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Effects of proteinase A on cultivation and viability characteristics of industrial *Saccharomyces cerevisiae* WZ65*

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Abstract: Proteinase A (PrA), encoded by *PEP4* gene, is a key enzyme in the vacuoles of *Saccharomyces cerevisiae*. We characterized the effects of PrA on cell growth and glucose metabolism in the industrial *S. cerevisiae* WZ65. It was observed that the lag phase of cell growth of partial *PEP4* gene deletion mutant (36 h) and PrA-negative mutant (48 h) was significantly extended, compared with the wild type strain (24 h) (P<0.05), but PrA had no effect on glucose metabolism either under shaking or steady state cultivations. The logistic model was chosen to evaluate the effect of PrA on *S. cerevisiae* cell growth, and PrA was found to promote cell growth against insufficient oxygen condition in steady state cultivation, but had no effect in shaking cultivation. The effects of glucose starvation on cell growth of partial *PEP4* gene deletion strain and PrA-negative mutant were also evaluated. The results show that PrA partial deficiency increased the adaption of *S. cerevisiae* to unfavorable nutrient environment, but had no effect on glucose metabolism under the stress of low glucose. During heat shock test, at 60 °C the reduced cell viability rate (RCVR) was 10% for the wild type *S. cerevisiae* and 90% for both mutant strains (P<0.01), suggesting that PrA was a negative factor for *S. cerevisiae* cells to survive under heat shock. As temperatures rose from 60 °C to 70 °C, the wild type *S. cerevisiae* had significantly lower relative glucose consumption rate (RGCR) (61.0% and 80.0%) than the partial mutant (78.0% and 98.5%) and the complete mutant (80.0% and 98.0%) (P<0.05), suggesting that, in coping with heat shock, cells of the PrA mutants increased their glucose consumption to survive. The present study may provide meaningful information for brewing industry; however, the role of PrA in industrial *S. cerevisiae* physiology is complex and needs to be further investigated.

Key words: Proteinase A (PrA), *PEP4* gene, *Saccharomyces cerevisiae* WZ65, Cell metabolism, Viability
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INTRODUCTION

The industrial *Saccharomyces cerevisiae* is widely used for biological production of ethanol. Proteinase A (PrA, EC 3.4.23.6), an aspartic proteinase, is located in the lysosome-like vacuoles of yeasts including *S. cerevisiae* (Li and Kane, 2009). PrA is considered essential for *S. cerevisiae* vacuolar proteolytic system during nutritional stress, sporulation, and vegetative growth (Simões and Faro, 2004; Parr *et al*., 2007). The lack of PrA leads to cell death under nutrient deficiency (Teichert *et al*., 1989). One reason for the role of PrA in starving cells might reside in its central function in the initiation of processing of vacuolar enzymes to their mature species (Teichert *et al*., 1989). The active vacuolar enzymes might be necessary for cell survival. The deletion of *PEP4*, the structural gene for PrA, leads to the accumulation of the pro-forms of vacuolar proteases including proPrB and proCPY in the vacuole (Stevens *et al*., 1982; van den Hazel *et al*., 2005). The mutation of *PEP4* gene results in 90%~95% reductions in the levels of several vacuolar hydrolyses in yeasts (Zubenko *et al*., 1983). In addition, PrA is reported to have negative effects on beer foam stability, especially in unpasteurized beer (Cooper *et al*., 2000; He *et al*., 2006).

Although the current understanding of structure

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and functions of PrA in *S. cerevisiae* is extensive, its roles in cell physiology are still complex. Information about effects of PrA on cell viability and substrate metabolism in *S. cerevisiae*, especially in the industrial *S. cerevisiae*, is limited and controversial. Zhang *et al*.(2008) reported that a partial deletion of *PEP4* gene in *S. cerevisiae* likely caused a long lag phase for cell growth. However, Wang *et al*.(2007) found that *PEP4* gene deletion had no negative effect on cell growth. Therefore, it is necessary to further clarify roles of PrA on cell viability and substrate metabolism in industrial *S. cerevisiae*.

In a previous study, in order to improve the stability of beer foam proteins in an industrial *S. cerevisiae*, we constructed a partial *PEP4* gene deletion *S. cerevisiae* strain and named it SC1 (Zhang *et al*., 2008; Wang *et al*., 2007). However, the cell growth and glucose metabolism of industrial *S. cerevisiae* need to be further illustrated using a PrA-negative strain. In the present study, based on SC1, we constructed SC2, in which the *PEP4* gene was completely deleted, and characterized roles of PrA on metabolism and cell viability in the industrial *S. cerevisiae* WZ65.

MATERIALS AND METHODS

Construction of PrA-negative mutant SC2

The industrial brewing yeast, *S. cerevisiae* WZ65 (wild type, named as SC), was provided by China Lion Brewery Group. The mutant strain SC1 with the *PEP4* gene partial deletion was constructed as previously described (Zhang *et al*., 2008). Briefly, one of *PEP4* alleles of *S. cerevisiae* WZ65 was replaced using bglS expression cassette. In the present study, the *PEP4* gene partial deletion strain SC1 was transformed with plasmid pSH47/ZEO to rescued Kan^R cassette, and a DNA fragment containing Kan^R cassette was inserted into the *PEP4* locus of the heterozygote SC1 to generate *PEP4* gene complete deletion strain, SC2.

The insert DNA fragment was prepared by polymerase chain reaction (PCR) with primers 5′-CATTATTGCCATTGGCCTTGTTGTTGGTCAGCGCCAACCAAGTTGCCCAGCTGAAGCTTCGTACGC-3′ and 5′-GCCAAACCAACCGCATTGTTGCCCAAATCGTAAATAGAATAGTATCGCATAGGCCACTAGTGGATCTG-3′, denoting the homologous regions located at upstream and downstream of *PEP4* coding sequences, respectively, with plasmid pUG6 as the template. The amplification of recombinant DNA was performed in a Bio-Rad thermal cycler. Cycling was carried out at 94 °C for 45 s, 58 °C for 40 s, and 72 °C for 2 min at 28 cycles.

The transformation of SC1 was carried out by electroporation as described by Sambrook *et al*. (2002). Transformants were screened on yeast extract peptone D-glucose (YEPD) plate containing 200 μg/ml G-418. To verify the insertion, yeast genomic DNA was extracted and PCR was performed using primers 5′-ACACGAGTTGTCCGATGAGATGAAAG-3′ and 5′-GTATTTACGCAAGAAGGCATCACCA-3′ for *PEP4* coding sequence (Fig.1a), and primer A 5′-TTGGGTAATTCGCTGCTATTT-3′, primer B 5′-AGAACCTCAGTGGCAAATCC-3′, and primer C 5′-ATGGTATTGATAATCCTGATATGAAT-3′, and primer D 5′-GCTTCTGCTTACATTGCTTTA-3′ for integration sites of the recombinant strain (Fig.1b). The amplification parameters were 94 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 52 °C for 40 s, and 72 °C for 1 min, and finally 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis (Figs.1a and 1b). The target 1072-bp fragment for *PEP4* gene was not observed in SC2, and a 720-bp fragment for the conjunction site between either *PEP4* upstream or downstream sequence and KanMX sequence was observed only in SC2, indicating that the Kan^R cassette was integrated in the *PEP4* gene locus, and the *PEP4* coding sequence in the recombinant strain SC2 was complete deleted.

Growth media and conditions

The wild type *S. cerevisiae* WZ65 (SC) was grown at 28 °C in YEPD medium (1% yeast extract, 2% peptone, and 2% glucose, w/v), the mutant strain SC1 was grown in the YEPD medium supplemented with zeocin at 50 mg/L after transformed with plasmid pSH47/ZEO, and SC2 was grown in the YEPD medium supplemented with G-418 at 200 mg/L after transformed with insert DNA fragment. The YEPD medium was also used for fermentation in flasks, while for nutrient-deficient cultivation, the SD medium (2% glucose and 0.67% yeast nitrogen base, w/v) and the carbon-limiting medium (1% yeast extract, 2% peptone, and 0.2%, 1%, or 2% glucose, w/v) were

used. The cultivation temperature was 28 °C, the shaking speed 120 r/min, and the inoculation size 5% (v/v).

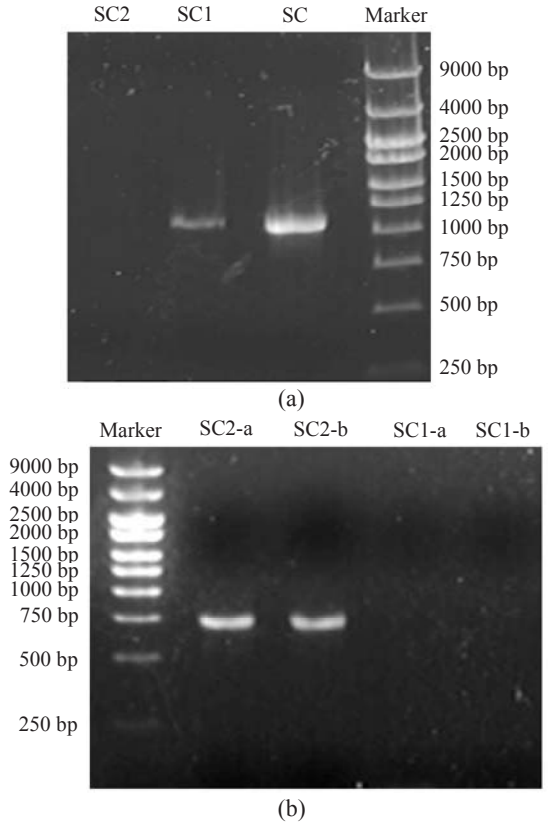

Fig.1 (a) Identification of *PEP4* gene of *Saccharomyces cerevisiae* strains by polymerase chain reaction (PCR). The PCR product (1072-bp length) was analyzed by 1% (w/v) agarose electrophoresis (SC: industrial *S. cerevisiae* WZ65; SC1: the partial *PEP4* deletion of *S. cerevisiae* WZ65 strain; SC2: the complete *PEP4* deletion of SC1 strain); (b) Identification of integration site of recombinant DNA in chromosome of recombinant strain. The PCR product (720-bp length) was analyzed by 1% (w/v) agarose electrophoresis (SC2-a: the PCR product composed of part of *PEP4* upstream sequence and part of KanMX sequence; SC2-b: the PCR product composed of part of *PEP4* downstream sequence and part of KanMX sequence; SC1-a and SC1-b: the PCR results amplified with the same primers to SC2-a and SC2-b as controls, respectively)

Nutrients starvation design

The yeasts were grown in the carbon starvation medium composed of YEPD with 0.2%, 1%, or 2% (w/v) glucose at 28 °C with shaking at 120 r/min. As nitrogen starvation design was carried out, the yeasts were inoculated into SD medium. The inoculated flasks were cultivated at 28 °C for 48 h with shaking at 120 r/min.

Heat shock

Pre-cultured yeasts were transferred to fresh YEPD media and then cultured for 12 h at 28 °C with shaking until log phase was achieved (A_{600}=1.0). In order to elicit heat shock, the mid-log cultured cells were exposed to 50, 60, 70, 80, and 90 °C for 30 min, respectively. Afterwards, the treated yeast cells were added to fresh YEPD media and then cultivated at 28 °C for 24 h. The cell growth was measured by the absorption value at the wavelength of 600 nm. Survival rate was estimated as the relative percentages of the normal cells without heat exposure.

Proteinase A activity assay

PrA activity was assayed with modified Bradford (1976)' method using 1% (w/v) casein (pH 2.0) as the substrate. Briefly, the 0.2 ml enzyme liquor (both yeast pellet disruption and supernatant were collected for PrA activity) was incubated with 2 ml 1% (w/v) casein (pH 2.0) at 37 °C for 20 min. The reaction was terminated by 2 ml 10% (w/v) trichloroacetic acid for 10 min, and the reaction mixture was centrifuged at 15000×*g* for 15 min. One unit of PrA hydrolyzes 1 mg of insulin chain B (oxidized) per minute at pH 6.0 (25 °C) (Wang *et al*., 2005). At 72 h culture, PrA activity was observed to be 27 U/ml in broth and 58 U/ml in cells in the wild type *S. cerevisiae* WZ65 (SC), and 10 U/ml in the broth and 28 U/ml in cells in the *PEP4* partial deletion strain SC1, but was not observed in the *PEP4* further deletion strain SC2 (Table 1). This indicates that the *PEP4* gene in SC2 has been completely deleted.

Table 1 PrA activity of *PEP4* mutant SC1 and SC2 and wild type *Saccharomyces cerevisiae* strains*

	PrA activity (U/ml)	
	Cells	Broth Culture
SC1	28	10
SC2	−	−
SC	58	27

* Culture duration 72 h and culture temperature 28 °C. −: no activity determined

Measurement of dry cell weight and residual reduced glucose

Cell growth was measured as the dry cell weight (DCW). The culture sample (10 ml) was centrifuged at 4000×*g* for 10 min, and cell pellet was washed twice with distilled water, dried to constant weight at

80 °C, and then weighed. The residual glucose concentration in the medium was measured using the dinitrosalicylic acid (DNS) method (Miller, 1959). Briefly, 1 ml culture supernatant was added into 1.5 ml DNS solution and then boiled for 5 min. The reaction solution was diluted to stop the reaction and measured by the wavelength at 520 nm. The blank control was set using distilled water replacing culture supernatant.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) by the Statistical Analysis System 8.0, followed by Duncan's multiple range test. The P values <0.05 were considered to be significantly different.

RESULTS AND DISCUSSION

Effects of proteinase A on cell growth and glucose metabolism

Brandberg *et al*.(2007) reported that, when the factor of aeration was considered, yeast strain possessed an increased ethanol tolerance and viability. We may, therefore, speculate that the PrA is important for the lag phase of *S. cerevisiae* cell growth in aeration condition. The cell growth of the three *S. cerevisiae* strains, SC, SC1, and SC2, was determined by DCW (Fig.2). Interestingly, it was observed that the lag phase of cell growth was 24, 36, and 48 h in the SC, SC1, and SC2 (P<0.05), respectively, indicating that PrA is responsible for the lag phase of *S. cerevisiae* cell growth. This observation further confirms our previous findings (Zhang *et al*., 2008). The glucose consumption rates (GCR) of the three *S. cerevisiae* strains were analyzed (Fig.3). In both the shaking and steady state conditions, the GCR fluctuated within the range of 93% to 98% for all the three, showing no significant difference between them. Glucose is utilized by yeast cells for proliferation, basic metabolism, and metabolite production. However, in the present study, we did not see PrA affect glucose consumption in *S. cerevisiae*.

Kinetics model simulation

The most popular kinetic expression for microbial growth is the Monod-equation. However, the

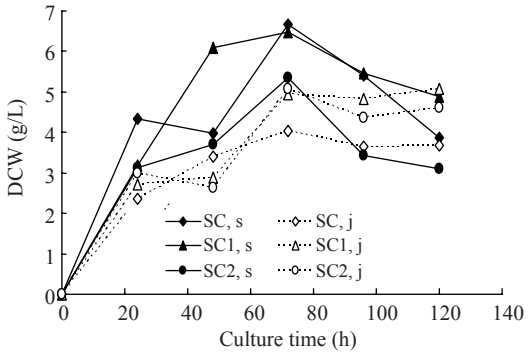

Fig.2 Effects of *PEP4* partial and complete deletions on cell growth of industrial *Saccharomyces cerevisiae* WZ65

DCW: dry cell weight; s: shaking cultivation; j: steady state cultivation

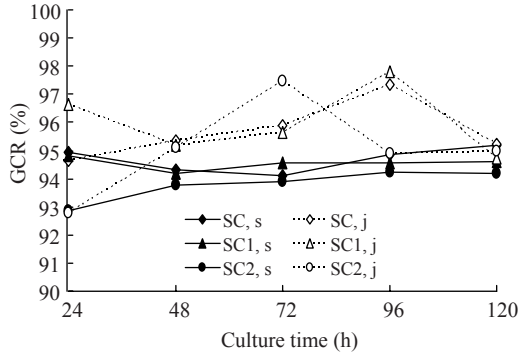

Fig.3 Effects of *PEP4* partial and complete deletions on glucose consumption rate of industrial *Saccharomyces cerevisiae* WZ65

GCR: glucose consumption rate; s: shaking cultivation; j: steady state cultivation

examination of the large number of experimental data (data not shown) obtained from batch fermentation showed that the model is not suitable for this particular system. Therefore, the logistic model was chosen as an alternative empirical equation for evaluating the effect of PrA on *S. cerevisiae* cell growth. In this model, the shaking and steady state conditions were simulated using data processing system (DPS) software (Tang and Feng, 1997). Table 2 presents the maximum specific growth rate (μ_{max}), the maximum biomass rate (X_{max}), and the initial biomass (X_0) values, among which the μ_{max} is the most important parameter for evaluating cell growth and glucose metabolism; the higher μ_{max}, the faster the cell growth and glucose metabolism. In the steady state condition, SC had the highest μ_{max} (0.18 h^{-1}) compared with SC1 (0.06 h^{-1}) and SC2 (0.06 h^{-1}), suggesting that PrA may play a role in promoting cell

growth against insufficient oxygen condition. In the shaking condition, there was no difference observed between the wild type SC (μ_{max}=0.31 h^{-1}) and the PrA-negative mutant SC2 (μ_{max}=0.30 h^{-1}), which may suggest that, except for extending the lag phase of cell growth, PrA did not affect the cell growth in oxygen-rich growth condition. However, it was noticed that, in shaking condition, the partial deletion mutant SC1 had the higher μ_{max} (0.81 h^{-1}) than both SC and SC2 (P<0.05). Since the industrial *S. cerevisiae* is facultative anaerobic strain, the partial *PEP4* gene deletion may be beneficial to cell growth.

Table 2 Comparison of SC1, SC2 and SC strain growth kinetics parameters using logistics model simulation

		X_{max} (g/L)	μ_{max} (h^{-1})	X_0 (g/L)
Shaking culture	SC	4.98	0.31	0.02
	SC1	5.74	0.81	0.005
	SC2	3.89	0.30	0.01
Steady state culture	SC	3.71	0.18	0.09
	SC1	5.08	0.06	0.60
	SC2	4.64	0.06	0.70

Effects of proteinase A on *Saccharomyces cerevisiae* cell growth and glucose metabolism under nutrients limitation

PrA possibly played an essential role in yeast survivability under the nutrient stress, especially under nitrogen deficiency (Teichert *et al*., 1989; Zaman *et al*., 2008). The effects of culture nutrients on cell growth and glucose metabolism were evaluated in the three *S. cerevisiae* strains. 0.2%, 1%, and 2% (w/v) glucose were applied to examine the effect of glucose starvation on cell growth (Fig.4). In 0.2% glucose, the maximum biomass value of each strain was evaluated by DCW: 3.7, 2.5, and 4.3 g/L in SC, SC1, and SC2, respectively. The period to reach the maximum growth biomass was significantly shorter in SC (24 h) than in SC1 and SC2 (48 h) (P<0.05). The results indicated that PrA deletion certainly led to extension of the lag phase in the industrial *S. cerevisiae* growth. However, SC2 had the maximum biomass when compared to the other two strains. It seems that PrA-deficiency may increase the adaption to unfavorable nutrient environment such as low glucose.

The effect of the limitation of carbon (which relies on the concentrations of glucose) on glucose metabolism was also discussed (Fig.5). The glucose

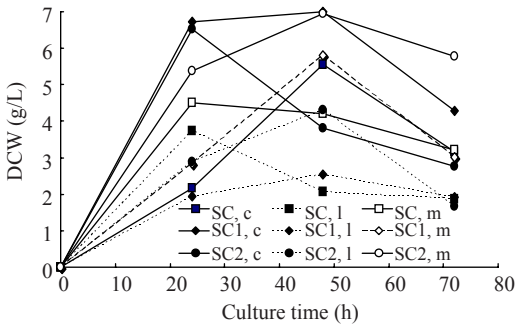

Fig.4 Effects of *PEP4* partial and complete deletions on cell growth of industrial *Saccharomyces cerevisiae* WZ65 under carbon-limiting conditions
DCW: dry cell weight; c: 2% glucose; l: 0.2% glucose; m: 1% glucose

consumption rates (GCRs) in 0.2% glucose in the three stains were generally lower than those in 1% and 2% glucose conditions. In 0.2% glucose, GCR at 48 h was 82.1% in SC1, 83.4% in SC2, and 84.6% in SC (P>0.05). The results suggest that, although PrA is crucial for maintaining normal cell growth under nutrients limitation (Cooper *et al*., 2000; Zubenko *et al*., 1983), it had no effect on glucose metabolism under the stress of lower glucose.

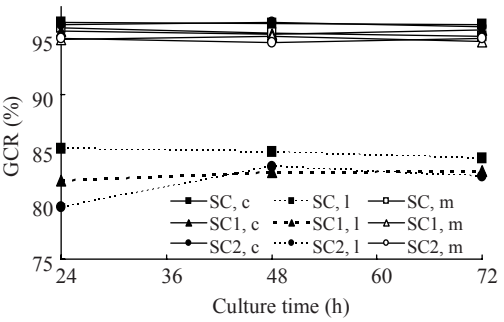

Fig.5 Effects of *PEP4* partial and complete deletions on glucose metabolism of industrial *Saccharomyces cerevisiae* WZ65 under carbon-limiting conditions
GCR: glucose consumption rate; c: 2% glucose; l: 0.2% glucose; m: 1% glucose

In order to further evaluate the role of PrA in the effect of nutrient starvation on industrial *S. cerevisiae* cell growth, SD medium was used. Fig.6 shows the DCW of the three *S. cerevisiae* strains grown in SD medium. At 72 h, the DCW dropped from 5.1 to 3.0 g/L in both SC and SC2, suggesting that PrA may not have an effect on cell growth in nitrogen starvation. Interestingly, SC1 was found to grow more quickly than the other two strains, with the DCW at 72 h being 6.11 g/L (P<0.05), indicating that, under the limitation

of medium nutrients, the partial *PEP4* gene deletion in the industrial *S. cerevisiae* may have a beneficial effect on cell growth. The glucose metabolism had no differences among the three tested *S. cerevisiae* strains (data not shown).

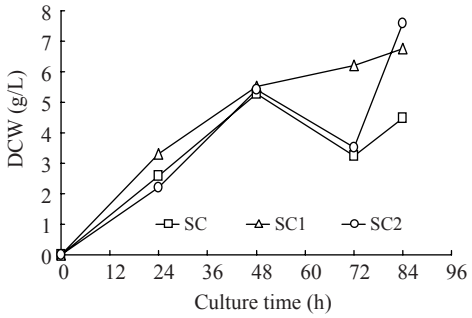

Fig.6 Effects of *PEP4* partial and complete deletions on viability of industrial *Saccharomyces cerevisiae* WZ65 under nitrogen starvation conditions
DCW: dry cell weight

Carbon and nitrogen limitation causes a lower biomass yield and a higher ethanol yield in chemostat cultures, and severe nitrogen limitation appears to uncouple the catabolic adenosine triphosphate (ATP) generation from the anabolic demand for ATP. Biosynthesis can be decreased by limiting the availability of nitrogen. In *S. cerevisiae*, the complete starvation of nitrogen has many consequences on its metabolism process, including the changes in the macromolecular composition, inactivation of the glucose transport system, increased turnover of proteins, and reduced fermentative capacity (Brandberg *et al*., 2007). However, there has been a lack of related studies on the effects of PrA on nitrogen metabolism in the industrial *S. cerevisiae*.

PrA and proteinase B (PrB) activities are clearly required for degradation of analog-containing proteins, starvation-induced vegetative protein degradation, and the ability to survive nitrogen starvation. It is unknown whether vacuolar proteases contribute to degradation of fructose 1,6-bisphosphatase during catabolite inactivation, since there are reports on both sides of the issue (Zaman *et al*., 2008). Vacuolar proteases appear not to be involved in other cases of catabolite inactivation that have been tested (Zaman *et al*., 2008). In response to rigid stress, they catalyzed massive amounts of protein degradation that facilitates cellular restructuring, particularly during sporulation (Jones, 1991). In yeasts, autophagy is induced at the absence of high-quality nitrogen and carbon sources (Zaman *et al*., 2008).

Effect of proteinase A on cell viability under heat shock

PrA was reported to be helpful to cell survival under stress environment (Wang *et al.*, 2007). We examined the effects of heat shock on the three *S. cerevisiae* strains. Five temperatures were used and the reduced cell viability rate (RCVR) was recorded (Fig.7). At 60 °C, the RCVR was 10% for the wild type SC, and 90% for both mutant strains (P<0.01). The high viability rate in the two mutant strains then gradually dropped to 10% at 90 °C. The results may suggest that PrA has no role in coping with heat shock, but rather has a negative influence. Similarly, it was observed that the vacuolar proteases were not essential to cell viability (Jones, 1991). Interestingly, two *PEP4* mutant strains, both partial and complete deletions, exhibited a high capability in resistance of heat shock, which may open a door for the genetic improvement of the industrial *S. cerevisiae*.

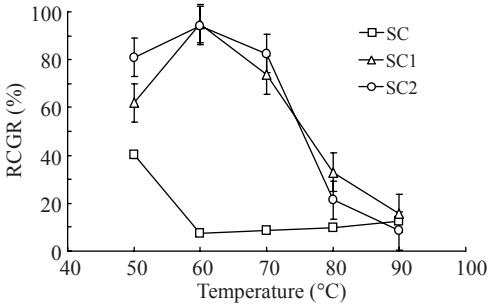

Fig.7 Effects of *PEP4* partial and complete deletions on cell viability of industrial *Saccharomyces cerevisiae* WZ65 strain under different heat shocks
RCGR: relative cell growth rate

The effect of heat shock on glucose metabolism was also evaluated in the three strains (Fig.8). As temperatures rose from 60 to 70 °C, SC had significantly lower relative glucose consumption rate (RGCR) (61.0% and 80.0%) than those of SC1 (78.0% and 98.5%) and SC2 (80.0% and 98.0%) (P<0.05). The higher RGCR in the PrA mutants may suggest that, in coping with heat shock, cells of the PrA mutants increased their glucose consumption to survive, which is in accordance with the general consideration that PrA is a beneficial factor for *S. cerevisiae* cells to survive under unfavorable environmental conditions (Jones, 1991).

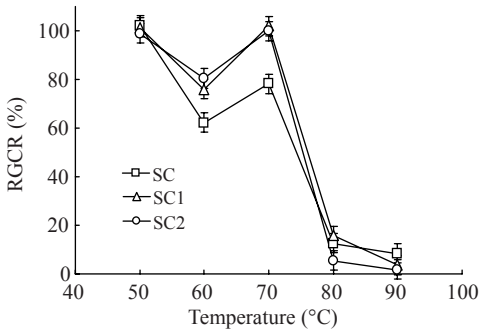

Fig.8 Effects of *PEP4* partial and complete deletions on glucose metabolism of industrial *Saccharomyces cerevisiae* WZ65 strain under different heat shocks
RGCR: relative glucose consumption rate

The optimum temperature of *S. cerevisiae* growth is within the range of 25 to 35 °C. In the range of 35 to 37 °C, yeast cells are moderately stressed but continue to grow, developing a protective tolerance against higher levels of lethal shock (Davidson and Schiestl, 2001a; 2001b). The oxidative stress under heat shock may induce a more profound stimulation of trehalose, antioxidant enzymes, and heat shock proteins, as well as other stimulators (Kim *et al*., 2006). When cells are subjected to abnormal conditions, the cells must carry out a rapid and effective genomic expression program in order to adapt, proliferate, or survive in new environment (Fernandes *et al.*, 2004). However, the role of PrA in industrial *S. cerevisiae* in coping with extreme environmental conditions needs to be further elucidated.

CONCLUSION

In the present study, we constructed the *PEP4* gene partial deletion and PrA-negative *S. ceverisiae* WZ65 strains to examine the effects PrA on the cell growth and glucose metabolism in *S. ceverisiae*. PrA was found to extend the lag phase of cell growth, but with no influence on glucose metabolism. Interestingly, it was noticed that the partial *PEP4* gene deletion had a beneficial effect on cell growth under aerobic cultivation and increased the adaption to unfavorable nutrient environment such as low glucose. In addition, both the *PEP4* partial and complete deletion mutants exhibited a higher capability in resistance of heat shock, compared with the wild type *S. ceverisiae* WZ65, suggesting an application for species improvement in brewing industry.

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