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Research Article

Efficient Optimization of *Gluconobacter oxydans* Based on Protein Scaffold-Trimeric CutA to Enhance the Chemical Structure Stability of Enzymes for the Direct Production of 2-Keto-L-gulonic Acid

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2-Keto-L-gulonic acid (2-KLG), the direct precursor of vitamin C, is produced by a two-step fermentation route from D-sorbitol in industry. However, this route is a complicated mix-culture system which involves three bacteria. Thus, replacement of the conventional two-step fermentation process with a one-step process could be revolutionary in vitamin C industry. The one-step fermentation of 2-keto-L-gulonic acid (2-KLG) has been achieved in our previous study; 32.4 g/L of 2-KLG production was obtained by the one-step strain *G. oxydans*/pGUC-*tufB-sdh*-GGGGS-*sndh* after 168 h. In this study, L-sorbose dehydrogenase (SDH) and L-sorbosone dehydrogenase (SNDH) were expressed in *G. oxydans* after the codon optimization. Furthermore, the trimeric protein CutA was used to improve the chemical structure stability of SDH and SNDH. The recombinant strain *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA* produced 40.3 g/L of 2-KLG after 168 h. In addition, the expression levels of the cofactor PQQ were enhanced to further improve 2-KLG production. With the stepwise metabolic engineering of *G. oxydans*, the final 2-KLG production was improved to 42.6 g/L. The efficient one-step production of 2-KLG was achieved, and the final one-step industrial-scale production of 2-KLG is drawing near.

1. Introduction

Vitamin C (L-ascorbic acid, L-AA) is widely used in pharmaceutical, food, beverages, cosmetics, and feed industries [1]. The most successful route for industrial production of vitamin C is the classical two-step fermentation process. The fermentation process contains one-step conversion from D-sorbitol to L-sorbose by *Gluconobacter oxydans*, followed by another step of converting L-sorbose to 2-keto-gulonic acid (2-KLG), the precursor of vitamin C, by a mixed culture system of *Ketogulonicigenium vulgare* and *Bacillus megaterium*. *K. vulgare* is difficult to culture alone and it possesses a rather low production capacity of 2-KLG. The accompany bacterium *B. megaterium* does not produce 2-KLG, but it can promote the growth and 2-KLG production of *K. vulgare* [2]. Over the years, researchers have attempted to improve fermentation processes in many ways; tremendous advances have been achieved in microbial production of vitamin C [2–5]. However, the culture broth from the "first step" by *G. oxydans* containing L-sorbose needs to be transferred to another bioreactor, added with other culture media, and sterilized for the second time. The addition process involves three microorganisms which significantly increases the cost of both raw materials and energy requirement [6]. Besides, the mix-culture system makes the process optimization difficult.

In consideration of problems presented above in the two-step fermentation process, using D-glucose or

D-sorbitol as a carbon source for the production of 2-KLG in one-step fermentation process would be more cost-effective and it will be a revolutionary advancement in the vitamin C industry. Although the strain which can catalyze D-glucose or D-sorbitol to 2-KLG efficiently by single strain fermentation has not been found, advances in biochemistry and recombinant DNA technology, together with the genomic revolution, have promoted the construction of the direct microbial processes to 2-KLG via genetic engineering [1, 7]. In the earlier study, membrane-bound sorbose dehydrogenase and cytosolic sorbosone dehydrogenase were cloned from *G. oxydans* T-100 and expressed in *G. oxydans* G624, which is able to accumulate L-sorbose. The recombinant strain produced 88 g/L of 2-KLG from D-sorbitol [8]. However, there was no research about one-step fermentation over the last decade, and the results obtained in our previous study showed that the expression of SDH and SNDH genes in *G. oxydans* could only result in a yield of 2-KLG of no more than 5 g/L [9].

G. oxydans is an industrially important bacterium for its ability to oxidize sugars and sugar alcohols at high levels. Many compounds such as acetic acid, D-gluconic acid, L-sorbose, and dihydroxyacetone have been produced successfully with *G. oxydans* [10–14]. In industrial 2-KLG synthesis, *G. oxydans* was initially found to be used in the Reichstein process for the oxidation of D-sorbitol to L-sorbose, which is a species of choice for the construction of genetically engineered strain that equipped the crucial dehydrogenases required for the conversion of D-sorbitol to 2-KLG [15]. *G. oxydans* WSH-003, in this study, is an L-sorbose-accumulating strain of industrial interest due to its powerful ability to oxidize D-sorbitol into L-sorbose with a high quantitative yield of over 98% on an industrial scale. *G. oxydans* WSH-003 was mutated by different methods from a wild-type strain for at least 90 times to improve the production of L-sorbose and the tolerance to saccharides and alditols. Finally, the industrial strain possessed both high L-sorbose productivity and extreme tolerance to saccharides and alditols [16]. In addition, in our previous study, it was identified that even 100 g/L of 2-KLG did not obviously affect the cell growth of *G. oxydans* WSH-003 and no obvious degradation of 2-KLG could be detected when *G. oxydans* WSH-003 was grown with 2-KLG [9].

Following the development of next-generation sequencing technology, the three bacteria involved in the classical two-step fermentation process for L-AA production have all been sequenced in my original laboratory. The gene clusters encoding D-sorbitol dehydrogenase and responsible for the synthesis of the cofactor pyrroloquinoline quinone (PQQ) were identified from the genome sequence of *G. oxydans* WSH-003 [16]. Meanwhile, in *K. vulgare* WSH-001, five L-sorbose dehydrogenases (SDHs) and two L-sorbosone dehydrogenases (SNDHs) were confirmed by expression of the DNA in *Escherichia coli*, which were a group of PQQ-dependent dehydrogenases for the catalysis of L-sorbose to L-sorbosone and further to 2-KLG [17]. In our previous study, five SDH genes and two SNDH genes were overexpressed in *G. oxydans* with different combinations, and by a series of biological engineering, the 2-KLG production increased to 32.4 g/L. In this study, the cross-linker protein CutA was used as a protein scaffold to improve the chemical structure stability of SDH and SNDH, which was the first time for the application of CutA in metabolic engineering. The recombinant strain pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA* produced 40.3 g/L of 2-KLG after 168 h, and the production was improved efficiently. Furthermore, the expression levels of the cofactor PQQ were enhanced to further improve 2-KLG production. With the stepwise metabolic engineering of *G. oxydans*, the final 2-KLG production was improved to 42.6 g/L, which was 5.7% higher than that by pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA*.

2. Materials and Methods

2.1. Strains and Plasmids. *K. vulgare* WSH-001 and *G. oxydans* WSH-003 were provided by Jiangsu Jiangshan Pharmaceutical Co., Ltd. *Escherichia coli* JM109 (Novagen, Darmstadt, Germany) was used as the host for plasmid construction. The pMD19-T vector was used for gene cloning (TakaRa, Dalian, China). All plasmids used in this study are provided in Table 1. PrimeSTAR HS DNA polymerase, restriction endonucleases, DNA gel extraction kit, and PCR reagents were purchased from TakaRa (Dalian, China). FastPure DNA kit and SanPrep Column Plasmid Mini-Preps Kit were purchased from Sangon (Shanghai, China). DNA Sanger sequencing was performed by Sangon (Shanghai, China).

2.2. Culture Conditions. *G. oxydans* strains were cultivated in a broth (15% D-sorbitol, 1.5% corn steep liquor, and 2% $CaCO_3$) at 30°C for 168 h. *G. oxydans* transformants were cultivated in medium containing 75 μg/mL ampicillin. *E. coli* strains were cultivated in the Luria-Bertani (LB, Oxoid) medium. 100 μg/mL of ampicillin was added to the LB medium to screening transformants with plasmids.

2.3. Codon Optimization of SDH and SNDH. The codons of the *sdh-sndh* gene (the two genes were fused with GGGGS linker peptide) were optimized based on the codon preference of *G. oxydans* using a codon algorithm with the GeMS software [18]. The codon-optimized gene was synthetized by Genewiz (Nanjing, China).

2.4. Expression of Trimeric Protein CutA in G. oxydans WSH-003. CutA is a small trimeric protein from *Pyrococcus horikoshii* (GenBank Accession number: BAA30089.1) [19]. In order to verify whether the expression of *cutA* could take effect in *G. oxydans*, *cutA* was optimized as stated above and was synthetized by Genewiz. The fragment of the codon-optimized *cutA* was digested and inserted into the *Kpn*I/*Bam*HI site of pGUC (the shuttle vector of *E. coli-G. oxydans* that constructed in our previous study). Then, the promoter of elongation factor TU (*tufB*) [20] was inserted into the *Sac*I/*Kpn*I site of pGUC-*cutA*, resulting in pGUC-*tufB-cutA*,

Table 1: Plasmids used in this study.

Plasmids	Relevant characteristics	Sources
pGUC	Ampr *E. coli-G. oxydans* shuttle vector	This study
pGUC-*tufB-sdh*-GGGGS-*sndh*	pGUC containing *tufB-sdh*-GGGGS-*sndh*	This study
pGUC-*tufB-cutA*	pGUC containing *tufB-cutA*	This study
pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA*	pGUC containing *tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA*	This study
pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA-tufB-pqq*ABCDE	pGUC containing *tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA-tufB-pqq*ABCDE	This study

and was transformed into *G. oxydans* WSH-003 by electroporation [21].

2.5. Fusion Expression of SDH and SNDH with CutA. For the ligation of SDH-GGGGS-SNDH with CutA, adaptor protein (*SH3*) and its ligand (*SH3*$_{lig}$) were used as the docking protein and docking station peptide, respectively [22–24]. Both *SH3* and *SH3*$_{lig}$-(GGGGS)$_2$-*cutA* were optimized as stated above and were synthetized by Genewiz. The *SH3* was fused with codon-optimized *sdh*-GGGGS-*sndh*, resulting in *SH3-sdh*-GGGGS-*sndh*. The *SH3*$_{lig}$-(GGGGS)$_2$-*cutA* was fused with the promoter *tufB*, resulting in *tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA*. The obtained *SH3-sdh*-GGGGS-*sndh* and *tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA* were further digested and inserted into the *Kpn*I/*Bam*HI and *Bam*HI/*Xba*I site of pGUC-*tufB*, respectively, resulting in pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA*, and were transformed into *G. oxydans* WSH-003 by electroporation [21]. The time courses of sorbitol oxidation by the recombinant strains were performed, every 12 h to take a sample, and were analyzed by HPLC. The mean value out of three independent experiments was calculated.

2.6. Overexpression of the Cofactor PQQ in G. oxydans WSH-003. *pqq*ABCDE was amplified using *G. oxydans* WSH-003 genomic DNA with the primers (Table 2). Because of the lack of restriction enzyme site, the promoter *tufB* was fused with *pqq*ABCDE, which was digested and inserted into the *Xba*I/*Pst*I site of pGUC, resulting in pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA-pqq*ABCDE. The construct was transformed into *G. oxydans* WSH-003 by electroporation [21].

2.7. Analysis Procedures. The optical density of the culture broth was measured using a Biospec-1601 spectrophotometer (Shimadzu, Kyoto, Japan) at 600 nm after an appropriate dilution. D-sorbitol, 2-KLG, and intermediate metabolites in the fermentation broth were determined by HPLC, using an Aminex HPX-87H column (Bio-Rad, Richmond, CA) at 35°C with a flow rate of 0.6 mL/min and 5 mmol/L H_2SO_4 as the eluent [25–27]. The concentration of PQQ in the culture supernatants was measured using LC-MS-IT-TOF under the conditions reported by Noji et al. [28].

3. Results and Discussion

3.1. Overexpression of SDH and SNDH in G. oxydans WSH-003 after Codon Optimization. *G. oxydans* WSH-003 is an industrial strain with high L-sorbose productivity and extreme tolerance to saccharides and alditols. The metabolic pathway of D-sorbitol in the recombinant *G. oxydans* strains is shown in Figure 1. In our previous study, five SDHs (KVU_pmdA_0245, KVU_2142, KVU_2159, KVU_1366, and KVU_0203) and two SNDHs (KVU_0095 and KVU_pmdB_0115) in *K. vulgare* WSH-001 were identified [17], which were introduced into *G. oxydans* WSH-003 in different combinations to construct the one-step strain. After a series of biological engineering, the production of 2-KLG was up to 32.4 g/L after 168 h of fermentation by *G. oxydans*/pGUC-*tufB-k0203*-GGGGS-*k0095* (*G. oxydans*/pGUC-*tufB-sdh*-GGGGS-*sndh*) [9].

Codon optimization is a key technique to achieve the efficient expression of heterologous proteins. Codon preference optimization strategy is the most commonly used codon optimization strategy at present, which mainly replaces the donor codon with the synonymous codon with the highest frequency in the host genome and uses the most abundant codon in the host to encode the amino acids in the optimized sequence [29]. In this study, codon preference optimization was conducted to further enhance the efficiency of expression of SDH and SNDH in *G. oxydans*. The *sdh*-GGGGS-*sndh* shares 82% similarity with its parental nucleotide sequence while maintaining the identical amino acid sequence. After 168 h of fermentation, the 2-KLG production reached 33.2 g/L. It was not significant for the enhancement of 2-KLG production by codon optimization, which may be due to the complex and varied factors, such as posttranslational folding and metabolic level, and thus, the expression of SDH and SNDH remains low [30].

3.2. Expression of Trimeric Protein CutA in G. oxydans WSH-003. CutA is a small trimeric protein from *P. horikoshii*, which is used as the cross-linker protein [19]. It has an extremely high denaturation temperature of nearly 150°C [31]. Furthermore, CutA retains its trimeric quaternary structure in a solution containing as much as 5 M GuHCl [32]. It was reported that the remarkably increased number of ion pairs in the monomeric structure contributes to the stabilization of the trimeric structure and plays an important

Table 2: Primers used in this study.

Primer	Sequences of primers (5′-3′)*	Restriction enzyme
tufB-F1	C**GAGCTC**GTACGATGGTAAGAAATCCACTG	*Sac*I
tufB-R1	CGG**GGTACC**CGTCTTTCTCCAAAACCCC	*Kpn*I
SH3-F	CGG**GGTACC**GCCGAGTATGTGCGCGCCCT	*Kpn*I
SH3-R	GGGTCGTGAGCTTCATGTACTTCTCCACGTACGGCACCG	—
sdh-GGGGS-*sndh*-F	GTACGTGGAGAAGTACATGAAGCTCACGACCCTGCTGC	—
sdh-GGGGS-*sndh*-R	CGC**GGATCC**TCACGCCGCGGAAATCCGC	*Bam*HI
tufB-F2	CGC**GGATCC**GTACGATGGTAAGAAATCCACTG	*Bam*HI
tufB-R2	GCAGGGCCGGCGGCGGCGTCTTTCTCCAAAACCCCGCT	—
$SH3_{lig}$-$(GGGGS)_2$-*cutA*-F	AGCGGGGTTTTGGAGAAAGACGCCGCCGCCGGCCCTGC	—
$SH3_{lig}$-$(GGGGS)_2$-*cutA*-R	CTAG**TCTAGA**TCACTTCTTCGTCTCCTCGATCAGC	*Xba*I
tufB-F3	CTAG**TCTAGA**TGCAGATCCGGTGGCCATGTTC	*Xba*I
tufB-R3	TTCGGCGTGTTCCAAGCCATCGTCTTTCTCCAAAACCCCGCT	—
*pqq*ABCDE-F	CGGGGTTTTGGAGAAAGACGATGGCTTGGAACACGCCG	—
*pqq*ABCDE-R	AAAA**CTGCAG**TTACATTCTTCGGTAAACAAAGT	*Pst*I

*Restriction sites used for cloning are in bold and are underlined.

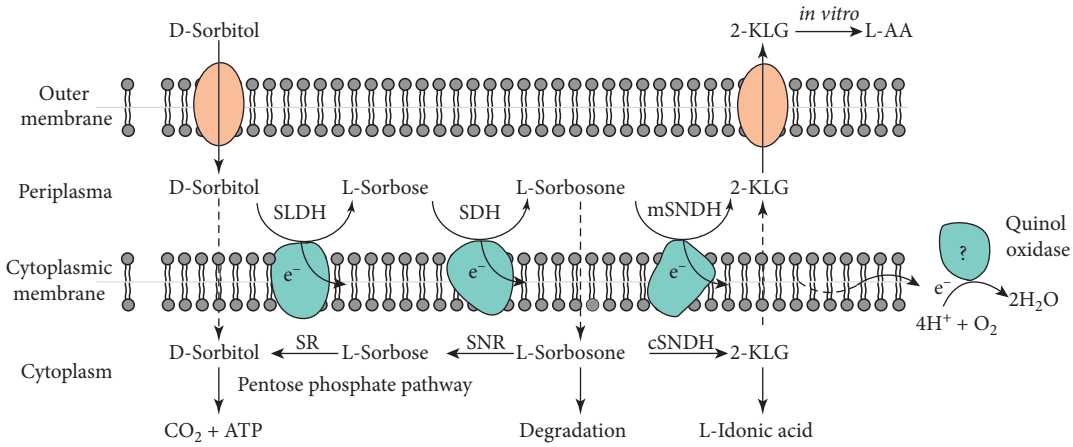

Figure 1: The metabolic pathway of D-sorbitol in the recombinant *G. oxydans* strains. SLDH, D-sorbitol dehydrogenase; SDH, L-sorbose dehydrogenase; SNDH, L-sorbosone dehydrogenase; mSNDH, membrane-bound SNDH; cSNDH, cytosolic SNDH; SR, L-sorbose reductase; SNR, L-sorbosone reductase.

role in enhancing the denaturation temperature up to 150°C [31].

Overexpression of CutA in *G. oxydans* WSH-003 was conducted to verify whether it would influence the growth of the strain. After the codon optimization of *cutA*, *G. oxydans* strain harboring pGUC-*tufB-cutA* was constructed. The growth curve of *G. oxydans*/pGUC-*tufB-cutA* and the wild strain was determined (Figure 2). The results showed that the growth was consistent with each other at 30°C, and *G. oxydans*/pGUC-*tufB-cutA* grew faster at 37°C and 42°C than the wild strain. After 12 h of fermentation, the OD_{600} of *G. oxydans*/pGUC-*tufB-cutA* and the wild strain at 37°C was 6.74 and 5.99, respectively, and at 42°C was 5.31 and 4.1, respectively. However, the growth of *G. oxydans*/pGUC-*tufB-cutA* and the wild strain was both inhibited at 45°C. It was found that the expression of the cross-linker protein CutA improved the heat resistance of the strain.

3.3. Overexpression of SDH and SNDH in G. oxydans WSH-003 Based on CutA. Ramirez et al. developed a novel self-assembling protein hydrogel with cross-linked protein CutA and formed a highly cross-linked protein network. The building blocks initiate an intein trans-splicing reaction that yields a hydrogel that is highly stable over a wide range of pH (6–10) and temperature (4–50°C) [22]. Inspired by this study, the stability of SDH and SNDH may be improved by generating a longer protein chain with the cross-linker CutA, and the catalytic efficiency of the enzymes may be increased further.

The trimeric protein CutA from *P. horikoshii* was able to be used as the protein scaffold, which may improve the stability of SDH and SNDH in *G. oxydans*. The adaptor protein SH3 and its ligand $SH3_{lig}$ were used as docking protein and docking station peptide, respectively. They possess high affinity for each other because of their relatively small size (56 and 11 amino acids, respectively) (Figure 3). After *SH3* and $SH3_{lig}$-$(GGGGS)_2$-*cutA* were optimized and synthetized, they were fused or ligated with the promoter *tufB* and codon-optimized *sdh*-GGGGS-*sndh*, and a recombinant plasmid pGUC-*tufB-SH3-sdh*-GGGGS-*sndh*-*tufB-*$SH3_{lig}$-$(GGGGS)_2$-*cutA* was constructed and was

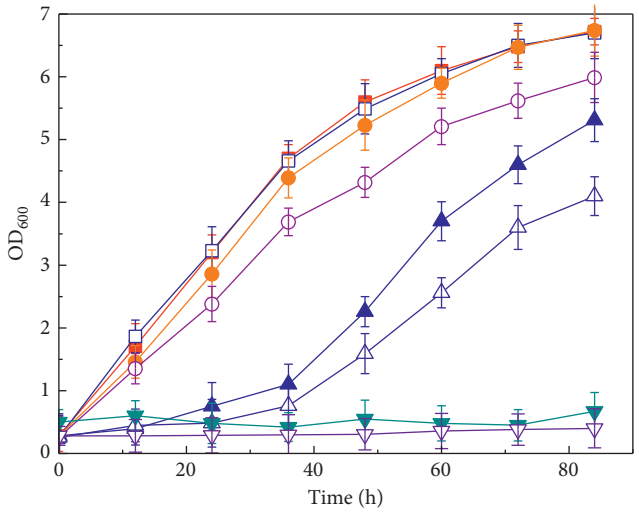

Figure 2: The effect on temperature tolerance of *G. oxydans* after the expression of CutA. The symbols ■, ●, ▲, and ▼ represent the growth curve of *G. oxydans*/pGUC-*tufB-cutA* at 30°C, 37°C, 42°C, and 45°C, respectively. The symbols □, ○, △, and ▽ represent the growth curve of *G. oxydans*/pGUC at 30°C, 37°C, 42°C, and 45°C, respectively. Error bars represent the standard deviation of three biological replicates.

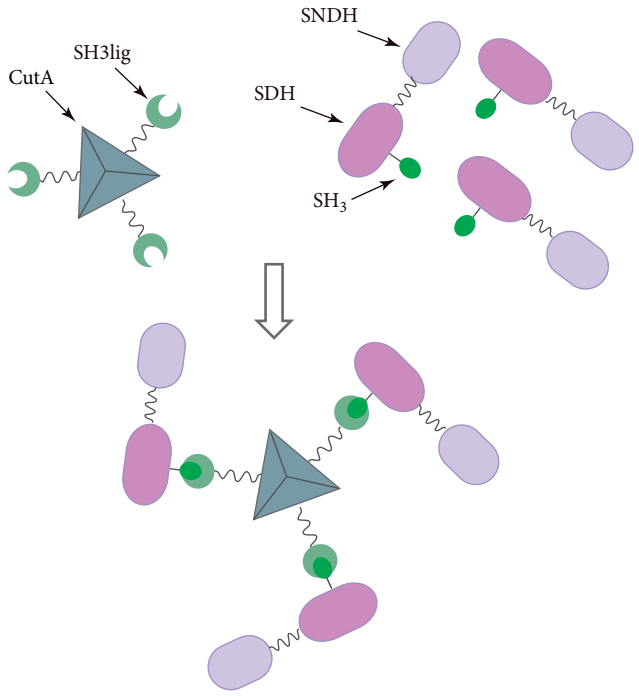

Figure 3: The scheme of protein expression based on the trimeric protein CutA. A longer protein chain with the trimeric CutA was generated under the use of the adaptor protein SH3 and its ligand $SH3_{lig}$ as docking protein and docking station peptide, respectively.

transferred into *G. oxydans* WSH-003. Comparison of optical densities at 600 nm (OD_{600}) and product formation in the recombinant strains is shown in Figure 4. *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA* grew basically consistent with *G. oxydans*/pGUC-*tufB-sdh*-GGGGS-*sndh*, and the 2-KLG production by the *G. oxydans* strain harboring pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA* was 40.3 g/L after 168 h of fermentation, which was 24.4% higher than that obtained by *G. oxydans*/pGUC-*tufB-sdh*-GGGGS-*sndh*, indicating that application of the cross-linker protein CutA efficiently increased the yield of 2-KLG.

3.4. Fermentation of Engineering G. oxydans at Different Temperatures. In the above study, it has been proved that the expression of CutA makes the strain more tolerable to temperature. Therefore, in order to investigate the fermentation performance at different temperatures, the recombinant strain *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA* was fermented at 30°C, 35°C, and 37°C, respectively. The recombinant strain *G. oxydans*/pGUC-*tufB-sdh*-GGGGS-*sndh* was used as control. The OD_{600} and the production of 2-KLG at different temperatures are summarized in Figure 5. The results showed that the growth of *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA* was nearly consistent with each other at these three temperatures, and it grew better at 35°C and 37°C than the control strain. The production of 2-KLG of *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA* was higher than the control at different temperatures. Furthermore, the specific activity of SDH and SNDH in the recombinant and the control strain at different temperatures is summarized in Figure 6. The results showed that the specific activity of SDH and SNDH was also higher than that of the control at different temperatures, which revealed that the stability and catalytic efficiency of the dehydrogenases may be improved after the expression of CutA. However, both strains of *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA* and the control produced less 2-KLG at 35°C and 37°C than at 30°C. This may be caused by that the enzyme activities were affected at higher temperatures.

3.5. Overexpression of pqqABCDE to Improve the 2-KLG Production. It has been confirmed that *G. oxydans* WSH-003 possesses a complete PQQ synthesis and regeneration system. However, the biosynthesis of 2-KLG is an oxidative process, and a large amount of the reduced cofactors, such as $PQQH_2$, should be rapidly regenerated for the following oxidation process. This means that the introduction of the extra PQQ-dependent dehydrogenases could lead to cofactor imbalances in metabolic pathways and significantly increase the burden for the global metabolic networks, thereby affecting 2-KLG production [33]. Cofactor engineering is often adopted to compensate the imbalance of cofactors to improve product synthesis [34]. In our previous study, SDH and SNDH from *K. vulgare* WSH-001 and SLDH from *G. oxydans* WSH-003 were all identified as PQQ-dependent dehydrogenases, and it was noted that increasing PQQ level could further improve the production of 2-KLG [9]. In this study, pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-$(GGGGS)_2$-*cutA-tufB-pqq*ABCDE was constructed. The production of 2-KLG by *G. oxydans* strain harboring

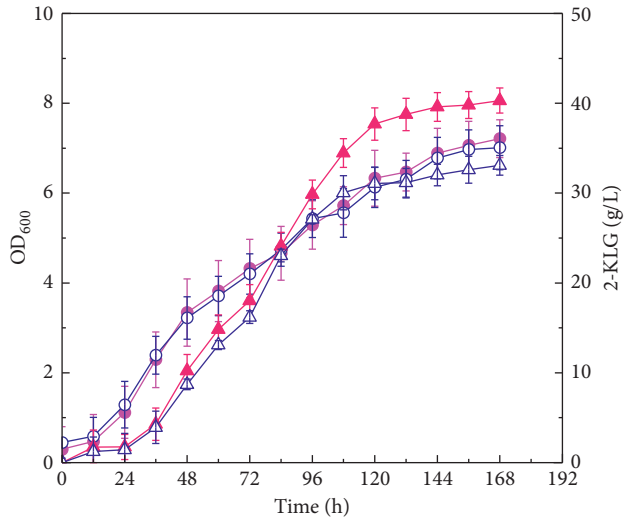

Figure 4: Time course of fermentation by CutA-based engineered strain. The symbols ▲ and △ represent the concentration of 2-KLG produced by *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA* and *G. oxydans*/pGUC-*tufB-sdh*-GGGGS-*sndh*, respectively. The symbols ● and ○ represent OD_{600} of *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA* and *G. oxydans*/pGUC-*tufB-sdh*-GGGGS-*sndh*, respectively. Error bars represent the standard deviation of three biological replicates.

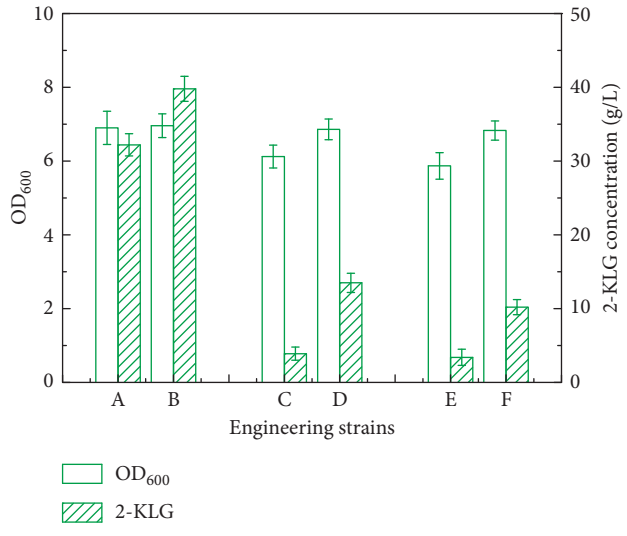

Figure 5: Production of 2-KLG by CutA-based engineered strain and the control strain at different temperatures. A, C, and E are OD_{600} and 2-KLG production of *G. oxydans*/pGUC-*tufB-sdh*-GGGGS-*sndh* at 30°C, 35°C, and 37°C, respectively. B, D, and F are OD_{600} and 2-KLG production of *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA* at 30°C, 35°C, and 37°C, respectively. Error bars represent the standard deviation of three biological replicates.

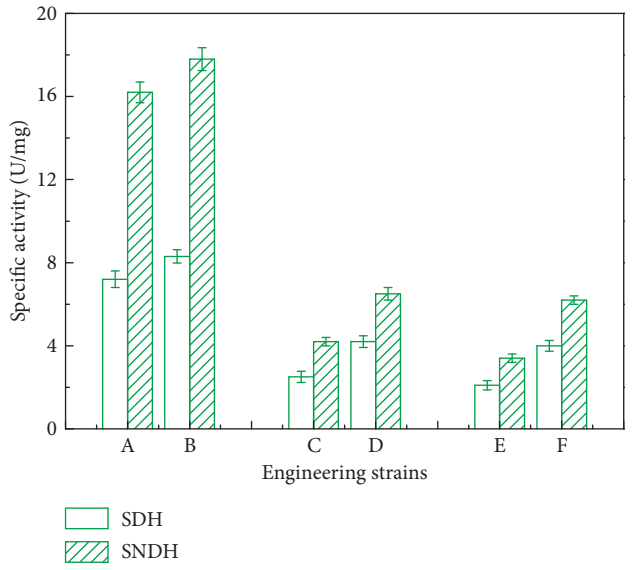

Figure 6: The specific activities of SDH and SNDH in the CutA-based engineered strain and the control strain at different temperatures. A, C, and E are SDH and SNDH specific activities of *G. oxydans*/pGUC-*tufB-sdh*-GGGGS-*sndh* at 30°C, 35°C, and 37°C, respectively. B, D, and F are SDH and SNDH specific activities of *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA* at 30°C, 35°C, and 37°C, respectively. Error bars represent the standard deviation of three biological replicates.

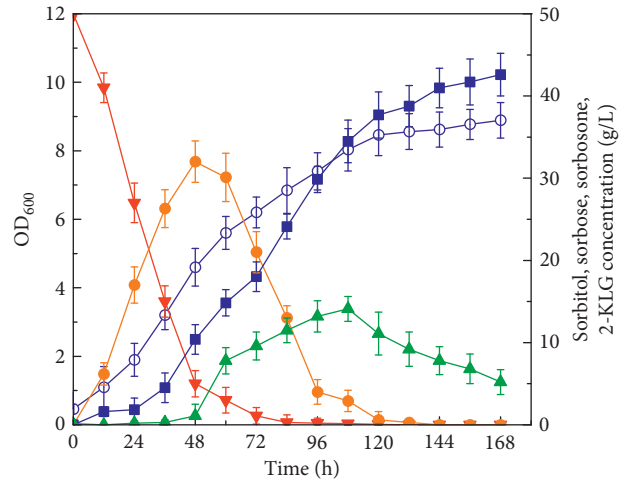

Figure 7: Time course of oxidative fermentation of D-sorbitol by the PQQ biosynthesis enhanced engineered strains. The symbols ▼, ●, ▲, and ■ represent the concentration of D-sorbitol, L-sorbose, L-sorbosone, and 2-KLG produced by *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA-tufB-pqq*ABCDE, respectively. The symbol ○ represents OD_{600} of *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA-tufB-pqq*ABCDE, respectively. Error bars represent the standard deviation of three biological replicates.

pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA-tufB-pqq*ABCDE after 168 h of fermentation is summarized in Figure 7. Overexpression of *pqq*ABCDE gene clusters enhanced PQQ production by 262.5% compared with the wild-type strain. Furthermore, overexpression of PQQ biosynthesis genes significantly enhanced cell growth, and the likely reason for this is that PQQ is also a signaling molecule in signal transduction pathways that affect bacterial growth by

neutralizing reactive species [35, 36]. The 2-KLG production by *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA-tufB-pqq*ABCDE reached 42.6 g/L, which was 5.7% higher than that by *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA*. Unfortunately, the overexpression of *pqq*ABCDE did not increase the yield significantly, but the fermentation cycle could be shortened.

In the past several decades, tremendous advances have been achieved in VC production by the classical two-step fermentation process. However, the further decreasing of VC price is significantly restricted because of many problems such as the two times of sterilization and mix-culture fermentation. Therefore, much more attention has been focused on the development of one-step fermentation route, but it is to be regretted that the progress is not ideal. In this study, the production of 2-KLG reached 42.6 g/L with the stepwise metabolic engineering of *G. oxydans*. Nowadays, with the development of biological technology, many new metabolic engineering methods were developed. The comprehensive optimization of metabolic engineering strategies and fermentation optimization should further facilitate the research process for VC production by one-step fermentation route.

4. Conclusions

In summary, SDH and SNDH were expressed in *G. oxydans* after the codon optimization. Furthermore, the trimeric protein CutA was used to improve the chemical structure stability of SDH and SNDH. The recombinant strain *G. oxydans*/pGUC-*tufB-SH3-sdh*-GGGGS-*sndh-tufB-SH3*$_{lig}$-(GGGGS)$_2$-*cutA* produced 40.3 g/L of 2-KLG after 168 h. In addition, the expression levels of the cofactor PQQ were enhanced to further improve 2-KLG production. With the stepwise metabolic engineering of *G. oxydans*, the final 2-KLG production was improved to 42.6 g/L. Efficient one-step production of 2-KLG was achieved.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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