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Influence of Heat Shock on Glycerol Production in Alcohol Fermentation

Marin Berovič,[1]* Aleksandra Pivec,[2] Tatjana Košmerl,[3] Mojmir Wondra,[3] and Štefan Čelan[2]

Department of Chemical, Biochemical and Environmental Engineering, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Askerceva 5, SI-1115 Ljubljana, Slovenia,[1] Scientific Research Centre Bistra Ptuj, Slovenski trg 6, SI-2250 Ptuj, Slovenia,[2] and Department of Enology, Biotechnical Faculty, Department of Food Technology, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia[3]

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The influence of single and double heat shocks induced during the exponential growth phase of the *Saccharomyces cerevisiae* fermentation of cultivar Sauvignon Blanc grape must was examined. Rapid temperature changes from 18°C to 34°C have been applied. The effect of the duration of exposure to a high temperature has been analyzed. By the applications of a single heat shock and a double heat shock, up to 8.2 g l^{-1} and 11.0 g l^{-1} glycerol have been produced, respectively. To prevent the evaporation of fine wine bouquet compounds during the temperature changes, reflux coolers on the top of bioreactors have been employed. By using this method, glycerol production was increased by up to 65%.

[**Key words:** wine fermentation, heat shocks, high glycerol production]

In wine fermentation, next to ethanol and carbon dioxide, glycerol is quantitatively the most important fermentation product. Because of to its nonvolatile nature, glycerol does not contribute to the aroma of wine, but it promotes the smoothness of wine (1). It contributes only indirectly to wine quality, but the overproduction of glycerol by wine yeast strains of *Saccharomyces cerevisiae* could markedly improve the sensory quality of wine (2). The increased biosynthesis of glycerol often gives wine a smoother mouth feel and an enhanced complexity (3).

The biosynthesis of glycerol in a cell is closely associated with osmotic cell regulation (4). The anaerobic conversion of glucose into ethanol by *S. cerevisiae* is redox neutral, that is, NAD^+, which is consumed initially in the Embden-Meyerhof-Parnas (EMP) pathway, is regenerated when ethanol is produced. However, when intermediates in the EMP pathway are withdrawn as precursors for the synthesis of the cellular material, this balance is disturbed, because the surplus of NADH produced is not converted back into NAD^+. This eventually causes metabolism to stop, unless other processes are employed for the regeneration of NAD^+. During the anaerobic growth of *S. cerevisiae*, NADH cannot be oxidized by oxygen, but must be disposed by the formation of reduced by-products such as glycerol. The accumulation of glycerol is caused by the need to maintain a favorable redox balance (4–9) by converting the excess NADH, which is generated during the biomass formation, to NAD^+. Glycerol formation requires the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (G-3-P) by dihydroxyacetone phosphate reductase, converting one molecule of NADH, which is generated in the oxidation of glyceraldehyde 3 phosphate, to NAD^+.

The reaction is catalyzed by G-3-P dehydrogenase (GPDH) and followed by the dephosphorylation of G-3-P to glycerol by glycerol-3-phosphatase. G-3-P is then dephosphorylated by glycerol phosphate phosphatase yielding glycerol (9). Glycerol formation may serve as the route for the generation of NAD^+ from NADH. However, the coupling of these two reactions does not generate net ATP (10), if all triose units are shunted to glycerol. It is therefore energetically unfeasible for the overproduction of glycerol to be an end product of glycolysis (9).

In glycerol biosynthesis, various growth and environmental factors such as fermentation temperature, strain selection, inoculation level, sulfite concentration, sugar concentration, osmotic stress, nitrogen source and concentration, pH, aeration, grape variety and ripeness have been reported to influence the amount of glycerol produced by yeast in wine (11–15). Several authors have also observed that glycerol is produced in response to solute stress in *S. cerevisiae* (12, 13). It has also been observed that the addition of even the smallest amount of SO_2 (100 ppm) can cause a significant increase in glycerol formation (16). Under controlled conditions, it has been shown that the yeast strain exerts a marked effect on the amount of glycerol produced (13). These findings have led to the distinction between strains within the *S. cerevisiae* species which are low- and high-glycerol producers (11, 13). Glycerol formation by free cells

* Corresponding author. e-mail: marin.berovic@fkkt.uni-lj.si
phone: +38-612419510 fax: +38-614760300

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as well as the effect of yeast cell immobilization on glycerol production in wine fermentation has also been studied (16, 17). Several studies have shown the improvement of wine quality related to various yeast strains, using genetic or molecular techniques to manipulate glycerol formation by wine yeasts (18–20). Gene engineering of the *S. cerevisiae* strain resulted in a 1.5- to 2.5-fold increase in glycerol production with only a slight decrease in ethanol formation in wine fermentation (18).

In many types of fermentation, temperature is one of the most important factors in metabolite biosynthesis. The interactions between the yeast strain, temperature and agitation also affects on glycerol production (12, 13, 21, 22). By using immobilized cells in wine fermentation at 15–30°C, glycerol concentration in the range of 11.9–14.9 g l^{-1} has been obtained. Glycerol concentration decreased with a decrease in temperature, but glycerol concentration was higher in fermentations using immobilized or free cells than in synthetic media (23, 24).

An investigation on alcohol fermentation carried out at different temperatures could also serve to test the natural stability of indigenous strains. This could be used as a criterion for the rapid selection of one of the several microbial strains, and at the same time act as a standard for the examination of strain resistance to temperature in a controlled situation in a laboratory environment. Temperature could also be a helpful tool for evaluating the effects of the dynamics of a known population of *S. cerevisiae* during alcohol fermentation (25).

The effect of heat shock treatment during the preculture stage on glycerol production in a primary culture has been studied with the *shochu* yeast *S. cerevisiae* BAW-6. Heat shock has been proven to be effective in stimulating glycerol production at 45°C and 50°C. The timing of heat shock treatment does not influence glycerol production. Furthermore, the increase in glycerol production induced by heat shock has also been observed in the sake yeast K-7, wine yeast W-3, beer yeast IF01167, and whisky yeast IF02363 (26). Heat shock treatment induces *S. cerevisiae* to produce heat shock proteins and alters cellular characteristics (27, 28). Cells subjected to heat shock acquire resistance against various stresses (29, 30). Many studies have shown that trehalose, which is involved in to the acquisition of various types of resistance, markedly accumulates in a cell by heat shock treatment (31). Wiemken *et al*. (32) have suggested that trehalose may actually be a stress protecting agent, because its accumulation and the acquisition of thermo tolerance during the heat shock are partially independent of protein synthesis in *S. cerevisiae*. Glycerol is an analogue of trehalose in that it acts as a redox balancing substance and osmoprotectant during osmotic stress (20, 26, 33).

The main objective of this study was to examine the effects influence of single and double heat shocks on *S. cerevisiae* and the effects influence on glycerol production in cultivar Sauvignon Blanc alcohol fermentation.

MATERIALS AND METHODS

Microorganism A commercial dry wine yeast strain (*S. cerevisiae*, Uvaferm SLO) was used in all of the experiments. Reactivation of the yeast was performed in 50 ml of water-diluted cultivar Sauvignon Blanc must (1 : 1) at 30°C. Fermentation processes were performed by inoculating 0.3 g of dry yeast per liter of the must.

Fermentation substrate Batch fermentation experiments were carried out on a grape juice of cultivar Sauvignon Blanc from the Ljutomersko-Ormoske Gorice wine growing region. The musts, fermented under laboratory scale measurement, were not sulfurized before the start of the fermentation. The initial sugar contents of the juice were 109.0 g l^{-1} of glucose and 102.83 g l^{-1} fructose, the pH was 3.16, and the total acidity was 7.87 g l^{-1}.

Fermentors All laboratory scale experiments were performed in identical standard type configuration, using 10-*l* working volume Stirred Tank Reactors (INFORS 210; Infors AG, Bottmingen, Switzerland and Bioengineering AG, Wald, Switzerland). All fermentors were equipped with reflux cooler columns cooled down to 4°C. These condensers enabled the condensing of the outlet gas phase, which was saturated with moisture and saturated with various volatile substances. The condensing of the outlet gas phase at 4°C was very effective that no volatile substances composing the typical cultivar Sauvignon Blanc bouquet were detected in the outlet fermentation gas during the entire fermentation. In all of the experiments, sterilizable Ingold pH and redox electrodes, an Industrial Lab pO_2 electrode, an Industrial Lab MFG 509 an automatic foam control and a temperature control unit were used. For the online measurements using the Infors Fermentor, the SHIVA control software (BIA d.o.o., Ljubljana, Slovenia) was used. Bioengineering AG Fermentor was equipped with a sterilizable Ingold pH electrode, Ingold redox (platinum as an indicator and a calomel electrode as a reference electrode were used) electrodes, Ingold pO_2 electrodes and pCO_2 electrodes, together with an automatic temperature control unit (Mettler Toledo, Ingelheim, Switzerland). The fermentors were aerated with inert nitrogen to prevent the oxidation of fermenting grape must. Three series of each experiment were performed. In all of the experiments, the mixing rate during the fermentation was 100 rpm. The control experiment fermentations were performed at the constant temperature of 18°C.

Analytical methods The major extra cellular metabolites, produced by an anaerobic culture of *S. cerevisiae* in cultivar Sauvignon Blanc grape must and wine, were determined by HPLC. Reducing sugars (glucose and fructose), glycerol, ethanol and some organic acids from the TCA cycle and other organic acids were analyzed according to validated methods proposed by Bio-Rad, Batton Rouge, USA (34). The samples were analyzed using a 300×7.8 mm Aminex HPX-87H organic acid cationic exchange column (Bio-Rad Laboratories, Batton Rouge, USA). Elution was performed at 65°C. The mobile phase was 2 mM H_2SO_4 in double distilled water. The pump was operating at a flow rate of 0.6 ml min^{-1}. The injection volume was 20 µl; the eluting compounds were monitored by a fixed wavelength ultraviolet (UV-VIS) detector at 210 nm. The detector was connected in the series with a refractive index (RI) detector. The samples were filtered through a 0.45-µm membrane. Ethanol, glycerol, glucose and fructose were detected by the RI detector, whereas all organic acids were detected by the UV-VIS detector.

RESULTS AND DISCUSSION

In relation to our previous findings, wherin a short heat shock was applied (35), in this present research, the effects of the durations of one and double heat shocks on the concentrations of glycerol and the other metabolites were examined. In the first group of experiments, heat shock was initiated 6 h after inoculation and the fermentation temperature of 34°C was maintained for 4 h. Wine yeast was ex-

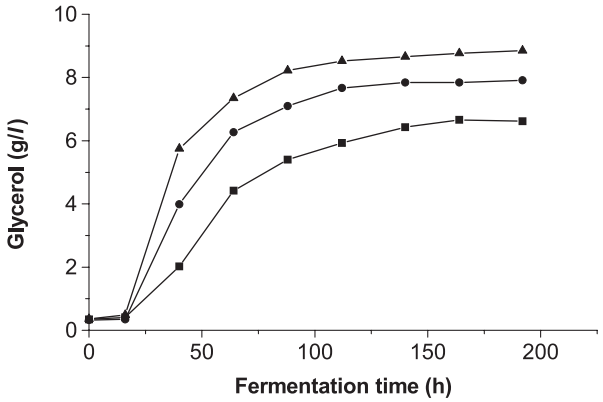

FIG. 1. Glycerol production at constant fermentation temperature of 18°C; squares, control; circles, single heat shock at 34°C for 4 h; triangles, single heat shock at 34°C for 24 h.

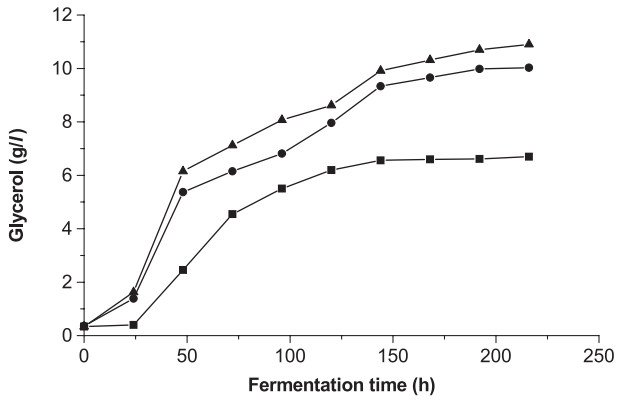

FIG. 2. Glycerol production at constant fermentation temperature of 18°C; squares, control; circles, double heat shock at 34°C (2×4 h); and triangles, double heat shock at 34°C (2×8 h).

posed to prolonged and fast temperature changes. Then, the fermentation broth was cooled back to 18°C, which was maintained until the end of fermentation at 192 h.

In the second group of experiments, 6 h after inoculation, the temperature was increased from 18°C to 34°C and it was maintained for 24 h at the same level. Then, the fermentation broth was cooled down to 18°C and maintained unchanged until the end of fermentation at 192 h.

In the third group of the experiments, the double heat shock was applied. In the first part of the experiment, 6 h after inoculation, the temperature was increased from 18°C to 34°C and it was maintained the same for 4 h. Then the fermentation broth was cooled down to 18°C and this temperature was maintained for another 4 h. After 4 h of fermentation at 18°C, rapid change in temperature back to 34°C was carried out and the temperature was maintained at 34°C for another 4 h. Then the fermentation temperature was lowered to the initial 18°C and was maintained until the end of fermentation.

In the fourth group of the experiments a longer double heat shock was applied. In the first part of the experiments, 6 h after inoculation, the temperature was increased from 18°C to 34°C and it was maintained at 34°C for 8 h. Then the fermentation broth was cooled down again to the initial 18°C, which was maintained for another 8 h. After 8 h of fermentation at 18°C, the temperature was rapidly increased back to 34°C and was maintained at the same level for another 8 h. The fermentation temperature was then decreased back to the initial 18°C and was steady maintained until the end of fermentation.

From the single heat shock experiments, the increase in the duration of heat shock from 4 to 24 h results in the slight increase in glycerol concentration from 7.9 to 8.4 g l^{-1} (Fig. 1).

Compared with the glucose concentration of 6.7 g l^{-1} of the experiments, there is an increase in glucose concentration of 25%. In the double heat shock experiments (Fig. 2), by increasing the double exposition time from 2×4 h, 10.00 g l^{-1} to 2×8 h, 10.9 g l^{-1}, the difference between both runs was even smaller, but compared with the control experiment glucose concentration of 6.7 g l^{-1} at 18°C, the increase was 62%.

At 18°C, after a shorter lag phase of 8 h and the exponential growth phase, the biomass concentration (Fig. 3) reached a maximum of 6.6 g l^{-1} (μ_{max}=0.032 h^{-1}). A single heat shock induced a faster and a higher biomass production of 9.7 g l^{-1} at 34 h (μ_{max}=0.086 h^{-1}), whereas with the application of double heat shock in the region of the first shock application growth rate was nearly the same (μ_{max}=0.080 h^{-1}).

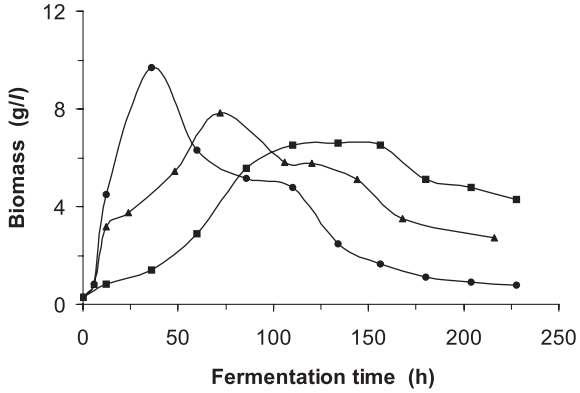

FIG. 3. Production of biomass at constant fermentation temperature of 18°C; squares, control; circles, single heat shock of 24 h at 34°C; and triangles, double heat shock heat shock (2×8 h) at 34°C.

After the second heat shock, however, specific growth rate after cooling down decreased to μ_{max}=0.030 h^{-1}. At 72 h, a final biomass concentration of 8.0 g l^{-1} was produced. Compared with the control, at a constant temperature of 18°C, where the maximum concentration of the biomass of 6.6 g l^{-1} was obtained, both heat shock experiments showed a higher amount of biomass were produced.

Using this method, metabolites such acetic acid (Table 1), which negatively contributes to the sensorial properties of wine were found only in low concentrations of up to 0.56 g l^{-1}.

Compared with the ethanol concentration of 86.9 g l^{-1} of the control, an increase to 94.0 g l^{-1} at single 1×24 h and an increase to 103.11 g l^{-1} at double 2×8 h were observed. The increase was associated with glycerol production that indicates the activity of both pathways. It was also surprising that under prolonged heat shock, single 1×24 h (0.41 g l^{-1}) and double 2×8 h (0.56 g l^{-1}), the concentration of acetic acid did not increase further. In relation to the other compounds

TABLE 1. Metabolites produced in fermentations at various temperature conditions

Fermentation	Reduc. sugars (g l^{-1})	Isoamyl alcohol (g l^{-1})	1-Propanol (g l^{-1})	Acet aldehyde (g l^{-1})	Ethanol (g l^{-1})	Glycerol (g l^{-1})	Acetic acid (g l^{-1})	Succ. acid (g l^{-1})	Citric acid (g l^{-1})	Malic acid (g l^{-1})	Tartaric acid (g l^{-1})
Control (18°C)	3.2	0.24	0.09	0.009	86.9	6.7	0.35	0.67	0.58	2.9	2.5
Single HS (4 h)	2.6	0.33	0.031	0.01	92	7.9	0.45	0.73	0.57	2.5	3.5
Single HS (24 h)	2.3	0.35	0.033	0.012	94	8.2	0.41	0.74	0.58	2.5	3.5
Double HS (2×4 h)	2	0.51	0.037	0.013	101	10	0.56	0.78	0.72	2.3	3.6
Double HS (2×8 h)	1.9	0.53	0.039	0.014	103.11	10.9	0.56	0.79	0.72	2.1	3.5

Single HS, Single heat shock for 4 h and single heat shock for 24 h; double HS, double heat shock for 2×4 h and double heat shock for 2×8 h.

acetaldehyde and tartaric acid concentrations were also under the limit concentrations. Malic acid concentration even decreased to 2.9 g l^{-1} at control, 2.5 g l^{-1} at 1×24 h and 2.1 g l^{-1} at double 2×8 h. However, in the case of a double temperature shock, a higher concentration of isoamyl alcohol of 0.53 g l^{-1} was mesured.

The application of heat shock is recognized as a simple method for effectively increasing glycerol concentration in wine. Because of its relatively high-specific gravity and viscosity, glycerol may contribute to the overall sensory perception of the wine body and thereby may enhance the fullness and sweetness of wine particulary in dry white wines. The most important parameter for achieving high glycerol production is a temperature change from 18°C to 34°C, which stimulates glycerol synthesis. A long duration of the high-temperature phase accounts for a higher glycerol production using single or double heat shocks, but the differences are rather slight. By using a single heat shock, the amount of glycerol increased by 24%, whereas in the case of a double heat shock, the increase was up to 65% (Figs. 1 and 2). The present method above for increasing glycerol production in grape must fermentation using heat shocks is protected by S.I. patent 21751, 2005.

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