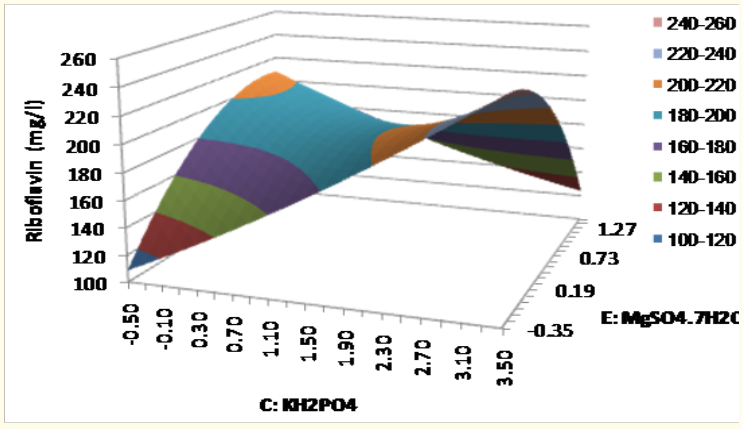


Figure 4B6: KH_2PO_4 and $MgSO_4.7H_2O$.

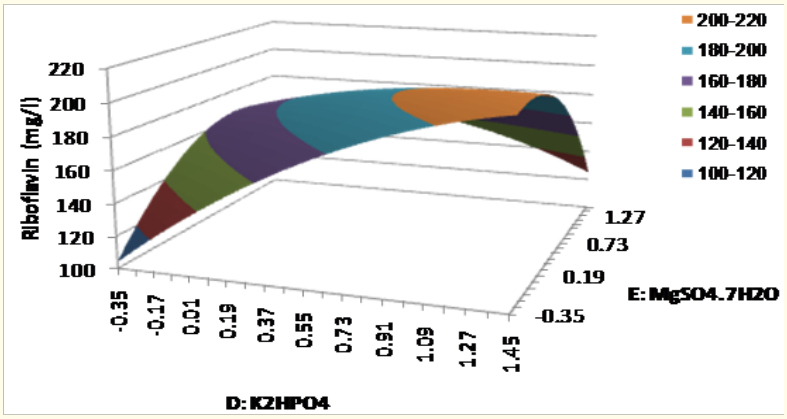


***Figure 4B7**: K_2HPO_4 and $MgSO_4.7H_2O$ for Bacillus tequilensis ASU_9.*

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