

Original Research Article

Construction of synthetic microbial consortia for 2-keto-L-gulonic acid biosynthesis

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ABSTRACT

Currently, the establishment of synthetic microbial consortia with rational strategies has gained extensive attention, becoming one of the important frontiers of synthetic biology. Systems biology can offer insights into the design and construction of synthetic microbial consortia. Taking the high-efficiency production of 2-keto-L-gulonic acid (2-KLG) as an example, we constructed a synthetic microbial consortium “*Saccharomyces cerevisiae*-*Ketogulonigenium vulgare*” based on systems biology analysis. In the consortium, *K. vulgare* was the 2-KLG producing strain, and *S. cerevisiae* acted as the helper strain. Comparative transcriptomic analysis was performed on an engineered *S. cerevisiae* (VTC2) and a wild-type *S. cerevisiae* BY4741. The results showed that the up-regulated genes in VTC2, compared with BY4741, were mainly involved in glycolysis, TCA cycle, purine metabolism, and biosynthesis of amino acids, B vitamins, and antioxidant proteases, all of which play important roles in promoting the growth of *K. vulgare*. Furthermore, Vitamin C produced by VTC2 could further relieve the oxidative stress in the environment to increase the production of 2-KLG. Therefore, VTC2 would be of great advantage in working with *K. vulgare*. Thus, the synthetic microbial consortium “VTC2-*K. vulgare*” was constructed based on transcriptomics analyses, and the accumulation of 2-KLG was increased by 1.49-fold compared with that of mono-cultured *K. vulgare*, reaching 13.2 ± 0.52 g/L. In addition, the increased production of 2-KLG was accompanied by the up-regulated activities of superoxide dismutase and catalase in the medium and the up-regulated oxidative stress-related genes (*sod*, *cat* and *gpd*) in *K. vulgare*. The results indicated that the oxidative stress in the synthetic microbial consortium was efficiently reduced. Thus, systems analysis confirmed a favorable symbiotic relationship between microorganisms, providing guidance for further engineering synthetic consortia.

1. Introduction

Oxidative stress could lead to macromolecular damage and disruption of redox signaling [1]. The accumulation of reactive oxygen species (ROS) is one of the main causes of oxidative stress [2]. The production of ROS can be scavenged by the cellular antioxidant system, including enzymatic components (superoxide dismutase, catalase, peroxidase, etc.) and non-enzymatic components (glutathione, NADPH, thioredoxin, glutaredoxin, vitamin C, etc.) [3]. Vitamin C is a water-soluble vitamin with important physiological functions, which has been proven to inhibit the generation of free radicals and reduce the oxidative damage

caused by ROS [4,5].

Currently, 2-keto-L-gulonic acid (2-KLG) is mainly produced through a two-step fermentation process developed in the 1980s [6]. The second step is to convert L-sorbose into 2-KLG by microbial co-cultivation, which is also the focus of microbial transformation research. 2-KLG-producing strain (*Ketogulonigenium vulgare*) and the helper strain (*Bacillus* sp.) are usually used as industrial strains for the synthesis of 2-KLG. Exploring the interaction between the two strains during co-culture has always been one of the hotspots in the field of 2-KLG fermentation. *K. vulgare* shows poor growth and low yield when cultured alone. Based on the research results of genomics, transcriptomics, proteomics,

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and metabolomics, scholars have done a lot of work in exploring the mechanism of interaction between the two strains from the perspective of amino acids, vitamins, and environmental stress [7–10]. *K. vulgare* lacks key enzymes for the synthesis of amino acids such as histidine, threonine, leucine, and isoleucine [11], while helper strains can synthesize amino acids lacking in *K. vulgare*. Our lab reconstructed the threonine biosynthetic pathway in *K. vulgare* to improve the biomass and 2-KLG production [12]. The expressions of genes related to amino acids transport and metabolism were significantly up-regulated in *K. vulgare* [13]. Studies have shown that *K. vulgare* needs the helper strain to provide B vitamins and purines to maintain normal metabolism [14,15]. In addition, the helper strain could secrete proteases (glutaredoxin, catalase, etc.) and up-regulate the expression of the antioxidant enzymes (superoxide dismutase, thioredoxin, etc.) in *K. vulgare* to alleviate oxidative stress caused by ROS metabolism disorder [16].

The construction of the synthetic microbial consortium is of great significance for the production of 2-KLG by *K. vulgare*. The helper strains for the industrial production of 2-KLG mainly include *Bacillus megaterium* [17], *Bacillus thuringiensis* [18], and *Bacillus cereus* [19], all of which are prokaryotic microorganisms. However, taking eukaryotic microorganisms as helper strains were rarely reported. *S. cerevisiae* is a conventional and well-studied model organism for which advanced genetic tools for metabolic pathway manipulation are available [20]. The co-cultured relationships between cells in synthetic microbial consortia are dynamically balanced, leading to greater adaptability and stability to variable environments, thus providing an important new field for synthetic biology. There are mainly two strategies for designing and constructing synthetic microbial consortia [21]. The first one is to construct microbial consortia based on genetic elements, modules, and metabolic pathways. The other one is to de novo design microbial consortia based on multiple omics analyses. Transcriptomic analyses can discover the dynamic expression and regulation mechanism of microbial genes, laying a foundation for exploring microbial community structure and ecological functions [22].

In this study, the synthetic microbial consortium was rationally designed based on system biology. Transcriptomic analyses were performed on the wild-type *S. cerevisiae* (BY4741) and the engineered *S. cerevisiae* (VTC2), which was a vitamin C producing strain constructed by our lab [4]. Taking advantage of VTC2, we constructed a synthetic microbial consortium system containing the *K. vulgare* and VTC2, which significantly increased the production of 2-KLG. This research could provide an effective reference for rational design of the synthetic microbial consortium to efficiently produce 2-KLG or other high value-added products.

2. Materials and methods

2.1. Strains

K. vulgare was an industrial strain, provided by LuWei Pharmaceutical Co. Ltd. *S. cerevisiae* BY4741 was a parent strain. *S. cerevisiae* VTC2 was a vitamin C-producing strain constructed by our laboratory [4]. Five exogenous modules from *Arabidopsis* were introduced into the chassis cells (BY4741) and the key gene *vtc2* was overexpressed to obtain the strain VTC2 harboring VC biosynthesis (Table S1).

2.2. Culture media and conditions

K. vulgare was grown in sorbose-corn steep liquor (CSL) medium containing 2% L-sorbose, 0.3% CSL, 1% peptone, 0.3% yeast extract, 0.3% beef extract, 0.1% urea, 0.1% KH_2PO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.1% CaCO_3 with pH 7.0, while *S. cerevisiae* was grown in yeast extract-peptone-dextrose (YPD) media containing 20 g/L of glucose, 10 g/L of yeast extract, and 20 g/L of peptone with pH 6.5. The medium of the co-cultured system was composed of 20 g/L of glucose and the CSL medium.

The seed culture of *K. vulgare* or *S. cerevisiae* was firstly prepared in 250 mL flasks with 50 mL seed medium at 30 °C, 220 rpm for 48 and 24 h, respectively. Then, the seed culture of *K. vulgare* or *S. cerevisiae* was transferred into another fresh seed medium to culture, acting as the final seed for subsequent fermentation, respectively. For mono-culture of *K. vulgare*, *S. cerevisiae*, or co-culture of *K. vulgare* and *S. cerevisiae*, the strains were inoculated into 250 mL flasks with 50 mL fresh seed medium with the initial OD_{600} of 0.1 and were cultured in the same conditions (30 °C, 220 rpm) for 72 h. The fermentation experiments were conducted in triplicates and data were shown as the means \pm S.D.

2.3. Biomass and metabolite analysis

Cell optical density (OD) was measured at 600 nm with TU-1810 spectrophotometer. The concentrations of extracellular L-sorbose and 2-KLG were detected by High Performance Liquid Chromatography (HPLC) (Waters, MA, USA), equipped with an Aminex HPX-87H column (Bio-Rad, CA, USA) and a refractive index detector. After fermentation, 2 mL of the fermentation broth was centrifuged at 12000 rpm for 10 min, and the supernatant was filtered through a 0.22 μm aqueous membrane for HPLC detection. 5 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.6 mL/min at 65 °C.

2.4. Transcriptomic analysis

Fermentation broths of VTC2 and BY4741 collected separately at 72 h were rapidly frozen and stored at -80 °C. RNA of these samples were extracted and transcriptomic analyses were carried out on the BGISEQ-500 platform. The sequencing data was filtered with SOAPnuke (v1.5.2) [23] to remove sequencing adapter, low quality, and unknown reads. Then the clean reads obtained and stored in FASTQ format were mapped to the references genome using HISAT2 (v2.0.4) [24]. Bowtie 2 (v2.2.5) [25] was applied to align the clean reads to the reference coding gene set. The expression level of genes was calculated by RSEM (v1.2.12) [26]. The significant differentially expressed genes (DEGs) were detected using DESeq2 (v1.4.5) [27] according to the criteria of the absolute value of \log_2 ratio ≥ 1 and Q value ≤ 0.05 . The distribution of DEGs in metabolic pathway was performed based on the Kyoto encyclopedia of genes and genome (KEGG) database [28].

2.5. Analysis of superoxide dismutase activity and catalase activity

The activities of superoxide dismutase (SOD) and catalase (CAT) were measured using commercially available kits. The SOD activity assay kit was from Sino Best Biological Technology Co. (Shanghai, China). The CAT activity assay kit was from Beyotime Co. (Nantong, China). These experiments were performed under the instructions of manufactures.

2.6. Analysis of gene expression by quantitative RT-PCR

The relative expression levels of related genes in *K. vulgare* were measured by quantitative PCR (qPCR). 1 mL of the 72 h fermentation broth from the mono-cultured and co-cultured shake flasks, respectively, was centrifuged at 4 °C and 4500 rpm to collect the supernatant, which was snap-frozen in liquid nitrogen. RNA was extracted using the RNAPrep pure Cell/Bacteria Kit (Tiangen). The extracted RNA was used for cDNA synthesis with the Transcriptor First Strand cDNA Synthesis Kit (Roche), following manufacturer's instructions. cDNA samples were diluted with ddH_2O and were used for qPCR. qPCR analysis was performed on the q225-0265 qPCR system using TransStart Top Green qPCR SuperMix (Transgen). The primers used for qPCR to quantify relevant genes were listed in Supplementary Table S2. The 16s rRNA was chosen as the internal reference gene and each sample was replicated three times. The relative expression of the genes was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [29].

3. Results

3.1. Transcriptomic analysis

The vitamin C-producing strain, *S. cerevisiae* VTC2, was constructed by introducing heterologous vitamin C biosynthetic pathways [4]. To investigate changes in the transcriptional levels of genes in the engineered VTC2, the transcriptomes of VTC2 and BY4741 were studied using RNA-sequencing. According to the criteria for judging DEGs [27], it was found that vitamin C production resulted in a significantly different expression of 2990 genes, 1492 of which were up-regulated and 1498 were down-regulated. KEGG pathway enrichment analysis of DEGs can help biologists better understand the functional relevance of DEGs [30]. KEGG analysis of DEGs for *S. cerevisiae* VTC2 vs. BY4741 were presented in Fig. 1. Based on the results of KEGG analysis, we specifically analyzed the important metabolic pathways related to the production of 2-KLG.

DEGs involved in the metabolic pathways were presented in Fig. 2 and Fig. 3. In the present study, the annotation of significant changes in transcript expression of genes related to glycolysis pathway and TCA cycle of VTC2 was shown in Fig. 2. By analyzing the gene expression profile of central carbon metabolism, it was found that most of the genes (*pgi1*, *pfk1*, *fba1*, *aps3*, *tdh2*, *gpm2*, *yor283w*, *err3*, *cdc19*, *pdcc6*, *acs2*, and *pdx1*) involved in glycolysis were significantly up-regulated in VTC2 compared with that of BY4741. In addition, most of the genes (*pyc1*,

pyc2, *adr2*, *mdh2*, *idh1*, *kgd1*, *gdh1*, and *sdh3*) throughout the TCA cycle were notably down-regulated. As shown in Fig. 2, several genes (*rki1*, *prs1*, *prs3*, and *prs4*) encoded by VTC2 in the pentose phosphate pathway (PPP) were significantly up-regulated, which enhanced the synthesis of ribulose-5-phosphate (Ru5P) and 5-phosphoribosyl 1-pyrophosphate (PRPP). In the metabolic pathway of fructose and mannose shown in Fig. 2, transcript levels of *pmi40* and *psa1* were observed to be up-regulated in VTC2, indicating that the carbon flux of the synthetic vitamin C precursor pathway was increased. However, the expressions of *gal1* and *gal3* were significantly down-regulated in the galactose metabolism pathway (Fig. 2), which might be related to the heterologous expression of the gene *gpp*, both of which catalyzed the conversion of L-galactose to galactose-1-phosphate. The step catalyzed by *gpp* was one of the rate-limiting steps in the synthesis of vitamin C [4]. Thus, overexpression of exogenous genes might result in decreased expression of endogenous genes.

Expression of genes related to amino acids synthesis pathway was up-regulated in VTC2 (Fig. 2), which mainly including *cys4*, *got2*, *hom3*, *hom6*, *thr4*, *iv3*, *bat1*, *leu2*, *arg3*, *arg4*, *arg7*, *lys12*, *pro1*, *ser1*, and *shm2*. In addition, several genes (*gcv2*, *car1*, *put1*, *agx1*, and *cha1*) related to the amino acid degradation were significantly down-regulated (Fig. 2). These genes were mainly involved in the synthesis of aspartate, threonine, isoleucine, leucine, glutamate, ornithine, arginine, valine, lysine, glycine, cysteine, proline, and serine. The gene *ykl069w*, involved in methionine synthesis, was up-regulated by 2.32-fold and catalyzed the reversible redox of the R-enantiomer of free methionine sulfoxide to methionine, which was involved in resistance to oxidative stress [31]. It was also found that the *uga1* and *uga2* genes were significantly up-regulated in the catabolic pathway of glutamate. γ -aminobutyric acid in the glutamate metabolic pathway of *S. cerevisiae* is further catabolized to succinate, producing the major intracellular antioxidant NAD(P)H, an essential contributor to the maintenance of intracellular antioxidant levels [32]. As shown in Fig. 3A, significantly up-regulated genes related to B vitamins were mainly concentrated in *thi5*, *ltp1*, *pho12*, *fsh3*, *bio2*, *bio4* and *npt1*. The transcriptional levels of genes involved in purines metabolisms were up-regulated, such as *gua1*, *imd2*, *imd3*, *ade13*, and *fap7* (Fig. 3B). As shown in Fig. 3C, the expression level of genes (*cta1*, *ure2*, *grx7*, *hyr1*, and *gtt1*) involved in the synthesis of antioxidant proteases were up-regulated in VTC2 compared with that of BY4741.

3.2. Design and construction of synthetic microbial consortium

The transcriptomic analyses showed that VTC2, compared with BY4741, had great advantages in the metabolism of carbon, amino acids, vitamins B, purines, and antioxidant proteases. Therefore, VTC2 might have the ability to promote the growth of *K. vulgare* and enhance 2-KLG production by providing metabolites and assisting in resisting environmental stress. From the perspective of rational design and construction, VTC2 and BY4741 were used as the helper strain of *K. vulgare*, respectively, to construct the synthetic microbial consortia.

Firstly, the effects of different carbon sources on *K. vulgare* and *S. cerevisiae* were investigated. As shown in Fig. 4A, *S. cerevisiae* could not grow in the medium with L-sorbose as the only carbon source, i.e. *S. cerevisiae* did not consume L-sorbose. Besides, the growth of *K. vulgare* had better performance in the mixture of glucose and L-sorbose compared to the sole L-sorbose, which indicated that *K. vulgare* could also metabolize glucose to promote its growth (Fig. 4A). However, *K. vulgare* contributed almost the same yield of 2-KLG whether there was glucose in the CSL medium or not, i.e. glucose did not have an effect on the yield of 2-KLG (Fig. 4A).

Compared with the mono-cultured system, both VTC2 and BY4741 had the ability to increase the yield of 2-KLG. As shown in Fig. 4A, in the VTC2-*K. vulgare* microbial consortium, *K. vulgare* produced 13.2 ± 0.52 g/L of 2-KLG, which was 1.49 times higher than the mono-cultured fermentation (8.5 ± 0.21 g/L). A 20% increase in production of 2-KLG was also observed in BY4741-*K. vulgare* microbial consortium

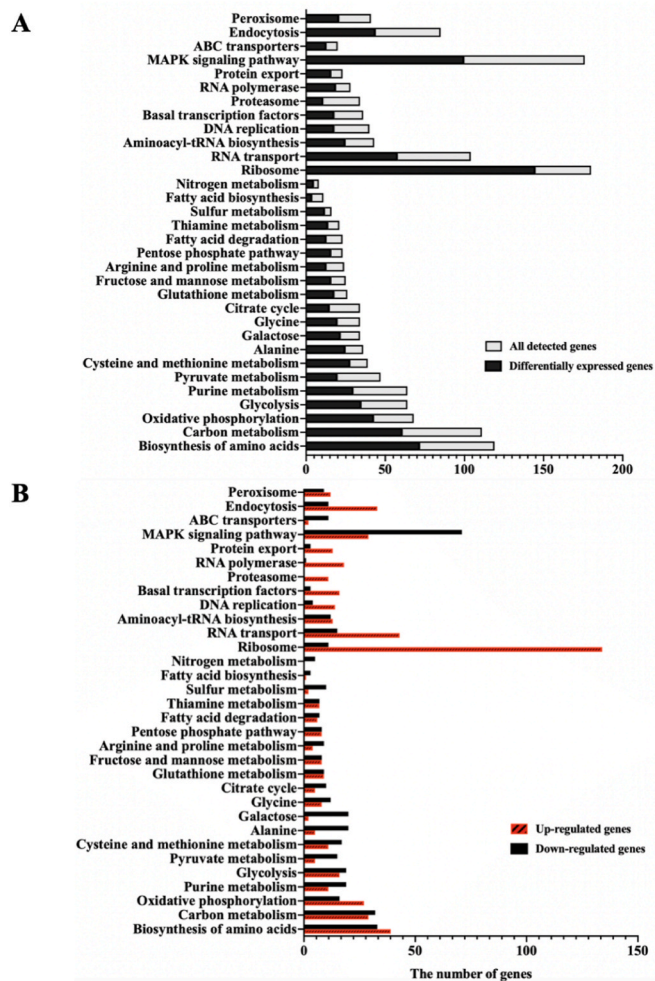


Fig. 1. KEGG pathway ontology enrichment analysis. (A) The numbers of all predicted genes and DEGs. (B) The numbers of up-regulated genes and down-regulated genes in DEGs.

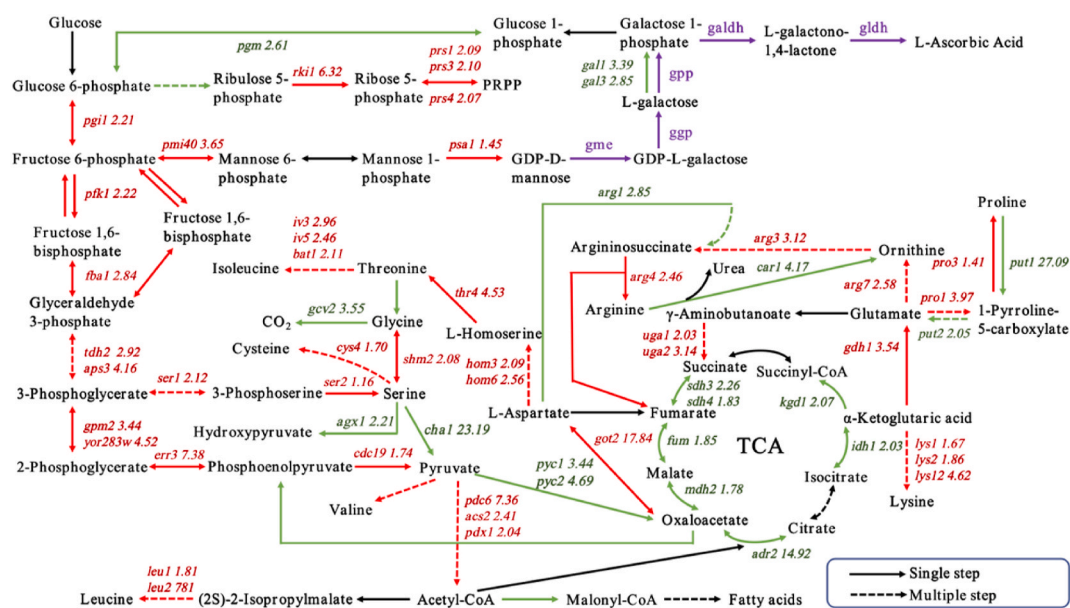


Fig. 2. Schematic diagram of DEGs involved in glycolysis, the TCA cycle, pyruvate metabolism, pentose phosphate pathway, amino acid biosynthesis, and vitamin C biosynthesis pathway. The numbers indicate the values of the ratios of the expression levels in VTC2 vs. BY4741. Red indicates significant up-regulation. Green indicates significant down-regulation. Black indicates non-significant change. Purple indicates the genes introduced exogenously.

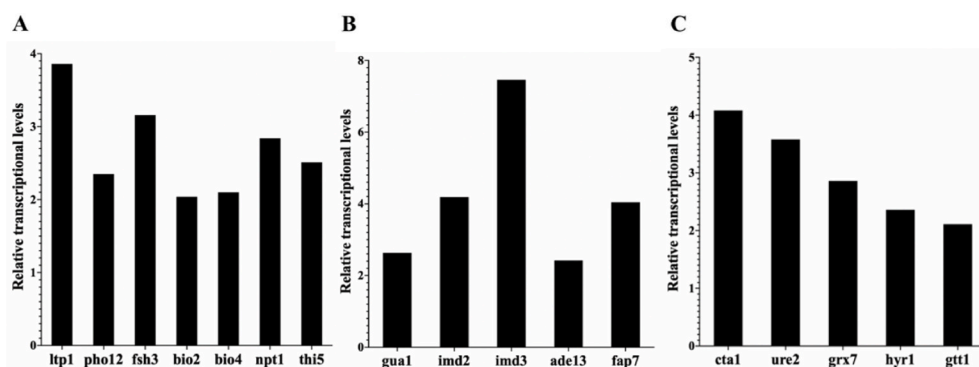


Fig. 3. The relative transcriptional levels of genes involved in different metabolic pathways. (A) B vitamins biosynthesis. (B) Purines metabolism. (C) Antioxidant proteases. The numbers indicate the values of the ratios of the expression levels in VTC2 vs. BY4741. The error bars showed the standard deviations of three biological replicates.

compared with that of mono-cultured system. Combined with the results of transcriptomic analyses, both VTC2 and BY4741, as helper strains, could provide an adaptive microenvironment through producing amino acids, vitamins, antioxidant proteases, and other substances to promote the cellular growth of *K. vulgare* (Fig. 4B). And this result could further promote the 2-KLG production of *K. vulgare*. However, in VTC2-*K. vulgare* microbial consortium, the yield of 2-KLG was 1.25 times higher than that of BY4741-*K. vulgare*. The higher yield appeared in VTC2-*K. vulgare* system might result from the higher transcriptional levels of key metabolic genes and the antioxidant effect of vitamin C.

3.3. Analysis of SOD and CAT activities in fermentation system

Superoxide dismutase (SOD) is a protective enzyme of antioxidants that can scavenge ROS [33]. Determination of SOD activities helps to understand the ability of cells to clear superoxide anions. The total activity of superoxide dismutase was shown in Fig. 5A, telling that there were significant differences in SOD activities between mono-cultured systems and co-cultured systems. The SOD values of BY4741-*K. vulgare* and VTC2-*K. vulgare* microbial consortia were 4 times and 6 times higher than those of *K. vulgare* mono-cultured system,

respectively. It was found that the cells of VTC2-*K. vulgare* microbial consortium had stronger superoxide anion scavenging abilities and could effectively protect Fe-S clusters from the attack of superoxide anion [34].

Catalase is an important antioxidant enzyme that can decompose hydrogen peroxide, a kind of ROS, into the water and molecular oxygen [35]. The CAT activities were also measured in this study to reflect the efficiency of removing H₂O₂ from the fermentation system. As shown in Fig. 5B, the CAT values of BY4741-*K. vulgare* and VTC2-*K. vulgare* microbial consortia were 1.5 times and 2.5 times higher than those of *K. vulgare* in the mono-cultured system, respectively.

The results of SOD and CAT activities showed that the total antioxidant capacity of microbial consortia was higher, which should be closely related to the antioxidant substances released by *S. cerevisiae*. In the presence of various antioxidant substances, ROS generated by cell metabolism and substrate conversion could be degraded and eliminated in time to alleviate the oxidative stress on the cells of *K. vulgare*. Since the vitamin C produced by VTC2 had great antioxidant effects, the total antioxidant capacity of the VTC2-*K. vulgare* system was stronger than that of BY4741-*K. vulgare* system.

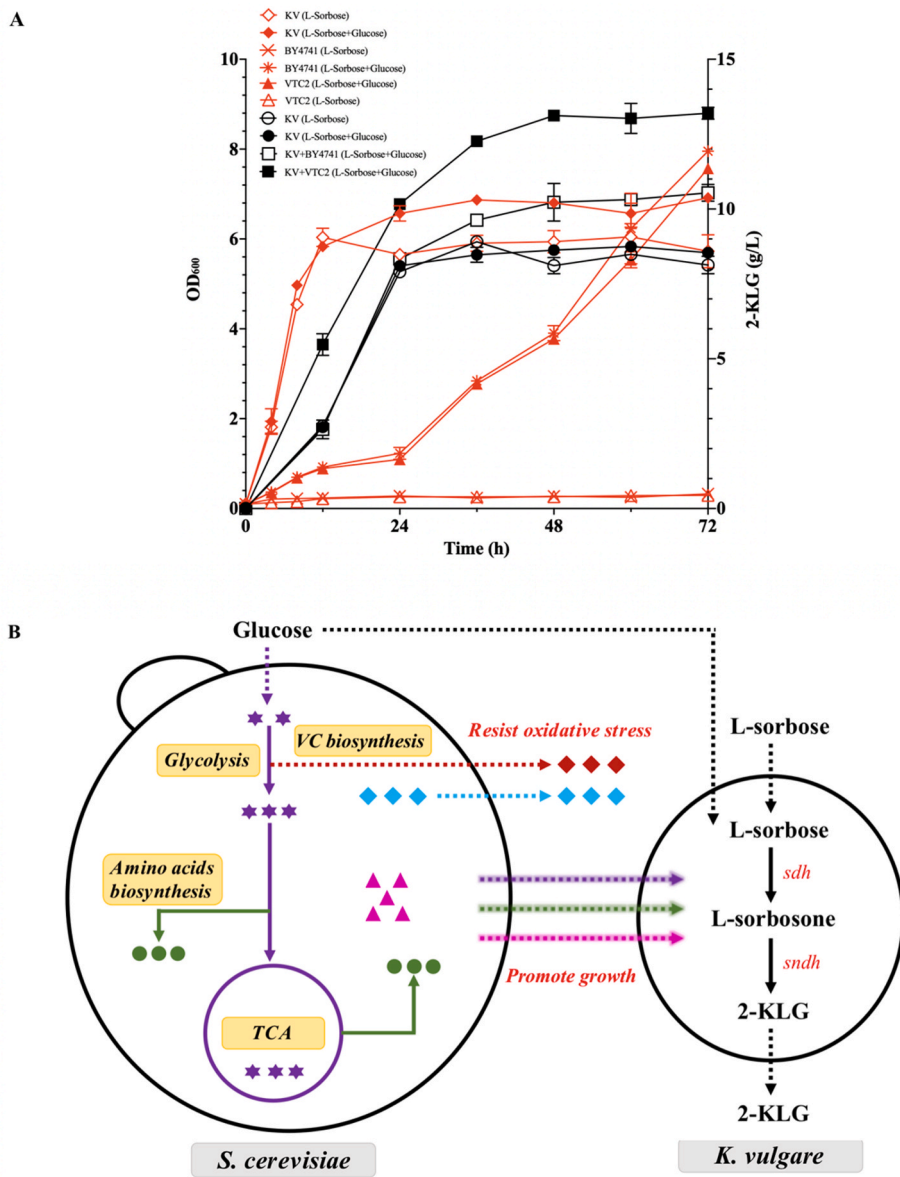


Fig. 4. The construction of the synthetic microbial consortium. (A) Time courses showed changes in cell concentration (red), and 2-KLG production (black). The content in brackets represents the type of carbon sources. KV: *K. vulgare*. The error bars showed the standard deviations of three biological replicates. (B) Schematic diagram of the interaction between *S. cerevisiae* VTC2 and *K. vulgare*. Purple stars: intermediate products of central carbon metabolism; Green circles: amino acids; Pink triangle: vitamins and purines; Blue rhombus: antioxidant proteases; Red rhombus: vitamin C.

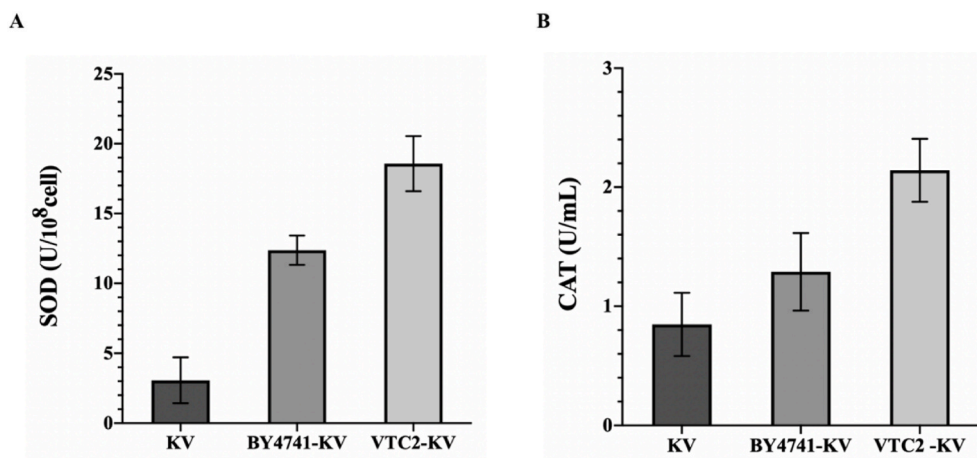


Fig. 5. Determination of antioxidant activities in different fermentation systems. (A) SOD activities (B) CAT activities. KV: *K. vulgare*. The error bars showed the standard deviations of three biological replicates.

3.4. Transcriptional analysis of *K. vulgare* in microbial consortium

To further investigate the significant increase in the production of 2-KLG in VTC2-*K. vulgare* microbial consortium, the transcriptional levels of key genes in the 2-KLG synthesis pathway, and oxidative stress-related genes in *K. vulgare* were simultaneously investigated by qPCR. As shown in Fig. 6, genes involved in the metabolic process of reducing sorbose and sorbosone to 2-KLG, such as *ssda1*, *ssda2*, *ssda3*, *ssdb*, and *sndh* were up-regulated by 1.62, 1.64, 1.47, 1.73, and 1.74 times, respectively.

Studies have shown that the pathways of glycolysis and TCA circle in *K. vulgare* were incomplete, and the carbon metabolism mainly relied on the pentose phosphate pathway (PPP) [36]. The PPP is accompanied by the production of a large amount of NADPH, which can provide cells with more reducing power. And *gpd* is the rate-limiting enzyme of the PPP pathway. In addition, *cat* and *sod* are common antioxidant genes. The expression levels of *gpd*, *cat*, and *sod* genes in the co-cultured system were up-regulated by 1.45, 2.84, and 2.67 times (Fig. 6), respectively, significantly higher than those in the mono-cultured system. Therefore, VTC2 induced the up-regulated expression of antioxidant-related genes in *K. vulgare* to eliminate the ROS metabolism disorder and enhanced the ability of *K. vulgare* to produce 2-KLG.

3.5. Effect of vitamin C addition on the production of 2-KLG

In order to investigate whether the oxidative stress on *K. vulgare* was relieved by vitamin C, a product of VTC2, different concentrations of vitamin C were exogenously added to the mono-cultured fermentation. The curves of the growth, the L-sorbose consumption, and the 2-KLG production of *K. vulgare* were indicated in Fig. 7. As shown in Fig. 7A and B, *K. vulgare* grew better and consumed L-sorbose more efficiently under exogenous addition of 20 mg/L vitamin C, and had the highest yield of 2-KLG (10.23 ± 0.15 g/L) (Fig. 7C). However, the yield of 2-KLG was not improved with the addition of 1 mg/L and 100 mg/L of vitamin C to the fermentation system. The antioxidant effect of low-concentration vitamin C was poor since Vitamin C is easily consumed by oxidation in the air [37]. However, the growth and 2-KLG production

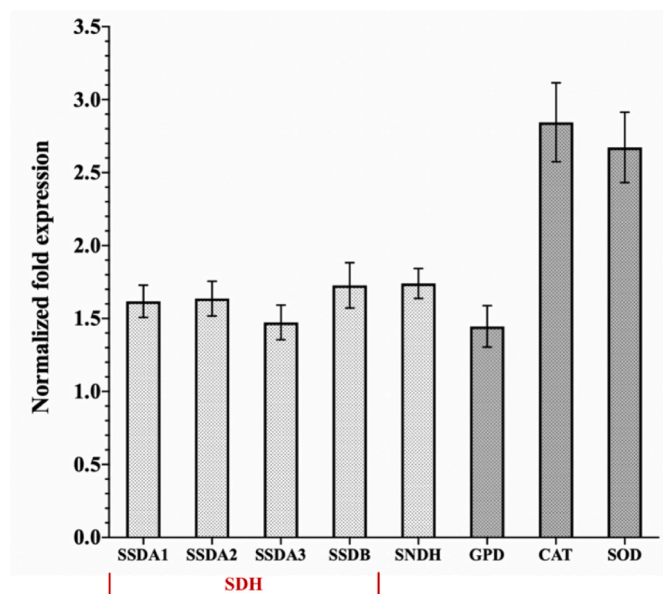


Fig. 6. Transcriptional level of relevant genes in *K. vulgare*. sorbose dehydrogenase: *ssda1*, *ssda2*, *ssda3*, *ssdb*; sorbosone dehydrogenase: *sndh*; 6-phosphogluconate dehydrogenase: *gpd*; catalase: *cat*; superoxide dismutase: *sod*. The values in the graphs indicate the ratio of gene expression levels of *K. vulgare* in co-culture vs. mono-culture. The error bars showed the standard deviations of three biological replicates.

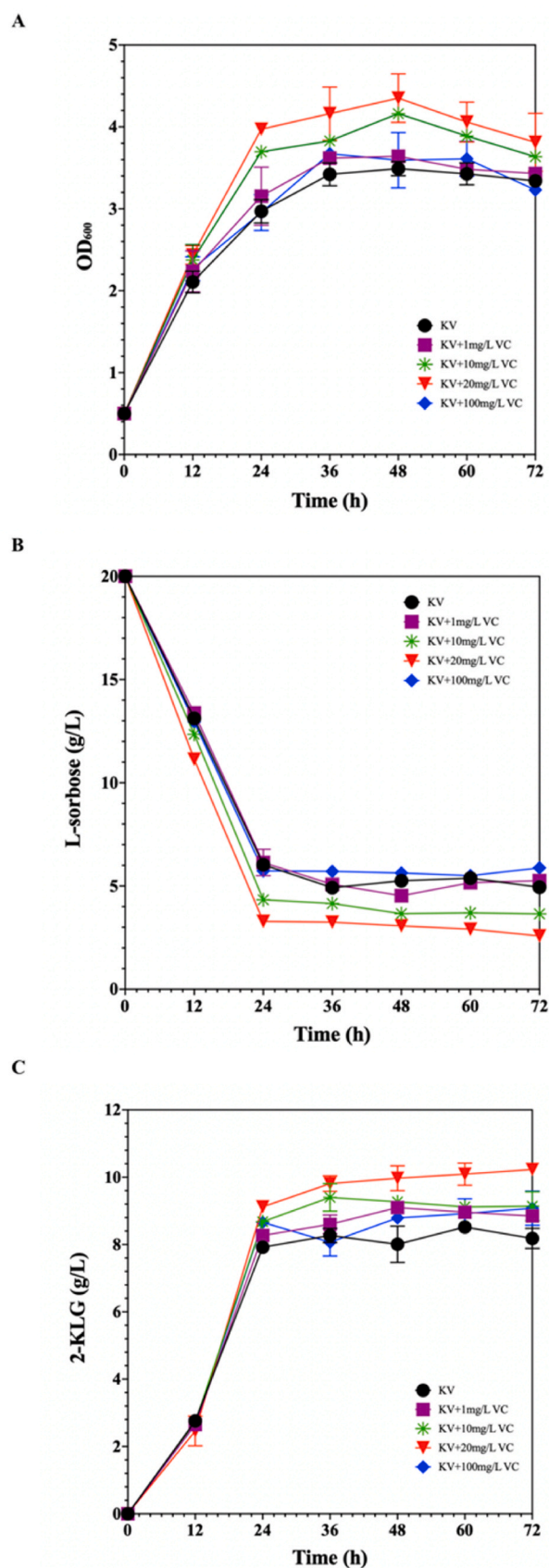


Fig. 7. Effect of vitamin C addition on *K. vulgare*. (A) Cell concentration, (B) L-sorbose consumption, (C) 2-KLG production. The error bars showed the standard deviations of three biological replicates.

of *K. vulgare* were not significantly enhanced when a high concentration of vitamin C was added exogenously, indicating that a high concentration of vitamin C was not suitable to be added in the fermentation system.

It was found that exogenous vitamin C with a certain concentration significantly promoted the growth and 2-KLG production of *K. vulgare*. This result indirectly confirmed that vitamin C produced by VTC2 helped to alleviate the oxidative stress suffered by *K. vulgare*. Therefore, the construction of this synthetic microbial consortium had practical implications.

4. Discussion

Synthetic microbial consortia could perform complicated tasks and endure the changeable environment, thus providing an important new frontier for synthetic biology [38]. Recent advances in multi-omics and automation technology have enabled researchers to focus on engineering the microbiome's metabolic network and microbial interactions. The design principles for synthesizing microbial consortia are mainly based on the patterns of microbial interactions, including cell-cell communications, and exchange of metabolites and energy, etc. To achieve predictions of microbiome function at a systems level, measurements of the microbiome's in situ metabolic network structure and activity are essential [39]. Systems biology approaches enable the objective description of genetic and metabolic pathways in microbial consortia, which facilitates the design and optimization of synthetic consortia [40]. Systems biology analysis [21], particularly meta-omics techniques (e.g. meta-genomics [41,42], meta-proteomics [16], meta-transcriptomics [43], and meta-metabolomics [8]), can be combined with bioinformatic tools to enable the analysis of individual species in the microbiome and global measurements of proteins and metabolites. This provides insights into the design and construction of functional genes, functional modules, and entire synthetic consortia. In this study, comparative transcriptome analyses of VTC2 and BY4741 were performed to objectively characterize the genetic and metabolic pathways of VTC2, which facilitated the design and optimization of the synthetic consortium.

As described in previous studies, the glycolysis pathway and the TCA cycle were central metabolic pathways of glucose metabolism in *S. cerevisiae* [44]. The genes involved in glycolysis were significantly up-regulated in VTC2 compared with those of BY4741. Thus, VTC2 had higher glucose utilization efficiency compared to BY4741 and could provide more precursors for the synthesis of vitamin C. The transcription levels of most genes were significantly down-regulated in VTC2 throughout the TCA cycle compared with that of BY4741, indicating a decrease in the production of reducing substances and ATP, i.e. the strains were in a stable growth phase at 72 h. And 2-ketoglutarate decarboxylase (encoded by *kgd1*) catalyzed the oxidative decarboxylation of 2-ketoglutarate to succinyl coenzyme A, a key reaction of the TCA cycle [45]. The down-regulation of *kgd1* transcription might lead carbon flux into the biosynthesis of glutamate, the source of 80% of cellular nitrogen [46]. The PPP is also a fundamental component of central carbon metabolism, maintaining carbon homeostasis. The PPP provided precursors for nucleotide and amino acid synthesis, and also provided reducing substances to eliminate oxidative stress [47,48]. The up-regulation of the transcription of key metabolic genes in the PPP pathway could lead to an increase in the synthesis of Ru5P and PRPP. Ru5P is a precursor for the synthesis of nucleotides. PRPP is an important precursor of de novo synthesis of purine nucleotide and pyrimidine nucleotide [49,50].

The establishment of symbiosis between strains via metabolites exchanges is a common mechanism in synthetic microbiota [51]. Modularity is introduced into the pathway design of microbial metabolite production, in which metabolic intermediates provided by one microorganism were transferred to the other one. This unidirectional interaction can stabilize the *Escherichia coli*-*S. cerevisiae* co-culture for high

production of oxygenated taxanes [52]. In addition, the mutualism is enhanced by the exchange of metabolites between strains. It is considered that cross-feeding of metabolites in synthetic consortia would facilitate the heterologous production of flavonoids [53]. The expression of vitamin C heterologous biosynthetic pathway in VTC2 resulted in the transcriptional up-regulation of genes involved in the biosynthesis of amino acids, B vitamins, purines and antioxidant proteases compared with BY4741.

Amino acids play an important role in cell growth, metabolism, and cell survival in severe environments. Proline is an essential stress protector in yeast cells and also participates in numerous important intracellular physiological processes, such as maintaining protein and membrane stability and scavenging ROS [54]. Valine, leucine, glycine, and serine are derived from the glycolysis pathway. Higher intracellular levels of amino acids derived from the glycolysis pathway reflect the higher glycolytic capacity of the cell [55]. Cysteine can be used to synthesize glutathione, which is the most important intracellular antioxidant. And cysteine oxidation can be used to control protein function and cell signaling pathways [56]. Previous studies have shown that *K. vulgare* itself could not synthesize B vitamins [15,57], whereas *S. cerevisiae* contained the B vitamins synthesis pathway and supplied the synthesized B vitamins to *K. vulgare* to maintain its normal growth metabolism in our constructed consortium. The B vitamins comprised a group of water-soluble vitamins (thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, biotin, etc.) that acted as cofactors, precursors, and substrates for biological processes [58]. Pyridoxine is a cofactor for more than 140 biochemical reactions in cells [59], most of which are related to amino acids biosynthesis. In addition, pyridoxine can also quench ROS [60]. Folic acid and its derivatives are also important factors for the growth of *K. vulgare* [61]. However, *K. vulgare* is unable to utilize folic acid for deficiency of folate reductase, while *S. cerevisiae* can metabolize folic acid to dihydrofolate and tetrahydrofolate that can be utilized by *K. vulgare*. In addition, adenine, guanine, xanthine, and hypoxanthine produced by helper strains could contribute to the resistance of the microflora to ROS [14]. Cellular redox systems included glutathione, thioredoxin, pyridine nucleotides, and NADPH [62]. Among them, antioxidant proteases mainly include superoxide dismutase, catalase, glutathione, and glutathione. In summary, the metabolites released by VTC2 could compensate for the defective growth of *K. vulgare*. In addition, the results that exogenous addition of vitamin C raised 2-KLG production also further confirmed that VTC2, producing high vitamin C, had better concomitant effects.

The consumption of the same carbon source by two strains can lead to competitive interactions and unstable symbiosis in microbial communities. Differences in nutrient metabolism in symbiotic systems can reduce or eliminate competitive exclusion, and make symbiosis possible. Therefore, avoiding carbon source competition is one of the strategies to achieve effective symbiosis. For example, a bacterial consortium consisting of a glucose-selective strain and a xylose-selective strain was constructed to achieve high yields of n-butanol [63]. Here, we used a dual carbon source of glucose and L-sorbose for fermentation. The results showed that *S. cerevisiae* did not compete with *K. vulgare* for L-sorbose, thus achieving optimal growth of *K. vulgare*. *S. cerevisiae*, as a model organism, is widely used in large-scale industrial production due to intensive research on its genetic and growth characteristics. The focus of this study was to explore the advantages of applying engineered *S. cerevisiae* to the microbial consortia. *K. vulgare* suffered from weak growth and low production due to oxidative stress and lack of nutrients required for growth. However, the results of the transcriptomic analysis indicated that VTC2 had the potential to promote the growth of *K. vulgare*. Therefore, this study took VTC2 as the helper strain with *K. vulgare* to form the synthetic microbial consortium, and successfully achieved a significant increase in the production of 2-KLG. In addition, ROS are harmful byproducts of aerobic metabolism. When ROS levels increase, oxidative stress will occur, destroying cellular biomolecules (DNA, proteins, and lipids) [2], limiting cellular growth and 2-KLG

production. In order to explore the antioxidant capacity of different 2-KLG fermentation systems, the SOD and CAT activities were evaluated, respectively. The results showed that the synthetic microbial consortium had a stronger antioxidant capacity compared to the mono-cultured system, which was associated with the high vitamin C production of VTC2. It was also demonstrated that VTC2, as the helper strain, induced a significant up-regulation of 2-KLG producing and antioxidant-related genes in *K. vulgare*. These results further validated the advantages of the VTC2 as the helper strain in the synthetic microbial consortium. This microbial consortium also has implications for continued research and improvement. For example, engineered *K. vulgare* could work with VTC2 to form a more efficiently microbial consortium for 2-KLG production. And transcriptomic analyses of the microbial consortium are indeed an important direction to obtain more information of the microbial consortium. In addition, the constructed consortium could be expanded in controlled fermenters to achieve a higher yield of 2-KLG.

5. Conclusions

In this study, systems biology analysis was applied to construct a meaningful synthetic microbial consortium. The yield of 2-KLG was significantly improved in the co-cultured system compared with that of the mono-cultured system. To the best of our knowledge, this is the first report on increasing 2-KLG production via taking engineered *S. cerevisiae* as helper strain. This study provided a reference for subsequent rational design and the construction of synthetic microbial consortia.

CRedit authorship contribution statement

Yan Wang: Conceptualization, Methodology, Investigation, Writing – original draft. **Hengchang Li:** Investigation, Methodology. **Yu Liu:** Investigation, Methodology. **Mengyu Zhou:** Investigation, Methodology. **Mingzhu Ding:** Conceptualization, Supervision, Project administration, Writing – review & editing. **Yingjin Yuan:** Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.synbio.2021.12.001>.

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