Enhanced production of \( \text{l}-\text{sorbose} \) by systematic engineering of dehydrogenases in \( \text{Gluconobacter oxydans} \)

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**A B S T R A C T**

\( \text{l}-\text{sorbose} \) is an essential intermediate for the industrial production of vitamin C (\( \text{l}-\text{ascorbic acid} \)). However, the formation of fructose and some unknown by-products significantly reduces the conversion ratio of D-sorbitol to \( \text{l}-\text{sorbose} \). This study aimed to identify the key D-sorbitol dehydrogenases in \( \text{Gluconobacter oxydans} \) WSH-003 by gene knockout. Then, a total of 38 dehydrogenases were knocked out in \( \text{G. oxydans} \) WSH-003, and 23 dehydrogenase-deficient strains could increase \( \text{l}-\text{sorbose} \) production. \( \text{G. oxydans} \)-30, wherein a pyrroloquinoline quinone-dependent glucose dehydrogenase was deleted, showed a significant reduction of a by-product with the extension of fermentation time. In addition, the highest conversion ratio of 99.60% was achieved in \( \text{G. oxydans} \) MD-16, in which 16 different types of dehydrogenases were inactivated consecutively. Finally, the gene \( \text{vhb} \) encoding hemoglobin was introduced into the strain. The titer of \( \text{l}-\text{sorbose} \) was 298.61 g/L in a 5-L bioreactor. The results showed that the systematic engineering of dehydrogenase could significantly enhance the production of \( \text{l}-\text{sorbose} \).

1. Introduction

\( \text{l}-\text{sorbose} \) is an essential precursor for the industrial fermentation production of 2-keto-\( \text{l}\)-gulonic acid (2-KLG), which is the direct precursor of \( \text{l}\)-ascorbic acid (vitamin C) [1,2]. The most used industrial method for producing \( \text{l}-\text{sorbose} \) is the conversion of D-sorbitol by \( \text{Gluconobacter} \) or \( \text{Acetobacter} \) species [3,4] in which membrane-bound D-sorbitol dehydrogenases are responsible for this enzymatic conversion. The catalytic activity center of membrane-bound D-sorbitol dehydrogenases is commonly exposed to the periplasmic space; therefore, the \( \text{l}-\text{sorbose} \) is generally produced in the periplasm and subsequently secreted into the culture broth [5,6]. In the classical vitamin C production process, the \( \text{l}-\text{sorbose} \) produced by \( \text{Gluconobacter} \) spp. was further converted into 2-KLG via a mixed fermentation of \( \text{Ketogulonicigenium vulgare} \) and \( \text{Bacillus megaterium} \) [7], and the resulting product 2-KLG was chemically esterified for vitamin C production [1,8].

\( \text{Gluconobacter} \) belongs to the family of \( \text{Acetobacteraceae} \) and is unsurpassed by other organisms in the capacity of incompletely oxidizing a great variety of carbohydrates, alcohols, and related compounds [4,9]. It can oxidize many substrates regio-selectively, hence is widely used for the industrial production of \( \text{l}-\text{sorbose} \), 6-amino-\( \text{l}\)-sorbose, \( \text{d}\)-gluconic acid, and \( \text{keto-gluconic acids} \) [4,9]. \( \text{l}-\text{sorbose} \) is an essential substrate in vitamin C production, and mainly two types of membrane-bound D-sorbitol dehydrogenase are involved in the oxidation of D-sorbitol. One is pyrroloquinoline quinone (PQQ) dependent D-sorbitol dehydrogenase with two subunits (sldB and sldA), and the other one is FAD dependent D-sorbitol dehydrogenase with three subunits (sldS, sldL, and sldC) [10,11]. In addition, some cytoplasmic D-sorbitol dehydrogenases or xylitol dehydrogenases in \( \text{G. oxydans} \) mainly catalyze D-sorbitol to form \( \text{d-fructose} \) for cell growth [12,13]. Some special NAD\(^+\)/NAD\(^\text{−}\)-dependent D-sorbitol dehydrogenases from \( \text{G. oxydans} \) G624 could also catalyze D-sorbitol to form \( \text{l-sorbose} \) efficiently [14]. The membrane-bound D-sorbitol dehydrogenases are usually considered as the main enzymes responsible for catalyzing D-sorbitol into \( \text{l-sorbose} \).

Genome deletion is expected to obtain genome-simplified chassis cells with rich application potential. Some studies on the simplification of the genomes of model microorganisms have been reported, including \( \text{B. subtilis} \), \( \text{Escherichia coli} \) [15], \( \text{Corynebacterium glutamicum} \) [16], and...
Table 1

| Strains or plasmids | Characteristics | Sources |
|---------------------|-----------------|---------|
| G. oxydans Δapp    | upp             | This study |
| G. oxydans A       | upp and sldB1A  | This study |
| G. oxydans B       | upp and sldB1A  | This study |
| G. oxydans C       | upp and slDLSC  | This study |
| G. oxydans D       | sldB1A and slDLSC | This study |
| G. oxydans E       | upp, sldB1A and slDLSC | This study |
| G. oxydans F       | upp, sldB2A and slDLSC | This study |
| G. oxydans G       | upp, sldB1A, sldB2A and slDLSC | This study |
| G. oxydans E       | upp, sldB1A and slDLSC | This study |
| G. oxydans L       | alcohol DH 1    | This study |
| G. oxydans M       | D-arabitol DH   | This study |
| G. oxydans N       | glucose 5-DH    | This study |
| G. oxydans K       | lactate DH      | This study |
| G. oxydans P       | i-lactate 5-DH  | This study |
| G. oxydans Q       | NAD(P)H DH (quinone) | This study |
|                      | NAD-dependent xylitol DH 2 | This study |
| G. oxydans S       | 2-hydroxyacetic DH | This study |
| G. oxydans T       | alcohol DH 3    | This study |
| G. oxydans U       | alcohol DH 4    | This study |
| G. oxydans V       | alcohol DH      | This study |
| G. oxydans W       | sorbose DH      | This study |
| G. oxydans X       | 2-sorbitol DH    | This study |
| G. oxydans Y       | sorbose DH      | This study |
| G. oxydans Z       | NAD-dependent xylitol DH | This study |
| G. oxydans AA      | NADH DH (ubiquinone) | This study |
| G. oxydans AB      | PQ-containing DH 2 | Failed |
| G. oxydans AC      | short chain DH  | This study |
| G. oxydans AD      | short chain DH 3 | This study |
| G. oxydans AE      | zinc-dependent alcohol DH | This study |
| G. oxydans AF      | zinc-type alcohol DH | This study |
| G. oxydans AG      | glucose12-DH    | This study |
| G. oxydans AH      | glucose-DH      | This study |
| G. oxydans AI      | NADH DHypell2   | Failed |
| G. oxydans AJ      | NADH DH type II | Failed |
| G. oxydans AK      | Aldehyde DH-like protein | This study |
| G. oxydans AL      | Glucose DH2     | This study |
| G. oxydans AM      | Mannitol DH     | This study |
| G. oxydans AN      | glucose DH(PQQ) | This study |
| G. oxydans AO      | Hydroxyacetic DH | This study |
| G. oxydans AP      | short chain DH reductase 2 | This study |
| G. oxydans AQ      | short chain DH reductase | This study |
| G. oxydans AR      | aldehyde DH 2   | Failed |
| G. oxydans AS      | alcohol DH      | This study |
| G. oxydans AT      | aldehyde DH (NAD(+)3) | This study |
| G. oxydans AU      | aldehyde DH 3   | Failed |
| G. oxydans AV      | alpha-hydroxy-acid oxidizing enzyme | Failed |
| G. oxydans AW      | glucose DH      | This study |
| G. oxydans AZ      | NADH-dependent alcohol DH | This study |
| G. oxydans BA      | NADH DH (quinone) | This study |
| G. oxydans BB      | PQ-containing DH 3 | This study |
| G. oxydans BC      | MD 1-glucose DH2 | This study |
| G. oxydans BD      | MD 2-NAD-dependent xylitol DH | This study |
| G. oxydans BE      | MD 3-NAD-dependent xylitol DH 2 | This study |
| G. oxydans BF      | MD 4-glucuronate 5-DH | This study |
| G. oxydans BG      | MD 5 Δ slDLSC  | This study |
| G. oxydans BH      | MD 6 Δ PTS      | This study |
| G. oxydans BI      | MD 7-glucose DH | This study |
| G. oxydans BJ      | MD 8-Mannitol DH | This study |
| G. oxydans BK      | MD 9 Δ PQQ-dependent DH 3 | This study |
| G. oxydans BL      | MD 10 Δalcohol DH | This study |
| G. oxydans BM      | MD 11Δaldehyde DH 3 | This study |
| G. oxydans BN      | MD 12Δaldehyde DH (NAD(+)3) | This study |
| G. oxydans BO      | MD 13Δglucanate2-DH | This study |
| G. oxydans BP      | MD 14Δaldehyde DH | This study |
| G. oxydans BQ      | MD 15Δaldehyde DH | This study |
| G. oxydans BR      | MD 16ΔPQQ-dependent alcohol DH | This study |
| G. oxydans BS      | MD 17Δlactonate2-DH | This study |
| G. oxydans BT      | MD 18Δmembrane-bound glucose DH | This study |
| G. oxydans BU      | MD 19Δlactonate DH | This study |
| G. oxydans BV      | MD 20Δaldehyde dehydrogenase2 | This study |

Strains or plasmids continued

| Strains or plasmids | Characteristics | Sources |
|---------------------|-----------------|---------|
| G. oxydans MD 22   | MD 21 Δalcohol DH | This study |
| G. oxydans MD 23   | MD 22 Δalcohol DH | This study |
| G. oxydans MD 24   | MD 23 Δalcohol DH | This study |
| G. oxydans MD 25   | MD 24 Δalcohol DH | This study |
| G. oxydans MD 26   | MD 25 Δalcohol DH | This study |
| G. oxydans MD 27   | MD 26 Δalcohol DH | This study |

Plasmids

| Plasmids | Characteristics | Sources |
|----------|-----------------|---------|
| p13tet   | shuttle vector, Tet<sup>a</sup> [23] |
| p13-tet-P<sub>pHb</sub> | P<sub>Hb</sub> promoter with pHb, Tet<sup>a</sup> | This study |
| p13-tet-P<sub>pHb</sub> | P<sub>Hb</sub> promoter with pHb, Tet<sup>a</sup> | This study |
| p13-tet-P<sub>Tat</sub>-vHb | P<sub>Tat</sub> promoter with vHb, Tet<sup>a</sup> | This study |
| p13-tet-P<sub>pHb</sub> | P<sub>Hb</sub> promoter with tet-vHb, Tet<sup>a</sup> | This study |

Streptomyces avermitilis [17]. For G. oxydans, studies were mainly performed to identify the function of some genes by gene knockout, such as the gene cluster for the biosynthesis of PQQ [18] and the characterization of membrane-bound dehydrogenases from G. oxydans 621H [19]. Knockout of eight membrane-bound dehydrogenases in G. oxydans 621H could improve the titer and conversion rate of L-erythrose to 242 g/L and 99%, respectively, in a dissolved oxygen (DO)-controlled stirred-tank bioreactor [20]. Thus, deleting enzymes unrelated to the L-sorbose formation may enhance the L-sorbose production by avoiding unnecessary energy consumption and providing limited membrane space to the most needed membrane-bound D-sorbitol dehydrogenase [21].

In this study, the critical membrane-bound D-sorbitol dehydrogenases in G. oxydans WSH-003 were identified by gene knockout. The genes of sldB1A and slDLSC encode major membrane-bound D-sorbitol dehydrogenases that catalyze the conversion of D-sorbitol into L-sorbose. On the contrary, the gene of sldB2A showed no catalytic activity for D-sorbitol, although it had high homology with sldB1A. After identifying the key D-sorbitol dehydrogenases of G. oxydans WSH-003, 38 predicted dehydrogenases unrelated to the synthesis of L-sorbose were knocked out. The results showed that 23 dehydrogenase-deficient strains could increase L-sorbose production and G. oxydans 30, a membrane-bound glucose dehydrogenase (mGDH)-deficient strain of them could significantly decrease a by-product in the fermentation broth with the extension of fermentation time. At the same time, G. oxydans MD-16, in which 16 kinds of dehydrogenases were knocked out consecutively, had the highest titer of L-sorbose (149.46 g/L) with a conversion rate of 99.60% in shake flasks. Besides, the overexpression of the gene vhb encoding Vitreoscilla hemoglobin (VHB) in G. oxydans MD-16, scaling up in a 5-L fermenter, the titer of L-sorbose reached 298.61 g/L in 300 g/L D-sorbitol medium.

2. Materials and methods

2.1. Strains, plasmids, and culture conditions

G. oxydans WSH-003 was obtained from Jiangsu Jiangshan Pharmaceutical Co., Ltd., and was sequenced in a previous study (GenBank Accession No. AHK100000001.0) [22]. E. coli JM109 (E. coli JM109) was purchased from Novagen (Darmstadt, Germany) and used as a host for plasmid construction and amplification. The pMD19-T simple vector was purchased from Takara (Dalian, China). The E. coli JM109 strains were cultivated in Luria-Bertani (LB) medium at 37 °C and 220 rpm. When required, tetracycline was added to a final concentration of 20 μg/mL. The G. oxydans strains were cultivated in D-sorbitol medium (80 g/L D-sorbitol and 10 g/L yeast extract) at 30 °C and 220 rpm. When required, fluorouracil (FU), kanamycin, cefoxitin, and tetracycline were added to a final concentration of 300 μg/mL, 50 μg/mL, 50 μg/mL, and 20 μg/mL, respectively.
2.2. Gene deletions and construction of recombinant strains

For knocking out dehydrogenases, the upstream and downstream homologous arms of dehydrogenases were amplified by PCR from the *G. oxydans* WSH-003 genome. The kanamycin resistance gene (kana) or kana-upp fusion segments was obtained by PCR amplification, and it was fused with the upstream and downstream homologous arms of dehydrogenases with resistance gene. Fragments (DHS-kana-DHX and DHS-kana-upp-DHX) corresponding to related dehydrogenases was obtained. Strains in which dehydrogenases was knocked out were generated by integrating the obtained homologous recombinant fragments into the *G. oxydans* WSH-003 parental strain. Membrane-bound D-sorbitol dehydrogenase genes of *G. oxydans* WSH-003 were deleted consecutively using the counter-selection marker of *upp* gene. D-sorbitol dehydrogenase knockout strains are listed in Table 1. The primers used to construct the D-sorbitol dehydrogenase knockout strains are given in Supplementary Table (Table S1). Dehydrogenases of unknown function of *G. oxydans* WSH-003 were deleted using the *kana* gene. Dehydrogenase of unknown function knockout strains are listed in Table 1. The primers used to construct the dehydrogenase knockout strains are given in Supplementary Table (Table S1). Dehydrogenase genes of *G. oxydans* WSH-003 were deleted consecutively using the counter-selection marker of the *upp* gene. Dehydrogenase combination knockout strains are listed in Table 1. The primers used to construct the combination knockout

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**Fig. 1.** Identification of key D-sorbitol dehydrogenase in *G. oxydans*.

(A) Agarose gel electrophoresis validation of *G. oxydans* WSH-003 D-sorbitol dehydrogenase knockout strains. M1, DL5000 DNA marker; M2, DL5000 DNA marker; 1-g1: Validation of *sldBA1* in *G. oxydans-Δupp*, *G. oxydans-*A, *G. oxydans-*B, *G. oxydans-*C, *G. oxydans-D*, *G. oxydans-E*, *G. oxydans-F*, and *G. oxydans-G*, respectively; 2-g2: Validation of *sldBA2* in *G. oxydans-Δupp*, *G. oxydans-*A, *G. oxydans-*B, *G. oxydans-C*, *G. oxydans-D*, *G. oxydans-E*, *G. oxydans-F*, and *G. oxydans-G*, respectively; 3-g3: Validation of *sldSLC* in *G. oxydans-Δupp*, *G. oxydans-*A, *G. oxydans-*B, *G. oxydans-C*, *G. oxydans-D*, *G. oxydans-E*, *G. oxydans-F*, and *G. oxydans-G*, respectively. (B) Growth curves of different D-sorbitol dehydrogenase-deficient strains. Square, *G. oxydans-Δupp*; circle, *G. oxydans-*A; up-triangle, *G. oxydans-*B; down-triangle, *G. oxydans-*C; rhombus, *G. oxydans-*D; left triangle, *G. oxydans-*E; right triangle, *G. oxydans-*F; pentagon, *G. oxydans-G*. (C) HPLC results of one sorbitol dehydrogenase-deficient strains. 1, *G. oxydans-*B; 2, *G. oxydans-D*; 3, *G. oxydans-C*; 4, D-sorbitol; 5, L-sorbose. (D) HPLC results of two sorbitol dehydrogenase-deficient strains. 1, *G. oxydans-*D; 2, *G. oxydans-E*; 3, *G. oxydans-F*; 4, D-sorbitol; 5, L-sorbose. (E) HPLC results of three sorbitol dehydrogenase-deficient strains. 1, *G. oxydans-G*; 2, D-sorbitol; 3, L-sorbose. (G) Comparison of L-sorbose titer of different D-sorbitol dehydrogenase-deficient strains. CK: *G. oxydans-Δupp*, A: *G. oxydans-*A, B: *G. oxydans-*B, C: *G. oxydans-*C, D: *G. oxydans-D*, E: *G. oxydans-E*, F: *G. oxydans-F*, G: *G. oxydans-G*. 
A total of 44 dehydrogenases of *G. oxydans* WSH-003 were selected to be knocked out, and dehydrogenase single knockout strain was used form l-sorbose from D-sorbitol (Fig. 1D). There was not l-sorbose produced in the fermentation broth, and still 74.16 g/L D-sorbitol left in the fermentation broth (Fig. 1G). The fermentation results of *G. oxydans*-G were the same as that of *G. oxydans*-E (Fig. 1E), and there was still 74.19 g/L D-sorbitol left in the fermentation broth (Fig. 1G).

l-sorbose is an essential intermediate for manufacturing high value-added products, such as vitamin C [2] and t-agatose [27]. In the fermentation of l-sorbose, the highly active D-sorbitol dehydrogenase is essential. Although in vitro characterization of D-sorbitol dehydrogenase was conducted by exogenous expression of D-sorbitol dehydrogenase in *E. coli* [14] or direct isolation and identification of D-sorbitol dehydrogenase from *G. oxydans* [11], these in vitro results could not directly represent their *in vivo* production capacity in *G. oxydans* because membrane-bound dehydrogenases require the channeling of electrons into the respiratory chain [28]. Thus, in the present study, the critical D-sorbitol dehydrogenase of *G. oxydans* WSH-003 was verified by gene knockout, demonstrating that D-sorbitol dehydrogenases *sldBA1* and *sldSLC* were key enzymes for l-sorbose formation, and one of them was sufficient to catalyze D-sorbitol to l-sorbose, may be used as a platform strain applied in the screening of high-activity D-sorbitol dehydrogenase in future studies.

### 3.2. Effects of dehydrogenase gene knockout on l-sorbose production

A total of 44 dehydrogenases of *G. oxydans* WSH-003 were selected to be knocked out, and dehydrogenase single knockout strain was used for synthesis and systems biotechnology, 7 (2022) 730-737

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**Table 2**

| Table 2 | Production of l-sorbose by various dehydrogenase knockout strains. |
|---------|---------------------------------------------------------------|
| **Strains** | l-Sorbose (g/L) | OD_{600} | l-Sorbose titer per OD_{600} |
| WSH-003 | 136.95 ± 3.04 | 2.96 ± 0.12 | 46.23 |
| *G. oxydans*-1 | 137.43 ± 3.50 | 2.95 ± 0.01 | 46.61 |
| *G. oxydans*-2 | 134.53 ± 5.27 | 2.94 ± 0.09 | 45.70 |
| *G. oxydans*-3 | 136.85 ± 4.74 | 2.99 ± 0.04 | 45.76 |
| *G. oxydans*-4 | 136.66 ± 4.11 | 3.10 ± 0.02 | 44.04 |
| *G. oxydans*-5 | 141.74 ± 4.03 | 2.75 ± 0.06 | 51.59 |
| *G. oxydans*-6 | 136.27 ± 4.83 | 3.51 ± 0.05 | 38.82 |
| *G. oxydans*-7 | 139.13 ± 0.62 | 2.88 ± 0.03 | 48.30 |
| *G. oxydans*-8 | 140.04 ± 3.50 | 2.71 ± 0.04 | 61.69 |
| *G. oxydans*-9 | 136.79 ± 4.82 | 3.27 ± 0.09 | 48.82 |
| *G. oxydans*-10 | 135.09 ± 3.81 | 2.69 ± 0.07 | 60.31 |
| *G. oxydans*-11 | 135.93 ± 4.69 | 2.90 ± 0.02 | 46.82 |
| *G. oxydans*-12 | 144.89 ± 1.89 | 2.73 ± 0.10 | 63.75 |
| *G. oxydans*-13 | 139.69 ± 5.07 | 3.29 ± 0.02 | 42.50 |
| *G. oxydans*-14 | 139.74 ± 4.66 | 2.92 ± 0.03 | 47.87 |
| *G. oxydans*-15 | 135.90 ± 4.08 | 2.29 ± 0.04 | 61.73 |
| *G. oxydans*-16 | 138.91 ± 4.10 | 3.56 ± 0.04 | 38.99 |
| *G. oxydans*-17 | 140.76 ± 2.21 | 2.83 ± 0.03 | 49.74 |
| *G. oxydans*-18 | 144.14 ± 1.37 | 2.26 ± 0.07 | 65.52 |
| *G. oxydans*-19 | 141.86 ± 0.25 | 3.34 ± 0.03 | 43.23 |
| *G. oxydans*-20 | 139.97 ± 0.67 | 2.76 ± 0.05 | 52.92 |
| *G. oxydans*-21 | 143.89 ± 5.34 | 2.71 ± 0.01 | 53.08 |
| *G. oxydans*-22 | 139.55 ± 0.77 | 2.19 ± 0.03 | 65.24 |
| *G. oxydans*-23 | 140.85 ± 0.80 | 2.25 ± 0.02 | 62.73 |
| *G. oxydans*-24 | 139.72 ± 1.20 | 2.93 ± 0.01 | 47.69 |
| *G. oxydans*-25 | 141.94 ± 1.86 | 3.27 ± 0.06 | 43.47 |
| *G. oxydans*-26 | 138.67 ± 3.15 | 3.29 ± 0.16 | 42.17 |
| *G. oxydans*-27 | 144.77 ± 2.60 | 2.81 ± 0.08 | 51.54 |
| *G. oxydans*-28 | 138.75 ± 3.38 | 3.30 ± 0.02 | 42.06 |
| *G. oxydans*-29 | 137.03 ± 0.50 | 3.22 ± 0.03 | 41.90 |
| *G. oxydans*-30 | 136.44 ± 5.48 | 2.70 ± 0.14 | 51.67 |
| *G. oxydans*-31 | 139.29 ± 0.53 | 3.21 ± 0.04 | 43.35 |
| *G. oxydans*-32 | 143.15 ± 0.49 | 3.30 ± 0.09 | 42.12 |
| *G. oxydans*-33 | 142.20 ± 1.80 | 3.08 ± 0.10 | 44.77 |
| *G. oxydans*-34 | 136.53 ± 0.23 | 2.73 ± 0.06 | 50.03 |
| *G. oxydans*-35 | 140.47 ± 0.75 | 3.00 ± 0.01 | 46.86 |
| *G. oxydans*-36 | 136.21 ± 0.31 | 3.10 ± 0.03 | 43.95 |
| *G. oxydans*-37 | 134.85 ± 2.59 | 2.82 ± 0.02 | 47.77 |
| *G. oxydans*-38 | 139.89 ± 0.31 | 2.64 ± 0.01 | 53.04 |
Fig. 2. Fermentation broth of different G. oxydans and their HPLC results. (A) Color of G. oxydans fermentation broth. a, G. oxydans WSH-003; b, G. oxydans-30. (B) HPLC results of G. oxydans WSH-003 and G. oxydans-30. A, G. oxydans-30; B, G. oxydans WSH-003; C, D-sorbitol; D, L-sorbose. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

to verify the effect of dehydrogenase knockout on the production of L-sorbose. Among the 44 dehydrogenases, 6 dehydrogenases (POQ-containing dehydrogenase 2, glucose dehydrogenase, NADH dehydrogenase type II, NADH dehydrogenase type II, glucose dehydrogenase, and aldehyde dehydrogenase 2) could not be successfully knocked out in G. oxydans WSH-003, indicating that they might be essential genes. Finally, 38 dehydrogenase genes were knocked out successfully. Among the 38 dehydrogenase-deficient strains, 23 dehydrogenase-deficient strains could increase L-sorbose production. Compared with G. oxydans WSH-003 (136.95 g/L), the L-sorbose production with G. oxydans-12, G. oxydans-18, and G. oxydans-31 increased obviously, reaching 144.89 g/L, 144.14 g/L, and 144.77 g/L, respectively (Table 2). Noticeably, the fermentation broth color of G. oxydans-30 was significantly different from that of G. oxydans WSH-003 with the extension of fermentation time (Fig. 2A). At the same time, an unknown by-product was obviously decreased in the culture broth of G. oxydans-30 using HPLC analysis (Fig. 2B). This by-product might be a reason why the strain could not reach a higher conversion rate.

The dehydrogenase promiscuity may allow for the use of cost-effective feedstock as substrates; however, these dehydrogenases may increase the formation of by-products and influence the yield and the downstream purification process. Therefore, reducing by-product formation through gene knockout is a valuable tool to demonstrate the function of a specific gene [29–31]. This study found that the number of by-products in the fermentation broth significantly reduced after membrane-bound glucose dehydrogenase was knocked out (Fig. 2). According to their protein sequences, these enzymes belonged to the quinoprotein GDH (EC 1.1.5.2) with PQQ as a cofactor [28]. The newly discovered membrane-bound glucose dehydrogenase with catalytic activity in L-sorbose conversion has expanded the substrate spectrum of the glucose dehydrogenase family. It can be used to produce other valuable products.

### 3.3. Effects of combinatorial knockout of dehydrogenase genes on L-sorbose production

The aforementioned results showed that some G. oxydans WSH-003 dehydrogenase-deficient strains increased the production of L-sorbose and reduced the formation of by-products. Therefore, a total of 27 genes of G. oxydans WSH-003 were knocked out by consecutively deleting one gene after the other using the upp gene as counter-selection markers [35]. Among the 27 G. oxydans WSH-003 dehydrogenase-deficient strains, the highest titer and yield of the strain G. oxydans MD-16 was 149.46 g/L and 99.60%, respectively, which increased by 6.54% compared with that of G. oxydans WSH-003 (93.06%) (Table 3). Regarding the growth of G. oxydans WSH-003 dehydrogenase-deficient strains, G. oxydans MD-1, G. oxydans MD-2, G. oxydans MD-3, G. oxydans MD-4, G. oxydans MD-5, and G. oxydans MD-15 grew better than G. oxydans Δupp. However, the final OD₆₀₀ of G. oxydans MD-26 and G. oxydans MD-27 were just 1.14 and 1.10, respectively, which was reduced by more than 50% and could not be further engineered for gene knockout (Table 3).

In some previous studies, L-sorbose production was enhanced by the overexpression of sldBA1 with a newly identified strong promoter [3, 36]. However, these methods did not increase the conversion rate of D-sorbitol to L-sorbose and reduce by-products in the fermentation.

### Table 3: Comparison of multi-deletion strains for L-sorbose production

| Strains          | L-Sorbose (g/L) | OD₆₀₀ | L-Sorbose titer per OD₆₀₀ |
|------------------|----------------|-------|--------------------------|
| G. oxydans Δupp  | 136.9 ± 0.38   | 2.84 ± 0.03 | 48.20                    |
| G. oxydans MD-1  | 144.43 ± 2.09  | 3.23 ± 0.01 | 44.72                    |
| G. oxydans MD-2  | 147.65 ± 4.14  | 3.29 ± 0.25 | 44.88                    |
| G. oxydans MD-3  | 144.04 ± 0.11  | 3.18 ± 0.06 | 45.30                    |
| G. oxydans MD-4  | 148.66 ± 0.76  | 3.29 ± 0.13 | 45.19                    |
| G. oxydans MD-5  | 144.47 ± 1.42  | 2.36 ± 0.49 | 43.00                    |
| G. oxydans MD-6  | 144.43 ± 0.79  | 2.67 ± 0.08 | 50.32                    |
| G. oxydans MD-7  | 145.89 ± 8.66  | 2.98 ± 0.07 | 48.96                    |
| G. oxydans MD-8  | 140.42 ± 0.87  | 2.94 ± 0.02 | 47.76                    |
| G. oxydans MD-9  | 148.55 ± 3.98  | 2.88 ± 0.19 | 51.58                    |
| G. oxydans MD-10 | 144.83 ± 0.74  | 2.85 ± 0.02 | 50.82                    |
| G. oxydans MD-11 | 147.61 ± 3.84  | 2.94 ± 0.01 | 50.21                    |
| G. oxydans MD-12 | 144.52 ± 0.07  | 2.96 ± 0.01 | 48.82                    |
| G. oxydans MD-13 | 144.25 ± 0.27  | 2.83 ± 0.11 | 50.97                    |
| G. oxydans MD-14 | 145.53 ± 2.53  | 2.46 ± 0.09 | 59.16                    |
| G. oxydans MD-15 | 141.11 ± 5.61  | 3.40 ± 0.11 | 41.50                    |
| G. oxydans MD-16 | 149.46 ± 1.53  | 2.80 ± 0.09 | 53.38                    |
| G. oxydans MD-17 | 144.22 ± 7.44  | 2.62 ± 0.03 | 55.05                    |
| G. oxydans MD-18 | 144.43 ± 0.60  | 2.66 ± 0.18 | 54.30                    |
| G. oxydans MD-19 | 133.12 ± 1.45  | 2.24 ± 0.10 | 59.43                    |
| G. oxydans MD-20 | 134.01 ± 0.27  | 2.10 ± 0.03 | 63.81                    |
| G. oxydans MD-21 | 137.33 ± 1.28  | 2.17 ± 0.06 | 63.29                    |
| G. oxydans MD-22 | 132.68 ± 0.15  | 1.67 ± 0.01 | 79.45                    |
| G. oxydans MD-23 | 133.86 ± 1.10  | 1.49 ± 0.10 | 89.84                    |
| G. oxydans MD-24 | 135.10 ± 0.38  | 1.12 ± 0.04 | 120.63                   |
| G. oxydans MD-25 | 135.43 ± 0.09  | 1.83 ± 0.16 | 74.01                    |
| G. oxydans MD-26 | 138.65 ± 4.14  | 1.14 ± 0.04 | 121.62                   |
| G. oxydans MD-27 | 135.04 ± 0.11  | 1.10 ± 0.05 | 122.76                   |
hydrogenases could indeed improve the conversion rate of D-sorbitol to l-sorbose and reduce the formation of by-products. The conversion rate of the strain L. oxydans MD-16 reached up to 99.60%, close to the theoretical conversion rate of D-sorbitol to l-sorbose.

3.4. Effects of vhb expression on l-sorbose production

During cell cultivation, the oxygen demand is extremely high because G. oxydans-mediated incomplete oxidation of D-sorbitol is closely coupled to the cellular respiration chain [38]. The intracellular and periplasmic vhb expression systems were constructed to improve the oxygen utilization efficiency. The results showed that both the final OD_{600} values of G. oxydans WSH-003 and G. oxydans MD-16 expressing vhb were about 3, and the expression of hemoglobin had no obvious effect on cell growth. Besides, the G. oxydans MD-16 with p13-tet-P_{2703}-Tat-vhb had the highest yield of l-sorbose (149.58 g/L), with the conversion ratio of 99.7% (Fig. 3A). The cell membrane imposed limits on the transfer of oxygen to intracellular VHB, and the twin-arginine translocase (Tat) pathway was to export active VHB into the periplasm [25]. The effect of VHB expression in the periplasm was further identified. The results showed that that overexpression of VHB in the periplasm space did not obviously affect the cell growth in shake flask fermentation and could slightly improve the titer of l-sorbose by G. oxydans WSH-003 with p13-tet. (B) Time course for the production of l-sorbose by G. oxydans MD-16 with p13-tet-P_{vhb}-Tat-vhb. Up-triangle, l-sorbose; down-triangle, D-sorbitol; square, OD_{600}.

Effective control of DO levels is essential to ensure that microbes maintain high cell densities during the fermentation process, which requires a constant increase in oxygen supply [39]. So far, various solutions have been investigated to increase the DO of the fermentation broth, including increasing agitation speed and oxygen partial pressure, using pure oxygen, or completely rebuilding fermentation vessels [40–42]. However, the aforementioned methods were usually expensive or led to cell damage. Alternatively, the overexpression of Vitreoscilla hemoglobin in microorganisms was used to improve cell growth and production [43–45]. In this study, the overexpression of hemoglobin VHB from Vitreoscilla increased oxygen availability and improved l-sorbose production without affecting cell growth (Fig. 3).

3.5. l-sorbose production in the 5-L bioreactor

Based on the effects of VHB expression in the shaking flask, the relatively high-production strain expressing VHB in the periplasm (G. oxydans MD-16 with p13-tet-P_{vhb}-Tat-vhb) was chosen to scale-up in a 5-L bioreactor. The G. oxydans strain harboring p13-tet was used as the control during the fermentation process (Fig. 4). The production of l-
Thus, the role of *G. oxydans* WSH-003 dehydrogenases in L-sorbosorb production was systematically investigated. The results proved that the engineering of dehydrogenases could further improve the titer and conversion rate of L-sorbosorb. By knocking out some dehydrogenase genes, the amounts of by-products of *G. oxydans* could be significantly reduced. Meanwhile, both the titer and the conversion rate of L-sorbosorb were improved. Additionally, by systematically knocking out dehydrogenases of *G. oxydans*, a high-performing strain achieved 149.4 g/L of L-sorbosorb titer and 99.60% theoretical yield in the flask. By over-expressing vbh in *G. oxydans* MD-16, the titer of L-sorbosorb reached 298.61 g/L in a 5-L bioreactor. The systematic characterization and engineering of dehydrogenases in *G. oxydans* could also provide references for more efficient biosynthesis of other compounds in this strain.

CRediT authorship contribution statement

Liu Li: Methodology, Investigation, Formal analysis, Writing – original draft. Yue Chen: Investigation, Writing – original draft, Validation. Shiqin Yu: Formal analysis, Writing – review & editing. Jian Chen: Funding acquisition. Jingwen Zhou: Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syntbio.2022.02.008.

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