Xia *et al. Annals of Microbiology* (2022) 72:23
https://doi.org/10.1186/s13213-022-01680-w

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

ORIGINAL ARTICLE

Open Access

The function of uridine diphosphate glucose pyrophosphorylase in the lyophilization-stress response of *Lactobacillus acidophilus*

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Abstract

Purpose: Uridine diphosphate glucose pyrophosphorylase (UGPase) plays an important role in glucose metabolism, catalyzing the reversible formation and decomposition of UDP-glucose (UDPG). In previous work, we found that UGPase is a key enzyme in lyophilization response for *Lactobacillus acidophilus* (*L. acidophilus*). However, its function and regulatory mechanism in the freeze-drying stress response are unknown. Herein, the effect of UGPase on freeze-drying survival rate of *Staphylococcus carnosus* (*S. carnosus*) was studied.

Methods: In this work, the genes *LBA*1719 encoding UGPase of *L. acidophilus* ATCC4356 were inserted into plasmid pMG-36e to construct the recombinant plasmid pMG-LBA1719 and then overexpressed in *S. carnosus*; the control group was *S. carnosus* transformed by pMG-36e. The lyophilization-survival rate of overexpressed *S. carnosus* was determined, and the differentially expressed genes (DEGs) were analyzed by transcriptome to disclose the mechanism of *LBA*1719 in regulating the lyophilization-survival rate.

Results: Compared with the control group, the UGPase activities of the overexpressed *S. carnosus* increased by 35.49%, while the lyophilization-survival rates decreased by 11.17% ($p < 0.05$). Overexpression of *LBA*1719 decreased the expression of genes *gap*A, *gap*B, and *pgi*A in carbohydrate metabolism and *dap*A, *dap*B, and *dap*E in amino acid metabolism, significantly changing the physiological characteristics of *S. carnosus* and decreasing its lyophilization-survival rate.

Conclusion: In summary, overexpression of UGPase accelerated the growth rate of *S. carnosus* and reduced its lyophilization-survival rates. *Gap*A, *gap*B, *pgi*A, *dap*A, *dap*B, and *dap*E are vital to lyophilization protection in lactic acid bacteria (LAB). These findings provide new theoretical basis for analyzing the regulatory and molecular mechanisms of lyophilization resistance in LABs.

Keywords: Uridine diphosphate glucose pyrophosphorylase, Lyophilization-survival rate, RNA-seq, Lyophilization stress

Key points

(1) UGPase regulation of lyophilization response in *L. acidophilus* is analyzed by RNA-seq.
(2) UGPase genes *LBA*1719 respond to lyophilization stress.

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(3) *LBA*1719 decreases survival rate by regulating carbohydrate and amino acid metabolism.
(4) *Gap*A, *gap*B, *pgi*A, *dap*A, *dap*B, and *dap*E are vital to lyophilization protection in LAB.

Introduction

Uridine diphosphate glucose pyrophosphorylase (UGPase) is widely distributed in nature, existing in the tissues of numerous organisms. Accordingly, since its first discovery by Munch-Petersen in 1953 (Munch-Petersen et al. 1953), UGPase has been isolated and purified from animals (Konishi et al. 1993), plants (Elling and Kula 1994), and microbes (Hossain et al. 1994). It has received considerable research attention as it is a crucial enzyme in glucose metabolism, catalyzing the reversible synthesis and decomposition UDP-glucose (UDPG), i.e., uridine triphosphate (UTP) + glucose-1-phosphate (Glc-l-P) ⇌ UDPG + inorganic pyrophosphate (PPi) (Aksamit and Ebner 1972). UDPG is an important form of uridine diphosphate, which is a key metabolite in the metabolic pathways of organisms and is a precursor of a variety of polysaccharides, including glycogen, trehalose, cellulose, and β-glucan (Yi and Huh 2015), which can be further converted to sucrose, hemicellulose, cellulose, colloids, glycoproteins, and proteoglycans. UDPG also plays important roles in the glycosylation of a series of secondary metabolites, such as steroids, flavonoids, phenylpropane, and guanidine.

The function of UGPase has been studied mainly by genetic engineering in fungus, protista, and plants. Zan et al. (Zan et al. 2020) found that silence UGPase gene significantly changed the mycelial growth and polysaccharide production of *Grifola frondosa*; the UGPase of *Phaeodactylum tricornutum* plays an important role in chrysolaminarin biosynthesis and carbon allocation (Zhu et al. 2016); Dae Gwan (Yi and Huh 2015) silenced *UGP*1 gene of *Saccharomyces cerevisiae* and found that trehalose content decreased. In the *Euglena gracilis* cells, the paramylon accumulation decreased by 60% after knockdown of the UGPase genes (Muchut et al. 2021). Meanwhile, the study of UGPase in plants is quite common. In *Arabidopsis thaliana*, UGPase activity is essential for both vegetative and reproductive phases (Jong-In et al. 2010); Balan et al.(Balan et al. 2018) determined the UGPase activity in four different thermotolerant varieties of wheat (*Triticum aestivum*), viz., and the kinetic was studied, and Gupta identified the UGPase in potato (Gupta 2017); Long (Long et al. 2017) found that the activity of UGPase has an important influence on starch biosynthesis in rice endosperm. However, little is known for the function of UGPase in lactic acid bacteria (LAB).

Lyophilization, more colloquially known as freeze-drying, is the preferred method of preserving microorganisms for transport, and it is currently widely used for preparing starters. However, bacteria can be damaged to some extent by lyophilization. In our previous work (Zhen et al. 2020), the lyophilization-survival rate of *Lactobacillus acidophilus* was significantly increased by heat-shock treatment at 45 °C for 30 min before freeze-drying. Our results revealed that the activity and protein expression of UGPase in the *L. acidophilus* were increased in the treated bacteria, indicating that UGPase is a key enzyme in lyophilization response. Nevertheless, the mechanism by which UGPase regulates the lyophilization-survival is still unknown.

In the present study, UGPase-encoding genes *LBA*1719 from *L. acidophilus* were cloned into an expression vector, over-expressed in *S. carnosus* which is effective gram-positive expression system as well as meat products fermentation starter. Transcriptomics plays an important role in revealing the differences of gene expression (Liu et al. 2015). Recent advances in RNA library preparation methods, platform accessibility, and cost efficiency have allowed high-throughput RNA sequencing (RNAseq) as the method of choice for transcriptome analyses (Han et al. 2015). Differentially expressed genes (DEGs) were identified by comparing RNA-seq data from *LBA*1719 over-expressing and non-overexpressing cells, revealing details of its regulatory mechanism in lyophilization.

Materials and methods

Bacterial strains and plasmids

L. acidophilus ATCC4356 and *S. carnosus* TM300 ACCC01657 were purchased from the Agricultural Culture Collection of China. *L. acidophilus* ATCC4356 was used as a source for the UGPase open reading frame. *S. carnosus* TM300 was a host strain for recombinant protein expression. pMG36e (Biosci Biotech, Hangzhou, China) was used as an expression vector.

Plasmid construction

The genes *LBA*1719 encoding UGPase were amplified by polymerase chain reaction (PCR), using the following primers: forward primer (5′-TCGACCTGCAGG CATGCAATGCACCATCATCACCATCATATGAAA GTAAGAAAAGCCATC-3′) and reverse primer (5′-GTTTTCAGACTTTGCAAGCTTTATTCCTTTTCA AGCTTCTT-3′) for *LBA*1719. Genomic DNA of *L. acidophilus* ATCC4356 was prepared using a Bacterial Genomic DNA Purification Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. PCR was performed at a volume of 50 μL with 10 μL of 5× PrimeSTAR Buffer (Takara, Dalian, China), 4 μL of dNTP mixture, 0.5 μL of PrimeSTAR

HS DNA Polymerase (Takara, Dalian, China), 2 μL of genomic DNA (50 ng), 1 μL (10 mM) of each forward and reverse primer, and 31.5 μL of ddH_2O. PCR conditions were set as follows: 1 cycle at 94 °C for 4 min followed by 30 cycles at 98 °C for 10 s, one cycle at 55 °C for 5 s, one cycle at 72 °C for 1 min, and 1 cycle for final extension at 72 °C for 5 min. The vector of pMG36e was digested with HindIII. Seamless cloning was used in this experiment. When designing primers, 18 homologous fragments were placed upstream of *LBA*1719, a start codon ATG and a 6× His-tag were added after the fragment, and 20 homologous fragments were placed downstream. The DNA fragment was cloned into the pMG36e vector using a Seamless Cloning Assembly Kit (0.05 pmol of PCR product, 0.025 pmol of pMG36e vector, 2.5 μL of 2× Assembly Mix (TransGen Biotech, Beijing, China), made up to 5 μL with ddH_2O). The ligation reaction was performed at 50 °C for 15 min. The generated recombinant plasmids were named pMG-LBA1719. DNA sequencing confirmed that UGPase genes *LBA*1719 were correctly inserted into the pMG-36e vector.

Transformation protocol

To obtain *S. carnosus* competent cells, a single colony was isolated from a lysogeny broth (LB) agar plate and inoculated into 50 mL of LB medium at 37 °C with agitation at 200 rpm. Then, 2% (*v/v*) was inoculated into 100 mL LB medium containing 0.5% glucose and 0.5% glycine. After the cultures had grown to an optical density at 600 nm (OD_{600}) of ca. 0.8, the cells were harvested by centrifugation at 4 °C and 3000 ×g for 10 min followed by two washes in 20 mL of sterile water and two washes in 20 mL pre-cooled solution A containing 10% glycerol and 10% sucrose. Finally, the cells were suspended in 0.5 mL of ice-cold solution A and then stored in aliquots at −80 °C until use.

The *S. carnosus* TM300 competent cells were thawed on ice. Portions (80 μL) were mixed with ca. 1 μg vector (pMG36e, used as control, pMG-LBA1719) and then transferred to an ice-cooled electroporation cuvette (0.1 cm electrode gap) and exposed to a single electrical pulse delivered by a Gene Pulser (Bio-Rad, USA) set at 25 μF, 200 Ω, and 1.5 kV for 5.1 ms. Then, 1 mL ice-cold LB medium was added immediately, and the cells were incubated at 37 °C for 2 h. Then, 100 μL portions were spread on LB plates containing 1 μg/mL erythromycin and incubated at 37 °C for 36 h to screen transformants. The recombinant strains were screened by bacterial liquid PCR and labeled *S. carnosus*-0 (containing vector pMG36e) and *S. carnosus*-1 (containing vector pMG-LBA1719).

Measuring UGPase activity

S. carnosus-0 and *S. carnosus*-1 were inoculated in 50 mL LB liquid medium containing 1 μg/mL erythromycin overnight to obtain seed culture solutions. The seed culture medium was inoculated in the above LB liquid medium with 2% (*v/v*) inoculum for 18 h. The OD value of *S. carnosus*-0 and *S. carnosus*-1 was adjusted to 1.4, 10 mL of each bacterial solution was centrifuged at 4 °C and 3000 ×g for 10 min, the supernatant was removed, and the cells were washed with physiological saline solution for 3 times. Finally, 1 mL of lysate (lysozyme dissolved in PBS, final concentration of 1 mg/mL) was added, and the solution was resuspended and stored on ice for 30 min. The cells were disrupted by a sonicator and centrifuged at 10,000 ×g for 10 min, and the supernatant was taken to obtain a protein loading solution.

The UGPase was purified with His-Bind resin chromatography. The chromatographic column was filled and balanced by Binding Buffer (10 mM imidazole, 20 mM Tris, 0.5 M NaCl, pH = 8.0), add 10 mL protein solution, the flow rate was adjusted to 0.5 ml/min and the column was balanced again with Binding Buffer, and the peak was observed under 280 nm absorption light. UGPase was eluted with Elution Buffer (250 mM imidazole, 20 mM Tris, 0.5M NaCl, pH = 8.0), collecting the eluent for SDS-PAGE detection.

UGPase activity of recombinant *S. carnosus*-0 and *S. carnosus*-1 was measured by UGP ELISA Kits (Ke Shun Biotech, Shanghai, China) according to the manufacturer's instructions and determination of absorbance (OD value) at 450 nm by microplate. Taking the OD value as the abscissa and the concentration value as the ordinate, according to the linear regression equation of the reference standards, the OD value of the sample is substituted into the equation to calculate the concentration of the sample.

Determination of growth curves

S. carnosus-0 and *S. carnosus*-1 were separately cultured in LB liquid medium overnight to obtain seed cultures, and 2% (*v/v*) was inoculated into larger LB liquid medium cultures. The OD600 value was measured every 2 h, the culture solution was thoroughly shaken before each sampling, and three replicates were performed.

Lyophilization-survival rate

After culturing for 18 h, *S. carnosus* cells entered the late logarithmic phase and the early stage of stability, which has the strongest freeze-drying resistance (Brashears and Gilliland 1995). Thus, *S. carnosus*-0 and *S. carnosus*-1 were lyophilized at this time. *S. carnosus*-0 and *S. carnosus*-1 were allowed to grow in 100 mL of LB medium

supplemented with 1 μg/mL erythromycin at 37 °C at an agitation speed of 200 rpm. The OD_{600} value was measured every 3 h to determine the growth rate of the bacteria. Then, 10 mL of the cells in the early stationary phase were harvested by centrifugation at 4 °C and 6000 ×g for 10 min. After three washes in 20 mL sterile 0.9% NaCl, the bacteria were resuspended in 1 mL of sterile H_2O, and 100 μL of the bacteria suspension was used to count the number of surviving bacteria on the LB agar plate, while the remaining 900 μL was used for lyophilization at −49 °C, 9 Pa, and 24 h (Eppendorf, Hamburg, Germany). After lyophilization, 900 μL sterile water was added to 100 μL bacterial suspension for cell counting according to the above method. The numbers of living cells before and after lyophilization were determined under the method of plate culture count, and the survival rate was calculated by the following formula: survival rate (%) = (number of living cells after lyophilization/number of living cells before lyophilization) × 100. Three parallel groups were set up, and the SPSS was used to analyze the data by one-way ANOVA to determine the significant difference.

RNA isolation, cDNA library construction, and RNA-seq

The total RNA of *S. carnosus*-0 and *S. carnosus*-1 after lyophilization were extracted using Bacterial RNA Kits (OMEGA, Norcross, Georgia, USA), and the residual DNA was removed with RNase-free DNase I according to the manufacturer's protocol. RNA degradation and contamination were monitored on 1.2% agarose gels. The quality and quantity of the RNA were assessed with Minidrop (Excell Bio, Shanghai, China). The integrity of the RNA was assessed with RNA Nano 6000 Assay Kits and a Bioanalyzer 2100 system (Agilent, California, USA). The high-quality RNA samples ($OD_{260/280}$ = 1.8–2.1, 28S/18S ≥ 1.5:1, RIN ≥ 7, ≥ 5 μg) were used for the construction of cDNA libraries. The first-strand cDNA was synthesized with random hexamer primers. The second-strand cDNA was synthesized and purified with AMPure XP beads; add base "A" at the end of 3′ prime. According to agarose gel electrophoresis, the cDNA with appropriate fragment size was selected for PCR amplification, and the cDNA libraries were established. The libraries were sequenced using the Illumina HiSeq™ 2500 platform. The RNA samples for the different treatment conditions were labeled S0 (*S. carnosus*-0 after lyophilizing) and S1 (*S. carnosus*-1 after lyophilizing).

RNA sequencing data analyses

The next-generation sequencing quality control (NGSQC) software (Patel and Jain 2012) was used to filter the raw data and obtain clean data. The low-quality reads (those exceeding 30% low-quality bases with Q ≤ 20) and reads with N contents in excess of 5% or containing adaptor sequences were removed. Sequence reads were further compared to the *S. carnosus* TM300 genome database, and *P*-value is calculated by hypergeometric distribution method. If $\log_2$fold change ≥ 1 and $p \leq 0.05$, these genes were defined as significantly differentially expressed genes (SDEGs). If $\log_2$fold change ≥ 1, the gene is upregulated. If not, it is downregulated. The SDEGs were selected for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses using the hypergeometric test. The GO terms and KEGG pathways were selected for $p < 0.05$, which was considered as defining significant enrichment.

Validation of RNA-seq data by real-time quantitative PCR (RT-qPCR)

To validate the RNA-seq data, the expression levels of 10 SDEGs for the two strains (*S. carnosus*-0 and *S. carnosus*-1) were determined by RT-qPCR. All the specific primers listed in Table 1 were designed using Primer Premier 5.0 software (Canada) and synthesized by Beijing Genomics Institute (BGI, China). Lyophilization and RNA isolation were achieved as described above. RNA was reverse transcribed into cDNA with an All-in-One First-Strand cDNA Synthesis SuperMix for qPCR Kit (TransGen Biotech, Beijing, China). Then, RT-qPCR was performed under the following conditions: 94 °C for 30 s, followed by 45 cycles of 94 °C for 5 s and 60 °C for 30 s, and then 37 °C for 10 min. The 16S rRNA gene was used

Table 1 Specific primers

Genes	Note	Primers
16S RNA	16S RNA	GTCGTGAGATGTTGGGTTA CTTTATGGGATTTGCTTGA
Sca_1902	Glutathione S-transferase	GGTCAAATCAACCCTAAT CCTTCTTCTACACCCATA
Sca_2009	Fructosamine-3-kinase	TCGTCGTTTAGATCACTTAC AATAGGGATGCCTTACTTT
alaS	Alanine–tRNA ligase	AGTCGCAACTGTTCCTAAA AATGAATTGTAATGCCTCTG
argR	Arginine repressor	TTGAAGGAACAGGGAACT TGTATCATCACCGCAAAT
clpC	ATP-binding subunit ClpC	AGAATCAATGTTCGGAGAC TGTTGAATACATCTGGGTG
cysK	Cysteine synthase	AAACCCTGCCAATCCACT ACGTACCGCCTGTACCAA
ddh	Lactate dehydrogenase	TAAAGGATTATGACGGAGTT GCTATCGAGCTTGGTGAA
gapB	Glyceraldehyde-3-phosphate dehydrogenase	GTTTCAGACCGTAATCCA TCTTCAACATCAAGGCTAT
katE	Catalase	AGACCAACCGATGACTACAA ACACGCTCAGGAATACGC
murA	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	GTATTGAAGCGGGAACA GAAGCCAGGATGAGGTA

as the internal reference gene. The RT-qPCR data are expressed as the averages from three independent experiments where each experiment was performed with three parallels. Relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

cDNA cloning and construction of a recombinant expression vector

Fragments of *LBA*1719 encoding UGPase from *L. acidophilus* ATCC4356 were successfully amplified via PCR (Fig. 1a) and cloned on the pMG36e vector. The full lengths of the *LBA*1719 genes are 885 bp, which are the same as the corresponding gene sequences. Recombinant expression vectors pMG-LBA1719 were constructed (Fig. 1b). The sequence lengths after PCR amplification are 944 bp for *LBA*1719, which are consistent with the theoretical values.

Expression of UGPase

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of the proteins in the supernatants of the three transformants (Fig. 1c) indicated that the fragment encoding *L. acidophilus* ATCC4356 UGPase was successfully transferred and expressed in *S. carnosus*. The molecular weight of the protein encoded by the *LBA*1719 gene is 33.075 kDa. There is a 6× His-tag at the N-terminus of the recombinant protein downstream to the strong promoter P32. These results show that both UGPase protein bands are all ca. 40 kDa, which is in line with the expected results.

The UGPase activity of *S. carnosus*-0 transferred into the unloaded plasmid is 328.97 IU/L, while that of *S. carnosus*-1 transferred into pMG-LBA1719 is 445.72 IU/L. Therefore, compared with *S. carnosus*-0, the UGPase activities of *S. carnosus*-1 are significantly increased by 35.49%.

Lyophilization-survival rate

The lyophilization-survival rate of *S. carnosus*-0 is 35.05%; however, the survival rate of *S. carnosus*-1 is 23.88%, which is 11.17% ($p < 0.05$) lower than that of *S. carnosus*-0 (Fig. 1d).

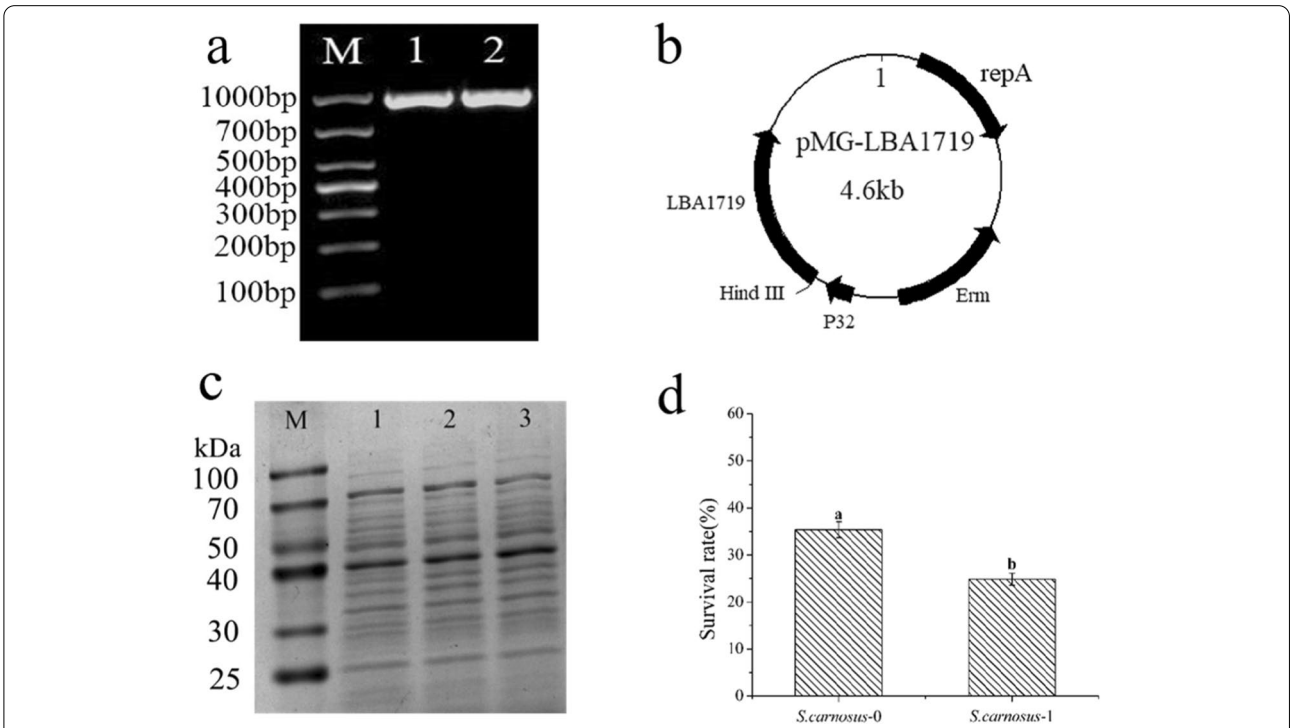

Fig. 1 Overexpression of *LBA*1719 and lyophilization-survival rates for *S. carnosus*. **a** Electrophoresis results for the PCR products of *LBA*1719. **b** Maps of recombinant expression plasmid pMG-LBA1719. (M, marker DL1000; lanes 1–2, products of full length *LBA*1719). **c** Expression of recombinant *S. carnosus* UGPase in transformants (M, protein markers; lane 1, *S. carnosus*-0 UGPase; lanes 2–3, *S. carnosus*-1 UGPase. **d** Lyophilisation-survival rates for *S. carnosus*-0 and *S. carnosus*-1

Comparison of growth in UGPase-overexpressing and non-overexpressing S. carnosus cells

From the growth curves in Fig. 2, it can be seen that the growth of both strains was similar. Between 8 and 18 h, bacterial cells proliferated exponentially, and *S. carnosus*-1 grew faster than *S. carnosus*-0. After culturing for 18 h, both recombinant strains reached the late logarithmic phase and the early stages of stability. The OD600 values of *S. carnosus*-0 and *S. carnosus*-1 strains were 1.3348 and 1.5002 ($p < 0.05$). These results indicate that overexpression of *LBA*1719 promotes the growth of *S. carnosus*.

Quality control of sequencing data

A total of 2 RNA samples generated from two biological replicates of two strains following lyophilization treatment were subjected to RNA-seq. A total of 36,765,152 and 38,218,207 clean reads were generated for each sample after quality filtering, with the clean rate ranging 98.58% and 98.82%. Among the clean bases, the ratio of bases with a mass value greater than 20 to the total number of bases (Q20) is in excess of 98%, while Q30 is more than 96% (Table 2). These results indicate that the Illumina sequencing platform captures most of the transcriptomics quickly and efficiently.

Numbers of DEGs

The DEGs were screened by comparing the sequencing data with the reference genome of *S. carnosus* TM300.

Table 2 Data filtering statistics

Samples	Raw reads	Clean reads	Clean rate (%)	Q20 (%)	Q30 (%)
S0	37286283	36765152	98.58	98.69	96.36
S1	36395704	35882939	98.59	98.73	96.45

For the S1 vs. S0 comparisons, 569 DEGs were identified. Among them, 210 DEGs are upregulated, and 359 DEGs are downregulated.

GO and KEGG enrichment analysis

GO enrichment analysis was performed for S1 vs. S0 comparisons to investigate the functions of the identified DEGs. GO has three basic classifications: biological processes, cellular components, and molecular engineering, which can be further divided into more detailed sub-function categories. And the number of functional sub-categories of each basic GO classifications for S1 vs. S0 is 13.9.11.

There are 395 DEGs with GO annotations for S1 vs. S0. Most of the DEGs in the biological process domain are associated with metabolic process, cellular process, and single-organism process subcategories. In the cellular component domain, the DEGs are mainly associated with the cell, cell part, membrane, and membrane part categories. In the molecular function domain, most of the DEGs are linked to catalytic activity, binding, and

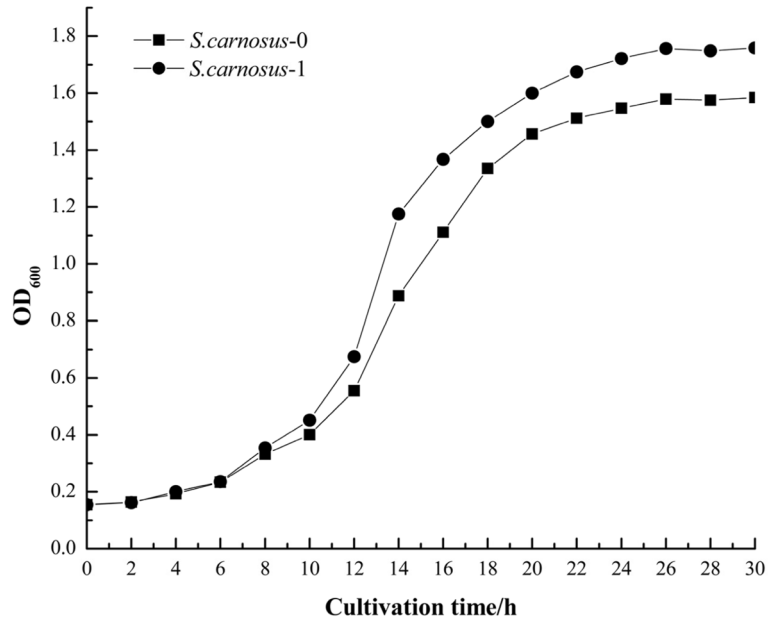

Fig. 2 Growth curves for *S. carnosus*-0 and *S. carnosus*-1

transporter activity. The main functional subcategories of the three functional types are basically the same for the two comparisons (Fig. 3).

KEGG enrichment analysis identified the DEGs involved in important metabolic and signal transduction pathways. Based on KEGG analysis, there are 520 genes annotated to 84 KEGG pathways. The most important pathway is the metabolic pathway with 94 DEGs (18.08%). The second most populated pathway is biosynthesis of secondary metabolites (44 DEGs, 8.46%) and then microbial metabolism in diverse environments (36 DEGs, 6.92%). Several categories only contain one DEG, such as phenylalanine metabolism, glutathione metabolism, and fatty acid biosynthesis (Fig. 4).

DEGs of transcriptional regulators

Microorganisms can quickly adapt to rapid environmental changes through transcription, translation, and gene expression when the environment and its resources are not conducive to survival. Most such adaptive responses are mediated by transcriptional regulators. For S1 vs. S0, 13 transcription regulators are differently expressed, with nine being upregulated and four downregulated (Table 3).

DEGs of carbohydrate metabolism and amino acid metabolism

The genes related to carbohydrate metabolism were screened by metabolic pathway. For S1 vs. S0, 21 DEGs are identified (Table 4). DEGs associated with glycolysis, including *pgi*A, *gap*A, *gap*B, *pgm*5, *eno*, and *ddh* encoding glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenases (GAPDHs), phosphoglycerate mutase, enolase, and lactate dehydrogenase (LDH), respectively, are downregulated (Fig. 5). The biosynthesis of lysine includes aspartate β-semialdehyde and pyruvic acid synthesis pathways. DEGs related to lysine metabolism include *lys*C, *dap*A, *dap*B, *dap*D, and *dap*E (Fig. 5). For S1 vs. S0, those DEGs are all downregulated, so the level of lysine synthesis declines (Table 5). The gene *trp*A, one of the Trp operons in the synthesis of tryptophan, is downregulated 1.16-fold. The expression of *cys*K encoding cysteine synthase is also downregulated.

DEGs result validation by qPCR

To confirm the accuracies and reliabilities of the expression profiles revealed by RNA-seq, 10 DEGs were detected by qPCR in S1 vs. S0. These 10 genes include three genes related to carbon metabolism (*ddh*, *gap*B, *Sca_2009*), three genes related to amino acids and proteins (*ala*S, *cys*K, *arg*R), three genes related to

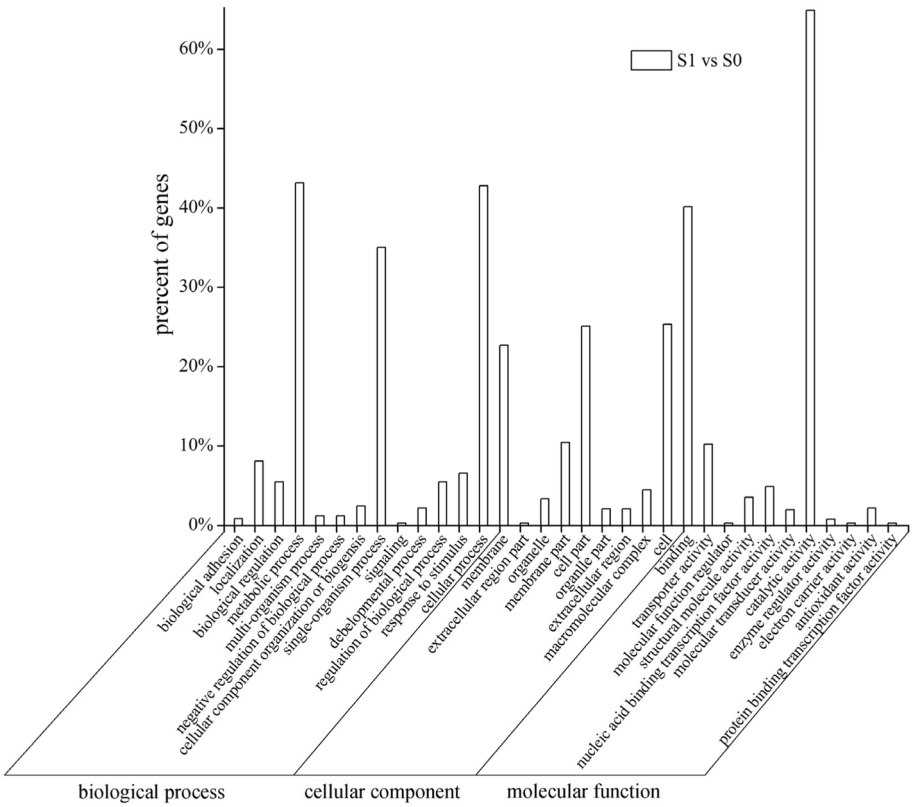

Fig. 3 GO categories of the DEGs

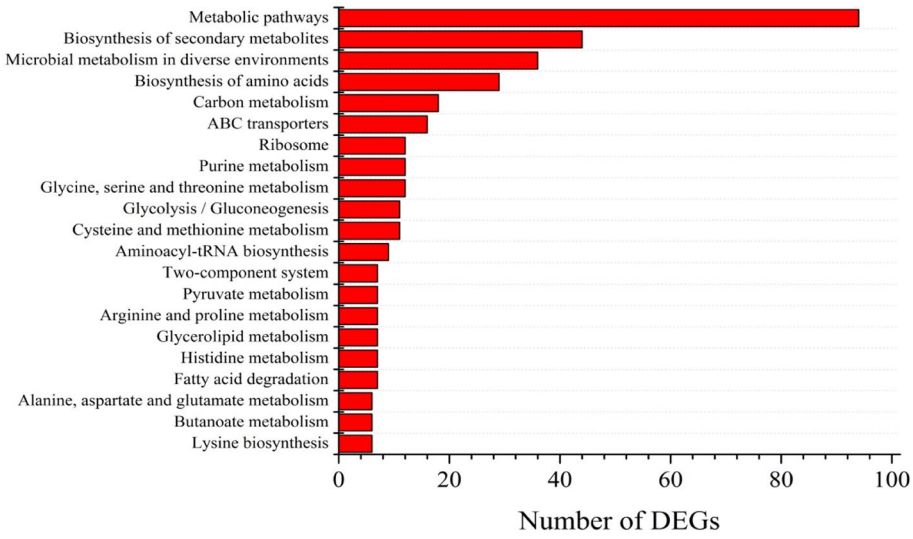

Fig. 4 KEGG classes of the DEGs for S1 vs. S0

Table 3 DEGs related to transcriptional regulators

Genes	Note	S1 vs S0
*glt*C	LysR family transcriptional regulator	1.107
Sca_0658	MarR family transcriptional regulator	1.341
Sca_0712	Transcriptional regulator	1.762
Sca_0724	Cro/CI family transcriptional regulator	2.271
rex	Transcriptional regulator	1.083
Sca_2137	RpiR family transcriptional regulator	1.033
Sca_2381	MerR family transcriptional regulator	1.759
Sca_2432	MarR family transcriptional regulator	2.001
*gnt*R	GntR family transcriptional regulator	1.762
*czr*A	Transcriptional regulator	−1.659
Sca_1830	LysR family transcriptional regulator	−1.289
Sca_2234	TetR family transcriptional regulator	−1.539
spxA	Transcriptional regulator Spx	−1.879

Table 4 DEGs related to carbohydrate metabolism

Gene	note	S1 vs S0
*lac*G	6-Phospho-beta-galactosidase	1.427
*pfl*B	Formate acetyltransferase	3.749
*pck*A	Phosphoenolpyruvate carboxykinase (ATP)	1.475
*tre*C	Glucohydrolase	1.047
Sca_2321	PTS sorbitol transporter subunit IIB	1.237
Sca_1047	Acylphosphatase	−1.719
*pgi*A	Glucose-6-phosphate isomerase	−1.491
Sca_2269	NADPH:quinone reductase	−2.009
*ppd*K	Pyruvate phosphate dikinase	−2.537
*adh*C	Alcohol dehydrogenase	−1.493
*ald*A	Aldehyde dehydrogenase	−1.372
Sca_1625	Aldehyde dehydrogenase	−1.145
eno	Enolase	−1.011
gdh	Glucose-1-dehydrogenase	−1.083
*mtl*D	Mannitol-1-phosphate 5-dehydrogenase	−1.075
gnd	6-Phosphogluconate dehydrogenase, decarboxylating	−1.396
*pgm*5	Phosphoglycerate mutase	−1.748
tkt	Transketolase	−1.153
*gap*B	Glyceraldehyde-3-phosphate dehydrogenase	−1.195
*gap*A	Glyceraldehyde-3-phosphate dehydrogenase	−1.290
ddh	Lactate dehydrogenase	−1.400

abiotic stress (*Sca_ 1902*, *clp*C, *kat*E), and one other genes (*mur*A). As shown in Fig. 6, these genes show similar expression patterns in the RNA-seq and qPCR analyses, confirming that the RNA-seq data was reliable.

Discussion

Overexpression of UGPase significantly promoted the growth of *S. carnosus*. Analysis of the transcriptome data from *S. carnosus*-1 and *S. carnosus*-0 identified genes altered in the *LBA*1719 strain. In *S. carnosus*-1, the DEGs is mainly concentrated in transcriptional regulators, carbohydrate metabolism, and amino acid metabolism.

Ramos et al. (2005) divided transcriptional regulators such as LysR, MerR, MarR, and GntR into 16 categories. They control the initiation, extension, and termination

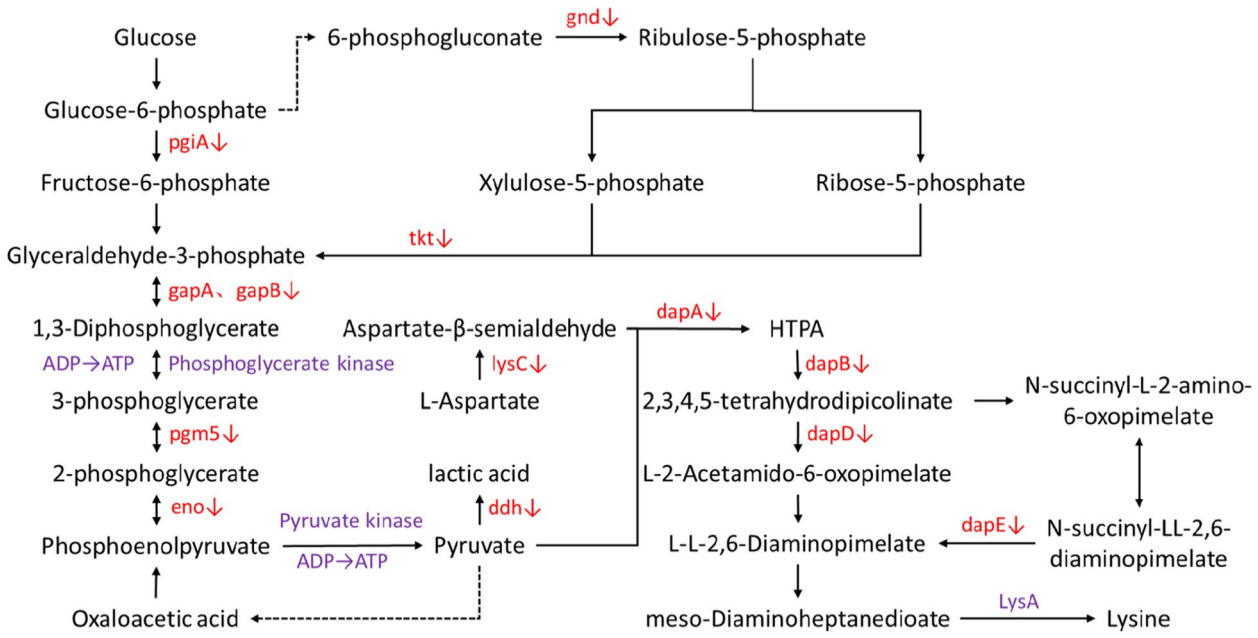

Fig. 5 DEGs related to carbohydrate metabolism and lysine synthesis. (HTPA = (2S, 4S)-4-hydroxy-2,3,4,5-tetrahydrodipicolinate)

of transcription and are an essential type of molecules in the transcription process. TetR proteins control genes whose products are involved in osmotic stress as osmoprotectants in hyperosmotically stressed cells. LysR is the transcriptional activator of *lys*A (encoding diaminopimelate decarboxylase and participating in the synthesis of lysine) (Maddocks and Oyston 2008). GntR regulators play roles in amino acid catabolism, fatty acids metabolism, and carbon catabolism (Hoskisson and Rigali 2009). MarR family proteins modulate the expression of genes encoding proteins involved in metabolic pathways and stress responses (Perera and Grove 2010).

Carbohydrate metabolism is the main pathway by which microorganisms acquire energy, such as that via gluconeogenesis and glycolysis (Wu et al. 2016). In *S. carnosus*-1, the gene expression related to sugar metabolism and amino acids was the most significantly altered. In *S. carnosus*-1, the gene gapA related to glyceraldehyde 3-phosphosphate dehydrogenases (GAPDH) synthesis is downregulated. GAPDH acts as an enzymes of glycolysis and participates in the glycolytic pathway to generate energy and involved in many cellular processes in addition to glycolysis, such as DNA repair and tRNA export (Schormann et al. 2020). One research found that GAPDH may play an important role in transcriptional regulation under stress (Vescovi et al. 2013). Meanwhile, the GAPDH in some cases undergoes different types of redox-regulated posttranslational modifications which encourage relocation to other cell compartments and interactions with other proteins, to execute non-glycolytic functions (Zaffagnini et al. 2013); Pachauri also found that GAPDH plays a role in secondary metabolism (Pachauri et al. 2019). The downregulation of *gap*A gene hinders GAPDH synthesis, and the GAPDH activity was decreased. GAPDH is an important factor for cell survival, and cell viability will decline sharply when its activity is reduced (Corcoran et al. 2005; Marceau et al. 2004), and the normal glycolysis process of bacteria is disturbed, which affects the normal energy supply of bacteria. LDH is divided into L-LDH (EC. 1.1.1.27) and D-LDH (EC. 1.1.1.28), which fall into two distinct enzyme families, and there is no correlation between them in their process of evolution. LDH uses the coenzyme NADH or NAD + as hydrogen transmission (Yi et al. 2009) to catalyze the reduction and oxidation between pyruvate and lactic acid in the body reaction (Al-Jassabi 2002). With the downregulation of *ddh* gene encoding LDH, the synthesis of ATP is blocked. Meanwhile, the pre-stage process of glycolysis does not produce energy and consumes two molecules of ATP, the decrease of LDH activity leading to production capacity being blocked, further exacerbated the reduction of bacterial energy supply. Furthermore, the decline in LHD activity is one of the main factors causing bacterial damage upon lyophilization (Kawai and Suzuki 2007). Under the joint action of the downregulation of a variety of glucose metabolism genes (Table 4), insufficient energy supply, abnormal bacterial metabolism, and ultimately a decrease in cell activities.

Table 5 DEGs related to amino acids and proteins

Genes	Note	S1 vs S0
*dap*A	4-Hydroxy-tetrahydrodipicolinate synthase	−1.886
*dap*B	4-Hydroxy-tetrahydrodipicolinate reductase	−1.687
*dap*D	2,3,4,5-Tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase	−1.594
*dap*E	Succinyl-diaminopimelate desuccinylase	−1.128
*cys*K	Cysteine synthase	−1.081
*trp*A	Tryptophan synthase alpha chain	−1.160
*trp*B	Tryptophan synthase beta chain	−1.835
*trp*C	Indole-3-glycerol-phosphate synthase	−1.233
*trp*D	Anthranilate phosphoribosyltransferase	−1.594
*trp*E	Anthranilate synthase component	−1.013
*inf*A	Translation initiation factor IF-1	−1.624
Sca_1619	Translation factor Sua5	−1.329
*rbs*K	Ribokinase	2.320
Sca_1907	Protein disulfide-isomerase	1.080
*rpl*K	50S ribosomal protein L11	1.020
*rpl*T	50S ribosomal protein L20	1.949
*rpl*V	50S ribosomal protein L22	−1.460
*rpl*X	50S ribosomal protein L24	−1.256
*rpm*G	50S ribosomal protein L33	−2.570
*rps*K	30S ribosomal protein S11	−1.125
*rps*Q	30S ribosomal protein S17	−1.149
*rps*S	30S ribosomal protein S19	−1.093
*rps*T	30S ribosomal protein S20	−1.471
Sca_0203	50S ribosomal protein L7/L12	−1.342
*ala*S	Alanine–tRNA ligase	−1.027
*tyr*S	Tyrosine–tRNA ligase	−1.560
*ser*S	Serine–tRNA ligase	−1.057
*gly*S	Glycine–tRNA ligase	−1.008
*his*S	Histidine-tRNA ligase	−1.053

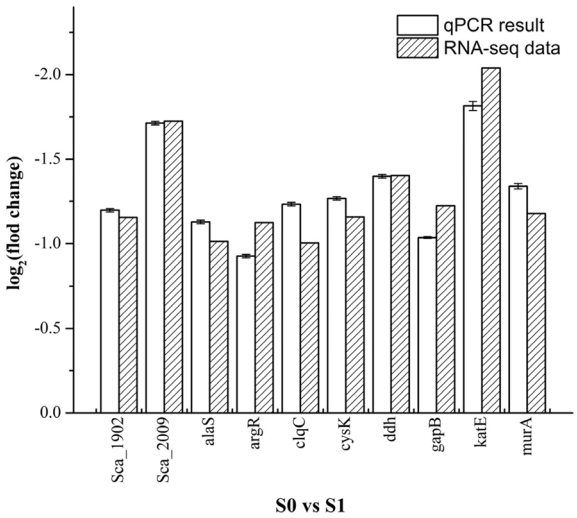

Fig. 6 Validation of RNA-seq data by q-PCR

In bacteria, tryptophan, lysine, cysteine, etc. can be used as osmoprotectants to protect cells from damage (Liu et al. 2015); the downregulation of genes encoding these amino acids is not conducive to the growth of bacteria. Lysine is not only an essential amino acid for bacterial survival; it is also vital for the structure of peptidoglycan in prokaryote cell walls containing diaminopimelate or lysine (Chamaillard et al. 2003). Lysine through aspartic-β-semialdehyde and acetone is condensed for biosynthesis, and aspartic-β-semialdehyde is produced by aspartate under the action of aspartokinase. For S1 vs. S0, *lys*C expression is downregulated, directly affecting the synthesis of aspartate-β-semialdehyde. During the condensation of aspartate-β-semialdehyde and acetone, the expression levels of *dap*A, *dap*B, *dap*D, and *dap*E are all downregulated, and the level of lysine synthesis inevitably decreases. Lysine acts as an osmoprotectant against hypertonic stress, so a reduction in lysine synthesis makes the ability of bacteria to resist stress decrease. Cysteine is one of the precursors in the synthesis of glutathione (GSH). GSH is a low-molecular-mass thiol compound that acts as an important antioxidant and free-radical scavenger in the body and can help bacteria resist external stress (Sies 1999). In addition, cysteine as well is being the primary sulfur source for a variety of other molecules, such as biotin, coenzyme A, and lipoic acid (Hicks and Mullholland 2018). Cysteine synthase is the rate-limiting enzyme for cysteine synthesis (Liu et al. 2008). It catalyzes the production of cysteine from O-acetyl serine. Cysteine acts as an osmotic-protective agent to prevent cell injury caused by osmotic stress (Liu et al. 2015). For S1 vs. S0, *cys*K associated with cysteine synthase is downregulated, which is detrimental to cell growth. Tryptophan is an essential amino acid, required for the production of serotonin (Di Pizio and Nicoli 2020); the inhibition of tryptophan synthesis often leads to the inhibition of bacterial growth (Islam et al. 2019). The synthases for tryptophan and DAHP are the two major rate-limiting enzymes in the tryptophan biosynthetic pathway (Matsui et al. 1987). There are five enzyme structural genes (*trp*E, *trp*D, *trp*C, *trp*B, and *trp*A) in the tryptophan operon, and these are responsible for the conversion from branched acids to tryptophan (Tribe et al. 1976). In S1 vs S0, the five genes are all downregulated (Table 5), especially *trp*B, directly reducing the biosynthesis of tryptophan. Furthermore, the gene *Sca_1907* encoding protein disulfide-isomerase, a kind of antioxidant enzyme, is upregulated. It is a chaperone capable of responding to the unfolding of proteins, catalyzing the formation and isomerization of cysteine bonds during protein folding (Karala et al. 2007; Winter

et al. 2007). The expression of glutathione S-transferase (*Sca_1902*), heat-shock protein (HSP, *Sca_1882*), and co-chaperone GroES (*groES*) (Kong et al. 2012), which can respond to abiotic stress, is downregulated. The regulation of related genes reduces the amino acid synthesis and affects the normal growth of *S. carnosus*. In general, the normal carbohydrate and amino acid metabolism are disturbed, which inhibits the energy supply and amino acid production of bacteria and reduces the tolerance of *S. carnosus* to external environmental stress.

Meanwhile, freeze-drying not only causes DNA damage; it also disrupts the correct synthesis of proteins. In order to repair this damage, the body will express certain stress proteins to protect and repair biological macromolecules. DNA repair proteins can repair DNA damage. The genes *rad*A and *rec*N encoding DNA repair proteins are at different levels of expression. The gene *rad*A is upregulated 1.52-fold, while *rec*N is downregulated 1.7-fold. This demonstrates that different types of repair protein exert different functions in cells and may be involved in cross-mechanisms to repair biological macromolecules.

The growth and metabolism of *S. carnosus* are a very complicated process (John et al. 2017). In this study, overexpression of *LBA*1719 in *S. carnosus* increased the enzyme activity of UGPase, accelerating the growth rate of *S. carnosus*. However, the lyophilization survival rate of *S. carnosus* decreased, and transcriptome analysis showed that the expression of many genes in bacteria was downregulated in varying degrees. We speculate that the overexpression of foreign gene *LBA*1719 increases the content of UGPase in bacteria compared with the normal level. UGPase plays an important role in glucose metabolism, and even minor UGPase overexpression can have a significant effect. In order to maintain the balance and stability of UGPase content in bacteria, *S. carnosus* inhibit the expression of UGPase gene in genome through negative feedback regulation. Consequently, the expression of related genes was downregulated, affecting the normal metabolic activities of bacteria, which in turn affects *S. carnosus* response to freeze-drying stress.

Conclusion

In summary, the results of this study indicate that UGPase plays an important role in lyophilization response. Gene *LBA*1719 encoding UGPase was separately ligated into an expression vector and electroporated into the *S. carnosus* host strain for overexpression. Overexpression of *LBA*1719 downregulated the gene expressions such as *gap*A, *pgi*A, and *dap*E, and also, the normal carbohydrate and amino acid metabolism were inhibited, which reduces the lyophilization-survival rates of the recombinant *S. carnosus*.

Acknowledgements
We acknowledge the financial support provided by the National Natural Science Foundation of China (32072195, 41406165, 41641052, 31972093), Science and Technology Department of Zhejiang Province (LGN19C200011, 2019C02085), and Ningbo Municipal Bureau of Science and Technology (202002N3068).

Authors' contributions
Designed of the study, XQZ; performed the experiments and prepared the manuscript, CRX and LYP; analyzed the data, LYP; reviewed and wrote the manuscript, CRX, XQZ, and LYP; helped improve the research plan, DDP; provided guidance on experimental techniques, ZW, YXG, and ZDC. The authors read and approved the final manuscript.

Funding
The National Natural Science Foundation of China (32072195, 41406165, 41641052, 31972093), Science and Technology Department of Zhejiang Province (LGN19C200011, 2019C02085), and Ningbo Municipal Bureau of Science and Technology (202002N3068).

Availability of data and materials
All data generated and analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 14 December 2021 Accepted: 1 June 2022
Published online: 21 June 2022

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