Saccharomyces cerevisiae HEAT SHOCK TREATMENT FOR ENHANCED GLYCEROL PRODUCTION IN WINE

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Abstract

Present research was focused in the development of new method for the regulation of *Saccharomyces cerevisiae* metabolism using thermal shocks on inoculum suspension of yeast cells. Heat shocks were applied at 10, 20 and 30 minute intervals at 45 °C. The most resistible cells that survived this thermal shock represented new active biomass that include also a memory effect that enables decreasing ethanol production by increasing for production of glycerol up to 33.3 % at reduction of ethanol for 5.7%.

Present results represent a new and easy method applicable for high glycerol production at reduction of ethanol,

Keywords: 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[1,2,3]. Extensive studies of *Saccharomyces cerevisiae* metabolism proved that that glycerol biosynthesis of glycerol is related to yeast strain, process condition and consistence of fermentation substrate[1,4,5] 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 is 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 However, when intermediates in the EMP pathway are withdrawn as precursors for synthesis of cellular material this balance is distributed since the surplus of produced NADH is not converted back into NAD^+ [9,10].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 fermentation temperature, strain selection, inoculation level, sulphite concentration, sugar concentration, osmotic stress, nitrogen source and concentration, pH, aeration, grape variety and ripeness[13,14].

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[15,16].

The effect of heat shock treatment in alcohol fermentation was investigated in the shochu yeast BAW-6, sake yeast K-7, wine yeast W-3, beer yeast IF01167, and whisky yeast IF02363[17].

The main objective of this work was to study the extend of heat shock intervals on glycerol production, applied on cell suspension of wine yeast *Saccharomyces cerevisiae* inoculum starter culture.

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 was then cooled to T 18 ºC and then exposed for 10, 20 and 30 minutes to temperature 45 ºC. After exposure cell suspension was immediately cooled down back to 18 ºC. 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 (before Tocai Friulano) from wine growing region Goriška Brda. Before the start of fermentation grape must was not previously sulphurized and not filtered. Initial sugar content of the juice was 110 g glucose /L and 114 g fructose/L, with pH 3.71 . As bioactivator to the initial substrate 0.40 g /L Fermaid E (Danstar Ferment AG) was added.

Fermentors

All laboratory scale experiments were performed in identical standard type configuration 10 L working volume Stirred Tank Reactors (Bioengineering AG, Switzerland). All of fermentors were equipped with reflux cooler columns cooled down to 4 ºC. This condensers were so effective that no volatile substances of typical cultivar Sauvignonasse bouquet were detected in outlet fermentation gas during the whole period of fermentation. In all of 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 ºC. The mobile phase was 2 mM 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 heat shocks on *Saccharomyces cerevisiae* inoculum where yeast cells suspension was exposed to 45 °C eliminated on one side too young or too old, unproductive and temperature unresitent cells, from further fermentation process. Using a viability test it was found that at 10 min exposure to 45°C *Saccharomyces cerevisiae* initial cell concentration was reduced for 33.4 % at 20 min 39.6 % and at 30 min for 42.6 % from the initial cell concentration (Fig.1). 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.

Fig.1

Table 1

The highest biomass amount 6.7 g/L was obtained at 30 min exposure and fermentation at 22°C (Fig. 2).

Fig. 2

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

Fig. 3

It was fascinated 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 and at 22 °C, 7.8 g glycerol /L was detected. The highest glycerol production was detected at 10 min heath shock and fermentation temperature 22 °C (Table 1.).

Fig. 4

The results in fermentation showed that the duration of the heath shock interval reasonable increase 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).

Table 2

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 one at lowest temperature at 18 °C.

During the exponential phase of the yeast growth, the ethanol production started and abruptly decrease of redox potential was detected. In the stationary phase redox potential become constant for a while. In this phase the accumulation of the ethanol already inhibits the microbial growth blocking

the yeast metabolism further fermentation process. In this phase redox potential reach its steady state (Fig. 5).

Fig. 5

According to the analysis organic acids were in general higher in fermentations with applied heath shocks, but still under the allowed limits. In the case of applied heat shock,a small 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 significantly contributes to a fullness and complexity especially in premium quality dry wines. Increasing of glycerol was found in all of the samples where yeast cells were exposed to heat shock.

Faster fermentation kinetics as well as higher amounts of glycerol 33.3 % at simultaneous reduction of ethanol for 5.7 %. This results show that during the yeast metabolism both fermentation pathways in glycerol and ethanol production were active.

Present results represent a new and easy method applicable for high glycerol production at reduction of ethanol, represent an applicable method for decreasing of ethanol production in the period of temperature globalization and high glucose production in the grapes.

Application of heat shock on wine yeast cells represents an effective and simple applicable method for increasing the glycerol concentration in wine also in large scale production. Additionally most of the secondary metabolites of fermentation (i.e. succinic and acetic acids) were found in in a permittable concentrations, that did not negatively impact on wine sensorial properties.

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References

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Figures

Fig. 1. The influence of heath shock on the population of yeast cells

Fig. 2. Biomass accumulation

■ temperature 18 °C; □ temperature 18 °C - 20 minutes heat shock; ▲temperature 22 °C; Δ temperature 22 °C - 20 minutes heat shock

Fig. 3. Accumulation of ethanol

□ temperature 18 °C; ■ temperature 18 °C - 20 minutes heat shock; Δtemperature 22 °C; ▲ temperature 22 °C - 20 minutes heat shock

Fig. 4. Accumulation of glycerol

■ temperature 18 °C; □ temperature 18 °C - 20 minutes heat shock; ▲temperature 22 °C; Δ temperature 22 °C - 20 minutes heat shock

Fig. 5. Redox potencial during fermentation

■ temperature 18 °C; □ temperature 18 °C - 20 minutes heat shock; ▲temperature 22 °C; Δ temperature 22 °C - 20 minutes heat shock

Tables

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

Table 2. Organic acids concentrations at various heat shock intervals

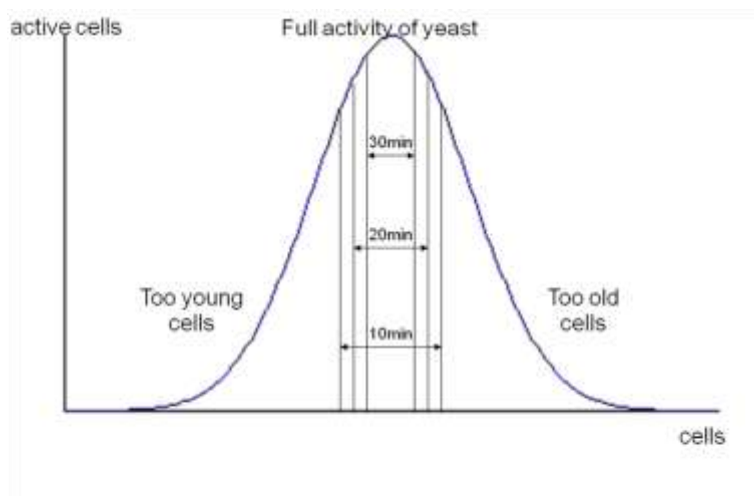

Fig. 1.

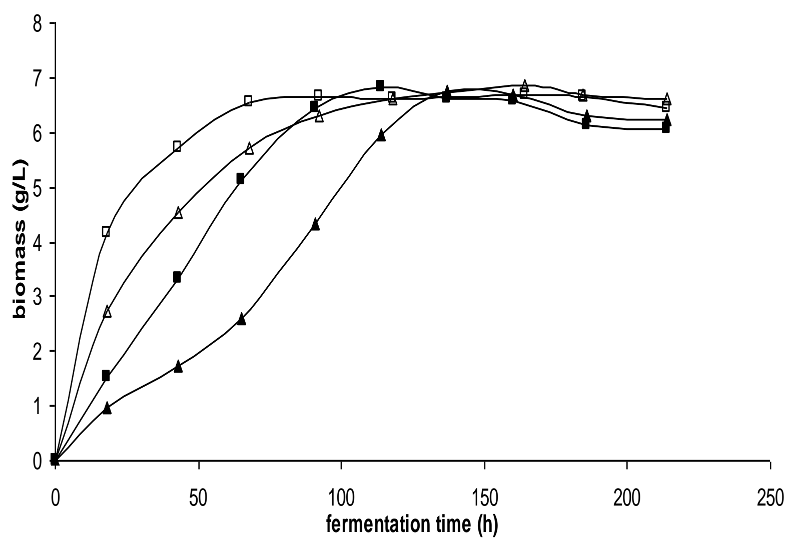

Fig. 2.

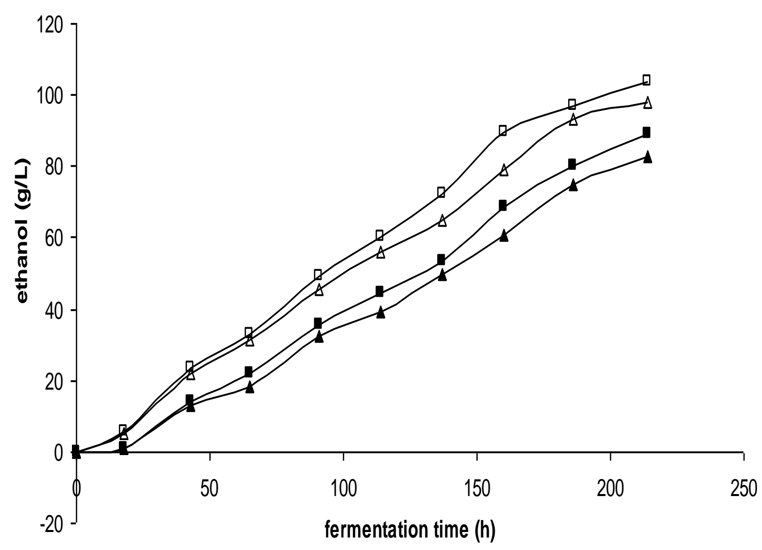

Fig. 3.

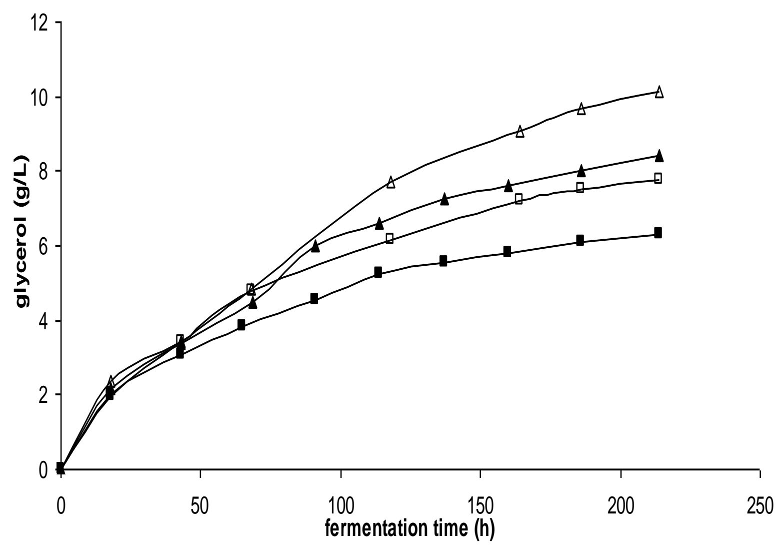

Fig. 4.

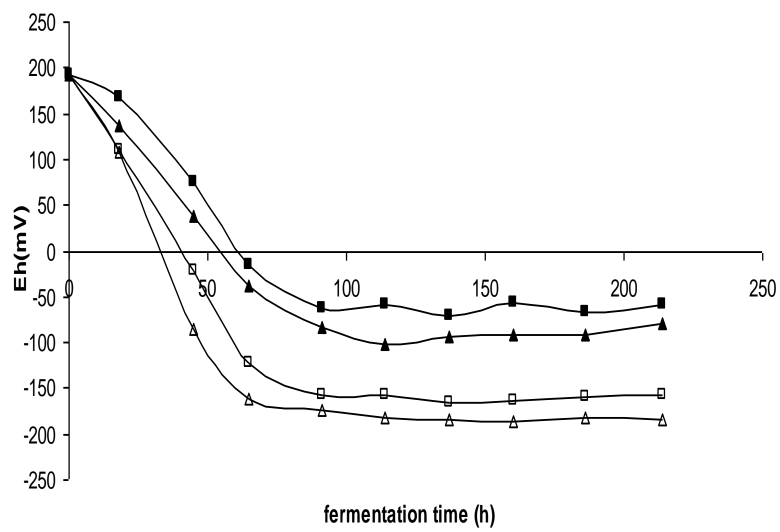

Fig. 5.

Table 1.

Temp. ***(° C)***	***Exposition*** ***(min)***	***Biomass*** ***(g/L)***	***Ethanol*** ***(g/L)***	***Glycerol*** ***(g/L)***
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

Table 2.

Temp. (°C)	*Exposition (min)*	*T.A. (g/L)*	*M.A. (g/L)*	*A.A. (g/L)*	*C.A. (g/L)*	*S.A. (g/L)*
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