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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.

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 Häen (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 his3Δ leu2Δ0 met15Δ0 ura3Δ0 bcy1-53::Kan-FLP-FRT) [33] and its cognate wild type strain BY4741 (MATa his3Δ leu2Δ0 met15Δ0 ura3Δ0) 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 10^6 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 μl of the YASF with 675 μl 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-μl aliquot was mixed with 1375 μl 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 μl of these final solutions were mixed with 25 μl 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 μm 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-MECK–LIF

method employed to analyze amino acids in different maize samples was used [35]. A comparison with the previous method [35] indicates that the group of amino acids to be separated in the present work is different to that studied in Ref. [35] and, therefore, the method needs to be adapted to separate these amino acids. The following derivatization conditions that gave higher amino acid signals together with lower FITC signal, that is, better derivatization yields, were selected: 200 μl of sample solution were mixed with 25 μl of a 3.75 mM FITC solution in acetone and let to react overnight. Results from four replicates confirmed the high repeatability of the procedure since no significant variation in the electropherogram profile was observed.

3.1. Chiral-MEKC–LIF analysis: figures of merit

Once the derivatization procedure was optimized, the MEKC–LIF separation conditions were further improved. To perform this study, a mixture of 10 different chiral amino acids, namely D/L-Arg, D/L-Asn, D/L-Ala, D/L-Glu, and D/L-Asp, was used. This selection was done considering the impossibility to find *ab initio* information on amino acid content of transgenic yeasts in the literature and, therefore, trying to cover a profile of amino acids as large as possible (i.e., including positively charged, neutral and negatively charged amino acids). Under these conditions, the published background electrolyte (BGE) developed to carry out the separation of chiral amino acids from maize was tested [35]. Namely, a BGE composed of 100 mM sodium tetraborate buffer with 80 mM SDS and 20 mM β-CD at pH 10.0 was used. It was observed that this buffer provided good separation of the amino acids, however, in order to speed up the separation the amount of SDS was decreased without compromising the resolution. Under these conditions, a BGE composed of 100 mM sodium tetraborate buffer with 30 mM SDS and 20 mM β-CD at pH 10.0 was finally selected providing the separation indicated in Fig. 1.

The optimized procedure including derivatization with FITC of the standards and chiral-MEKC–LIF analysis under the mentioned conditions provided the separation of the D- and L-forms of Arg, Asn, Ala, Glu and Asp in less than 30 min with efficiencies up to 800,000 plates/m, resolution values ranging from 1.0 to 5.3 and sensitivities in the nM range (e.g., the limit of detection LODs for D-Arg was 40 nM considering a signal to noise ratio of three). Next,

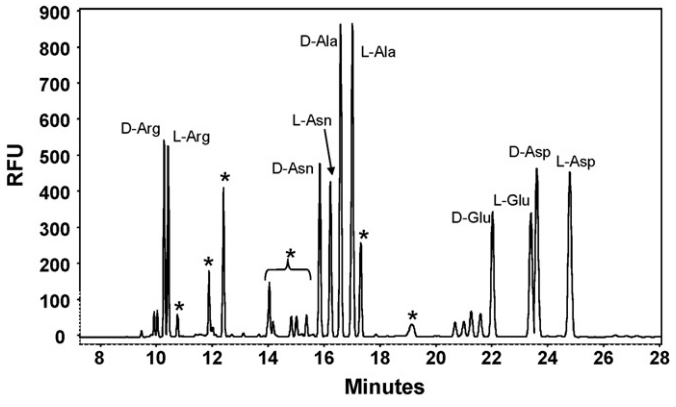

Fig. 1. Chiral-MEKC–LIF electropherogram of a standard mixture of 10 D/L-amino acids (D-, L-Arg; D-, L-Asn; D-, L-Ala; D-, L-Glu; D-, L-Asp) obtained under the selected MEKC–LIF conditions. Peaks marked with an asterisk correspond to FITC. Background electrolyte: 100 mM sodium tetraborate, 30 mM SDS, 20 mM β-CD at pH 10.0. Sample: standard mixture of FITC derivatized amino acids injected for 6 s at 0.6 psi. Bare fused-silica capillary: 60 cm total length, 50 cm detection length and 50 μm i.d.; running voltage: 20 kV; LIF detection: Ar^+ laser at 488 nm (excitation wavelength) and 520 nm (emission wavelength).

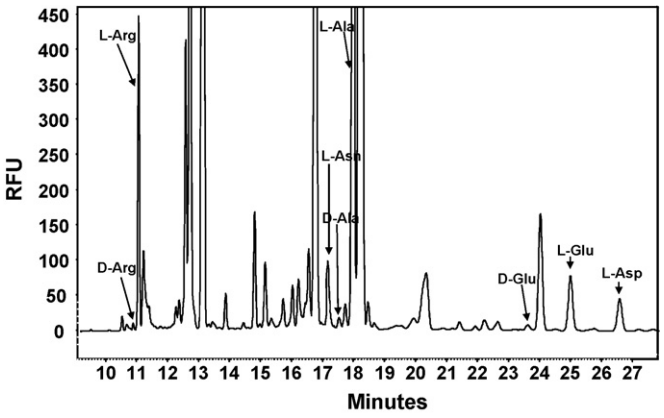

Fig. 2. Chiral-MEKC–LIF electropherogram of amino acids from a transgenic yeast (LS11). All the conditions as in Fig. 1.

the reproducibility of the chiral-MEKC–LIF method was determined injecting the same standard sample five times. The $\%RSD_{n=5}$ values obtained for corrected peak area reproducibility varied between 6.2% for L-Arg and 6.7% for L-Asp. On the other hand, $\%RSD_{n=5}$ values obtained for migration time reproducibility varied between 0.17% for L-Ala and 0.62% for L-Arg, corroborating the good capabilities of this approach for qualitative and quantitative analysis.

3.2. Transgenic vs. wild type yeast

Using this method, D- and L-amino acids were extracted, derivatized and analyzed in both yeasts (i.e., the transgenic LS11 and its isogenic wild type control, BY4741). An example of the typical electropherogram obtained under these conditions is given in Fig. 2. It is interesting to remark the time shift systematically observed between the real samples and the standard electropherograms (compare Figs. 1 and 2). This is very likely due to a matrix effect observed in the amino acid extracts from yeasts. Thus, the higher ionic strength of these samples brings about a lower electrophoretic mobility and as a result longer migration times compared to the standard sample. To overcome this limitation, an spiking procedure adding FITC derivatized amino acids was followed to identify the D/L-aa in the real samples.

Autolysis of the two *S. cerevisiae* strains (the transgenic LS11 obtained after a partial deletion of a gene encoding a regulatory subunit of cAMP-dependent protein kinase A and the wild type BY4741) were next monitored in time using the approach proposed in this work. Namely, the chiral-MEKC–LIF method was used to identify and quantify the main D- and L-aa released from the wild *S. cerevisiae* yeast (BY4741) and its corresponding genetically engineered variety (LS11) after 2, 5, 8, 11, 14, 18 and 21 days, obtaining the results shown in Fig. 3.

Release of amino acids usually starts very soon in these conditions, even before the end of sugar consumption (that takes about 1 week under these experimental conditions), and is faster for LS11 than for the wild type. Global amino acid content reached a maximum after 8 days of incubation for the recombinant LS11 strain, or after 14 days for the wild type control, and remained approximately constant up to the end of the experiment (see Fig. 4). This pattern of global amino acid release was paralleled, with more or less accuracy, by most of the L-amino acids analyzed in this work, i.e., L-Ala, L-Asp, L-Arg and L-Glu. Curiously, no differences were observed between both strains for L-Asn release during the 21 days of incubation, and a clear reduction in L-Asn content was observed after day 15 for both stains. The main contribution to amino acid release under these accelerated autolysis conditions is expected to

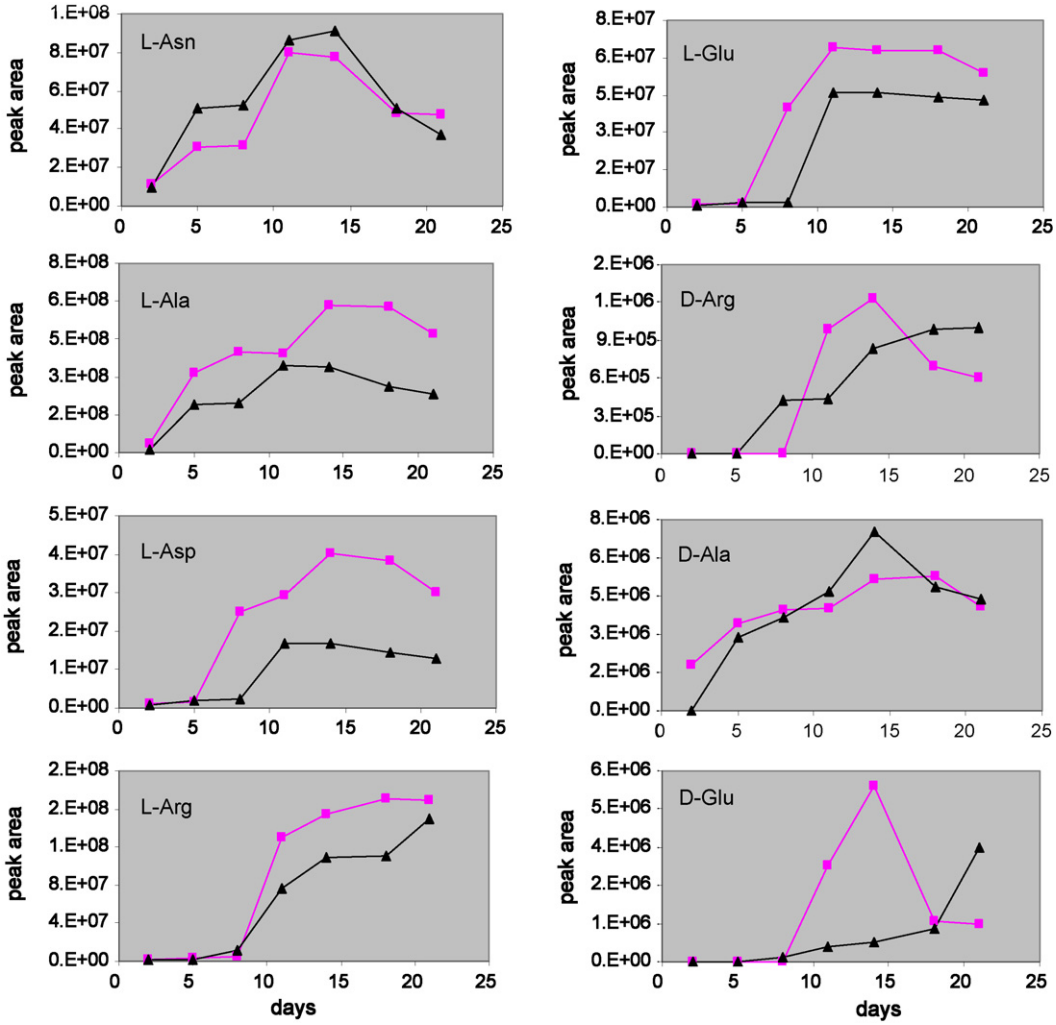

Fig. 3. Comparison of the different D- and L-amino acids released from the transgenic LS11 yeast (squares) and its isogenic wild counterpart (BY4741, triangles) during their autolysis. All the conditions as in Fig. 1.

be proteolysis, but free amino acids released from the cytoplasmic and vacuolar compartments might also contribute to the increase of amino acid content in the YASF. Release of L-Asn by mechanisms different to the rest of the amino acids, not affected by the genetic modification in LS11, would explain the aberrant behavior of this amino acid. However, since the internal free L-Asn content of yeast cells is not higher than that of other amino acids [36], we do not have a clear hypothesis to explain this observation. Amino acid content in the YASF is the result of different processes, mainly release from yeast cells, and degradation or to other spontaneous chemical reactions involving amino acids. This would explain the reduction with time of L-Asn concentration in YASF from both strains.

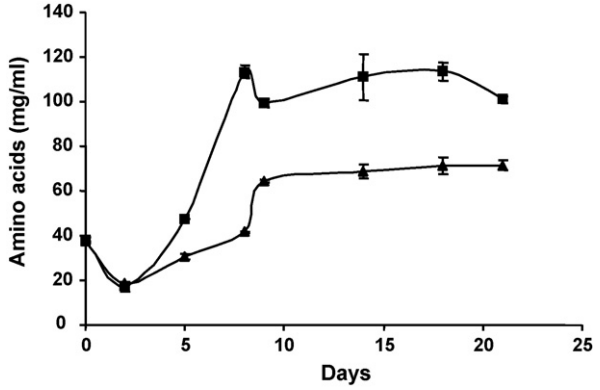

Fig. 4. Comparison of the total amino acids released from the transgenic LS11 yeast (squares) and its isogenic wild counterpart (BY4741, triangles) during their autolysis. See Section 2 for details.

The patterns of accumulation of D-amino acids is in accordance to the expected pleiotropic effects of *BCY* deletion in LS11 [16–19]. Indeed, accelerated autolysis, in the main *BCY1* deletion-linked phenotype of industrial interest [33], is not directly linked to defective autophagy but is a secondary consequence of accelerated cell death under stationary phase or starvation conditions. The transient increase in D-Arg and D-Glu observed after the end of the fermentation for the mutant strains would be indicative of the acceleration of other not yet identified metabolic pathways. The fact that, by the end of the experiment, the levels of D-Arg and D-Glu were higher for the control strain than for LS11 suggests that a similar metabolic process would be taking place in the unmodified strain, albeit at a lower rate. So, analysis of chiral amino acids seems to reveal unintended effects of the genetic modification. The biological and technological significance of these unexpected effects of *BCY1* deletion remains to be addressed. Interestingly, these D-amino acids have already been detected in other natural matrices.

Thus, D-Ala and D-Glu, were previously reported in wine, fortified wine, grape juice, yeast extract and vinegar [37–40] while D-Arg has already been found in vinegar [40].

4. Conclusions

In this work, it is demonstrated that the chiral analysis of amino acids in transgenic yeasts, apart of having interest from a nutritional and food safety point of view, can provide additional information for assessing the existence (or not) of unexpected modifications in other metabolic pathways linked to the amino acids profile. Thus, this methodology can highlight details on the changes induced (if any) on some parts of the metabolic network by the presence of significant modifications in the L/D content of amino acids that could be linked to the gene deleted in the original genome. In the case of *BCY1* deletion, it is shown that the genetic modification brings a faster autolysis of the yeast, releasing a higher amount of L-aa to the medium in a short time. Moreover, some unexpected effects of the genetic modification were also revealed, that could be in accordance with the pleiotropic effect expected for mutations involving the PKA pathway.

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