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A new method for the rapid separation of magnetized yeast in sparkling wine

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ABSTRACT

A novel method for the rapid magnetic separation of wine yeast cells from sparkling wine was developed. The cells were made responsive to a magnetic field by the absorption of superparamagnetic nanoparticles of iron oxide maghemite (γ-Fe_2O_3) coated with a thin layer of silica and grafted with (aminoethylamino)propylmethyldimethoxysilane (APMS). The terminal amino groups of the APMS molecules provide a positive charge on the nanoparticles' surfaces and promote their electrostatic absorption onto the negatively charged surfaces of the cells. The optimal mass ratio between the magnetic nanoparticles and the wine yeast was determined to be 1:10. The separation of the "magnetized" biomass in the magnetic field as well as the influence of the magnetic nanoparticles on the yeast metabolism was studied. Scanning and transmission electron microscopy showed that the magnetic nanoparticles remained fixed at the microbial cell surfaces, even after fermentation. The results of the chemical analysis demonstrated that besides the faster microbial kinetics there were no negative influences on the cell metabolism. The same results were confirmed in sensorial analyses of the sparkling wine. The separation of the magnetized waste biomass in the bottle neck using relatively weak magnetic-field gradient can be successfully completed in approximately 15 min.

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1. Introduction

Classic sparkling-wine production is one of the most sophisticated technologies in the wine industry and results in several millions of bottles every year. Although there are three methods to produce sparkling wines, i.e., *Methode Charmat* [1,2], *Méthode Classic Traditional* [2,3] and the continuous method [4], the most well-known is the *Méthode Classic Traditional* or *Méthode Champenoise*, a secondary fermentation in the bottle that leads to the production of CO_2.

In the final step the spent yeast biomass has to be removed from the bottle. Traditional separation is based on rotating and simultaneously inclining the bottle gradually until all the yeast cells settle into the neck of the bottle. This needs up to 60 days of rotation for each bottle, which is mostly done manually [1,3], or by the use of expensive rotating pallets [2,5]. After the sedimentation, the bottle neck is submerged into freezing liquid and the sediment is frozen. When the bottle is opened the frozen yeast sediment plug is expedited from the bottle by the internal gas pressure (550 kPa) [1,3,6].

Several techniques have been applied to shorten these time and energy consuming processes. One involves wine yeast immobilization in natural gels as mono-layer beads of 2% calcium alginate with 10^9 cells/g of the bead [7]. With this technique it was found that the critical factor is the leakage of the cells from the gel beads in the bottle [8–11]. The way to avoid this is by double immobilization, coating the beads with a gel layer without microorganisms [12]. Yeast immobilized within double-layer alginate beads was introduced in commercial sparkling wine production [10,13]. The immobilization of champagne yeasts was also made by inclusion into cryogels of polyvinyl alcohol that prevented cell release from the carrier matrix into the fermented wine [14]. The influence of the yeast strain immobilization and of the influence of ageing time on the changes to the free amino acids and the amino acids in peptides of bottle-fermented sparkling wines was also studied [15].

The immobilized-yeast technology for sparkling-wine production compared with the traditional *Méthode Champenoise* has several advantages related to the cost, the ability to control the fermentation and minimize its duration as a result of sharply reducing or even eliminating the riddling and disgorging steps, resulting in

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less storage space being needed in the winery. It also facilitates the yeast fermentation under a CO_2 pressure with the yeast cells partially protected from the toxicity of the ethanol [5].

In present research magnetic separation of wine yeast cells "magnetized" with absorption of magnetic nanoparticles onto their surfaces was used. Magnetic separation is a complex procedure based on the selective attachment of targeted species, i.e., ions, molecules or cells, onto magnetic particles. In further this "magnetisation" enables separation of targeted species from the mixture by using a magnetic field [16]. When the magnetic particle is placed in a magnetic field gradient, the magnetic force attracts it in the direction of the increasing magnetic-field density. For this purpose the magnetic material, simple magnetic iron oxides, i.e., maghemite (γ-Fe_2O_3) or magnetite (Fe_3O_4), is often used as a source for the nanoparticles applied in magnetic separation [17]. In recent years, large research efforts have been dedicated to investigating superparamagnetic iron-oxide nanoparticles in relation to their potential biomedical applications [17–19]. These iron-oxide nanoparticles are inexpensive and their synthesis is relatively simple. They are considered to be non-toxic and were even approved for in vivo medical applications by the Food and Drug Administration (FDA) [17].

Magnetic nanoparticles have already been used in biotechnology to separate products [20,21] or immobilized enzymes from the reaction mixtures [22], to separate cells and microorganisms [23], including yeast [24]. Dauer and Dunlop adsorbed ferromagnetic, submicron-sized, acicular maghemite particles onto yeast in order to separate it using high-gradient magnetic separation (HGMS) [25]. Yavuz et al. studied the biosorption of the heavy metal Hg on magnetically modified yeast using a water-based suspension containing magnetite nanoparticles stabilized by perchloric acid [26]. The magnetically modified yeast was applied in water purification.

The aim of this research was to develop fast method for used yeast biomass separation from the bottles of the sparkling wine. The method includes magnetization of the yeast cells starter culture using the absorption of superparamagnetic nanoparticles of iron oxide maghemite on the cell membranes and their separation from the bottle neck in a magnetic-field gradient.

2. Materials and methods

2.1. Fermentation procedures

2.1.1. Microorganism

In the primary fermentation for the base wine production wine yeast *Saccharomyces cerevisiae* (Daystar Ferment AG, Switzerland) was used. In the secondary fermentation in the bottle for a production of CO_2 bubbles in the sparkling wine traditionally used strain *Saccharomyces bayanus* (18-2627, Epernay, France) was used. Both strains were applied in magnetized form.

2.1.2. Fermentation substrate

The primary fermentation was carried out on not previously sulphurized and filtered grape juice of the cultivar Sauvignonasse (ex. *Tocai Friulano*) from the wine-growing region of Goriška Brda, Slovenia. The initial sugar content of the juice was 110 g glucose/L and 114 g fructose/L, at pH 3.7. As a bioactivator to the initial substrate, 0.40 g/L Fermaid E (Danstar Ferment AG, Switzerland) was added. The secondary fermentation was performed with primary fermented wine in 0.75 L pressure bottles. A total of 18 g/L of glucose was added to the basic wine as a source of carbohydrates for CO_2 production in the secondary fermentation.

2.1.3. Bioreactor

As the fermentor a 15 L stirred tank reactor (STR) in a standard configuration was used. It was equipped with a reflux cooler column, *on-line* sensors – pH electrode (HA-405-DPA-SC-S8) and a redox electrode (Pt4805-DPA-SC-S8) (Mettler Toledo, Switzerland), a temperature control unit and agitation control (Infors AG, Switzerland). For the *on-line* process control, SHIVA control software (BIA d.o.o., Slovenia) was used. The fermentor's head space was bubbled with N_2 to prevent oxidation of the fermenting grape must.

2.1.4. Fermentation

In the primary fermentation 10 L of grape must was inoculated at 22 °C and 100 rpm with 20 mL of *S. cerevisiae* yeast-cells suspension at a concentration of 2×10^7 cells/mL. The experiments were carried out in triplicate and the averages of the three runs were calculated. In the secondary fermentation the reactivation of 3.0 g of dry magnetized *S. bayanus* yeasts (applied magnetic nanoparticles-to-yeast ratio of 1:10) was suspended in 20 mL of grape juice of cultivar Sauvignonasse (ex. *Tocai Friulano*) diluted (1:1) at 38 °C with water for 20 min. A total of 0.3 g/L of the magnetized yeast was used for the inoculation of each bottle. The secondary fermentation in the pressure bottles proceeded for 24 days at 17 °C, being covered with stainless-steel crown stoppers. For the analysis a total of 100 mL of sample was taken from each bottle at the end of the secondary fermentation.

2.2. Nanoparticles

2.2.1. Synthesis of the Nanoparticles

The synthesis of the amino-functionalized maghemite nanoparticles is described in detail elsewhere [27,28]. The magnetic nanoparticles were precipitated from an aqueous solution of Fe(II) and Fe(III) sulphates using aqueous ammonia. The XRD pattern of the as-synthesized nanoparticles corresponded to the spinel structure, whereas their chemical analysis showed that less than 3% of the Fe was in the oxidation state 2+, proving that they were maghemite (γ-Fe_2O_3) nanoparticles. They had a globular shape with the average size being estimated from transmission electron microscopy (TEM) images to be 13.7 ± 2.9 nm. The nanoparticles exhibited zero magnetic coercivity, in agreement with their superparamagnetic nature, and a relatively high saturation magnetization of 66 Am^2/kg [27].

In order to coat the individual nanoparticles with a thin layer of silica, they were dispersed in water using citric acid as the surfactant [29]. The thin silica layer was then coated onto the surfaces of the dispersed nanoparticles using hydrolysis and the polycondensation of tetraethylorthosilicate (TEOS). The formed silica nucleated and grew on the surface of the nanoparticles, resulting in an approximately 1-nm-thick, homogeneous coating. The size of the silica-coated nanoparticles was measured to be 16 ± 5 nm, whereas their magnetization decreased to 46 Am^2/kg because of the presence of non-magnetic silica [27]. Finally, the silica-coated maghemite nanoparticles were grafted with 3-(2-aminoethylamino)propylmethyldimethoxysilane (APMS) to introduce amino groups onto their surfaces. The amino-functionalized maghemite nanoparticles were thoroughly washed with distilled water and dispersed in water. This aqueous suspension of nanoparticles was completely stable [27].

2.2.2. Absorption of the superparamagnetic amino-functionalized maghemite nanoparticles onto the yeast cells

The suspension of wine yeast was exposed to the magnetic nanoparticles in a separate step. A total of 200 mL of water suspension of the yeast, at a concentration of 10^7 cells/mL, was intensively stirred and the suspension with the magnetic nanoparticles was added. In the previous preliminary experiments, designed to determine an optimal mass ratio between the nanoparticles and the yeast, allowing efficient magnetic separation, the ratio between

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