بهینه سازی محیط کشت برای تولید استوین در سویه Bacillus subtilis GB03 با استفاده از طراحی آماری آزمایش

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چکیده

مقدمه: تولید استوین توسط باکتری‌های فراریشه نقش مهمی در افزایش رشد و مقاومت گیاهان به بیمارگرهای گیاهی دارد. هدف این پژوهش، بهینه سازی تولید استوین در B. subtilis GB03 با استفاده از روش‌های طراحی آزمایش بود.

مواد و روش‌ها: اهمیت هشت جزء مواد غذایی و شرایط کشت بر تولید استوین با استفاده از روش Plackett–Burman ارزیابی شد. از روش بالاترین درجه فراز و نشیب برای دستیابی به محدوده بهینه سه فاکتور منتخب استفاده شد و در نهایت سطح بهینه آنها توسط طرح مرکب مرکزی تعیین شد.

نتایج: نتایج نشان داد که گلوکز، فسفات آمونیوم و سرعت هم‌زنی اثر قابل توجه ای در تولید استوین داشتند. سطح بهینه فاکتورها مقدار ۷۵/۹۱ گرم گلوکز، ۵/۷۹ گرم فسفات آمونیوم و سرعت هم زنی ۲۱۳ دور در دقیقه بود. بیشینه تولید استوین به مقدار ۲۶/۱ گرم در لیتر و در زمان ۴۰ ساعت حاصل شد که ۴۳ درصد بیشتر از محیط پایه بود. البته ترکیبات فرار آزاد شده از باکتری در محیط بهینه شده دارای فعالیت گیاهسوزی روی گیاهچه های Arabidopsis thaliana بود. نتایج نشان داد که غلظت یک قسمت در میلیون بیشترین اثر را در افزایش رشد گیاه داشت..

بحث و نتیجه‌گیری: روش های آماری طراحی آزمایش ابزاری ارزشمند در بهینه سازی شرایط فرمانتاسیون برای افزایش تولید استوین هستند. اما به هر حال ترکیبات فرار تولید شده توسط باکتری در محیط بهینه شده گیاهسوز هستند و نیاز است قبل از مصرف غلظت بهینه آنها تهیه شود.

واژه‌های کلیدی: استوئین، باسیلوس، روش سطح پاسخ، رشد گیاه، گیاهسوزی،

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Optimization of acetoin production by biological control strain *Bacillus Subtilis* GB03 using statistical experimental design

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Abstract

Introduction: Acetoin production, by beneficial rhizobacteria, plays an important role in plant growth and resistance to plant pathogens. The aim of this work was to optimize the nutritional conditions using statistically based experimental designs for the production of *B. subtilis* GB03 acetoin.

Materials and methods: Eight components of the medium and cultivation conditions were examined for their significance on the production of acetoin using the Plackett–Burman experimental design. Steepest ascent experiments were employed to approach the optimal region of the three factors and a central composite design was applied to determine their optimal levels.

Results: Results indicated that Glucose, ammonium phosphate and agitation speed had significant effects on the acetoin production. The significant medium components in our optimization medium were 75.91 g/l of glucose, 5.79 g/l of ammonium phosphate, and 213rpm of agitation speed. The maximum acetoin concentration of 26.1 g/l at 40 h was obtained. Volatiles emitted from bacteria on optimized medium showed phytotoxicity on Arabidopsis seedlings. Result revealed that 1 ppm of acetoin was the best for the promotion of plant growth.

Discussion and conclusion: Statistical experimental designs appeared to be an influential tool for optimization of fermentation conditions to enhance the acetoin production. However, volatile components secreted from the bacteria in the optimized medium showed some degree of phytotoxicity on Arabidopsis seedlings.

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Key words: Culture Media, Experimental Design, Phytotoxicity, Plant Growth.

Introduction

Plant growth promoting rhizobacteria (PGPR) produces specific volatiles that have a positive impact on plant growth and could induce systemic resistance against plant diseases (1, 2). Ryu et al. (3) has demonstrated that 2, 3-butanediol and acetoin as its precursor produced by *Bacillus subtilis* GB03 could efficiently trigger systemic resistance against *Pectobacterium carotovorum* subsp. *carotovorum* in *Arabidopsis*. Rudrapa et al. (4) proved that acetoin is responsible for induced-resistance against *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*. Complementary work performed by (5) showed that long time exposure to GB03 volatiles could result in sustained beneficial effects on *Arabidopsis* growth and significantly increase seed yield compared with water as the control. These findings encouraged researchers to apply synthetic volatile compounds for field treatment. Field treatments with synthetic 2, 3-butanediol, and acetoin resulted in 15.2% and 12.4%, higher weights of Pepper than negative control, respectively (6, 7).

Despite the abundance of chemical approaches for the synthesis of acetoin, natural acetoin generated through fermentation and enzymatic reactions appears to be more promising since it is the consumer and environmentally friendly (8, 9). However, the most of common used strains for the purpose of acetoin production give low-yield in general media. Thereby, a lot of efforts have been made to optimize nutrient conditions in growth medium in order to enhance acetoin production using candidate strains (10-12). Xiao et al. (12, 13) based on the literature reviews showed that *B. subtilis* was one of the best reported species for acetoin production. *Bacillus subtilis* CICC 10025 was able to produce 37.9 g/L acetoin using an optimized medium (12), demonstrating the importance of medium optimization.

Nutritional and physiological factors such as media component, agitation, pH, and temperature of the cell culture are crucial for operating fermentative processes. In previous reports (14, 15), oxygen supply was an essential factor for acetoin and 2,3-butanediol production. Moes et al. (15) reported that oxygen level has a profound impact on acetoin/2,3-butanediol ratio production during fermentation, with acetoin and 2,3-butanediol being excreted under high oxygen levels and under low oxygen levels, respectively. Ji et al. (14) examined the acetoin and 2,3-butanediol production process at different oxygen supply conditions by changing agitation speed, and similar results were found.

The objective of this study is to optimize fermentation conditions maximizing acetoin yield from *Bacillus subtilis* GB03. The optimization of acetoin production was carried out using a stepwise approach. In the first step, medium composition and physiological factors significantly influential on the acetoin production would be initially screened using the Plackett–Burman design (16, 17). Subsequently, the optimization of the most significant components would be performed by

steepest ascent method and the Response Surface Methodology (RSM) design (12). Finally, the suggested models would be verified.

Materials and Methods

Microorganism and culture conditions: The bacterium used throughout the study, *Bacillus subtilis* GB03, is an active ingredient of commercial biocontrol product Kodiak® (kindly provided by prof. Kleopper from Auburn University). The strain was grown on fresh Luria-Bertani Medium (LB) and sub-cultured every month in LB agar. Bacterium maintained in 20% glycerol at −80°C for long-term storage. The production of acetoin was studied in 50ml of a basic fermentation medium containing glucose 45 g/l, $K_2HPO_4$4 g/l, $(NH_4)_2$ HPO_4 3.5 g/l, $FeSO_4$ 0.01 g/l, $MgSO_4$ 0.45 g/l. The medium pH was adjusted to 6.5 before sterilization. The cultivation was performed in a 250-ml flask incubated in a shaker incubator at 180 rpm, 30˚C for 40 h.

Thereafter, In order to estimate microbial population, one milliliter of the fermented sample was taken and relevant optical density (OD) was recorded using a UV-visible spectrophotometer (Lambda, Perkin-Elmer, USA) at 600 nm. Bacterial population was also determined by standard plate count method (SPC) and reported as Colony Forming Units (CFU) per milliliter. For analyzing acetoin concentration, one milliliter of cultivated cells was centrifuged at 10,000×g for 10 min. The supernatant was analyzed by the Romick and Fleming (18) method.

Plackett–Burman design: The Plackett–Burman (PB) design was used as an efficient way for screening the important factors among a large number of variables (17). This design is based on the first-order model:

$$\text{Eq. 1} \qquad Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i$$

Where Y is the response (acetoin production); β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. Each variable is represented at two levels, high and low, which are denoted by (+1) and (−1), respectively. A total of eight parameters including glucose (X1), glycerol (X2) and $(NH_4)_2$ HPO_4 (X3) concentration, agitation speed (X4), pH (X5), temperature (X6), $MgSO_4$ (X7) and $FeSO_4$ (X8) were included for the selection of the most important factors. Table 1 illustrates the levels of each variable and the design details. All experiments were carried out in triplicates.

Table 1- Plackett–Burman matrix for screening of significant factors affecting acetoin production. Variables provided in coded and real values

Run	Coded (real)Variable levels								Acetoin (g/l)
	X1	X2	X3	X4	X5	X6	X7	X8	
1	-1(30)	1(15)	1(5)	-1(100)	-1(5.5)	1(34)	1(0.02)	1(0.6)	18.3
2	1(60)	-1(10)	1(5)	-1(100)	-1(5.5)	-1(26)	1(0.02)	-1(0.3)	20.5
3	1(60)	-1(10)	1(5)	1(300)	1(7.5)	1(34)	-1(0.00)	1(0.6)	22.8
4	1(60)	1(15)	-1(2)	-1(100)	1(7.5)	1(34)	-1(0.00)	-1(0.3)	18.6
5	-1(30)	1(15)	1(5)	-1(100)	1(7.5)	-1(26)	-1(0.00)	-1(0.3)	17.1
6	1(60)	1(15)	-1(2)	1(300)	-1(5.5)	1(34)	1(0.02)	-1(0.3)	21.2
7	-1(30)	1(15)	-1(2)	1(300)	1(7.5)	-1(26)	1(0.02)	1(0.6)	16.9
8	1(60)	1(15)	1(5)	1(300)	-1(5.5)	-1(26)	-1(0.00)	1(0.6)	24.1
9	-1(30)	-1(10)	-1(2)	-1(100)	-1(5.5)	1(34)	-1(0.00)	1(0.6)	15.2
10	-1(30)	-1(10)	-1(2)	1(300)	-1(5.5)	-1(26)	-1(0.00)	-1(0.3)	16.6
11	-1(30)	-1(10)	1(5)	1(300)	1(7.5)	1(34)	1(0.02)	-1(0.3)	18.9
12	1(60)	-1(10)	-1(2)	-1(100)	1(7.5)	-1(26)	1(0.02)	1(0.6)	18.4

Path of steepest ascent: The method of steepest ascent was generated by the first order model derived from PB design to move serially along the direction of the maximum increase in the response. The zero level of selected variables in the PB design was chosen as the base in the steepest ascent path. The direction of steepest ascent was the direction in which Y increased most rapidly. Experiments were performed along the steepest ascent path until the response did not increase anymore. Thus, the steepest ascent method allowed coming closer to the optimal level and locating a new experimental region (10).

Response surface methodology: Response surface methodology (RSM) was used to optimize acetoin production based on central composite design. The three factors screened by PB design, with five coded levels, were used to determine the most significant factors for enhancing acetoin production. The level of each variable and the design matrix are given in Table 4. The data obtained from RSM on acetoin production were subjected to the analysis of variance (ANOVA) and the role of each variable, their interactions, and statistical analysis to obtain predicted yields are explained by applying the following quadratic equation:

Eq. 2 $$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2$$

Where Y is the predicted response, X_i and X_j are the independent variables, β_0 is the intercept term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient. For statistical calculations according to the following equation:

Eq. 3 $$X = (U - U_0)/\Delta U$$

Where X is the coded value of the independent variable, U is the real value of the independent variable, U_0 is the real value of the independent variable on the center point, and ΔU is the step change value.

Validation of the experimental model: In order to validate the model, experiments were carried out at optimal levels of the most significant variables and middle level of other medium components in 250-ml flasks. All experiments were carried out in triplicates and averages of the results were taken as the response. Correlation coefficient (R^2) between actual and predicted data determines accuracy of model.

Plant growth promotion bioassay: The experiment was performed using the I-plate method described by Ryu et al. (3). 30 µl of 10^8 CFU/ml *B. subtilis* GB03 suspension or 0, 0.001, 0.01, 0.1, 1, 10 and 100 ppm of acetoin inoculated on paper disc on the optimized medium. Two-day-old Arabidopsis seedlings were transplanted into other part of I-plate. Seedling fresh weight was measured after 14 days.

Statistical Analysis: The experimental designs and regression analysis of the experimental data were conducted using Design-Expert, Version 7.0 (STAT-EASE Inc., Minneapolis, USA). All experiments were repeated three times. The analysis of variance (ANOVA) was used to estimate the statistical parameters. In PB design, the variables with less significant effect (confidence level less than 95%) were not included in the optimization experiments. However, in the case of negative and positive factors, they were used at their low and high levels, respectively. The fit of the obtained regression model was checked using the adjusted coefficient of determination R^2, and its statistical significance was determined by Fisher's test. Surface plots of responses were obtained using the same software.

Results

Selection of significant variables by Plackett–Burman design: PB design was

used to identify the variables with significant impact on acetoin production. In this section, a twelve-experiment PB design was used for eight variables in acetoin production including glucose, glycerol, $(NH_4)_2$ HPO_4, agitation speed, pH, temperature, $MgSO_4$ and $FeSO_4$, based on preliminary experiments and related reports (10, 12, 17). The experimental data analysis indicated that there was a wide variation in acetoin concentrations from 15.2 to 24.1 g/L in the twelve-experiment PB design. After regression analysis, the parameters ($P<0.05$) such as glucose, agitation speed and ammonium phosphate concentration showed P values less than 0.05 and were consequently considered as significant factors on the production of acetoin. Despite the insignificant effect of $FeSO_4$, $MnSO_4$ and pH on acetoin production ($P>0.05$), they were adjusted at their low levels according to their negative impact. Factors such as temperature and glycerol had non-significant positive effects and were set at their high levels. Teixeira et al. (11) showed that pH has non-significant negative effect on acetoin production but the effect of temperature was reported to be positive. $MgSO_4$ exhibited a negative impact on both acetoin and butanediol production (12, 17). Table 2 shows the effects of the variables on the response and the significant levels.

To approach the optimum response, the fitted first-order model equation for acetoin production was obtained from the PB design experiments:

Eq. 4 $Y = 5.172 + 0.085X_1 + 0.073X_2 + 0.122X_3 + 0.027X_4 - 0.369X_5 + 0.133X_6 - 5.417X_7 - 2.683X_8$

The coefficient of each variable in Eq. 4 represents the strength of the effect of this variable on acetoin production. Statistical testing was carried out using Fisher's test for ANOVA according to the experimental data. The F and p values were 11.36 and 0.0163, respectively. The test model was statistically significant at the 98.8% level of significance. The coefficient R^2 of the first-order model was 0.957, which indicated that only about 4.3% of the total variations were not explained by the model. The value of the adjusted determination coefficient (Adj R^2 = 87.3%) was also very high to advocate for a high significance of the model.

Table 2- statistical analysis of Plackett–Burman design

Variable	Coefficient	Standard error	F value	*P* value
Intercept	5.172	0.085	19.93	0.006*
X1	0.085	0.085	82.63	0.001*
X2	0.073	0.085	3.74	0.125
X3	0.122	0.085	8.14	0.047*
X4	0.027	0.085	50.25	0.002*
X5	-0.037	0.085	3.85	0.121
X6	0.133	0.085	6.57	0.62
X7	-5.417	0.085	0.08	0.787
X8	-2.683	0.085	4.57	0.099

$R^2 = 0.957$, R^2 (Adj) = 0.873
* Significant at 95%

Path of steepest ascent: The path of steepest ascent was determined to find the correct direction of changing variables by increasing the value of the main factors. Based on the model obtained using Eq. 4, the path of steepest ascent was employed to move rapidly towards the optimum response, i.e., increasing the glucose, ammonium phosphate and agitation speed to improve acetoin yield. From the glucose, ammonium phosphate and agitation speed coefficients (0.0855, 0.122 and 0.0267), which were approximately equivalent (0.7, 1, 0.22), the glucose concentration would increase 0.7 design units (10 g/L) if the ammonium phosphate concentration increased 1 unit (1.5 g/L). The center point of the PB design (no. 1 in Table 3) was considered the origin path. The design and responses of the steepest ascent experiment are shown in Table 3. The yield of acetoin increased along the path reaching the peak of 25.28 g/L when the concentration of glucose, ammonium phosphate and agitation speed was selected at 75, 5.5 and 240 g/l, respectively. It

suggested that this point was an appropriate center point for response surface methodology.

Table 3 Experiment design and results of the steepest ascent path

Run	Variable levels			Acetoin (g/l)
	Glucose (g/l)	$(NH_4)_2HPO_4$ (g/l)	Agitation (RPM)	
1	45	3.5	200	17.42
2	60	4.5	220	21.64
3	75	5.5	240	25.28
4	90	6.5	260	22.36
5	105	7.5	280	21.17

Response surface analysis: Based on the PB design and the path of steepest ascent, RSM using CCD was employed to determine the optimal levels of the three selected variables (glucose, ammonium phosphate and agitation speed) which significantly influenced the acetoin production. The design matrix of the variables based on central composite design and the responses of the experiments are listed in Table 4.

By applying multiple regression analysis on the experimental data, the response variable and the test variables were related by the following second-order polynomial equations (Eq. 5).

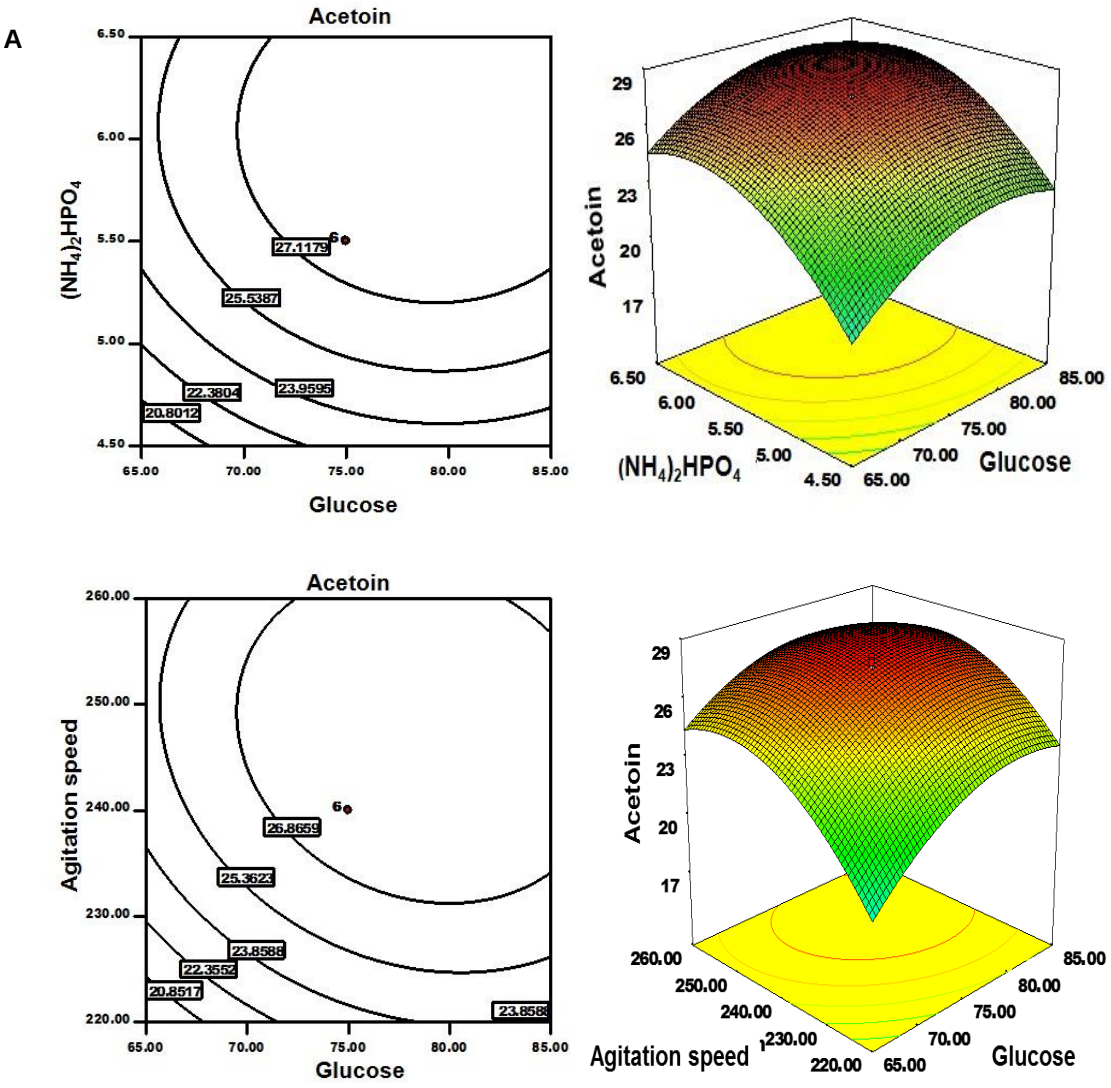
Eq. 5 $Y=27.86+1.55X_1+2.53X_2+1.95X_3-0.19X_1X_2-0.61X_1X_3+0.73X_2X_3-1.82X_1^2-2.42X_2^2-2.46X_3^2$

Where Y is the predicted acetoin production (g/l); X1, X2 and X3 are the coded values of glucose, ammonium phosphate and agitation speed, respectively. The model predicted the optimum values of the three most significant factors to be X_1 = 1.55, X_2 = 2.53 and X_3 = 1.95. Correspondingly, the values of glucose, ammonium phosphate and agitation speed were 75.9 g/l, 5.79 g/l and 213 rpm, respectively. The maximum predicted concentration of acetoin was 28.1 g/l. By optimization of culture conditions, acetoin production was enhanced from 15.2 to 28.1 g/l. The goodness of the model was checked by coefficient of determination (R^2) and the adjusted determination coefficient (Adj R^2). R^2 value was 0.984, indicating that 98.4% of the variability in the response could be explained by the model. The adj. R^2 was 0.963, indicating high significance of the model. On the basis of the experimental values, statistical testing was performed using Fisher's test for ANOVA (Table 5). F value of 68.24 in the model indicated that the model was significant ($p<0.0001$). There is only a 0.01% probability that could occur due to noise. The "lack of fit of 0.65 indicated that there was a 6.51% chance, such that a large "lack of fit F value" could be happen due to noise. The model was found to be adequate for prediction within the range of variables used. This suggests that the combined effects of all the independent factors significantly contributed to maximizing the acetoin production. All variables analysis are shown in Table 5. A P-value of less than 0.05 indicates that the model terms are significant. In this case, the independence variables of X_1, X_2 and X_3 and the interaction variables of X_1X_3, and X_2X_3 were significant model terms. They were proven to have important impact for acetoin production.

The response surfaces curves are plotted to explain the interaction of the variables (Fig. 1). Each figure demonstrates the effect of two factors while the other factor was fixed at its optimum level and the other two varying factors within their experimental ranges**.** As shown in Fig. 1a, the maximum response for acetoin production occurs at 75 and 5.5 g l^{-1} glucose and ammonium phosphate concentrations, respectively.

It can be concluded from Fig. 1b that an increase in acetoin production could be significantly achieved by the increase in glucose concentration and agitation speed. This suggests that both variables have a very substantial effect on the acetoin production. As the agitation speed

increased, the maximum response appeared near the middle of the glucose level. The response also varied noticeably at different levels of glucose along the axis, thus, suggesting a considerable interaction between these two factors. Fig. 1c depicts the combined effects of ammonium phosphate concentration and agitation speed.



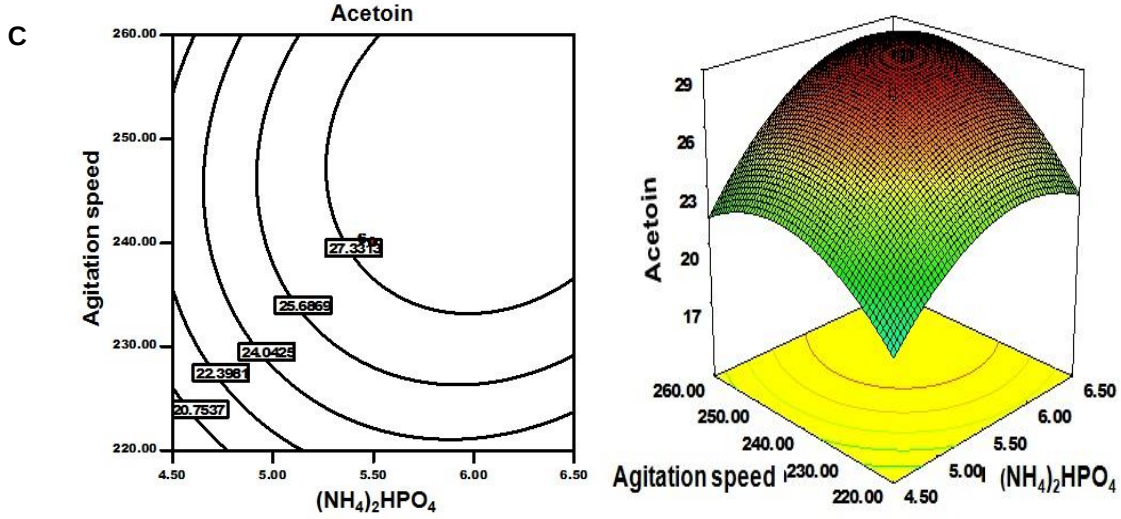


Fig. 1- Response surface and its corresponding counter plot of acetoin production from *B. subtilis* GB03 showing the interaction between glucose and ammonium phosphate (a), glucose and agitation speed (b), and ammonium phosphate and agitation speed (c).

Table 4- the results of the central composite experiment in coded units and real values

Run	Coded variable levels				Real variable levels			Acetoin (g/l)
	X1	X3	X4		Glucose	$(NH_4)_2HPO_4$	Agitation	
1	1	-1	-1		85	4.5	230	17.1
2	-1	1	-1		65	6.5	230	18.7
3	1	-1	1		85	4.5	290	18.9
4	-1	1	1		65	6.5	290	22.1
5	1	1	-1		85	6.5	230	17.3
6	-1	-1	-1		65	4.5	230	19.8
7	-1	-1	1		65	4.5	290	21.9
8	1	1	1		85	6.5	290	20.5
9	-1.68	0	0		60	5.5	260	20.5
10	1.63	0	0		90	5.5	260	16.4
11	0	-1.63	0		75	3.8	260	17.8
12	0	1.63	0		75	7.1	260	19.1
13	0	0	-1.63		75	5.5	210	20.2
14	0	0	1.63		75	5.5	310	25.9
15	0	0	0		75	5.5	260	26.1
16	0	0	0		75	5.5	260	25.8
17	0	0	0		75	5.5	260	26.1
18	0	0	0		75	5.5	260	25.7
19	0	0	0		75	5.5	260	25.9
20	0	0	0		75	5.5	260	25.6

Table 5- Significance test of regression coefficient and quadratic model

Variable	Coefficient	Standard error	F value	*P* value
Intercept	27.86	0.31		
X_1	1.55	0.21	55.26	0.001
X_3	2.53	0.21	148.15	0.000
X_4	1.95	0.21	87.59	0.000
X_1X_3	-0.19	0.27	0.83	0.507
X_1X_4	-0.61	0.27	5.06	0.048
X_3X_4	0.73	0.27	7.32	0.022
X_1^2	-1.82	0.20	80.61	0.000
X_3^2	-2.42	0.20	142.55	0.000

X_4^2	-2.46	0.20	146.74	0.000
Model			97.64	0.000
Lack of fit			0.65	0.676

Validation: The model has been approved for the three factors (glucose, ammonium phosphate and agitation speed) within the design space. A random set of six variable combinations was prepared and examined for the acetoin production (Table 6). The actual acetoin production values were found to be in good agreement with the statistically predicted values (R^2=0.94), approving the model's validity.

Table 6- experimental vs. predicted values of acetoin

Run	Variable levels				Acetoin (g/l)	
	Glucose	$(NH_4)_2HPO_4$	Agitation		Actual	Predicted
1	60	6.5	290		19.42	19.81
2	65	7	260		19.13	18.83
3	85	5.5	300		21.82	22.59
4	75	4.5	230		22.28	21.45
5	65	6.5	260		21.36	21.68
6	75	5.5	260		25.35	25.73

Pearson correlation coefficient=0.94

Plant growth promotion bioassay: Volatiles emitted from *B. subtilis* GB03 grown on basal medium increased plant fresh weight up to 4 times. However, optimized medium not only did not improved plant growth promotion activity but also showed phytotoxicity on Arabidopsis seedlings (Fig. 2). In the next step, the investigations on the effect of different concentrations of acetoin exhibited that 1 ppm is the best concentration for the promotion of plant growth. Further increase in acetoin concentration decreased the plant growth promotion activity. 100 ppm acetoin level exhibited some phytotoxicity in leaf margins.

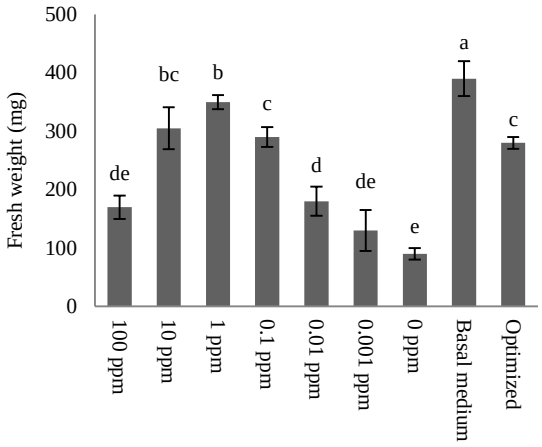


Fig. 2- Plant growth promotion in Arabidopsis seedlings by exposure to volatiles of *Bacillus subtilis* GB03 inoculated on basal or optimized medium and different concentration of pure acetoin.

Discussion and conclusion

The medium for acetoin production by *B. subtilis* GB03 was optimized by employing statistical methodologies based on the Plackett–Burman design, the steepest ascent method, and RSM. This optimization resulted in a maximum yield of 28.1 g/L acetoin.

According to our findings, glucose, ammonium phosphate and agitation speed significantly influenced the acetoin production. According to the literature, carbon and nitrogen source follow similar

patterns for diol, acetoin and butanediol, production, but agitation speed presented an opposite role in the production of acetoin and butanediol. Sun et al. (10) showed that sucrose like carbon source is a key medium component for acetoin production and led to 41 g/l acetoin concentration in *Serratia marcescens*. However, Glucose was the best carbon source for *B. subtilis* SF4-2 (19). Glucose and sucrose have similar impact on acetoin production and are favored compared to other carbon sources (20). Teixeira et al. (11) reported that 63 g/l glucose was the optimum concentration for acetoin production by *Hanseniaspora guilliermondii* but in our study, 75.9 g/l glucose was reported as the optimum level required by *Bacillus subtilis* GB03 for acetoin production. Although, some *Bacillus* strains can response to as high as 200 g/l glucose in the medium (11). In addition, the production of acetoin and butanediol has been shown to be sensitive to different nitrogen sources. Among the eight variables tested by PB experiments, soybean meal, corn steep liquor and ammonium citrate were identified as the most significant nitrogen sources for butanediol production (10). In other study, $(NH_4)_2$ HPO_4 was reported as the most important factor affecting the butanediol accumulation (21). It is suggested that $(NH_4)_2$ HPO_4 could provide phosphorus source required for cell growth and metabolites formation by the microorganism. Garg and Jain (22) reported that phosphate could trigger diols fermentation. They proposed that the effects on diol yields was due to the effect of phosphate, which stimulated the complete metabolism of the bacterium. The study of Sun et al. (10) also approved the substantial role of $(NH_4)_2$ HPO_4 as nitrogen and phosphorous sources for the production of diols.

Moes et al. (15) discussed the effects of oxygen levels on diol production by *B. subtilis*. They found that acetoin and 2,3-butanediol were produced at oxygen level above 100 ppb and below 100 ppb, respectively. The product concentration ratio changed quickly in the range of 80–90 ppb oxygen concentrations. Varying oxygen levels might cause the conversion of one product into another in a reversible mode. Nakashimada et al. (23) investigated the impact of oxygen supply on diol production by *P. polymyxa* ATCC. They showed that, much more 2,3-butanediol than acetoin was produced at low airflow rates. But 2,3- butanediol decreased rapidly to trace quantities and acetoin increased quickly to a high level when the airflow rate was raised from 400 to 500 ml min−1. Sun et al. (10) proposed a two-stage agitation speed for enhanced acetoin production by *Serratia marcescens***,** in which the agitation speed was fixed at 700 rpm during the first 8 h and then decreased to 600 rpm. In this process 44.9 g/l of acetoin was obtained.

In conclusion, we conducted a series of statistical experimental designs to improve the medium for acetoin production by *B. subtilis* GB03. Glucose, $(NH_4)_2$ HPO_4 and agitation speed had significant impacts on acetoin production and the optimal values of these three key variables were 75.91 g/l, 5.79 g/land 213 rpm, respectively. After the optimization process, the acetoin concentration was improved to 28.1 g/l after40 h of fermentation incubation time. It was revealed that statistical experimental designs positively influenced acetoin production optimization. However, volatile components secreted from the bacteria in the optimized medium showed some degree of phytotoxicity on Arabidopsis seedlings. Ann et al. (24) revealed that high concentration of volatiles have negative effect on plant growth. In this study, 100 ppm of acetoin exhibited phytotoxicity symptom on Arabidopsis seedlings.

Statistical experimental designs

appeared to be an influential tool for the optimization of fermentation conditions to enhance the acetoin production. Since there are a few reports on the medium and fermentation process optimization or for acetoin production using statistical experimental designs, this study could be a reference work for further relevant researches aiming for acetoin production.

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