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Enhancement of 5-keto-D-gluconate production by a recombinant *Gluconobacter oxydans* using a dissolved oxygen control strategy

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The rapid and incomplete oxidation of sugars, alcohols, and polyols by the gram-negative bacterium *Gluconobacter oxydans* facilitates a wide variety of biological applications. For the conversion of glucose to 5-keto-D-gluconate (5-KGA), a promising precursor of the industrial substance L-(+)-tartaric acid, *G. oxydans* DSM2343 was genetically engineered to strain ZJU2, in which the GOX1231 and GOX1081 genes were knocked out in a markerless fashion. Then, a secondary alcohol dehydrogenase (GCD) from *Xanthomonas campestris* DSM3586 was heterologously expressed in *G. oxydans* ZJU2. The 5-KGA production and cell yield were increased by 10% and 24.5%, respectively. The specific activity of GCD towards gluconate was 1.75 ± 0.02 U/mg protein, which was 7-fold higher than that of the *sld*AB in *G. oxydans*. Based on the analysis of kinetic parameters including specific cell growth rate (μ), specific glucose consumption rate (q_s) and specific 5-KGA production rate (q_p), a dissolved oxygen (DO) control strategy was proposed. Finally, batch fermentation was carried out in a 15-L bioreactor using an initial agitation speed of 600 rpm to obtain a high μ for cell growth. Subsequently, DO was continuously maintained above 20% to achieve a high q_p to ensure a high accumulation of 5-KGA. Under these conditions, the maximum concentration of 5-KGA reached 117.75 g/L with a productivity of 2.10 g/(L·h).

[**Key words:** 5-Keto-D-gluconate; *Gluconobacter oxydans*; Secondary alcohol dehydrogenase; Pyruvate decarboxylase; Dissolved oxygen control strategy]

Gluconobacter is an important genus of acetic acid bacteria. Rather than fully oxidizing a wide variety of substrates to CO_2, it incompletely oxidizes substrates using an apparently energetically wasteful process (1). Because of these features, *Gluconobacter* strains have been exploited industrially for decades (2) for the production of vitamin C (ascorbic acid), gluconic acid, dihydroxyacetone, and vinegar (3–6). More recently, new processes using *Gluconobacter* strains for the synthesis of compounds such as L-ribulose, D-tagatose, miglitol, and chiral aldehydes and acids have been developed (2). The majority of these oxidative reactions are catalyzed by membrane-bound dehydrogenases, whose reactive centers are oriented to the periplasmic space so that the metabolic materials are accumulated in the medium and product recovery is relatively easy (7,8).

5-Keto-D-gluconate (5-KGA) is an attractive compound because it can be converted to L-ascorbic acid (9), 4-hydroxy-5-methyl-2, 3-dihydrofuranone, a flavor compound (10), and xylaric acid (11). Furthermore, 5-KGA is also an important precursor of L-(+)-tartaric acid (12,13), which is used as an antioxidant in the food industry, as an acidic reducing agent in the textile industry and as a chiral reagent in organic synthesis (14). Currently, 5-KGA is primarily produced via incomplete oxidation of glucose by *Gluconobacter oxydans* in a fermentation process (8,15,16). This conversion of glucose to gluconic acid (GA) is catalyzed by the membrane-bound pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (mGDH, GOX0265) and then further to ketogluconates, i.e., 2-keto-D-gluconate (2-KGA) and 5-KGA by the membrane-bound FAD-dependent gluconate-2-dehydrogenase (GA2DH, GOX1230-1232) and PQQ-dependent sorbitol dehydrogenase (SLDH, GOX0855-0856), respectively (17). During such incomplete fermentation process, the co-production of 2-KGA is the main isomer against 5-KGA. To avoid possible side-effects, the GA2DH must directly be inactivated. In addition, the reaction by the membrane bound dehydrogenases, such as mGDH, SLDH, can transfer the electrons to the respiratory chain and generate the energy, while *heme* c or FAD serve as the prosthetic group (17,18). The other glucose oxidation system is located in the cytosol and consists of the soluble $NADP^+$-dependent glucose dehydrogenase (sGDH, GOX2015), and gluconate: NADP 5-oxidoreductase (GNO, GOX2187) (7,17), which occurs only via pentose phosphate pathway (PPP) and Entner-Doudoroff pathway (EDP).

In order to achieve high yield of 5-KGA, numerous attempts have been explored by the genetically engineering, for instance expressing GNO (19), knocking out GA2DH (14,20), optimizing the media conditions (16), and overexpressing SLDH and simultaneously inactivating GA2DH (8,15). To date, the highest yield achieved of 5-KGA was 240–295 mM (about 60 g/L) in a batch fermentation process over periods as long as 72 h with a productivity of 0.83 g/(L·h) (15).

A secondary alcohol dehydrogenase of *Xanthomonas campestris* DSM3586 was earlier presumed to encode a quinoprotein glucose dehydrogenase (GCD). In previous study (21), to identify enzymes

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that can efficiently oxidize GA to 5-KGA, a BLAST search of the GenBank database, using combined sequences of small and large subunits of SLDH, obtained a number of homologues, among which several glucose dehydrogenases, e.g., those from *Escherichia coli* and *Acinetobacter calcoaceticus* (both having about 38% identity). The closest (48% sequence identity) uncharacterized homologue was found in *X. campestris* DSM3586. However, it was found to have no glucose dehydrogenase activity but to be active on many different polyols and diols, aliphatic alcohols, certain aldonic acids and amino-sugars. Based on the study of the substrate specificity and other basic enzymatic properties of GCD, it was found that the product of GA oxidation was 5-KGA and the relative activity was 6–7 fold higher than SLDH in *G. oxydans*, which had the biological potential to be used for 5-KGA production.

Dissolved oxygen (DO) is one of most important factors in many fermentation processes not only in the case of *G. oxydans*, however, in its natural habitat *G. oxydans* is likely subject to low-oxygen stress conditions, due to the rapid oxygen consumption by its own metabolism. In the previous study, it was found that DO concentration was a critical factor for the high levels of production by *G. oxydans* (22). But higher DO concentration in the last stage of fermentation leaded to the high expenses. While the oxygen was limitation (0–15%), the oxidize reaction by the membrane bound dehydrogenases were inhibited, as well as the genes expression were changed, of those representing 20% of the chromosome (22). Hence, the oxygen dependency was key characteristics of *G. oxydans*. Taken together, this information demonstrates the positive or negative effect of oxygen supply on the 5-KGA production by the incomplete oxidation process of *G. oxydans*. Therefore, it was necessary to set up a proper oxygen supply strategy to ensure efficient 5-KGA production with high concentration, high yield and high productivity.

In the present study, the recombinant strain *G. oxydans* ZJU2 was constructed by knocking out GA2DH and pyruvate decarboxylase (PDC, GOX1081). Subsequently, the GCD from *X. campestris* DSM3586 was heterologous expressed in *G. oxydans* ZJU2. And a simple oxygen supply method based on agitation speed control was obtained with an aim to achieve high yield and productivity of 5-KGA according to the kinetic analysis of batch processes.

MATERIALS AND METHODS

Microorganisms, plasmids, media, and growth conditions The bacterial strains, plasmids and primers used in this study are listed in Table 1. *E. coli* strains were cultivated in Luria–Bertani (LB) medium or LB agar plates at 37°C. When required, kanamycin (50 μg/mL) or gentamicin (50 μg/mL) was added to the final concentration indicated. *G. oxydans* strains were routinely cultivated on mannitol medium (MP) containing 5 g/L yeast extract, 3 g/L peptone, and 25 g/L mannitol at 30°C. *G. oxydans* possesses natural resistance against cefoxitin, thus, as a precaution to prevent bacterial contamination, cefoxitin was added to the media at 50 μg/mL.

Vector construction Routine molecular biological techniques were performed according to standard procedures (23). Based on the sequence of *G. oxydans* 621H (DSM2343, accession number CP000009), oligonucleotide primers, 1231_HindIII_F /1231_Fus_R and 1231_Fus_F/1231_XbaI_R, were designed to amplify the upstream (1050 bp) and downstream (1041 bp) fragments of GOX1231. Then, the fragments were fused together via fusion PCR according to a modified long flanking homology PCR protocol (24), using primers 1231_HindIII_F and 1231_XbaI_R. Then, the fusion fragment and pK18mobsacB vector were both digested with *Hin*dIII and *Xba*I, purified and ligated together, and transformed into *E. coli* DH5α cells via chemical transformation. Vector pΔGOX1231 was verified by DNA sequence. Deletion vector pΔGOX1081 was also constructed in the same manner with the corresponding primers in Table 1.

The GCD gene was amplified with PrimerSTAR HS DNA polymerase (Takara, Dalian, China) from the genomic DNA of *X. campestris* DSM3586, using oligonucleotides primers gcd_XbaI_F/gcd_EcoRI_R. The resulting fragment and vector pBBR1MCS-5 were all digested with *Xba*I and *Eco*RI (MBI Fermentas). Subsequently, they were ligated together using T4 ligase (MBI Fermentas) to generate plasmid pBB5-*gcd*. To insert the GOX0169 promoter sequences (25), pBB5-*gcd* was digested with the restriction endonucleases *Xba*I and *Sac*I. The native GOX0169 promoter was amplified with PrimerSTAR HS DNA polymerase using the primers 0169_SacI_F/0169_XbaI_R. The amplificates were digested with the restriction endonucleases *Xba*I and *Sac*I, and the fragments were ligated into the prepared vector to construct

TABLE 1. Strains, plasmids and oligonucleotides used in this study.

Strain/plasmid/oligonucleotide	Properties	Source
Strain		
E. coli DH5α	F^-, *endA1*, *hsdR17* (rk-mk-),*supE*44, *thi*1, *recA*1, *gyrA*, (Nalr), *relA*1, D(*lacZYAargF*), U169, and F80*lacZ*DM15	Invitrogen
E. coli S17-1	*Thi*, *pro*, *hsdR*$^-$, *hsdM*$^+$, *recA*$^-$, 284 *recA*, chromosomally integrated RP4 derivative	Invitrogen
E. coli HB101	F^-, *hsdS*20 (r-B, m-B), *supE*44, *ara*-14, *galK*-2, *lacY*1, *proA*2, *rpsL*20, *xyl*-5, *mtl*-1, *recA*13, Kan^R, *oriColE*1, *RK2-Mob*$^+$, *RK2-Tra*$^+$, and *mH*-1 with plasmid pRK2013	Invitrogen
G. oxydans DSM2343	Wild type, Cef^R	DSMZ[a]
G. oxydans ZJU1	Gluconate 2-dehydrogenase deletion strain derived from *G. oxydans* DSM2343, Cef^R	This work
G. oxydans ZJU2	Pyruvate decarboxylase deletion strain derived from *G. oxydans* ZJU1, Cef^R	This work
Plasmid		
pK18mobsacB	Suicide vector, *sacB*, and Km^R	36
pΔGOX1231	Deletion vector based on pK18mobsacB for deletion of GOX1231, Km^R	This work
pΔGOX1081	Deletion vector based on pK18mobsacB for deletion of GOX1081, Km^R	This work
pBBR1MCS-5	Broad-host-range (bhr) vector, Gm^R	37
pBB5-P_{0169}-*gcd*	*gcd* gene expression vector derived from pBBR1MCS-5, inserted P_{0169} promoter, Gm^R	This work
Oligonucleotide		
1231_HindIII_F	ataAAGCTTagccaaaggcggaaagacggc	This work
1231_Fus_R	catttcaggggagaccgcttaaatgaagtggccgctggtcatc	This work
1231_Fus_F	gatgaccagcggccacttcatttaagcggtctcccctgaaatg	This work
1231_XbaI_R	ataTCTAGAcgccggcactttcttctacc	This work
1231_Seq_F	gtgccccccatggaaccgacactgaac	This work
1231_Seq_R	tcagttcagtgagaccgcatcatctgc	This work
1081_HindIII_F	cccAAGCTTctcgtctgggcgattcatg	This work
1081_Fus_R	cctgaggtactgaaatcatgacaaagcgtctgatccttcc	This work
1081_Fus_F	ggaaggatcagacgctttgtcatgatttcagtacctcagg	This work
1081_SalI_R	acgcGTCGACaggcatgagacctacctga	This work
1081_Seq_F	tctggatccggaacatcagg	This work
1081_Seq_R	tccggagacacgatgact	This work
gcd_XbaI_F	GCTCTAGAatgtcgacattgcttcgtcc	This work
gcd_EcoRI_R	TTAGAATTCttaccgctgcggcaacgcgt	This work
0169_SacI_F	ATAGAGCTCtgaaagcggctggcgcgt	This work
0169_XbaI_R	GCTCTAGAgcggaaggcgttataccctga	This work

[a] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.