J Ind Microbiol Biotechnol (2014) 41:1793–1802
DOI 10.1007/s10295-014-1517-1

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Mechanism of proanthocyanidins-induced alcoholic fermentation enhancement in *Saccharomyces cerevisiae*

Jingyuan Li · Hongwei Zhao · Weidong Huang

Received: 14 July 2014 / Accepted: 23 September 2014 / Published online: 2 October 2014

Abstract Our previous work revealed proanthocyanidins (PAs) could pose significant enhancement on the activity of H^+-ATPase and fermentation efficiency after a transient initial inhibition (Li et al in Am J Enol Vitic 62(4):512–518, 2011). The aim of the present work was to understand the possible mechanism for this regulation. At Day 0.5 the gene expression level of *PMA1* in AWRI R_2 strain supplemented with 1.0 mg/mL PAs was decreased by around 54 % with a 50 % and a 56.5 % increase in the concentration of intracellular ATP and NADH/NAD^+ ratio, respectively, compared to that of control. After the transient adaptation, the gene expression levels of *PMA1* and *HXT7* in PAs-treated cells were enhanced significantly accompanied by the decrease of ATP contents and NADH/NAD^+ ratio, which resulted in the high level of the activities of rate-limiting enzymes. PAs could pose significant effects on the fermentation via glucose transport, the energy and redox homeostasis as well as the activities of rate-limiting enzymes in glycolysis.

Keywords Proanthocyanidins · *Saccharomyces cerevisiae* · Alcoholic fermentation · Glycolysis · Wine

J. Li and H. Zhao contributed equally to this work.

J. Li
College of Food Science and Engineering, Qingdao Agricultural University, Qingdao, China

H. Zhao · W. Huang (✉)
College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China
e-mail: manuscriptsubmit@126.com

W. Huang
Center for Research and Development of Viticulture and Enology, Beijing, China

W. Huang
Beijing Key Laboratory of Viticulture and Enology, Beijing, China

Introduction

Phenolic compounds are widely distributed within the pulp, skin, seed and stem of the grapes, which are partially extracted during winemaking [15]. Those chemicals play an important role in the sensory properties of red wines, such as astringency and color [4, 40]. In addition, as reported in several studies, many of the phenolic compounds show important biological activities related to their antioxidant properties, reactive oxygen and nitrogen species scavenging, modulate and immune function [1, 10, 36]. The composition of the phenolic compounds is very complex and changes over the fermentation and aging phases by the condensation of some of those compounds, especially proanthocyanidins (PAs) and anthocyanins, which could affect the modification in the color and taste [5, 39]. Maceration time [38], fermentation temperature [14] and storage time [29] could impact the extraction content and structure of phenolic compounds. Furthermore, interactions between the phenolic compounds and the yeast cells have been studied previously and are proved to affect the formation of new polymeric pigments and yeast metabolisms. It is well known that polyphenols could be adsorbed by yeast cells by Van der Waals bonds and H-bonds during alcoholic fermentations [22, 42]. This could explain that the yeasts can modify the color of the wine either through establishing weak and reversible interactions between anthocyanins and yeast walls or through periplasmic β-glucosidase activity targeted to the 3-*O*-glucoside part of anthocyanins [6]. Salmon et al. [35] reported that the spherical morphology of yeast cells could be maintained by the interaction of

yeast lees with wine polyphenols, although degradation of the yeast cell wall occurs during autolysis. As polysaccharides released during the autolysis process are thought to exert a significant effect on the sensory qualities of wines [21], the presence of polyphenols during aging should strongly affect both the released polysaccharides and the sensory properties of the final wines. Furthermore, Vivas et al. [44] studied the effects of phenolic compounds on growth, viability, and malolactic activity of lactic acid bacteria. They found that different phenolic compounds possess favorable or un-favorable influence on the growth and metabolism of *Leuconostoc oenosmay*. Hervert-Hernández et al. [12] reported that hydroxycinnamic acids and their esters could be metabolized during the growth phase of some lactic acid bacteria species. However, at high concentrations, phenolic compounds are toxic for the cells, which could cause inhibition of their growth and fermentation [30]. It indicated that the phenolic compounds act in a structure-dependent and dose-dependent manner to impact the metabolism of yeast and bacterial cells.

Alcoholic fermentation phase in winemaking plays an important role in the formation of sensory properties of the wines. The interactions between yeast cells and the grape must-complexes will be started at the initial stage of the fermentation and be maintained throughout the whole process. Based on the above literature survey, the effect of the yeast on the composition, absorption and structure evolution of phenolic compounds has been demonstrated properly. However, few studies focus on the influences of the phenolic compounds on the growth and alcoholic fermentation of the yeast.

As principal phenolic compounds in red wine, PAs consist of more than 90 % of the total phenolic content and the concentration varies from 0.2 to 0.8 mg/mL in different wine types [28]. In our previous study, PAs showed different effects on the yeast metabolism at the initial phase and the mid-exponential phase of fermentation [17]. At the beginning of the fermentation, PAs inhibited the activity of H^+-ATPase, followed by decreased profiles of the cell growth rate, sugar consumption rate, and ethanol production rate. In contrast, PAs enhanced the cell growth and alcoholic fermentation rate at the mid-exponential phase of fermentation (Table 1). In general, the fermentation was shortened by PAs supplementation. It indicated that PAs at the optimal concentration could be considered as a kind of protectant to protect the yeast cells from environmental stress damages (e.g. osmotic stress, ethanol stress, heat stress). The mechanism of the PAs-induced alcoholic fermentation improvement should be clarified for the quality control of winemaking.

In this study, we carried out the alcoholic fermentation in a model synthetic medium supplemented with elevated concentrations of PAs. The gene expression levels of *PMA1* and *HXT7*, encoding the plasma membrane ATPase and yeast hexose transporter, respectively, were measured to evaluate the effect of the PAs on glucose uptake. Furthermore, the intracellular ATP contents, NADH/NAD^+ ratio and the activities of the rate-limiting enzymes in glycolysis pathway were also measured to study the regulation mechanisms of the PAs. Our study could be beneficial for fully understanding of the response mechanism of wine yeasts under environmental stresses and the protective role of PAs. In addition, the information is also helpful for the improvement of fermentation and wine quality.

Materials and methods

Strains and culture conditions

Two *S. cerevisiae* strains, one commercial wine strain AWRI R_2 (Maurivin, Queensland, Australia) and one laboratory-isolated strain BH_8, were used in this study. Strain BH_8 was isolated from naturally fermented juice of the *Beihong* grape (a hybrid of *Muscat Hamburg* and *V. amurensis*). The colony of the strain was hemispherical in shape with cream color and smooth surface on YPD agar plates, which was similar to the characteristics of *S. cerevisiae* strain. Internal transcribed spacer (ITS) regions of the rDNA of the strain BH_8 and strain S288C (a model strain

Table 1 Effect of PAs on the growth and fermentation ability of strain AWRI R_2 and BH_8

	AWRI R_2			BH_8		
PAs concentration (mg mL^{-1})	0	0.1	1	0	0.1	1
Viable cell counts (lg cfu)	6.57 ± 0.07	6.97 ± 0.02	7.10 ± 0.031	6.79 ± 0.072	6.95 ± 0.047	7.08 ± 0.027
[a]Sugar consumption rate (gL^{-1} h^{-1})	12.4 ± 0.23	14.2 ± 0.45	16.5 ± 0.19	12.4 ± 0.22	14.0 ± 0.29	16.4 ± 0.18
[b]Ethanol volumetric production rates (gL^{-1} h^{-1})	0.89 ± 0.021	1.03 ± 0.058	1.25 ± 0.051	0.9 ± 0.029	1.04 ± 0.048	1.58 ± 0.032

[a] Sugar consumption rates were calculated by dividing the volumetric consumed sugar by the time reaching the minimum sugar concentration in the media

[b] Ethanol volumetric production rates were calculated by dividing the maximum ethanol concentration by the fermentation time required to reach that concentration

Table 2 Primers used in this study

Primer name	Sequence
PMA1[a] -forward	TTTTCCCACAACATAAATACAGAGT
PMA1-revese	CATCAATAATAGCAGATAGACCAGG
HXT7[b] -forward	CTGCATCCATGACTGCTTGTAT
HXT7-revese	TCAGCGTCGTAGTTGGCACCTC
Actin[c] -forward	GATTCTGAGGTTGCTGCTTTGG
Actin-revese	GACCCATACCGACCATGATACC

[a] Gene encoding plasma membrane H^+-ATPase

[b] Gene encoding hexose transporters

[c] Internal reference gene for relative expression level calculation in real-time PCR

of *S. cerevisiae*) were sequenced, respectively. There were no significant differences in the base sequence between the two strains. Therefore, strain BH_8 could be classified as *S. cerevisiae*. Yeast cells were maintained on YPD medium slants (1 % yeast extract, 2 % peptone, 2 % glucose, 2 % agar). To prepare the inoculum, one loop of yeast cell colony was transferred to 60 mL aqueous YPD media in 250 mL flask. The culture was incubated aerobically for 14 h in a shaker set at 28 °C and 120 rpm.

Fermentation

Model synthetic medium (MSM) was used for fermentation in this study. The MSM simulated a standard grape juice with a chemically well-defined medium. The compositions are as follows: carbon source and nitrogen source: glucose (100 g), fructose (100 g), tartaric acid (3 g), citric acid (0.3 g), L-malic acid (0.3 g), $MgSO_4$ (0.2 g), KH_2PO_4 (2 g), $(NH_4)_2SO_4$ (0.3 g) and asparagine (0.6 g); mineral salts: $MnSO_4 \cdot H_2O$ (4 mg), $ZnSO_4 \cdot 7H_2O$ (4 mg), $CuSO_4 \cdot 5H_2O$ (1 mg), KI (1 mg), $CoCl_2 \cdot 6H_2O$ (0.4 mg), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1 mg), H_3BO_3 (1 mg); vitamins: meso-inositol (300 mg), biotin (0.04 mg), thiamin (1 mg), pyridoxine (1 mg), nicotinic acid (1 mg), pantothenic acid (1 mg), p-amino benzoic acid (1 mg); fatty acids: palmitic acid (1 mg), palmitoleic acid (0.2 mg), stearic acid (3 mg), oleic acid (0.5 mg), linoleic acid (0.5 mg), linolenic acid (0.2 mg). The above chemicals were dissolved in 1 L deionized water thoroughly and the pH value was adjusted to 3.3 [20]. PAs were purchased from Biofine International Inc. at Vancouver, BC, Canada, and purified to 98 % as described in our previous work [17]. The purified PAs were supplemented to MSM to give final concentrations of 0, 0.1 and 1.0 mg/mL. The media were sterilized by filtration (nitrate cellulose membrane, 0.45 μm) before inoculation.

Yeast strains were pre-cultured aerobically in YPD to late-exponential phase. Initial yeast inoculums were adjusted to 5×10^5 cfu/mL using hemocytometer in 400 mL medium in 500 mL flasks equipped with glass capillary stoppers for the anaerobic condition. The MSM cultures were incubated in shaker at 28 °C and 120 rpm. Samples were taken at the specified time intervals for the following analysis.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated using the TAKARA Universal RNA Extraction Kit purchased from Takara Biotechnology (Dalian) Co., LTD. The quality of the isolated RNA was determined by agarose gel electrophoresis. Reverse transcription for the cDNA synthesis was carried out in Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) with Promega A3500 Reverse Transcription System as per the manufacturer's protocols. The primers used in this study are listed in Table 2. The cDNA products were stored at −80 °C.

Real-time quantitative PCR

Real-time quantitative PCR was performed in the GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Grand Island, NY) using SYBR green detection. Briefly, a 10-fold dilution series of the cDNA products containing the tested genes were used as the templates for real-time quantitative PCR to generate a plot of log copy numbers of the tested genes at different dilutions versus the corresponding cycle threshold (Ct). The *Actin* was employed as the reference gene to calculate the relative expression value of the tested genes. Thus, the quantity of *PMA1* and *HXT*, relative to the reference gene *Actin*, can be calculated using the formula $2^{-\Delta Ct}$, where $\Delta Ct = (Ct_{PMA1 \text{ or } HXT} - Ct_{Actin})$ [37].

Measurement of the intracellular ATP, NADH and NAD^+ contents

The concentrations of intracellular ATP, NADH and NAD^+ were measured using waters 2695 HPLC system (Waters Corp., Milford, MA) based on the methods described previously [18, 26]. 10 mL of each sample was taken and divided into two groups equally for the calculation of ATP concentration and dry cell mass (DCW). For extracting ATP, cells were frozen in liquid nitrogen for 60 s ensuring rapid cooling and effective inhibition of cell metabolism.Then the samples were boiled with equal volumes of 0.3 M trichloroacetic acid for 5 min followed by cooling down rapidly. The mixture was centrifuged for 10 min at 10,000 × g to collect the supernatant. The final volume of the supernatants was adjusted to 10 mL for HPLC analysis. For extracting the NAD^+, the cell pellets were homogenized

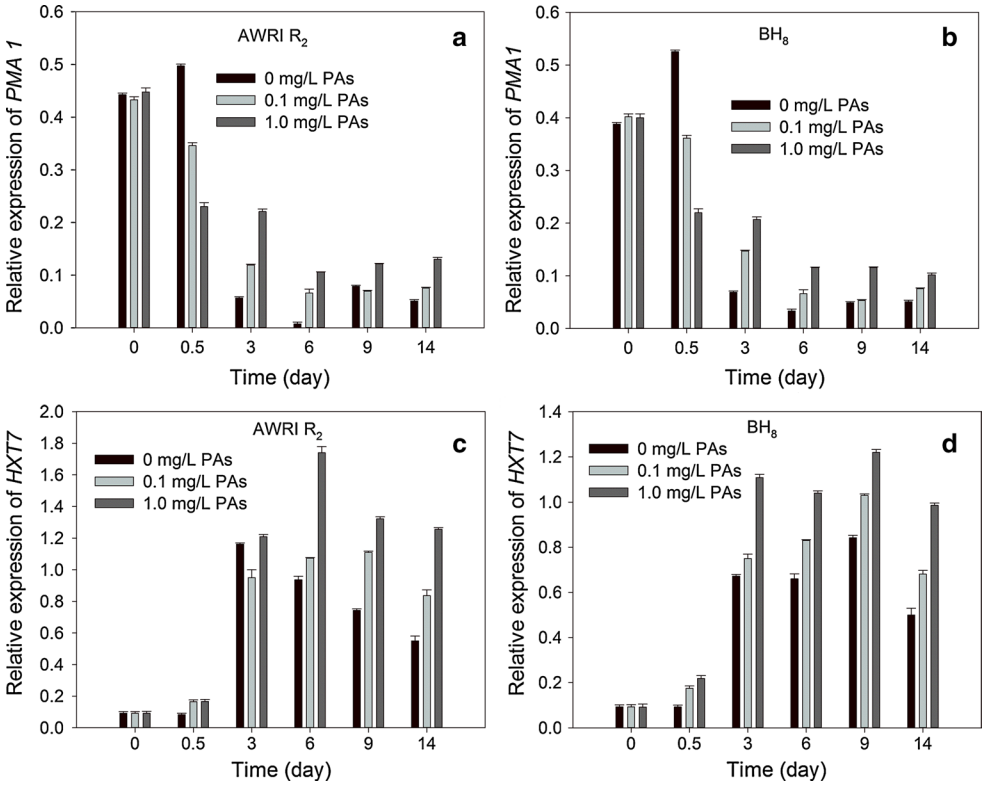

Fig. 1 Effects of PAs on the expression levels of *PMA1* and *HXT7*, encoding plasma membrane H^+-ATPase and hexose transporters, respectively. AWRI R_2 and BH_8 strains were cultivated in model synthetic medium (MSM) supplemented with 0, 0.1 and 1.0 mg/mL PAs at 28 °C and 120 rpm anaerobically. The total RNA of the sampled cells was isolated and used as templates of the reverse transcription for the cDNA synthesis. The *Actin* gene was employed as the internal reference gene. Real-time PCR were performed and the relative expression levels of the *PMA1* (**a**, **b**) and *HXT7* (**c**, **d**) were calculated using the formula $2^{-\Delta Ct}$, where $\Delta Ct = (Ct_{PMA1\ or\ HXT} - Ct_{Actin})$ described by Schmittgen et al. [37]. All Results were the mean ± SD of three independent experiments

with 1 mL of acid buffer (0.02 N H_2SO_4 + 0.1 M Na_2SO_4) and heated at 60 °C for 45 min. For extracting the NADH, the cell pellets were homogenized with 1 mL of alkaline buffer (0.02 N NaOH + 0.5 mM DTT) and heated at 60 °C for 10 min. The mixtures containing NAD^+ and NADH were centrifuged for 10 min at 10,000×*g* to get the supernatants, respectively.

The waters 2695 HPLC system was equipped with a waters 2996 ultraviolet detector and LiChrospher 100RP-18e (250 mm × 4.0 mm i.d., 5 μm, Merck). Mobile phase for ATP separation was prepared by mixing with 0.04 M potassium dihydrogen orthophosphate and 0.06 M dipotassium hydrogen buffer; mobile phase for NAD^+ and NADH separation was prepared by mixing with 80 % 10 mM potassium dihydrogen orthophosphate and 20 % methanol. The pH of the mobile phases was adjusted to 7.0 with sodium hydroxide and filtered through a 0.22 μm filter. The injection volume was 10 μl and detection was monitored at 260 nm. Column temperature was maintained at 25 °C. The total separation time was 10 min to ensure full separation.

Measurement of activities of the key enzymes in the glycolysis pathway

5 mL of each sample was taken at specified time intervals and washed twice with cold de-ionized water. The cell pellets were homogenized with 2 mL of potassium phosphate buffer (0.1 M, pH 7.5) and sonicated with 1.5 mL glass beads (0.5 mm diam.; Sigma) on ice bath for 160 3 s intervals. The mixtures were centrifuged at 4 °C at 12,000 rpm for 10 min to obtain the crude enzymes. The activities of the hexokinase, phosphofructokinase and pyruvate kinase were measured as described by Bär et al. [3], Murcott et al. [23] and Martinez-Barajas, Randall [19] with some slight modifications, respectively, and expressed as μmol products min^{-1} mg^{-1} protein.

Results

Effect of the PAs on the expression of *PMA1* and *HXT7*

The relative expressions of *PMA1* and *HXT7* genes were evaluated by real-time PCR. As shown in Fig. 1, both *PMA1* and *HXT7* genes isolated either from AWRI R_2 strain or BH_8 strain performed different expression levels over the fermentation time. At the initial period, the expression of *PMA1* remained in the higher level; however, it decreased sharply from Day three to the end of the fermentation. In contrast, the expression of *HXT7* genes performed in low level at the beginning of the fermentation but in high level three days later. PAs supplementation posed significant effects on the expression of the two genes in both strain AWRI R_2 and strain BH_8 ($P < 0.05$). The gene expression level of *PMA1* decreased at the

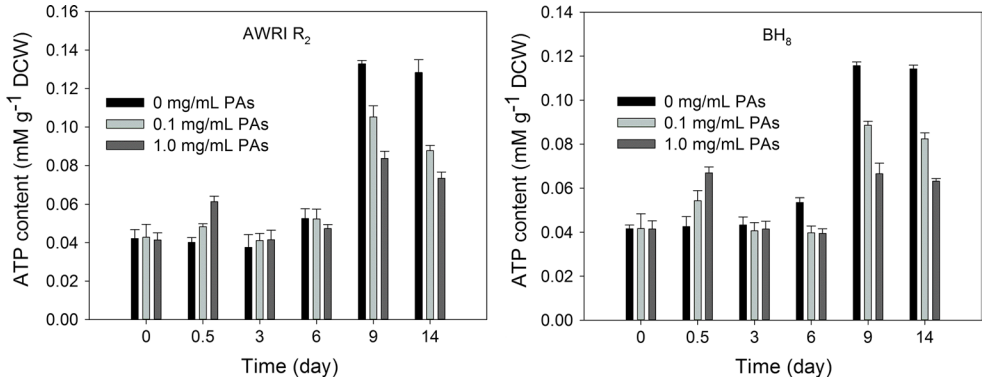

Fig. 2 Content of the intracellular ATP during alcoholic fermentation. AWRI R_2 and BH_8 cells were cultivated in model synthetic medium (MSM) supplemented with 0, 0.1 and 1.0 mg/mL PAs at 28 °C and 120 rpm anaerobically. 10 mL of each treated sample was taken at Day 0, 0.5, 3, 6, 9, and 14 and divided into two groups equally for calculation of the ATP concentration and dry cell mass (DCW), respectively. For ATP concentration measurement, the cells were frozen in liquid nitrogen for 60 s immediately. The intracellular ATP content were extracted and measured based on the method previously described [26]. The results were shown as mean mM ATP g^{-1} DCW ± SD of three independent experiments

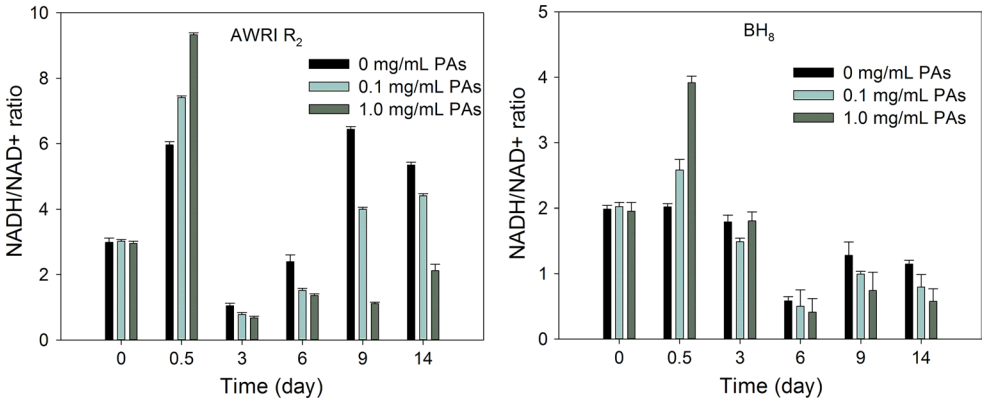

Fig. 3 Ratio of intracellular NADH/NAD^+ during alcoholic fermentation using AWRI R_2 and BH_8 strains. Fermentation were carried out in model synthetic medium (MSM) supplemented with 0, 0.1 and 1.0 mg/mL PAs at 28 °C and 120 rpm anaerobically. 10 mL of each treated sample was taken at Day 0, 0.5, 3, 6, 9, and 14 and frozen in liquid nitrogen for 60 s immediately. The intracellular NADH and NAD^+ were extracted and measured, respectively, as described by Liu et al. [18]. The NADH/NAD^+ ratios were calculated and the data were the mean ± SD of three independent experiments

beginning of the fermentation; however, it was increased significantly by the PAs supplementation compared to the control group three days later (Fig. 1a, b). In addition, dose-dependent effect was monitored in the gene expression regulation. For example, the expression level of *PMA1* gene in strain AWRI R_2 increased by around 140 and 260 % with 0.1 and 1.0 mg/mL PAs supplementation compared to the control, respectively (Fig. 1a). Referred to the *HXT7* genes, similar effects of the PAs supplementation on the gene expression were found during the fermentation. The maximal expression level appeared at Day 6 for AWRI R_2 and Day 9 for BH_8, in which the gene expression levels were improved by around 87 and 20 % with 1.0 mg/mL PAs supplementation compared to the control, respectively (Fig. 1c, d).

Effect of the PAs on the intracellular ATP, NADH and NAD^+ contents

The gene expression level of *PMA1* and *HXT7* could be positively affected by PAs supplementation in the fermentation media, which indicated that the hexose transport ability of the cells was improved by PAs. Apart from the hexose transport from external to cytosol, the intracellular metabolites such as ATP, NADH and NAD^+ could also play an important role in the regulation of the glycolysis pathway. To better understand the regulation mechanisms, the contents of the intracellular ATP, NADH and NAD^+ were measured during the fermentation. The results are shown in Figs. 2 and 3. From the initiation of fermentation to Day six the concentrations of intracellular ATP remained at a

low level ranging from 0.04 to 0.06 mM g^{-1} DCW in both strains AWRI R_2 and BH_8; whereas, the amounts of the intracellular ATP increased sharply from Day nine to Day fourteen. In addition, PAs supplementation could decrease the intracellular ATP contents with a dose-dependent effect (Fig. 2). For example, the lowest concentration of intracellular ATP (0.07 mM g^{-1} DCW) was found in strains AWRI R_2 supplemented with 1.0 mg/mL PAs at Day 14; however, the ATP content increased by 28.6 and 82.9 % compared to that of cells supplemented with 0.1 mg/mL PAs (0.09 mM g^{-1} DCW) and control (0.128 mM g^{-1} DCW), respectively. Similar changes of the intracellular ATP contents were found in BH_8 strain.

NADH/NAD^+ ratio was calculated based on the intracellular concentrations of NADH and NAD^+. As shown in Fig. 3, an obvious fluctuation in NADH/NAD^+ ratio was monitored during the whole fermentation in strains AWRI R_2 and BH_8. For example, the largest ratio (~9.33) of strain AWRI R_2 was found at Day 0.5 supplemented with 1.0 mg/mL PAs, whereas it decreased sharply to about 1 at Day 3 and then performance increased progressively. Similar trends were monitored in strain BH_8. Besides, an obvious dose-dependent effect of PAs on NADH/NAD^+ ratio was also observed in the both strains. Cells treated with higher PAs concentration performed at a lower NADH/NAD^+ ratio (Fig. 3).

Effect of PAs on the activities of the key enzymes in the glycolysis pathway

Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) are considered as the three key enzymes which effectively catalyze the irreversible reactions in the glycolysis pathway. The steps catalyzed by the three enzymes are rate-limiting steps which regulate the glycolysis flux in response to conditions both inside and outside the cells. To understand how PAs impact the glycolysis flux, the activities of HK, PFK and PK were determined during the fermentation. As shown in Fig. 4, specified trends in the enzymes activities were observed between the cells supplemented with PAs and the control. After a transient inhibition effect, the enzyme activity of HK in strain AWRI R_2 sharply reached to a high level ranging from 0.0035 to 0.0055 U mg^{-1} proteins and then fluctuated over time in the fermentation. The enzyme activity was increased by PAs supplementation with a dose-dependent effect from Day 3 to Day14. The maximal activity (0.0065 U mg^{-1} proteins) appeared in the cells supplemented with 1.0 mg/mL PAs at Day 9, in which the activity significantly enhanced by 14 % compared to that of the control (0.0057 U mg^{-1} proteins) (Fig. 4a). As compared to the strain AWRI R_2, the activity of HK in strains BH_8 showed a similar trend during the fermentation. At Day 3 the enzyme activity (0.0057 U mg^{-1} proteins) of 1.0 mg/mL PAs treated cells increased by more than 100 % compared to that of control group (0.0025 U mg^{-1} proteins) (Fig. 4b). Referred to PFK, a significant inhibition was also monitored in both of the two strains at the initial phase of the fermentation. 6 days later the PFK activities of the PAs-treated cells exceeded that of the control with an obvious dose-dependent effect. The maximal enzyme activities were 0.0086 U mg^{-1} proteins for strain AWRI R_2 and 0.0068 U mg^{-1} proteins for strains BH_8 at Day 9, respectively. The transient inhibition was also found in the change of enzyme activity of PK. From Day 0.5, PAs enhanced the enzyme activity significantly in both strains with dose-dependent effect. The maximal activities appeared at Day 9 for strain AWRI R_2 (0.0069 U mg^{-1} proteins) and Day 3 for BH_8 (0.0056 U mg^{-1} proteins), increased by 138 and 155 % compared to the control, respectively.

In general, after the transient initial inhibition, the PAs could enhance the activities of the key enzymes in glycolysis pathway with a dose-dependent effect.

Discussion

The quality control plays an important role in winemaking, not only depending on the quality of the grapes but the interactions between yeasts and compositions of grape musts [34]. The grape musts consist of 70–80 % water and many dissolved solids to provide a very complex environment for the yeast growth. Following sugars and acids, phenolic compounds are the most abundant constituents present in grapes, which play a key role in sensory attributes of wine including color intensity and stability, mouth-feel, astringency and taste [5, 9]. After the initiation of the alcoholic fermentation, the interaction between the yeasts and phenolic compounds will be maintained during the whole process and the quality of the wine will be significantly influenced during this period. Yeast cells could impact the composition, absorption and structure evolution of phenolic compounds during the fermentation, which have been demonstrated properly in many previous studies [22, 35].

Referred to the effect of phenolic compounds on the yeast alcoholic fermentation, we have proved that PAs, the principal constitute in the phenolic compounds, could influence the growth and metabolism of yeast cells in a dose and time-dependent manner [17]. To better understand the mechanism of this positive effect performed by PAs, we focused on transport of glucose from external to cytosol, the variation of intracellular key metabolites contents (ATP, NADH and NAD^+) and three key enzymes activities (HK, PFK, PK) in glycolysis pathway in this work. We presented several types of data which support the idea that the PAs supplementation enhanced the alcoholic fermentation with a dose-dependent and time-dependent manner.

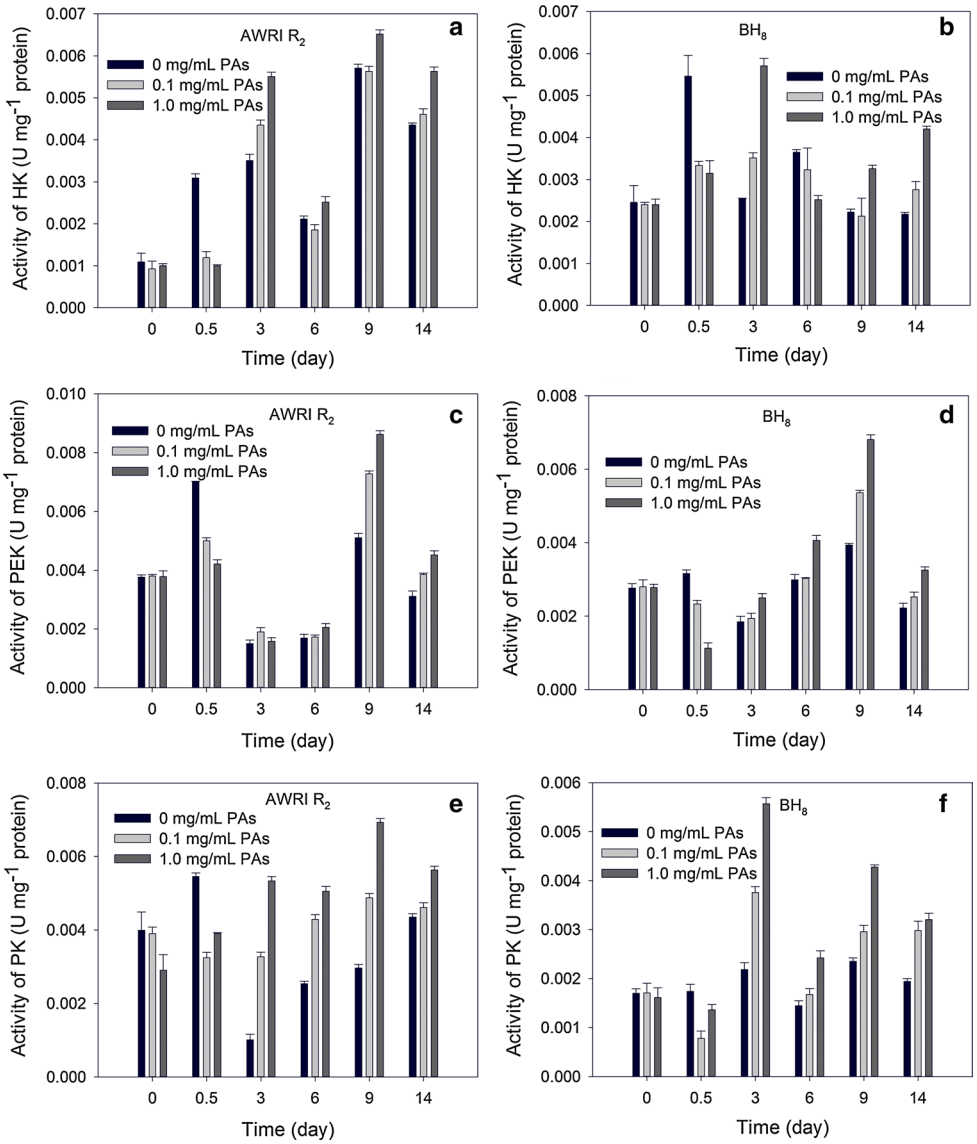

Fig. 4 Effect of PAs on the activities of hexokinase (HK), phosphofructokinase (PKF) and pyruvate kinase (PK). The cells were cultivated in model synthetic medium (MSM) supplemented with 0, 0.1 and 1.0 mg/mL PAs at 28 °C and 120 rpm anaerobically. The harvested cells were washed twice with cold de-ionized water and crushed by using ultrasound sonication treatment on ice bath for 160 3-s intervals. The mixtures were centrifuged at 4 °C at 12,000 rpm for 10 min to obtain the crude enzymes. The activities of HK (**a**, **b**), PKF (**c**, **d**) and PK (**e**, **f**) were measured according to the methods described by Martinez-Barajas and Randall [19], Bär et al. [3] and Murcott et al. [23] with slight modifications, respectively. Data were shown as the mean ± SD of three independent experiments

First, the gene expression levels of *PMA1* and *HXT7* were measured using real-time PCR to evaluate the effect of the PAs on the transport of glucose from external to cytosol. At the initial phase of the fermentation, *PMA1* gene expression was at a high level (Fig. 1a, b). The phenomena could be explained from the adaptation mechanisms [17]. After the initiation of the fermentation, yeast cells need to adapt to the new external environment immediately for survival, which related to a series of gene expressions and other physiological changes [7]. Any change in the external environment could generate stress-induced damage to the cell. For the response of the environmental stress, a large amount of energy, mostly in the form of ATP, will be required for the intracellular metabolisms [13]. Therefore, *PMA1*expression level increased for the requirement of the large amount of plasma membrane H^+-ATPase. It is well known that the plasma membrane H^+-ATPase couples hydrolysis of ATP to pump protons to generate transmembrane electrochemical gradient ($\Delta\mu H^+$). Therefore, during this process, a large amount of ATP will be consumed to provide a source of the energy for secondary solute transport systems and playing an important role in maintenance of ion homeostasis in the yeast cell [27]. This could explain the lower concentrations of ATP at the earlier phase of the fermentation (Fig. 2).

PAs could eliminate the negative effect of the environmental stress. Therefore, the PAs-treated cells could recover faster from the new environment and trigger the fermentation process. This could explain the lower level of *PMA1*expression compared to the control at the initial fermentation period (Fig. 1a, b). After the transient inhibition, the expression level of *PMA1* in the PAs-treated cells exceeded control group due to the higher intracellular metabolisms such as biomass growth, glucose consumption and ethanol yields (Table 1).

Facilitated diffusion is the main mechanism of the sugar uptakes in yeast cells under high sugar concentration [33]. During this period, transporter proteins were required. Hexose transporters are involved in the uptake of glucose into the cell, which are encoded by the *HXT* gene family [8, 24]. The *HXT7* encodes one of many transporters and the expression of the gene could be repressed by high glucose level [32]. As shown in Fig. 1c, d, the expression of *HXT7* gene was repressed at the initial fermentation phase and these results were in agreement with other previous findings [31, 32]. In addition, the repression effect could be eliminated by PAs supplementation, especially in the strain of BH_8 (Fig. 1d). As the concentration of glucose decreased to around 100 mg/mL, the repression effect disappeared. Higher expression level of *HXT7* gene provided sufficient hexose transporters for the requirement of efficient intracellular metabolisms [25]. PAs could enhance the expression level of *HXT7* gene significantly with a dose-dependent manner, although the regulation mechanisms are not known.

Apart from the glucose transport, the energy and redox homeostasis is a fundamental requirement for sustained metabolism and growth in all biological system [16, 43]. Therefore, we measured the concentration of intracellular ATP, NADH and NAD^+, as well as the activities of the rate-limiting enzymes in glycolysis pathway. Not only used as a source of energy, the intracellular ATP is also the substrate in signal transduction pathways by kinases, in which the ATP was used to produce the second messenger molecule cyclic AMP to regulate the metabolic pathway [11]. At the beginning of the fermentation, the concentrations of intracellular ATP in PAs-treated cells were significantly higher than that of control (Fig. 2). It indicated that ATP production rate exceeded the ATP consumption rate in PAs-treated cells compared to the control. Our previous data revealed that the activity of the membrane H^+-ATPase was lower in the PAs-treated cells than that in control at Day 0.5 [17]. It could explain the reason why ATP production rate exceeded the ATP consumption rate in PAs-treated cells. Under anaerobic condition, the dominant intracellular ATP was generated via glycolysis pathway. Higher ATP content is also known to inhibit hexokinase, phosphofructokinase as well as pyruvate kinase [16]. It was also proved in our current study. Under the conditions of 0.1 or 1.0 mg/mL PAs in the medium, the activities of the hexokinase, phosphofructokinase and pyruvate kinase in the PAs-treated cells were significantly lower than that of control at the beginning of the fermentation; however, since entering into the mid-phase of the fermentation, the activities of the key enzymes in glycolysis were higher than that of control significantly (Fig. 4). An obvious negative correlation was found between the enzyme activity and concentration of intracellular ATP (Fig. 2). PAs acted as an external regulator in the glycolysis pathway to control the consumption rate and production rate of intracellular ATP. Meanwhile, the glycolysis flux could be regulated by the ATP feedback to keep the intracellular energy homeostatic.

The intracellular redox potential, primarily determined by the NADH/NAD^+ ratio, poses significant effect on the energy metabolism and product formation [41]. During the fermentation, the acetaldehyde is converted into ethanol using alcohol dehydrogenase with the re-oxidation of NADH to NAD^+. The generated NAD^+ could be used in the glycolysis pathway for ATP synthesis to maintain the cell metabolism [2]. Higher concentration of intracellular NAD^+ coupled with higher alcoholic fermentation rate. In other words, the ratio of NADH/NAD^+ could be considered as an index to measure the alcoholic fermentation efficiency. At the beginning of the fermentation, the NADH/NAD^+ ratio in PAs-treated cells was higher than that of control (Fig. 3), which could provide sufficient ATP for the adaptation of the new environment depending on the oxidative phosphorylation pathway. After entering into the mid-phase of the fermentation the dissolved oxygen has been consumed completely and acetaldehyde, the intermediate of alcoholic fermentation phase, became the only electron acceptor to oxidize the NADH to NAD^+ to maintain the glycolysis pathway. PAs could accelerate the alcoholic fermentation rate to consume much more NADH with a large amount of NAD^+ yields, which could drive the glycolysis flux to produce sufficient NADH to keep the redox homeostasis [43].

Conclusions

Based on the results presented in our study, we suggested after a transient adaptation to new environment PAs could stimulate the gene expression of *PMA1*and *HXT7* to accelerate the glucose transport; meanwhile, depending on the intracellular metabolites signaling (ATP, NADH and NAD^+), PAs enhanced the activities of rate-limiting enzymes in glycolysis pathway to improve the alcoholic fermentation.

Acknowledgments This research was supported by National Natural Science Foundation of China (No. 30611468), China Scholarship Council (No. 201306350115), Doctoral Program of Higher Education for Young Teacher (20070019049) and Innovation Fund for Graduate Student of China Agricultural University (KYCX2010065).

Conflict of interest The authors declare that there are no conflicts of interest.

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