Heat shock on *Saccharomyces cerevisiae* inoculum increases glycerol production in wine fermentation

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Abstract

A heat shock was applied to *Saccharomyces cerevisiae.* 5 min change from 18 to 45 ºC and then maintenance at later temperature for 20 min followed by cooling to 18 ºC. Such a treated inoculum when was used in an alcoholic fermentation of Welsch Riesling grape must at 18 ºC gave up to 12 g glycerol $l.^{-1}$ This is a new and easy method for high glycerol production in large scale wine production.

Introduction

Glycerol, as a secondary product in the fermentation process, is an important product of the determinant of premium wine quality. Its usual concentration in wines does not proceed 6 g l^{-1}. Because of its high specific gravity and viscosity, high concentrations of glycerol significantly contributes to fullness and complexity of dry wines (Nordstrom 1968,

Nieuwoudt *et al.* 2002). The increased biosynthesis of glycerol often gives wine a smoother mouth feel and an enhanced complexity (Karasu and Ozbas 2003). 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 However, when intermediates in the EMP pathway are withdrawn as precursors for the synthesis of the cellular material, this balance is disturbed because the surplus of NADH produced is not converted back into NAD^+.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 O_2, but must be disposed by the formation of reduced by-products such as glycerol (Oura 1977, Jackson 2000). The accumulation of glycerol is caused by the need to maintain a favourable redox balance (4 to 9) by converting the excess NADH.

In glycerol biosynthesis many growth and environmental factors can influence the amount produced by yeast in wine: temperature, strain selection, inoculation level, sulfite concentration, sugar concentration, osmotic stress, nitrogen source and concentration, pH, aeration, grape variety and ripeness (Ribereau-Gayon *et al.* 2000, Carrasco *et al.* 2001).

Fermentation temperature is one of the most influential factors affecting the 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 (Jesus Torija *et al.* 2003). The effect of heat treatment on *Saccharomyces cerevisiae* has been investigated in various alcohol fermentations (Omori *et al.*1996).

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

Materials and methods

Yeast

Saccharomyces cerevisiae wine yeast (Fermicru, AR2Val de Loire, France) 2g dry wgt. was reactivacted for 20 min at 34 °C in a 20 ml Welsch Riesling must diluted with water (1:1 v/v). The suspension of yeast, 10^7 cells ml^{-1} was cooled to 18 ºC and over 5 min heated to 45 ºC and then held for 20 min at the latter temperature after which the cell suspension was quickly cooled to 18 ºC. For the inoculation of grape must 20 ml suspension were used.

Fermentation substrate

Fermentations were carried out on a grape juice of cultivar Welsh Riesling from Ljutomersko-Ormoske Gorice. Before the start of fermentation, the grape must was neither sulfurized nor filtered. Initial sugar content of the juice was 109 g glucose l^{-1} and 103 g

fructose l^{-1}, with a pH 3.15 and a total amount of organic acids of 7.8 g l^{-1}. As bioactivator to the initial substrate, 30 g Fermaid E (Danstar Ferment AG) to 100 l of the grape must was added .

Fermentors

All experiments were performed in 10 l stirred tank reactors (Bioengineering AG, Switzerland) of standard configuration. Fermentors were equipped with reflux cooler columns, Ingold pH and redox electrodes, a temperature control unit and were stirred at 100 rpm.. For *on-line* measurements, SHIVA control software (BIA d.o.o., Slovenia) was applied. The fementors' head space was filled with N_2 to prevent oxidation of the fermenting grape must. 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. (Table 1)

Analytical methods.

The major extracellular 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 analyzed using a 300 mm × 7.8 mm Aminex HPX-87H organic acid cationic exchange column (Bio-Rad Laboratories, USA) at 65ºC. The mobile phase was 2

mMol H_2SO_4 in double distilled water at 0.6 ml min^{-1}. The injection volume was 20 µl; the elution was monitored by 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

Fermentations proceeded for 192 h at 18 ºC. Comparing the results of the heat shocked inoculum to the control fermentation, a 65 % higher consumption of reducing sugars and a higher production of yeast metabolites occurred. Most pronounced, however, was the production of glycerol, up to 12.25 gl^{-1}, that represents an 80 % increase or a 1.93 molar yield increase (Fig.1). Residual sugar concentration was 2.4 g l^{-1} and ethanol concentration 90 g l^{-1} .

(Fig.1)

Biomass production was faster with the control inoculum reaching 6.7 g l^{-1} in 144 h compared to the heat-shocked inoculum that reached 7.2 g l^{-1} in 168 h (Fig.2).

(Fig.2)

Ethanol increased from 85 gl^{-1} in the control up to 90. Succinic and malic acids slightly increased but tartaric acid decreased (see Table 1). The control fermentation had lower glycerol concentration, at the end of fermentation compared with the heat shock fermentation. Only slightly increase of succinic acid from 0.62 to 0.78 gl^{-1} and malic acid from 3.30 to 3.40 gl^{-1} , but a decrease of 0.28 g tartaric acid l^{-1} was indicated (Table 1).

(Table 1)

The temperature of fermentation was constant at 18 ºC. Only the inoculum has been heathed. Heat shock treatment induces *S.cerevisiae* to produce heat shock proteins with altered cellular characteristics (Nevoigt & Stahl 1997, Roustan & Sablayrolles 2005). Cells subjected to heat shock acquire resistance against various stresses (Attfield 1987, Odumeru *et al.* 1992). Fast changes in fermentation temperature result in a rapid response in yeast metabolism. Yeast cells pushed out of their balance and forced into, new temperature environment, started a pronounced glycerol secretion to reach another stable redox balance suitable for further microbial growth (Suetterlin *et al.* 2001). A fast temperature change induced activation of the enzyme triose phosphate isomerase, which displayed higher affinity to dihydroxyacetone phosphate than to glycerol aldehyde 3-phosphate, which was converted in a two-step reaction to glycerol (Scanes *et al.* 1998).

The yeast inoculum heat shock represents a new and easy applicable method to achieve higher glycerol (molar yield 1.93) and ethanol production (molar yield 1.07) in grape must alcohol fermentation. Using this method only a slightly increase of the undesirable acetic acid was detected. Similar indications were observed with other metabolites. Using this method, the fermenting grape must is not exposed to a higher temperature and thus there is no significant evaporation of the fine wine bouquet compounds. The main relevance of higher content of glycerol is its high specific gravity and viscosity and its sweet character that significantly contributes to a fullness and sweetness especially in dry wines.

The present method has its relevant practical application and it could be easily applied in large scale wine production.

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Table 1. Metabolites produced by a control and at heat shocked inoculum

Fermentation parameter	**Control (18 °C) (g l^{-1})**	**Heat Shock (20 min / 45 °C) (g l^{-1})**
Biomass	6.7 ± 0.02	7.2 ± 0.02
Reducing sugars	6.5 ± 0.23	2.4 ± 0.09
Acetic acid	0.28 ± 0.06	0.34 ± 0.02
Succinic acid	0.63 ± 0.11	0.75 ± 0.12
Malic acid	3.3 ± 0.16	3.4 ± 0.17
Tartaric acid	2.5 ± 0.18	2.2 ± 0.09
Ethanol	84.7 ± 2.18	91.8 ± 0.93
Glycerol	6.8 ± 0,06	11.8 ± 0,26

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FIGURES

Fig 1: Production of glycerol in fermentation with ■ 20 min 45 °C heat shock exposed inoculum and ♦ control fermentation at constant fermentation temperature 18 ºC; Results are the average of three separate fermentations.

Fig 2: Biomass production in fermentation with ■ 20 min 45 °C heat shock exposed inoculum and ♦ control fermentation at constant fermentation temperature 18 ºC; Results are the average of three separate fermentations.

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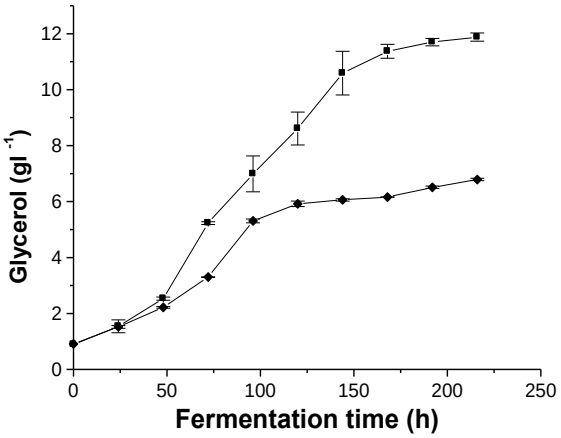

Fig.1

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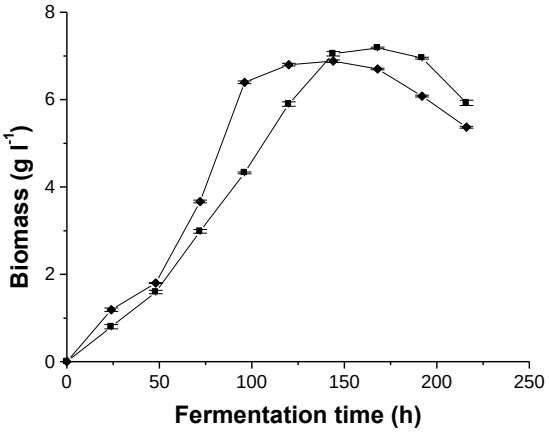

Fig.2

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