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Screening for L-sorbose and L-sorbosone dehydrogenase producing microbes for 2-keto-L-gulonic acid production

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Acetic acid bacteria incompletely oxidize L-sorbose to 2-keto-L-gulonic acid (2KLG) by L-sorbose- and L-sorbosone dehydrogenases. In order to isolate novel microorganisms with these enzyme activities, a new screening method has been studied with a presumption that microorganisms reuse their metabolic products when principal carbon sources are exhausted. When various keto-aldonic acid-producing microorganisms were tested for the ability to grow in minimal media containing such products as 2,5-diketo-gluconic acid, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid or 2-keto-L-gulonic acid, they grew with these keto-aldonic acids as the sole carbon source. By enriching the isolates collected from screening samples for their growth in minimal medium containing 2KLG as the sole carbon source, as much as 50% of selected strains showed L-sorbose- and L-sorbosone dehydrogenase activities. In spite of the presence of these enzymes, no significant amount of 2KLG was detected in the culture broth, possibly due to 2KLG reductase activity, indicating that the direct screening for 2KLG producer microorganisms would be less successful. These results suggest that the screening strategy using 2KLG as a carbon source is a useful method for the selective screening of microorganisms with L-sorbose- and L-sorbosone dehydrogenases, and that a similar strategy may be applied to other cases.

Keywords: 2-keto-L-gulonate; L-sorbose dehydrogenase; L-sorbosone dehydrogenase; acetic acid bacteria; screening

Introduction

2-Keto-l-gulonic acid (2KLG) is readily converted to l-ascorbic acid by a one-step chemical procedure in the Reichstein process for l-ascorbic acid production [10]. As the microbial oxidation process of d-sorbitol to l-sorbose has been integrated in the current Reichstein process, further biological oxidation of l-sorbose to 2KLG provides an attractive option for the improvement of the process [9]. Although 2KLG may be produced from glucose via 2,5-diketo-d-gluconic acid (25DKG) either by a two-step fermentation [25] or by recombinant *Erwinia herbicola* [2], the bioconversion of l-sorbose to 2KLG shows a higher conversion yield [16,19,20] and appears to integrate more easily into current ascorbic acid production plants [10].

In 1961 Tengerdy reported the microbial process using UV-irradiated mutant strains of *Pseudomonas* for the oxidation of l-sorbose to 2KLG [28]. Oxidation of l-sorbose to 2KLG is widely distributed in several genera: *Acetobacter*, *Alcaligenes*, *Aerobacter*, *Azotobacter*, *Bacillus*, *Escherichia*, *Gluconobacter*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, *Serratia* and *Xanthomonas* [13,17]. In 1972, Tsukada and Perlman reported conditions for the bioconversion of l-sorbose to 2KLG by *G. melanogenus* IFO 3293 and proposed the direct conversion of l-sorbose to 2KLG [30]. A commercial-scale production of 2KLG has been established in China by using, for the first time, a mixed culture of *Gluconobacter* sp and *Bacillus* sp [14,16]. Ning *et al* obtained more than 5% 2KLG in a medium containing 7% l-sorbose [19]. Sugisawa *et al* used the *G. oxydans* U-13 strain to produce 2KLG with a 64% yield [26]. A novel strain, *Pseudogluconobacter saccharoketogenes*, which has the ability to produce 2KLG at a higher yield (76%), has been isolated from soil [20]. In 1990, Hoshino *et al* elucidated the metabolic pathway for 2KLG production in *G. suboxydans* IFO 3293, which is catalyzed by a membrane-bound l-sorbose dehydrogenase and a cytoplasmic l-sorbosone dehydrogenase [11,12]. The l-sorbosone dehydrogenase requires either NAD or NADP as a cofactor [11].

Recently, genes encoding l-sorbose and l-sorbosone dehydrogenase were cloned and separated from *G. oxydans*, which showed that both enzymes are monomeric [22,23]. Introduction of l-sorbose- and l-sorbosone dehydrogenase genes to l-sorbose-producing bacteria such as *G. oxydans* resulted in enhanced production of 2KLG [22]. Choi *et al* purified the membrane-bound sorbitol dehydrogenase from *G. suboxydans* [7], and cloning and sequencing of the corresponding gene has been recently completed (Choi, Korea Research Institute of Bioscience and Biotechnology, personal communication). We were interested in isolating a novel host for 2KLG production and analyzing the exact metabolic pathways for 2KLG overproduction. By finding the novel l-sorbose and l-sorbosone dehydrogenase genes and cloning them with the sorbitol dehydrogenase gene into an 'inert' host such as *E. coli*, it would be possible to exploit well-established fermentation process technologies in 2KLG production.

However, in contrast to an earlier report [13], strains producing a significant level of 2KLG appear to be distributed very rarely in nature [9]. Prior workers indicated that the

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overproduction of 2KLG could be achieved only with a mixed culture of a 2KLG-producing strain and other growth-stimulating strains such as *Bacillus megaterium* or other counterpart microorganisms [19,20]. In a pure culture of *Gluconobacter* sp, the yield of 2KLG was very low [16,19], indicating the complex physiology and metabolism involved in 2KLG overproduction. Moreover, most 2KLG-producing strains, *Gluconobacter* sp, require undefined growth factors [27] and grow very poorly (Yin, Shanghai Research Center of Biotechnology, personal communication). A 2KLG-producing strain may catabolize 2KLG without accumulating 2KLG in the culture medium, making the direct isolation of 2KLG-overproducing strains difficult.

We reasoned that direct screening for 2KLG-producing strains would not be easy and it would be necessary to develop a new method of enrichment for strains showing l-sorbose- and l-sorbosone dehydrogenase activities. Many microorganisms produce extracellular (by)products and reuse them when the principal carbon sources are exhausted. For example, *E. coli* produces acetate during growth and reuses it in the stationary phase [8]. It is also the case for 2-keto-d-gluconate (2KDG) in *Serratia* sp [5,6], 5-keto-d-fructose in *G. cerinus* [18] and 25DKG in *Erwinia* sp [29]. With the idea that the keto-aldonic acid-producing microorganisms may have a higher potential to grow on their products as the sole carbon source, we first checked the growth of various microorganisms producing keto-aldonic acids and showed that they were growing on their products. We report here that 50% of the strains growing on 2KLG showed l-sorbose- and l-sorbosone dehydrogenase activities, which are required for 2KLG production.

Materials and methods

Microorganisms for the test of ability to grow on keto-aldonate

Acetobacter sp ATCC 21409, *A. diazotrophicus* ATCC 49037, *Enterobacter aerogenes* ATCC 13048, *E. pyrinus* ATCC 49851, *Erwinia cypripedii* ATCC 29267, *E. herbicola* ATCC 21998, *E. rhapontici* ATCC 29283, *Gluconobacter cerinus* ATCC 12302, *G. oxydans* ATCC 23776, *G. oxydans* ATCC 621, *G. oxydans* ATCC 9324, *G. oxydans* ATCC 9937, *Klebsiella pneumoniae* KCTC 1560, *Pseudogluconobacter saccharoketogenes* IFO 14464, *Pantoea citrea* ATCC 31623, *Pseudomonas putida* ATCC 8209 and *P. putida* ATCC 21025 were obtained from the Korean Collection for Type Cultures, Taejon, Korea (KCTC). *Acinetobacter johnsonii* 11-2, *Bacillus cereus* 16-1, *B. cereus* 17-1, *B. cereus* 18-9, *B. laterosporus* 24-5, *B. megaterium* 10-2, *B. megaterium* 11-5, *B. megaterium* 15-2, *B. megaterium* 15-6, *B. thuringiensis* 17-4, *Curtobacterium flaccumfaciens* 16-2, *Cytophaga johnsonii* 18-2, *C. johnsonii* 18-4, *Serratia marcescens* SEM1 were isolated in our laboratory for the ability to produce keto-aldonates.

Media and culture conditions

Two media were designed for the selection of acid-producing bacteria and 2KLG-producing bacteria. The medium for the selection of acid-producing bacteria (ethanol selection medium), contained 0.5% yeast extract, 2.0% ethanol, 0.5% of $CaCO_3$ and 2.0% agar. In the medium designed to select bacteria showing l-sorbose and l-sorbosone dehydrogenases, 0.5% l-sorbose or sodium 2KLG was included in a mineral salts (MS) medium containing: 0.1% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.3% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 1.0% sodiumethanesulfonic acid, 0.01% yeast extract, 0.004% nicotinic acid, 0.005% calcium pantothenate, 2.0% of agar and 1 ml of trace mineral solution per liter. The trace mineral solution contained: 1.32% $CaCl_2 \cdot 2H_2O$, 0.84% $FeSO_4 \cdot 7H_2O$, 0.24% $MnSO_4 \cdot 4H_2O$, 0.24% $ZnSO_4 \cdot 7H_2O$, 0.048% $CuSO_4 \cdot 5H_2O$, 0.048% $CoCl_2 \cdot 6H_2O$, 0.024% $Na_2MoO_4 \cdot 2H_2O$ and 0.006% $K_2B_4O_7 \cdot H_2O$ in 1 N HCl. The medium for evaluating enzyme activities contained 0.3% yeast extract, 0.5% peptone, 0.5% glycerol, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% l-sorbose and 0.1% d-glucose. To test for 2KLG production, isolates were cultivated at 30°C for 72 h in a medium containing 0.5% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 1.0% $CaCO_3$, 0.5% glycerol and 7.0% l-sorbose. Cell growth was followed by measuring the optical density at 600 nm.

Growth on keto-aldonate

To test for growth on keto-aldonate as sole carbon source (Table 1), the microorganisms pre-grown on complex medium containing 0.3% yeast extract, 0.5% peptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% d-glucose and 2.0% agar were transferred to MS agar plates containing 2,5-diketo-gluconic acid, 2-keto-d-gluconic acid, 5-keto-d-gluconic acid or 2-keto-l-gulonic acid. Growth was checked after 72 h at 30°C. The production of keto-aldonate was assayed by HPLC after cultivation at 30°C for 24 h in a complex medium containing 0.5% peptone, 0.3% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 5.0% glucose or l-sorbose.

Screening samples

Samples of fruits (strawberries, kiwis, grapes etc), soil, flowers and weeds were taken for screening the strains growing on 2KLG. The samples were sliced into small particles and suspended in 0.95% NaCl solution. After centrifugation at $4500 \times g$, the supernatant was discarded and the remaining pellet was resuspended in 0.95% NaCl solution and spread on 2.0% ethanol selection medium. After 24–48 h incubation at 30°C, colonies were picked and the selected isolates were tested for their growth in a minimal salts medium containing l-sorbose or 2KLG as the sole carbon source.

Enzyme assays

Strains which grew on l-sorbose or 2KLG were tested for the presence of l-sorbose- and l-sorbosone dehydrogenases. Cell pellets were separated from 10 ml cultures by centrifugation at $4500 \times g$ for 15 min and washed twice with 0.02 M phosphate buffer (pH 6.4). The washed pellet was suspended in 10 ml of 0.02 M phosphate buffer (pH 6.4) and sonicated for 3 min with 20-s intervals. The sonicated cell suspensions were centrifuged at $4500 \times g$ for 15 min to remove cell debris. The resulting supernatant was used for the assay of l-sorbosone dehydrogenase and cytoplasmic reductase activities.

For l-sorbose dehydrogenase, whole cells were used. The activity of dehydrogenase was tested by following the dichlorophenol-indophenol (DCIP) extinction at 520 nm in a 1-ml assay mixture containing 50 mM of sodium acetate buffer (pH 5.0), 10 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM phenazine-methosulfate, 0.15 mM DCIP, 50 μl 10% Triton-X100, 100 mM substrate and 20 μl enzyme solution. Glyoxal was used instead of l-sorbosone for the measurement of l-sorbosone dehydrogenase as reported by Hoshino *et al* [11]. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the reduction of 1 μmol of DCIP per minute. The activities of l-sorbose-, l-sorbosone- and 2KLG reductase were tested spectrophotometrically by following the oxidation of NADPH at 340 nm in 1 ml standard reaction mixture containing 280 mM Tris-HCl buffer (pH 7.0), 0.3 mM NADPH, 20 μl enzyme and 10 mM substrate: l-sorbose, glyoxal or 2KLG. One unit of enzyme activity is the amount of enzyme which catalyzed the oxidation of 1 μmol of NADPH per minute. Protein concentrations were determined by the Bradford method using a protein assay kit (Bio-Rad Lab, Hercules, CA, USA), using bovine serum albumin as the standard.

Aldonic and keto-aldonic acids

The presence of aldonic and keto-aldonic acids in the culture broth were tested by HPLC [15]. A Bio-Rad Aminex HPX-87H column was used with a refractive index detector (Gilson, Villiers Le Bel, France). 5KDG and 2KDG were purchased from Sigma and 2KLG was kindly supplied by G Yin (Shanghai Research Center of Biotechnology, China). 25DKG was produced by *E. herbicola* ATCC 21998 in a medium containing 0.3% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 3.0% $CaCO_3$, 0.5% glycerol, 5.0% glucose and 1 ml of trace mineral solution per liter. After cultivation, 25DKG powder was obtained by methanol precipitation.

Results and discussion

Most keto-aldonic acid producing microorganisms grow on their products as the sole carbon source

We first checked the growth of various microorganisms on their products, eg such keto-aldonic acids as 25DKG, 2KDG, 5KDG, or 2KLG. Most keto-aldonic acid-producing microorganisms grew on their products such as 25DKG,

Table 1 Growth of keto-aldonate producing microorganisms on mineral salts media containing glucose, 25DKG, 2KDG, 5KDG or 2KLG as sole carbon source

Strains	Carbon source					Products	Reference
	Glu	25DKG	2KDG	5KDG	2KLG		
Acetobacter sp ATCC 21409	++	++	+	–	–	25DKG	a,b
A. diazotrophicus ATCC 49037	++	++	+++	++	+	2KDG, 25DKG	a, [1,3]
Acinetobacter johnsonii 11-2	+++	–	+++	–	–	2KDG	a
Bacillus cereus 16-1	+++	+++	+++	–	+++	2KDG, 2KLG	a
B. cereus 17-1	+++	++	++	+	–	2KDG	a
B. cereus 18-9	+++	+++	+++	+	+++	5KDG	a
B. laterosporus 24-5	+++	+++	+++	+	+++	5KDG	a
B. megaterium 10-2	+++	+++	+++	+	+++	–	a
B. megaterium 11-5	+++	++	+++	+	–	–	a
B. megaterium 15-2	+++	+++	+++	+	+++	2KLG	a
B. megateriuim 15-6	+++	++	++	+	–	–	a
B. thuringiensis 17-4	+++	+++	+++	+	+++	2KDG, 5KDG	a
Curtobacterium flaccumfaciens 16-2	+++	+	+	–	++	2KLG, 2KDG	a
Cytophaga johnsonii 18-2	+++	+++	+++	+	+++	–	a
C. johnsonii 18-4	+++	+++	+++	+	+++	2KDG, 5KDG	a
Enterobacter aerogenes ATCC 13048	++	++	++	±	±	2KDG, 5KDG	a
E. pyrinus ATCC 49851	+++	++	++	–	±		
Erwinia cypripedii ATCC 29267	+++	+++	+++	+++	++	2KDG, 25DKG	a
E. herbicola ATCC 21998	++	±	+	++	++	5KDG, 25DKG	a
E. rhapontici ATCC 29283	+++	++	++	++	++		
Gluconobacter cerinus ATCC 12302	+++	+	+++	–	–		
G. oxydans ATCC 23776	++	++	++	+	–	25DKG	a
G. oxydans ATCC621	+++	+++	+	+	–	2KDG, 25DKG	a
G. oxydans ATCC 9324	++	–	–	–	–	2KLG	
G. oxydans ATCC 9937	+	–	–	–	–	5KDG, 25DKG	a, [31]
Klebsiella pneumoniae KCTC 1560	++	++	+	–	±	25DKG	a
Pseudogluconobacter saccharoketogenes IFO 14464	+	–	+	–	–	2KLG	a, [20]
Pantoea citrea ATCC 31623	+++	++	++	++	++	2KDG, 25DKG	[24]
Pseudomonas putida ATCC 8209	+++	++	+++	+	+++	2KDG, 25DKG	a,b
P. putida ATCC 21025	+++	–	+++	±	–	2KDG, 25DKG	a,b
Serratia marcescens SEM1	+++	+++	+++	+++	+	2KDG	a

+++ Excellent growth; ++ good growth; + growth; ± merely growth; – no growth. Growth was defined as colony size on an MS agar plate containing 0.5% glucose, 25DKG, 2KDG, 5KDG or 2KLG as sole carbon source after cultivation at 30°C for 72 h. The test for keto-aldonate was carried out by HPLC after cultivation at 30°C for 24 h in a complex medium containing 0.5% peptone, 0.3% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 5.0% glucose or l-sorbose. Letter 'a' in the reference column indicates the strains for which the production test was carried out in this work and 'b' indicates strains cited as the keto-aldonate producers in ATCC catalog.

2KDG, 5KDG, or 2KLG as the sole carbon source (Table 1). Thus, microorganisms growing on 2KLG should have enzymes required for 2KLG production, indicating that a screening method based on growth on 2KLG as the sole carbon source may be applied to screen for novel 2KLG-producing strains. *G. oxydans* IFO 3293 and *Pseudogluconobacter saccharoketogenes* IFO14464, which produce 2KLG, were not able to grow in the selective media because in order to be a 2KLG accumulating strain, catabolic pathways for 2KLG and related keto-aldonic acids have to be minimized (Figure 1). In addition, *Gluconobacter* sp requires an unknown growth factor [27] and 2KLG producers grow very poorly in a general ethanol isolation medium (Yin, personal communication). Thus, it is predicted that traditional screening methods for direct isolation of 2KLG overproducers from natural samples would not be easy.

Microorganisms growing on 2KLG show L-sorbose- and L-sorbosone dehydrogenase activities

In order to select strains with 2KLG production pathways, samples of fruits, soils, flowers, weeds and grapes were prepared and spread on ethanol selection medium containing calcium carbonate as an indicator for acid production. Colonies with halos were selected for their ability to produce acid(s) before being plated on agar plates containing l-sorbose, 2KLG or l-sorbose plus 2KLG. In the ethanol selection medium, more than 800 colonies were isolated which were checked for abilities to grow on plates containing 2KLG and/or l-sorbose as a sole carbon source. The number of microorganisms which grew was higher on plates containing l-sorbose plus 2KLG than in l-sorbose or 2KLG alone (Table 2). l-sorbose dehydrogenase (SDH) and l-sorbosone dehydrogenase (SNDH) were assayed in strains selected from l-sorbose, 2KLG or l-sorbose plus 2KLG. Ten out of 45 strains selected from l-sorbose media showed both SDH and SNDH activities while along the 16 strains selected from 2KLG media, eight strains showed both SDH and SNDH activities ranging from 0.03 to 0.15 unit/OD_{cell} and 0.05–0.1 unit/OD_{cell} respectively. Only three of 212 strains selected from l-sorbose plus 2KLG media had both SDH and SNDH activities. The results indicate that while a larger number of isolates growing on 2KLG plus l-sorbose yielded low numbers of positive strains (1.5%), a relatively small number of isolates growing on 2KLG yielded a higher number of positive strains (50%). For all three selective media, the distributions of SNDH were more widely distributed than SDH. It is also notable that a higher distribution of SDH and SNDH activities has been detected in isolates taken from flowers and weeds.

Microorganisms with L-sorbose- and L-sorbosone dehydrogenase activities did not accumulate 2KLG

Among strains selected for the presence of both SDH and SNDH, 10 strains (five each from isolates selected from l-sorbose and 2KLG) showing relatively high activities were tested for production of 2KLG from l-sorbose. None of the isolates produced detectable levels of 2KLG in culture broth when assayed by HPLC (Table 3). All strains showed 2KLG reductase activity, which converts 2KLG to l-idonic acid. The strains grew weakly on 5KDG, indicating that the metabolic activities related to catabolism of 2KLG are via l-idonic acid, before proceeding to 5KDG and then to d-gluconate (Figure 1).

Conclusions and further studies

Screening of potential 2KLG producers is facilitated by initial screening of the microorganisms growing on the target product, 2KLG, followed by determining whether the corresponding enzymes are present in the cells. This strategy is applicable to cases where overproducer microorganisms for the target products are found very rarely in nature either due to the inherently weak growth competition on the selective plates or due to the metabolic pathway structure that will not allow significant overproduction. Once microorganisms showing l-sorbose- and l-sorbosone dehydrogenase activities are selected, blocking of catabolic pathways for 2KLG consumption would quite easily generate the 2KLG overproducers. However, catabolic pathways

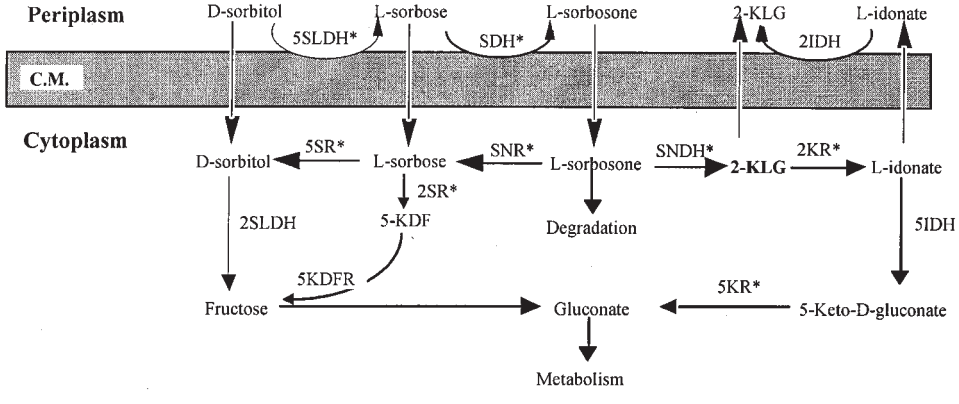

Figure 1 Proposed pathway for l-sorbose and 2-keto-l-gulonate metabolism in selected strains on mineral salts medium containing l-sorbose or 2KLG as carbon source. '*' indicates the metabolic pathways checked in this work. The degradation of l-sorbosone was cited in a paper by Shinjoh *et al* [23]. Abbreviations: 5SLDH, d-sorbitol 5-dehydrogenase [11,21–23]; SDH, l-sorbose dehydrogenase [11,22]; SNDH, l-sorbosone dehydrogenase [11,22,23]; 2IDH, l-idonate 2-dehydrogenase [11,21]; 5IDH, l-idonate 5-dehydrogenase [4,11,29]; 2SR, l-sorbose 2-reductase [21]; 5SR, l-sorbose 5-reductase [21,23]; SNR, l-sorbosone reductase [11,23]; 2KR, 2-keto-l-gulonate reductase [11]; 5KR, 5-keto-d-gluconate reductase [1,4,29]; 5KDFR, 5-keto-d-fructose reductase [18,21]; 2SLDH, sorbitol 2-dehydrogenase [21]; 2KLG 2-keto-l-gulonate; 5KDF, 5-keto-d-fructose.

Table 2 Distribution of sorbose dehydrogenase (SDH) and l-sorbosone dehydrogenase (SNDH) in strains from minimal salts (MS) medium containing l-sorbose, 2KLG or l-sorbose plus 2KLG as the carbon source

Samples	Carbon source											
	MS sorbose medium				MS 2KLG medium				MS 2KLG plus sorbose medium			
	Strains isolated[a]	SDH[b]	SNDH[b]	SDH & SNDH[b]	Strains isolated[a]	SDH[b]	SNDH[b]	SDH & SNDH[b]	Strains isolated[a]	SDH[b]	SNDH[b]	SDH & SNDH[b]
Fruits	0	0	0	0	0	0	0	0	2	0	0	0
Soil	0	0	0	0	0	0	0	0	3	0	0	0
Flowers	2	1	1	1	2	2	2	2	13	0	0	0
Weeds	22	8	8	8	6	4	4	4	167	5	0	0
Grapes	21	2	15	1	8	3	6	2	27	5	20	3
Total	45	11	24	10	16	9	12	8	212	10	20	3

[a]Number of isolates growing on MS medium containing l-sorbose, 2KLG or l-sorbose plus 2KLG as the carbon source.
[b]Number of isolates showing activities of SDH or SNDH.

Table 3 2KLG production and distribution of l-sorbose, l-sorbosone and 2KLG reductase, and growth on 5KDG in strains isolated from minimal salt medium containing l-sorbose (S) or 2KLG (L) as the carbon source (– not detected; + detected or growth)

Strain	l-sorbose reductase (U mg^{-1} protein)	l-sorbosone reductase (U mg^{-1} protein)	2KLG reductase (U mg^{-1} protein)	2KLG production	Growth on 5KDG
S4	–	0.525	0.117	–	+
S5	–	0.201	0.011	–	+
S7	–	0.167	0.015	–	+
S9	–	0.270	0.016	–	+
S31	–	0.146	0.100	–	+
L18	–	0.018	0.064	–	+
L19	–	0.025	0.107	–	+
L20	–	0.246	0.674	–	+
L23	–	0.756	0.560	–	+
L25	–	0.245	0.069	–	+

of their own (by)products appear to have a significant physiological role in producer microorganisms: a knock-out mutant of *Erwinia cypripedii* in gluconate dehydrogenase, which was recently cloned in our laboratory [32], was not viable and 2KLG-overproducing microorganisms grew very poorly (Pan *et al*, unpublished results; Yin, personal communication). Analysis of metabolic fluxes in *Acetobacter diazotrophicus*, an acetic acid bacterium producing 25DKG, indicated that these products are functioning possibly as a redox balance of the NADPH cofactor flux ([18]; Lee and Pan, in preparation). Obviously these microorganisms provide a unique source for the novel l-sorbose- and l-sorbosone dehydrogenase genes, which are required for reconstruction of the 2KLG overproduction pathway in an inert host such as *E. coli*. By using *E. coli* with *yiaE* mutation showing no 2KLG reductase [33], it would be possible to achieve a higher 2KLG conversion yield, which is a critical optimization point in using a *G. melanogenus* mutant [12].

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