Production enhancement of the glycopeptide antibiotic A40926 produced by an engineered *N. gerenzanensis* lcu1

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Research Article

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Abstract

Objective

To enhance the production of A40926 by implementing a strategy of the combination of genetically engineered strain construction and medium optimization.

Results

The engineered strain of *Nonomuraea gerenzanensis* presented an increment of 30.6 percent in A40926 production compared with that of the parent strain. Subsequently, an assembling medium, which was defined as M9 medium and mainly comprised glucose, maltodextrin, soybean meal, peptone, L-valine, and other inorganic salts, was determined as the optimal medium among the tested nine media. The optimum concentration of medium components was glucose 10 g/l, maltodextrin 37.9 g/l, soybean meal 34.5 g/l, peptone 30.0 g/l, and L-valine 4.3 g/l, respectively. The optimized medium was verified experimentally, and A40926 yield increased significantly from 257 mg/l to 332 mg/l, as compared to the non-optimized medium. The strategy brought a significant increase of A40926 yield by 65.2 percent.

Conclusions

The engineered mutant with the genetic attributes of the co-expression of the *dbv3* and *dbv20* genes and the deletion of the *dbv23* gene could obviously enhance the production of A40926. In addition, the optimization of medium was an effective and essential tool for the improvement of the secondary metabolites in Actinomyces.

Introduction

The antibiotic A40926 belongs to the teicoplanin family of glycopeptides antibiotics (GPAs), which remain a tool of last resort for treating resistant, “superbug” bacterial infections in clinic (Yim et al. 2014). It is the precursor of dalbavancin, a second-generation glycopeptide, and consists of a heptapeptide skeleton, two sugar residues, two chlorine atoms, and an acyl chain (Marschall et al. 2019). The *dbv* (from *dalbavancin*) cluster, is involved in the biosynthesis of A40926, had been isolated and characterized from *N. gerenzanensis* ATCC 39727. It spans approximately 71 kb and consists of 37 open reading frames (ORFs) (Sosio et al. 2010).

Until now, fermentation has still been the only approach for the production of A40926 in industrial scale. Therefore, how to improve A40926 titer is one of the essential researches about the rare actinomyces *N. gerenzanensis*. Originally, A40926 yield of the wild-type *N. gerenzanensis* strain was only 5 mg L⁻¹ in an early fermentation study using chemically defined media with high concentrations of phosphate (Gunnarsson et al. 2003). Later studies demonstrated that initially high inorganic phosphate or

ammonium concentrations inhibited A40926 production (Gunnarsson et al. 2004). Technikova-Dobrova reported that L-Gln, which replaced ammonium or nitrogen as the sole nitrogen in MM-103 medium, could improve the A40926 yield to 123 mg L⁻¹ (Technikova-Dobrova et al. 2004). Chen and colleagues developed a higher-producing strain by mutagenesis with UV irradiation and diethyl sulfate treatment, which presented the highest yield thus far, 1 g L⁻¹ of A40926. This group also confirmed that the addition of L-leucine could improve A40926 production. Other studies confirmed that the addition of branched-chain amino acids promotes the production of A40926 and teicoplanin (Chen et al. 2016).

In our recent studies, the positive impacts of the deletion of *dbv23* and the overexpression of regulatory *dbv3* and *dbv20* on A40926 production had been verified, respectively (Yue et al. 2020a). Based on the above findings, we constructed a multiple genetic operating strain laboring the knockout of *dbv23* and the co-expression of *dbv3* and *dbv20*. Furthermore, central composition design was carried out to optimize the medium for obtaining the high yield of A40926 by the mutant.

Methods

Bacterial Strains, Plasmids, and Media

All bacterial strains and plasmids used in this work are listed in Table 1. *N. gerenzanensis* and its derivatives were grown on Mannitol-Soy-agar (MS) solid medium at 30°C (Marcone et al. 2010). MS medium supplemented with 20 mM MgCl₂ was used for intergeneric conjugation between *Escherichia coli* and mycelia of *N. gerenzanensis*. For seed culture, 50 mL of VSP medium in 250-mL shake flasks were inoculated with a single colony from solid MS media and cultured at 30°C, 220 rpm for 72 h. For mycelial collection used for intergeneric conjugation, the above seed culture was then transferred into 75 mL of VSP medium in 500-mL shake flask with 4% inoculum for continuous culture at 30°C for 36 h. For A40926 production, a set of media listed in Table 3 were chosen to evaluate their abilities of A40926 production, and which were conducted in the 500-mL shake flasks with four baffles at 30°C and 220 rpm for 168 h.

The *E. coli* strains were cultivated on LB agar medium or in LB liquid medium at 37°C. Apramycin (50 µg L⁻¹), kanamycin (50 µg L⁻¹), chloramphenicol (25 µg L⁻¹) and nalidixic acid (25 µg L⁻¹), were supplemented when necessary. The *E. coli* strain ET12567/pUZ8002 was used as a donor for intergeneric conjugation and delivered recombinant plasmids into *N. gerenzanensis*.

Plasmids Construction

The sequence of promoter *gapdh* was synthesized with *Bam* HI/*Nde* I sites and further cloned into pIJ8660, yielding pIJ8660-P*gapdh*. The DNA sequence of the promoter *gapdh* was provided in Supporting information. Isolation of genomic DNA from *N. gerenzanensis* were carried out according to instructions of commercial kits (TIANGEN Biotech Co., Ltd, China). The *dbv3* and *dbv20* genes were amplified from the genome DNA of *N. gerenzanensis* using the corresponding primers listed in Table 2, respectively. These fragments were cloned into the *Eco* RV site of pUC57 (Vazyme Biotech Co., Ltd, China),

respectively. These plasmids carrying different *dbv* gene were digested with *Nde* I and *Not* I, and the obtained fragment was cloned into respective sites of pIJ8660-*Pgapdh* to produce pIJ8660-*Pgapdh-dbv3* and pIJ8660-*Pgapdh-dbv20*, respectively. To co-overexpress *dbv3* and *dbv20*, the *dbv3* fragments with promoter *gapdh* were amplified from pIJ8660-*Pgapdh-dbv3* and ligated into pIJ8660-*Pgapdh-dbv20*, generating pIJ8660-*Pgapdh-dbv3-Pgapdh-dbv20*. The expression of *dbv3* and *dbv20* was driven by the promoter *grpdh* separately.

Genotype Confirmation of The Exconjugants by PCR

The exconjugants on the MS medium were transferred into the VSP medium containing 50 µg mL⁻¹ apramycin and cultured at 30°C and 200 rpm for 48 h. The mycelia were collected and treated for extracting genome DNA by commercial kit. Primers listed in Table 2 were used for confirmative PCR of *gapdh-dbv3* and *gapdh-dbv20* fusion fragments.

Optimization of A40926 Production Utilizing Central Composite Design (CCD)

Here, the optimization process of A40926 production was firstly carried out to identify the preferred media for A40926 production based on the literature (Table 3). A novel medium was designed based on the above optimization results. Subsequently, response surface methodology (RSM) was carried out by the central compost design (CCD) to optimize the redesigned medium, which is a complicated statistical method for determining the optimum experimental conditions that require the minimum number of tests. A software Design Expert 11 (Stat-Ease, Inc., USA, Windows operating system) was applied to perform the experimental design matrix and its statistical experimental design analysis. All assays were performed in triplicate.

Three-dimensional curves of the response surfaces were obtained by using Design Expert 11 to visualize individual effects and interaction between significant parameters. The model was evaluated using Fisher's statistical test for analysis of variance (ANOVA).

Shake Flask Fermentation

For the shake flask cultivation, the inoculum was prepared by inoculating the mycelia of *N. gerenzanensis* in 50 mL VSP medium in a 500-ml shake flask. The preculture was incubated at 30°C and 200 rpm, sustaining for 48 h. Based on the literature reports and novel design, nine different production media (Table 3) were selected for the shake flask tests to evaluate their effects on the A40926 production. All experiments were performed in 500-mL shake flasks, with 75 mL of medium and a 10% (v/v) inoculum prepared as described above. The initial pH was set to 7.2 by the addition of 10% ammonium hydroxide or 1 M HCl. Cultivation was performed at 30°C and 220 rpm, for 144 h. At the end of the experiments, the cultivation broth was collected and used to detect the A40926 yield using high-performance liquid chromatography (HPLC). Three replicated tests were conducted for each medium.

Analytical Methods

The A40926 analysis was similar to that described by Gunnarsson with modifications (Gunnarsson et al. 2003). Briefly, the fermentation broth sample was adjusted to pH 11.3 by adding NaOH solution, and then sonicated at 50°C for 1 h. Subsequently, the sample was centrifuged at 5,000 rpm for 5 min. The supernatant was filtered through a 0.45 µm Millipore filter for analysis. A40926 was analyzed by HPLC equipped with a SHIMAZU Shim-pack GIST C₁₈ column (5 µm, 250 × 4.60 mm) and a UV detector (220 nm) (Technikova-Dobrova et al. 2004). The column temperature was 25°C, the injection volume was 10 µL, and the flow rate was 1.0 mL min⁻¹. A gradient with two eluents (eluent A: 10% acetonitrile, 90% 12.5 mM NaH₂PO₄, pH 7.8; eluent B: 50% acetonitrile, 50% 12.5 mM NaH₂PO₄, pH 7.8) was applied as follows: (% eluent B): 0 min, 20%, 5 min 50%, 32 min 65%, 35 min 20%.

Statistical Analysis

The statistical analyses One-way and Two-way ANOVA were performed using GraphPad Prism version 7.

Results

Construction of The Engineered Strain Previous studies had verified that the overexpression of positive regulatory gene *dbv3* or the deletion of *dbv23*, encoding an acetyltransferase, could enhance the production of A40926 (Lo Grasso et al. 2015; Sosio et al. 2010; Yue et al. 2020a). In addition, we found that the overexpression of *dbv20* gene encoding a mannosyltransferase also improved the A40926 production (Dong et al. 2020). In this study, a strategy of multiple genetic operations referring to co-expressing *dbv3* and *dbv20* in a *dbv23*-deleted mutant was adopted to improve the strain. The two *dbv* genes were separately and simultaneously expressed under the control of each promoter *gapdh* (Fig. 1A). The recombinant plasmid was transferred to the mutant $\Delta 23$ by conjugation, which was expected to obtain the final mutant verified by PCR. As a result, the fragments of *Pgapdh-dbv3* and *Pgapdh-dbv20* were successfully obtained and about 0.7 kb size (Fig. 1B). Furthermore, we compared the A40926 yield of mutant with that of the other control strains including the original strain ($\Delta 23$), the wild-type strain, the *dbv3*-overexpressed strain (*op3*) and the *dbv20*-overexpressed strain (*op20*). As expected, the co-expression of *dbv3* and *dbv20* further improved the A40926 yield, which increased from 159 to 262 mg L⁻¹ compared with the wild-type strain (Fig. 1C). Media Screening Experiments Previous studies had reported some suitable media for A40926 production (Table 3). In this work, we applied these media to evaluate their capabilities for A40926 production, and further redesigned medium and optimized the components. The fermentation results demonstrated that the more kinds of natural components, such as maltodextrin, soybean meal, peptone, and corn starch, were contained in the medium, and the higher the yield of A40926 was (Fig. 2). Based on the results, a novel combination medium was designed and named M9, which was composed of carbon resource of glucose and maltodextrin, nitrogen resource of peptone and soybean meal, the amino acid of L-valine, and other inorganic salts. In contrast to media from literature, the engineered strain gave the highest yield of A40926 in M9 medium as expected. It was supposed that the proper ratio of carbon and nitrogen resource, the relative lower viscosity of the medium, and more various inorganic salts were beneficial to improve the A40926 production.

Optimization of Medium Components for A40926 Production by CCD To further optimize the components

of M9 medium, central composition design was applied. The optimization process focused on investigating the impact of five independent variables: glucose (A) and maltodextrin (B) as carbon sources, soybean meal (C) and peptone (D) as nitrogen sources, L-valine as a precursor (E), on the yield of A40926, using response surface methodology based on central composite design (Table 4). A statistically designed study was performed to evaluate the individual and the interactive effect of five medium ingredients on the A40926 yield. The values of response from the 32 experiments are presented in Fig. 3. The first set of optimal statistical conditions, maximizing A40926 yield by *N. gerenzanensis* were obtained with 28-32 standard tests (Supplementary Table 1) which corresponded to the medium composition including glucose 10 (g L⁻¹), maltodextrin 35 (g L⁻¹), soybean meal 30% (g L⁻¹), peptone 20 (g L⁻¹), and L-valine 3.5 (g L⁻¹). Here, RSM is a five factorial design where 3D contour plots of surface curves (Fig. 4 and Supplementary Fig. 1) can be generated by linear effects, quadratic effects and two-way interactions between the factors. From these profiles, a semi-empiric model (Eq.1) can be derived that best fits the experimental data. The parameters and results of the CCD experiments are presented in Table 4, Fig. 3 and 4, and Supplementary Table 1 and Fig 1. The statistical significance of the quadratic model was tested by F- and p- values (Table 5). The results from ANOVA indicated that the quadratic regression used to produce a second-order model was significant, as revealed from the p- and F-values: the calculated Model F-value of 37.53 and the p-value of <0.0001 indicate that the model is significant. The Lack of Fit F-value of 0.45 implies that the Lack of Fit is not significant relative to the pure error. There is 81.52% chance that a Lack of Fit F-value this large could occur due to noise. The second-order polynomial equation of the model fitted for A40926 production before eliminating the non-significant terms is: A40926 production yield (mg/l)= 64.4097+0.929924*A+3.31465*B+9.69432*C+0.885795*D+8.7298*E-0.0275*AB+0.03375*AC-0.02875*AD+0.175*AE-0.00208333*BC-0.00958333*BD+0.0472222*BE-0.006875*CD+0.0541667*CE+0.179167*DE-0.0404545*A²-0.0389394*B²-0.145114*C²-0.0126136*D²-2.22727*E² (Eq. 1) (Degree of freedom=20; F-value=37.53; p-value <0.0001; R²=0.9856) In this study, the model terms with the values of p-value Prob > F (Table 5) lower than 0.05 was considered as the significant terms. On the contrary, if the p-value Prob > F is higher than 0.1, the model terms are non-significant. In this case, the model terms B (maltodextrin), C (soybean meal), E (L-valine), DE, B², C², and E² were considered significant. The R² value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The closer the R² value to 1.00, the stronger the model is, and the better it predicted the observed response. It was suggested that the R² value should be at least 0.80, for a good model fitness (Venkatachalam et al. 2020). Here, the calculated R² value of 0.9543 (Supplementary Table 2), indicated that 4.57% of the total variation could not be explained by the empirical model; this expresses a good enough quadratic fit to navigate the design space. Thus, the response surface model developed in this study for predicting the A40926 production may be considered satisfactory (Eq. 2). The signal to noise ratio was measured by Adeq Precision value of 33.0287, which indicated that this model could be used to navigate the design space. From the above, the second-order polynomial equation of the model fitted for A40926 production, after eliminating the non-significant terms (Supplementary Table 2), is A40926 production yield (mg L⁻¹)=323.91+3.37*B+13.04*C+2.63*E+2.69*DE-8.59*B²-14.34*C²-4.84*E² (Eq. 2) (Degree of freedom=7; F-value=71.54; p-value <0.0001; R²=0.9543) Whereby the F-value increased, meaning that the mean

squares of the model are larger than the square residual average. Thus, with a higher the F-value, the more significant p-value for ANOVA and the more significant the model is. Effect of Significant Components on A40926 Production 3D response surface graphs (Fig. 4 and Supplementary Fig. 1) were plotted to illustrate the interaction of the different paired factors and to determine the optimum of each paired factor for maximum response. Each graph represents the combinations of two test factors in relation to A40926 production yield. The data in Fig. 4 indicate that the increase in significant carbon source (maltodextrin) and amino acid/nitrogen sources (soybean meal, peptone, and L-valine) resulted in increased A40926 production. From the combined effect of maltodextrin and soybean meal concentration (Fig. 4A), the highest production was obtained by the appropriate soybean meal concentration and the proper maltodextrin concentration. Similar results were obtained with the combined effect of L-valine and maltodextrin (Fig. 4B). The effects of L-valine and peptone concentrations, and of L-valine and soybean meal, on A40926 production yield are illustrated in Fig. 4C and 4D. Yield increased as the concentrations of soybean meal and L-valine increased and decreased, respectively. The main medium combination of L-valine and peptone (DE, Table 5) showed the highest p-value for Prob > F (0.0264) and therefore represented a more significant model term combination. L-valine is a well-known precursor for A40926 production and meanwhile is a source of nitrogen (Alduina et al. 2018; Beltrametti et al. 2004; Yue et al. 2020b). It is possible that higher concentrations of these nutrients could have led to a substrate growth inhibition and/or affected A40926 production. The optimal concentrations of the four factors excluding factor glucose that maximized A40926 production yield were predicted using the optimization function of the statistical experimental designs Design Expert 11. Glucose 10 (g L⁻¹), maltodextrin 37.9 (g L⁻¹), soybean meal 34.5 (g L⁻¹), peptone 30.0 (g L⁻¹), and L-valine 4.3 (g L⁻¹) were chosen as the optimal concentrations, resulting in the highest A40926 yield of 328.6 mg L⁻¹. The predicted medium composition roughly coincided with experiment trials from 27 to 32 (Supplementary Table 1). No statistical differences were observed between the predicted maximum production yield and the experimental results ($p > 0.05$). The optimized results were also confirmed ($p > 0.05$) by conducting a further fermentation experiment in triplicate at the above-optimized values, resulting in production yield of 332 ± 13 mg L⁻¹. A Parity plot illustrating the distribution of experimental (actual) and predicted (model) values is shown in Fig. 5. Data points are scattered along the diagonal line, also suggesting that the model is adequate to explain A40926 production within the experimental range studied. The process profiles of A40926 production shown in Fig. 6 obtained by the cultivation of the engineered strain lcu1 in the non-optimized and optimized media, respectively. Here, the strain lcu1 was denominated by the abbreviation of Liaocheng University number one, and which has the genetic characteristics of the dbv23 gene deletion and the dbv3-dbv20 gene co-expression. During the first 48 h of culture, there was no difference in the two media for A40926 production. Subsequently, the productivity of A40926 in the optimized medium was always higher than that in the non-optimized medium. In particular, the biosynthesis of A40926 stagnated and even decreased in the last 24 h of fermentation. The yield of A40926 in the optimized medium reached 332 mg L⁻¹ at the end of fermentation, which was significantly higher than that of 257 mg L⁻¹ in the non-optimized medium.

Discussion

In this study, we combined the genetic manipulation of *dbv* genes and the traditional media optimization to enhance the production of A40926. The strategy brought a significant increase of A40926 yield by 65.2 percent, which exceeded our expectations. The previous researches referring to the *dbv* cluster gave us a comprehensive understanding of the biosynthesis of A40926. For instance, the pathway-specific regulatory gene *dbv3* and *dbv4* were verified to play positive roles in the A40926 production (Alduina et al. 2007; Lo Grasso et al. 2015). The post-modified genes in *dbv* cluster exerted significant roles in the biosynthesis of A40926, which included seven genes such as *dbv8* encoding an acyltransferase (Kruger et al. 2005), *dbv21* encoding a deacetylase (Ho et al. 2006), *dbv23* encoding an acetyltransferase (Sosio et al. 2010), and *dbv29* encoding a hexose oxidase (Liu et al. 2011). Typically, the absence of *dbv23* distinctively promoting the production of A40926 had been confirmed by Sosio's and our previous studies (Sosio et al. 2010; Yue et al. 2020a). Recently, the overexpression of the *dbv20* gene, encoding a mannosyltransferase, was verified to improve the A40926 production (Dong et al. 2020). Here, we constructed an engineered strain with the deletion of *dbv23* and the co-expression of *dbv3* and *dbv20* to improve the A40926 yield actually.

To further improve the A40926 production, the preferred nutrients, including carbon and nitrogen sources for A40926 production, were determined based on the literature and the preliminary fermentation assays. As shown in the results (Fig. 3), the delayed natural carbon and nitrogen sources such as maltodextrin and soybean meal were beneficial to producing A40926. Nevertheless, it should be noted that the higher concentration of these delayed carbon and nitrogen sources, such as corn starch and soybean meal, resulted in a decrease of A40926 yield, which might be caused by the limitation of the dissolved oxygen transfer. Therefore, we designed a novel medium M9 based on the comprehensive consideration of viscosity related to oxygen transfer and the mixture of the delayed and quick-acting carbon and nitrogen sources. The M9 medium led to the highest yield of A40926 among the screened media.

Furthermore, response surface methodology with central composite design was carried out to obtain a mathematical model to identify the optimum concentrations of the significant components for the improvement of A40926 production. The most optimal concentrations of significant medium components were glucose 10 g L^{-1} , maltodextrin 37.9 g L^{-1} , soybean meal 34.5 g L^{-1} , peptone 30.0 g L^{-1} , and L-valine 4.3 g L^{-1} . The maximum yield 332 mg L^{-1} of A40926 was obtained. Therefore, the response surface statistical methods should be effective for optimizing the medium nutrients for the production of secondary metabolites from microorganisms.

Declarations

Conflict of interest statement

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1 Strains and plasmids used in this work

Strains or plasmids	Characteristics ^a	Sources or reference
<i>Escherichia coli</i>		
DH5α	Routine subcloning host	Invitrogen
ET12567/pUZ8002	Derived from the methylation-defective strain ET12567 carrying plasmid and donor strain for <i>E. coli</i> - <i>Actinomyces</i> conjugation	(Gust et al. 2003)
<i>Actinomyces</i>		
<i>N. gerenzanensis</i> ATCC 39727	Wild-type, glycopeptide A40926 producer	(Goldstein et al. 1987)
Δ23	<i>N. gerenzanensis</i> mutant with the knockout of <i>dbv23</i> gene	(Yue et al. 2020a)
lcu1	Δ23 strain laboring pIJ8660- <i>gapdhp-dbv3-gapdhp-dbv20</i>	This study
Plasmids		
pIJ8660	A pSET152 derivative. Apr ^r	(Sun et al. 1999)
pIJ8660-P <i>gapdh</i>	pIJ8660 plasmid with promoter <i>gapdh</i>	This study
pIJ8660-P <i>gapdh-dbv3</i> -P <i>gapdh-bv20</i>	pIJ8660 plasmid with two promoter <i>gapdh</i> , <i>dbv3</i> and <i>dbv20</i>	This study

a: Apr^r, apramycin resistance

Table 2 Oligonucleotides and Primers used in this work

Primers	Sequence (5'-3')	Description
p1/p2	tgaaaggggatacgccatatggtgctgttcgggcgagatcgtg acgggctgcagccgggcgccgcctacagccgcactgcctcacg	Amplification for <i>dbv3</i>
p3/p4	tgaaaggggatacgccatatgatgtcgcacatcaccatgactc acgggctgcagccgggcggtacctcagccccgggtgtccg	Amplification for <i>dbv20</i>
p5/p6	gctgctccttcggtcggacgtgcgtctacg cacggcccagatcgccacacctcctccgg	Verification of lcu1 strain
p7/p8	gctgctccttcggtcggacgtgcgtctacg tccggtacatcaccagcaccgagatcacgc	Verification of lcu1 strain

Table 3 Media used for the cultivation of the engineered *N. gerenzanensis* lcu1 in this study

Component (g L ⁻¹)	M1	M2	M3	M4	M5	M6	M7	M8	M9
Glucose	10	20	20	50	20	5	30	5	10
Maltodextrin			50		15	30			30
Peptone			10						15
Yeast extract			10			5	8		
Soybean meal			20	10		10	30	30	20
Corn starch					40	30		40	
Soluble starch						10			
Tryptone					15				
Cottonseed meal				10		5			
Soybean protein				5	40	5			
Meat peptone						10			
Soybean oil				10	10				
Fish powder/peptone								15	
Malt extract							15		
Casein								2	
L-Leu		0.5	3	1					
L-Gln	2			1.5					
L-Val		0.5				1	1	1	5
L-Tyr				0.5					
L-Ile		0.5							
K ₂ HPO ₄	0.5								
KH ₂ PO ₄		0.65		1.5					0.5
MgSO ₄ ·7H ₂ O	0.2	0.45		0.2					0.4
FeSO ₄ ·7H ₂ O	0.01								0.2
(NH ₄) ₂ SO ₄		3.65		2					
CaCO ₃			5				4	4	3
NaCl			2						2

CuSO ₄	0.03				0.03				
pH	7.0-7.2	7.5	7.0	7.0	6.5	7.0	7.4	7.0	7.2
References	a	b	c	d	e	f	g	h	This study

a, (Technikova-Dobrova et al. 2004); b, (Jovetic et al. 2008); c, (Chen et al. 2016); d, (Yan L 2013); e, (Liu ZY 2018); f, (Huang LL 2012); g, (Marcone et al. 2014); h, (Chen CF 2015).

Table 4 Levels of carbon and nitrogen resources selected for the experimental central composite design

Factor	Name	Units	Min	Max	Low	High	Mean
A	Glucose	g L ⁻¹	0.0	20.0	5.0	15.0	10.0
B	Maltodextrin	g L ⁻¹	5.0	65.0	20.0	50.0	35.0
C	Soybean meal	g L ⁻¹	10.0	50.0	20.0	40.0	30.0
D	Peptone	g L ⁻¹	0.0	40.0	10.0	30.0	20.0
E	L-valine	g L ⁻¹	0.5	6.5	2.0	5.0	3.5

Table 5 Analysis of variance (ANOVA) for the quadratic model based on Response Surface Method^a

Source	Sum of Squares	Df	Mean Square	F-value	p-value (prob. >F)	
Model	13199.26	20	659.96	37.53	< 0.0001	significant
A-Glucose	26.04	1	26.04	1.48	0.2491	
B-Maltodextrin	273.37	1	273.37	15.54	0.0023	significant
C-Soybean meal	4082.04	1	4082.04	232.11	< 0.0001	significant
D-Peptide	77.04	1	77.04	4.38	0.0603	
E-L-valine	165.38	1	165.38	9.40	0.0107	significant
AB	68.06	1	68.06	3.87	0.0749	
AC	45.56	1	45.56	2.59	0.1358	
AD	33.06	1	33.06	1.88	0.1977	
AE	27.56	1	27.56	1.57	0.2366	
BC	1.56	1	1.56	0.0888	0.7712	
BD	33.06	1	33.06	1.88	0.1977	
BE	18.06	1	18.06	1.03	0.3326	
CD	7.56	1	7.56	0.4300	0.5255	
CE	10.56	1	10.56	0.6006	0.4547	
DE	115.56	1	115.56	6.57	0.0264	significant
A ²	30.00	1	30.00	1.71	0.2182	
B ²	2251.67	1	2251.67	128.03	< 0.0001	significant
C ²	6177.00	1	6177.00	351.23	< 0.0001	significant
D ²	46.67	1	46.67	2.65	0.1316	
E ²	736.67	1	736.67	41.89	< 0.0001	significant
Residual	193.45	11	17.59			
Lack of Fit	68.62	6	11.44	0.4581	0.8152	not significant
Pure Error	124.83	5	24.97			
Corrected Total	13392.72	31				
R ²					0.9856	
Adjusted R ²					0.9593	

Predicted R²

0.8503

Adequately
Precision

24.7638

a, before eliminating the non-significant terms.

Figures

Figure 1

The recombinant plasmid pIJ8660-Pgapdh-dbv3-Pgapdh-dbv20 (A), PCR verification (B), and fermentation assay of the different strains (C).

Figure 2

Comparison of A40926 yield produced by engineered *N. gerenzanensis* strain in different media.

Figure 3

The response values of A40926 from 32 tests.

Figure 4

Three-dimensional response surface curves for A40926 production yield presenting the mutual interactions of independent variables. (A) soybean meal and maltodextrin; (B) L-valine and maltodextrin; (C) L-valine and peptone; (D) L-valine and soybean meal.

Figure 5

Correlation of predicted values versus experimental values of the response surface methodological model developed by central composite design.

Figure 6

Fermentation profiles of A40926 production in shake flask experiments using the optimized media.

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