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; 32.4g/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-SH3lig-(GGGGS)2-cutA* produced 40.3g/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-sorbose 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)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)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 JM110 (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% CaCO3) 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 KpnI/BamHI 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 SacI/KpnI site of pGUC-cutA, resulting in pGUC-tufB-cutA,
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 (SH3lig) were used as the docking protein and docking station peptide, respectively [22–24]. Both SH3 and SH3lig (GGGGS)2-cutA were optimized as stated above and were synthesized by Genewiz. The SH3 was fused with codon-optimized sdh-GGGGS-sndh, resulting in SH3-sdh-GGGGS-sndh. The SH3lig-(GGGGS)2-cutA was fused with the promoter tufB, resulting in tufB-SH3lig-(GGGGS)2-cutA. The obtained SH3-sdh-GGGGS-sndh and tufB-SH3lig-(GGGGS)2-cutA were further digested and inserted into the KpnI/BamHI and BamHI/Xbal site of pGUC-tufB, respectively, resulting in pGUC-tufB-SH3-sdh-GGGGS-sndh-tufB-SH3lig-(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. pqqABCDEFG 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 pqqABCDEFG, which was digested and inserted into the XbaI/PstI site of pGUC, resulting in pGUC-tufB-SH3-sdh-GGGGS-sndh-tufB-SH3lig-(GGGGS)2-cutA-pqqABCDEFG. 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 H2SO4 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

| 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-SH3lig-(GGGGS)2-cutA | pGUC containing tufB-SH3-sdh-GGGGS-sndh-tufB-SH3lig-(GGGGS)2-cutA | This study       |
| pGUC-tufB-SH3-sdh-GGGGS-sndh-tufB-SH3lig-(GGGGS)2-cutA-tufB-pqqABCDEFG | pGUC containing tufB-SH3-sdh-GGGGS-sndh-tufB-SH3lig-(GGGGS)2-cutA-tufB-pqqABCDEFG | This study       |

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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 OD600 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.

3.3.1. Construction of recombinant plasmids. 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 SH3lig 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 SH3lig-(GGGGS)2-cutA were optimized and synthesized, 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-SH3lig-(GGGGS)2-cutA was constructed and was

| Table 2: Primers used in this study. |
|--------------------------------------|
| Primer | Sequences of primers (5′-3′)* | Restriction enzyme |
| tufB-F1 | CGAGGTCGTACGATTGTAAGAAATCCACTG | SacI |
| tufB-R1 | CGGGGTACCGGTGCTTCTCCAAAAACCC | KpnI |
| SH3-F | CGGGATCCCTCTACGCGGAAATCCGC | — |
| SH3-R | GGGTCCGTGACCTGTACCTCTCCACGTTACGTC | — |
| sdh-GGGGS-sndh-F | GTACGTTGAGAAAGCTACGAGTGTAAGAAATCCACTG | — |
| sdh-GGGGS-sndh-R | CGGGATCCCTCTACGCGGAAATCCGC | BanHI |
| cutA-F2 | GCACGGTTGAGGACGAGCGCGCGTCTTTCTCAGAAACCCCGCT | — |
| cutA-R2 | GACGCGTTGAGGACGAGCGCGCGTCTTTCTCAGAAACCCCGCT | XbaI |
| cutA-F3 | CGGTGTATGTGCGCGCCCT | — |
| cutA-R3 | GGGTCGTGAGCTTCATGTACTTCTTCGTCGTTTCGCGGCGC | — |
| pqqABCDE-F | CGGGGTGGAGAAAGACGATGGCTTGGAACACGCCG | — |
| pqqABCDE-R | AAAACTCGAGTTACCTCTCCGTAAACAAAGT | PstI |

*Restriction sites used for cloning are in bold and are underlined.
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 PQ 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 SDLH 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-pqqABCDE was constructed. The production of 2-KLG by G. oxydans strain harboring
Overexpression of the pqqABCDE 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*<sub>lig</sub>-(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. Unfortunately, the overexpression of *pqqABC* 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*<sub>lig</sub>-(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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