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Production of Rapamycin in *Streptomyces hygroscopicus* from Glycerol-Based Media Optimized by Systemic Methodology S

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Rapamycin, produced by the soil bacterium *Streptomyces hygroscopicus*, has the ability to suppress the immune system and is used as an antifungal, anti-inflammatory, antitumor, and immunosuppressive agent. In an attempt to increase the productivity of rapamycin, mutagenesis of wild-type *Streptomyces hygroscopicus* was performed using ultraviolet radiation, and the medium composition was optimized using glycerol (which is one of the cheapest starting substrates) by applying Plackett-Burman design and response surface methodology. Plackett-Burman design was used to analyze 14 medium constituents: M100 (maltodextrin), glycerol, soybean meal, soytone, yeast extract, $(NH_4)_2SO_4$, L-lysine, KH_2PO_4, K_2HPO_4, NaCl, $FeSO_4{\cdot}7H_2O$, $CaCO_3$, 2-(*N*-morpholino) ethanesulfonic acid, and the initial pH level. Glycerol, soytone, yeast extract, and $CaCO_3$ were analyzed to evaluate their effect on rapamycin production. The individual and interaction effects of the four selected variables were determined by Box-Behnken design, suggesting $CaCO_3$, soytone, and yeast extract have negative effects, but glycerol was a positive factor to determine rapamycin productivity. Medium optimization using statistical design resulted in a 45% (220.7 ± 5.7 mg/l) increase in rapamycin production for the *Streptomyces hygroscopicus* mutant, compared with the unoptimized production medium (151.9 ± 22.6 mg/l), and nearly 588% compared with wild-type *Streptomyces hygroscopicus* (37.5 ± 2.8 mg/l). The change in pH showed that $CaCO_3$ is a critical and negative factor for rapamycin production.

Keywords: *Streptomyces hygroscopicus*, immunosuppressant, rapamycin production, Plackett-Burman design, Box-Behnken design, response surface methodology

Introduction

Rapamycin, produced by *Streptomyces hygroscopicus*, is a 31-membered macrocyclic natural product exhibiting various biological and pharmacological activities, including antifungal, immunosuppressive, antitumor, neuroprotective, and anti-aging activities [5, 23]. Its potent activity, unique mode of action, and low toxicity have led to a great deal of interest in its potential applications in human medicine [18]. It is also reported that rapamycin can extend the lifespan of mice [3]. Thus, there has been great interest in increasing the yield of rapamycin, and many researchers have mainly focused on increasing rapamycin titers [1, 9, 22, 25] and the production of rapamycin analogs to enhance the efficacy of rapamycin [16]. A number of metabolic engineering approaches have been tried so far; however, these efforts have not been successful as the titer is still too low to produce rapamycin efficiently and economically [8,

18, 21]. As a result, mutagenesis is still a feasible solution to improve the bacterial strain for higher productivity and ultaviolet (UV) radiation induces different evolutionary changes on bacteria that would otherwise not be possible by metabolic engineering. However, it still needs optimization because it is strongly influenced by media compositions [20].

One way to overcome or avoid this is to optimize the rapamycin production conditions, and there is information on optimization of the rapamycin production using a small number of factors and examination of different carbon sources [6]. Although this information is very helpful to design media composition, the effects from the different medium components were not well evaluated for many factors that may cause interactive effects on the final production.

Glycerol is one of the cheapest starting materials as a carbon source, and has been found to support the growth and antibiotic production [10]. Glycerol has attracted the attention of scientific and industrial communities owing to its generation in bulk quantities as a byproduct of biofuel industries. With the rapid growth of these industries in recent years, glycerol is frequently treated as a very low-value byproduct or even a waste product with a disposal cost associated to it. Glycerol is not only abundant and inexpensive but can also generate more reducing equivalents than glucose or xylose [2], and thus media optimization of glycerol-based media seems to be a very promising and economical route to produce rapamycin repeatedly and stably. To identify the effects and optimal medium for rapamycin production, information of other components were gathered from previous reports and employed in the Plackett-Burman design and response surface methodology for glycerol-based media. The Plackett-Burman design can help to find important factors by eliminating many experiments and avoiding the drawbacks of one variable at a time [14, 17, 18]. Dual application of the Plackett-Burman and Box-Behnken designs resulted in dramatic enhancement of rapamycin production and the finding of the correlation to several key factors, such as $CaCO_3$ and glycerol. In addition, this study showed an important correlation of final pH to the amount of rapamycin produced.

Materials and Methods

Microorganism and Culture Conditions

The mutant strain of *Streptomyces hygroscopicus* ATCC 29253 used in this study was maintained on TSB agar plates at 30°C. Analytical chemicals were obtained from BD (San Jose, CA, USA) or Sigma-Aldrich (St. Louis, MO, USA). For the fermentation, *S. hygroscopicus* was cultivated at 30°C in 50 ml [12] of production medium (30 g M100, 30 g glycerol, 10 g soybean meal, 10 g soytone, 6.5 g yeast extract, 5 g $(NH_4)_2SO_4$, 6.5 g L-lysine, 0.7 g KH_2PO_4, 1.14 g K_2HPO_4, 5 g NaCl, 0.05 g $FeSO_4 \cdot 7H_2O$, and 42.6 g MES, pH 5.5, per liter) with constant shaking at 200 rpm. This medium is called the unoptimized medium in this experiment. For UV random mutagenesis, we used UV-mutagenesis to improve the strain by the direct plate irradiation protocol [20], and the spore suspension was diluted with sterile water and placed in an uncovered petri dish and irradiated under a UV lamp (kill rate 99%) [26].

Assay for Rapamycin Production

For rapamycin extraction, after centrifugation of 500 µl of cultured cells, the supernatant was discarded. Then 500 µl of methanol was added to the cells in a 1.7 ml Eppendorf tube, and the supernatant was analyzed by high-performance liquid chromatography (HPLC, YL-9100, Korea) using a Waters C18 reverse phase column, acetonitrile-water (80:20 (v/v)) as the mobile phase, and 1 ml/min flow rate with detection at 277 nm [22].

Statistical Analysis

Minitab 16.0 (Minitab Inc., Pensylvania, USA) was used for the experimental designs and subsequent regressional analysis of the experimental data. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically by the coefficient of determination R^2, and its statistical significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test. In this case, glycerol, soytone, $CaCO_3$, and yeast extract all had a significant effect on rapamycin yield ($p < 0.05$).

Screening of Essential Medium Components Using the Plackett–Burman Design

The Plackett-Burman design was used to analyze important factors. Twenty experiments were conducted in duplicate to evaluate 14 factors. A total of 14 components (independent variables k = 14, Table 1) were selected for the study, with each variable being represented at two levels, high (+) and low (-), as well as two dummy variables in 20 trials (Table 2). The two dummy variables were used to calculate the standard error.

Box-Behnken Design and Response Surface Methodology

The significant variables identified from the previous experiments were optimized using Box-Behnken design at different levels (Table 3), while the other variables of nonsignificance were fixed at the initial medium level. In developing the regression equation, the relation between the coded values and actual values are described in Eq. (1) below: [11]

$$X_i = \frac{(A_i - A_0)}{\Delta A_i} \quad (1)$$

Table 1. High(-1) and low(+) values of the independent variables in the Plackett-Burman design.

Factor	Levels of factor	
	-1	1
M100 (X_1, g/l)	10	30
Glycerol (X_2, g/l)	10	30
Soybean meal (X_3, g/l)	1	10
Soytone (X_4, g/l)	1	10
Yeast extract (X_5, g/l)	1	6.5
$(NH_4)_2SO_4$ (X_6, g/l)	1	5
L-Lysine (X_7, g/l)	1	5
KH_2PO_4 (X_8, g/l)	1	2.5
K_2HPO_4 (X_9, g/l)	1	2.5
NaCl (X_{10}, g/l)	1	5
$FeSO_4 \cdot 7H_2O$ (X_{11}, g/l)	0	0.1
$CaCO_3$ (X_{12}, g/l)	1	5
MES (X_{13}, g/l)	21	42.6
pH (X_{14}, g/l)	5	5.5
Dummy1 (D_1, g/l)	-1	1
Dummy2 (D_2, g/l)	-1	1

where X_i is the coded value of the *i*th variable, A_i is the actual value of the *i*th variable, A_o is the actual value of the *i*th variable at the center point, and ΔA_i is the step change value of the *i*th variable. The correlation between the response and the four variables were fitted to a predictive quadratic polynomial equation as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad i = 1,2,3, \ldots \ldots k \qquad (2)$$

where Y was the predicted response, β_0 is the intercept term, β_i is the linear coefficient, β_{ii} is the squared coefficient, and β_{ij} is the interaction coefficient. X_i, X_j represented the independent factors (medium component) in the form of coded values. The accuracy and general ability of the above polynomial model could be

Table 3. Coded and real values of factors in the Box-Behnken design.

Factor	Level of factor		
	-1	0	1
Glycerol (X_2, g/l)	10	30	50
Soytone (X_4, g/l)	1	10.5	20
Yeast extract (X_5, g/l)	1	5.5	10
$CaCO_3$ (X_{12}, g/l)	0	5	10

Table 2. Combinations of variables and responses in the Plackett–Burman design experiment.

Run	Independent variables														Dummy variables		Rapamycin (mg/l)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	D_1	D_2	
1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	29.8 ± 0.7
2	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	12.55 ± 2.15
3	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	77.35 ± 2.25
4	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	5.95 ± 0.05
5	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	41.95 ± 1.55
6	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	40.4 ± 18.2
7	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	61.4 ± 5.5
8	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	30.1 ± 0.4
9	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	43.7 ± 10
10	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	12.15 ± 0.85
11	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	43.8 ± 0.2
12	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	36.95 ± 9.05
13	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	12.55 ± 2.85
14	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	42.9 ± 3.2
15	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	19.45 ± 0.95
16	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	45.5 ± 10
17	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	31.3 ± 5.4
18	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	30.5 ± 0.8
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	44.2 ± 12.2
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	36.85 ± 2.95

evaluated by the coefficient of determination R^2 [4]. Each experimental design was carried out in duplicate, and the mean values were used for further anlaysis. The factors that were significant at 95% confidence ($p < 0.05$) from the regression analysis were considered to have greater effects on the rapamycin production and were further optimized by response surface methodology using the Box-Behnken design.

Results

Screening of Essential Medium Components Using the Plackett–Burman Design

Fourteen different carbon and nitrogen sources and inorganic salts factors were evaluated for their suitability to sustain increased rapamycin production by *Streptomyces hygroscopicus*. The Plackett-Burman design was used for initial screening of medium components. Tables 1 and 2 show the effects of the 14 components, and Table S1 shows their significant levels. Seven components, as displayed in Fig. 1, were found to have a significant effect on rapamycin production (*i.e.*, soytone, $CaCO_3$, yeast extract, glycerol, pH, $FeSO_4{\cdot}7H_2O$, and soybean). The components were screened at a confidence level of 95% on the basis of their effects. Soybean, $FeSO_4{\cdot}7H_2O$, and the intial pH were excluded because the effects by soybean and $FeSO_4{\cdot}7H_2O$ were relatively weak and the initial pH had no significant effects on rapamycin production. Variables showing effects with a confidence level of 99.1% (glycerol), 100% (soytone), 99.2% (yeast extract), and 100% ($CaCO_3$) were identified as important factors for rapamycin production. Thereafter, the exact optimal values for the individual factors were determined using the Box-Behnken design.

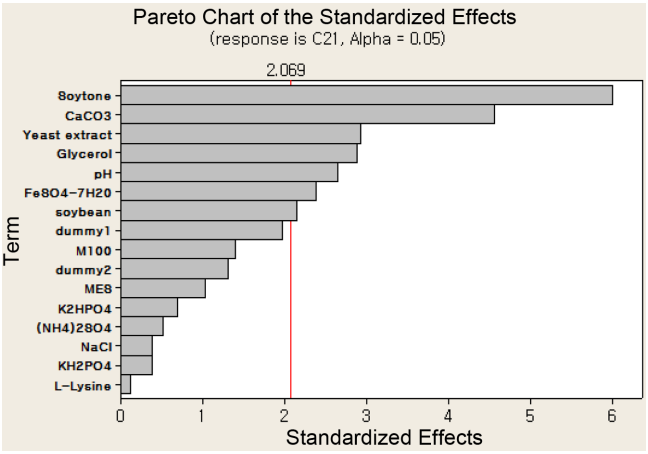

Fig. 1. Pareto chart of the 14-factor standard effects on rapamycin production.

Optimization of Screened Medium Component Using the Box-Behnken Design

The four most significant factors (glycerol, soytone, $CaCO_3$, and yeast extract) among seven were selected and examined in a Box-Behnken design in 27 runs (Table 4). Rapamycin production was evaluated to find the combined effect of the four factors in their specific ranges. The variables showing effect with a confidence level of 99.1% (glycerol), 100% (soytone), 99.2% (yeast extract), and 100% ($CaCO_3$) (Table S1) in the Plackett-Burman design were selected, and further optimization was achived using a Box-Behnken design. A contour plot was drawn and the data revealed that $CaCO_3$ is a critical factor to determine rapamycin productivity, whereas soytone and yeast extract showed negative effect at certain ranges on rapamycin production, and glycerol showed a positive effect (Fig. 2).

$$\text{Rapamycin (mg/l)} = 58.800 - 23.633\ A - 16.934\ B + 112.738\ C - 8.534D + 20.691\ B^2 - 18.605\ D^2 \quad (3)$$

(A: soytone; B: $CaCO_3$; C: glycerol; D: yeast extract)

Rapamycin production is the predicted response and A, B, C, and D are the coded variables for soytone, $CaCO_3$,

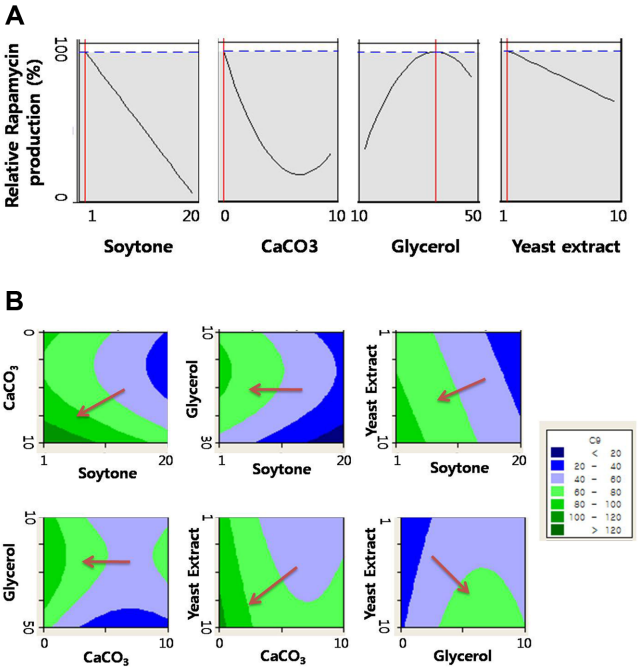

Fig. 2. Countour plots showing the effects of independent variables (soytone, yeast extract, glycerol, and calcium carbonate concentration) on rapamycin production.

Table 4. Box-Behnken design matrix with experimental values of rapamycin production and final pH.

Run	Soytone (g/l)	$CaCO_3$ (g/l)	Glycerol (g/l)	Yeast extract (g/l)	Final pH	Rapamycin (mg/l)
1	1	0	30	5.5	5.0 ± 0.0	128.4 ± 11.8
2	20	0	30	5.5	5.9 ± 0.0	60.7 ± 7.9
3	1	10	30	5.5	5.7 ± 0.1	90 ± 2.0
4	20	10	30	5.5	5.9 ± 0.0	35.7 ± 1.2
5	10.5	5	10	1	5.5 ± 0.0	33.5 ± 6.4
6	10.5	5	50	1	5.6 ± 0.1	67.5 ± 2.1
7	10.5	5	10	10	6.1 ± 0.0	17.9 ± 5.8
8	10.5	5	50	10	5.7 ± 0.0	25.2 ± 24.5
9	1	5	30	1	5.0 ± 0.1	68.9 ± 0.3
10	20	5	30	1	6.0 ± 0.1	30.8 ± 2.5
11	1	5	30	10	5.6 ± 0.1	57.8 ± 6.4
12	20	5	30	10	6.2 ± 0.1	24.0 ± 3.0
13	10.5	0	10	5.5	5.7 ± 0.0	45.2 ± 3.6
14	10.5	10	10	5.5	5.9 ± 0.2	27.5 ± 0.3
15	10.5	0	50	5.5	5.4 ± 0.0	101 ± 2.1
16	10.5	10	50	5.5	6.1 ± 0.1	52.5 ± 2.6
17	1	5	10	5.5	6.0 ± 0.1	75.2 ± 0.9
18	20	5	10	5.5	6.6 ± 0.0	12.5 ± 0.2
19	1	5	50	5.5	5.4 ± 0.1	66.8 ± 24.8
20	20	5	50	5.5	6.1 ± 0.1	39.7 ± 4.1
21	10.5	0	30	1	5.4 ± 0.1	116.1 ± 4.9
22	10.5	10	30	1	5.7 ± 0.1	66.0 ± 4.4
23	10.5	0	30	10	6.2 ± 0.0	89.6 ± 1.0
24	10.5	10	30	10	5.9 ± 0.1	66.0 ± 0.9
25	10.5	5	30	5.5	5.5 ± 0.0	73.2 ± 0.9
26	10.5	5	30	5.5	5.5 ± 0.0	71.5 ± 1.3
27	10.5	5	30	5.5	5.6 ± 0.1	68.0 ± 1.4

glycerol, and yeast extract respectively (Eq. (3)). Soytone, $CaCO_3$, and yeast extract had negative effects in the rapamycin production media, whereas glycerol showed positive effects on rapamycin productivity. The expected maximum rapamycin production was 130.4 mg/l using media containing glycerol 36.2 g/l, soytone 1 g/l, yeast extract 1 g/l, and no $CaCO_3$.

Effect of pH on Rapamycin Production

The effect of the initial pH value on rapamycin production was examined in the Plackett-Burman design. The effects from initial pH were smaller than the other significant factors, and no significant effects were observed when the initial pH was between 6 and 8 in the flask trials [24]. Thus, we excluded the initial pH factor from the effect factors. However, the final pH was observed for correlation

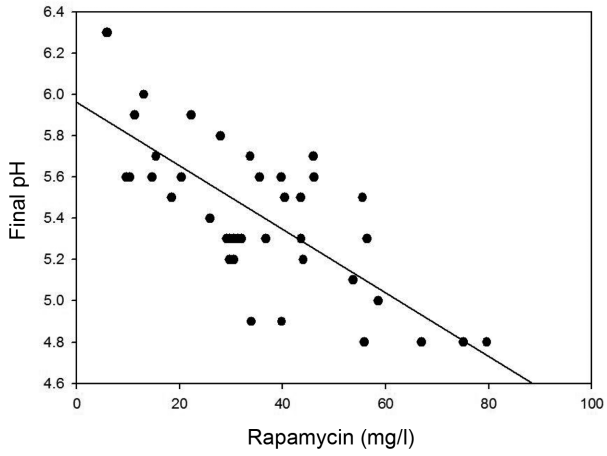

Fig. 3. Correlation of final pH and rapamycin production on data used for the Plackett-Burman design.

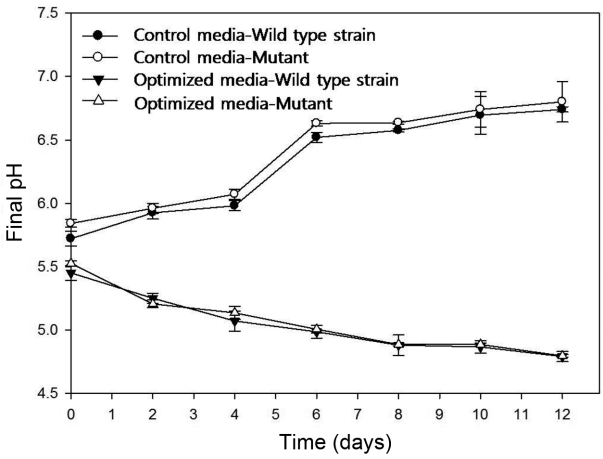

Fig. 4. Time course data of pH during rapamycin production in optimized and unoptimized media for wild-type and mutant-type *Streptomyces hygroscopicus*.

between the pH and production of rapamycin (Fig. 3). We found rapamycin production increased linearly with the decrease in pH. For this reason, final pH was statistically a significant factor; namely, a decrease in pH levels leads to an increase in rapamycin production (Fig. 4).

$$pH = 0.0154\ (\text{rapamycin}) + 5.963 \quad (4)$$

$$\text{rapamycin} = 64.94\ pH - 387.21\ (4.8 < pH < 6.3) \quad (5)$$

$CaCO_3$ is a determining factor for the change of pH, and so the decrease of rapamycin production with an increase in pH indicated that $CaCO_3$ is a critical and negative factor for rapamycin production. The maximum rapamycin production reached 220.7 ± 5.7 mg/l in the optimized media from *S. hygroscopicus* mutant, whereas 151.9 ± 22.6 mg/l rapamycin production was recorded in the unoptimized media. For the wild strain, 76.2 ± 4.7 and 37.5 ± 2.8 mg/l rapamycin production were observed in optimized and unoptimized media, respectively (Fig. 5). We observed an increase in the production of rapamycin by 5.88-fold compared with-the wild type *S. hygroscopicus*.

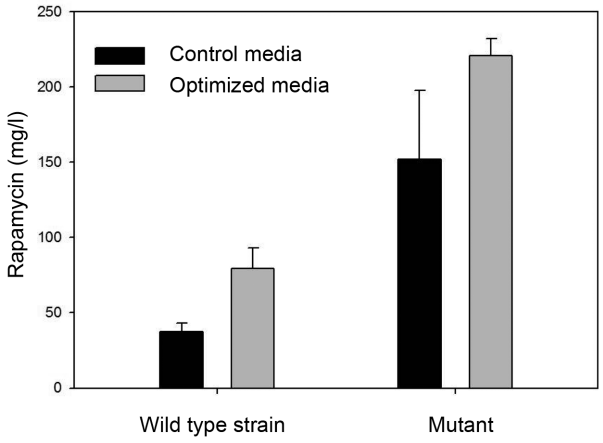

Fig. 5. Comparison of rapamycin production by *Streptomyces hygroscopicus* in optimized and unoptimized media.

Discussion

Rapamycin is a potent immunosuppressive secondary metabolite that has been increasingly of interest for clinical treatments. However, the low titers of rapamycin limited its availability for expanded industrial use [19]. The present report is an attempt to formulate a glycerol-based medium for rapamycin production with *S. hygroscopicus*. A systematic study of rapamycin improvement through medium optimization, and the effects of various medium components at different concentrations were investigated using a Plackett-Burman experiment. From the 14 factors, soytone, glycerol, $CaCO_3$, and yeast extract were selected for their effect on rapamycin production in a Box-Behnken design for further optimization. We found that $CaCO_3$, soytone, and yeast extract showed negative effects on rapamycin production, whereas the glycerol showed a positive effect. $CaCO_3$ modulated the dissociated-undissociated equilibrium and prevented a reduction in pH during the fermentation [15]. Omission of $CaCO_3$ and a lower amount of nitrogen resulted in the medium having a lower final pH [13]. The glycerol showed positive effect, and importantly the final pH also showed correlation with rapamycin production. Glycerol is potentially also an economical feedstock for fermentation, and it can be consumed more quickly after adding ammonium sulfate in the rapamycin biosynthesis phase, which will lead to an increase of the pool of rapamycin biosynthesis. This unique characteristic of glycerol offers a tremendous opportunity for its biological conversion to valuable products at higher yield. The total amount of rapamycin produced was correlated to final pH and it was found that the acidic pH might have triggered initiation of the stationary or secondary metabolite production phase [7]. Although the exact mechanism was not revealed, different metabolites related to the TCA cycle, fatty acids metabolism, and fatty acid degradation seemed to trigger the drop in pH, resulting in pH shock and contributing to activation of the TCA cycle and production of secondary metabolites [7, 23]. Finally, it is expected that an acidic pH strongly promoted

production of rapamycin, as the pH can affect the production of rapamycin complex by changing the electron interaction of the rings.

In conclusion, this research is a systematic optimization for rapamcyin production by a *S. hygroscopicus* mutant. By applying statistical design, we have desgined a medium for rapamycin production, resulting in an increase of rapamycin production by nearly 40% compared with an unoptimized production medium.

Acknowledgments

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