Pak. J. Bot., 50(6): 2443-2448, 2018.

A BIOSYNTHESIS METHOD TO PRODUCE RESVERATROL IN *SACCHAROMYCES CEREVISIAE* WITH SECRETION EXPRESSION OF GRAPE ANTI-OXIDANT RESVERATROL SYNTHASE GENE DURING WINE FERMENTATION

YANGBO SONG[1], LI FENG[1], DONGQING YE[1], YUYANG SONG[1, 2], YI QIN[1, 2], YANLIN LIU[1, 2, *] AND SHUWEN LIU[1, 2, *]

[1]*College of Enology, Northwest A&F University, Yangling, Shaanxi, 712100, P. R. China;*
[2]*Shaanxi Engineering Research Center for Viti-Viniculture, Yangling, Shaanxi, 712100, P. R. China*
[*]*Corresponding authors email: yanlinliu@nwsuaf.edu.cn, liushuwen@nwsuaf.edu.cn*

Abstract

Resveratrol (Res), a stilbenic compound, recently has become the focus of a number of studies in medicine and plant physiology, and *Saccharomyces cerevisiae* (*S. cerevisiae*) has become a popular host for production of non-native compounds. As the last and key enzyme of the biosynthesis pathway, the amount and expression level of Resveratrol Synthase (RS) positive correlation on the production of Res. In this study, a signal peptide sequence, secreted by α-factor of *S. cerevisiae*, was connected with the RS gene, resulting a pYCα secretory plasmid construction. The RS gene was secretory expressed in *S. cerevisiae* successfully. Subsequently, a white wine fermentation was carried out by the recombinant strain. The Res concentration of the white wine fermented by the recombinant strain was 36% higher than the control. This has great interests for establishing a controllable way to produce RS and lays the foundation for exploring the Res biosynthesis method.

Key words: Resveratrol synthase, *Saccharomyces cerevisiae*, Secretion expression, Resveratrol.

Introduction

In wine, Resveratrol (Res) has been reported to have health benefits including anti-oxidant effects, life span extension, inhibiting obesity and cancer prevention (Gambini *et al.,* 2015; Tomé-Carneiro *et al.,* 2013; Juan *et al.,* 2005; Park *et al.,* 2001; Sgambato *et al.,* 2001). Since Res has various health benefits and physiological roles above, it has a wide range of applications in health food, cosmetics and pharmaceuticals, and it is also an active principle found in traditional Chinese and Japanese medicine used to treat ailments related to the liver, skin, heart, and lipid metabolism (Pan *et al.,* 2012). Previous studies have shown that the gene encoding stilbene synthase is transcribed only when induced by stimuli, to improve the immunity of plants and against pathogenic invasion under harsh conditions and environmental deterioration. Res has been found in more than 70 species of plants, such as *polygonum cuspidatum*, grapevine, and peanuts (Pan *et al.,* 2012; Baur *et al.,* 2006; Gruber *et al.,* 2007; Howitz *et al.,* 2003). Res biosynthesis occurs via a pathway that branches off from the phenylalanine/ polymalonate pathway (Damianaki *et al.,* 2000; Jeandet *et al.,* 2012; Vos *et al.,* 2015) as shown in Fig. 1. Malonyl-CoA, one of the substrates required for the production of Res, is present in the yeast and is actively involved in fatty acid biosynthesis. The other substrate for RS, para-coumaroyl-CoA, can be produced from para-coumaric acid, which is found in small quantities in grape must (Goldberg *et al.,* 1998) and has been shown to be accumulated by yeast (Smit *et al.,* 2003). There are four enzymes in the pathway: phenylalanine ammonia lyase (PAL), cinnamate 4 hydroxylase (C4H), 4 coumarate: coenzyme A ligase (4CL), and RS. RS is the key enzyme which catalyzes the reaction of Malonyl-CoA with para-coumaroyl-CoA, formed CoASH, CO_2 and Res. As the essential enzyme at last step of biosynthesis, the amount and the expression level of RS positive correlation on the production of Res. If the RS gene is absent in plants, the Res cannot be synthesized (Fig. 1). Meanwhile, several different related RS genes have been isolated and characterized in various plant species (Hain *et al.,* 1993; Kobayashi *et al.,* 2000; Kodan *et al.,* 2001; Preisig-Muller *et al.,* 1999; Schwekendiek *et al.,* 1992; Sparvoli *et al.,* 1994; Thomzik *et al.,* 1997). Knowledge of Res biosynthetic route thus paves the way for metabolic engineering in microorganisms and plants. At present, commercial production of Res from plant sources such as *Ploygonum cuspidatum* is complicated by low product yield, slow growth, low efficient, difficult purification procedures and inconsistent performance (Andrea *et al.,* 2010). Res has been also biosynthesized through introducing exogenous genes into microorganisms. Moreover, the engineering of yeast or bacteria has been optimized in encoding sequences and combination of genes with suitable fermentation process control (Becker *et al.,* 2003; Kang & Back, 2009). The biosynthetic pathway of Res and similar compounds have been reconstructed in heterologous microbial systems before, mainly in *E. coli* and yeast (Pitera *et al.,* 2007; Sydor *et al.,* 2010; Trantas *et al.,* 2009; Vannelli *et al.,* 2007; Ververidis *et al.,* 2007). Many exogenous genes can secretory expressed by yeast. Secretion expression can increase expression levels and simplify the purification steps of expression products. It can also make expression products complete a series of processing and modification by the secretory pathway, which is important to ensure the natural activity of the expressed products (Michaelis & Barrowman, 2012). If the RS gene were combined with secretion signal peptide coding sequence and secretory expressed in *S. cerevisiae*, the full functionality protein would be obtained directly.

In this study, the RS gene was cloned from *Cabernet Sauvignon*. The RS gene was connected with a signal peptide sequence, which was secreted by α-factor of *S. cerevisiae*. After constructing the pYCα secretory plasmid, the RS was secretory expressed in *S. cerevisiae* successfully, as an ectoenzyme react on zymolyte in grape juice. Furthermore, the recombinant yeast strains were tested for the expression of RS and for its ability to produce Res in white wine fermentation.

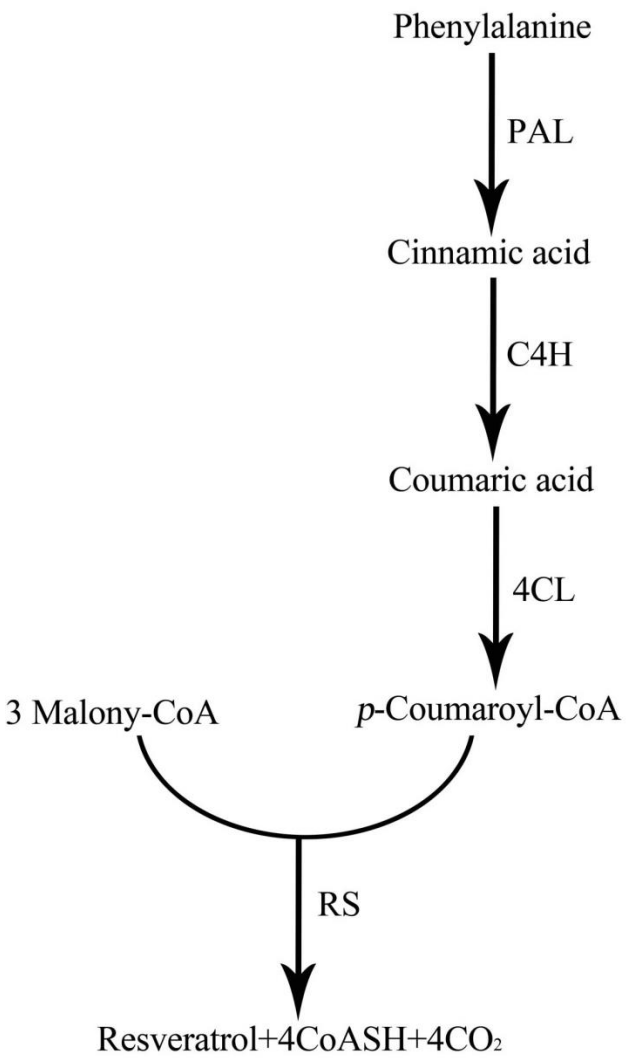

Fig. 1. Biosynthesis pathways towards resveratrol in grape. Malonyl-CoA is required for the production of Res. The other substrate for RS, p-coumaroyl-CoA, can be produced from p-coumaric acid. There are four enzymes in the pathway: phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate: coenzyme A ligase (4CL), and RS. RS is the key enzymes which catalyzes the reaction of Malonyl-CoA with p-coumaroyl-CoA, formed CoASH, CO_2 and resveratrol.

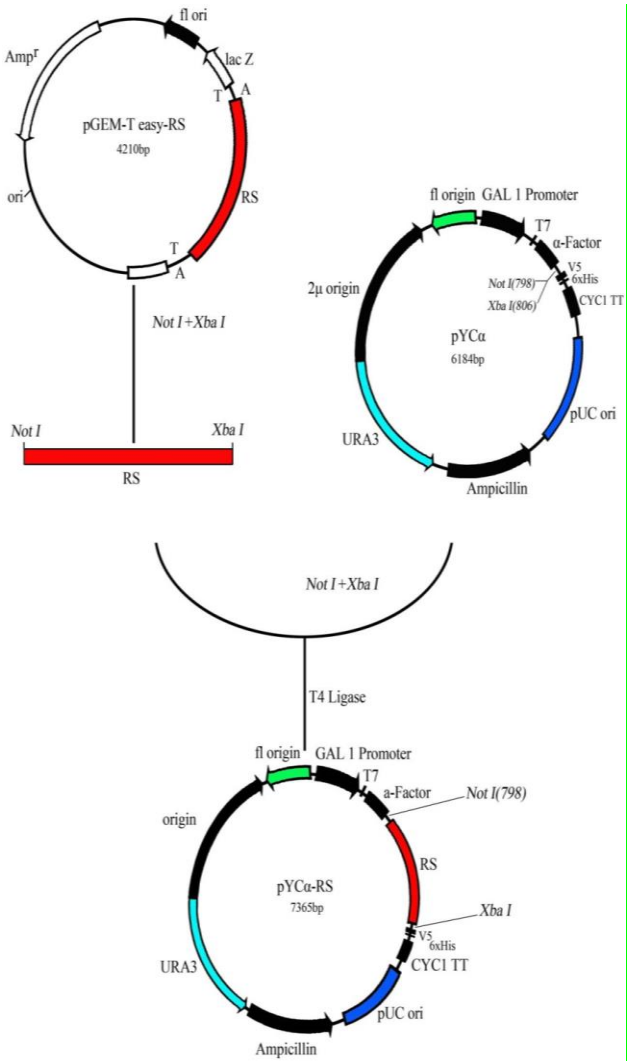

Fig. 2. Construction for the secretory expression vector: pYCα-RS T-Easy-RS plasmid was digested, meanwhile secretory expression plasmid pKCα was double digested by *Not I* and *Xba I* and obtained a about 6000bp fragment. Then, the RS target gene was inserted to pYCα fragment with T4 DNA ligase and screened. The results showed that RS fragments were successfully inserted into pYCα secretory expression plasmid. The pYCα-RS plasmid had been constructed.

Materials and Methods

Grape leaves: *Cabernet Sauvignon* grape leaves were collected from the grape resource garden of the college of Enology in Shaanxi, China (34.2701°N, 108.0744°E). After collection, the grape leaves of flowering phase were wrapped in aluminum foil and frozen in liquid nitrogen quickly, saved at -80°C.

Strains and plasmids: *S. cerevisiae* YS58 (Institute of Microbiology, Beijing, China) was used as a host strain for Res production. *Escherichia coli* DH5α (TIANGEN, Haidian, Beijing, China) was used for all the recombinant DNA experiments. The pGEM ®-T Easy vector (Promega, Madison, Wisconsin, USA) was used for construction of T-Easy-RS plasmid. The plasmid pKCα (α-factor signal peptide; GAL1, T7 promoter; CYC1terminator; 6 His tags; Amp resistance screening gene; URA3 defective screening gene) was used for the transforming DNA, which was donated by Dr. Hu and Dr. Yao (Institute of Microbiology, Jinan University, China). *S. cerevisiae* YS58 was transformed with the secretory expression plasmid pKCα-RS, which was constructed to constitutively express RS, thus leading to the production of Res. The RS gene was from grape.

Culture conditions: The yeast strain YS58 was grown on YPD medium (yeast extract 1%, peptone 2%, glucose 2% agar 2%). *E. coli* DH5α was cultivated at 37°C in LB medium (0.5% yeast extract, 1% tryptone and 1% sodium chloride) with or without 2% agar. The *E. coli* transformants were selected on LB solid medium containing 100 g/mL ampicillin. The selective solid medium SD-Ura (yeast nitrogen base 0.67% without amino acid, 0.19% yeast synthetic drop-out medium supplement without Uracil, agar 2% and glucose 2%) was

used for the *S. cerevisiae* transformants screening. The induced medium SD-Ura-gal (0.67% yeast nitrogen base without amino acid, 0.19% yeast synthetic drop-out medium supplement without Uracil, 2% agar, 2% glucose and 2% galactose) was used for the induction of RS.

Genetic manipulation and test of genetic stability: The RS gene (NCBI GenBank accession no. DQ 366301) from *Vitis vinifera* was amplified by PCR from cDNA using oligonucleotides 5'- ATAAGAAT GCGGCCGC G ATGGCTTCAGTCGAGGA and 5'- GC TCTAGA TTAATTTGTAACCATA (Restriction enzyme sites *Not I* and *Xba I* were underlined). Amplification consisted of 38 cycles of 10 s at 95°C and 15s at 55°C with a last polymerization step of 1min 20s at 72°C. Amplified fragment was connected to the pGEM®-T Easy vector (Promega, Madison, Wisconsin, USA), resulting T-Easy-RS plasmid then it was transformed into *E. coli*. The T-Easy-RS was verified by sequencing and Blast in NCBI database. The confirmed T-Easy-RS and pYCα were double digested to construct secretory expression pYCα-RS plasmid. The ligation product was transformed into *E. coli* and screened by LB/Amp medium. Then pYCα-RS plasmid was transformed into *S. cerevisiae* YS58 and the engineered strain were screened on selective mediums.

The engineered strain was successively cultivated on plates for 10 generations. Each generation of the strain was cultivated at 28°C for 24 h. The 10th generation strain was used for plate-streaking. After two days' cultivation at 28°C, 100 randomly chosen single-grown colonies were transferred to 0.5 ml of sterilized water to starve for 4 h at room temperature. Single colonies after starvation was inoculated onto SD-Ura plates and kept at 28°C for 2 days.

Induced expression and identification of RS: The positive transformants were selected on SD-Ura agar. A shaking flask fermentation of this strain in SD-Ura liquid culture medium was performed. Then the RS gene was expressed in SD-Ura-GAL culture medium induced by galactose. After centrifugation at 13,000 rpm for 10 min at 4°C, the supernatant was recovered. Subsequently, the proteins in the supernatant were separated on a 12% SDS-PAGE and then blotted onto a Hybond-C membrane. Separated proteins in the gels were electrophoretically transferred onto nitrocellulose membrane at 150 mA for 1h 20min. The blotted membrane was blocked with 5% skimmed milk in PBS containing 0.05% Tween 20 (TBS-T buffer) for 2h. After blocking the membrane, anti-his antibody (1 mg/mL), diluted in PBS, was added and incubated for overnight at 4°C. After washing the membrane with TBS-T, HRP-goat anti-mouse IgG (H+L) (0.4mg/mL), diluted in PBS-T, was added and incubated for 2h at room temperature. Finally, washed the membrane and stained by DAB reagent.

Microvinification experiments and determination the content of Res: Fermentation assays were carried out using 2 liters of sterilized *Chardonnay* (Sugar content 200 g/L, total acid 6.88 g/L (tartaric acid), pH 3.89). The selected pYCα-RS transformant was inoculated into must to produce wine in a microvinification process. A parallel control fermentation using the YS58 strain was performed. During the fermentation, 40mL fermentation liquid was taken per 24h for the extraction and determination of Res. In order to confirm the effect of RS on Res synthesis, a fermentation also has been carried out. The enzyme solution of pKCα-RS transformant was added to must, then YS58 was inoculated into must. Periodically sampled for the determination of Res' s content during the fermentation. The enzyme solution of pYCα-RS transformant was not added to the must as a control. All microvinification experiments were in triplicate.

Res was extracted twice from samples and cell lysates with equal volumes of pure ethyl acetate. Then the organic phase was washed three times using 15 mL 3% $NaHCO_3$ solution. The residues were filtered after drying over anhydrous sodium sulfate and concentrated to a volume of 2 mL with a rotary evaporator (CENCO). The samples were stored at 20°C until the HPLC analysis. All of the above operations carried out in the dark.

Res concentration was determined by HPLC (Shimadzu Corporation) with a Wondasil C18 column (Shimadzu, Kyoto, Japan). The mobile phase consisting of solvent A (acetonitrile) and solvent B (distilled water). Start: 5% A, 95% B, remain 5min; 28min: 60% A, 40% B 33min: 85% A, 15% B, remain 2min; 40 min: 5% A, 95% B; Finally, the pole was balanced. It was set at a flow rate of 1.0 mL/min. UV detection was performed at 360nm of wavelength. Extraction and analysis of Res were performed in triplicate.

Results

Construction of pKCα-RS recombinant plasmid: Secretory expression plasmid pKCα was double digested by *Not* I and *Xba I* and obtained a about 6000bp fragment. Electrophoretic identification analysis reveals that the digestion of pYCα was a success. Similarly, the plasmid T-Easy-RS was double digested by *Not I* and *Xba I* and RS target gene fragment was recycled. In the next step, the RS target gene was connected to pYCα fragment for 12 hours at 4°C with T4 DNA ligase. The ligation product was transformed into *E. coli* and screened by LB/Amp medium.

The single colony was cultured for 12h. Electrophoresis analysis was carried out after plasmids were extracted. The 1179bp insert was found via electroporosis and it was further confirmed by sequencing. The results showed that RS fragments were successfully inserted into pYCα secretory expression plasmid. The pYCα-RS plasmid had been constructed successfully. The process of constructing the secreting expression vector is shown in Fig. 2.

Transformation, screening of yeast and test of genetic stability: Under the control of the T7 promoter, the RS protein encoded by the target gene can be secreted into the extracellular matrix guided by the α-factor signal peptide. C-terminal of the α-factor signal peptide in pYCα-RS contains yeast endoprotease recognition site (Lys-Arg). It can remove the signal peptide in the process of secreting into the extracellular matrix.

Because both pYCα-RS and pYCα contain Ura3 genes, transformants can grow on the SD-Ura (lacking uracil) medium. At this time, SD-Ura solid medium was used for the yeast transformants screening.

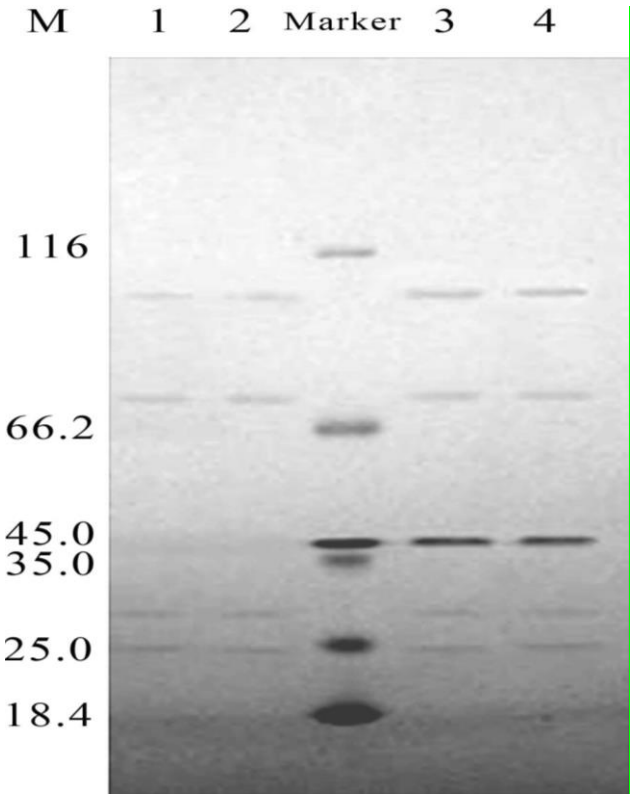

Fig. 3. SDS-PAGE analysis of the secretion expression of RS, upper panel yeast culture media after secretory expression in *S. cerevisiae* (10× concentrated media supernatants). 1, 2: Protein samples of pYCα-RS/YS58 without galactose induced medium. 3, 4: Protein samples of pYCα-RS/YS58 galactose induced medium. Maker: Protein molecular weight marker.

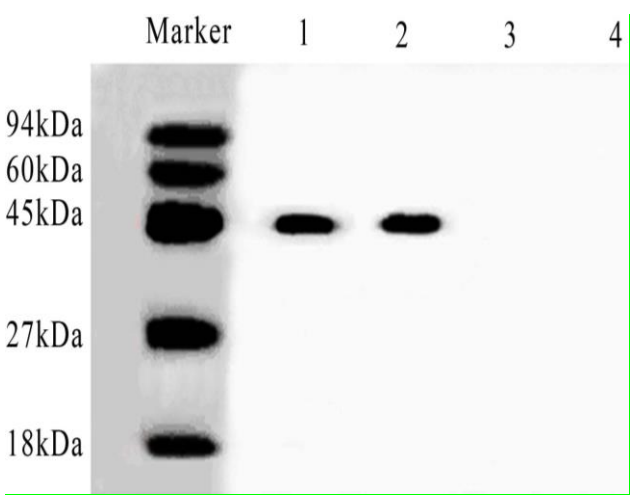

Fig. 4. Western blot detection of protein samples, Western blot experiments using monoclonal antibodies against RS. The proteins of RS expressed by pYCα-RS/YS58 (45kD). Lanes: 1, 2: The culture supernatant of pYCα-RS/YS58 galactose induced medium. 3, 4: The culture supernatant of pYCα-RS/YS58 without galactose induced medium. Maker: Protein molecular weight marker.

Both pYCα-RS and pYCα were transformed into competent cells of *S. cerevisiae* YS58 through electric shock. Recombinant plasmid transformants pKCα-RS/YS58 were screened on mediums later. Finally, these transformants were identified by colony PCR. Electrophoresis showed that the band size is 1179bp, so pYCα-RS has been inserted into the chromosome of *S. cerevisiae* YS58 successfully.

All of the 100 single colonies of the tenth generation of engineered strain could grow on SD-Ura plates, suggesting that the engineered strain was genetically stable.

Induced expression of secretory expression plasmid in *S. cerevisiae*: The recombinants were cultured by galactose induced medium and the negative control was without galactose induced medium. After induction, the supernatants were collected and a certain band was shown in the SDS-PAGE analysis. SDS-PAGE analysis revealed that the recombinant yeast cells expressed the predicted 45kDa RS. The result was shown in Fig. 3.

There was a strong cross-reaction well above the background and corresponding to a polypeptide with a molecular weight expected for the RS protein of about 45kDa, was detected (lanes 1, 2). While for the transformant grown without galactose-induced medium, had no same polypeptides detected (lanes 3, 4). No noticeably sharper specific bands appeared at 45kDa.The analysis revealed that the protein secretory expressed by recombinant yeast cells is RS. The RS was appeared in the supernatants of recombinant yeast. Then, we did western-blot according antigen specificity. The result was shown in Fig. 4 this proved that we have got specific RS protein.

Microvinification experiments by the recombinant wine yeast strain: The wine-making processes monitored by weightlessness. During the fermentation, 40mL samples were collected per 24h for the extraction and determination of Res by HPLC. Every microvinification experiment was carried out in triplicate (Fig. 5).

At the beginning, the concentration of Res is low in Chardonnay grape juice, lower than 5 μg /L. There was an upward trend on the Res content overall for them with the fermentation process been carried out to completion. It can be seen in Fig. 5, the concentration of Res in the wine produced by the pYCα-RS/YS58 transformant was always higher than that produced by the YS58 strain during the wine-making. In the case of the fermentation caused by pYCα-RS/YS58 transformant, the Res content increased rapidly at the beginning of fermentation. Subsequently, the concentration increased gradually with fermentation carrying out and finally remained unchanged. While, the concentration of Res increased slowly throughout the whole fermentation for the YS58 strain. At the end of fermentation, the Res content of pYCα-RS/YS58 reached 31 ± 2 μg/L which was 36% higher than it. The results clearly showed there was a significant increase (p<0.05) in the Res content in wine produced by the pYCα-RS/YS58 transformant.

In order to confirm the effect of RS on Res synthesis, another fermentation was carried out. The enzyme solution of pYCα-RS transformant was added to must, then YS58 was inoculated into must. During the fermentation, 40mL samples were collected for the extraction and determination of Res by HPLC in initial stage, prophase, metaphase and anaphase. The results were shown in Fig. 6. When the enzyme solution of pYCα-RS transformant was added to the must, the concentration of Res was higher than the control. The results clearly showed that the addition of the enzyme solution has promoted the generation of Res during the fermentation (Fig. 6).

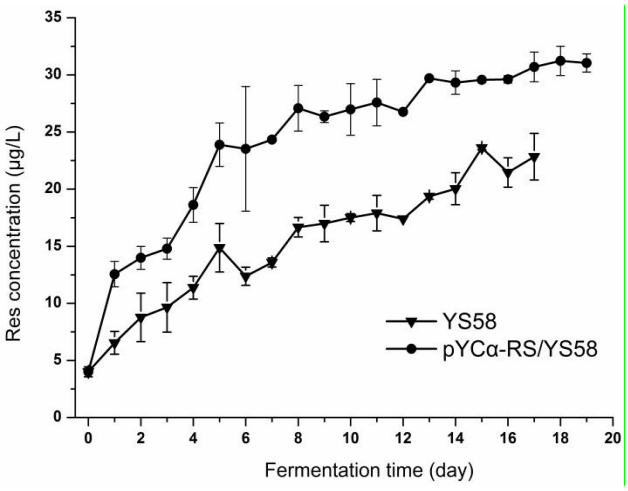

Fig. 5. Time course of Res production during experimental fermentation. Two curves represent fermentation conducted with yeast strain YS58 and pYCα-RS/YS58, respectively. There was significant difference in the concentration(mg/L) of resveratrol during Chardonnay fermentation.

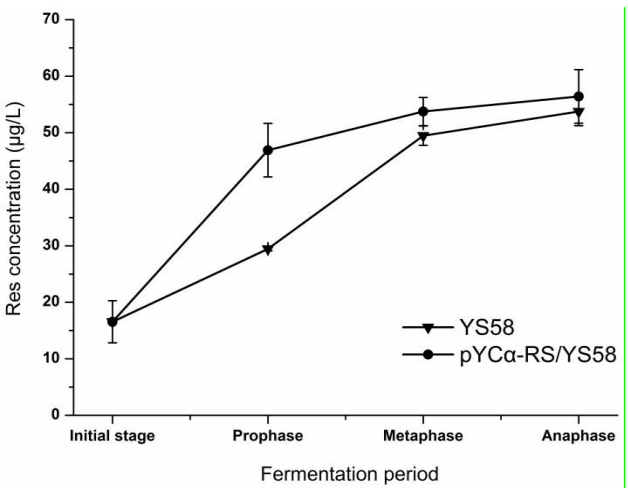

Fig. 6. Fermentation curves for treatment containing added enzyme solution of pYCα-RS transformant and control. Different treatments plotted as the mass difference of the concentration of resveratrol during fermentation. These two curves are fermentation using strain YS58. Treatment containing enzyme solution of pYCα-RS transformant were produced significantly faster as compared to non-treatment.

Discussi on

If plants were not stimulated by some inducing factors such as microbial infection, UV radiation, tissue damage, they cannot produce inducible the RS enzyme. Under normal conditions, it is difficult for plants to synthesis the Res by their own. So far, Res only has been found in 72 kinds of plants of 12 families, 31 genera, and the concentration in grapes is the highest in all fruits (Jeandet *et al.,* 2012). Many articles reported that the precursors of Res is widely present in plants, but they also cannot synthesize Res due to the lack of RS. As can be seen from that, the RS is the critical enzyme in the synthesis process of Res.

Constructing engineered strains by yeast and other microorganisms is still a hotspot for the biosynthesis of Res. Due to limitations of genetic manipulation methodologies and tools, *E. coli* or *S. cerevisiae* has been widely applied for construction currently. While, because of superior performance and biosafety eukaryotic expression system, yeast has become the best engineered strain and has good prospects for the development and application. So, yeast is the best option to construct the engineered strain in this study. In order to remove the introns in DNA sequence and obtain 392 amino acids of RS correctly, the RS gene was isolated by the reverse transcription of RNA.

In this study, a signal peptide sequence, secreted by α-factor of *S. cerevisiae*, was connected with RS gene, resulting a pYCα secretory plasmid construction. The RS gene was secretory expressed in *S. cerevisiae* successfully. Then the white wine fermentation was carried out by recombinant strain. The Res concentration of the white wine fermented by recombinant strain was higher than the control. Furthermore, the biological activity of secretory expressed product has been approved. Moreover, it can be seen that the addition of the enzyme solution has promoted the generation of Res during the fermentation. The present study describes a first practical application for wine production of a *S. cerevisiae* that was engineered for novel production of Res during the processing which makes grape juice to wine. The relationship between specific growth rate and biomass productivity is a significant parameter in the control of aerobic fed-batch processes for yeast product formation. We observed a strong positive correlation between biomass productivity and specific growth rate in these experimental fermentation, limited nutrient, and the environment cultures of an engineered, Res-producing yeast strains of *S. cerevisiae*. Similar positive correlations between biomass productivity and growth rate relations as identified in this study were found for heterologous production of proteins by engineered yeasts and for production of ethylene by a *S. cerevisiae* strain expressing a heterologous ethylene-forming enzyme (Johansson *et al.,* 2013). In view of the key role of many of the involved precursors in central metabolism, breaking this correlation represents a major challenge for metabolic engineers and synthetic biologists.

Other studies have shown that Res due to its potent anti-oxidant and anti-inflammatory properties are cardio-protective, chemotherapeutic, neuroprotective, and display anti-aging effects. We observed it from fermentation that pYCα-RS/YS58 have two days longer lifespan than YS58. Combining with other previous metabolic studies, when it comes to the end of stationary phase, the living condition of *S. cerevisiae* becomes tough, because of the rising of alcohol and lacking of nutrient content. So, it is a proof that yeast with RS gene live a longer life, meanwhile, this result verified that Res has many potential and beneficial properties to yeast.

This recombinant strain might offer a viable way to meet the demands of health-conscious wine consumers. In the future, we can obtain Res and increase the concentration of Res in wine by the recombinant strain, meanwhile, the study also laid the foundation for the potential commercialization of Res, as a bioactive food additive.

Acknowledgements

We are grateful for financial supported by Shaanxi Province Scientific and Technological Transformative Project (201604D132034) and National Natural Science Fund Program of China (Grant No. 31571812, 31501463). YANGBO SONG, SHUWEN LIU and YANLIN LIU designed the research. YANGBO SONG and LI FENG performed this research. YANGBO SONG and LI FENG collected and analyzed data. YUYANG SONG, YI QIN, and DONGQING YE discussed the data, and also DONGQING YE edited and modified those pictures. LI FENG and YANGBO SONG wrote the paper. All authors have read, edited and approved the current version of the manuscript.

References

Andrea, M., C. Cinzia, L. Sergio, A. van Beek Teris, G. Luca, R.S. Francesco and B. Jules. 2010. Production of novel antioxidative phenolic amides through heterologous expression of the plant's chlorogenic acid biosynthesis genes in yeast. *Metab. Eng*., 12: 223-232.

Baur, J.A., K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin and A. Kalra. 2006. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature*, 444: 337-342.

Becker, J.V.W., G.O. Armstrong, M.J. Van der Merwe, M.G. Lambrechts, M.A. Vivier and I.S. Pretorius. 2003. Metabolic engineering of Saccharomyces cerevisiae for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res*., 4: 79-85.

Damianaki, A., E. Bakogeorgou, M. Kampa, G. Notas, A. Hatzoglou & S. Panagiotou. 2000. Potent inhibitory action of red wine polyphenols on human breast cancer cells. *J. Cell. Biochem*., 78: 429-441.

Gambini, J., M. Ingles, G. Olaso, R. Lopez-Grueso, V. Bonet-Costa, L. Gimeno-Mallench, C. Mas-Bargues, K.M. Abdelaziz, M.C. Gomez-Cabrera, J. Vina and C. Borras. 2015. Properties of Resveratrol: *In vitro* and *In vivo* Studies about metabolism, bioavailability, and biological effects in animal models and humans. *Oxid Med Cell Longev*., 6: 837042.

Goldberg, D.M., E. Tsang, A. Karumanchiri and G.J. Soleas. 1998. Quercetin and p-coumaric acid concentrations in commercial wines. *Am. J. Enol Viticult.,* 49: 142-151.

Gruber, J., S.Y. Tang and B. Halliwell. 2007. Evidence for a trade-off between survival and fitness caused by resveratrol treatment of Caenorhabditis elegans. *Ann. Ny Acad Sci.,* 1100: 530-542.

Hain, R., H.J. Reif, E. Krause, R. Langebartels, H. Kindl and B. Vornam. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. *Natur*e, 361: 153-156.

Howitz, K.T., K.J. Bitterman, H.Y. Cohen, D.W. Lamming, S. Lavu and J.G. Wood. 2003. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature*, 425: 191-196.

Jeandet, P., B. Delaunois, A. Aziz, D. Donnez, Y. Vasserot and S. Cordelier. 2012. Metabolic Engineering of Yeast and Plants for the Production of the Biologically Active Hydroxystilbene, Resveratrol. *J. Biomed. Biotechnol.,* 2012: 1-14.

Johansson, N., P. Quehl, J. Norbeck and C. Larsson. 2013. Identification of factors for improved ethylene production via the ethylene forming enzyme in chemostat cultures of Saccharomyces cerevisiae. *Microbial cell factories.,* 12.

Juan, M.E., E. Gonzalez-Pons, T. Munuera, J. Ballester, J.E. Rodriguez-Gil and J.M. Planas. 2005. trans-Resveratrol, a natural antioxidant from grapes, increases sperm output in healthy rats. *J. Nutr.,* 135: 757-760.

Kang, K. and K. Back. 2009. Production of phenylpropanoid amides in recombinant *Escherichia coli*. *Metab. Eng*., 11: 64-68.

Kobayashi, S., C.K. Ding, Y. Nakamura, I. Nakajima and R. Matsumoto. 2000. Kiwifruits (*Actinidia deliciosa*) transformed with a Vitis stilbene synthase gene produce piceid (resveratrol-glucoside). *Plant Cell Rep*., 19: 904-910.

Kodan, A., H. Kuroda and F. Sakai. 2001. Simultaneous expression of stilbene synthase genes in Japanese red pine (Pinus densiflora) seedlings. *J. Wood Sci*., 47: 58-62.

Michaelis, S. and J. Barrowman. 2012. Biogenesis of the *Saccharomyces cerevisiae* pheromone a-factor, from yeast mating to human disease. *Microbiol. Mol. Biol. Rev*., 76: 626-651.

Pan, M.H., C.S. Lai, M.L. Tsai, J.C. Wu and C.T. Ho. 2012. Molecular mechanisms for anti-aging by natural dietary compounds. *Mol. Nutr. Food Res*., 56: 88-115.

Park, J.W., Y.J. Choi, M.A. Jang, Y.S. Lee, D.Y. Jun and S.I. Suh. 2001. Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells. *Cancer Lett*., 163: 43-49.

Pitera, D.J., C.J. Paddon, J.D. Newman and J.D. Keasling. 2007. Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. *Metab. Eng*., 9: 193-207.

Preisig-Muller, R., A. Schwekendiek, I. Brehm, H.J. Reif and H. Kindl. 1999. Characterization of a pine multigene family containing elicitor-responsive stilbene synthase genes. *Plant Mol. Biol*., 39: 221-229.

Schwekendiek, A., G. Pfeffer and H. Kindl. 1992. Pine stilbene synthase Cdna, a tool for probing environmental-stress. *FEBS Lett*., 301: 41-44.

Sgambato, A., R. Ardito, B. Faraglia, A. Boninsegna, F.I. Wolf and A. Cittadini. 2001. Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. *Mutat. Res*., 496: 171-180.

Smit, A., R.R.C. Otero, M.G. Lambrechts, I.S. Pretorius and P. Van Rensburg. 2003. Enhancing volatile phenol concentrations in wine by expressing various phenolic acid decarboxylase genes in Saccharomyces cerevisiae. *J. Agric. Food Chem*., 51: 4909-4915.

Sparvoli, F., C. Martin, A. Scienza, G. Gavazzi and C. Tonelli. 1994. Cloning and Molecular Analysis of Structural Genes Involved in Flavonoid and Stilbene Biosynthesis in Grape (*Vitis vinifera* L). *Plant Mol. Biol*., 24: 743-755.

Sydor, T., S. Schaffer and E. Boles. 2010. Considerable increase in resveratrol production by recombinant industrial yeast strains with use of rich medium. *Appl. Environ. Microbiol*., 76: 3361-3363.

Thomzik, J.E., K. Stenzel, R. Stocker, P.H. Schreier, R. Hain and D.J. Stahl. 1997. Synthesis of a grapevine phytoalexin in transgenic tomatoes (*Lycopersicon esculentum* Mill.) conditions resistance against Phytophthora infestans. *Physiol. Mol. Plant Pathol*., 51: 265-278.

Tomé-Carneiro, J., M. Larrosa, A. González-Sarrías, F.A. Tomás-Barberán, M.T. García-Conesa and J.C. Espín. 2013. Resveratrol and clinical trials: The crossroad from *In vitro* studies to human evidence. *Curr. Pharm. Des*., 19: 6064-6093.

Trantas, E., N. Panopoulos and F. Ververidis. 2009. Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in Saccharomyces cerevisiae. *Metab. Eng*., 11: 355-366.

Vannelli, T., W.W. Qi, J. Sweigard, A.A. Gatenby and F.S. Sariaslani. 2007. Production of p-hydroxycinnamic acid from glucose in *Saccharomyces cerevisiae* and *Escherichia coli* by expression of heterologous genes from plants and fungi. *Metab. Eng*., 9: 142-151.

Ververidis, F., E. Trantas, C. Douglas, G. Vollmer, G. Kretzschmar and N. Panopoulos. 2007. Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part II: Reconstruction of multienzyme pathways in plants and microbes. *Biotechnol J*., 2: 1235-1249.

Vos, T., P. de la Torre Cortés, W.M. van Gulik, J.T. Pronk and P. Daran-Lapujade. 2015. Growth-rate dependency of de novo resveratrol production in chemostat cultures of an engineered *Saccharomyces cerevisiae* strain. *Microb. Cell. Fact*., 14: 133.

(Received for publication 21 December 2017)