Saccharomyces cerevisiae Inoculum Heat Shock Treatment – New Method for Enhanced Glycerol Production in Wine

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The present research was focused on developing a new method to enhance glycerol production for the benefit of ethanol reduction in alcohol fermentation of grape must metabolism using thermal shocks on inoculum suspension of *Saccharomyces cerevisiae* yeast cells. The influence of applied heat shocks at intervals of 10, 20 and 30 minutes at 45 °C is presented. The significance of this method is in the homogenization of the quality and efficiency of the resistible cells that survived thermal shock. They represent a new active biomass that includes also a memory effect of heat exposure that enables increasing the production of glycerol from 6.3 to 8.4 g L^{-1} at 18 °C and 7.8 to 10.3 g L^{-1} at 22 °C. In the same experiments, the concentration of ethanol decreased from 89 to 81 g L^{-1} at 18 °C and from 104 to 97 g L^{-1} at 22 °C.

These results represent a new and easily applicable method for high glycerol production at reduction of ethanol even in large scale.

Key words:
Wine fermentation, heat shock, high glycerol production

Introduction

Due to its high specific gravity and viscosity, glycerol significantly contributes to a fullness and complexity especially in premium quality dry wines.[1–3] Extensive studies of *Saccharomyces cerevisiae* metabolism proved that the biosynthesis of glycerol is related to yeast strain, process conditions and consistence of the fermentation substrate[1,4,5] and 4 to 10 % available glucose is converted to glycerol.[6]

Under anaerobic conditions, glycerol is formed in order to reoxidize NADH formed in anabolism and in the synthesis of organic acids.[2,7,8] The anaerobic conversion of glucose into ethanol by *Saccharomyces cerevisiae* is redox neutral, i.e. NAD^+ consumed initially in the Embden-Meyerhof-Parnas (EMP) pathway and regenerated when ethanol is produced. However, when intermediates in the EMP pathway are withdrawn as precursors for synthesis of cellular material this balance is distributed since the surplus of produced NADH is not converted back into NAD^+.[9,10] This will eventually cause metabolism to stop unless other processes exist for the regeneration of NAD^+. During anaerobic growth of *Saccharomyces cerevisiae* NADH cannot be oxidized by oxygen but must be disposed by formation of reduced by-products like glycerol.[7,11,12]

In glycerol biosynthesis in wine many growth and environmental factors have been reported to influence the amount of glycerol produced as oscillation of fermentation temperature,[13–15] strain selection, inoculation level, sulphite concentration, sugar concentration, osmotic stress, nitrogen source and concentration, pH, aeration, grape variety and ripeness.[16,17]

Fermentation temperature is in many technologies one of the most influential factors affecting process of various metabolite biosynthesis. Not only does temperature directly and indirectly influence yeast metabolism, but it is also one of the features over which the winemaker has the greatest control.[18,19]

The effect of heat shock treatment already applied in alcohol fermentation, was investigated in shochu yeast BAW-6, sake yeast K-7, wine yeast W-3, beer yeast IF01167, and whisky yeast IF02363.[20]

The main objective of this work was to develop a suitable method for regulating wine yeast cells *Saccharomyces cerevisiae* metabolism for higher glycerol production. The extent of the heat shock interval, applied on inoculum, suspension of wine yeast cells *Saccharomyces cerevisiae,* and its influence on glycerol and ethanol production in alcohol fermentation of grape must was studied.

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Materials and methods

Microorganism

Dry wine yeast strain (*Saccharomyces cerevisiae*, ANCHOR NT202 South Africa) was used in all of the experiments. Reactivation of 3 g of dry yeasts was performed 20 min at 30 °C in a 20 mL of water diluted (1:1) grape juice of cultivar sauvignonasse. Suspension of wine yeast in concentration of 10^7 cells mL^{-1} was then cooled to T = 18 °C and exposed for 10, 20 and 30 minutes to temperature 45 °C. After exposure, the cell suspension was immediately cooled back to 18 °C. For inoculation of grape must in further fermentation 20 mL of suspension was used.

Fermentation substrate

Fermentations were carried out on a grape juice of cultivar Sauvignonasse (former Tocai Friulano) from the wine growing region Goriška Brda. Before the start of fermentation, the grape must was not previously sulphurized or filtered. Initial sugar content of the juice was 110 g L^{-1} glucose and 114 g L^{-1} fructose, with pH 3.71. As bioactivator, 0.40 g L^{-1} of Fermaid E (Danstar Ferment AG) was added to the initial substrate.

Fermentors

All laboratory scale experiments were performed in identical standard type configuration 10 L working volume Stirred Tank Reactors (Bioengineering AG, Switzerland). All fermentors were equipped with reflux cooler columns cooled to 4 °C. These condensers were so effective that no volatile substances of typical cultivar Sauvignonasse bouquet were detected in the outlet fermentation gas during the whole period of fermentation. In all the experiments Bioengineering AG fermentors were equipped with: Ingold pH and redox electrodes, Industrial Lab pO_2 electrode MFG 509 and temperature control unit (Mettler Toledo). For on-line measurements SHIVA control software (BIA d.o.o., Slovenia) was applied. Fermentors head space was aerated through with the inert gas N_2 to prevent the oxidation of fermenting grape must. In all of experiments, mixing of 100 rpm was applied during fermentation. Fermentor head space was continuously aerated with nitrogen.

Fermentations were performed at temperatures 18 and 22 °C. Three series of each experiment were performed. The F ratio and Student's t test were used to determine if the samples were statistically significant at a 95 % confidence level.

Analytical methods

100 mL of samples were taken every 24 hours. After filtration, the major extra cellular metabolites were determined by HPLC. The reducing sugars (glucose and fructose), glycerol, ethanol and organic acids were analysed according to validated methods proposed by BIO-RAD 1997. The samples were analysed using 300 mm × 7.8 mm Aminex HPX-87H organic acid cationic exchange column (Bio-Rad Laboratories USA). Elution was performed at 65 °C. The mobile phase was 2 mmol L^{-1} H_2SO_4 in bi-distilled water. The pump was operating at flow rate 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. Detector was connected in series with a refractive index (RI) detector. Samples were filtered through 0.45 μm membrane. Ethanol, glycerol, glucose and fructose were detected by the RI detector. Organic acids were detected by the UV-VIS detector.

For determination of viable cells optical microscopy in conjunction with Bürker – Türk hemocitometer and methylene blue dye for labeling the dead cells were used. Number of viable cells/mL = $X \cdot R \cdot f$ (X – total number of cells, R – dilution factor, f – Bürker – Türk constant $2.5 \cdot 10^5$).

Results and discussion

Various duration of the heat shocks on *Saccharomyces cerevisiae* inoculum where yeast cells suspension was exposed to 45 °C eliminated on one side the too young or too old, unproductive and temperature nonresistant cells, from the further fermentation process. Using a viability test it was found that at 10 min exposure to 45 °C *Saccharomyces cerevisiae* initial cell concentration reduced by 33.4 % at 20 min 39.6 % and at 30 min by 42.6 % from the initial cell concentration (Fig. 1).

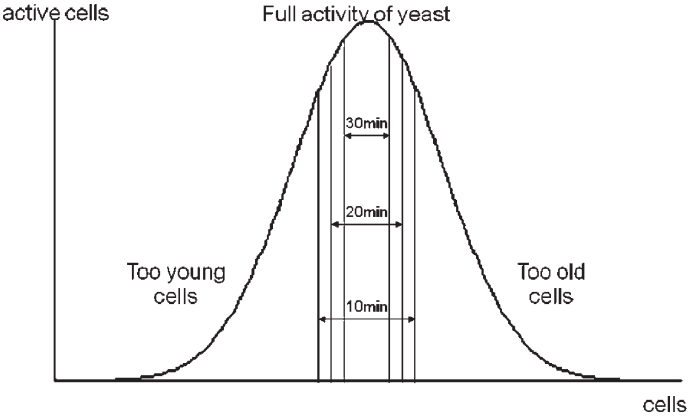

Fig. 1 – *The influence of heat shock on the population of yeast cells*

Comparing production of biomass (Table 1) higher biomass was obtained at the same fermentation temperatures in experiments with applied heath shock than those in control.

Table 1 – *Saccharomyces cerevisiae biomass, ethanol and glycerol concentrations at various heat shock intervals*

Temp. (° C)	Exposition (min)	Biomass (g L^{-1})	Ethanol (g L^{-1})	Glycerol (g L^{-1})
18	0	6.0	89	6.3
18	10	6.1	83	8.4
18	20	6.3	83	8.4
18	30	6.3	81	8.2
22	0	6.4	104	7.8
22	10	6.5	98	10.3
22	20	6.6	98	10.1
22	30	6.7	97	10.0

The highest biomass amount 6.7 g L^{-1} was obtained at 30 min exposure and fermentation at 22 °C (Fig. 2).

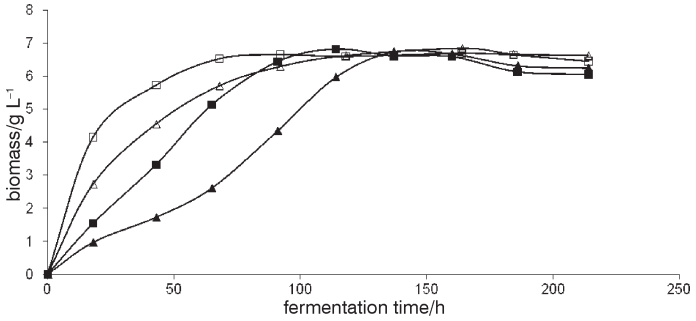

Fig. 2 – *Biomass accumulation:* ■ *temperature 18 °C;* □ *temperature 18 °C – 20 minutes heat shock;* ▲ *temperature 22 °C;* ∆ *temperature 22 °C – 20 minutes heat shock*

Ethanol production was the most expressed at control fermentations where 104 g L^{-1} in fermentation at 22 °C and 89 g L^{-1} at 18 °C were produced. On the other side, in fermentations where heat shock on inoculum was made, significant differences were observed. At 22 °C there were actually no differences between the control and the duration of exposure. (Fig. 3).

It was a fascinating discovery that at high temperature exposure the survived cells created a memory effect. Temperature shock resistant cells in further fermentation process protected themselves with expressed production of glycerol. In both control at 18 °C, 6.3 g L^{-1} and at 22 °C, 7.8 g L^{-1} glycerol

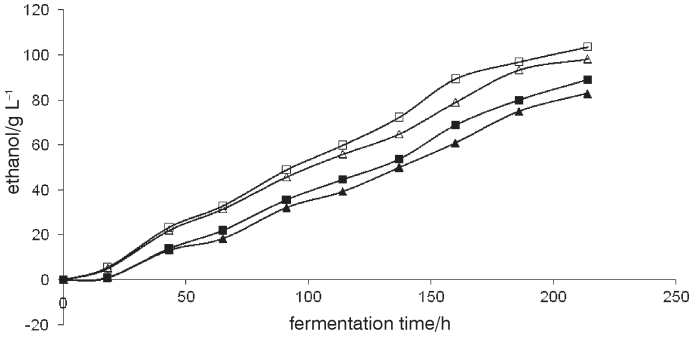

Fig. 3 – *Accumulation of ethanol:* □ *temperature 18 °C;* ■ *temperature 18 °C – 20 minutes heat shock;* ∆ *temperature 22 °C;* ▲ *temperature 22 °C – 20 minutes heat shock*

was detected. The highest glycerol production was detected at 10 min heat shock and fermentation temperature 22 °C (Table 1).

The results in fermentation showed that the duration of the heat shock interval reasonably increased the biosynthesis of glycerol (Fig. 4), while higher initial fermentation temperature promotes the rate of kinetic processes in biosynthesis of various organic acids (Table 2).

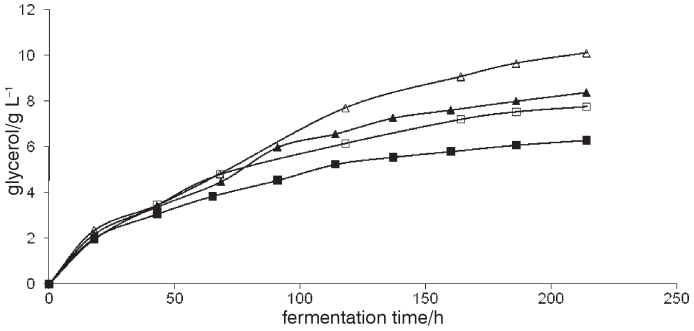

Fig. 4 – *Accumulation of glycerol:* ■ *temperature 18 °C;* □ *temperature 18 °C – 20 minutes heat shock;* ▲ *temperature 22 °C;* ∆ *temperature 22 °C – 20 minutes heat shock*

Table 2 – *Organic acids concentrations at various heat shock intervals*

Temp./ °C	Exposition/ min	T.A./ g L^{-1}	M.A./ g L^{-1}	A.A./ g L^{-1}	C.A./ g L^{-1}	S.A./ g L^{-1}
18	0	2.33	2.09	0.215	0.276	0.804
18	10	2.20	2.15	0.269	0.276	0.845
18	20	2.28	2.19	0.28	0.280	0.897
18	30	2.18	2.13	0.279	0.270	0.88
22	0	2.85	1.81	0.304	0.373	0.955
22	10	2.75	1.97	0.351	0.383	1.023
22	20	2.63	1.94	0.362	0.380	1.037
22	30	2.69	1.92	0.342	0.399	1.060

T.A. – tartaric acid, M.A. – malic acid, A.A. – acetic acid, C.A. – citric acid, S.A. – succinic acid

For effective monitoring of *Saccharomyces cerevisiae* microbial activity during the fermentation *on-line* redox potential measurement was used as main monitoring parameter in all of the experiments. Through fermentation, yeast cells adjust their redox balance to the conditions in the must producing adequate amounts of ATP, maintaining favourable redox and ionic balances, and synthesize the necessary metabolic intermediates.[7] Within a short interval in the period of the lag phase at the beginning of the fermentation processes, redox potential was constant. The shortest lag phase was detected at the higher temperature 22 °C and the longest at the lowest temperature of 18 °C.

During the exponential phase of the yeast growth, the ethanol production started and an abrupt decrease of redox potential was detected. In the stationary phase, redox potential becomes constant for a while. In this phase, the accumulation of the ethanol already inhibits microbial growth blocking the yeast metabolism of the fermentation process. In this phase, redox potential reaches its steady state (Fig. 5).

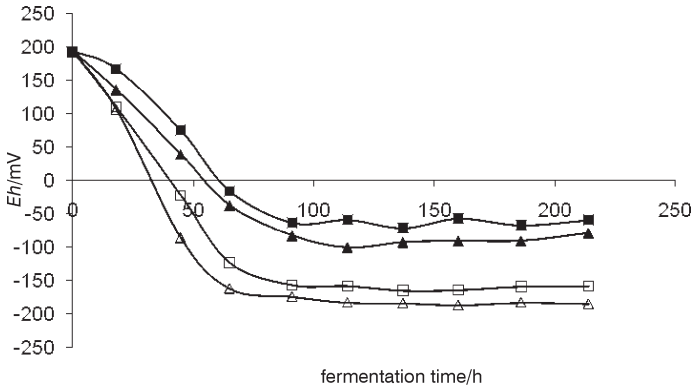

Fig. 5 – *Redox potencial during fermentation:* ■ *temperature 18 °C;* □ *temperature 18 °C – 20 minutes heat shock;* ▲ *temperature 22 °C;* ∆ *temperature 22 °C – 20 minutes heat shock*

According to the analysis, organic acids were generally higher in fermentations with applied heat shocks, but still below permitted limits. In the case of applied heat shock, a slightly enlarged production of the secondary metabolites (succinic and acetic acid) was detected (Table 2).

Conclusions

Glycerol as the main osmoregulator and redox balancing substance due to its high specific gravity and viscosity is one of the most remarkable qualities significantly contributing to the fullness and complexity especially in premium quality dry wines. Increasing of glycerol by fermentation is one of the most attractive tasks in comprehensive wine process technology.

In our previous research, the effect of temperature oscillation as well as heat shock on the wine yeast cells were studied. Fast temperature changes from 18 to 34 °C have been applied.[14,15] The effect of fermentation temperature oscillation was found as a useful parameter for improving glycerol production in small scale, while the heat shock on suspension of the yeast cells was found to be a simple and suitable method in large scale wine production.[13] In this article, the duration of heat shock interval as well as the height of the applied temperature shock on the yeast cells were examined.

New method for enhancement of glycerol production for the benefit of ethanol reduction in alcohol fermentation of grape must metabolism using thermal shock on inoculum suspension of *Saccharomyces cerevisiae* yeast cells was developed. The influence of applied heat shocks at intervals of 10, 20 and 30 minutes at 45 °C is presented. Heat shock on inoculum enabled homogenization of the quality and efficiency of the resistant cells that survived thermal shock. They represented new active biomass that include also a memory effect of heat exposure that enables increased production of glycerol from 6.3 to 8.4 g L^{-1} at 18 °C and 7.8 to 10.3 g L^{-1} at 22 °C. In the same experiments, the concentration of ethanol decreases from 89 to 81 g L^{-1} at 18 °C and from 104 to 97 g L^{-1} at 22 °C.

Heat shock at 45 °C actually reduced heterogenity of the wine yeast cell population, eliminating too young and too old unproductive cells increasing the homogeneity and productivity of the survived cells. These cells include also a memory effect of previous heat exposure. That is reflected in their increased productivity of osmotic protecting and balancing agent – glycerol up to 33.3 % at consecutive reduction of ethanol for 5.7 %.

Additionally, most of the primary (organic acids) as well as secondary metabolites of fermentation (i.e. succinic and acetic acids) were found in permissible concentrations, that did not negatively impact the wine's sensorial properties.

These results represent a new and easy method also for high glycerol production and reduction of ethanol applicable even in the large scale wine fermentation.

List of symbols

NADH – Nicotinamide adenine dinucleotide
EMP – Embden-Meyerhof-Parnas
UV – ultraviolet
RI – refractive index

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