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Simultaneous improvement of lincomycin A production and reduction of lincomycin B levels in *Streptomyces lincolnensis* using a combined medium optimization approach

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Abstract

Purpose: The current study aimed to optimize the culture and production parameters of industrial production of lincomycin A by *Streptomyces lincolnensis* using a statistical approach that could also reduce unwanted by-products.

Methods: The Plackett-Burman design, steepest ascent method, and response surface design were used to evaluate different factors that affect lincomycin A production.

Results: Using an optimized *S. lincolnensis* fermentation medium, lincomycin A production was increased up to 4600 mg/L in shaking flasks, which indicated a 28.3% improvement over previous production in an un-optimized medium (3585 mg/L). Additionally, the concentration of lincomycin B by-product was reduced to 0.8%, which was 82.2% lower than that in the un-optimized medium. Further, quantitative real-time PCR analysis revealed the optimized medium improved lincomycin A production by stimulating key genes in the lincomycin A biosynthesis pathway, as well as an osmotic stress gene.

Conclusions: Based on the results, the sequential optimization strategy in this study provides powerful means for the enhancement of lincomycin A with less by-product. We found that osmotic stress reduced the concentration of lincomycin B, which could also help reduce fermentation by-product yields in other actinobacteria.

Keywords: Streptomyces lincolnensis, Lincomycin, Medium optimization, Osmotic stress, qRT-PCR

Background

Streptomyces species produce a wide range of biologically active secondary metabolites, including antitumor agents, immunosuppressants, and antibiotics with clinical, agricultural, and veterinary uses (Bibb 2005). Among these products, lincomycin is a natural antimicrobial that belongs to the lincosamides class of antibiotics, which was first discovered in *Streptomyces lincolnensis* from Lincoln, Nebraska (Chang and Weisblum 1967; Hornish et al. 1987). Lincomycin is one of the most widely used antibiotics administered to animals and humans against Gram-positive bacteria; it exerts its antibacterial action by inhibiting protein synthesis and blocking the peptidyl transferase reaction on the 50S ribosome subunit in sensitive bacteria (Brahme et al. 1984; Spížek and Řezanka 2004). Recent research has focused on maximizing lincomycin production using *S. lincolnensis* by establishing fermentation conditions for commercial applications (Lee et al. 2014). The world market for lincomycin is at the scale of hundreds of tons per year. According to the ESVAC report, sales of lincosamides were approximately

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300 tons in the EU, which represented approximately 5% of the total sales of veterinary pharmaceuticals in 2017 (EMA/294674/2019).

In the industrial fermentation of *S. lincolnensis*, desirable lincomycin A, a 3C 4-alkyl-L-proline derivative (APD), is the main product. The structure of lincomycin A consists of a methylthiolincosaminide (MTL) moiety and a 4-propyl-l-proline (PPL) moiety (Figure S1). Molecular mechanism of lincomycin biosynthesis becomes clearer based on the experimental efforts made in recent years. The MTL is derived from a transaldol reaction using three sugars from the pentose phosphate pathway (Sasaki et al. 2012), while the PPL originates from hydroxylation of L-tyrosine catalyzed by LmbB2 (Neusser et al. 1998). Then the PPL and MTL are condensed by two small molecule thiols ergothioneine and mycothiol (Spížek and Řezanka 2017). Finally, lincomycin A is synthesized (Najmanová et al. 2013; Spížek and Řezanka 2017). However, lincomycin B, which has a 2C instead of a 3C APD moiety, is an undesirable by-product that often appears as a minority component in production cultures (Argoudelis et al. 1964; Pang et al. 2015; Janata et al. 2018). It exhibits only 25% of the antibiotic activity as compared with lincomycin A and has a high toxicity. According to the guidelines from Pharmacopoeias in the USA and China, lincomycin B content in a lincomycin formulation should be less than 5% (Pang et al. 2015). During fermentation, undesirable lincomycin B usually accounts for 7–10% of the total lincomycin content and must be removed by complicated downstream purification processes before it can be used as a marketable medicine or active pharmaceutical ingredient. Thus, it is necessary to maximize lincomycin A and minimize lincomycin B production through process optimization during fermentation.

To date, few studies have focused on reducing the production of lincomycin B in the upstream steps of fermentation. At present, reduction of lincomycin B production in the fermentation broth has mainly been through genetic engineering. In the biosynthesis of lincomycin, *lmbW* is an important methylase gene that is responsible for the C-methylation of 4-n-propyl-L-proline and determines whether 4-n-propyl-L-proline synthesizes lincomycin A or lincomycin B (Pang et al. 2015). The lincomycin B content was reduced to 4.41% by overexpressing both *lmbW* and *metK* genes, which regulate APD and propylproline biosynthesis of lincomycin (Pang et al. 2015). The *lmbB1* gene encodes for a 2,3-extradiol cleavage enzyme that breaks down the L-3,4-dihydroxyphenylalanine (L-DOPA) aromatic ring (Novotná et al. 2004). Replacing the tyrosinase involved in melanin synthesis, LmbB1 is assumed to be a dioxygenase that catalyzes the cleavage of 2,3-extradiol in the aromatic ring of L-3,4-dihydroxyphenyl alanine (Zhong et al. 2017). *LmbB1* is a key gene that determines whether L-DOPA follows the lincomycin or the melanin biosynthesis pathway. The overexpression of *lmbB1* (structural gene) alone only reduced the content of lincomycin B to 2.5% (Yang et al. 2020). However, few studies have been conducted on reduction of lincomycin B content in the fermentation broth through fermentation engineering.

Microorganisms cope with environmental stress factors during industrial fermentation processes, such as osmotic, temperature, and oxidative stress stresses, which can significantly impact their primary and secondary metabolism (Lee et al. 2005; Li et al. 2009). Osmotic stress has an important effect on the secondary metabolism of *Streptomyces*. For example, disruption of either *osaBSa*, which encodes a response regulator protein, led to increased production of oligomycin up to 200%, and avermectin up to 37% (Godinez et al. 2015). Regulation of the osmotic stress response can impact both development and antibiotic production in the model streptomycete, *S. coelicolor* (Bishop et al. 2004; Martínez et al. 2010). Bacteria have two families of mechanosensitive (MS) channels: small conductance (MscS) and large conductance (MscL) channels (Blount and Moe 2005; Blount and Iscla 2012). The majority of bacteria contain a single copy of *mscl*, which is highly conserved between species (Wray et al. 2019). In bacteria, MS channels act as emergency release valves; when bacteria are exposed to high osmotic pressure, they will transport (K^+, glutamate, betaine, and proline) and synthesize (glutamate, trehalose, proline, and betaine) solutes to balance the increase in external osmotic pressure to maintain high cell turgor, which is a requisite for cell growth and division.

Carbon sources as well as nitrogen sources are the most important factors in lincomycin production using *S. lincolnensis*. The preferred carbon sources for the production of lincomycin by *Streptomyces* were glucose (Bergy et al. 1967; Bhattacharyya et al. 1998). Similarly, corn steep liquor was among the preferred nitrogen sources for lincomycin production (Bergy et al. 1967; Bhattacharyya et al. 1998). Glucose, which is metabolized through glycolysis or pentose phosphate pathway, is not only essential for cell growth and energy source, but also can supply abundant of precursor for the desired product. The amount of the glucose is essential for lincomycin's production (Singh et al. 2017). Corn steep liquor is a good nitrogen source that is easily utilized by microorganisms because it is rich in amino acids, reducing sugars, phosphorus, trace elements, and biotin (Chu and Li 2007). Therefore, corn steep liquor is an essential key raw material in the fermentation process of lincomycin. However, excessive corn steep liquor in the medium will lead to high concentration of NH_4^+, which might cause

the ammonium ion suppression and subsequently reduce lincomycin A production by decreasing the specific activity of antibacterial glutamine synthetase (Jin et al. 2001).

Production of secondary metabolites depends on the strain and species of microorganisms used as well as on the fermentation medium, which can significantly affect product yield. The traditional "one-factor at a time" optimization strategy requires a considerable amount of time and labor, and often leads to unreliable results and inaccurate conclusions. As an alternative, statistical strategy is an efficient approach to accounting for a large number of variables and has been effectively applied to optimize cultivation processes (Mansouriieh et al. 2019; Fridous et al. 2020; Eskandari and Etemadifar 2021).

For example, Plackett-Burman design (PBD), the steepest ascent method, and Box-Behnken Design (BBD) have all been successfully applied for determining the components or variables that have significant effects on the production of primary or secondary metabolites and have statistically optimized metabolic production (Di et al. 2017; Wang et al. 2019). By optimizing media components, the yield of lincomycin A was increased from 1 g/L to 3.5 g/L (Luthra et al. 2019). Moreover, adding phosphorus improved the fermentation titer of lincomycin A (Li et al. 2007). However, few papers have addressed the use of a design method for medium formulation to improve lincomycin A productivity while minimizing lincomycin B accumulation.

For the strain used in this study was a mutagenic strain, we must optimize the conditions in the shake flask for the strain firstly. This study describes the successful optimization of a culture medium to produce lincomycin A with minimal lincomycin B production by an industrial lincomycin-producing strain of *S. lincolnensis* using statistical mixture designs. This was achieved by implementing PBD, steepest ascent, and BBD methodologies. Finally, we used transcription profiling to identify genes involved in the lincomycin biosynthetic pathway and osmotic pressure to improve our understanding of the mechanism underlying lincomycin accumulation in *S. lincolnensis*.

Materials and methods

Microorganism and culture conditions

The strain *S. lincolnensis* 24 was kindly provided by Topfond Pharmaceutical Co., Henan, China. *S. lincolnensis* 24 strain was activated on slant medium for approximately 7 days at 30 °C. Subsequently, approximately 0.5 m^3 strains from the solid medium were inoculated into 25 mL of seed medium for 48 h in Erlenmeyer flasks (250 mL) at 30 °C and at 220 rpm as the first-grade seeds. Then, 10% of first-grade seed was transferred into 25 mL fermentation medium at 30 °C and at 220 rpm for 7 days.

The media used in this study included (1) slant medium: 19 g/L soluble starch, 5 g/L soybean powder, 0.5 g/L NaCl, 1 g/L KNO_3, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 0.01 g/L $FeSO_4$, 12 g/L agar. (2) seed medium: 20 g/L soybean powder, 10 g/L glucose, 20 g/L corn starch, 30 g/L corn steep liquor, 2 g/L $(NH_4)_2SO_4$ and 5 g/L $CaCO_3$; (3) initial fermentation medium (control): 25 g/L soybean powder, 2 g/L corn steep liquor, 5 g/L NaCl, 8 g/L $NaNO_3$, 0.2 g/L KH_2PO_4, 8 g/L $(NH_4)_2SO_4$, 100 g/L glucose, 8 g/L $CaCO_3$. (4) optimize fermentation medium (OP): except for corn steep liquor (1.4 g/L) and glucose (126 g/L), other components were maintained constant as initial fermentation medium (4) Medium-1 (M-1): except for NaCl (10 g/L), other components were maintained constant as initial fermentation medium. (5) Medium-2 (M-2): except for glucose (126 g/L), other components were maintained constant as initial fermentation medium. (6) Medium-3 (M-3): except for corn steep liquor (1.4 g/L), other components were maintained constant as initial fermentation medium.

Analytical method

Lincomycin A and lincomycin B were quantified by high-performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA, USA) on Eclipse XDB-C18 column (5 μm, i.d. 4.6 × 250 mm). The samples were eluted with 60% (*v/v*) methanol and 40% (*v/v*) 50 mM ammonium acetate at a flow rate of 0.6 mL/min (30 °C). The lincomycin peak was separated and detected by UV absorbance at 210 nm. The peak areas were proportional to the concentration of lincomycin. The injection volume was 20 μL, and the retention time of lincomycin was about 15 min. The supernatant of the fermentation broth is chemically analyzed for reducing sugar level via the Fehling reagent (Xu et al. 2004).

The packed mycelia volume (PMV) value was the percentage of precipitate volume to broth volume (Zhang et al. 2019).

Supernatant samples were obtained by centrifugation at 3000 rpm for 10 min. The 600 μL centrifuged supernatant of a lincomycin culture or broth was transferred to EP tube. Then the osmotic pressure of the fermentation broth was determined with an automatic freezing point osmometer FM-8P (Shanghai, China) (Wucherpfennig et al. 2011; Song et al. 2018).

Quantitative RT-PCR analysis

The sample time for qRT-PCR was set at 48 h. Spin Column Bacteria Total RNA Purification Kit (Sanhon Biotech, China) was used for the total RNA extraction and HiFiScript gDNA Removal RT MasterMix (Kangwei Century Biotechnology Co., Ltd., Taizhou, China) for cDNA synthesis, following procedures recommended by

the manufacturers. The reverse transcription procedure was as follow: 37 °C for 15 min, 85 °C for 5 s. Quantitative Real-time PCR was performed using the Bio-Rad CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Ultra SYBR Mixture (Kangwei Century Biotechnology Co., Ltd.) was used to prepare the reaction solution at low temperature, according to the instructions. PCR was performed using the following protocol: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 1 min. qRT-PCR was conducted in triplicate for each sample. With the transcription level of each gene in the control group as the reference unit, relative gene quantification was originally performed using the comparison $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) and normalized to 16S rDNA.

Plackett-Burman design

The Plackett-Burman design was used to screen the most important medium components for lincomycin A production. In general, this design is a two-factorial design, which identifies the significant influencing factors required for improved targeted production by screening n variables in $n + 1$ experiments (Burman and Plackett 1946). In this experiment, 8 independent and three dummy variables were selected for the screening in 12 trials. Each variable was represented at two levels high and low (Table 1). The variables chosen for the present study were soybean powder (X_1), corn steep liquor (X_2), NaCl (X_3), $NaNO_3$ (X_4), KH_2PO_4 (X_5), $(NH_4)_2SO_4$ (X_6), glucose (X_7), and $CaCO_3$ (X_8).

Statistical experimental designs

During medium optimization, response surface methodology (RSM) can assess the relationships between the independent variables and the responses with useful mathematical and statistical techniques. The two most common designs used in RSM are the central composite design (CCD) and Box-Behnken design (BBD). Compared to the CCD method, the BBD is considered the most suitable for evaluating quadratic response surfaces, and is much easier to realize because it requires less factor levels, (Lee et al. 2011). BBD is a rotatable three-level design, which is used for the investigation of response surface method and the constructing a second-order polynomial model. The respective levels for the three variables are shown in Table S1, and the concentrations of the other variables were set at zero levels. The results were described using the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_7 + \beta_{11} {X_2}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_7}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_7 + \beta_{23} X_2 X_7, \quad (1)$$

where Y represents the dependent variable (lincomycin A or lincomycin B content), β_0 represents the model constant, β_1, β_2, and β_3 represent the linear coefficients, β_{11}, β_{22}, and β_{33} represent the squared coefficients, and β_{12}, β_{13}, and β_{23} represent interaction coefficients (Chen et al. 2020).

Table 1 Three-level design of Plackett-Burman experiment

Source	Levels (g/L)		
	−1	0	+1
X_1	20	25	30
X_2	1.6	2	2.4
X_3	4	5	6
X_4	6.4	8	9.6
X_5	0.16	0.20	0.24
X_6	6.4	8	9.6
X	80	100	120
X_8	6.4	8	9.6

X_1 soybean powder, X_2 corn steep liquor, X_3 NaCl, X_4 $NaNO_3$, X_5 KH_2PO_4, X_6 $(NH_4)_2SO_4$, X_7 glucose, X_8 $CaCO_3$

Results and discussion

Plackett-Burman design to improve lincomycin A production in S. lincolnensis fermentation

PBD was developed using Design-Expert software (Wang et al. 2019). The design matrix selected for the screening of significant variables for lincomycin A and lincomycin B production (Table 2). The confidence level was set at 5%; therefore, the variables that scored a probability (p) value less than 0.05 were considered significantly influential factors that could affect lincomycin production. The statistical analysis of the model was used an analysis of variance (ANOVA) to evaluate the significance and effectiveness of the design. The ANOVA for lincomycin A content showed that soybean powder, corn steep liquor, glucose, and $CaCO_3$ were components of the culture medium that significantly affected lincomycin A production, as their p values were less than 0.05 (Table S2). ANOVA analysis of lincomycin B content showed that soybean powder, corn steep liquor, glucose, and $NaNO_3$ were components that had a significant effect on lincomycin A production, as their p values were also less than 0.05 (Table S2). Lincomycin A and lincomycin B content was significantly affected in media that contained soybean powder, corn steep liquor, and glucose. Thus, the important factors identified by the initial Plackett-Burman screening method, which influenced lincomycin A and lincomycin B content, were soybean powder, corn steep liquor, and glucose.

Table 2 Results of Plackett-Burman experiment

Run	X_1 (g/L)	X_2 (g/L)	X_3 (g/L)	X_4 (g/L)	X_5 (g/L)	X_6 (g/L)	X_7 (g/L)	X_8 (g/L)	Lincomycin A (mg/L)	Lincomycin B (%)
0	0	0	0	0	0	0	0	0	3555	4.23
1	+ 1	− 1	+ 1	+1	− 1	− 1	− 1	+ 1	2884	1.54
2	− 1	+ 1	+ 1	+ 1	+ 1	+ 1	− 1	− 1	2625	1.89
3	− 1	− 1	− 1	− 1	− 1	− 1	− 1	− 1	3706	5.43
4	+ 1	− 1	− 1	+ 1	+ 1	+ 1	+ 1	+ 1	3937	4.36
5	+ 1	+ 1	+ 1	+ 1	− 1	− 1	+ 1	− 1	3083	1.08
6	− 1	+ 1	− 1	+ 1	+ 1	− 1	− 1	+ 1	3124	2.15
7	+ 1	+ 1	− 1	− 1	− 1	+ 1	− 1	+ 1	1837	1.66
8	+ 1	− 1	+ 1	− 1	+ 1	+ 1	− 1	− 1	1568	2.62
9	− 1	− 1	− 1	− 1	− 1	+ 1	+ 1	− 1	4364	4.69
10	+ 1	+ 1	− 1	− 1	+ 1	− 1	+ 1	− 1	2624	4.38
11	− 1	− 1	+ 1	− 1	+ 1	− 1	+ 1	+ 1	4542	4.83
12	− 1	+ 1	+ 1	− 1	− 1	+ 1	+ 1	+ 1	4439	4.62

The values were obtained from three independent experiments

X_1 soybean powder, X_2 corn steep liquor, X_3 NaCl, X_4 $NaNO_3$, X_5 KH_2PO_4, X_6 $(NH_4)_2SO_4$, X_7 glucose, X_8 $CaCO_3$

Table 3 Three-factors steepest ascent experiment

Run	X_1 (g/L)	X_2 (g/L)	X_7 (g/L)	Lincomycin A (mg/L)	Lincomycin B (%)
0	25	2	100	3536 ± 82	4.4 ± 0.2
1	15	1.2	140	3421 ± 102	5.3 ± 0.2
2	20	1.3	133	3875 ± 48	2.8 ± 0.1
3	25	1.4	126	4606 ± 101	0.8±0.1
4	30	1.5	119	3671 ± 152	2.4 ± 0.1
5	35	1.6	112	3415 ± 72	3.7 ± 0.2
6	40	1.7	105	2299 ± 96	3.6 ± 0.1
7	45	1.8	98	1029 ± 22	7.2 ± 0.3

The values were obtained from three independent experiments

X_1 soybean powder, X_2 corn steep liquor, X_3 NaCl, X_4 $NaNO_3$, X_5 KH_2PO_4, X_6 $(NH_4)_2SO_4$, X_7 glucose, X_8 $CaCO_3$

Steepest ascent improved culture conditions for optimizing lincomycin A production

Because soybean powder and corn steep liquor exerted negative effects on lincomycin A and glucose exerted a positive effect (Table S2), the direction of steepest ascent indicated that the concentration of glucose should increase and the concentrations of soybean powder and corn steep liquor should decrease for optimal experimental conditions that maximize lincomycin A production. Five sets of experiments using the steepest ascent and corresponding experimental results showed that the optimum value area was located in group Run3 (Table 3).

These results helped define the optimal concentrations as 25 g/L soybean powder, 1.4 g/L corn steep liquor, and 126 g/L glucose; further suggesting that lincomycin A and lincomycin B were proximal to the region of maximum production. Accordingly, the concentrations of the three medium ingredients in the third out of five experiments were considered the center point of BBD.

Box-Behnken design experimental optimization and response surface analysis of growth conditions that affect lincomycin production

BBD is a well-known optimization method based on the establishment of a mathematical model that assesses the statistical significance of the effects of different factors on the final response (Annadurai and Sheeja 1998). The BBD method was used to assess the effect of soybean powder, corn steep liquor, glucose concentrations on lincomycin A, and B production. A total of 17 experiments with different combinations of nutrient concentrations were performed and their effects on lincomycin A and B production are presented in Table 4. Multiple regression analysis of the experimental data used a second-order polynomial equation derived for the lincomycin A yield and lincomycin B content using the significant terms:

$$Y_1 = 4569.6 - 220.88 \times X_1 + 54.87 \times X_2 - 345.75 \times X_7 - 236.25 \times X_1 - 236.50 \times X_1X_7 + 114.50 \times X_2X_7 - 829.93 \times X_12 + 78.57 \times X_22 - 368.17 \times X_72 \quad (2)$$

$$Y_2 = 0.96 + 0.20 \times X_1 + 0.013 \times X_2 - 0.19 \times X_7 + 0.15 \times X_1X_2 - 0.05 \times 10^{-3} \times X_1X_7 + 0.17 \times X_2X_7 + 1.18 \times X_12 + 0.56 \times X_22 + 1.76 \times X_72 \quad (3)$$

where Y_1 (mg/L) is the predicted lincomycin A yield; Y_2 (%) is the predicted lincomycin B content; and X_1,

Table 4 Box-Behnken design experimental design

Run	X_1 (g/L)	X_2 (g/L)	X_7 (g/L)	Lincomycin A (mg/L)	Lincomycin B (%)
0	25	2	100	3519 ± 71	4.8 ± 0.2
1	25	1.68	151.2	4078 ± 39	3.0 ± 0.1
2	20	1.40	151.2	3464 ± 64	3.6 ± 0.1
3	25	1.68	100.8	4511 ± 36	3.5 ± 0.2
4	30	1.40	100.8	3752 ± 75	4.3 ± 0.2
5	20	1.12	126	3712 ± 61	2.8 ± 0.2
6	30	1.68	126	3452 ± 54	2.9 ± 0.1
7	20	1.68	126	4375 ± 122	2.6 ± 0.2
8	30	1.40	151.2	2558 ± 102	4.3 ± 0.2
9	25	1.12	100.8	4711 ± 58	3.9 ± 0.1
10	30	1.12	126	3734 ± 193	2.5 ± 0.2
11	25	1.12	151.2	3820 ± 19	2.7 ± 0.1
12	20	1.40	100.8	3712 ± 15	3.4 ± 0.1
13	25	1.40	126	4500 ± 41	1.0 ± 0.1
14	25	1.40	126	4580 ± 46	0.8 ± 0.1
15	25	1.40	126	4608 ± 78	1.3 ± 0.1
16	25	1.40	126	4612 ± 43	1.2 ± 0.1
17	25	1.40	126	4548 ± 57	0.8 ± 0.1

The values were obtained from three independent experiments. *X1* soybean powder, *X2* corn steep liquor, *X3* NaCl, *X4* NaNO3, *X5* KH2PO4, *X6* (NH4)2SO4, *X7* glucose, *X8* CaCO3

X_2, and X_7 are the coded values of soybean powder, corn steep liquor, and glucose, respectively. Statistical significance of the response surface model and all the coefficient estimates were assessed with ANOVA (Tables S3 and S4). The high F value (180.64) and a very low p-value (< 0.0001) suggested that the model was highly significant, while an insignificant lack of fit ($p = 0.2201 > 0.05$) revealed the effectiveness of the regression analysis, suggesting that the regression model could fit the effect of the three culture factors on lincomycin A content. The ratio of the explained and total variation indicated that the coefficient of determination (R^2) could be used to assess the goodness of the model. The value of R^2 was 99.57%, which indicated that only 0.43% of the lincomycin A content variability could not be explained by the predicted equation of the model. The Adj-R^2 value of 99.02% further validated the significance of this model. A low coefficient of variation (CV) (CV = 1.44%) value revealed that the deviations between the predicted and experimental values were low, and it displayed a high degree of precision and reliability in the conducted experiments. "Adeq Precision" provides the signal-to-noise ratio and a ratio greater than four is desirable. In this study, a ratio (47.512) greater than 4 indicated the use of this model in future studies will be supported.

The ANOVA showed that the model for lincomycin B content had a p value = 0.0006, suggested that the model was highly significant (Table S3). The lack of fit ($p = 0.1252 > 0.05$) was insignificant, suggesting that the regression model could fit the effect of the three culture factors on lincomycin B content.

The R^2 was 0.9628, Adj-R^2 was 0.9149, and the ratio was 14.541; these results indicated a high degree of precision and high reliability for this model, which supported its use in future studies. The content of lincomycin A and lincomycin B changed with changes in soybean powder, corn steep liquor and glucose concentrations, and their corresponding 3D response surfaces were generated to better determine the interaction of variables with the corresponding variables (Fig. 1). The maximum lincomycin A and lincomycin B contents were 4569.6 mg/L and 0.96%, respectively, with 25 g/L soybean powder (X_1), 1.40 g/L corn steep liquor (X_2), and 126 g/L glucose (X_7).

Fermentation was conducted and lincomycin production in broth was evaluated by HPLC (Fig. 2). The results for the validation experiment showed that the values for the two responses of lincomycin A and lincomycin B contents were in close agreement with the predicted values. When 25 g/L soybean powder, 1.40 g/L corn steep liquor, and 126 g/L glucose were used, the content of lincomycin A increased from 3585 ± 110 mg/L to 4600 ± 134 mg/L, and that of lincomycin B decreased from 4.5% ± 0.2% to 0.8 ± 0.1%. These concentrations verified the accuracy of the statistical model.

Assay of lincomycin A, lincomycin B, and fermentation parameters

Glucose is not only a carbon source but can also affect the osmotic stress of a culture medium at certain concentrations, while corn steep liquor provides various amino acids, vitamins, and metal ions, etc. To explore possible causes of the increased lincomycin A and decreased lincomycin B after medium optimization, the fermentation parameters for *S. lincolnensis* 24 were measured (Fig. 3) using samples collected every 24 h.

The optimized medium not only influenced the nutrient content of the culture, such as sugar (Fig. 3A), it also affected the growth environment, such as osmotic stress and pH parameters (Fig. 3A, B). As shown in Fig. 3A, B, reducing the sugar and osmotic stress in the optimized medium were always higher than the initial medium before 144 h. At the end of 168 h of fermentation, the reducing sugars in and osmotic stress of the optimized medium were the same as the initial medium, with the reducing sugar consumed completely. These results indicated that changes in glucose affected the reducing sugar and osmotic stress of the medium. We hypothesized that these variations in the environment and nutrients affected the primary metabolism of *S. lincolnensis* and caused PMV changes (Fig. 3C), as well as changes in the

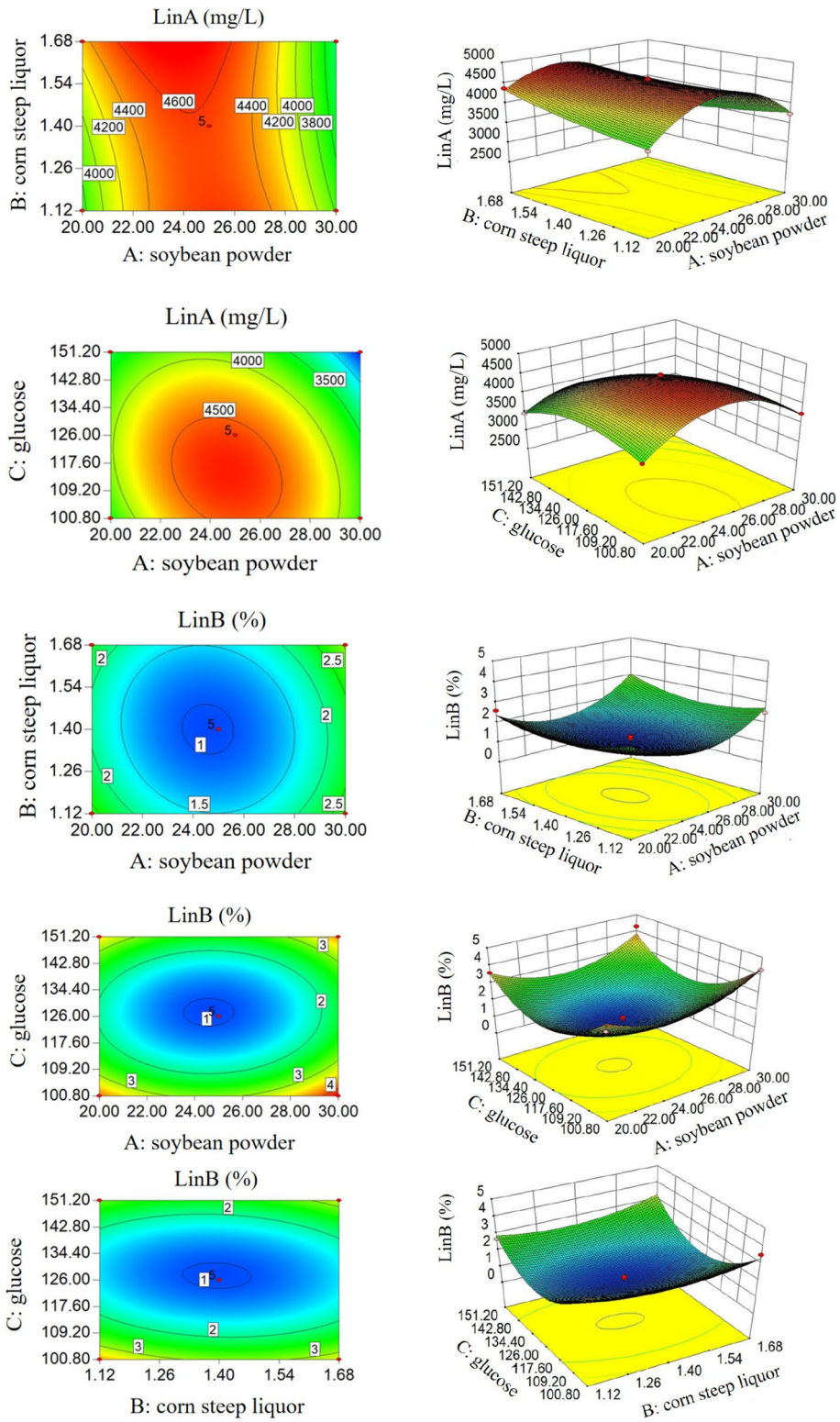

Fig. 1 Contour (left) and response surface (right) plots of the effects of soybean powder, corn steep liquor, and glucose on lincomycin A (**A**) and lincomycin B (**B**) concentration

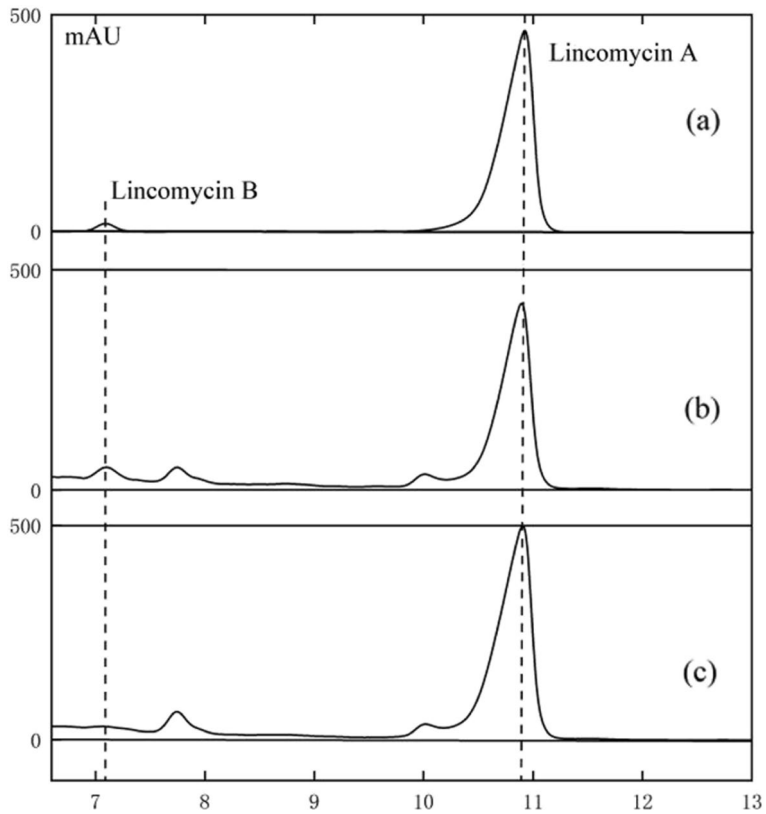

Fig. 2 HPLC analysis of lincomycin production during *Streptomyces. lincolnensis* fermentation. **a** Standard lincomycin A and lincomycin B. **b** Lincomycin A and lincomycin B produced by the initial medium. **c** Lincomycin A and lincomycin B produced by the optimized medium

secondary metabolism of *S. lincolnensis*, which improved lincomycin A quantity and quality (Fig. 3D). As shown in Fig. 3D, the optimized medium produced lincomycin A before 48 h, while the initial medium produced lincomycin A after 48 h. For lincomycin B, only the initial medium produced lincomycin B before 48 h. For both the initial and optimized media, both quickly increased lincomycin A and decreased lincomycin B in the 72–96-h stage.

Influence of corn steep liquor, glucose, and osmotic stress on the optimized medium conditions

In the present study, the increased glucose concentration affected the osmotic stress environment of the fermentation, which benefited *S. lincolnensis* growth and the reduction of lincomycin B accumulation (Fig. 3). We hypothesized that the change in osmotic stress had a significant influence on cell growth and lincomycin synthesis. Therefore, to explore the role of osmotic stress in lincomycin fermentation, NaCl was used to regulate osmotic stress in the initial medium. To determine the effect of salt-stress sensitivity on lincomycin production, *S. lincolnensis* was grown in the initial medium (control, containing 5 g/L NaCl), optimized medium (OP, initial medium with 1.4 g/L corn steep liquor and 126 g/L glucose), initial medium with 10 g/L NaCl (M1), initial medium with 126 g/L glucose (M2), or initial medium with 1.4 g/L corn steep (M3). The osmotic pressure of the OP and M1–M2 media was similar. As shown in Fig. 4, the OP medium produced the highest lincomycin A and lowest lincomycin B concentrations. Increasing the salt stress with 10 g/L NaCl in the initial medium (M1) resulted in a dramatic decrease in lincomycin B production. Similarly, under glucose (M2) and corn steep liquor (M3) salt-stress conditions, a knockdown of about 31.8–63.6% was achieved for lincomycin B production when compared with the control medium. These observations indicated that the osmotic conditions decreased lincomycin B concentrations during shake flask fermentation.

Transcriptional analysis of lincomycin biosynthesis-related genes

To further understand the observed variations between lincomycin production in initial and optimized media, quantitative real-time PCR (qRT-PCR) gene expression

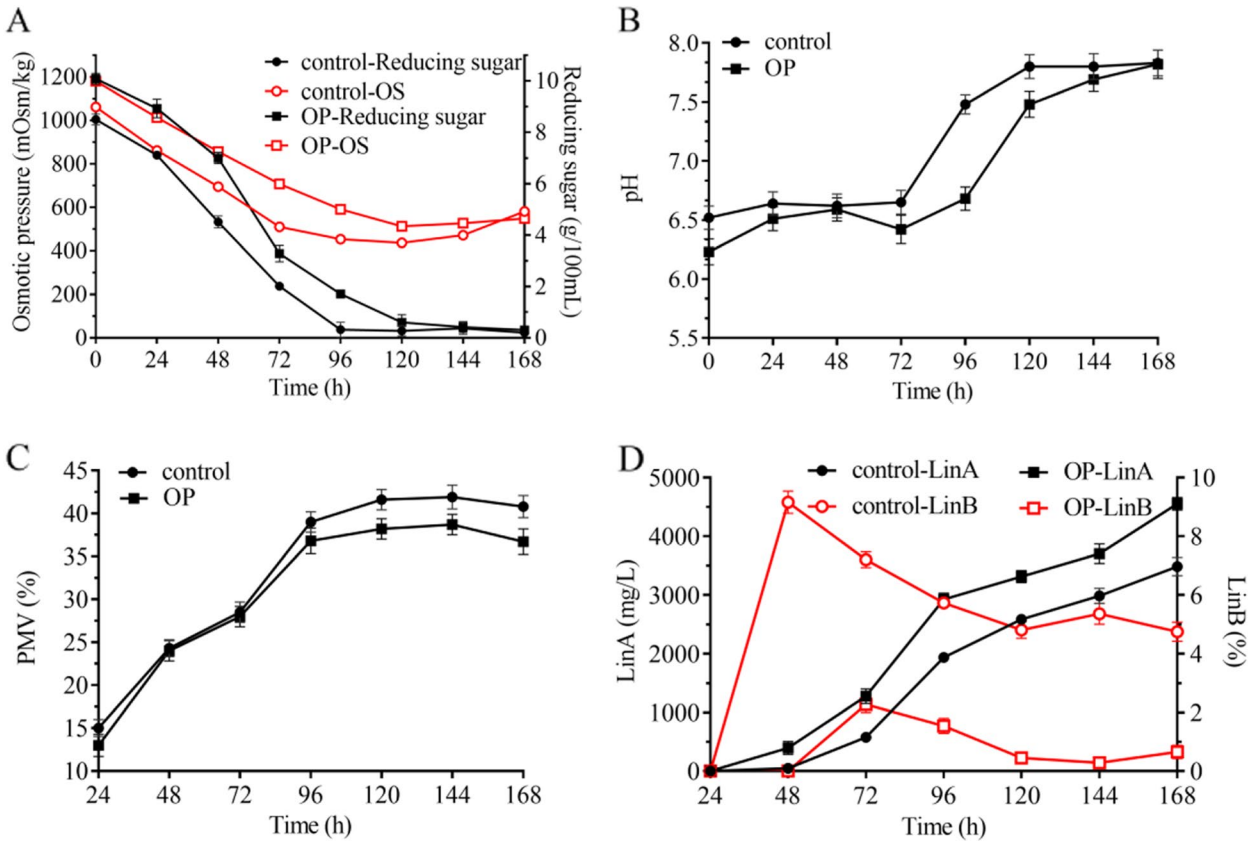

Fig. 3 Fermentation time-course profile of *Streptomyces lincolnensis* grown in the optimized and the initial media. **A** Reducing sugar concentration and osmotic stress level during fermentation. **B** pH evolution. **C** PMV evolution; and **D** production of lincomycin A and lincomycin B in initial and optimized media. Control: initial medium; OP: optimized medium; OS: osmotic stress

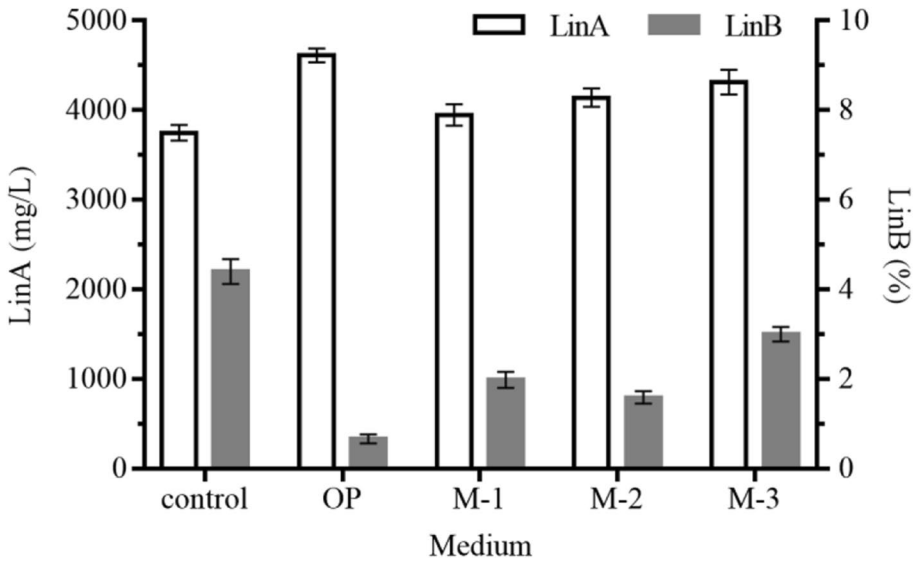

Fig. 4 Effects of different media on lincomycin A yield and lincomycin B concentration. Control: initial medium. The experimental media included the same components as the initial medium except for the following differences: OP: optimized medium and 1.4 g/L corn steep liquor and 126 g/L glucose; M-1: 10 g/L NaCl; M-2: 126 g/L glucose; and M-3: 1.4 g/L corn steep liquor

analysis was performed. In the biosynthesis of lincomycin, *lmbW* determines whether 4-n-propyl-L-proline synthesizes lincomycin A or lincomycin B (Pang et al. 2015). The regulatory genes in lincomycin synthesis are divided into the lincomycin biosynthesis gene cluster (*lmb* cluster) and non-*lmb* genes, which are *lmbU* and *SLCG_Lrp*, respectively. These genes play positive regulatory roles in lincomycin biosynthesis (Hou et al. 2018; Xu et al. 2020). The *lmbB1* gene is a key gene that determines whether L-DOPA follows the lincomycin or the melanin biosynthesis pathway. Overexpression of *lmbB1* can increase lincomycin A and decrease lincomycin B and melanin (Yang et al. 2020). These genes were selected based on their roles in the synthesis of lincomycin A and lincomycin B. Furthermore, osmotic stress in the optimized medium was higher than that of the initial medium (Fig. 3A); therefore, the transcript level of the osmotic stress related gene (*mscl*; GenBank: ANS65846.1) was also determined.

For 48 h is the key point for the transformation of primary metabolism to secondary metabolism, it is also the period when the production of lincomycin B changes significantly (Fig. 3D). Moreover, stress responses are integral to reprogramming the physiology of *Streptomyces* as they transition from primary to secondary metabolism (Godinez et al. 2015). Thus, RNA was isolated from both initial and optimized media samples collected after 48 h of fermentation. qRT-PCR was conducted in triplicate for each sample, using 16S rRNA as an internal control (RT primers are listed in Table 5). The qRT-PCR data showed that *lmbU*, *SLCG_Lrp*, *lmbW*, and *lmbB1* expression was significantly higher in the optimized medium than in the initial medium (2- to 4-fold increase; Fig. 5). One study recently reported that LmbU is a significant pleiotropic transcriptional regulator in lincomycin biosynthesis that activates the *lmb* cluster, including *lmbW* and *lmbB1* in *S. lincolnensis* (Lin et al. 2020). Another study showed that SLCG_Lrp is a positive regulator for lincomycin biosynthesis through the direct induction of *lmb* cluster genes, such as *lmbU* (Xu et al. 2020). Our results indicated that increased lincomycin A production in the optimized medium was likely to be associated with the overexpression of the key genes involved in the lincomycin biosynthesis pathway, as well as some of the key regulators of the pathway.

In this study, the optimized medium enhanced the osmotic stress of the fermentation condition and led to a 6.2-fold increase in the transcription of *mscl* when compared with the initial medium (Fig. 5). The expression levels of *lmbU* were 3.9-fold up-regulated in the optimized medium after 48 h compared to those of initial medium (Fig. 5). There are many reports in the literature that the *lmbU* gene can regulate the lincomycin biosynthesis gene cluster and some extra-cluster genes during the production of lincomycin streptomycin (Hou et al. 2018; Lin et al. 2020; Xu et al. 2020). Recent studies have suggested LmbU can bind to the regions upstream of the *lmbA* and *lmbW* genes at the consensus and palindromic sequence 5′-CGCCGGCG-3′ (Hou et al. 2018). By sequence alignment, this palindromic sequence was located 184 bp upstream of the *mscl* gene. Therefore, when the expression levels of *lmbU* and *mscl* genes were both upregulated when the osmotic pressure increased after the medium was optimized, we guessed that the osmotic pressure gene may also be regulated by *lmbU* (Fig. 6). The laboratory is doing follow-up advance.

LmbU binds directly to the regions upstream of *lmbW* and activates transcription. Thus, LmbU may also actively inhibit the production of lincomycin B (Hou et al. 2018; Pang et al. 2015). Higher osmotic stress might result in the down-regulation of lincomycin B biosynthesis (Fig. 4) due to LmbU targeting *mscl* and affecting the expression of *lmbW* during lincomycin B biosynthesis.

As shown in Fig. 3D, under the optimized conditions, the emergence time of lincomycin A was enhanced, which effectively reduced the fermentation period. Previous studies showed that the pneumocandin B_0 fermentation period was significantly shorter when *Glarea lozoyensis* were cultured in a high-osmolarity medium (Song et al. 2018); thus, osmotic stress could shorten the fermentation period in other bacteria. Moreover, simultaneously with the enhanced production time of lincomycin A, that of lincomycin B was delayed by approximately 24 h (Fig. 3D). These results suggest a temporal order and interconversion of lincomycin A and lincomycin B biosynthesis that are related to osmotic stress. The mechanism stated for lincomycin A and lincomycin B interconversion has not been reported. This study provides a foundation for interconversion of lincomycin A and lincomycin B in *S.*

Table 5 Primer sequences

Name	Primer sequences 5′→3′
SLCG_Lrp	(F)-TCGTCGTACAGCCGCTGGTAG (R)-GATCGCGGAAGTGGTGGATGC
lmbU	(F)-GCGTAGTTGCGGATCGTCTGG (R)-ACTCATCGGCCTGGTGTCTGG
mscl	(F)-CCTCATGGTCCTGCCGATGT (R)-AGCTCGCTCACCTCGATGAC
lmbW	(F)-A G C T G C T G G C C G A G G G C G T A (R)-G C C G C C G G A C T T G G A C G A C A
lmbB1	(F)-AGTAAAGTCAATGCCGCCCGTATC (R)-GAATGTGTCGAGGGTCCAGGAAAC
16S rRNA	(F)-GCATCTGTGGTGGTTGAAAG (R)-CGTGTCTCAGTCCCAGTGTG

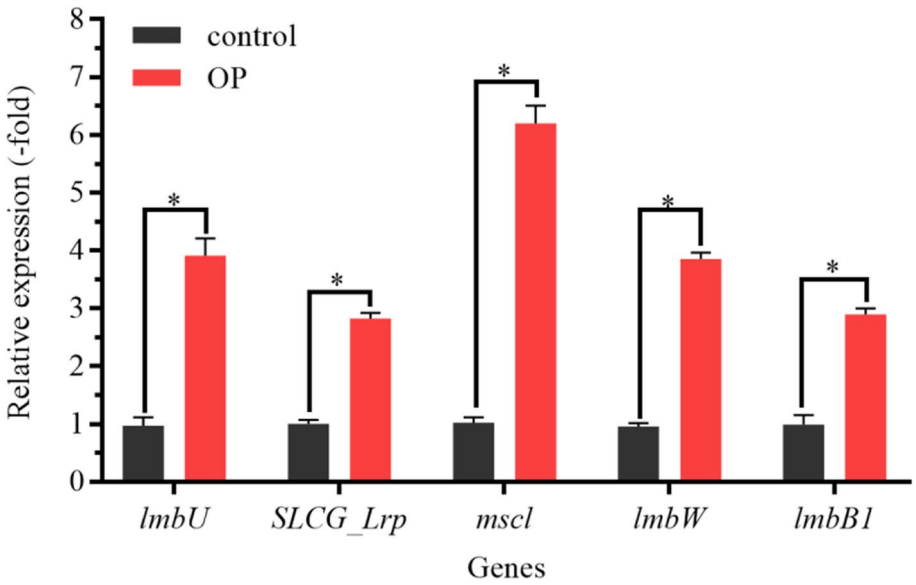

Fig. 5 Transcriptional analysis of the lincomycin biosynthetic genes and osmotic stress gene in *Streptomyces lincolnensis* grown in initial and optimized media. All gene transcription levels in the control were set to 1. * indicates statistical significance based on the two-tailed test ($p < 0.05$). Control: initial medium; OP: optimized medium

lincolnensis, although further research is needed to develop its application potential.

Conclusion

In the present study, for the strain used in this study is a mutagenic strain, we must optimize the conditions in the shake flask for the strain firstly. we aimed to increase lincomycin A production, while simultaneously reducing lincomycin B level using a medium optimization approach. For this purpose, PBD methodologies were used to evaluate the influence of soybean powder, corn steep liquor, NaCl, $NaNO_3$, KH_2PO_4, $(NH_4)_2SO_4$, glucose, and $CaCO_3$, on lincomycin production using the industrial strain of *S. lincolnensis*. The results show that soybean powder, corn steep liquor and glucose have the most significant effects on lincomycin. In terms of medium optimization, we chose soybean powder, corn steep liquor, and glucose for follow-up steepest ascent and response surface design, and finally found the most suitable conditions for the production of lincomycin. After 168 h of culture in an optimized medium, the lincomycin A titer was significantly increased, while the unwanted lincomycin B byproduct decreased.

In our optimized medium composition, the total amount of corn steep liquor was reduced from 2 g/L to 1.4 g/L, which might help to reduce the cost of the medium. For natural raw materials, such as corn steep liquor, using in industrial large-scaled cultivation might bring uncertainty to industrial production, the effect of different batches of corn steep liquor on the production of lincomycin A and lincomycin B was investigated. The result showed that the lincomycin concentrations were not significantly different among the various batches of corn steep liquor, suggesting that different batches of this natural origin is less effect in the present study (Figure S2). This needs to be further scaled up and verified on the fermentation tank to prepare for industrial application. There are many problems (such as stirring speed, mass transfer, etc.) during the fermentation scale-up process for lincomycin production. It should take a long time to optimize the bioreactor conditions.

The enhancement of lincomycin A production in the optimized medium could be attributed to the expression of the key genes in the lincomycin biosynthesis pathway as well as other regulator genes. Furthermore, Fig. 6 shown that there was a palindrome sequence to which *lmbU* could bind in the upstream region of *mscl* gene. The question of whether the up-regulation of osmotic pressure gene is regulated by *lmbU* can be studied later. In the genome of the model streptomycete *Streptomyces coelicolor*, Martínez had identified osmoregulation gene encoding a member of a novel family of regulatory proteins (Martínez et al. 2010). In this experiment, we found that after the optimization of the medium, the osmotic pressure increased, and the expression of *lmbW*, which is closely related to the synthesis of lincomycin B, also increased. Therefore, it is speculated that the *mscl* gene may be used as a regulatory

Fig. 6 Nucleotide sequence of the region upstream of *mscl* and the predicted LmbU binding site (yellow)

gene to regulate *lmbW*, and *lmbW* is regulated by *lmbU* (Hou et al. 2018), so the osmotic gene may also regulate the *lmbU* gene. This is not reported, and further research is needed. And the reduced lincomycin B accumulation could be related to increased osmotic stress, which should be the focus of future studies.

The structural difference between lincomycin A and B implies the different biosynthetic pathways of proline derivative moieties (Argoudelis et al. 1964; Pang et al. 2015; Janata et al. 2018). It was proposed that the C-methylation of two-carbon side chain of proline derivative moiety would lead to propylproline (PPL); otherwise ethylproline (EPL) would be produced (Brahme et al. 1984). However, the conversion mechanism between PPL and EPL is not yet clear. This study found that 48 h was the key point for the transformation of primary metabolism to secondary metabolism, it was also the period when the production of lincomycin B changes significantly (Fig. 3D). Therefore, it was speculated that exploring the conversion of PPL and EPL in fermentation prophase may have some gains.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-022-01672-w.

Additional file 1: Table S1. Three-level design of Box-Behnken Design experiment. **Table S2**. The regression analysis of variance for Plackett-Burman factorial model for the content of lincomycin A (Y_1) and lincomycin B (Y_2). **Table S3.** ANOVA for response surface quadratic model. **Table S4.** Feasible analysis of model. **Figure S1**. Putative lincomycin biosynthetic pathway in *Streptomyces lincolnensis*. MTL: methylthiolincosaminide; PPL: 4-propyl-l-proline. **Figure S2**. Effect of different batches of corn steep liquor on lincomycin A and lincomycin B concentration. Control: initial medium; OP: optimized medium; CSL: corn steep liquor.

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Not applicable.

Authors' contributions
This research was conducted under the guidance of RFY and FXH. QHD was the assistant who assisted XHZ in completing the experiment. The industrial strains used in the experiment will be provided in the HZZ and SL. The authors read and approved the final manuscript.

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Availability of data and materials
The data sets used and/or analyzed during the current study are available from the corresponding author on a reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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