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Short communication

Overproduction of 2-phenylethanol by industrial yeasts to improve organoleptic properties of bakers' products

Rafael Dueñas-Sánchez [a], Ana G. Pérez [b], Antonio C. Codón [a], Tahía Benítez [a], Ana María Rincón [a,*]

[a] Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, E-41080, Sevilla, Spain
[b] Departamento de Fisiología y Tecnología de Productos Vegetales, Instituto de la Grasa-CSIC, Avenida de Padre García Tejero 4, E-41012, Sevilla, Spain

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ABSTRACT

2-Phenylethanol (PEA), an important alcohol derived from phenylalanine, is involved in aroma and flavour of bakers' products. Four spontaneous mutants of an industrial bakers' yeast, V1 strain, were isolated for their resistance to p-fluoro-DL-phenylalanine (PFP), a toxic analogue of L-phenylalanine. Mutants overproduced this amino acid and showed variations in their internal pool for several other amino acids. Moreover, a rise in PEA production after growth in industrial medium (MAB) was observed in three of the mutants, although their growth and fermentative capacities were slightly impaired. However, concentration of PEA remained higher during dough fermentation and also after baking, thus improving taste and aroma in bread.

1. Introduction

Fusel alcohols, which are compounds related to the sensory properties of fermented food, derive from the metabolism of amino acids by yeasts (Dickinson et al., 1997, 1998, 2003; Hazelwood et al., 2008). Among them, phenylalanine derivative 2-phenylethanol (PEA) is particularly interesting, due to its rose-like flavour, and its relevance in the aroma and taste of bakers' products, especially bread (Etschmann et al., 2002, 2003; Rehman and Awan, 2012). An increase in production of PEA by *Saccharomyces cerevisiae* mutants that overproduce phenylalanine to improve organoleptic features of sake has been reported (Fukuda et al., 1991a, 1991b). However, no one has ever attempted to do that with regard to bakers' yeasts.

In yeast, aromatic amino acids are synthesized in a multi-branched pathway that consists of several highly regulated steps (Braus, 1991). Regulation takes place at two levels, namely, a transcriptional control mediated by Gcn4p, the general activator of amino acid biosynthetic genes, and allosteric inhibition/induction by end-product of the enzymes involved in the first step of principal branches (Braus, 1991; Luttik et al., 2008). These enzymes are mainly the 3-deoxy-D-arabino-heptulosonate-7-phosphate (DHAP) synthases encoded by *ARO3* and *ARO4* genes, and are inhibited by phenylalanine and tyrosine respectively (Braus, 1991; Luttik et al., 2008).

The utilization of toxic amino acid analogues to select amino acid overproducing mutants is a common practise, since an increase in the mimicked amino acid concentration usually counteracts the negative effect of the toxic analogue (Gasent-Ramírez and Benítez, 1997; Martínez-Force and Benítez, 1992; Ramos and Calderón, 1992). p-fluoro-DL-phenylalanine (PFP), a toxic analogue for L-phenylalanine, strongly inhibits DHAP synthase; its use allows selection of mutants that accumulate a higher amount of this amino acid and, consequently, of its derivative PEA (Fukuda et al., 1991a, 1991b).

The aim of the present study was to isolate *S. cerevisiae* bakers' mutants that are resistant to PFP and that overproduce L-phenylalanine and PEA, and to evaluate their capacity to increase flavour and aroma in final product.

2. Materials and methods

2.1. Strains, media and growth conditions

The strain used in this study was *S. cerevisiae* V1, an industrial bakers' yeast kindly provided by Compañía General de Levadura (Valladolid, Spain).

Media such as YPD (1% yeast extract; 2% bacto peptone; 2% glucose), SD (0.17% Difco yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 2% glucose) and SDP (SD with 1% proline as single nitrogen source instead of ammonium sulphate) were used. When necessary, different amounts of p-fluoro-DL-phenylalanine (Sigma) were added to SDP. Beet molasses diluted to 3.6% sucrose and

* Corresponding author. Tel.: +34 954 557109; fax: +34 954 557104.
E-mail addresses: rduenas@us.es (R. Dueñas-Sánchez), agracia@cica.es (A.G. Pérez), accodon@us.es (A.C. Codón), tahia@us.es (T. Benítez), amrincon@us.es (A.M. Rincón).

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supplemented with biotin (0.5 mg/L) and diammonium phosphate (0.5 g/L) was employed as an industrial medium (MAB). Media were solidified by addition of 2% agar.

Flasks containing 50 mL media were inoculated with a yeast pre-inoculum to reach initial optical density at 660 nm (OD_{660}) of 0.1, and incubated at 200 rpm, at 30 °C. Growth was determined by measuring an increase of turbidity at OD_{660} in laboratory media, and at 690 nm (OD_{690}) in MAB, using a Beckman DU640 (Brea, CA, USA) spectrophotometer. Flasks (5 L) containing 1.5 L MAB medium were used to produce yeasts for baking.

2.2. Quantification of internal pool of amino acids

Strains were grown in SD to exponential phase for the screening of phenylalanine overproducing mutants, or in MAB to stationary phase for industrial characterization. Cells were centrifuged and collected, washed three times with bi-distilled water and then resuspended in 1 mL bi-distilled water and stored at −20 °C. Cell suspensions were boiled for 15 min and centrifuged for 10 min at 10,000 *g*. Supernatants were collected, and concentrations of amino acids and total proteins were determined. Amino acids were analysed by HPLC, following Waters AccQ-Tag instruction manual (Millipore Corporation, Milford, Mass, USA). A chromatographer equipped with a Waters AccQ-Tag column, an automatic injector (Waters Alliance 2695), and a scanning fluorescence detector (Waters 474) were used. Data integration and processing were performed with the aid of Waters Millennium 32 software.

Data were normalized using protein concentration of boiled supernatants. Total protein was determined according to the Bradford method (1976), using Bio-Rad protein assay dye reagent, and bovine serum albumin as protein standard.

2.3. Biomass production and sugar consumption

Evaluation of yeast biomass production was carried out as described in Dueñas-Sanchez et al., 2010. It is defined as the amount of yeast produced per litre of medium in dried cell weight.

For glucose and sucrose measurements, cells were grown at 30 °C in MAB and, at specified times, 0.5 mL aliquots were taken and centrifuged at 16,000 ×*g* for 5 min. To determine sugar concentration, supernatants were first incubated with – in the case of sucrose – or without invertase, for 1 h at 30 °C. Reducing sugar was measured by the Somogyi–Nelson procedure (Nelson, 1957; Somogyi, 1952).

2.4. Leavening activity

4 g wheat flour (specific deformation work [alveograph value] W180 × 10^3 ergs for plain doughs), 2.5% (wet weight) yeast (grown in molasses until stationary phase) and 7 mL distilled water were mixed in 20 mL tubes (Barber et al., 1987). Tubes were incubated without shaking at 30 °C. An increase in volume was monitored every 15 min for 2–3 h (Rincón et al., 2001; Rincón and Benítez, 2001).

2.5. Baking

Wheat flour (W180), 2.5% yeast (wet weight) of either V1 or mutants, 2% salt, and water were mixed, and doughs were incubated for 1 h 40 min at 30 °C and then baked at 200 °C for 15 to 20 min.

2.6. Analysis of volatile compounds in yeast, doughs and bakery products

Samples for volatile compound detection consisted of 1 g yeast (wet weight) obtained after culture in MAB for 48 h resuspended in 1 mL aqueous saturated $CaCl_2$ solution, or 2 g dough after 1 h 40 min fermentation at 30 °C, or 2 g bread after cooking fermented dough for 10 min at 200 °C. Vials with samples were adjusted to room temperature and then placed in a vial heater at 40 °C. After 10 min equilibration, volatile compounds from headspace were adsorbed on a SPME fibre DVB/Carboxen/PDMS 50/30 μm (Supelco Co., Bellefonte, PA, USA). Sampling time was 50 min at 40 °C. Desorption of volatile compounds trapped in SPME

Table 1
Amino acid internal pools of PFP-resistant mutants and wild type growing during exponential phase in SD medium.

Amino acid	V1	V8.2	V10.5	V10.12	V900
Asp	2.0 ± 1.0	2.3 ± 0.2	2.3 ± 0.8	1.8 ± 0.8	2.8 ± 1.1
Ser	5.7 ± 0.9	3.4 ± 0.0	3.9 ± 0.7	3.0 ± 0.9	4.2 ± 0.1
Glu	39.1 ± 4.7	31.7 ± 5.2	33.0 ± 4.9	23.7 ± 4.0	32.9 ± 1.1
Gly	6.4 ± 2.4	3.6 ± 0.3	4.2 ± 0.3	3.6 ± 1.2	5.0 ± 0.7
Arg	20.6 ± 4.6	**51.3 ± 2.8**	**57.1 ± 9.2**	**39.9 ± 6.0**	**47.6 ± 3.9**
Thr	24.8 ± 7.7	12.7 ± 7.2	13.6 ± 6.2	16.4 ± 5.3	13.9 ± 7.2
Ala	14.7 ± 2.8	14.0 ± 1.8	15.1 ± 1.5	9.8 ± 0.2	**28.8 ± 0.4**
Tyr	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	**0.5 ± 0.2**
Val	5.0 ± 2.2	3.6 ± 0.8	4.2 ± 1.3	2.8 ± 0.7	7.7 ± 0.7
Met	1.7 ± 0.6	0.9 ± 0.0	1.1 ± 0.3	0.8 ± 0.2	1.4 ± 0.2
Lys	3.6 ± 0.8	4.6 ± 0.1	5.3 ± 0.7	4.2 ± 1.1	2.4 ± 0.2
Ile	1.3 ± 0.3	1.0 ± 0.0	1.2 ± 0.2	0.9 ± 0.2	1.6 ± 0.0
Leu	1.2 ± 0.3	1.1 ± 0.0	1.3 ± 0.2	1.0 ± 0.2	1.8 ± 0.1
Phe	0.6 ± 0.1	**2.1 ± 0.2**	**2.6 ± 0.5**	**2.3 ± 1.0**	**3.6 ± 0.2**

Concentrations are indicated in nmol amino acid per μg protein. Average data of three independent experiments with standard deviation are represented.

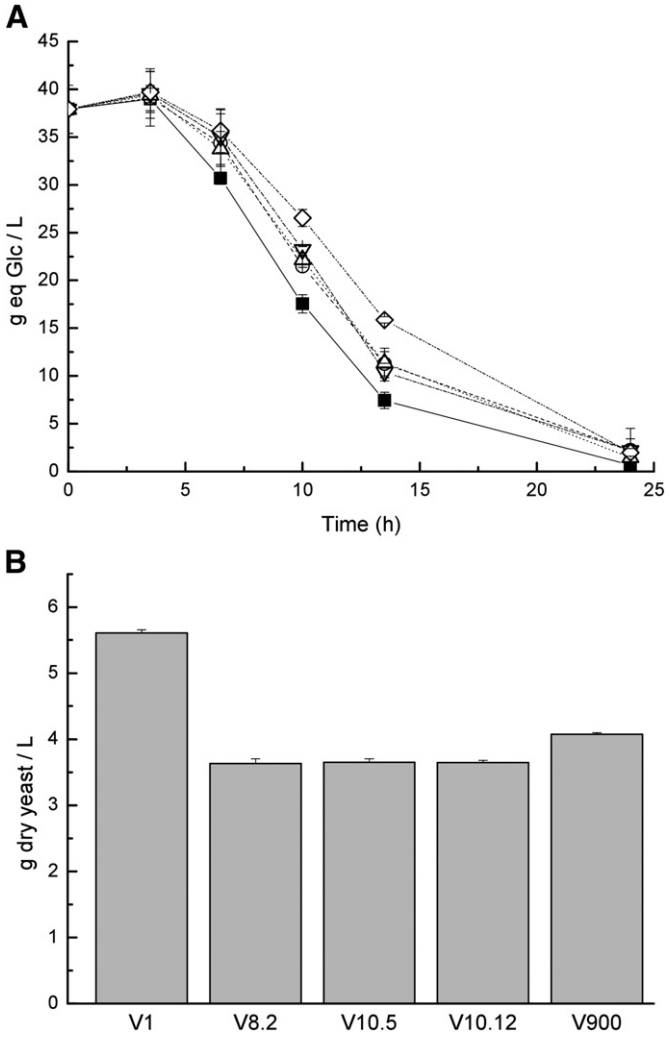

Fig. 1. Residual sugar concentrations (A) and final biomass production (B) of bakers' strains after growth in MAB. V1 (■), V8.2 (○), V10.5 (△), V10.12 (▽) and V900 ( ). Results are average, and standard deviation of two experiments in duplicate. Sugar concentration was estimated as glucose equivalent per litre.