Appl Microbiol Biotechnol (2001) 55:296–300
DOI 10.1007/s002530000506

ORIGINAL PAPER

A. Eden · L. Van Nedervelde · M. Drukker
N. Benvenisty · A. Debourg

Involvement of branched-chain amino acid aminotransferases in the production of fusel alcohols during fermentation in yeast

Received: 24 March 2000 / Received revision: 23 July 2000 / Accepted: 1 September 2000 / Published online: 4 January 2001

Abstract Organoleptic compounds produced by yeast during the fermentation of wort have a great impact on beer smell and taste. Among them, fusel alcohols are the major abundant volatile compounds. The availability of *Saccharomyces cerevisiae* mutants in which the genes coding for the two branched-chain amino acid aminotransferases have been deleted offers the possibility of further defining the role of these enzymes in the formation of higher alcohols. Comparing the production profiles of different strains, it is clear that they are not all influenced in the same way by branched-chain amino acid aminotransferase mutations. First of all, as propanol is synthesised from α-ketobutyrate, the first metabolic intermediate in the anabolic pathway of isoleucine, neither the *eca39* nor *eca40* mutations have any effect on the production of this higher alcohol. On the other hand, it can be concluded that the *eca40* mutation has a drastic effect on the production of isobutanol. To a certain extent, the same conclusion can be made for the production of active amyl alcohol and isoamyl alcohol, although the results suggest that another route could lead to the formation of these two higher alcohols.

Introduction

Beer organoleptic characteristics depend on different compounds that originate from raw materials or are produced during fermentation. Nevertheless, the organoleptic compounds produced by yeast during the fermentation of wort have the greatest impact on beer smell and taste.

One of the main features of fermentation and beer flavour maturation is the adjustment of the concentration of all volatile compounds. Among them fusel or higher alcohols are the major abundant organoleptic compounds in beer, contributing along with the related acetate esters to the overall flavour of beer.

The isoleucine–leucine–valine (ILV) pathway has been shown to contribute to the formation of higher alcohols. The higher alcohols are formed during fermentation from keto acid produced either catabolically, involving degradation of an amino acid via the so-called Ehrlich pathway (Ehrlich 1904), or anabolically via the biosynthetic route from the carbon source (Hammond 1993).

Both anabolic and catabolic pathways have been shown to be important in brewery fermentation. Although some work has been done to evaluate the relative contribution of the catabolic and anabolic pathways during fermentation (Oshita et al. 1995), the role of the different enzymes catalysing the different steps has never been studied in detail, mainly due to a lack of mutants in some of the key enzymes.

Recent studies on the catabolism of the branched-chain amino acids in *Saccharomyces cerevisiae* have shown that the first step is transamination. Moreover, studies on the catabolism of leucine and valine by *S. cerevisiae* have shown that there are differences in the enzymes involved in the decarboxylation of the keto acids to the corresponding higher alcohols (Dickinson et al. 1997, 1998).

In order to clearly identify the contribution of the anabolic pathway in the production of higher alcohols during fermentation of a rich medium such as wort, it is necessary to avoid any transamination of amino acids present in the growth medium. Two genes, *ECA39* and *ECA40*, have been identified as coding for mitochondrial and cytosolic branched-chain amino acid aminotransferases, respectively (Eden et al. 1996). The availability of *S. cerevisiae* mutants in which the genes coding for these two BCAA aminotransferases have been deleted

A. Eden and L. Van Nedervelde contributed equally to this work.

A. Eden · M. Drukker · N. Benvenisty
The Hebrew University of Jerusalem,
Institute of Life Sciences, Department of Genetics,
Givat-Ram, Jerusalem 91904, Israel

L. Van Nedervelde · A. Debourg (✉)
Haute Ecole Lucia de Brouckère, Institut Meurice,
Department of Brewing Sciences and Fermentation Technology,
Avenue E Gryson 1, 1070 Brussels, Belgium
e-mail: adebourg@meurice.heldb.be
Tel.: +32-2-526 73 51, Fax: +32-2-524 38 59

Table 1 Strains of *Saccharomyces cerevisiae* used in this work

Strains	Genotype
MD101	*trp1Δ63–LEU2–URA3–his3Δ200*
MD22	*trp1Δ63–LEU2-eca39Δ::URA3–his3Δ200*
MD133	*trp1Δ63–LEU2–URA3–eca40Δ::HIS3*
MD124	*trp1Δ63–LEU2–eca39Δ::URA3–eca40Δ::HIS3*
MD138	*ilv2Δ::TRP1–LEU2–eca39Δ::URA3–eca40Δ::HIS3*
AE55	*trp1Δ63–leu2Δ1-URA3–his3Δ200*
AE16	*trp1Δ63–leu2Δ1–eca39Δ::URA3–his3Δ200*
AE25	*trp1Δ63–leu2Δ1–URA3–eca40Δ::HIS3*
AE89	*trp1Δ63–leu2Δ1–eca39Δ::URA3–eca40Δ::HIS3*

offers the possibility of further defining the role of these enzymes in the formation of higher alcohols.

The aim of this study is to evaluate the influence of both BCAA aminotransferase mutations on the production of the different higher alcohols derived from the ILV pathway. Therefore the production of these compounds will be monitored during growth of wild type, *eca39*, *eca40* and *eca39-eca40* mutant strains on YPD medium.

Materials and methods

Yeast strains and media

The strains of *S. cerevisiae* used in this study are listed in Table 1. Fermentation experiments were carried out on YPD medium with the following composition: 40 g/l glucose, 20 g/l bactopeptone and 10 g/l yeast extract. The initial pH of the fermentation medium was 5.5.

Construction of the yeast strains AE55, AE16, AE25 and AE89 was previously described (Eden et al. 1996; Schuldiner et al. 1996; Eden and Benvenisty 1998). MD101, MD22, MD133, MD124 and MD138 yeast strains were constructed by replacing the mutant *leu2Δ1* gene by a wild type *LEU2* gene using the by one-step replacement methodology (Rothstein 1991).

Fermentation experiments

The strains were precultured in liquid YPD medium at 30°C to provide sufficient biomass to inoculate at a rate of 1.10^6 cell/ml the fermentation flasks containing 500 ml of YPD. Flasks were incubated under shaking at 30°C for the total duration of the experiment.

At regular intervals, 10 ml samples were taken to allow analyses to be carried out as described in the next section. The results are means of three reproductible experiments performed independently.

Chromatographic analysis of higher alcohols

Samples from fermentation were centrifuged at 4,000 *g* to eliminate yeast cells. The supernatant was collected and a 2-ml sample was placed in a septum-sealed vial for gas chromatography analysis.

Fusel alcohols were separated and quantified by headspace gas chromatography using a flame ionisation detector (FID). Analyses were performed on a Perkin Elmer 8500 gas chromatograph equipped with an HS-40 headspace sampler. The chromatography column was a Carbowax 400 (50 m×0.32 mm i.d.) (Chrompack). The carrier gas was He (200 kPa) and the FID detector gases were H_2 (150 kPa) and air (120 kPa). Headspace equilibration conditions were 20 min at 60°C. The chromatography conditions were: injection temperature 110°C; transfer temperature 130°C; oven temperature 55°C isothermal; analysis time 15 min and detection temperature FID at 250°C.

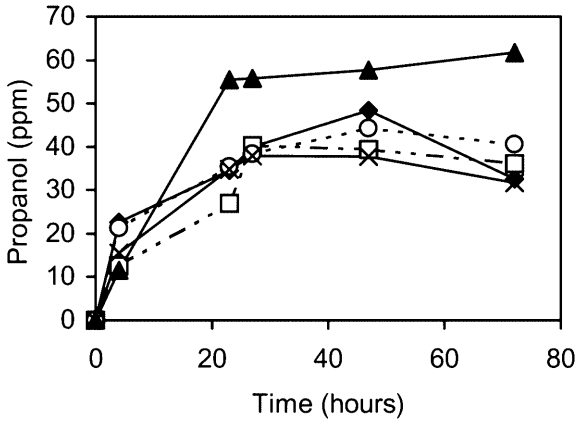

Fig. 1 Evolution of the propanol concentration during growth on YPD medium of different *Saccharomyces cerevisiae* strains: ♦ MD101 *ECA39-ECA40-LEU2*; □ MD22 *eca39-ECA40-LEU2*; ○ MD133 *ECA39-eca40-LEU2*; × MD124 *eca39-eca40-LEU2*; ▲ MD138 *eca39-eca40-LEU2-ilv2*

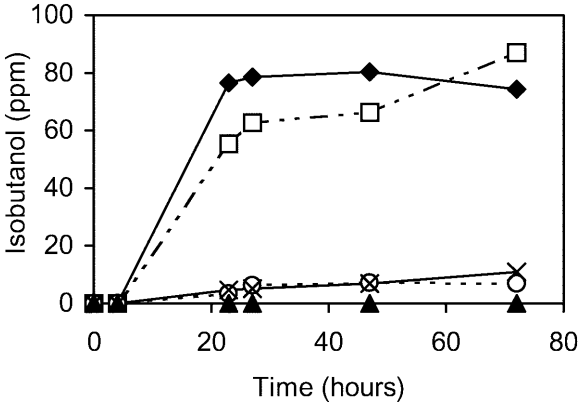

Fig. 2 Evolution of the isobutanol concentration during growth on YPD medium of different *Saccharomyces cerevisiae* strains: ♦ MD101 *ECA39-ECA40-LEU2*; □ MD22 *eca39-ECA40-LEU2*; ○ MD133 *ECA39-eca40-LEU2*; × MD124 *eca39-eca40-LEU2*; ▲ MD138 *eca39-eca40-LEU2-ilv2*

Results

Influence of *eca39* and *eca40* mutations on the production of higher alcohols

To study the involvement of *ECA39* and *ECA40* in the catabolism of the BCCA, we previously disrupted the genes in *S. cerevisiae* (Eden et al. 1996; Schuldiner et al. 1996; Eden and Benvenisty 1998). These mutant strains were generated on the *leu2Δ1* background. Isogenic strains to all of these mutants where the *LEU2* gene replaced the mutant one were also created.

The evolution of the concentration of the major higher alcohols derived from the ILV pathway during growth of the different strains is illustrated in Figs. 1, 2, 3 and 4.

Comparing the concentration evolution profiles for the different higher alcohols, it can immediately be seen that they are not all influenced in the same way by the

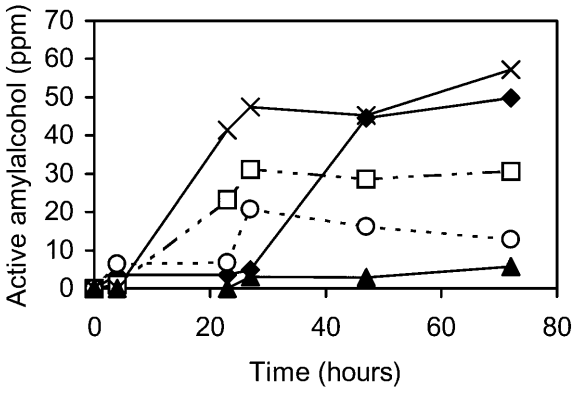

Fig. 3 Evolution of the active amyl alcohol concentration during growth on YPD medium of different *Saccharomyces cerevisiae* strains: ♦ MD101 *ECA39-ECA40-LEU2*; □ MD22 *eca39-ECA40-LEU2*; ○ MD133 *ECA39-eca40-LEU2*; ✕ MD124 *eca39-eca40-LEU2*; ▲ MD138 *eca39-eca40-LEU2-ilv2*

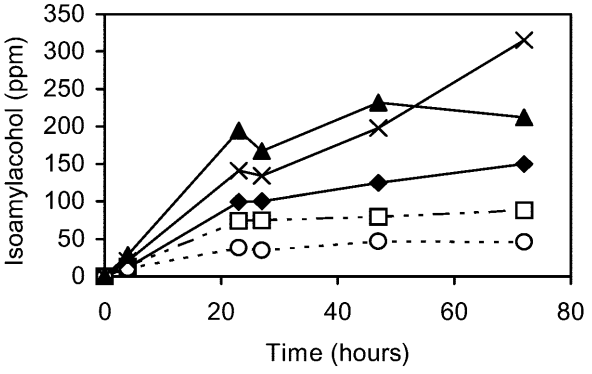

Fig. 4 Evolution of the isoamyl alcohol concentration during growth on YPD medium of different *Saccharomyces cerevisiae* strains: ♦ MD101 *ECA39-ECA40-LEU2*; □ MD22 *eca39-ECA40-LEU2*; ○ MD133 *ECA39-eca40-LEU2*; ✕ MD124 *eca39-eca40-LEU2*; ▲ MD138 *eca39-eca40-LEU2-ilv2*

BCAA aminotransferase mutations. As expected, the production of propanol, illustrated in Fig. 1, is not significantly affected by any of the BCAA aminotransferase mutations, the precursor being α-ketoisobutyrate, the first metabolic intermediate in the synthesis of isoleucine.

On the other hand, the production of the three other higher alcohols investigated in this work is drastically modified by the *eca39* and/or *eca40* mutations. As the *eca39* has no significant effect on the production of isobutanol, the mutation *eca40* leads to an important decrease in the isobutanol concentration as illustrated in Fig. 2. The production patterns of active amyl alcohol and isoamyl alcohol by the different strains are identical, Figs. 3 and 4 indicating that, although *eca39* or *eca40* mutation alone reduces the level of these higher alcohols, an unexpectedly higher concentration is observed in the double *eca39–eca40* mutant.

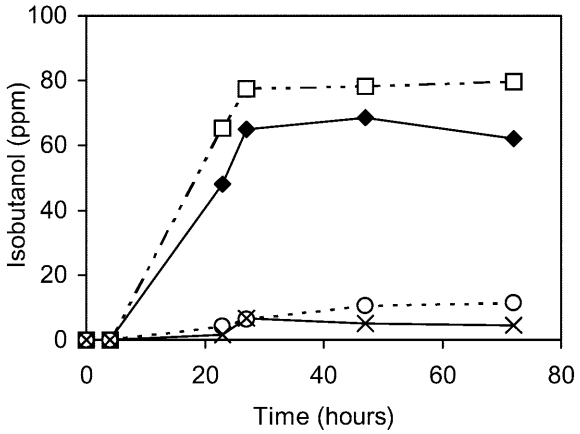

Fig. 5 Evolution of the isobutanol concentration during growth on YPD medium of different *Saccharomyces cerevisiae* strains: ♦ AE55 *ECA39-ECA40-leu2*; □ AE16 *eca39-ECA40-leu2*; ○ AE25 *ECA39-eca40-leu2*; ✕ AE89 *eca39-eca40-leu2*

Influence of a mutation in the isoleucine–valine biosynthetic pathway

In order to investigate the additional effect of a mutation in the isoleucine–valine biosynthetic pathway, the higher alcohol production by strain MD138 was also followed.

This *ilv2* mutant has no acetolactate synthase (ILV2) activity, the first common enzyme in the isoleucine–valine parallel biosynthetic route. As a consequence of this mutation, the accumulation of one of the substrates of the *ilv2* enzyme, α-ketobutyrate, leads to an important increase in the concentration of propanol as seen in Fig. 1. As observed in Fig. 2, the total blocking of the isoleucine–valine biosynthetic pathway in addition to *eca39* and *eca40* mutations in mutant MD138 completely eliminated the production of isobutanol. On the other hand, the effect of the *ilv2* mutation on the production of active amyl alcohol and isoamyl alcohol was different. Indeed, comparing active amyl alcohol concentrations obtained with MD124 and MD138 (see Fig. 3), it is evident that the *ilv2* mutation led to a drastic decrease in the production of this higher alcohol. On the other hand, although the final concentration of isoamyl alcohol obtained with the *ilv2* mutant was somehow reduced, it was still higher than that of the reference strain MD101, as illustrated in Fig. 4.

Influence of a mutation in the leucine biosynthetic pathway

Since leucine is synthesised from the keto acid isocaproate, which is one immediate precursor of valine, the additional effect of a *leu2* mutation in the leucine biosynthetic pathway was also investigated. Propanol being produced from α-ketobutyrate, the first intermediate in the isoleucine biosynthetic pathway, the *leu2* mutation has no effect on the production of this higher alcohol. As illustrated in Fig. 5, the production of isobutanol by the

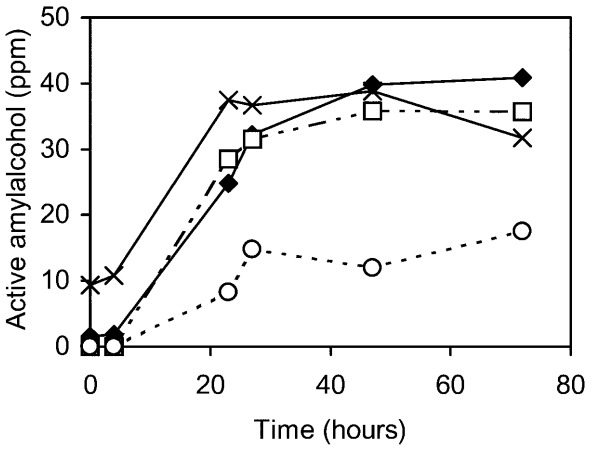

Fig. 6 Evolution of the active amyl alcohol concentration during growth on YPD medium of different *Saccharomyces cerevisiae* strains: ♦ AE55 *ECA39-ECA40-leu2*; □ AE16 *eca39-ECA40-leu2*; ○ AE25 *ECA39-eca40-leu2*; ✕ AE89 *eca39-eca40-leu2*

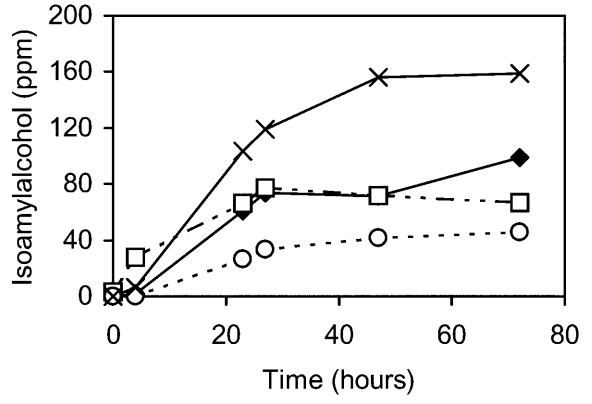

Fig. 7 Evolution of the isoamyl alcohol concentration during growth on YPD medium of different *Saccharomyces cerevisiae* strains: ♦ AE55 *ECA39-ECA40-leu2*; □ AE16 *eca39-ECA40-leu2*; ○ AE25 *ECA39-eca40-leu2*; ✕ AE89 *eca39-eca40-leu2*

different strains bearing the *leu2* mutation is only significantly affected by the *eca40* mutation. As far as the production of active amyl alcohol is concerned, only the *eca40* mutation led to a decreased concentration as shown in Fig. 6. Moreover, isoamyl alcohol is still produced in strain AE89 having the double BCAA aminotransferase *eca39* and *eca40* mutations and the *leu2* mutation, as illustrated in Fig. 7.

Discussion

Analysis of the production of higher alcohols by the different yeast strains suggested different roles of the BCAA aminotransferase activities in the synthetic pathways of these compounds. First of all, as propanol is synthesised from α-ketoisobutyrate, the first metabolic intermediate in the anabolic pathway of isoleucine, neither *eca39* nor *eca40* mutations have any effect on the production of this higher alcohol. Nevertheless, the *ilv2* mutation in strain MD138 leads to an accumulation of α-ketoisobutyrate with, as a consequence, an increase in propanol concentration.

On the other hand, the production of the three other higher alcohols, isobutanol, active amyl alcohol and isoamyl alcohol, was affected by the *eca39* and/or *eca40* mutations. The two genes *ECA39* and *ECA40* have been identified as coding for mitochondrial and cytosolic BCAA aminotranferases, respectively (Eden et al. 1996). As all other ILV enzymes have been localised in the mitochondria (Ryan and Kohlan 1974), it can be assumed that the ECA39 activity is more involved in ILV amino acids biosynthesis whereas ECA40 activity could be a catabolic enzyme. From our first series of fermentations, it can be concluded that the *eca40* mutation has a drastic effect on the production of isobutanol. Under our fermentation conditions, the ECA40 enzymatic activity is responsible for the transamination of valine to α-ketoisovalerate, precursor of isobutanol (Dickinson et al. 1998). The small amount of isobutanol produced by strains MD124 and MD133 derives from the anabolic flux in the isoleucine and valine pathway since *ilv2* mutation in strain MD138 leads to a total absence of isobutanol. To a certain extent, the same conclusion can be made for the production of active amyl alcohol. Indeed, the *eca40* mutation in strain MD133 leads to an important decrease of active amyl alcohol concentration in the fermentation medium. Nevertheless, its production pattern by the *eca39–eca40* double mutant is different from that of isobutanol, having a concentration identical to the wild type. This can be explained by the fact that, unlike α-ketoisovalerate, the precursor of isobutanol, which is also an intermediate metabolite of the leucine pathway, the α-keto-β-methylvalerate has no other known metabolic route than the conversion into the corresponding higher alcohol. Therefore, in the double BCAA aminotransferase mutant strain MD124, the anabolic flux to isoleucine leads to an accumulation of the α-keto-β-methylvalerate and thus to the production of the active amyl alcohol. This is further confirmed by the production profile observed for strain MD138, the *ilv2* additional mutation leading to a very low concentration of active amyl alcohol. Moreover, its presence in MD138 strain fermentation medium, although at a low concentration, suggests that another route could lead to the formation of small amounts of active amyl alcohol.

A similar conclusion can be made concerning the role of the BCAA aminotransferases in the production of isoamyl alcohol. Indeed, in strain AE89, lacking both BCAA aminotransferases activities and LEU2 activity, neither the anabolic nor the catabolic pathway could explain the production of the α-ketoisocaproate, known precursor of the isoamyl alcohol. This unexpected result indicates that one other route for the production of isoamyl and maybe active amyl alcohols exists in yeast.

These findings are not in opposition with previous work that proposed potential metabolic routes for the formation of higher alcohols. It is, as far as we are

aware, the first time BCAA aminotransferase mutants have been used to block the transamination step that allows us to identify other routes. Indeed, if yeast cells are cultured in a medium containing leucine or valine, yeast could convert the amino acids into the corresponding keto acids and it becomes impossible to evaluate other metabolic pathways.

References

Dickinson JR, Lanterman MM, Danner DJ, Pearson BM, Sanz P, Harrison SJ, Hewlins M (1997) A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J Biol Chem 272:26871–26878

Dickinson JR, Harrison SJ, Hewlins JE (1998) An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae.* J Biol Chem 273:25751–25756

Eden A, Benevisty N (1998) Characterization of a branched-chain amino acid aminotransferase from *Schizosaccharomyces pombe*. Yeast 14:189–194

Eden A, Simchen G, Benvenisty N (1996) Two yeast homologs of *ECA39*, a target for c-Myc regulation, code for cytosolic and mitochondrial branched-chain amino acid aminotransferases. J Biol Chem 271:20242–20245

Ehrlich F (1904) Uber das natürliche isomere des leucins. Ber Dtsch Chem Ges 37:1809–1840

Hammond JRM (1993) Buwei's yeasts In: Rose AH, Harrison JS (eds) The yeasts, vol 5: yeast technology. Academic Press, London, pp 8–67

Oshita K, Kubota M, Uchida M, Ono M (1995) Clarification of the relationship between fusel alcohol formation and amino acid assimilation by brewing yeast using ^{13}C-labeled amino-acid. Proc 25th EBC Congr 387–394

Rothstein R (1991) Targeting, disruption, replacement and allele rescue: integrative DNA transformation in yeast. Methods Enzymol 194:281–301

Ryan ED, Kohlan GB (1974) Subcellular localization of isoleucine–valine biosynthetic enzymes in yeast. J Bacteriol 120: 631–637

Schuldiner O, Eden A, Ben-Yosef T, Yanuka O, Simchen G, Benvenisty N (1996) *ECA39*, a conserved gene regulated by c-Myc in mice, is involved in G1/S cell cycle regulation in yeast. Proc Natl Acad Sci USA 93:7143–7148