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Initial *Saccharomyces cerevisiae* concentration in single or composite cultures dictates bioprocess kinetics

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Abstract

The growth dynamics of three non-*Saccharomyces* strains in combination with *Saccharomyces cerevisiae* during fermentation of a sterile grape juice have been studied. The influence of the initial concentrations of *S. cerevisiae* on the whole yeast community was the main purpose of this research. The progression of *S. cerevisiae* within the first 5 days of fermentation was monitored by enumeration on selective and non-selective media. The population of each species was evaluated by morphological criteria. After 24 h, *Hanseniaspora uvarum* represented more than 50% of the whole yeast community, including ferments with the highest initial concentration of *S. cerevisiae*. As the population of *S. cerevisiae* increased, *H. uvarum* decreased. *Metchnikowia pulcherrima* was more inhibited by *S. cerevisiae* than *H. uvarum*, whereas the growth of *Candida stellata* was less inhibited. After thirty days, irrespective of the initial concentration of *S. cerevisiae*, only *S. cerevisiae* was detected in all ferments. Conversion of sugars to ethanol correlated with the initial population of *S. cerevisiae*. Glucose was almost completely exhausted in all cases, independent of the initial *S. cerevisiae* concentration used. Grape juices inoculated with composite inocula of non-*S. cerevisiae* and *S. cerevisiae*, with its initial concentration lower than 5 cfu/ml did not produce wines according to wine regulations. The concentration of ethanol in the wines did not reach the minimum amount of 9 vol%. Samples of wines fermented with a composite inoculum the concentration of *S. cerevisiae* represented 50 cfu/ml of grape juice, were judged to have the best sensorial properties.

Keywords: Population dynamics; Co-inoculation; Non-*Saccharomyces*; *Saccharomyces cerevisiae*; Yeast interactions

1. Introduction

The difference between wine fermentations carried out by inoculation with pure cultures and those performed by indigenous yeasts is a long-debated subject in the oenology field. Naturally presented micro-organisms whose origin can be attributed to grape berries or cellar equipment enable ''traditional'' wine fermentation. The apiculate yeasts, having a low fermentation power and belonging to genera *Hanseniaspora* and *Kloeckera* or other genera such as *Candida*, *Pichia* and *Metschnikowia*, carry out the first stage of a spontaneous fermentation (Fleet, 2003; Povhe Jemec et al., 2001; Fleet and Heard, 1992). The diversity and composition of yeast flora can influence the fermentation performance and chemical composition of the wine (Zagorc et al., 2001; Sipiczki et al., 2001; Pretorius 2000). It is generally accepted that some species play a beneficial role in wine production, while others act detrimentally as spoilage organisms. Despite the difficulty of spontaneous wine fermentations some wine-makers are willing to admit the risk involved to achieve a stylistic distinction. Several research papers (Romano et al., 2003; Egli et al., 1998; Romano and Marchese, 1998) stressed the ability of non-*Saccharomyces* yeasts to produce more glycerol and other polyols.

The fact that spontaneous fermentation takes longer than induced fermentation and that the outcome is highly unpredictable, represents one reason for the expansion of the yeast starter cultures. However, to

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ensure the completion of spontaneous fermentation, inoculation with a mixed culture of selected yeast might be a possible solution (Soden et al., 2000; Gil et al., 1996; Moreno et al., 1991). This approach could utilize new and desirable non-*Saccharomyces* yeast species in conjunction with a highly fermentative strain of *Saccharomyces cerevisiae*. To achieve the desired sensorial properties of fermented wine, different inoculation strategies had been tested in past experiments, such as the co-inoculation of the non-*Saccharomyces* yeasts at a 10-fold higher concentration than *S. cerevisiae*, or primary inoculation of the non-*Saccharomyces* yeast, followed by a super-inoculation with *S. cerevisiae* yeast (Soden et al., 2000).

It should be noted that the use of the selected *S. cerevisiae* starter cultures might not necessarily prevent the growth and metabolic activity of some species of, e.g. *Hanseniaspora* and *Candida* (Heard and Fleet, 1985), and they can be found in later stages as well, or even at the end of wine fermentations. During fermentation beside the well-known yeast interactions (toxic effect of ethanol, consumption of sugars, nitrogen, oxygen, vitamins), many less known interactions exist. One of them is the inhibitory effect of pulcherric acid produced by *Metchnikowia pulcherrima* which is active against various species (Nguyen and Panon, 1998). The second one is the protease activity of *Hanseniaspora uvarum*, providing low molecular weight peptides and amino acids utilizable by other yeasts, such as *S. cerevisiae* (Romano et al., 2001; Dizy and Bisson, 2000).

Many researches in the past attempted to reveal the influence of non-*Saccharomyces* yeasts on the fermentation efficiency and chemical composition of the fermented wines (Romano et al., 2003; Gil et al., 1996; Moreno et al., 1991; Heard and Fleet, 1988). These papers focus mainly on a detailed chemical analyses of fermented must, carried out by high concentration of the *S. cerevisiae* yeast associated with one of the non-*Saccharomyces* yeast species.

In our study, the influence of the initial *S. cerevisiae* cell concentration on yeast population dynamics and fermentation kinetics in pure and composite cultures with non-*Saccharomyces* yeast (*H. uvarum*, *Candida stellata* and *M. pulcherrima*) has been studied. The complex population dynamics was monitored in order to ascertain the quantity of the *S. cerevisiae* yeast in a composite inoculum needed to ensure the efficient fermentation kinetics.

2. Materials and methods

2.1. Overall experimental design

The study consists of three experiments. In the first experiment, monoculture fermentations were conducted with single strains of *S. cerevisiae*, *H. uvarum*, *M. pulcherrima* and *C. stellata*. In the second and third experiment, fermentations were inoculated with inocula of *S. cerevisiae* alone or in combination with non-*Saccharomyces*. The concentrations of inoculated *S. cerevisiae* varied between 400 and 0.04 cfu/ml, while the non-*Saccharomyces* part of inoculum was in the same concentration as established in previous experiments, analysing the indigenous yeast community of Malvasia grape must (Povhe Jemec et al., 2001).

The non-*Saccharomyces* inocula were composed of *H. uvarum* at 23%, *C. stellata* at 67% and *M. pulcherrima* at 10% of the total, as estimated by counting yeast cells in a single strain inoculum by Bürker–Türk slide. A composite inoculum was added in the total initial concentration of approx. 5×10^7 cfu/ml.

2.2. Grape must and its treatment

Grapes of Malvasia (*Vitis vinifera* L. cv. 'Malvasia') vintage 2000, produced in the coastal region of Slovenia, were crushed and the grape juice was stored in a freezer at –20 °C until used. After thawing, particulate material was removed by cross-flow filtration (0.45 μm). Sterile grape juice was achieved by membrane filtration (0.2 μm, Sartorious, Goettingen, Germany). To minimize browning, grape juice was fully filled into sterile glass bottle. No antioxidants were added.

Unsulphured grape juice (500 ml) was aseptically divided into sterile fermenters with fermentation locks and sampling ports. The initial pH value of the grape juice was 3.22 and the concentration of sugars was 186 g/l.

2.3. Yeast cultivation

Four yeast strains, *H. uvarum* (ZIM 1847), *C. stellata* (ZIM 1842), *M. pulcherrima* (ZIM 1850) and *S. cerevisiae* (ZIM 1927), were cultivated separately with a shaking speed of 200 rpm in a cultivation medium at 28 °C. The medium was a composite of one part of Malvasia must (total sugar: 186 g/l, pH 3.22) and four parts of water. To reconstitute the growth factor and nitrogen components, 1 g/l of yeast extract (Biolife, Italia) and 0.25 g/l of ammonia bisulphite (Merck, Germany) were added. The yeast cells for the inocula preparation were harvested from the middle of the exponential phase, which was estimated by measuring optical density of a sample with a photometer at $\lambda = 650$ nm (Photometer MA 9510, Iskra, Slovenia). After cell counting of each strain by Bürker–Türk slide, the required volume of the cultivated yeast was taken. The density of the single-strain inoculated in the fermenter in the first fermentation experiment was the same as the density of the individual strain in the fermenters inoculated with the composite inoculum. The