



Chiral analysis of amino acids from conventional and transgenic yeasts[☆]

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ABSTRACT

Autolysis of *Saccharomyces cerevisiae* yeast is the main source of molecules that contribute to the quality of sparkling wines made by the traditional method. In this work, a genetically modified yeast (LS11) is compared to its isogenic wild type strain (BY4741) after autolysis. Chiral micellar electrokinetic chromatography with laser-induced fluorescence detection (chiral-MEKC-LIF) is used to identify and quantify the main D- and L-amino acids from both strains after accelerated autolysis. The procedure includes amino acids extraction, derivatization with FITC and chiral-MEKC-LIF separation in a background electrolyte composed of 100 mM sodium tetraborate, 30 mM SDS, 20 mM β -CD at pH 10.0. The D- and L-forms of Arg, Asn, Ala, Glu and Asp, corresponding to the major amino acids found in these samples, are separated in less than 30 min with efficiencies up to 800,000 plates/m and high sensitivity (i.e., LODs as low as 40 nM were obtained for D-Arg for a signal to noise ratio of three). From these results it is corroborated that the genetic modification brings a faster autolysis of the yeast, releasing a higher amount of L-amino acids to the medium in a short time. Interestingly, the pattern of release of D-amino acids was also different between the transgenic and the conventional yeast strains.

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1. Introduction

Nowadays, the use of genetically modified organisms (GMOs) has seen a great increase in agriculture and food industry. Thus, genetic engineering can be used e.g., to improve resistance of crops to plagues or pesticides, to provide better nutritional properties, etc. [1,2].

In spite of the aforementioned advantages, the use of GMOs in foods is not commonly accepted in many countries [3]. This situation has led to the implementation in some countries of different regulations regarding the development, growing and/or commercialization of genetically modified products [4]. At present, research on how the different genetic modifications can impact on chemical composition is of great interest since it has repeatedly been demonstrated the existence of unexpected modifications in GMOs [5]. Moreover, new strategies will be required to study the nutritional, safety assessment and chemical composition of the coming new generation of GMOs (e.g., with increased fatty acids or amino acids content, polyphenols, vitamins, reduced undesirable constituents,

etc.), requiring the development of more powerful and informative analytical procedures [6–8].

Among the GMOs proposed for being used in food science, the development of transgenic yeasts occupies an important place. Thus, the contribution of yeasts to the properties of sparkling wines during “prise de mousse” takes place in two steps. First, a secondary fermentation of the added sucrose leads to the production of ethanol, carbon dioxide, and minor secondary products [9]. After fermentation has been completed, there is an aging period. During aging, yeast cells die and undergo autolysis, which releases intracellular compounds, mostly peptides and amino acids, into the external medium. Improvement in the sensorial quality of the wines has been correlated with the products of the hydrolytic degradation of yeast cells, including free amino acids, peptides, mannoproteins, nucleic acid derivatives, and lipids [10–13]. However, autolysis in enological conditions is a very slow process that leads to long aging periods involving costly storages of the wines. A novel procedure for accelerating autolysis is the use of transgenic autolytic yeast strains that can improve the quality of sparkling wines by accelerating the acquisition of aging-like characteristics [10,14,15].

In this work, an original analytical strategy is proposed able to provide new information on the composition of transgenic yeasts based on the enantioselective determination of amino acids released after autolysis from a transgenic variety compared

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to its corresponding isogenic wild type strain. Thus, although multiple genetic procedures have been proposed to get transgenic autolytic yeasts [16–24], to our knowledge, a work like that proposed here has not been carried out so far. Thus, chiral analysis of amino acids is a remarkable methodology that can provide important information allowing a better understanding on the chemistry, nutrition, safety, microbiology, metabolic pathways, etc. of the organisms in which these molecules are found [25–32].

In spite of this interest, no method has been developed so far to separate and identify the main L- and D-aa found in transgenic yeasts. The goal of this work is, therefore, to carry out the profiling of the main D- and L-aa released by a transgenic autolytic yeast strain and the unmodified control strain. To do this, a CE method is developed that combines micellar electrokinetic chromatography (MEKC) with a chiral selector and laser-induced fluorescence (LIF) to analyze a group of L- and D-aa in yeast. The developed chiral-MEKC–LIF method is fast and reproducible and allows the quantification of the enantiomers with high efficiency and sensitivity.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. β -Cyclodextrin (β -CD) from Fluka Buchs (St. Louis, MO) was used as chiral selector for the MEKC running buffer together with sodium dodecyl sulphate (SDS) from Acros Organics (New Jersey, U.S.A.) and boric acid from Riedel-De H  en (Seelze, Germany). A water solution containing 5 mol/l sodium hydroxide from Panreac Quimica S.A. (Barcelona, Spain) was used to adjust the pH of the buffers and 0.1 mol/l NaOH was used to rinse the capillary. The buffer was stored at 4 °C and warmed at room temperature before use. Water was purified by using a Milli-Q system (Millipore, Bedford, MA). Fluorescein isothiocyanate (FITC, from Fluka Buchs, St. Louis, MO) was dissolved in acetone analytical grade (Merck, Darmstadt, Germany). Standard L- and D-aa were from Sigma (St. Louis, MO, USA). Trichloroacetic acid (TCA, from Merck, Darmstadt, Germany) as well as sodium deoxycholate (minimum 97%, from Sigma, Madrid, Spain) were used for amino acids extraction.

2.2. Yeast samples: obtention and autolysis

The autolytic *Saccharomyces cerevisiae* strain LS11 (MATa his3 Δ leu2 Δ 0 met15 Δ 0 ura3 Δ 0 bcy1-53::Kan-FLP-FRT) [33] and its cognate wild type strain BY4741 (MATa his3 Δ leu2 Δ 0 met15 Δ 0 ura3 Δ 0) were used in this study. Cells were cultivated in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or synthetic base wine (0.17% Yeast Nitrogen Base without amino acids and ammonium sulphate (Difco), 0.6% malic acid, 0.3% tartaric acid, 0.03% citric acid, 0.05% ammonium sulphate, 4% ethanol, 2% sucrose, 41 mg/ml histidine, 25 mg/ml leucine, 41 mg/ml methionine, 83 mg/ml uridine, pH was adjusted to 3.5 with KOH). Both strains were submitted to accelerated second fermentation conditions as next described: briefly, the strains were grown overnight at 30 °C and 180 rpm on YPD medium, and the cells washed three times with saline solution (NaCl 0.87% p/v). Then 10⁶ cells/ml were inoculated in 50 ml of synthetic base wine. Cultures were maintained at 30 °C without agitation and samples were withdrawn at different time points. Yeast cells were removed by centrifugation and the supernatant, yeast autolysis soluble fraction (YASF) was stocked at –20 °C until amino acid extraction.

2.3. Extraction of amino acids from yeast

For the extraction of free amino acids, 0.05 ml of 2 M TCA was added to 0.2 ml of every sample of YASF. The mixture was vortexed for 1.5 min. Then, 0.1 ml of 3.6 mM sodium deoxycholate was added to make possible the protein precipitation. The mixture was left to stand for 10 min and then, a 15 min centrifugation at 3000 g was carried out. The supernatant was separated and submitted to another 1 h centrifugation process at 4500 g. Total amino acids were quantified by the modified Cd–Ninhydrin method [34]. Again, the supernatant was collected and employed for the derivatization procedure described below.

2.4. Derivatization procedure

The FITC derivatization procedure was optimized as described next. Namely, the selected procedure consisted of mixing an aliquot of 625 μ l of the YASF with 675 μ l of water and 1.5 ml of 355 mM sodium tetraborate buffer at pH 10. This mixture was adjusted to pH 10 by adding 1 M sodium hydroxide. For amino acid standards derivatization, a 625- μ l aliquot was mixed with 1375 μ l of water and 7 ml of 355 mM sodium tetraborate buffer at pH 10. Again, this mixture was adjusted to pH 10 by adding 1 M sodium hydroxide and water was added until a final volume equal to 10 ml. 200 μ l of these final solutions were mixed with 25 μ l of a 3.75 mM FITC solution in acetone. The reaction took place overnight in darkness at room temperature. After derivatization, samples were diluted with water prior to their injection in the MEKC–LIF.

2.5. MEKC–LIF conditions

All analyses of L- and D-aa were carried out in duplicate using a P/ACE MDQ CE apparatus from Beckman Instruments (Fullerton, CA, USA) equipped with an Ar⁺ laser at 488 nm (excitation wavelength) and 520 nm (emission wavelength) also from Beckman Instruments to detect FITC-amino acids. Bare fused-silica capillary was purchased from Composite Metal Services (Worcester, England). The capillary dimensions were 50 cm of detection length, 60 cm of total length and 50 μ m i.d. and was held at a constant temperature of 25.0 °C. Injections were made at the anodic end at 0.6 psi (4.14 kPa) for 6 s (approximate hydrodynamic injection of 7.11 nl) and the applied voltage was +20 kV. The P/ACE MDQ CE instrument was controlled by a PC running the System Karat32 software from Beckman.

Before first use, new capillaries were preconditioned by rinsing with 0.1 M NaOH for 30 min. The washing protocol between runs was optimized to obtain adequate reproducibility, selecting the following conditions: at the beginning of each run, the capillary was rinsed with 0.1 M NaOH for 1 min, followed by 2 min with Milli-Q water, and then equilibrated for 5 min with the optimized running buffer (100 mM sodium tetraborate, 30 mM SDS and 20 mM β -CD at pH 10.0). At the end of the day, the capillary was rinsed with Milli-Q water for 10 min.

3. Results and discussion

Previously to the chiral-MEKC–LIF analysis, the derivatization procedure was studied in order to achieve the maximum amino acid signal with the lowest number of interferences from the derivatizing reagent FITC, since it is known to produce a high number of interfering fluorescent compounds [28]. To do so, different volumes of the YASF (obtained using the amino acids extraction protocol described under Section 2) and FITC solutions were mixed at different ratios to carry out the derivatization reaction. To monitor the results of this initial optimization, a published chiral-MEKC–LIF

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