Chapter 12
Metabolic Engineering of Microorganisms for Vitamin C Production

Jingwen Zhou, Guocheng Du, and Jian Chen

Abstract Vitamin C, an important organic acid, is widely used in the industries of pharmaceuticals, cosmetics, food, beverage and feed additives. Compared with the Reichstein method, biotechnological production of vitamin C is an attractive approach due to the low cost and high product quality. In this chapter, biosynthesis of vitamin C, including one-step fermentation processes and two-step fermentation processes are discussed and compared. Furthermore, the prospects of the biotechnological production of vitamin C are also presented.

Keywords Vitamin C • 2-keto-L-gulonic acid • Fermentative production • One-step fermentation

Abbreviations

AA-2G	2-O-alpha-D-glucopyranosyl-L-ascorbic acid
2,5-DKG	2,5-diketo-D-gluconate
2,5-DKGR	2,5-diketo-D-gluconic acid reductase
GUL oxidase	L-gulono-1,4-lactone oxidase
2-KLG	2-keto-L-gulonic acid
2-KLGR	2-keto-L-gulonic acid reductase
ORFs	open reading frames
PQQ	pyrro-quinoline quinone
SDH	L-sorbose dehydrogenase
SLDH	D-sorbitol dehydrogenase
SNDH	L-sorbosone dehydrogenase

J. Zhou.G. Du. J. Chen (✉)
School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, China
e-mail: jchen@jiangnan.edu.cn

X. Wang et al. (eds.), *Reprogramming Microbial Metabolic Pathways*, Subcellular Biochemistry 64, DOI 10.1007/978-94-007-5055-5_12,

Table 12.1 Application of vitamin C and its derivatives

Fields	Usages
Pharmaceutical industry	For treatment of encephalitis convulsions, idiopathic thrombocytopenic purpura, atherosclerosis, viral myocarditis, cancer, secondary erythroderma, nitrite poisoning, bronchialasthma and acute viral hepatitis;
Food industry	Inhibit oxidation and browing; change the flavor of food; food color protection; inhibit corrosion of the tank wall; inhibit lipid oxidation.
Beverage industry	Antiseptic preservation function as an additive;
Cosmetic industry	For treatment of chloasma; free radical scavenging, anti-aging;
Feed industry	Improve animal anti-stress ability, enhance immune function, accelerate bone growth, improve the rate of reproduction;
Biochemical reagent	Used for the determination of phosphorus.

12.1 Introduction

Vitamin C, also named as L-ascorbic acid, is a kind of essential vitamin and antioxidant for human beings and some primates (Zhang et al. 2011). Vitamin C is widely used in pharmaceutical, foods, beverages, cosmetics and feeds industries (Table 12.1). Along with the expansion of vitamin C in different field, the market is still growing. The world market for vitamin C and its direct derivatives (such as calcium salt, potassium salt, sodium salt, and glycosylation derivatives) is beyond 0.5 billion US dollars.

The earliest commercial vitamin C production process began from the Reichstein route (Table 12.2), which is developed by Reichstein in 1934, Germany. Reichstein developed a strategy for conversion of glucose to 2-keto-L-gulonic acid (2-KLG) with five steps of chemical reactions and one step of bio-reaction (Reichstein and Grussner 1934). The 2-KLG was further converted into vitamin C with esterification (Fig. 12.1). The process was industrialized and dominated the vitamin C market for over 60 years. However, the Reichstein process has many disadvantages, such as high-energy consuming, high-amount of organic solvent requirement and serious environment pollution. Therefore, many researchers find out several different processes to improve the performance of classical Reichstein process. One of the most successful routes is the "two-step" fermentation process, which was applied in industrial scale for more than 40 years. Along with the development of industrial biotechnology, researchers further developed several other processes to improve the economic and environmental aspects of the two-step fermentation process.

According to the production process, vitamin C production by biotechnology can now be divided into two-step fermentation and one-step fermentation. The two-step fermentation including tandem fermentation process that uses glucose as substrate and the fermentation process that uses D-sorbitol as substrate (Fig. 12.2). For the

Table 12.2 Manufacturing methods of vitamin C

Routes	Fermentative products	Substrates	Microbial/chemical methods	Characteristics			References
				Concentration (g/L)	Intensity (g/L/h)	Yield (wt%)	
Reichstein routes	2-KLG	D-Glucose	5 steps of chemical reactions, 1 step of bioreaction	—	—	50	Bremus et al. (2006)
Biotechnology methods	2-KLG	D-gluconic acid	*G. oxydans* ATCC 9937 and *Corynebacterium* sp. ATCC 31090	9.43	0.13	38	Ji and Gao (1998)
		D-Sorbitol	*G. melanogenus* Z84	60	0.42	60	Sugisawa et al. (1990)
			G. oxydans NB6939/pSDH-tufB1	88	1.22	88	Saito et al. (1997)
		L-Sorbose	*G. melanogenus* U13	60	0.42	60	Sugisawa et al. (1990)
			G. oxydans IGO112 and *B. megaterium* IBM302	75.8	1.58	94.8	Xu et al. (2004)
			G. oxydans SCB329 and *B. thuringiensis* SCB933	130.92	2.85	90	Yin et al. (1997)
		L-Sorbone	*G. oxydans* U13(p7A6 4)	32.7	0.2725	83.4	Shinjoh et al. (1995)
	Ca-KLG	D-Glucose	*Erwinia* sp. SHS 2629001 and *Corynebacterium* sp. SHS 752001	106.3	1.16	84.6	Sonoyama et al. (1982)
	Vitamin C	D-Glucose	*Xanthomonas campestris* 2286	20.4	0.408	5.1	Rao and Sureshkumar (2000)
		D-Sorbitol	*K. vulgare* DSM 4025^{TP}	0.09	0.0038	0.11	Sugisawa et al. (2005)
		L-Sorbose	*K. vulgare* DSM 4025^{TP}	0.908	0.045	1.14	Sugisawa et al. (2005)
		L-Sorbone	*K. vulgare* DSM 4025^{TP}	1.37	0.34	27.4	Sugisawa et al. (2005)
		D-Galacturonic acid	*Candida norvegensis*	1.3	0.027	8.7	Petrescu et al. (1992)
		L-galactose	*Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*	0.1	6.7×10^{-4}	40	Sauer et al. (2004)

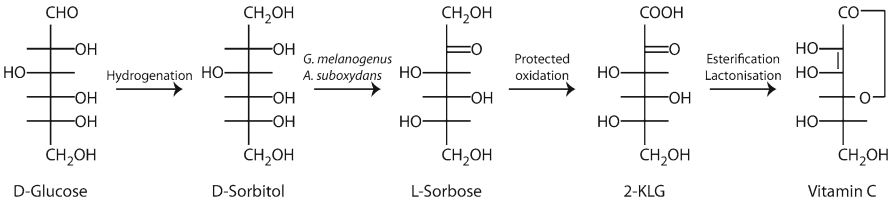

Fig. 12.1 Reichstein process for vitamin C production. The D-glucose was hydrogenated to form D-sorbitol. The D-sorbitol was converted into L-sorbose by acetic bacteria. The L-sorbose was further oxidized with protection to form 2-KLG. The 2-KLG was then esterified and lactonized to form vitamin C

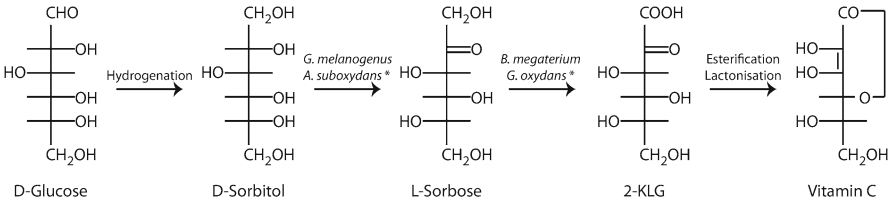

Fig. 12.2 Classical two-step fermentation process. The D-glucose was hydrogenated to form D-sorbitol. The D-sorbitol was converted into L-sorbose by acetic acid bacteria. The L-sorbose was further oxidized with a mixture culture system with *B. megaterium* and *K. vulgare* to form 2-KLG. The 2-KLG was then esterified and lactonized to form vitamin C. The only difference between the classical two-step process and the Reichstein process is the replacement of low efficient protective oxidation with a fermentation process. *The *G. oxydans* here was further identified to be *K. vulgare*

former, D-glucose is converted into 2,5-diketo-D-gluconate (2,5-DKG) by *Erwinia sp.*, which is subsequently converted into 2-KLG by *Corynebacterium*. The latter was studied the earliest and most intensively. In this process, L-sorbose is formed from D-sorbitol by *Gluconobacter oxydans*, and then by *Ketogulonicigenium vulgare* with *Bacillus megaterium* as the associated bacterium, L-sorbose is converted into 2-KLG (Zhang et al. 2011). *K. vulgare* is a 2-KLG producing bacterium, its growth is a lengthy, inefficient process with a low 2-KLG yield when cultured alone. *B. megaterium* is an associated bacterium which can promote *K. vulgare* growth and acid production but it does not produce 2-KLG (Liu et al. 2011a).

The optimization of the fermentation process has been continuing ever since, tremendous work has been done to improve the process stability and yield, such as the medium optimization, the relationship between two bacteria, the ecological regulation, etc. (Zhang et al. 2010). One-step fermentation is based on the bacteria of the two-step fermentation, it aims to achieve one-step fermentation from glucose or sorbitol to 2-KLG by using genetic engineering techniques to construct the genetically engineered bacteria.

12.2 Elucidation of the Mechanisms in Mutualism Between *Bacillus megaterium* and *Ketogulonicigenium vulgare*

12.2.1 Previous Research on the Classical Two-Step Fermentation Process

Because of co-culture of *K. vulgare* and *B. megaterium* during the classical two-step fermentation period of vitamin C, the optimal culture condition not only very suitable for growing condition of two bacteria but the excretion of acid by *K. vulgare* (Zhang et al. 2011). Zhou et al. discovered that carbon source, nitrogen source, dissolved oxygen, growth factors of seed culture medium and environmental factors etc., could regulate the growth and metabolism of strains effectively, and make the organisms grow under an optimal condition (Zhou et al. 2002). Therefore, there would be considerable impacts on the fermentation. The metabolism of mixed bacteria in vitamin C fermentation was more complex compared with that of a single strain.

The commonly used urea and corn steep liquor were nitrogen source in the industrial production of vitamin C by the classical two-step fermentation process. The metabolic properties were listed as follow: (1) There were two kinds of roles with urea addition, one was as a physiological alkaline substance to adjust pH, the other was providing the nitrogen sources for bacterial metabolism; (2) The protein content of the system was increasing with fermentation time; (3) The 17 kinds of amino acids in corn steep liquor were divided into three categories (Li et al. 1996). The large number of unclear natural components and the different batches of corn steep liquor can also cause the instability of 2-KLG production and fermentation cycle. The specific growth factors of cell growth and acid production were a prerequisite to control the stability of 2-KLG production.

Dissolved oxygen was also an important factor in the fermentation process of vitamin C. Giridhar and Srivastava (2000) found that in the batch and fed-batch fermentation processes of D-sorbitol to L-sorbose, adding 4% of the oxygen vector (N-hexadecane) could shorten the period for 2 and 5 h, and increase the production by 17 and 26%, respectively.

12.2.2 Elimination the Inhibition Effects of Substrate to Improve the Production and Yield of 2-KLG

The microbial transformation of D-sorbitol to L-sorbose is the most important industrial fermentation process of vitamin C in “Reichstein process” and two-step fermentation. Microorganisms are inhibited severely by high concentration of

D-sorbitol. The oxidation rate of D-sorbitol decrease drastically, which lead to the low concentration of L-sorbose in broth. Thereby, it will increase the downstream cost of the separation and purification. Control of initial concentration of sorbitol with 200 g/L, the production and yield of L-sorbose were 200 g/L and 14.2 g/L/h (Giridhar and Srivastava 2002). Besides this, with constant speed feeding of 600 g/L D-sorbitol, the concentration of L-sorbose was 1.6 times greater than batch fermentation; whereas 272.37 g/L L-sorbose achieved with linear feeding strategy (Giridhar and Srivastava 2001); exponential feeding of 700 g/L D-sorbitol after 10 h batch fermentation with 200 g/L D-sorbitol made 290 g/L L-sorbose (Srivastava and Lasrado 1998).

When L-sorbose was further converted to 2-KLG, huge amount of L-sorbose will make the bacteria metabolize abnormally, meanwhile the production and yield reduced. With the fermentation of 80 g/L sorbose for 35–40 h, the concentration of 2-KLG was 65–75 g/L. In the industrial process, constant feeding strategy was used widely. During the whole fermentation process, the curve of acids producing rate shaped a saddle while an ideal curve should be only a peak. Feeding sorbose according to the curve of fermentation rate would make the curve of acids producing fit the ideal model closer. Feeding L-sorbose at 10 and 20 h during fermentation, L-sorbose was 100 and 140 g/L, the concentration of 2-KLG reached 120–135 g/L, and the transformation efficiency was 90%.

12.2.3 Improvement of High Temperature Tolerance

Previous researches showed that *B. megaterium* could grow very well under 33 °C. However, *K. vulgare* could only grow under 30 °C. Therefore, the energy cost could be significantly improved by improving the higher temperature tolerance of *K. vulgare*. Because the mechanisms inside the tolerance of bacteria to high temperature are obscure, the rational metabolic engineering of *K. vulgare* to improve its tolerance to higher temperature is difficult. Current improvement of the high temperature tolerance of *K. vulgare* is mainly focused on the random mutagenesis (Yan et al. 2006) or *de novo* screening from nature (Moonmangmee et al. 2000). Yan et al. improved the performance of the mix-culture system consists of *K. vulgare* G0 and *B. megaterium* B0 under 33 °C by obtaining of mutant GI13 by ion implantation. The conversion ratio of the mutant GI13 at 33°C was improved to 94%. Besides, the L-sorbose dehydrogenase (SDH) activity of the mutant strain was further improved by 100% compared to the wild-type strain G0 (Yan et al. 2006).

G. oxydans, which is used for the conversion of the D-sorbitol to L-sorbose, could only grow under 32 °C (Yang and Lim 1997). Moonmangmee et al. (2000) screened a *G. oxydans* CHM54 from plants. The strain could grow under 37 °C well and has a conversion ratio of 80%.

12.2.4 Inhibition of the Degradation Pathway of 2-KLG

During the fermentation process of the mix-culture of *Pseudomonas putida* ATCC 21812 and *Gluconobacter melanogenus* IFO 3293 for the 2-KLG production, it was discovered that there was an enzyme, which needs NAD(P)H as cofactor, could further convert the 2-KLG to L-idonic acid. Furthermore, a similar enzyme was also found in *K. vulgare* WB0104 (Yang et al. 2006). Besides, the 2-ketoaldonate reductase A and B in *Erwinia herbicola* SCB125 could also degrade 2,5-DKG and 2-KLG. These downstream pathways could inhibit the further accumulation of 2-KLG. Therefore, knockout of these genes is essential for the further improvement of 2-KLG production. Chen et al. (2000) knocked out the *thrA* gene with a streptomycin resistance gene and successfully decreased the 2-KLG degradation.

12.2.5 Effects of the Relationship Between Two Bacteria in Mixed Culture on Vitamin C Production

The two-step fermentation process which uses D-sorbitol as the substrate is a classic example where a mixed-culture method produces a metabolic product. The second step fermentation is completed by mixed fermentation of 2-KLG-producing bacteria *K. vulgare* and associated bacteria *B. megaterium*. *K. vulgare* is difficult to culture alone and 2-KLG production capacity is very low simultaneously. *B. megaterium* does not produce 2-KLG, but it can promote the growth and acid production of *K. vulgare*. There are many kinds of associated bacteria, In addition to the commonly used *B. megaterium*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus thuringiensis*, and some yeast strains also possess the role.

It is a collaborative symbiotic relationship between acid producing bacteria and associated bacteria (Zhou et al. 2002). The enzyme activity that synthesizes of 2-KLG is directly proportional to the amount of *K. vulgare* cells and has nothing to do with the amount of *B. megaterium* cells. Further studies have shown that the 30–50 KDa (Feng et al. 2000) and >100 KDa (Lv et al. 2001) biological active substances of *B. megaterium* released in the metabolic process, participating in the growth and acid production process of *K. vulgare*. The extracellular active substances are proteins.

Three bacteria were involved in the classical two-step fermentation process, for example *G. oxydans*, *B. megaterium* and *K. vulgare*. The denomination of the bacteria for *G. oxydans* and *K. vuglare* is highly speculative because of the lack of efficient strain identification methods. Furthermore, there are also some updates on the two strains. In early times, *G. oxydans* were named as *G. melanogenus*, *Acetobacter suboxydans*, or *A. melanogenus*. Because most of these stains could not

be sufficiently distinguished for further confirmation, all these strains that could efficiently catalyze D-sorbitol to L-sorbose are now designated as *G. oxydans*. The species classified as *K. vulgare* were finally defined as *K. vulgare*. The bacterium was previously known as *G. oxydans* or some other *Acetobacter* sp. strains. However, the strain that could only grow well with *B. megaterium* or other *Bacillus* sp. is now referred to as *K. vulgare*.

In the classical two-step fermentation process, the D-sorbitol was synthesized by hydrogenation from D-glucose by chemical reaction. The D-sorbitol was then converted into L-sorbose by the sorbitol dehydrogenase (encoded by *sldh*) in *G. oxydans*. The culture broth containing L-sorbose was then transferred to another bioreactor, added with other raw materials and sterilized for the second time. Then the L-sorbose was converted in to L-sorbosone by the L-sorbose dehydrogenase (encoded by *sdh*) in *K. vulgare*. The L-sorbosone was further converted into 2-KLG by L-sorbone dehydrogenase (encoded by *sndh*) in *K. vulgare*.

The fantastic phenomenon in the classical two-step fermentation process is the second step, which consists of the *B. megaterium* and *K. vulgare*. The *K. vulgare* itself could not grow well after supplement of different kinds of substrates. The addition of *B. megaterium*, *B. thuringiensis* or *B. cereus* could significantly enhanced both the cell growth and 2-KLG production by the *K. vulgare*. There are many reports aiming to demonstrate the mutualism between the two bacteria. In the past few decades, much effort has been devoted to elucidating the detailed mechanism by which *B. megaterium* enhances *K. vulgare* growth and 2-KLG production (Bremus et al. 2006; Shinjoh et al. 1995; Sugisawa et al. 2005; Tsukada and Perlman 1972). Studies have showed that both internal and external metabolites from *B. megaterium*, through biochemical and molecular methods and certain proteins or amino acids, play a role in this process (Zhang et al. 2011; Zhao et al. 2008). Further research has shown that *B. megaterium* secretes two kinds of proteins of about 30–50 kDa and >100 kDa that improve *K. vulgare* growth and increase 2-KLG productivity (Lu et al. 2003; Xu et al. 2004; Zhao et al. 2008). However, researchers have not specifically determined which proteins play a role in promoting *K. vulgare* growth and 2-KLG production. Based on these results, Zhang et al. (2010) used lysozyme to damage the *B. megaterium* cell wall structure to release the intracellular components, as a consequence, the growth rate of *K. vulgare*, its sorbose consumption rate, and 2-KLG productivity increased 27.4, 37.1, and 28.2%, respectively. Because most of the companion strains for *K. vulgare* are *Bacillus* sp., it was proposed that the spore formation of these *Bacillus* strains should have some impact on the mutualism process. Zhu et al. (2012) first revealed that not only the spore formation, but also the spore stability, played key role in the mutualism process.

Based on the development of next-generation high-throughput sequencing technologies, all the genomes of three strains involved in the classical two-step fermentation process were obtained in last several years. One of the *B. megaterium* was sequenced. The 4.14-Mb genome of *B. megaterium* WSH-002 contains four replicons, a circular chromosome (4.04 Mb) encoding 5,186 predicted open reading frames (ORFs), and three circular plasmids, named pBME_100 (0.074 Mb),

pBME_200 (9,699 bp), and pBME_300 (7,006 bp), with mean GC contents of 39.1, 36, 32.2, and 33.2%, respectively. There are 5,482 protein-encoding genes, 99 tRNAs, and 10 rRNA operons. Among them, 2,460 functional descriptions, 1,327 gene abbreviations, and 856 EC numbers were assigned to the WSH-002 genome by function annotation. Furthermore, 782 genes were assigned according to the Kyoto Encyclopedia of Genes and Genomes metabolic pathways (Liu et al. 2011c).

There are two *K. vulgare* strains independently. According to phylogenetics, both *K. vulgare* Y25 and WSH-001 has the same ancestor. The two strains were used by the two main vitamin C manufactures in China, North China Pharmaceutical Group Corporation and Jiangsu Jiangshan Pharmaceutical Company, respectively. However, the multiple-mutagenesis with different methods may lead to some significant differences in these two strains. The genome of *K. vulgare* Y25 consists of a circular chromosome and two plasmids. The chromosome is composed of 2,776,084 bp, with a GC content of 61.72%. One plasmid contains 268,675 bp, with a GC content of 61.35%, and the other contains 243,645 bp, with a GC content of 62.63%. There are a total of 3,290 putative ORFs (2,807 [chromosome], 256 [pYP1], and 227 [pYP2]) using Glimmer, giving a coding intensity of 91.05% (Xiong et al. 2011). The complete genome sequence of *K. vulgare* WSH-001 is composed of a circular, 2,766,400-bp chromosome and two circular plasmids named pKVU_100 (267,986 bp) and pKVU_200 (242,715 bp) with mean GC contents of 61.69, 61.33, and 62.58%, respectively. There are 2,604 protein-encoding genes, three rRNA operons, and 51 tRNA-encoding genes in the chromosome and 246 and 215 protein-encoding genes in plasmids pKVU_100 and pKVU_200, respectively. 2497 functional descriptions, 1,279 gene abbreviations, and 820 EC numbers were assigned in the WSH-001 genome by function annotation. Among them, the genes for the 2-KLG synthesis pathway from L-sorbose were annotated; four genes encoding L-sorbose/L-sorbosone dehydrogenase, responsible for converting L-sorbose to L-sorbosone, are highly homologous to *ssdA1* (AB092515), *ssdA2* (AB092516), *ssdA3* (AB092517), and *ssdB* (AB092518) of *K. vulgare* strain DSM 4025. The *sndh* gene that is responsible for the conversion of L-sorbosone to 2-KLG was located in plasmid pKVU_200 (Liu et al. 2011b). It seems that the genome of *K. vulgare* (~2.7 M) is much smaller than other common bacteria. Function annotation indicated that the *K. vulgare* lacks of most of the genes or gene clusters for biosynthesis of many kinds of amino acids, nucleotides and cofactors.

Based on the release of genome sequences for *K. vulgare* and *B. megaterium*, and the integration of other -omics data, the understanding of the mechanisms in the mutualism were further illuminated (Liu et al. 2011b, c; Xiong et al. 2011). Based on the global pathway analysis, Zhang et al. (2011) reported most of the essential amino acids for the dependent growth of *K. vulgare* and developed a definite culture medium for both *K. vulgare* growth and 2-KLG production (Zhang et al. 2011). Furthermore, Liu et al. (2011a) reconstructed the biosynthesis pathways for amino acids in *K. vulgare* and found out many gaps in the amino acid metabolic pathways,

and consequently developed a novel strategy with gelatin supplement, which contains the several kinds of key amino acids that could not be *de novo* biosynthesized in *K. vulgare*, and significantly enhanced the *K. vulgare* growth and 2-KLG productivity.

Zhou et al. (2011) found that the microorganisms interact by exchanging a number of metabolites with time-of-flight mass spectrometry. Both intracellular metabolism and cell-cell communication via metabolic cooperation were essential in determining the population dynamics of the ecosystem. The contents of amino acids and other nutritional compounds in *K. vulgare* were rather lower in comparison to those in *B. megaterium*, but the levels of these compounds in the medium surrounding *K. vulgare* were fairly high, even higher than in fresh medium. Erythrose, erythritol, guanine, and inositol accumulated around *B. megaterium* were consumed by *K. vulgare* upon its migration. The oxidization products of *K. vulgare*, including 2-KLG, were sharply increased. Upon co-culturing of *B. megaterium* and *K. vulgare*, 2,6-dipicolinic acid (the biomarker of sporulation of *B. megaterium*), was remarkably increased compared with those in the monocultures. Therefore, the interactions between *B. megaterium* and *K. vulgare* were a synergistic combination of mutualism and antagonism.

12.3 Metabolic Engineering Based on the Two-Step Vitamin C Fermentation Process

The classical two-step fermentation process is the most successful route for vitamin C production for its high yield of 2-KLG on D-sorbitol. Though there are two fermentation process, the yield of L-sorbose on D-sorbitol and the yield of 2-KLG on L-sorbose could achieve to more than 99.5 and 97%, respectively. Few of the industrial process could achieve this level. Therefore, the metabolic engineering on the classical two-step fermentation process is always undergoing.

12.3.1 Interruption of the Downstream Metabolism Pathways

It has already been reported that the 2-KLG synthesized by *K. vulgare* could be further degraded into iduronic acid by 2-KLG reductase in the bacteria. Therefore, the earliest metabolic engineering for the classical two-step fermentation process is to knockout the gene that encodes the 2-KLG reductase. Saito et al. (1997) obtained a *K. vulgare* strain NB6939 (it was named as *G. oxydans* in the original literature) with interrupted L-iduronic acid by mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine, the 2-KLG concentration of the resultant strain was improved to 31 g/L, which was 200% of the wild-type strain.

12.3.2 Enhancement of the Cofactor Metabolism Pathway

Conversion of D-glucose or D-sorbitol to 2-KLG or vitamin C involves a number of dehydrogenases that need different kinds of cofactors. These dehydrogenase include D-sorbitol dehydrogenase (SLDH), L-sorbose dehydrogenase (SDH), L-sorbosone dehydrogenase (SNDH), 2,5-diketo-D-gluconic acid reductase (2,5-DKGR), 2-keto-L-gulonic acid reductase (2-KLGR). The SDH needs FAD or Pyrroloquinoline Quinone (PQQ) as cofactor in different strains. SNDH from *G. melanogenus* UV10 use NAD(P) as cofactor. 2,5-DKGR in *Corynebacterium* ATCC 31090 (Anderson et al. 1985) and 2-KLGR in *K. vulgare* (former *G. oxydans*) (Jiang et al. 1997) use NAD(P)H as the cofactor.

Therefore, rational regulation of the existence, concentration or preference of cofactors could improve 2-KLG production. Banta et al. (2002) showed that there were two kinds of 2,5-DKGR in *Corynebacterium* sp., i.e., 2,5-DKGR A and 2,5-DKGA B. The 2,5-DKGR A could use both NADH and NADPH as cofactor. However, the dissociation constant (K_d) of the enzyme with NADH is 260 times of that with NADPH as cofactor. Therefore, the 2,5-DKG A tend to use NADPH as cofactor. However, the NADH concentration is three times higher than the NADPH and has higher stability. Sanli et al. decreased the K_d of 2,5-DKGR A with NADH to 1/3 compared to the wild-type enzyme using double mutagenesis of F22Y and A272G (Sanli et al. 2001). The tetra-mutagenesis of the 2,5-DKGR A (F22Y/K232G/R238H/A272G) decreased the K_d to only 1/4 of the wild-type enzyme (Banta et al. 2002). This will significantly decreased the cost for 2-KLG production by 2,5-DKGR A in vitro.

Primary analysis showed that the *K. vulgare* could not synthesize folate, which referred to a series of folic acid derivatives that is distinguished by the state of oxidation, one-carbon substitution of the pteridine ring, and the number of glutamate residues (Sybesma et al. 2003). Folate is a kind of important cofactor, which donates one-carbon compounds in many reactions, and is crucial in the *de novo* biosynthesis of amino acids, purines, and pyrimidines (Sybesma et al. 2003). Leduc et al. (2004) showed that the derivatives of folate could enhanced the cell growth and 2-KLG production of *K. vulgare* LMPP-20356, while the vitamins, glutathione, hemin, PQQ, pyrimidines could not achieve the same results. By introducing a folate biosynthesis genes cluster found in *Lactococcus lactis* MG1363, which encodes enzymes catalyzing reactions involved in the assembly of folate and its various derivatives, into *K. vulgare*, both the cell density and 2-KLG production by the classical two-step fermentation process were significantly enhanced (Cai et al. 2012).

12.4 One-Step Vitamin C Fermentation Process

Previously, researchers from both industry and academia have invariably noted the inherent disadvantages of the two-step fermentation vitamin C production process, such as long period fermentation, additional sterilizing, control of the mix-culture

system, which lead to increased consumption for both raw materials and energy. Compared to other similar large block fermentation products, the manufacturing cost for vitamin C is much higher than others according to its final concentration of more than 100 g/L. The higher cost is mostly caused by its two-step fermentation process.

Therefore, if the vitamin C or its direct precursor, 2-KLG, could be produced by one single step fermentation process, the final cost of the vitamin C will be significantly decreased. Current research on the one-step fermentation process is focused mainly on the following three aspects: (1) Classical two-step based one-step fermentation process for 2-KLG production; (2) Innovative two-step based one-step fermentation process 2-KLG; (3) Direct production of vitamin C from glucose.

12.4.1 Classical Two-Step Based One-Step Fermentation Process

The *K. vulgare* contains the genes and proteins for the *sdh* and *sndh*, which could convert the L-sorbose to 2-KLG. The cofactor requirements of the *sdh* and *sndh* is somehow confused. However, it could be confirmed that at least one of the dehydrogenase is PQQ dependent. The PQQ is a kind of cofactor that was discovered by Hauge as the third redox cofactor after NAD^+ and FAD^+ in bacteria (Hauge 1964). Anthony and Zatman (1967) found the unknown redox cofactor in alcohol dehydrogenase and named it methoxatin (Anthony and Zatman 1967). In 1979, Salisbury and colleagues as well as Duine and colleagues extracted this prosthetic group from methanol dehydrogenase of methylotrophs and identified its molecular structure (Salisbury et al. 1979; Westerling et al. 1979). The existence of PQQ in *Acetobacter* sp. was first reported by Adachi's group (Ameyama et al. 1981).

Because the *G. oxydans* has *sldh* gene and the complete PQQ *de novo* biosynthesis and regeneration genes, if the *sdh* and *sndh* from *K. vulgare* was introduced into the *G. oxydans*, the resultant *G. oxydans* strain should produce 2-KLG from D-sorbitol by one-step fermentation process. Early research had already found out that the *G. oxydans* T100 could produce 2-KLG from sorbitol directly. However, both the final 2-KLG concentration and yield of 2-KLG on sorbitol was relatively low; 7.0 g/L and 13.1%, respectively. Overexpression of the sorbitol dehydrogenase gene *sldh* and sorbitone dehydrogenase gene *sndh* from *G. oxydans* T100 into the strain *G. oxydans* G624 with expression vector pSDH155 could significantly improve the 2-KLG production (Fig. 12.3). The final concentration of 2-KLG and the yield of 2-KLG on sorbitol was improved to 16 g/L and 30%, respectively (Saito et al. 1997). Furthermore, by inhibition of the L-idose pathway in *G. oxydans* by chemical mutagenesis and the replacement of *tufB1* promoter from *E. coli*, the recombinant strain could finally produce 2-KLG with a concentration of 88 g/L and a yield of 2-KLG on sorbitol of 82.6% (Saito et al. 1998).

G. oxydans is a kind of Gram-positive bacteria and lacks of investigation in the genetic engineering for both multi-gene overexpression and gene knockout.

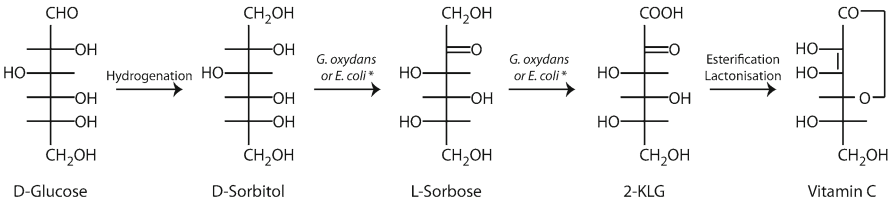

Fig. 12.3 Classical two-step based one-step fermentation process (*Recombinant strains with different dehydrogenase). The D-glucose was hydrogenated to form D-sorbitol. The D-sorbitol was directly converted into 2-KLG with a *G. oxydans* strain or a *E. coli* strain (or any other potential strains) with the three different kinds of dehydrogenases. The 2-KLG was then esterified and lactonized to form vitamin C

Another choice is to metabolic engineer other model microorganisms, such as *E. coli* or *S. cerevisae*. Because most of the dehydrogenase genes are located on the cell membrane, the difference between prokaryotes and eukaryotes makes *S. cerevisiae* a poor choice. Therefore, the aim would be to express all of the dehydrogenase and PQQ metabolism related pathways in *E. coli* for one-step biosynthesis of 2-KLG from D-sorbitol. Previous reports had already achieved the *de novo* biosynthesis of PQQ in *E. coli* by overexpression of the PQQ biosynthesis gene cluster *pqqABCDE* from *G. oxydans* (Yang et al. 2010). However, the regeneration system of the PQQ is still not well elucidated. Therefore, current works are mainly focused on the bacteria that has its own PQQ metabolism system, such as *G. oxydans* and *Paracoccus denitrificans* (Xia et al. 2003). To solve this problem, it is essential to elucidate the PQQ regeneration system on gene level.

However, after the report of the innovative two-step fermentation process, the research on the classical two-step based one-step fermentation process seems to be suspended. Few literatures about metabolic engineering of *G. oxydans* for one-step vitamin C production could be found after then.

12.4.2 Innovative Two-Step Based One-Step Fermentation Process

Though the classical two-step fermentation process has got highly promoted results, it still needs D-sorbitol as substrate, which need additional hydrogenation step by chemical process. Therefore, the direct fermentation of D-glucose to 2-KLG is still presumed. There have been some attempts to add an additional step that could convert the D-glucose to D-sorbitol, in order to supplement the classical two-step fermentation process. However, the lack of efficient enzyme makes this impossible.

Therefore, an innovative two-step fermentation process were discovered to resolve this problem. In the new innovative two-step fermentation process, the D-glucose was converted into 2,5-diketo-gluonic acid (2,5-DKG) by *Erwinia herbicola* ATCC 21988 or some similar strains with glucose dehydrogenase, gluconate dehydrogenase

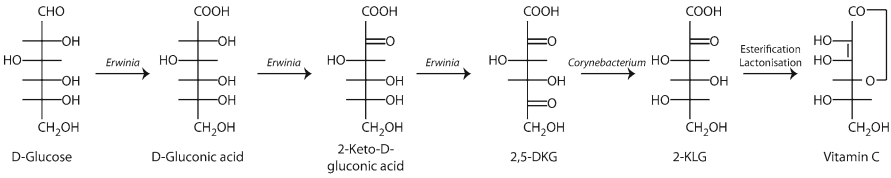

Fig. 12.4 Innovative two-step based one-step fermentation process. The D-glucose was converted into 2,5-DKG by *Erwinia* sp. The 2,5-DKG was converted into 2-KLG by *Corynebacterium* sp. The 2-KLG was then esterified and lactonized to form vitamin C. The innovative two-steps fermentation process need not chemical hydrogenation process

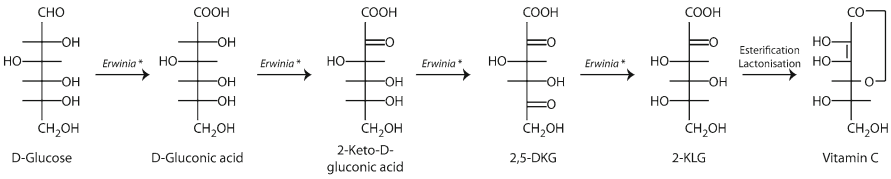

Fig. 12.5 Innovative two-step based one-step fermentation process. The D-glucose was directly converted into 2,5-DKG by a metabolic engineered *Erwinia* sp. strain containing a 2,5-DKGR from *Corynebacterium* sp. The 2-KLG was then esterified and lactonized to form vitamin C

and 2-keto-D-gluconate dehydrogenase. Then the 2,5-DKG could be transformed into 2-KLG by *Corynebacterium* ATCC 31090 or other similar strains with 2,5-DKG reductase. With this innovative two-step fermentation process, the D-glucose could be transformed into 2-KLG within two steps (Anderson et al. 1985) (Fig. 12.4). However, 2,5-DKG is highly unstable during the fermentation process. Additional sterilization could completely destroy this chemical.

Based on this innovative two-step fermentation process, Anderson et al. (1985) has expressed a 2,5-DKG reductase from *Corynebacterium* ATCC 31090 into the *Erwinia herbicola* ATCC 21988 with the expression vector ptrp1-35. The final recombinant *E. herbicola* strain could produce 1 g/L of 2-KLG from saturated D-glucose solution. By the protoplast fusion, Lin et al. (1999) fused an *Erwinia herbicola* and a *Corynebacterium* strain. The resultant strain could produce 2.07 g/L of 2-KLG (Fig. 12.5).

12.4.3 Direct Production of Vitamin C from D-Glucose

The vast majority of animals and plants are able to synthesize vitamin C through a sequence of four enzyme-driven steps, which convert D-glucose to vitamin C (Fig. 12.6). In eukaryotes, vitamin C biosynthesis occurs via two highly divergent biochemical pathways. In rats and other mammals, glucose is converted into L-gulono-1,4-lactone via a complex pathway involving uronic acid derivatives and

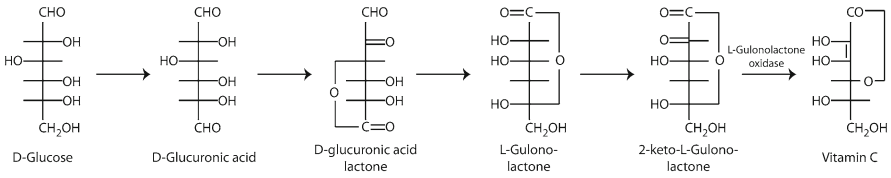

Fig. 12.6 Vitamin C production in higher eukaryotes

the 'inversion' of the original carbon skeleton (Isherwood and Cruickshank 1954; Touster 1962). The widespread occurrence of this pathway in the animal kingdom is deduced by the ubiquity of its terminal enzyme L-gulono-1,4-lactone oxidase (GUL oxidase). The gene coding for this enzyme is mutated in humans and some other animals, resulting in the loss of vitamin C synthesizing capacity.

Vitamin C biosynthesis generally exists in most of the plant cells (Zhang et al. 2007). It plays a crucial role in the detoxification of peroxide, ozone and free radicals and is essential for photosynthetic activity via the regeneration of membrane-soluble antioxidants (α-tocopherol), zeaxanthin and the pH-mediated modulation of PS II activity. Plant cells can accumulate large amount of vitamin C, particularly in green tissues and storage organs (Smirnoff 2000). Because plant cells have the whole pathway for the synthesis of vitamin C from D-galactose, attempts have been made for exploiting plant cell or microalgae cultures for the commercial production of vitamin C via fermentation.

Running et al. (2002) isolated *Chlorella pyrenoidosa* strains that were capable of producing over 70-fold more vitamin C than wild-type strains (0.64 mg/g dry cell weight for wild-type compared with 45 mg/g dry cell weight for the most-productive mutants) (Running et al. 2002). These improvements were achieved through repeated rounds of chemical mutagenesis and optimization of fermentation process conditions. Further claims were made regarding a method for the extracellular stabilization of vitamin C by reduction of medium pH (Running et al. 2002), resulting in the majority of vitamin C becoming harvestable in the fermentation medium. Yields of vitamin C microalgal cultures are not yet competitive with the traditional processes and it is likely that full economic exploitation of these systems for one-step fermentation strategies for the synthesis of vitamin C will require genetic engineering of the pathway. However, progress in the definition of the vitamin C biosynthetic pathway in plants has been singularly slow. Original models for the biosynthetic pathway in plants were based on the animal model (Isherwood and Cruickshank 1954). However, plant cells do not contain GUL oxidase and do not invert the glucose carbon skeleton during its conversion into vitamin C (Smirnoff 2000). Over the past years several pathways were proposed to accommodate these observations, but direct evidence of operation of any of these pathways has not been obtained. Only recently, with the identification of vitamin C-deficient *Arabidopsis* mutants (Conklin et al. 1996), the pathway in plants has been resolved (Wheeler et al. 1998). This made the direct fermentation production from glucose or even starch or cellulose achievable. However, because most of the genes from plants or animals had weaker

reactive dynamics characteristics, how to improve the productivity by involving these pathways from higher organisms is the key bottleneck for this route.

Furthermore, though the direct production of vitamin C from D-glucose seems to be an ideal process, vitamin C is renowned for its instability and susceptibility to oxidation. Therefore, a ventilation fermentation process may destroy most of the vitamin C. Some vitamin C derivatives, such as 2-O-alpha-D-glucopyranosyl-L-ascorbic acid (AA-2G), are relatively stable and perform almost identical physiological functions. In consequence, AA-2G is now widely used in luxury cosmetics and some of beverages. It can survive for a longer period than in subjects administered ascorbic acid 2-phosphate, which is a conventional vitamin C derivative. Therefore, the simultaneously glycosylation of vitamin C during the fermentation process may be a direct route for vitamin C production.

12.5 Perspectives

Based on the current reports, though there are many researches on the strain screening and mutagenesis, process optimization, relationships between *B. megaterium* and *K. vulgare*, metabolic engineering of *K. vulgare*, development of new two-step fermentation process to avoid mix-culture of *B. megaterium* and *K. vulgare*, development of one-step fermentation process based on both classical two-step fermentation process on new two-step fermentation process, the significant proceedings in the new processes are still sought for industrial applications for different reasons. It seems that evolution of the classical two-step fermentation process of vitamin C seems to have encountered many problems.

Based on current results, the further development of vitamin C production by microorganisms with metabolic engineering should be focused on the following area: (1) Demonstration of mutual relationships between the *B. megaterium* and *K. vulgare* and therefore enhancing cell growth and 2-KLG production by *K. vulgare*; (2) Elucidation of the genes involved in the biosynthesis and transportation of 2-KLG and its intermediate metabolites in both classical two-step and new two-step fermentation steps; (3) Investigation of the mechanisms of PQQ-dependent incompletion oxidation dehydrogenases and the regeneration of PQQ; (4) Discovery of efficient metabolic pathways for the direct fermentation of vitamin C and even its stable derivatives.

References

Ameyama M, Matsushita K, Ohno Y, Shinagawa E, Adachi O (1981) Existence of a novel prosthetic group, PQQ, in membrane-bound, electron transport chain-linked, primary dehydrogenases of oxidative bacteria. FEBS Lett 130:179–183

Anderson S, Marks CB, Lazarus R, Miller J, Stafford K, Seymour J, Light D, Rastetter W, Estell D (1985) Production of 2-keto-L-gulonate, an intermediate in L-ascorbate synthesis, by a genetically modified *Erwinia herbicola*. Science 230:144–149

Anthony C, Zatman LJ (1967) The microbial oxidation of methanol. The prosthetic group of the alcohol dehydrogenase of *Pseudomonas* sp. M27: a new oxidoreductase prosthetic group. Biochem J 104(3):960–969

Banta S, Swanson BA, Wu S, Jarnagin A, Anderson S (2002) Optimizing an artificial metabolic pathway: engineering the cofactor specificity of *Corynebacterium* 2,5-diketo-D-gluconic acid reductase for use in vitamin C biosynthesis. Biochemistry 41:6226–6236

Bremus C, Herrmann U, Bringer-Meyer S, Sahm H (2006) The use of microorganisms in L-ascorbic acid production. J Biotechnol 124:196–205

Cai L, Yuan MQ, Li ZJ, Chen JC, Chen GQ (2012) Genetic engineering of *Ketogulonigenium vulgare* for enhanced production of 2-keto-L-gulonic acid. J Biotechnol 157:320–325

Chen F, Chen CS, Li Y, Yin GL (2000) Studies on gene knocking out of 2-keto aldose reductases from *Erwinia* sp. SCB125. Acta Microbiol Sin 40(5):475–481

Conklin PL, Williams EH, Last RL (1996) Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. Proc Natl Acad Sci U S A 93:9970–9974

Feng S, Zhang Z, Zhang CG, Zhang ZZ (2000) Effect of *Bacillus megaterium* on *Gluconobacter oxydans* in mixed culture. Chin J Appl Ecol 11:119–122

Giridhar R, Srivastava AK (2000) Fed-batch cultivation of *Acetobacter suboxydans* for the microbial oxidation of D-sorbitol to L-sorbose. Bioproc Eng 23:575–577

Giridhar R, Srivastava AK (2001) Computer coupled substrate feeding strategies for efficient conversion of D-sorbitol to L-sorbose in fed-batch culture. Proc Biochem 36:829–834

Giridhar RN, Srivastava AK (2002) Productivity improvement in L-sorbose biosynthesis by fedbatch cultivation of *Gluconobacter oxydans*. J Biosci Bioeng 94:34–38

Hauge JG (1964) Glucose dehydrogenase of bacterium anitratum: an enzyme with a novel prosthetic group. J Biol Chem 239:3630–3639

Isherwood FA, Cruickshank DH (1954) Chromatographic separation and analysis of mixtures of pyruvic, oxalacetic and α-ketoglutaric acids. Nature 173:121–122

Ji AG, Gao PJ (1998) Synthesis of 2-keto-L-gulonic acid from gluconic acid by co-immobilized *Gluconobacter oxydans* and *Corynebacterium* sp. Biotechnol Lett 20:939–942

Jiang YY, Guo ZY, Zhang CG (1997) Study on the purification of 2-keto-L-gulonate reductase and its physical, chemical and enzymic properties. Chin J Biotechnol 13:400–405

Leduc S, de Troostembergh JC, Lebeault JM (2004) Folate requirements of the 2-keto-L-gulonic acid-producing strain *Ketogulonigenium vulgare* LMP P-20356 in L-sorbose/CSL medium. Appl Microbiol Biotechnol 65:163–167

Li Q, Diao JY, Xiang BT, Cao ZA (1996) Studies on metabolism of nitrogen source in fermentation of 2-keto-L-gulonic acid. Acta Microbiol Sin 1:19–24

Lin HY, Chen CS, Yin GL (1999) Intergeneric cell fusion of *Corynebacterium* and *Erwinia*. Chin Microbiol 26:3–6

Liu LM, Chen KJ, Zhang J, Liu J, Chen J (2011a) Gelatin enhances 2-keto-L-gulonic acid production based on *Ketogulonigenium vulgare* genome annotation. J Biotechnol 156:182–187

Liu LM, Li Y, Zhang J, Zhou ZM, Liu J, Li XM, Zhou JW, Du GC, Wang L, Chen J (2011b) Complete genome sequence of the industrial strain *Ketogulonicigenium vulgare* WSH-001. J Bacteriol 193:6108–6109

Liu LM, Li Y, Zhang J, Zou W, Zhou ZM, Liu J, Li XM, Wang L, Chen J (2011c) Complete genome sequence of the industrial strain *Bacillus megaterium* WSH-002. J Bacteriol 193:6389–6390

Lu SJ, Jun W, Yao JM, Yu ZL (2003) Study on the effect of mutated *Bacillus megaterium* in two-stage fermentation of vitamin C. Plasma Sci Technol 5:2011–2016

Lv SX, Feng S, Zhang ZZ, Liu Y, Xie ZW, An HY (2001) The effect of *Bacillus megaterium* in vitamin C two-step fermentation. Acta Microbiol Sin 28:10–13

Moonmangmee D, Adachi O, Ano Y, Shinagawa E, Toyama H, Theeragool G, Lotong N, Matsushita K (2000) Isolation and characterization of thermotolerant Gluconobacter strains catalyzing oxidative fermentation at higher temperatures. Biosci Biotechnol Biochem 64:2306–2315

Petrescu S, Hulea SA, Stan R, Avram D, Herlea V (1992) A yeast strain that uses D-galacturonic acid as a substrate for L-ascorbic acid biosynthesis. Biotechnol Lett 14:1–6

Rao YM, Sureshkumar GK (2000) Direct biosynthesis of ascorbic acid from glucose by *Xanthomonas campestris* through induced free-radicals. Biotechnol Lett 22:407–411

Reichstein T, Grussner A (1934) A productive synthesis of L-arcorbic acid (C-vitamin). Helv Chim Acta 17:311–328

Running JA, Severson DK, Schneider KJ (2002) Extracellular production of L-ascorbic acid by *Chlorella protothecoides*, *Prototheca species*, and mutants of *P. moriformis* during aerobic culturing at low pH. J Ind Microbiol Biotechnol 29(2):93–98

Saito Y, Ishii Y, Hayashi H, Imao Y, Akashi T, Yoshikawa K, Noguchi Y, Soeda S, Yoshida M, Niwa M, Hosoda J, Shimomura K (1997) Cloning of genes coding for L-sorbose and L-sorbosone dehydrogenases from *Gluconobacter oxydans* and microbial production of 2-Keto-L-Gulonate, a precursor of L-ascorbic acid, in a recombinant *G-oxydans* strain. Appl Environ Microbiol 63:454–460

Saito Y, Ishii Y, Hayashi H, Yoshikawa K, Noguchi Y, Yoshida S, Soeda S, Yoshida M (1998) Direct fermentation of 2-keto-L-gulonic acid in recombinant *Gluconobacter oxydans*. Biotechnol Bioeng 58:309–315

Salisbury SA, Forrest HS, Cruse WB, Kennard O (1979) A novel coenzyme from bacterial primary alcohol dehydrogenases. Nature 280:843–844

Sanli G, Blaber SI, Blaber M (2001) Reduction of wobble-position GC bases in *Corynebacteria* genes and enhancement of PCR and heterologous expression. J Mol Microbiol Biotechnol 3:123–126

Sauer M, Branduardi P, Valli M, Porro D (2004) Production of L-ascorbic acid by metabolically engineered *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. Appl Environ Microbiol 70:6086–6091

Shinjoh M, Tomiyama N, Asakura A, Hoshino T (1995) Cloning and nucleotide sequencing of the membrane-bound L-sorbosone dehydrogenase gene of *Acetobacter liquefaciens* IFO 12258 and its expression in *Gluconobacter oxydans*. Appl Environ Microbiol 61:413–420

Smirnoff N (2000) Ascorbic acid: metabolism and functions of a multi-facetted molecule. Curr Opin Plant Biol 3:229–235

Sonoyama T, Tani H, Matsuda K, Kageyama B, Tanimoto M, Kobayashi K, Yagi S, Kyotani H, Mitsushima K (1982) Production of 2-keto-L-gulonic acid from D-glucose by two-stage fermentation. Appl Environ Microbiol 43:1064–1069

Srivastava AK, Lasrado PR (1998) Fed-batch sorbitol to sorbose fermentation by *A suboxydans*. Bioproc Eng 18(6):457–461

Sugisawa T, Hoshino T, Masuda S, Nomura S, Setoguchi Y, Tazoe M, Shinjoh M, Someha S, Fujiwara A (1990) Microbial production of 2-keto-L-gulonic acid from L-sorbose and D-sorbitol by *Gluconobacter melanogenus*. Agr Biol Chem 54:1201–1209

Sugisawa T, Miyazaki T, Hoshino T (2005) Microbial production of L-ascorbic acid from D-sorbitol, L-sorbose, L-gulose, and L-sorbosone by *Ketogulonicigenium vulgare* DSM 4025. Biosci Biotechnol Biochem 69:659–662

Sybesma W, Starrenburg M, Kleerebezem M, Mierau I, de Vos WM, Hugenholtz J (2003) Increased production of folate by metabolic engineering of *Lactococcus lactis*. Appl Environ Microbiol 69:3069–3076

Touster O (1962) Carbohydrate metabolism. Ann Rev Biochem 31:407–450

Tsukada Y, Perlman D (1972) The fermentation of L-sorbose by *Gluconobacter melanogenus*. I. General characteristics of the fermentation. Biotechnol Bioeng 14:799–810

Westerling J, Frank J, Duine JA (1979) The prosthetic group of methanol dehydrogenase from Hyphomicrobium X: electron spin resonance evidence for a quinone structure. Biochem Biophys Res Commun 87:719–724

Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 393:365–369

Xia ZX, Dai WW, He YN, White SA, Mathews FS, Davidson VL (2003) X-ray structure of methanol dehydrogenase from *Paracoccus denitrificans* and molecular modeling of its interactions with cytochrome c-551i. J Biol Inorg Chem 8:843–854

Xiong XH, Han S, Wang JH, Jiang ZH, Chen W, Jia N, Wei HL, Cheng H, Yang YX, Zhu B, You S, He JY, Hou W, Chen MX, Yu CJ, Jiao YH, Zhang WC (2011) Complete genome sequence of the bacterium *Ketogulonicigenium vulgare* Y25. J Bacteriol 193:315–316

Xu A, Yao J, Yu L, Lv S, Wang J, Yan B, Yu Z (2004) Mutation of *Gluconobacter oxydans* and *Bacillus megaterium* in a two-step process of L-ascorbic acid manufacture by ion beam. J Appl Microbiol 96:1317–1323

Yan B, Xu A, Zhang W, Zhou W, Wang J, Yao JM, Yu ZL (2006) Accumulation of 2-keto-L-gulonate at 33 degrees C by a thermotolerant *Gluconobacter oxydans* mutant obtained by ion beam implantation. Plasma Sci Technol 8:237–241

Yang FC, Lim YH (1997) Kinetic study of the bioconversion of D-sorbitol to L-sorbose by *Acetobacter pasteurianus*. Proc Biochem 32:233–236

Yang F, Jia Q, Xiong Z, Zhang X, Wu H, Zhao Y, Yang J, Zhu J, Dong J, Xue Y, Sun L, Shen Y, Jin Q (2006) Complete genome analysis of *Ketogulonigenium* sp. WB0104. Chin Sci Bull 51(8):941–945

Yang XP, Zhong GF, Lin JP, Mao DB, Wei DZ (2010) Pyrroloquinoline quinone biosynthesis in *Escherichia coli* through expression of the *Gluconobacter oxydans pqqABCDE* gene cluster. J Ind Microbiol Biotechnol 37:575–580

Yin GL, He JM, Ren SX, Song Q, Ye Q, Lin YH, Chen CS, Guo XY (1997) Production of vitamin C precursor-2-leto-L-gulonic acid from L-sorbose by a novel bacterial component system of SCB329-SCB933. Ind Microbiol 27:1–7

Zhang L, Wang Z, Xia Y, Kai G, Chen W, Tang K (2007) Metabolic engineering of plant L-ascorbic acid biosynthesis: recent trends and applications. Crit Rev Biotechnol 27:173–182

Zhang J, Liu J, Shi ZP, Liu LM, Chen J (2010) Manipulation of *B-megaterium* growth for efficient 2-KLG production by *K-vulgare*. Process Biochem 45:602–606

Zhang J, Zhou JW, Liu J, Chen KJ, Liu LM, Chen J (2011) Development of chemically defined media supporting high cell density growth of *Ketogulonicigenium vulgare* and *Bacillus megaterium*. Bioresour Technol 102:4807–4814

Zhao SG, Yao LM, Su CX, Wang T, Wang J, Tang ML, Yu ZL (2008) Purification and properties of a new L-sorbose dehydrogenase accelerative protein from *Bacillus megaterium* bred by ion-beam implantation. Plasma Sci Technol 10:398–402

Zhou B, Li Y, Liu YP, Zhang ZZ, Zhu KL, Liao DM, Gao YT (2002) Microbiological eco-regulation in Vc two-step fermentation. Chin J Appl Ecol 13:1452–1454

Zhou J, Ma Q, Yi H, Wang LL, Song H, Yuan YJ (2011) Metabolome profiling reveals metabolic cooperation between *Bacillus megaterium* and *Ketogulonicigenium vulgare* during induced swarm motility. Appl Environ Microbiol 77:7023–7030

Zhu YB, Liu J, Du GC, Zhou JW, Chen J (2012) Sporulation and spore stability of *Bacillus megaterium* enhance *Ketogulonigenium vulgare* propagation and 2-keto-L-gulonic acid biosynthesis. Bioresour Technol 107:399–404