Unlocking the potential of *Vibrio natriegens* for L-lysine production

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Abstract
L-lysine is an essential amino acid widely used for feed additive and pharmaceutical purposes. It is produced via microbial fermentation, mainly using Corynebacterium glutamicum. Recently, Vibrio natriegens has become a promising host in the bioindustry, thanks to its rapid growth, versatility in substrate utilization and high salt-tolerance. The aim of this study is to evaluate the feasibility of L-lysine production in V. natriegens.

Introduction of six genes encoding core metabolic enzymes and transporter involved in lysine biosynthesis from C. glutamicum into V. natriegens genome and deletion of endogenous pckA gene enabled the production of L-lysine with a titer of 4.6 g/L (nearly 80-fold increase in comparison with the wild-type) and a yield of 152 mmol/mol glucose in the defined synthetic medium after 40 hours of fermentation in shake flask. We reported the feasibility of L-lysine biosynthesis in V. natriegens.

Keywords: *Vibrio natriegens*, metabolic engineering, genetic engineering, synthetic biology.

Introduction

L-lysine represents an important component of feed additive. The global lysine market in 2022 was estimated at nearly 3.0 million tons to meet the demand in the animal feed and pharmaceutical industries[4]. Currently, L-lysine production predominantly relies on *Corynebacterium glutamicum*, which is widely recognized as the most effective producer of amino acids[1]. Recently, *Vibrio natriegens* has emerged as a promising alternative host for industrial biotechnology due to its extremely rapid growth rate with a reported doubling time of less than 10 min, non-pathogenic nature, genetic tractability and ability to utilize a variety of carbon sources[11].

Additionally, *V. natriegens* exhibits high salt-tolerance, allowing it to be cultivated under non-sterile condition[7,10]. Notably, *V. natriegens* has been successfully modified by metabolic engineering to produce the amino acid alanine, with a high yield of 0.73 g/g glucose[5]. In this preliminary study, we aimed to investigate the feasibility of L-lysine production in *V. natriegens.*

Material and Methods

Materials: *V. natriegens* strain TND1964, kindly offered by by Prof. Ankur Dalia from Indiana University, Bloomington, USA, was used as initial strain[3]. *Corynebacterium glutamicum* ATCC 13032 was obtained from the American Type and Culture Collection. The tRNA genes for TCC and CGG codons and T7 RNA polymerase gene were synthesized by Genscript. All other reagents were obtained from Macrogen, Thermo Scientific, New England Biolabs, Merck, Qiagen and Bio Basic, unless otherwise specified.

Generation of transfer DNA (tDNA) construct: The tDNA-I construct (Figure 1) was generated by assembling 11 fragments through two sequential Gibson assembly reactions by NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs). These component fragments were amplified by PCR in 50 µL reaction containing 0.5 µM of specific primers (Table 1), 0.2 mM dNTPs and 0.02 U/µL of the Q5 Hot Start High-Fidelity DNA Polymerase in 1X Q5 reaction buffer under the conditions described in table 1. The PCR products were verified by agarose gel electrophoresis and purified by Monarch® PCR and DNA Cleanup Kit (New England BioLabs). In the first round, six fragments (*dns*-UHA-PT7a-*dapA*-*dapB*-PT7b-*ddh*) were assembled to form tDNA-IA construct and the remaining five fragments (*lysA*-tRNA-*Kan*^{R}-*lysE*-*dns*-DHA) were assembled to form tDNA-IB construct. In the second round, two resulting products were combined to generate the complete tDNA-I construct. Similarly, the tDNA-II construct (Figure 1) was generated through a seven-fragment Gibson assembly reaction, using primers listed in table 2.

Natural transformation of tDNA constructs into *V. natriegens*: To generate the final mutant strains, the two tDNA constructs were sequentially introduced into the genome of *V. natriegens* strain TND1964 using natural transformation method[3]. Briefly, a single colony of *V. natriegens* TND1964 was grown in LBv2 medium supplemented with 100 µM IPTG at 30°C, 200 rpm overnight. LBv2 is LB-Miller supplemented with v2 salts (204 mM NaCl, 4.2 mM KCl and 23.14 mM $MgCl_2$). Subsequently, 10^8 CFUs of the overnight culture were diluted into 350 µL of instant ocean medium supplemented with 100 µM IPTG and tDNA construct (50 ng) was added and the mixture was incubated at 30°C for 5 hours.

Then, 1 mL LBv2 fresh medium was added and the mixture was incubated at 30°C, 200 rpm for 2 hours. Transformants were selected on LBv2 agar plates supplemented with ampicillin (25 µg/mL) and kanamycin (200 µg/mL) for transformants with tDNA-I construct and ampicillin (25 µg/mL), kanamycin (200 µg/mL) and chloramphenicol (25 µg/mL) for transformants with tDNA-II construct.

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Table 1
Primers used for generation of tDNA-I construct

Primer name	Primer Sequence (5'→3')	Thermocycling conditions
Primers for generating construct		
Vna-deldns-F1	TGTACTAGCAAGCGTGGTATTCAGCA	98°C-30 s; 30 cycles of 98°C-10 s, 67°C-30 s, 72°C-30 s.
Vna-deldns-R1	AGCTGGTGGGATGATCTTGA	
PT7a-F	tcaagatcatcccaccagct CTCGATCCCGCGAAATTAAT	98°C for 30 s; 6 cycles of 98°C-10 s, 61°C-30 s, 68°C-30 s; 9 cycles of 98°C-10 s, 68°C-1 min.
PT7a-R	aaacctgtgctcatagagtt CATATGTATATCTCCTTCTTAAAGTTAAACA	
Cglu-dapA-gb-F	AACTCTATGAGCACAGGTTTAAC	98°C-30 s; 30 cycles of 98°C-10 s, 63°C-30 s, 72°C-30 s.
Cglu-dapA-gb-R	tatgctttctcctctttaat GCGATTTCGGGAATCATTCA	
Cglu-dapB-gb-F	attaaagaggagaaagcata ATGGGAATCAAGGTTGG	98°C-30 s; 6 cycles of 98°C-10 s, 65°C-30 s, 72°C-40 s; 24 cycles of 98°C-10 s, 72°C-1 min 20 s.
Cglu-dapB-gb-R	cgggatcgagatctcgggca TGAGCCTTTACAGGCCTAG	
PT7b-F	TGCCCGAGATCTCGATCC	98°C-30 s; 6 cycles of 98°C-10 s, 62°C-30 s, 68°C-30 s; 9 cycles of 98°C-10 s, 68°C-1 min.
PT7b-R	ttgtatttctcctctttaat TATTTCTAGAGGGAAACCGT	
Cglu-ddh-gb-F	attaaagaggagaaatacaaa ATGACCAACATCCGCG	98°C-30 s; 6 cycles of 98°C-10 s, 67°C-30 s, 72°C-30 s; 24 cycles of 98°C-10 s, 72°C-1 min.
Cglu-ddh-gb-R	tattatttctcctctttaat TAGACGTCGCGTGCGATC	
Cglu-lysA-gb-F	attaaagaggagaaataatag ATGGCTACAGTTGAAAATTTCA	98°C-30 s; 6 cycles of 98°C-10 s, 63°C-30 s, 72°C-45 s; 24 cycles of 98°C-10 s, 69°C-30 s, 72°C-45 s.
Cglu-lysA-gb-R	aggactgagctagctgtc aaGAAACCCAGAAACCCAAAACCG	
tRNA-gb-F	TTGACAGCTAGCTCAGTCCT	98°C-30 s; 15 cycles of 98°C-10 s, 66°C-30 s, 68°C-30 s.
tRNA-gb-R	gttccactgagcgtcaga ccTCACAACAGCTAGTCAGAGA	
KanR-gb-F	GGTCTGACGCTCAGTGGAAC	98°C-30 s; 6 cycles of 98°C-10 s, 65°C-30 s, 72°C-30 s; 9 cycles of 98°C-10 s, 69°C-30 s, 72°C-30 s.
KanR-gb-R	catgatcacatctccttc ttCTTAGAAAAACTCATCGAGCATCA	
Cglu-lysE-gb-F	aagaaggagat gtgatcatgGAAATCTTCATTACAGGT	98°C-30 s; 6 cycles of 98°C-10 s, 64°C-30 s, 72°C-40 s; 24 cycles of 98°C-10 s, 72°C-1 min 20 s.
Cglu-lysE-gb-R	cgattgtcgcgattggtgag CGAAAACTAACCCATCAACATCA	
Vna-deldns-F2	CTCACCAATCGCGACAATCG	98°C-30 s; 30 cycles of 98°C-10 s, 67°C-30 s, 72°C-30 s.
Vna-deldns-R2	GCTTCAAGCATCATGGCAAAGCTAGA	
Vna-deldns-F1	TGTACTAGCAAGCGTGGTATTCAGCA	98°C-30 s; 15 cycles of 98°C-10 s, 70°C-30 s, 72°C-4 min.
Vna-deldns-R2	GCTTCAAGCATCATGGCAAAGCTAGA	
Primers for screening transformants		
Cglu-dapA-sc-F	ACAGCATTACGTGAGTTGT	98°C-30 s, 30 cycles of 98°C-10 s, 62°C-30 s, 72°C-30 s.
Cglu-dapB-sc-R	AGCGTTAGGAGTGGTGA	
kanR-sc-F	TGGAACTGCCTCGGTGAG	98°C-30 s, 30 cycles of 98°C-10 s, 66°C-30 s, 72°C-30 s.
Cglu-lysE-sc-R	ACAGGTAAGCGATGCCAC	
Sequencing primers		
Dns-seq-F	TCCGTCCTCATGGCAAAG	*dapA* sequencing primer
Cglu-dapB-seq-R	ACTTCAGCTGATTCGAAGA	*dapA-dapB* sequencing primer
Cglu-dapB-seq-F	TCTTTTCCAAGCAGGCTG	*dapB-ddh* sequencing primer
Cglu-ddh-seq-F	AGCACCAGCAGCACACCT	*ddh-lysA* sequencing primer
Cglu-lysA-seq-F1	TAATAGATGGCTACAGTTGAAA	*lysA* sequencing primer
Cglu-lysA-seq-F2	ACAACATCCGCCCAGCA	*lysA*-tRNA sequencing primer
KanR-seq-F	GGTCTGACGCTCAGTGGAAC	*kanR-lysE* sequencing primer
Cglu-lysE-seq-F	GTGATCATGGAAATCTTCATTAC	*lysE* sequencing primer

Table 2
Primers used for generation of tDNA-II construct

Primers for generating construct		
Vna-delpckA-F1	AGGTCATATGGTGATCACCATCGAACCT	98°C-30 s; 30 cycles of 98°C-10 s, 69°C-30 s, 72°C-30 s.
Vna-delpckA-R1	GTGTTGCAGCCTTTGTATGTTCCA	
lacI-gb-F	atggaacatacaaaggctgca acATTAATTGCGTTGCGCT	98°C-30 s; 6 cycles of 98°C-10 s, 64°C-30 s, 72°C-40 s; 9 cycles of 98°C-10 s, 72°C-1 min 20 s.
lacI-gb-R	agccgatgattaattgtc aaGGGAGAGCGTCGAGA	
T7RNAP-gb-F	TTGACAATTAATCATCGGCTCGT	98°C-30 s; 15 cycles of 98°C-10 s, 65°C-30 s, 72°C-1 min 30 s.
T7RNAP-gb-R	tctccctatagtgagtcgtattagc TAGCATTACGCGAACGCGAAGTCC	
Cglu-lysC-gb-F1	gctaatacgactcactatagggaga AAAGAGGAGAAAACAAAG ATGGCCCTGGTCGTACAGA	98°C-30 s; 6 cycles of 98°C-10 s, 70°C-30 s, 72°C-30 s; 24 cycles of 98°C-10 s, 72°C-1 min.
Cglu-lysC-gb-R1	cggaacgagggcaggtgaag ATGATGTCGGTGGTGCCGT	
Cglu-lysC-gb-F2	TCATCTTCACCTGCCCTCGTTC	98°C-30 s; 30 cycles of 98°C-10 s, 62°C-30 s, 72°C-30 s.
Cglu-lysC-gb-R2	AAAACTTTAGCGTCCGGT	
CmR-gb-F	gcaccggacgctaaagtttt ACCTGTGACGGAAGATCA	98°C-30 s; 6 cycles of 98°C-10 s, 64°C-30 s, 72°C-30 s; 9 cycles of 98°C-10 s, 72°C-1 min.
CmR-gb-R	caacattagtcaagctgtgg ATTCAGGCGTAGCACCAGG	
Vna-delpckA-F2	CCACAGCTTGACTAATGTTGTGCG	98°C-30 s; 30 cycles of 98°C-10 s, 68°C-30 s, 72°C-30 s.
Vna-delpckA-R2	AGCGGAAGTGAAGCATCCT	
Vna-delpckA-F1	AGGTCATATGGTGATCACCATCGAACCT	98°C-30 s; 15 cycles of 98°C-10 s, 68°C-30 s, 72°C-4 min.
Vna-delpckA-R2	AGCGGAAGTGAAGCATCCT	
Primers for screening transformants		
Ptac-sc-F	ATCGGCTCGTATAATGTGT	98°C-30 s; 30 cycles of 98°C-10 s, 61°C-30 s, 72°C-30 s.
T7RNAP-sc-R	TATTGTCGGCTGACGTTAGA	
Cglu-lysC-sc-F	TGAATACGCTCGTGCATT	98°C-30 s; 30 cycles of 98°C-10 s, 61°C-30 s, 72°C-30 s.
Cglu-lysC-sc-R	GCAGAGCTTCCATGAACT	
Sequencing primers		
T7RNAP-seq-R	TGTGAGTACGCACTAACG	T7RNAP sequencing primers
T7RNAP-seq-F1	CTCTCCGATGTTCCAACC	
T7RNAP-seq-F2	ACGAAGTAGTTACAGTGACC	
Cglu-lysC-seq-F	GAAGCCAAAGTAACCGTTC	*lysC* sequencing primers
Cglu-lysC-seq-R	GCAGAACCATGTCAATGTT	

Transformants were then screened by colony PCR with specific primer pairs. Genomic DNA from positive lines was extracted and amplified with Vna-deldns-F1 and Vna-deldns-R2 primers, Vna-delpckA-F1 and Vna-delpckA-R2 primers. Purified PCR products were sent for DNA sequencing to verify the construct accuracy.

Shake Flask Fermentation: Two independent transformants were inoculated in 5 mL of LBv2 medium supplemented with ampicillin (25 µg/mL), kanamycin (200 µg/mL) and chloramphenicol (25 µg/mL) and cultured at 30°C, 200 rpm overnight. Subsequently, 0.5 mL of the overnight culture was reinoculated into 50 mL of LBv2 medium in a 250 mL Erlenmeyer flask and incubated at 30°C, 200 rpm until OD_{600} reached 0.6-0.8. The cultures were then induced with IPTG (0.1 mM – 1 mM) at 30°C for 5 hours. After centrifuging at 3000 × *g* for 5 min at 4°C, the supernatants were discarded. The remaining cell pellets were resuspended in 50 mL of modified M9 medium (222 mM glucose, 224.3 mM NH_4Cl, 150 mM Na_2HPO_4, 75 mM KH_2PO_4, 30 mM NaCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM $FeCl_3$, 0.05 mM $ZnSO_4$, 0.05 mM H_3BO_3, 0.8 µM $CuSO_4$, 0.15 µM $CoCl_2$, 0.15 µM Na_2MoO_4, 1 µg/mL biotin and 1 µg/mL thiamine), cultured at 30°C, 200 rpm for 48 hours and supernatants were collected and stored at -80°C until lysine quantification. Reducing sugars in culture supernatants were quantified using dinitrosalicylic acid (DNS) method[13].

Lysine quantification by high-performance liquid chromatography (HPLC): Lysine was quantified using the method described by Vuong et al[12]. Briefly, samples were derivatized with o-phthalaldehyde in the presence of 2-mercaptoethanol for 2 minutes prior to separation on a C18 Zorbax Eclipse column (5 µm, 4.6 × 150 mm; Agilent, USA). Detection was carried out using a diode-array

detector (DAD) at 338 nm on an Agilent 1200 Series HPLC system. Separation was achieved through a 25-minute gradient elution program with two mobile phases: (A) 40 mM sodium phosphate buffer (pH 7.8) and (B) a mixture of methanol, acetonitrile and deionized water (45:45:10, v/v/v). The program began with 100% phase A, transitioning to 100% phase B over 15 minutes. This was followed by a 5-minute isocratic elution with 100% phase B.

Results and Discussion

To enhance lysine production in *V. natriegens*, a metabolic engineering strategy (Figure 2) was implemented by generating two transfer DNA (tDNA) constructs for integration into *V. natriegens* genome (Figure 1). The tDNA-I construct was generated to introduce five core genes involved in the L-lysine biosynthesis pathway from *C. glutamicum*. In this construct, while *dapA*, *dapB*, *ddh*, *lysA* were under the control of the strong T7 promoter, *lysE* was driven by kan^R promoter to prevent excessive expression of the lysine exporter, which may disrupt membrane integrity. Of note, genes from *C. glutamicum* abundantly contain two codons CGG and TCC, that are rare in *V. natriegens*.

To ensure the translation of the introduced genes, two genes coding for $tRNA^{CGG}$ and $tRNA^{TCC}$ under the control of T7 promoter were included in the tDNA-I construct. Overall, a total of 11 component fragments was needed (Figure 3A and Figure 3B) and sequential assembly was used to generate the tDNA-I construct. Briefly, the tDNA-IA fragment (*dns*-UHA-*dapA*-*dapB*-*ddh*) was generated as shown in figure 3A and the tDNA-IB fragment (*lysA*-$tRNA^{CGG,TCC}$-Kan^R-*lysE*-*dns*-DHA) was produced as shown in figure 3B. Subsequently, the full-length of tDNA-I construct was successfully generated by assembly of tDNA-IA and tDNA-IB fragments (Figure 3C).

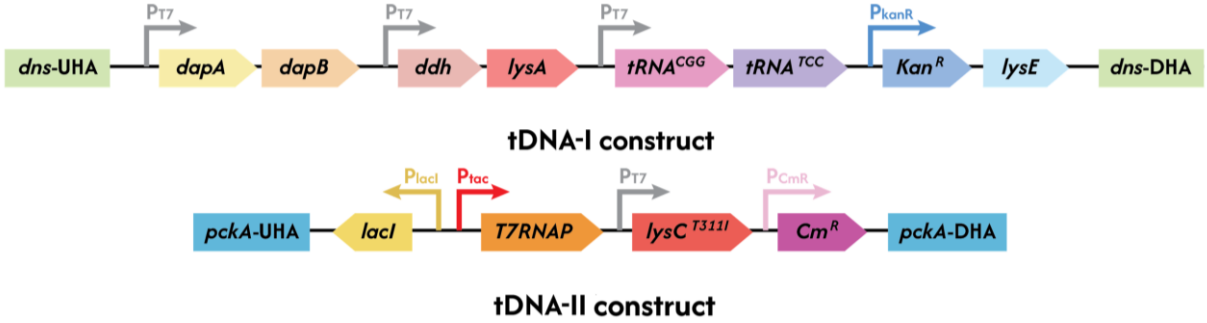

Figure 1: Schematic depiction of the integration of the two transfer DNA (tDNA) constructs into *V. natriegens* genome in this study.
UHA: upstream homologous arm; DHA: downstream homologous arm.

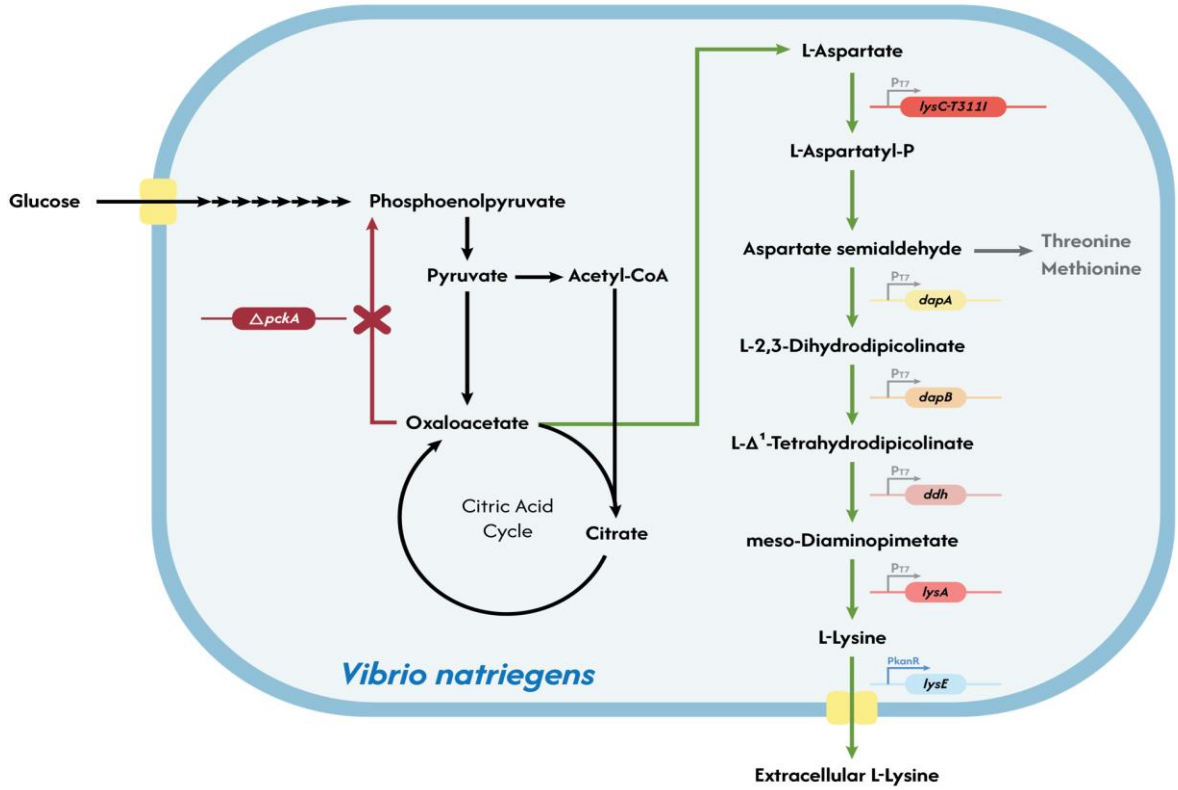

Figure 2: Metabolic engineering strategy for improvement of L-lysine production in *V. natriegens* used in this study

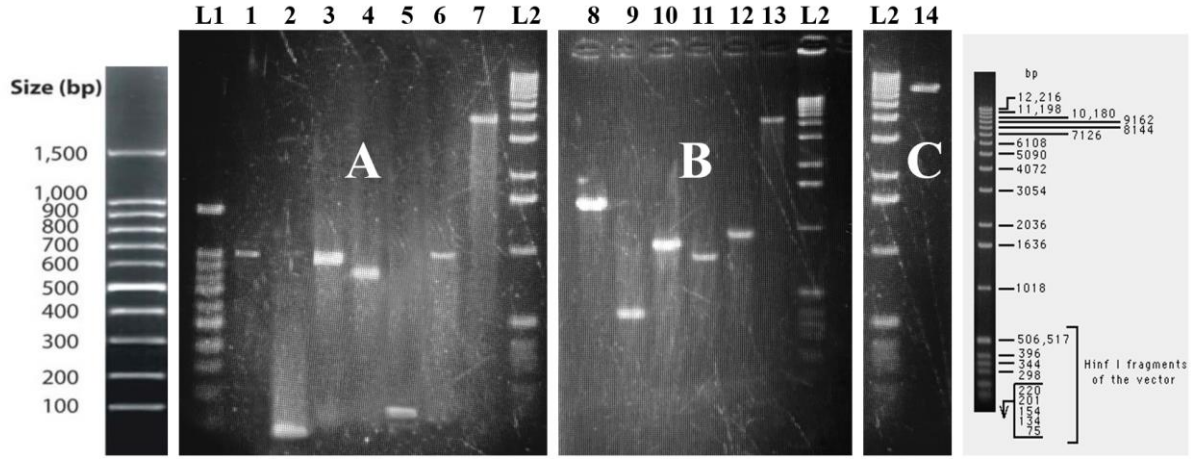

Figure 3: Agarose gel analysis for verification of PCR products during the generation of tDNA-I construct by Gibson assembly.

(A) Generation of tDNA-IA construct: (1) dns-UHA fragment; (2) PT7b fragment; (3) dapA fragment; (4) dapB fragment; (5) PT7a fragment; (6) ddh fragment; (7) Gibson assembly product amplified by Vna-deldns-F1 and Cglu-ddh-gb-R primers. **(B) Generation of tDNA-IB construct:** (8) lysA fragment; (9) tRNA fragment; (10) KanR fragment; (11) lysE fragment; (12) dns-DHA fragment; (13) Gibson assembly product amplified by Cglu-lysA-gb-F and Vna-deldns-R2 primers. **(C) Generation of the full-length tDNA construct:** (14) Full-length product of tDNA-I construct amplified by Vna-deldns-F1 and Vna-deldns-R2 primers. L1: 100 bp DNA ladder (Mebep Bioscience); L2: 1 kb DNA ladder (Invitrogen). UHA: upstream homologous arm; DHA: downstream homologous arm.

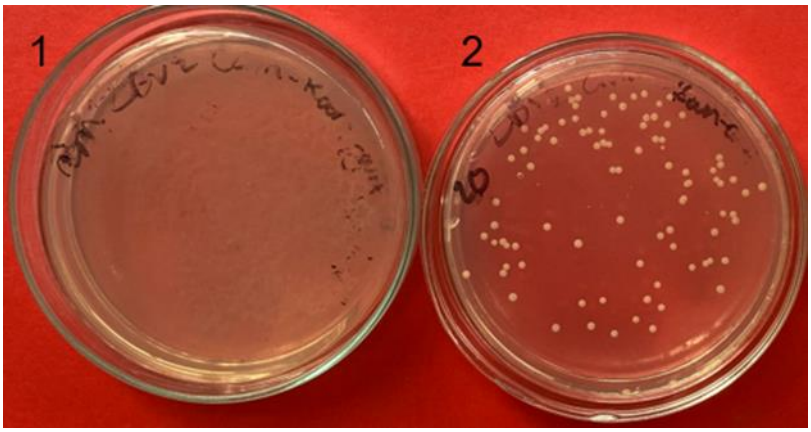

Figure 4: Colonies transformed with tDNA-I construct on selection LBv2 agar supplemented with ampicillin and kanamycin.

(1) Selection plate with wild-type control; (2) Selection plate with transformants.

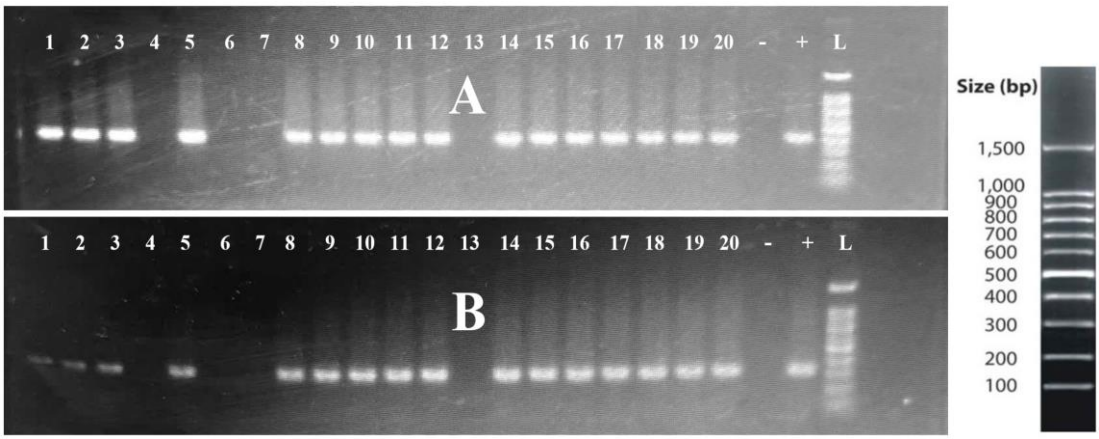

Figure 5: Screening results for colonies transformed with tDNA-I construct by PCR with (A) Cglu-dapA-sc-F and Cglu-dapB-sc-R, (B) kanR-sc-F and Cglu-lysE-sc-R.

(-) no-template control; (+) positive control; 1-20: colony PCR products; L: 100 bp DNA ladder (Mebep Bioscience).

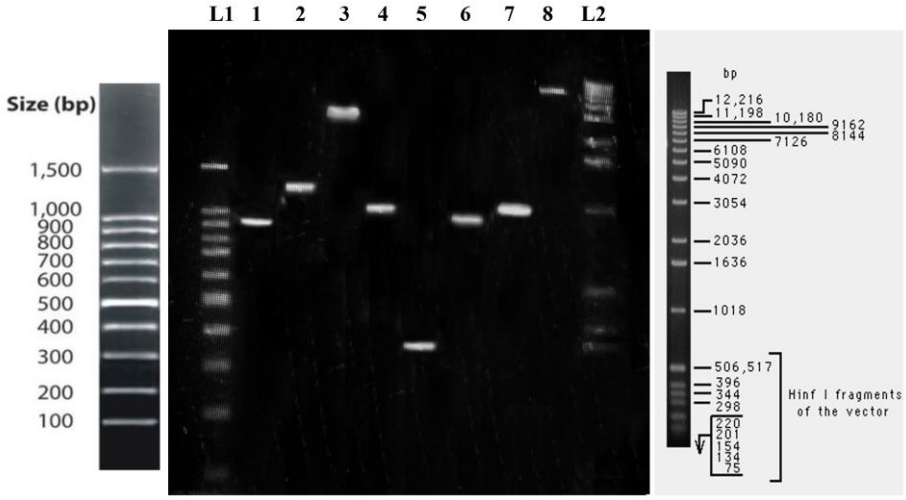

Figure 6: Agarose gel analysis for verification of PCR products during the generation of tDNA-II construct by Gibson assembly.
(1) pckA-UHA fragment; (2) lacI fragment; (3) T7RNAP fragment; (4) lysC fragment 1; (5) lysC fragment 2; (6) Cm^R fragment; (7) pckA-DHA fragment (8) Full-length product of tDNA-II construct amplified by Vna-delpckA-F1 and Vna-delpckA-R2 primers. L1: 100 bp DNA ladder (Mebep Bioscience); L2: 1 kb DNA ladder (Invitrogen). UHA: upstream homologous arm; DHA: downstream homologous arm.

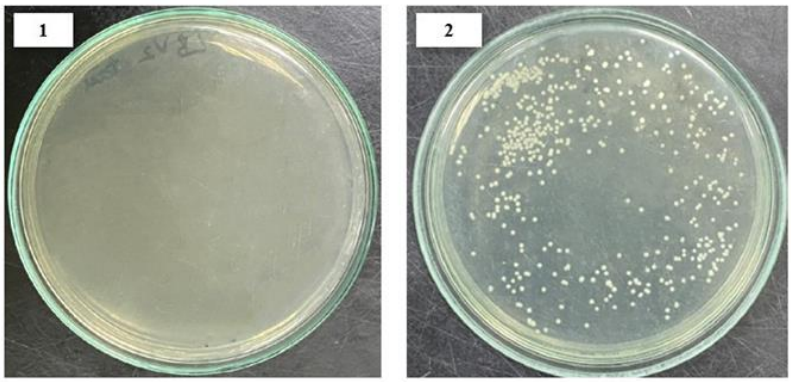

Figure 7: Colonies transformed with tDNA-II construct on selection LBv2 agar supplemented with ampicillin, kanamycin and chloramphenicol.
(1) Selection plate with parental strain 1D9; (2) Selection plate with transformants with tDNA-II construct.

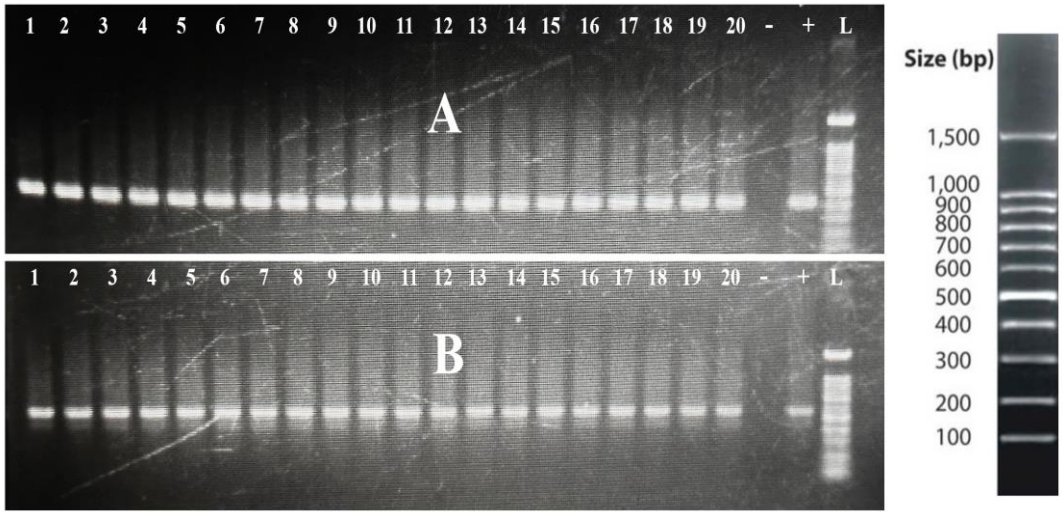

Figure 8: Screening results for colonies transformed with tDNA-II construct using PCR with (A) Ptac-sc-F and T7RNAP-sc-R primers, (B) Cglu-lysC-seq-F and Cglu-lysC-seq-R.
(-) no-template control; (+) positive control; 1-20: colony PCR products; L: 100 bp DNA ladder (Mebep Bioscience).

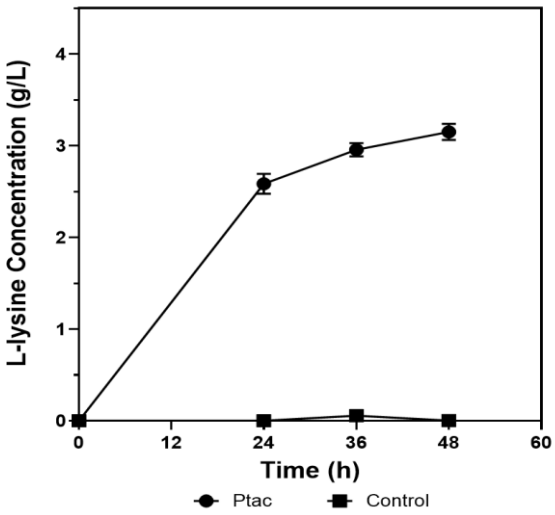

Figure 9: Comparison of L-lysine titer between *V. natriegens* wild-type strain and transformants carrying the tDNA-I and tDNA-II constructs (induced by 0.1 mM IPTG).

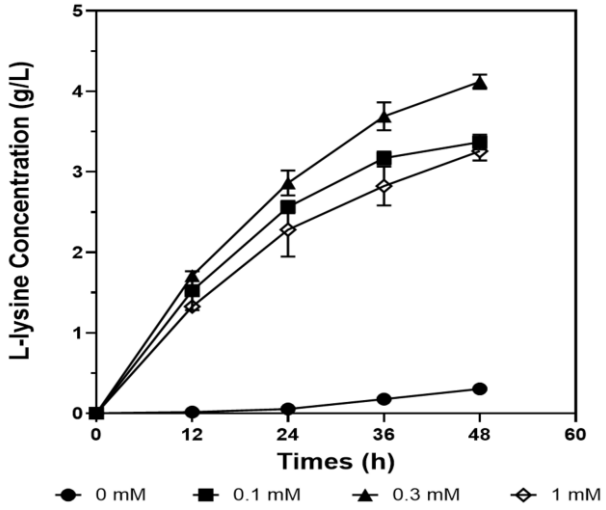

Figure 10: Effect of IPTG concentration on L-lysine titer of *V. natriegens* transformants carrying the tDNA-I and tDNA-II constructs.

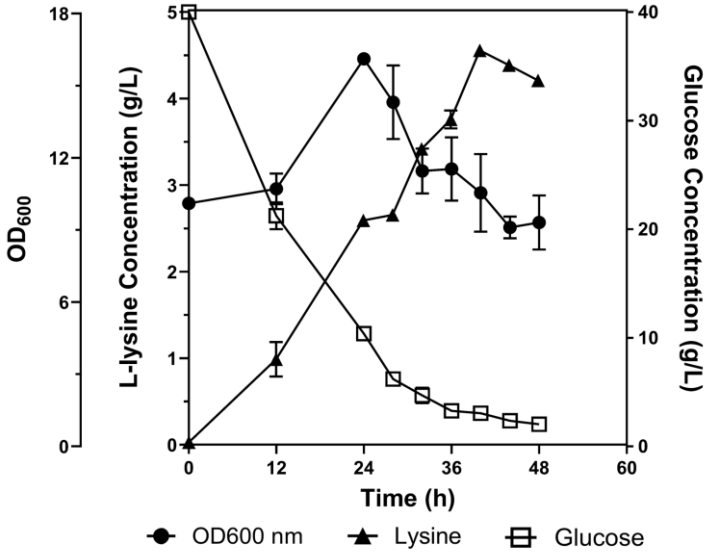

Figure 11: L-lysine production performance, reducing sugars and growth kinetics of *V. natriegens* transformants carrying the tDNA-I and tDNA-II constructs.

In the same manner, the tDNA-II construct (Figure 1) was engineered to carry a T7 RNA polymerase gene under the control of the inducible promoter *tac*, along with a feedback-resistant variant of *lysC* gene encoding aspartokinase from *C. glutamicum*. This construct was designed to integrate to the *pckA* locus, which encodes phosphoenolpyruvate carboxykinase A, in order to block the conversion of oxaloacetate (OAA), a key precursor for lysine production, into phosphoenolpyruvate (PEP). A seven-fragment Gibson assembly reaction was performed to generate the tDNA-II construct. Amplified products were verified by agarose gel electrophoresis, each fragment and the full-length of tDNA-II construct were observed as single bands at expected size (Figure 6). Finally, the tDNA-I and tDNA-II constructs were sequentially introduced into *V. natriegens* TND1964.

Transformants with tDNA-I construct were obtained on LBv2 agar plates supplemented with ampicillin (25 µg/mL) and kanamycin (200 µg/mL) (Figure 4). After screening by colony PCR, 16 out of 20 colonies were positive with both Cglu-dapA-sc-F and Cglu-dapB-sc-R primers (Figure 5A) and kanR-sc-F and Cglu-lysE-sc-R primers (Figure 5B). Subsequently, genomic DNA from two positive colonies was amplified for sequencing and the clone 1D9 with correct sequence was used for second round of transformation. Transformants with tDNA-II construct were obtained on LBv2 agar plates supplemented with ampicillin (25 µg/mL), kanamycin (200 µg/mL) and chloramphenicol (25 µg/mL) (Figure 7).

Screening results showed that 20 out of 20 colonies were positive with Ptac-sc-F and T7RNAP-sc-R primers (Figure 8A), Cglu-lysC-sc-F and Cglu-lysC-sc-R primers (Figure 8B). The whole construct of positive clones was amplified for sequencing and transformants with validated sequence were used for further experiments. Two independent transformants carrying the tDNA-I and tDNA-II constructs were assessed for production ability of L-lysine. As shown in figure 9, L-lysine titer secreted by the transformants increased rapidly, reached nearly 2.6 g/L after 24 hours and peaked at 3.1 g/L after 48 hours, reflecting 55-fold increase compared to the wild-type strain. These results demonstrated that the strategy used in this study to produce L-lysine was effective.

In addition, to fine-tune the expression of the introduced genes, IPTG concentration was optimized (Figure 10). As shown, L-lysine production in the non-induced condition reached 0.3 g/L after 48 hours, while that in the induced conditions was above 3 g/L, suggesting slightly leaky expression of the introduced genes. Among the concentrations tested, 0.3 mM IPTG was optimal, resulting in the highest L-lysine production of 4.1 g/L after 48 hours of fermentation.

Based on these results, the kinetic of L-lysine production in the mutant strain was in-depth evaluated (Figure 11). While glucose was rapidly consumed in the first 24 hours, it coincided with the growth phase of the mutant, extracellular L-lysine gradually increased over 40 hours, reaching a maximum titer of 4.6 g/L (nearly 80-fold higher compared to wild-type strain) before observing a slight decline.

This trend is consistent with previous observations in *C. glutamicum*, in which L-lysine titer peaked during the stationary phase[9]. The decrease in L-lysine concentration after 40 hours may be explained by the exhaust of glucose, which results in the consumption of L-lysine by *V. natriegens* as an alternative nutrient source. Fed-batch fermentation will be considered in further work to remediate this issue and improve L-lysine titer. Notably, the yield of L-lysine production of the strains created in this study reached 152 mmol/mol glucose, comparable to the values obtained for *C. glutamicum* LYS-6 strain reported in the pioneer study by Becker et al[2]. Of note, the LYS-6 strain contains $lysC^{T311I}$ and *dapB* genes under the control of strong promoters, additional copy of the *ddh* and *lysA* genes and deletion of *pckA* gene. The strains created in the present study received two more additional genes involved in the biosynthesis of L-lysine in *C. glutamicum* that are *dapA* and *lysE*. The main limitation of our study is the lack of the data concerning the bottlenecks in metabolic pathways of *V. natriegens* related to lysine production. This is the reason all the genes encoding core metabolic enzymes for L-lysine production used in this study were from *C. glutamicum*, of which the L-lysine metabolic pathway has been elucidated.

Furthermore, flux analysis revealed that the carbon flux directed into oxidative pentose phosphate pathway (PPP) in *V. natriegens* is 33% lower than those in *E. coli*[6]. As the synthesis of one mole of lysine requires four moles of NADPH[8], a deficiency in NADPH, due to the low activity of PPP, may limit L-lysine production in *V. natriegens*. In addition to stimulating PPP activity, other strategies could be investigated in further study such as overexpression of pyc^{P485S}, encoding pyruvate carboxylase, to boost the intracellular level of the precursor OAA, as well as inhibition of methionine and threonine biosynthesis to concentrate the carbon flux toward L-lysine.

Conclusion

In summary, this study provides strong evidence for the feasibility of L-lysine biosynthesis in *V. natriegens*.

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