J Ind Microbiol Biotechnol (2015) 42:897–904
DOI 10.1007/s10295-015-1616-7

BIOTECHNOLOGY METHODS

L-sorbose is not only a substrate for 2-keto-L-gulonic acid production in the artificial microbial ecosystem of two strains mixed fermentation

Mandlaa[1] · Weichao Yang[1] · Chengbin Liu[1] · Hui Xu[1]

Received: 18 December 2014 / Accepted: 28 March 2015 / Published online: 10 April 2015

Abstract The co-culture system of the fermentation process of vitamin C can be regarded as an artificial microbial ecosystem (AME). To extend our understanding of this AME, an investigation of the relationship between strains, substrate and product was carried out in this study. The results showed that both *Ketogulonicigenium vulgare* and 2-keto-L-gulonic acid (2-KLG, the precursor of vitamin C) can inhibit the growth of the helper strain, while the helper strain promoted the growth of *K. vulgare* and 2-KLG production. Moreover, L-sorbose is not only a substrate for 2-KLG production in the AME, but also a promoter of *K. vulgare* and an inhibitor of the helper strain. In the earlier stage of fermentation, the inhibition of L-sorbose on the helper strain's growth is a key factor for ensuring an efficient fermentation. In the condition of adding the extra helper strain (OD: 0.57, ratio of inoculation: 2 %), the yields of 2-KLG is increased by 9 % in the 14 % L-sorbose medium. To the best of our knowledge, this is the first report about the inhibition of substrate in the AME of 2-KLG production.

Keywords Artificial microbial ecosystem · Vitamin C · 2-Keto-L-gulonic acid · L-sorbose · Helper strain · *Ketogulonicigenium vulgare*

Introduction

Vitamin C (or L-ascorbic acid), a water-soluble vitamin, is widely applied in food, beverages, animal feed and pharmaceutical industry [1]. It is estimated roughly that 110,000 t of vitamin C are industrially produced per year and more than 80 % of it is produced by two-step fermentation process [13], which has been applied more than 30 years [25]. During this process, L-sorbose is bio-converted to 2-keto-L-gulonic acid (2-KLG, the precursor of vitamin C) by a two-strain co-culture system in the second-step fermentation. The two strains consist of a 2-KLG-producing strain *Ketogulonicigenium vulgare* (previously identified as *G. oxydans*) [14, 18] and a helper strain (mostly belongs to *Bacillus*) [1]. *K. vulgare* has a complete enzyme system for converting L-sorbose to 2-KLG [7], but its growth is very poor and the 2-KLG yield is very low when *K. vulgare* is cultured without the helper strain [2].

Nowadays, the two-step fermentation process has been developed into a more mature process for vitamin C production by years' efforts. However, the conversion rate of L-sorbose to 2-KLG has been kept at the relatively low level (88–90 %) for years due to the reasons such as fermentation instability. Hence, the two-strain mixed fermentation process with higher fermentation efficiency is in great need for 2-KLG industrial production. To enhance 2-KLG productivity, the relationship between *K. vulgare* and the helper strain, as well as the mechanism of 2-KLG biosynthesis, should be elucidated comprehensively. Therefore, many efforts have been made to try to resolve above

Weichao Yang and Hui Xu contributed equally to this work.

✉ Weichao Yang
yangweichao@iae.ac.cn

✉ Hui Xu
xuhui@iae.ac.cn

Mandlaa
mandlaa@foxmail.com

Chengbin Liu
389887229@qq.com

[1] Institute of Applied Ecology, Chinese Academy of Sciences, 72 Wenhua Road, Shenyang 110016, China

problems in following two main research directions: the mutual relationships of the two microbes in the co-culture system and optimization of the fermentation process for enhanced 2-KLG production.

In the research direction of interrelationships between *K. vulgare* and the helper strain, it is reported that the helper strain can generate and release some metabolites, which stimulate the propagation of *K. vulgare* and accumulation of 2-KLG during the co-culture process [8, 20]. The mechanism of the interaction between the two strains in the co-culture system is suggested by Zhou and his co-workers [23]. They observed that the helper strain is attracted by exogenous metabolites from *K. vulgare*, meanwhile, *K. vulgare* also benefits from erythrose, erythritol and inositol secreted by the helper strain. Moreover, sporulation of the helper strain is an important factor for promoting *K. vulgare* growth and 2-KLG biosynthesis in the co-culture system [10, 24]. After sporulation, purine substrates from the cell lysis of the helper strain might satisfy the growth of *K. vulgare* [10]. These studies have shown light on the relationships between *K. vulgare* and the helper strain in molecular level. However, up to now, there is no clear definition about the mechanisms underlying the interactions between *K. vulgare* and its helper strain [13].

In the research direction of optimization of the fermentation process for enhanced 2-KLG production, Zou [25] suggested that the co-culture system should be optimized systematically because it can be regarded as an artificial microbial ecosystem (AME). Zhang [20] introduced a strategy of lysozyme addition in which the cell wall of helper strain was damaged by lysozyme, and subsequently the helper-strain's intracellular components were released. This strategy manipulated the growth of *K. vulgare* and increased 2-KLG productivity by 28.2 %. Moreover, addition of folate [4], glutathione [9, 19] and gelatine [6] also enhanced the 2-KLG production. Therefore, in this AME, not only the relationship of the helper strain and *K. vulgare*, but also the environmental and nutritional factors, including the substrate and the product, may be the determinants for the 2-KLG production. Therefore, optimizing only one or two factors for improving 2-KLG production could not be an effective approach. Optimizing the AME multi-factorially might be a better choice. However, the relationships among the two microbes, substrate and product in AME, have not been systematically investigated up to now.

In this study, a comprehensive investigation of the relationships among the two microbes, product (2-KLG) and substrate (L-sorbose) is carried out from an ecological perspective to get a further understanding of the process of 2-KLG production in AME. To the best of our knowledge, this is the first report concerning the effects of product and substrate on the AME.

Materials and methods

Microbial strains and media

Two strains, *Ketogulonicigenium vulgare* (2-KLG-producing strain) and *Bacillus cereus* (the helper strain), which were stored in Institute of Applied Ecology, Chinese Academy of Sciences, were used for 2-KLG fermentation in this study.

Fermentation medium (g/L): L-sorbose 80, corn-steep liquor 10, carbamide 12, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 0.2, $CaCO_3$ 1 and pH 6.7–7.0 (L-sorbose and carbamide are sterilized separately). Isolation medium (g/L): L-sorbose 20, yeast extraction 3, corn-steep liquor 3, carbamide 1, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 0.3, $CaCO_3$ 1, agar 15 and pH 6.7. The pH was adjusted with NaOH solution (40 %, w/v) before sterilization. Minimal medium (g/L): Glucose 5, $(NH_4)_2SO_4$ 2, Sodium Citrate 1, $MgSO_4{\cdot}7H_2O$ 0.2, $K_2HPO_4{\cdot}3H_2O$ 4, KH_2PO_4 6. All the media were sterilized at 115 °C for 30 min. After adjusting pH of 2-KLG solution to 7.0 by NaOH solution (40 %), the 2-KLG solution was sterilized by filter sterilization through a 0.22 μm filter.

Preparation of seeds

The seed of *K. vulgare*: About 800 colonies of *K. vulgare* in isolation medium were collected into a tube containing 5 ml sterilized water and inoculated into petri dishes that contained 30 ml fresh isolation medium. After 3 days cultivation at 29 °C, the lawns of *K. vulgare* from 15–20 petri dishes were re-collected into a 250 ml shake flask containing 40 ml sterilized water. The seed of the helper strain: One colony of the helper strain was inoculated into a shake flask that contained 20 ml minimal medium. The flask was then cultivated at 29 °C on a rotary shaker at 180 rpm for 24 h. 1 ml of the broth was inoculated into another 250 ml shake flask that contained 40 ml minimal medium and cultivated at 29 °C on a rotary shaker at 180 rpm for 12 h. The preparation of the co-culture seed was according to the report of Mandlaa [11].

Design of experiment

The single-factor experiment was applied to investigate the influence of L-sorbose or 2-KLG on the growth of helper strain and *K. vulgare* in fermentation medium by using flask fermentation. The seed of *K. vulgare* was inoculated into 250 ml shake flasks containing the fermentation medium with different concentration of L-sorbose (0–10 %, w/v) or 2-KLG (0–10 %, w/v). The inoculation ratio was 20 % (v/v) and the flasks were cultivated at 29 °C, 180 rpm. After 50 h cultivation, the OD of *K. vulgare* was determined.

Similarly, the influence of L-sorbose or 2-KLG on the growth of helper strain was studied by inoculating the seed of the helper strain into flasks containing the fermentation medium with different concentration of L-sorbose (0–10 %, w/v) or 2-KLG (0–10 %, w/v). The inoculation ratio and the cultivation conditions were the same as the above description on investigation of *K. vulgare*. After 12 h cultivation, the OD of the helper strain was determined.

To investigate the effects of combination of L-sorbose and 2-KLG on the growth of the helper strain during the fermentation process, the concentrations of L-sorbose and 2-KLG in the medium was designed to be changed by gradient. The proportion of L-sorbose and 2-KLG in the medium (20 ml in 250 ml shake flask) was 10: 0, 8:2, 6: 4, 4: 6, 2: 8 and 0:10 % (w/v), respectively. The inoculation ratio of helper strain was 20 % (v/v) and the cultivation condition was 29 °C and 180 rpm for 12 h. The OD of the helper strain was determined at the 0 h and 12 h cultivation, respectively.

The pure culture of the helper strain was applied as the control treatment to investigate the influence of *K. vulgare* on growth of the helper strain in flask fermentation. The ratio of *K. vulgare* to the helper strain was 5 % (v/v) before the inoculation. The cultivation condition was 29 °C and 180 rpm. The OD of helper strain was determined at 0, 12, 24 h and 60 h of fermentation, respectively.

The effect of L-sorbose on the co-culture system was studied by adding L-sorbose (8 %, w/v) or not in flask fermentation. The inoculation of co-culture system was 15 % (v/v) and the fermentation condition was 29 °C and 180 rpm. The OD of the helper strain and *K. vulgare*, the concentrations of L-sorbose and 2-KLG, were measured at 0, 12, 24 h and 60 h, respectively.

To investigate the effect of adding extra helper strain to the co-culture system on the 2-KLG production, two levels of L-sorbose (8 and 14 %, w/v) and three levels of adding extra helper strain (OD: 0.570; inoculation ratio: 0, 2 and 4 %, v/v) were designed and the flask fermentation with different treatment was carried out. The fermentation condition was 29 °C and 180 rpm. After 45 h cultivation, the 2-KLG concentration was determined.

Assay methods

Determinations of 2-KLG and L-sorbose, the Optical Density (at 660 nm, OD) of *K. vulgare* and the helper strain in the fermentation broth were according to previously published literatures [6, 15, 17]. Three replications were performed in all treatments. Differences between two groups were regarded as statistically significant if $P < 0.05$. Microsoft office excel 2007 was applied to analyze the data and draw the Figures.

Results

The effects of L-sorbose and 2-KLG on the growth of *K. vulgare*

L-sorbose, the substrate in the second-step fermentation of vitamin C production, is converted into 2-KLG by *K. vulgare* in the co-culture system. The effect of L-sorbose on the growth of *K. vulgare* is investigated by adding various concentrations of L-sorbose and the results are shown in Fig. 1a. After 50 h cultivation, the growth of *K. vulgare* was significantly different under the condition of various concentrations of L-sorbose (0–10 %). When there were a lower concentration of L-sorbose (i.e., 2–6 %), the growth of *K. vulgare* was promoted by L-sorbose. Compared to the control treatment (no L-sorbose in the medium), the OD value of *K. vulgare* are increased by 64, 50 and 16.5 % when the concentration of L-sorbose was 2, 4 and 6 %, respectively. However, in the medium with a higher L-sorbose concentration (8–10 %), the promoting ability of L-sorbose on growth of *K. vulgare* got weak. These results indicate that L-sorbose can promote the growth of *K. vulgare* when the concentration of substrate was 2–10 %, especially for 2–6 %.

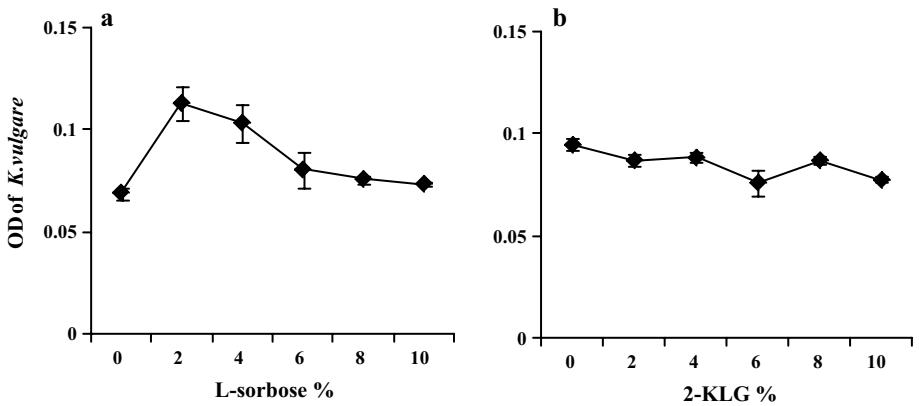

Fig. 1 Variation of the growth of *K. vulgare* in different concentrations of L-sorbose and 2-KLG. **a** Variation of the growth of *K. vulgare* in different concentration of L-sorbose; **b** variation of the growth of *K. vulgare* in different concentration of 2-KLG

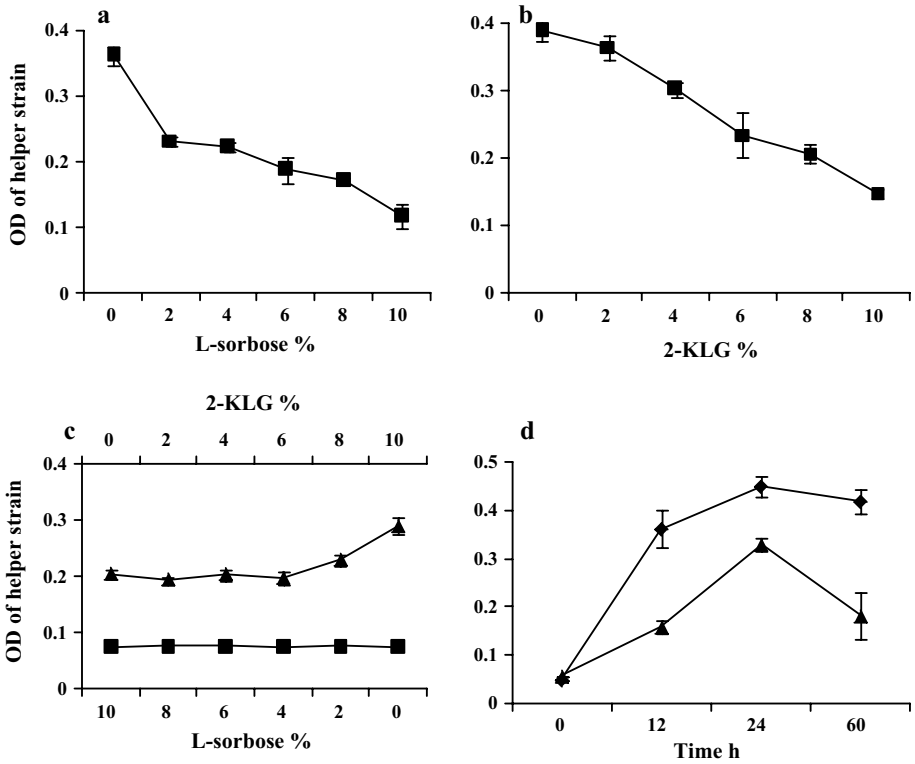

Fig. 2 The effects of L-sorbose, 2-KLG and *K. vulgare* on the growth of helper strain. **a** The effect of L-sorbose on the growth of helper strain; **b** the effect of 2-KLG on the growth of helper strain; **c** the effect of simulation of the fermentation process by decreasing L-sorbose and increasing 2-KLG on the growth of helper strain (*filled triangle* 12 h; *filled square* 0 h); **d** the effect of *K. vulgare* on growth of helper strain

2-KLG is the only target product in the second-step fermentation of vitamin C production. The effect of 2-KLG on the growth of *K. vulgare* is shown in Fig. 1b. The OD of *K. vulgare* gradually decreased (but not significantly) with the increasing 2-KLG concentration (2–10 %), which indicates that 2-KLG have inhibiting effect on the growth of *K. vulgare* if the concentration of 2-KLG increased.

The effects of L-sorbose, 2-KLG and *K. vulgare* on the growth of helper strain

In the fermentation medium after 12 h cultivation, the OD of the helper strain gradually decreased with the increasing L-sorbose (2–10 %) and 2-KLG (2–10 %) concentrations, respectively (Fig. 2a, b). The significant negative correlation between the OD of the helper strain and the concentration of L-sorbose or 2-KLG (L-sorbose: $y = -0.041x + 0.359$, $R^2 = 0.869$; 2-KLG: $y = -0.05x + 0.449$, $R^2 = 0.985$) indicates that both L-sorbose (substrate) and 2-KLG (product) can inhibit the growth of the helper strain. However, the simulation of the fermentation process (Fig. 2c), in which there were a gradually decreased L-sorbose concentration and a gradually increased 2-KLG concentration, showed that the inhibiting ability of L-sorbose and 2-KLG are different. The OD of the helper strain increased with the decreasing of L-sorbose and increasing of 2-KLG after 12 h cultivation. The OD of the helper strain in treatment of 10 % (w/v) 2-KLG was higher than that in treatment of 10 % (w/v) of L-sorbose (0.29 ± 0.02 and 0.20 ± 0.01, respectively; Fig. 2c), despite their concentration are the same. This results demonstrated that L-sorbose has stronger ability to inhibit the growth of the helper strain than 2-KLG.

The influence of *K. vulgare* on the growth of the helper strain is investigated by using the pure helper strain as the control (Fig. 2d). Under the condition of the same inoculation of the helper strain, the OD of helper strain in its monoculture was significantly higher than that of the helper strain in the co-culture system (56.5, 26.9 and 56.9 % higher than control treatment after 12, 24 and 60 h cultivation, respectively). These results manifested that *K. vulgare* retarded the growth of helper strain in the AME.

The effects of L-sorbose on the growth of helper strain and *K. vulgare* in the co-culture system

The effects of L-sorbose on the growth of helper strain and *K. vulgare* in the co-culture system were studied by addition of L-sorbose or not, and the results are shown in the Fig. 3. The OD of the helper strain is significantly higher under the treatment of no L-sorbose in the earlier stage of fermentation (0–12 h). But the OD of the helper strain under the treatment of no L-sorbose got lower than that the treatment of 8 % L-sorbose after 24 h fermentation. The OD of helper strain in the treatment of 8 % L-sorbose was 93.3 and 166.7 % higher than that treatment of no L-sorbose at 24 and 60 h, respectively (Fig. 3a).

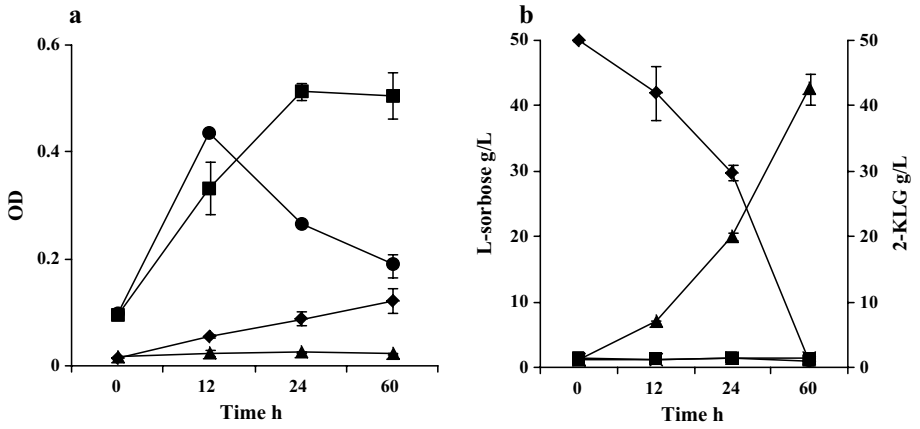

Fig. 3 The effect of L-sorbose on the co-culture system. **a** The effect of L-sorbose on the growth of the helper strain and *K. vulgare* in the co-culture system (*filled square*: OD of helper strain in the medium of 8 % L-sorbose; *filled circle*: OD of helper strain in the medium of no L-sorbose; *filled diamond*: OD of *K. vulgare* in the medium of 8 % L-sorbose; *filled triangle*: OD of *K. vulgare* in the medium of no L-sorbose;); **b** the effect of L-sorbose on the variation of L-sorbose and 2-KLG in the co-culture system (*filled diamond*: L-sorbose in treatment of 8 % L-sorbose; *filled square*: L-sorbose in the treatment of no L-sorbose; *filled triangle*: 2-KLG in the treatment of 8 % L-sorbose; *filled circle*: 2-KLG in the treatment of no L-sorbose)

The OD of *K. vulgare* in treatment of 8 % L-sorbose was significantly higher than the treatment of no L-sorbose from 12 to 60 h. After 60 h fermentation, the OD of *K. vulgare* in the treatment of 8 % L-sorbose was four times higher than that of the no L-sorbose treatment (Fig. 3a).

The variations of L-sorbose and 2-KLG during the fermentation are shown in Fig. 3b. L-sorbose was gradually consumed from concentration of 49.8 ± 0 g/L and converted into 2-KLG in the treatment of 8 % L-sorbose. After 60 h fermentation, the concentration of 2-KLG was 42.6 ± 2.4 g/L and the concentration of residual L-sorbose was 1.3 ± 0.2 g/L. The molarity of L-sorbose was 0.277 mol/L at the starting of fermentation (A) and 0.007 mol/L after 60 h fermentation (B). The molarity of 2-KLG is 0.006 mol/L at the start of fermentation(C) and 0.220 mol/L after 60 h fermentation (D). The L-sorbose (X) lost in 1 L fermentation broth was 0.056 mol, which was calculated by formula: A + C = B + D + X. This results indicate that a part of L-sorbose is metabolized by another pathway instead of biosynthesizing 2-KLG.

The effects of addition of the extra helper strain on the 2-KLG production in the co-culture system

The effects of raising the concentration of L-sorbose and addition of extra helper strain on the 2-KLG production were investigated and the results are shown in Fig. 4. Under 8 % of L-sorbose and incubated for 45 h, addition of 2 (v/v) and 4 % (v/v) of the helper strain in its pure culture (its OD was 0.57) decreased the yields of 2-KLG by 9 and 18 %, respectively. However, under condition of 14 % of L-sorbose, addition of helper strain (2 and 4 %) showed no significant influence on the yields of 2-KLG. Compared with 8 % L-sorbose, addition of 2 % pure culture of helper strain into the medium containing 14 % L-sorbose increased the yields of 2-KLG by 9 % after 45 h fermentation. Under the same fermentation conditions, the 2-KLG productivity was 1.62 g/L/h in 8 % L-sorbose, while it was 1.77 g/L/h in 14 % L-sorbose by addition of 2 % helper strain. These results indicate that, in the two-strain co-culture system, more 2-KLG can be produced in high concentration of substrate L-sorbose (14 %) by addition of extra helper strain.

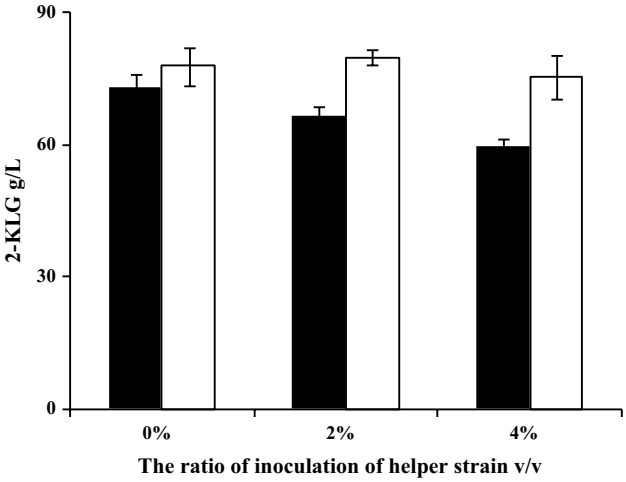

Fig. 4 Variation of the 2-KLG production under addition of the extra helper strain in different concentration of L-sorbose. *Filled square*: 8 % L-sorbose, *open square*: 14 % L-sorbose

Discussion

In the AME of vitamin C fermentation, it was a consensus that the helper strain could promote the growth of *K. vulgare* and production of 2-KLG [2, 8, 18, 25]. Therefore, the relationship between *K. vulgare* and the helper strain had become a research hotspot in the field of the vitamin C fermentation [10, 23, 25]. However, up to now, there has been no report regarding the effects of L-sorbose and 2-KLG on the two microbes in this AME. Based on the above concern, a comprehensive analysis of the AME was launched by investigating the relationships of the helper strain, *K. vulgare*, product and substrate in this study.

In the industrial production of vitamin C by two-step fermentation process, L-sorbose is converted to 2-KLG in the AME [18]. Theoretically, 1 mol L-sorbose could be converted to 1 mol 2-KLG and the amount of L-sorbose at the beginning of the fermentation should be equal to the sum of amounts of the L-sorbose and 2-KLG at the end of the fermentation. However, a small amount of L-sorbose was lost in the fermentation process as showed in Fig. 3b. According to a pioneering literature on the mixed culture [18], we realized that the 2-KLG-producing strain, *K. vulgare*, was first isolated with a medium in which the L-sorbose was a unique carbon source [18]. This fact showed the capability of *K. vulgare* utilizing L-sorbose as a carbon source. Therefore, if the preferential carbon sources for *K. vulgare*, rather than L-sorbose, were exhausted during the later period of fermentation process, L-sorbose should be utilized as carbon source for *K. vulgare*. This might be one of the reasons for the inequality of mole number of total L-sorbose and the 2-KLG after fermentation. Perhaps, the problem of "losing L-sorbose" could be solved by interdiction of the metabolic pathway of L-sorbose as carbon source in *K. vulgare*. However, the pathway has not been illustrated.

Moreover, L-sorbose in low concentration (2–6 %), besides as the substrate and carbon source, could promote the growth of *K. vulgare* and had no significant inhibitory effect on the growth of *K. vulgare* in higher concentration (6–10 %). But in the AME, *K. vulgare* could not grow without L-sorbose, despite the other carbon source was sufficient (Fig. 3a). From our own perspective, there were three possible interpretations about this phenomenon. First, L-sorbose (2–10 %) could inhibit the growth of helper strain significantly (Fig. 2a). In the AME without L-sorbose, the helper strain could not be inhibited by L-sorbose and reproduced quickly, which consequently inhibited the growth of *K. vulgare* by means of nutrition competition in the medium. Second, L-sorbose might induce some products from helper strain to promote the growth of *K. vulgare*. Third, L-sorbose also showed a capability of promoting the growth of *K. vulgare* in low concentration (2–6 %). Therefore, these three aspects might give a well interpretation on the phenomenon that *K. vulgare,* when co-cultured with a helper strain, cannot grow in the medium without L-sorbose.

The results in this study imply that L-sorbose plays an important role in keeping the ratio of *K. vulgare* and the helper strain in AME. On one side, as our results (Fig. 1a), L-sorbose promoted the growth of *K. vulgare*. On the other side, L-sorbose showed the growth-inhibiting ability for helper strain. Moreover, the inhibiting strength of L-sorbose was higher than 2-KLG in the same concentration (10 %, w/v) (Fig. 2b, Fig. 2c). Especially in the earlier period of fermentation (0–12 h), because of the low concentration of 2-KLG and the small number of *K. vulgare*, the growth of the helper strain was mainly inhibited by L-sorbose. Therefore, the numbers of *K. vulgare* and the helper strain during the fermentation process can be adjusted by L-sorbose in the medium, which might balance the proportion of the helper strain and *K. vulgare* and lead to a feasible state for 2-KLG production in AME.

Generally speaking, raising the concentration of substrate was a feasible method to enhance the fermentation efficiency. However, the concentration of L-sorbose was kept at 8–9 % in the fermentation medium of the industrial 2-KLG fermentation due to the inhibition of a higher substrate concentration (over 9 %) [15, 16, 22, 26]. Based on the ecological function of L-sorbose in the AME, a novel method for enhancement of 2-KLG production was proposed in this work. This method, by adding the extra helper strain incubated alone in advance, increased the 2-KLG yield by 9 % in the medium with a 14 % concentration of L-sorbose than that with an 8 % concentration of L-sorbose. Therefore, the results further suggested that

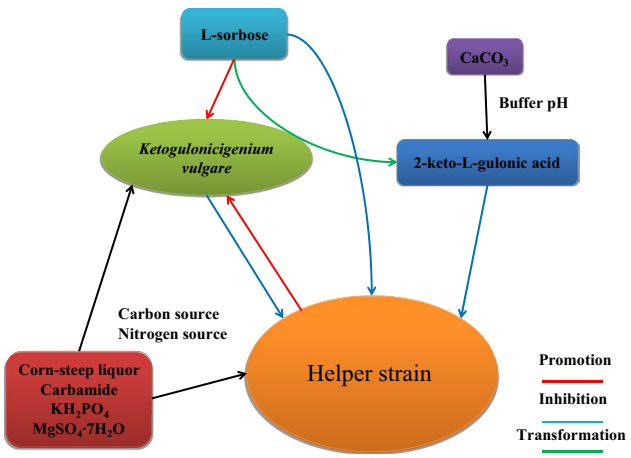

Fig. 5 The relationships of the artificial microbial ecosystem in vitamin C fermentation

the 2-KLG production was regulated by both the helper strain and L-sorbose. Hence, if this method is applied in the industrial production of 2-KLG with a higher concentration (such as 14 %) in the future, the proportion of *K. vulgare* and helper strain must be optimized again based on the ecological relationships among *K. vulgare*, helper strain and L-sorbose.

The relationships of components in AME of the vitamin C fermentation process were demonstrated in Fig. 5. In this AME, *K. vulgare* could convert L-sorbose to 2-KLG (indicated as the green arrow) and the helper strain could promote the growth of *K. vulgare* (indicated as the red arrow) and 2-KLG production [2, 8, 13]. It was reported, which is in accordance with our results, that the helper strain promoted the 2-KLG production by promoting the growth of *K. vulgare* [3]. On the contrary, *K. vulgare,* L-sorbose and 2-KLG could inhibit the growth of helper strain (indicated as the blue arrow). The promotion of the helper strain to *K. vulgare* growth and the inhibition of *K. Vulgare*, L-sorbose and 2-KLG to helper strain, might be the reason that the growth of helper strain and *K. vulgare* was in a harmonious status and keep the 2-KLG fermentation more stable. In this AME, L-sorbose played an important role. First, it was the substrate for 2-KLG conversion. Second, it promoted the growth of *K. vulgare* in some concentration (2–6 %). Finally, it inhibited the growth of the helper strain, which might adjust the ratio of two strains and make a stable fermentation in the AME.

In the fermentation process, the pH in medium was kept at 6.5–7.0 by $CaCO_3$. The carbon and nitrogen sources mainly came from the corn-steep liquor and carbamide in the medium (indicated as the black arrow) [5, 21]. In the corn-steep liquor, glycine, serine, biotin, proline, nicotinic acid and threonine were the key components for growth of microbes and 2-KLG production by *K. vulgare* in the AME [21].

Ecology can be defined as the study of interactions or relationships between living organisms and their environment [12]. Therefore, in the two-strain mixed fermentation for 2-KLG production, interactions among the two microbes and the environmental conditions were the key factors for sustainability of the ecosystem. However, there were few studies on these interactions in the AME. In this study, a comprehensive investigation was carried out, and the results may help us in providing some new theoretical and applied acknowledges about this AME.

In conclusion, L-sorbose played an important role in the AME. Besides, as the substrate L-sorbose could ensure the fermentation to proceed by regulating the proportion of the helper strain and *K. vulgare* in the AME. In addition, the efficiency of 2-KLG production could be enhanced by adding the extra helper strain in the medium with high L-sorbose concentration.

References

1. Bremus C, Herrmann U, Bringer-Meyer S, Sahm H (2006) The use of microorganisms in L-ascorbic acid production. J Biotechnol 124:196–205
2. Feng S, Zhang Z, Zhang CG, Zhang ZZ (2000) Effect of *B. megaterium* on *Gluconobacter oxydans* in mixed culture. J Appl Ecol 11:119–122 (In Chinese)
3. Jiao YH, Zhang WC, Xie L, Yuan HJ, Chen MX (2002) Effects of *Bacillus cereus* on *Gluconobacter oxydans* in vitamin C fermentation process. Microbiol 29:35–38 (In Chinese)
4. Leduc S, Troostembergh JC, Lebeault J (2004) Folate requirements of the 2-keto-L-gulonic acid-producing strain *Ketogulonigenium vulgare* LMP P-20356 in L-sorbose/CSL medium. Appl Microbiol Biot 65:163–167
5. Li Q, Diao JY, Xiang BT, Cao Z (1996) Study on metabolism of nitrogen source in fermentation of 2-keto-L-gulonic acid. Acta Microbiologica Sinica 36:19–24 (In Chinese)
6. Liu LM, Chen KJ, Zhang J, Liu J, Chen J (2011) Gelatin enhances 2-keto-l-gulonic acid production based on *Ketogulonigenium vulgare* genome annotation. J Biotechnol 156:182–187
7. Liu LM, Li Y, Zhang J, Zhou ZM, Liu J, Li XM, Zhou JW, Du GC, Wang L, Chen J (2011) Complete genome sequence of the industrial strain *Ketogulonicigenium vulgare* WSH-001.J. J Bacteriol 193:6108–6109
8. Lv SX, Niu JS, Ma D, Zhang L, Chen HQ, Zhang ZZ (2011) Effect of different components of *Bacillus megaterium* on *Gluconobacter oxydans* in mix-cultured of vitamin C fermentation. J Food Sci Biotechnol 30:700–704 (In Chinese)
9. Ma Q, Zhang WW, Zhang L, Qiao B, Pan CS, Yi H, Wang LL, Yuan YJ (2012) Proteomic analysis of *Ketogulonicigenium vulgare* under glutathione reveals high demand for thiamin transport and antioxidant protection. PLoS One 7:e32156
10. Ma Q, Zhou J, Zhang WW, Meng XX, Sun JW, Yuan JY (2011) Integrated proteomic and metabolomic analysis of an artificial microbial community for two-step production of vitamin C. PLoS One 6:1–9
11. Mandlaa, Yang WC, Han LT, Wang ZY, Xu H (2013) Two-helper-strain co-culture system: a novel method for enhancement of 2-keto-l-gulonic acid production. Biotechnol Lett 35:1853–1857
12. Pandhal J, Noirel J (2014) Synthetic microbial ecosystems for biotechnology. Biotechnol Lett 36(6):1141–1151
13. Pappenberger G, Hohmann H (2014) Industrial production of L-ascorbic acid (Vitamin C) and D-isoascorbic acid. Adv Biochem Eng Biot 143:143–188
14. Urbance JW, Bratina BJ, Stoddard SF, Schmidt TM (2001) Taxonomic characterization of *Ketogulonigenium vulgare* gen. nov., sp. nov. and *Ketogulonigenium robustum* sp. nov., which oxidize L-sorbose to 2-keto-L-gulonic acid. Int J Syst Evol Micr 51:1059–1070
15. Xu A, Yao JM, Yu L, Lv S, Wang J, Yan B, Yu ZL (2004) Mutation of *Gluconobacter oxydans* and *B. megaterium* in a two-step process of L-ascorbic acid manufacture by ion beam. J Appl Microbiol 96:1317–1323
16. Yang WC, Han LT, Mandlaa M, Chen HQ, Jiang MY, Zhang ZZ, Xu H (2013) Spaceflight-induced enhancement of 2-keto-L-gulonic acid production by a mixed culture of *Ketogulonigenium vulgare* and *Bacillus thuringiensis*. Lett Appl Microbiol 57:54–62
17. Yin GL, He JM, Ren SX, Song Q (1997) Production of vitamin C precursor-2-keto-L-gulonic acid from L-sorbose by a novel bacterial component system of SCB329-SCB933. J Ind Microbiol 27:1–7 (In Chinese)
18. Yin GL, Tao ZX, Yu LH, Wang DS (1980) Studies on the production of vitamin C precursor—2-keto-L-gulonic acid from

L-sorbose by fermentationI. Isolation, screening and identification of 2-Keto-L-gulonic acid producing bacteria. Acta Microbiologica Sinica 20:246–251 (In Chinese)
19. Yuan YJ, Yi H, W LL, Zhou J, Ma Q, Xue J (2009) A method of increased production of 2-keto-L-gulonic acid by *Gluconobacter oxydans*. China Patent CN200910069697.7
20. 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
21. 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*. Bioresource Technol 102:4807–4814
22. Zhang XG, Jiang JX, Wu H, Yuan WK, Chen MH (1988) Study on bioconversion of L-sorbose into 2-keto-L-gulonic acid (2KGA) (1) characteristics of reaction and analysis of affecting factors. Chem React Eng Technol 4:45–52 (In Chinese)
23. Zhou J, Ma Q, Yi H, Wang LL, Hao S, Yuan YJ (2011) Metabolome profiling reveals metabolic cooperation between *Bacillus megaterium* and K*etogulonicigenium vulgare* during induced swarm motility. Appl Environ Microb 77:7023–7030
24. 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. Bioresource Technol 107:399–404
25. Zou W, Liu LM, Chen J (2013) Structure, mechanism and regulation of an artificial microbial ecosystem for vitamin C production. Crit Rev Microbiol 39:247–255
26. Zou Y, Hu ML, Lv YJ, Wang Y, Song H, Yuan YJ (2013) Enhancement of 2-keto-gulonic acid yield by serial subcultivation of co-cultures of *Bacillus cereus* and *Ketogulonigenium vulgare*. Bioresource Technol 132:370–373