Improved Organoleptic and Nutritive Properties of Bakery Products Supplemented with Amino Acid Overproducing *Saccharomyces cerevisiae* Yeasts

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Spontaneous yeast mutants isolated in continuous culture as resistant to toxic amino acid analogues, able to increase up to 40 times their free amino acid pool of Thr, up to 160 times their pool of Met, or up to 20 times their pool of Lys, were characterized with regard to properties of industrial interest. Growth rate, μ (h^{-1}), and biomass yield, Y (g/L), of the amino acid overproducing mutants (AA^S) were in many cases similar to those of the wild type, whereas their free amino acid content was substantially increased in laboratory and industrial media (molasses). Doughs fermented with 3% baker's yeast and 0.5% AA^S mutants produced bakery products that displayed texture similar to those fermented with 3.5% baker's yeast, but the former had a considerable improvement of their taste and aroma. On the other hand, bread content of the essential amino acids Lys, Met, and Thr provided by yeast was also increased.

Keywords: *Bakery products; organoleptic properties; nutritive properties; amino acid enrichment; amino acid overproducing yeasts*

INTRODUCTION

Numerous feeding experiments have shown that yeasts are a valuable source of protein and vitamins, especially B-group vitamins. Furthermore, Lys and, to a lesser extent, Met and Thr have been long known to be limiting amino acids in cereal products (*1*−*3*). Hence, many bakery products are currently being prepared with the addition of inactive dried yeasts (*1*, *3*−*7*). Amino acid-enriched yeasts could also be added to cereals to improve their nutritive value.

The fact that toxic amino acid analogues are known to block essential functions of physiological amino acids in cells and that most of their biochemical effects are reversed by the administration of these amino acids has led to the use of resistance to these analogues as a method to isolate mutants which overproduce the corresponding amino acids (*8*−*11*). The increase in analogue resistance gave rise to the isolation of mutants that accumulated 160 times more Met, 40 times more Thr, or 20 times more Lys than the wild types (*8*, *10*, *11*).

In addition, the aroma of bread is created during baking by thermal reactions within some of its individual components formed by yeast fermentation and between these compounds and other dough constituents such as amino acids (Ala, Val, Leu, Met, Gly, and Phe) (*3*, *12*, *13*). When there is an excess of amino acids, they are degraded to aromatic carbonyl compounds (*5*). This is an oxidative deamination and decarboxylation of amino acids to an aldehyde or ketone: Leu to 2-methylbutanal, Ile to 3-methylbutanal, Met to methional, and Phe to phenylethanal (*3*). Other amino acids do not form aroma compounds but condense to melanoidin (*5*).

There is a tendency to reduce the time of dough fermentation, with the consequent decrease in flavor and aroma (*4*, *5*, *7*). There have been attempts to produce fermentation flavors that could be used in leavened products (*14*, *15*). Among them, amino acids such as Thr are used extensively in bakeries and other food industries as flavor enhancers, antioxidants, and nutritional supplements as feed and food additives (*4*, *5*). Hence, the use of Thr and/or other amino acid-overproducing yeasts would also make it possible to obtain bread enriched in amino acids, essential in the contribution of flavors and aroma.

Bread manufacturers have an ongoing interest in new strains of yeasts, especially those that increase the quality of the final product (*5*). Recombinant DNA technology and classical genetics (*7*) have led to the construction of new baker's yeasts, the introduction of which to manufacturing may have a great economic impact. However, these so-called genetically modified organisms are not acceptable to the consuming public (*7*). Also, leavening properties, dough rheology, and bread characteristics have hardly been tested for the new strains derived from baker's yeast (*14*).

In this study the characterization of baker's and amino acid-overproducing yeast mutants, including their capacity for dough fermentation and bread production, has been attempted. These mutants share some characteristics of interest for their inmmediate commercial application: they are spontaneous stable mutants and, therefore, they have not been subjected to recombinant DNA technology (*8*), and also they possess the most desirable property of industrial strains, name, genetic stability, which guarantees the reproducibility of properties in the final product (*7*).

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10.1021/jf001130u CCC: $20.00 © 2001 American Chemical Society
Published on Web 03/17/2001

MATERIALS AND METHODS

Strains. The following *Saccharomyces cerevisiae* strains were used: baker's strains V1 and V2 were chosen for their high fermentative capacities (*16*, *17*) as well as for their high frequency of sporulation, tetrad formation (>50%<) and spore viability (∼60%) as compared with other baker's strains (*18*, *19*); strain SL1 was a spontaneous Lys overproducing mutant (Lys^S mutant) derived from the baker's yeast V2 and isolated in continuous culture as resistant to the Lys toxic analogue aminoethylcysteine (*10*); strain IFI256 was a wine yeast isolated from white wine (Albariño, Galicia, Spain) and was chosen for its high fermentative capacity (*29*); strains HNVBC5 and ETHCC7 were spontaneous mutants derived from IFI256, which overproduced Thr and Met, respectively (Thr^S and Met^S mutants), and which were isolated in continuous culture as resistant to the Thr and Met toxic analogues hydroxynorvaline and ethionine, respectively (*8*); finally, a commercial yeast (L'Hirondelle, Lesaffre, France) was used as a control of baking abilities.

Media. Yeasts were grown in complete YPD medium (1% Difco yeast extract, 2% bactopeptone, and 2% glucose) or in minimal medium (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose) (SD) or this medium with ammonia substituted by 5 mM proline (SDP). Beet molasses (72% sucrose) obtained from Unión Alcoholera Española, S.A. (Granada, Spain), diluted 20 times (3.6% sucrose) was also used. Biotin (0.5 mg/L) and diammonium phosphate (0.5 g/L) were added to the molasses (MBA medium). Media were solidified by the addition of 2% agar.

Enzymes and Chemicals. Acetonitrile was obtained from Fluka (Buchs, Switzerland); methanol was purchased from Probus (Barcelona, Spain) and tetrahydrofuran from Merck (Darmstadt, Germany). *o*-Phthalaldehyde, β-mercaptoethanol, and individual crystalline amino acid standards, or a mixture, as well as all other chemicals used, were purchased from Sigma Chemical Co. (St. Louis, MO).

Culture Conditions. Yeasts were inoculated into 20 mL tubes containing 5 mL of YPD and incubated with rotatory shaking in a New Brunswick incubator at 30 °C until the stationary phase was reached (∼10^8 cells/mL). Flasks of 250 mL−5 L with 100 mL−2.5 L of media were prepared. The flasks were inoculated with the stationary phase culture to reach an initial optical density at 660 nm (A_{660}) of 0.05. After inoculation, the flasks were incubated at 30 °C with shaking. When minimal media were used, the precultures were centrifuged and the cells washed twice with distilled water before inoculation.

Growth Rate. Growth was determined by measuring the increase in turbidity in laboratory media at 660 nm (A_{660}) and in molasses at 690 nm (A_{690}) using a Spectronic 20 (Bausch and Lomb) spectrophotometer. An exponential increase in A_{660} or A_{690} between 0.1 and 0.5 was used to determine the growth rate, μ (h^{-1}). Previously, a linear relationship between cell number and absorbance at 660 nm (A_{660}) ranging from 0.1 to 0.5 was observed.

Cell Number and Viability. Cell number was estimated by diluting the samples in water, measuring the absorbance at 660 nm (A_{660}), and counting cells under the microscope. Viability, defined as the percentage of cells able to form colonies on YPD, was determined by taking samples at intervals over a period of time or at the end of the incubation and spreading them on YPD. After 3−4 days at 30 °C, colonies were counted to calculate the frequency of viable cells.

Amino Acid Determination. Amino acid samples were prepared from cells after they had been collected by centrifugation. Cells were washed five times with glass-distilled deionized water and resuspended in $^1/_{20}$ of the initial volume. The suspension was boiled in a water bath for 15 min and centrifuged, and the pellet was discarded. The supernatant, which contains the internal pool of amino acids, was filtered through a Millipore filter of 0.45 μm pore size, and the filtrate was kept at −20 °C for further amino acid analysis. Primary amino acids were derivatized with *o*-phthalaldehyde and β-mercaptoethanol and detected by absorbance at 340 nm. The derivatization mixture was prepared by mixing 4.5 mL of sodium borate (0.4 M, pH 10) with 0.5 mL of *o*-phthalaldehyde (54 mg of *o*-phthalaldehyde/mL of methanol, maintained at −20 °C and protected from light) and 0.2 mL of β-mercaptoethanol. The mixture was filtered through a 0.45 μm pore size Millipore filter and maintained at −20 °C. Amino acid samples (filtered media or filtered supernantant from boiled cell suspensions) were derivatized by mixing 25 μL of samples with 75 μL of the derivatization mixture. After a 2 min incubation at room temperature, the derivatized samples were separated using an acetonitrile/phosphate buffer gradient (*21*) by reverse-phase high-performance liquid chromatography (HPLC) following methods described previously (*8*, *9*).

Protein Determination. Total protein was determined in permeabilized cells following the procedure of Lowry et al. (*22*). In some experiments, protein was determined according to the Bradford method (*23*) using the Bio-Rad protein assay dye reagent and bovine serum albumin as a protein standard.

Determination of the Capacity To Leaven Dough. Stationary phase cultures were inoculated into 100 mL−5 L of YPD or molasses flasks to reach an initial optical density of 0.05. The flasks were incubated with rotatory shaking at 30 °C until early stationary phase [(∼1−5) × 10^8 cells/mL]. The cells were then filtered through Millipore filters of 0.45 μm and washed twice with distilled water. Finally, wet weight (ww) was determined for all of the cultivated strains—baker's yeasts are mostly sold as fresh, compressed yeasts rather than dry yeasts (*24*). To determine leavening capacity under laboratory conditions, 20 mL tubes containing 7 mL of distilled water plus 4 g of wheat flour were inoculated with 0.1 g (ww, 1.5 × 10^7 cells/mg) of the yeasts previously grown in YPD (2.5% ww yeast with regard to the flour's weight). The tubes were incubated without shaking at 30 °C, and the increase in volume was monitored every 10 min for 2 h.

Baking. Yeast were grown in molasses (MBA) or in YPD medium until late stationary phase. Elaboration of bakery products was carried out by mixing 1 kg of wheat flour (180 W) with 2% salt and 3.5% yeast (ww) with regard to the flour's weight, and 500 mL of water, following standard protocols (*5*). Each dough was mechanically divided into pieces and then weighed. The mixture was incubated at 33 °C and 85% humidity for 2.5 h and finally baked at 220 °C for 20 min, the last 10 min with aeration. The products were assessed with regard to their texture and organoleptic properties by 30−40 nonexperts following standard procedures for quality evaluation (*24*, *25*).

All data shown are the average of three to six experiments, with standard deviations of <10%.

RESULTS AND DISCUSSION

Characterization of Baker's Yeasts V1 and V2, Wine Yeast IFI256, and Amino Acid Overproducing Mutants (AA^S Mutants) Derived from V2 and IFI256. Genetic Stability. Genetic stability of industrial strains could guarantee the reproducibility of best quality in final products (*7*). The three AA^S mutants (*8*, *10*) were maintained at −80 °C and at 4 °C for at least three years and periodically checked for their karyotype and their capacity to tolerate toxic amino acid analogues or to overproduce the physiological amino acids. The three of them were very stable and maintained these characteristics (Tables 1 and 2 and data not shown), although the karyotype of the Lys^S mutant indicated that severe reorganizations had taken place in its genome during the selection procedure (*10*, *11*) and that there were numerous changes in the number and position of the chromosomal bands with regard to its V2 parental strain. Met^S and Thr^S mutants showed alteration of the kinetic properties and of the activity of some enzymes involved in the biosynthesis of these

Table 1. Growth Rate, μ (h^{-1}), of Strains Cultivated in Laboratory (SD, SDP, YPD) and Industrial (MBA) Media[a]

strain	media			
	SD	SDP	YPD	MBA
IFI256	0.31[a]	0.30[a]	0.39[a]	0.20[a]
Thr^S mutant	0.28[a]	0.27[a]	0.45[b]	0.31[b]
Met^S mutant	0.27[a]	0.25[b]	0.40[a]	0.10[c]
V1	0.27[a]	0.15[c]	0.46[b]	0.31[b]
V2	0.31[a]	0.17[c]	0.47[b]	0.36[b]
Lys^S mutant	0.16[b]	0.13[d]	0.19[c]	0.16[d]

[a] Data represent mean values with $n = 6$ and standard error of <10%. Mean values in the same column followed by the same letter do not differ significantly ($p < 0.01$) according to the Scheffe *S* test (*30*).

Table 2. Free Amino Acid Pool (Millimolar) of Different Yeast Strains Grown in Laboratory (SD, SDP, and YPD) and Industrial (MBA) Media

amino acid/strain	SD	SDP	YPD	MBA
lysine				
IFI 256	1.0[a]	1.0[a]	2.0[a]	2.1[a]
Thr^S mutant	0.8[b]	0.9[a]	2.0[a]	1.5[b]
Met^S mutant	0.7[b]	0.8[a]	1.8[a]	1.5[b]
V2	1.0[a]	5.0[b]	2.0[a]	2.0[a]
Lys^S mutant	**18**[c]	**86**[c]	**38**[b]	**42**[c]
methionine				
IFI 256	0.2[a]	0.2[a]	1.0[a]	0.5[a]
Thr^s mutant	3.0[b]	3.5[b]	6.0[b]	2.9[b]
Met^S mutant	**35**[c]	**33**[c]	**112**[c]	**80**[c]
V2	0.2[a]	0.5[d]	1.0[a]	0.8[d]
Lys^S mutant	0.3[d]	1.0[e]	1.9[d]	1.9[e]
threonine				
IFI 256	2.2[a]	5.8[a]	11.7[a]	3.4[a]
Thr^S mutant	**120**[b]	**214**[b]	**280**[b]	**123**[b]
Met^S mutant	18[c]	15[c]	35[c]	23[c]
V2	2.2[a]	2.0[d]	1.2[d]	3.0[a]
Lys^S mutant	2.5[a]	2.0[d]	1.5[e]	1.7[d]
isoleucine				
IFI 256	0.6[a]	1.2[a]	2.6[a]	17.2[a]
Thr^S mutant	**6.0**[b]	**7.5**[b]	**10**[b]	**78**[b]
Met^S mutant	6.0[b]	5.1[c]	12.5[c]	51[c]
V2	0.6[a]	1.0[a]	2.5[a]	15[d]
Lys^S mutant	0.8[a]	1.5[d]	3.0[d]	19[a]

[a] Data represent mean values with $n = 6$ and standard error of <10%. Mean values of either Lys, Met, Thr, or Ile concentration in the same column followed by the same letter do not differ significantly ($p < 0.01$) according to the Scheffe *S* test (*30*).

amino acids such as aspartokinase, homoserine dehydrogenase, and homoserine kinase (*26*, *27*). The Lys^S mutant displayed alterations of enzymes involved in Lys biosynthesis such as homocitrate synthase, aminoadipate reductase, or saccharopine reductase (*10*, *28*). However, chromosomal rearrangements (*10*, *11*) and the alteration of enzyme kinetics (*26*, *27*) did not result in changes of properties of industrial interest such as growth, yield, viability, or baking abilities.

Growth Rate and Yield in Different Media. Growth rate, μ (h^{-1}), and biomass yield, Y (g/L), were determined for the IFI256 wine strain, V1 and V2 baker's strains, and the mutants in laboratory media (SD, SDP, and YPD) and in molasses (MBA). The yields (28−30 g/L, ww) in YPD and MBA media were similar in the parental IFI256 and the Met^S and Thr^S mutants. The growth rates were also similar in IFI256 and the Thr^S mutant. The Lys^S mutant grew more slowly than its parent and more slowly than the V1 strain (Table 1); the yield was 25 g/L. Alteration of some amino acid concentrations (Table 2) may account for this lower growth rate (*29*), because the Lys^S mutant accumulated Lys (Table 2) but had altered concentrations of Glu and Arg (*10*). However, an inhibitory effect on the growth rate of wild-type strains V1 and V2 caused by Lys, probably due to the accumulation of the toxic intermediate aminoadipate semialdehyde (*29*), was detected. The accumulation of Lys in the Lys^S mutant may also result in toxicity, as reflected in its low growth rate (Table 1) and decreased leavening ability (see below).

Free Amino Acid Pool. The free amino acid pool of IFI256, V1, V2, and the mutants was measured in laboratory and industrial media (Table 2). Molasses lacked His, Met, and Phe, whereas Glu, Leu, Thr, and Lys were at very low concentrations (*21*). Furthermore, when molasses is supplemented with diammonium phosphate, there is probably an inactivation of the general amino acid permease caused by ammonia, so that amino acid uptake is prevented (*21*, *29*). Despite that, the concentration of Met, Thr, Ile, or Lys was very high in the mutants grown in MBA as compared with the parental strains (Table 2). The amino acid pool varies depending on the growth medium, with relatively poor levels of Met being produced when yeasts are grown in minimal medium (SD) with glucose as the carbon source (Table 2). The total amino acid pool also changed with the nitrogen source (SD versus SDP, Table 2). However, the free amino acid pool seemed to depend on the availability of carbon intermediates of the most active metabolic pathways because the three AA^S mutants had high levels of their overproduced amino acid in all media tested (Table 2).

When the protein content of cells cultivated in MBA or YPD media was monitored, this value varied between 46 and 53% dry matter (16−18% of ww). Protein contents of ∼50% have been reported for other strains grown in molasses (*5*, *21*, *24*). The free amino acid pool of IFI256, V1, and V2 strains was measured. This pool represented 4.6 and 4.3% of total amino acids (protein-bound and free pool) present in yeast cells grown in MBA and YPD, respectively (*21*). Data on protein content and on free amino acid pools (and of free Thr, Met, or Lys pools specifically) with regard to total amino acids were also measured in the AA^S mutants grown in MBA and YPD media. Protein content in the mutants was similar to that in the parental strains. The percentages of free amino acid pool and, above all, of the specific amino acids Thr, Met, and Lys were substantially increased in the AA^S mutants as compared to the parental strains (Tables 2 and 3).

Leavening Ability. The leavening capacity of the V1 and V2 strains, and of the Lys^S mutant, was also tested. IFI256 and the Thr^S and Met^S mutants derived from it were not checked because they were not baker's strains. Both V1 and V2 strains produced similar final amounts of liberated CO_2, measured as volume increase. However, the Lys^S mutant possessed the wild-type capacity to increase the volume of the dough and to reach the same maximal value, but both V1 and V2 initiated dough leavening after a 20 min lag and reached maximal volume after 80 min of incubation, whereas the Lys^S mutant did so ∼1 h later.

Baking. The V1 strain and the Thr^S mutant were grown in MBA medium until late stationary phase (∼30 g/L ww). Met^S and Lys^S mutants were grown in YPD (because their growth rate in MBA was low) (Table 1). Their yields were of 30 and 25 g/L (ww), respectively.

Experiments of baking were carried out by supplementing baker's yeasts with any of the mutants because the Lys^S mutant which derived from a baker's strain

Table 3. Estimation of Amino Acid (AA) Content (Lysine, Threonine, Methionine, and Isoleucine) (Milligrams per Kilogram of Flour) Provided by Yeast of Doughs Fermented with 3.5% Baker's Strains or with a Mixture of 3% of These Strains and 0.5% of Amino Acid Overproducing (AA^S) Yeast[a]

	doughs fermented with 3.5% baker's yeast			doughs fermented with 3% baker's yeast plus 0.5% AA^S yeast[b]			
	protein-bound AA	free AA pool	total AA	protein-bound AA	free AA pool	total AA	(Δ)[c]
Lys	425	1.9[a] (0.5%)[d]	426.9	425	7.1[b] (1.7%)[d]	432.1	(1.2%)
Met	75	0.4[a] (0.5%)	75.4	75	8.2[b] (9.8%)	83.2	(9.3%)
Thr	250	2.5[a] (1%)	252.5	250	14.5[b] (5.5%)	264.5	(4.5%)
Ile	275	13.8[a] (5%)	288.8	275	20.5[b] (7%)	295.5	(2%)

[a] Data represent mean values with $n = 6$ and standard error of <10%. Mean values in the same row followed by the same letter do not differ significantly ($p < 0.01$) according to the Scheffe *S* test (*30*). [b] The AA^S strains used were Lys^S for lysine enrichment, Met^S for methionine enrichment and Thr^S for threonine and isoleucine enrichment. [c] Increase in bread of the total concentration of a specific amino acid contributed by the AA^S yeasts. [d] Percentage of free pool with regard to total concentration of a specific amino acid (protein-bound plus pool) in the yeast cells.

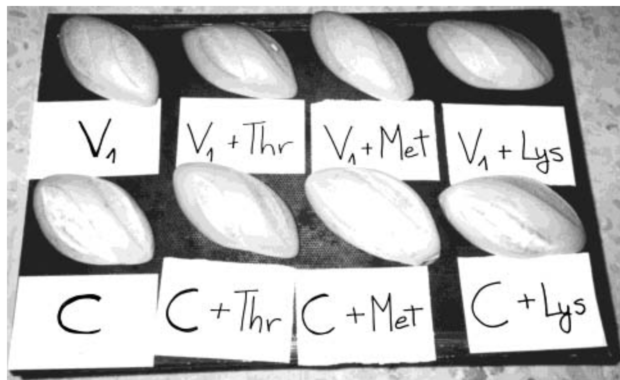

Figure 1. Bread fermented with 3.5% baker's yeast V1 (V1) or commercial baker's yeast (C) or 3% of these yeasts and 0.5% of Thr^S (V1 + Thr or C + Thr), Met^S (V1 + Met or C + Met) or Lys^S yeast mutants (V1 + Lys or C + Lys).

was slower at fermenting doughs than its parental and Thr^S and Met^S mutants derived from a wine yeast. Yeast mixtures were the following: 35 g of yeast of either the parental strain V1 or the commercial baker's yeast (strain C) used as control, or mixtures of 30 g of either V1 or commercial baker's yeast and 5 g of each of the AA^S mutants per kilogram of flour, as for standard industrial protocols (*5*). Each dough was divided into 15 pieces (Figure 1). The weight of each piece varied between 85 and 90 g in all cases.

Yeast biomass is quite a complete additive (3% baker's yeast and 0.5% yeast additive in bakery products) (*5*) that can fortify a diet with different nutritional components as well as proteins (*5*, *7*, *14*, *15*). As an excellent food additive, yeast would contribute polyunsaturated fatty acids, different vitamins, glutathione, and, above all, Lys- and Thr-rich proteins to the diet (*5*, *12*, *13*). Yeast aminograms have in general a fairly high level of Lys and relatively poor levels of Thr and sulfur-containing amino acids (*3*, *4*, *28*). These levels can be increased by using amino acid overproducing mutants. In this study, yeast cell protein content (46−53%), protein-bound amino acids (95.7%), free amino acid pools (4.3%), and a free pool of specific amino acids per gram of yeast cell proteins (0.01 g of Thr, 0.003 g of Met, 0.008 g of Lys, and 0.055 g of Ile) of V2 parental strain grown in MBA medium were measured. The contribution of 3.5% yeast to the amino acid content of bread was then estimated (Table 3). Protein content (46−53%) and protein-bound and free amino acid pools, as well as a free pool of Thr, Met, Lys, and Ile specifically (per gram of yeast cell proteins), were also measured in the AA^S mutants grown in YPD (Met^S and Lys^s mutants) or MBA media (Thr^S mutant), respectively (Table 3).

Table 4. Order of Preference and Marks of Bakery Products Fermented with Different Baker's Yeasts with Regard to Their Organoleptic Properties (Flavor and Taste) or Their Texture[a]

strain order	organoleptic properties	mark	texture	mark
1	V1 + Thr^s mutant	7.94[a]	commercial (C)	8.06[a]
2	V1	7.01[b]	V1	7.25[b]
3	C + Met^S mutant	6.60[b]	V1 + Thr^S mutant	6.90[b]
4	C + Thr^S mutant	6.55[b]	C + Met^S mutant	6.38[c]
5	C + Lys^S mutant	6.40[b]	C + Thr^S mutant	6.15[c]
6	V1 + Met^S mutant	6.24[c]	C + Lys^S mutant	5.98[d]
7	V1 + Lys^S mutant	5.92[c]	V1 + Met^S mutant	5.90[d]
8	commercial (C)	5.28[d]	V1 + Lys^S mutant	5.88[d]

[a] Bakery products were tried by 30−40 nonexperts following procedures already described for quality evaluation (*24*, *25*). Data represent mean values with $n = 6$ and standard error of <10%. Mean values of quality evaluation (organoleptic properties or texture) followed by the same letter do not differ significantly ($p < 0.01$) according to the Scheffe *S* test (*30*).

The contribution of 3.5% yeast (3% of the parental V2 plus 0.5% of either of the AA^S mutants) to the amino acid content of bread was also estimated (Table 3). Whereas the mutants derived from strain IFI256 had up to 40 times more Thr or 160 times more Met than the wild type (Table 2), and they behaved just like their parental strain (Table 1), the Lys^S mutant was able to increase the free pool of Lys only 20 times (Table 2), as it had been already reported (*10*, *21*). This could be due to the toxic effects of the intermediate amino adipate, as previously discussed (*28*, *29*). As a consequence, the increase in total Lys (protein-bound and free pool) apported by the mixture of baker's yeast and Lys^S mutant, compared to the baker's strain alone, is only of 9.5%, whereas that of Met is 46.5% and that of Thr, 30% (Table 3). On the other hand, Met and Thr biosynthetic pathways are partly shared (*29*). For this reason, the Thr^S mutant also overproduces Met, and the Met^S mutant overproduces Thr (Table 2), as already reported (*8*). These two amino acids are essential for the aroma and organoleptic properties of bread (*5*). Ile is not an essential dietary amino acid, but it is very important in the aroma of and for the organoleptic properties of bread (*5*). Isoleucine derived from the Thr/Met pathway (*29*) so that both Thr^S and Met^S mutants possessed increased levels of Ile (Table 2).

Once the pieces were baked, they were tried by 30−40 nonexperts, and their taste, flavor, and texture were evaluated (three separate scores, one for texture, one for flavor, and one for taste; data given in Table 4 are texture score and average of flavor and taste scores) following procedures already established (*24*, *25*, *30*). The mixture of V1 plus the Thr^S mutant (which also

overproduced Met and Ile, Table 2), was chosen as the best with regard to the organoleptic properties (taste and flavor), on a scale from 1 to 10 (Table 4), statistically different from the other strains (*24*, *25*, *30*). The commercial strain alone got the lowest mark (Table 4). This commercial strain got the highest mark with regard to the texture (browning, volume and density, elasticity, color, consistency, suitability for slicing, and regularity and size of the alveoli), followed by V1 and then by either C or V1 strain, plus the AA^S mutants (Table 4). From the latter, the mixture of V1 plus the Thr^S mutant was best evaluated with regard to texture. When the average of the three combined scores was taken into account, bread fermented with V1 plus the Thr^S mutant and therefore enriched in Thr, Met, and Ile was chosen as the best. These were sponteaneus mutants isolated in continuous culture, so they can be immediately used in bakery or as food and feed additives.

Conclusions. This work reports on the characterization of baker's and AA^S yeast mutants, including the production of bread fermented with them. These mutants grow with similar or even higher growth rates in MBA media (as under industrial conditions) and have similar biomass yields as the wild type. Mixtures of baker's and AA^S strains ferment doughs the same as the wild type. The bakery products obtained with these mixtures were enriched in Lys, Met, Ile, and/or Thr and, in most cases, their organoleptic properties were also improved. Due to the spontaneous character of their improved features, the mutants, which are genetically very stable, can be immediately used for industrial processes.

ABBREVIATIONS USED

YPD, complete (yeast/peptone/dextrose) medium; SD, minimal (sulfate/dextrose) medium, ammonia as a nitrogen source; SDP, minimal (sulfate/dextrose/proline) medium, proline as a nitrogen source; MBA, molasses (molasses/biotine/ammonia) medium supplemented with biotin and diammonium phosphate; μ, growth rate (h^{-1}); *Y*, biomass yield (g/L); AA^S mutants, amino acid overproducing mutants; Lys^S mutant, lysine overproducing mutant; Met^S mutant, methionine overproducing mutant; Thr^S mutant, threonine overproducing mutant; Ile^S mutant, isoleucine overproducing mutant.

ACKNOWLEDGMENT

We thank Emilia Hernández and Burns Philp Food Co. (Villarrubia, Córdoba, Spain) for technical assistance and Diego Benítez Moreno and the Nueva Florida Co. (Alcalá de Guadaira, Sevilla, Spain) for advice and help in the baking experiments.

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Received for review September 14, 2000. Revised manuscript received February 1, 2001. Accepted February 1, 2001. This research was supported by CICYT Projects ALI96-0938, FD97-0820, PTR940022, and PTR95-0198 and by Junta de Andalucía PAI CVI-107.

JF001130U