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Effect of threonine, cystathionine, and the branched-chain amino acids on the metabolism of *Zygosaccharomyces rouxii*☆

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Abstract

Zygosaccharomyces rouxii is an important yeast in the formation of flavor in soy sauce. In this study, we investigated the separate effects of exogenous threonine, cystathionine, and the branched-chain amino acids on the metabolism of *Z. rouxii*. The addition of these amino acids had significant effects on both *Z. rouxii* growth and glycerol and higher alcohol production. It also seemed that *Z. rouxii* displayed the Crabtree effect, which was independent of the added amino acids. Furthermore, we investigated the regulation of the metabolism of α-ketobutyrate, which is a key-intermediate in *Z. rouxii* amino acid metabolism. Threonine and cystathionine were introduced separately to stimulate the formation rate of α-ketobutyrate and the branched-chain amino acids to inhibit its conversion rate. Enzyme activities showed that these amino acids had a significant effect on the formation and conversion rate of α-ketobutyrate but that the α-ketobutyrate pool size in *Z. rouxii* was in balance all the time. The latter was confirmed by the absence of α-ketobutyrate accumulation.

Keywords: *Zygosaccharomyces rouxii*; Metabolism; Amino acids; Alpha-ketobutyrate; Enzyme activities; Higher alcohols; Glycerol; Crabtree-effect; Ehrlich pathway

1. Introduction

Zygosaccharomyces rouxii, a salt-tolerant yeast, is important for the flavor development in soy sauce. In soy sauce, *Z. rouxii* produces ethanol, higher alcohols, and 4-hydroxy-2 (or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF). HEMF is considered as one of the important compounds for soy sauce flavor [1]. Much research has been done to understand the biosynthesis of these flavors by *Z. rouxii* [2–4]. Nevertheless, there is still a lack of knowledge about the metabolism of *Z. rouxii*. For this reason, we are studying the metabolism of *Z. rouxii* to gain more insight into it. To facilitate this, we assumed in this work, unless otherwise stated, that *Z. rouxii* and *Saccharomyces cerevisiae* share common metabolic pathways. The found differences and similarities between the metabolism of *Z. rouxii* and *S. cerevisiae* are amply discussed.

In our research, we are especially interested in the regulation of the α-ketobutyrate metabolism in *Z. rouxii* because α-ketobutyrate is a key intermediate in the amino acid metabolism of *S. cerevisiae* (Fig. 1). In *S. cerevisiae*, some α-ketobutyrate is needed for the synthesis of isoleucine but intracellularly accumulated α-ketobutyrate might inhibit the growth as shown in *Salmonella typhimurium* [5,6] and *Corynebacterium glutamicum* [7]. In this work, we studied the regulation of α-ketobutyrate metabolism in *Z. rouxii* at the enzyme level. We did this by increasing the formation rate of α-ketobutyrate or decreasing its conversion rate.

In *S. cerevisiae*, α-ketobutyrate arises in the biosynthesis of at least two amino acids, namely isoleucine and cysteine, as can be seen in Fig. 1 [8]. In the isoleucine biosynthesis, the first enzyme is threonine deaminase (L-threonine hydrolyase [deaminating]; EC 4.2.1.16), which catalyzes the deamination of threonine to α-ketobutyrate and ammonia.

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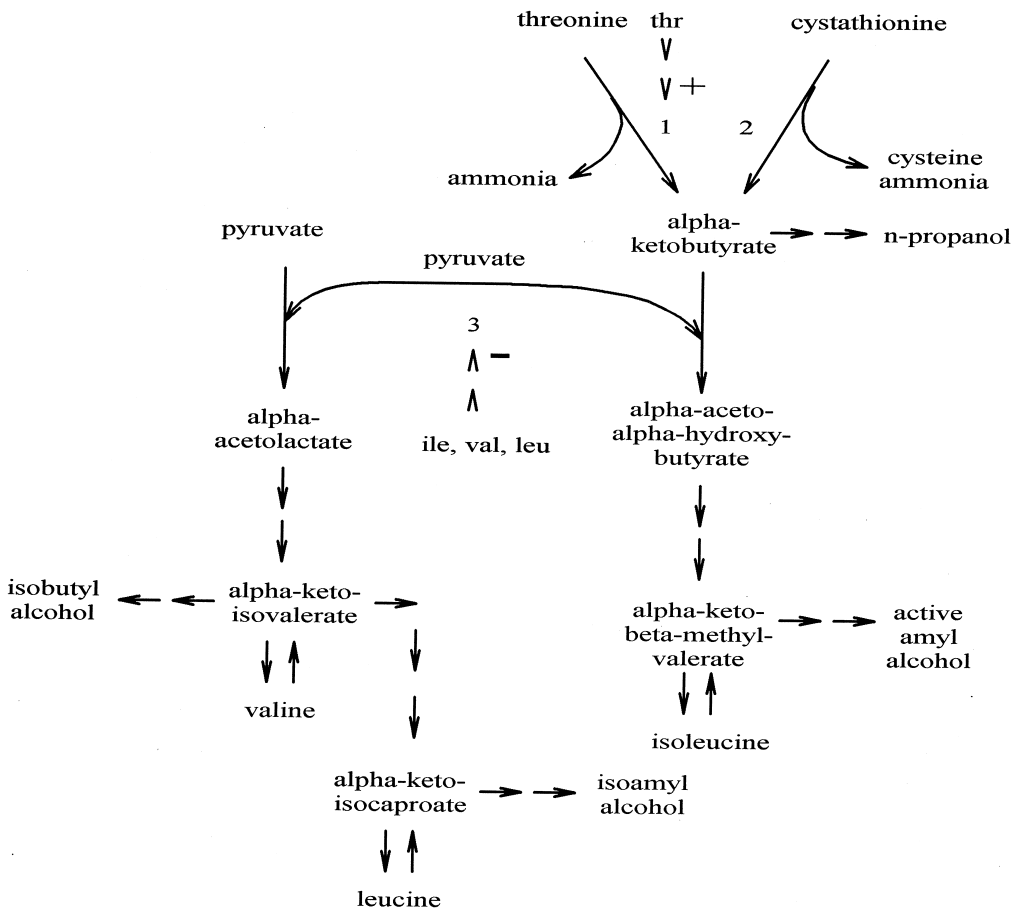

Fig. 1. Biosynthetic pathways of isoleucine, valine, leucine, and cysteine in *S. cerevisiae*. Enzymes: 1, threonine deaminase; 2, cystathionine γ-lyase; 3, acetohydroxy acid synthase. Regulation at the enzyme level (≫): + thr, induction by threonine; − ile, val, leu, repression by isoleucine, valine, and leucine together.

In the transsulfuration pathway for synthesizing cysteine, cystathionine γ-lyase (L-cystathionine cysteine-lyase [deaminating]; EC 4.4.1.1) catalyzes the deamination of cystathionine to cysteine, α-ketobutyrate, and ammonia. The enzyme acetohydroxy acid synthase (acetolactate pyruvate-lyase [carboxylase]; EC 4.1.3.18) catalyzes the flow of α-ketobutyrate and pyruvate towards isoleucine by forming α-aceto-alpha-hydroxybutyrate. The same enzyme also catalyzes the flow of two molecules pyruvate towards valine and leucine by forming alpha-acetolactate. In Fig. 1, the formation of the higher alcohols *n*-propanol, isobutyl alcohol, active amyl alcohol, and isoamyl alcohol is also shown [9]. These higher alcohols are derived either from the deamination or transamination of extracellular amino acids (Ehrlich pathway) or directly from amino acid biosynthetic pathways. In the Ehrlich pathway, the uptake and transamination of isoleucine, valine, and leucine results in the formation of α-keto-β-methylvalerate, α-keto-isovalerate and α-keto-isocaproate, respectively, which are converted into active amyl alcohol, isobutyl alcohol, and isoamyl alcohol, respectively. Recently, it is suggested that the Ehrlich pathway is not the only pathway involved in the catabolism of the branched-chain amino acids (isoleucine, valine, and leucine) [10,11].

A way to increase the formation rate of α-ketobutyrate is by increasing the specific activity of threonine deaminase and/or cystathionine γ-lyase and to decrease the conversion rate of α-ketobutyrate by decreasing the specific activity of acetohydroxy acid synthase (Fig. 1). The specific activity of threonine deaminase in *S. cerevisiae* can be increased by threonine, which induces the synthesis of threonine deaminase [12]. Whether cystathionine increases the specific activity of cystathionine γ-lyase is not known, because a lack of knowledge about the regulation of cysteine biosynthesis exists in *S. cerevisiae* [8]. On the other hand, it is known that the specific activity of acetohydroxy acid synthase in *S. cerevisiae* can be decreased by the branched-chain amino acids, all three of which are necessary to repress the synthesis of acetohydroxy acid synthase [13,14].

In the research presented here, the separate effects of threonine, cystathionine, and the branched-chain amino acids on the metabolism of *Z. rouxii* were studied. For this, the consumption of substrates and the growth and production of ethanol, glycerol, and higher alcohols were monitored. Ad-

ditionally, to study the regulation of α-ketobutyrate metabolism, the effect of these amino acids on the specific activities of threonine deaminase, cystathionine γ-lyase, and acetohydroxy acid synthase in *Z. rouxii* and the accumulation of α-ketobutyrate by *Z. rouxii* were determined.

2. Materials and methods

2.1. Yeast strain

Z. rouxii CBS 4021 (Centraalbureau voor Schimmelcultures, Delft, The Netherlands) was used in all experiments. The cells were maintained on a mixture of glycerol and skimmed milk at −80°C.

2.2. Inoculum cultures

Inoculum cultures were made in 300-ml Erlenmeyer flasks, containing 100 ml of GPY medium, on a rotary shaker at 28°C and 200 rev./min. GPY medium has the following composition per liter of demineralized water: 40 g of glucose.$1H_2O$, 5 g of pepton, and 5 g of yeast extract. The components were separately autoclaved at 120°C for 20 min. The cells were used for inoculation of the bioreactor when they were in the exponential growth phase (between 20 and 40 h cultivation).

2.3. Bioreactor cultures

Batch cultures were carried out in a bench–scale autoclavable bioreactor with a maximum volume of 2 l. The height and the diameter of the bioreactor were 0.20 and 0.12 m, respectively. The bioreactor was stirred by using a six-blade Rushton turbine stirrer with a diameter of 45 mm. The exponentially growing cells from the inoculum culture were inoculated (about 10% [v/v]) in a defined medium with the following composition per liter demineralized water: 22 g of glucose.$1H_2O$, 7.65 g of NH_4Cl, 2.81 g of KH_2PO_4, 0.59 g of $MgSO_4 \cdot 7H_2O$, 10 ml of trace metal solution, and 2 ml of vitamin solution. The trace metal solution contained per liter demineralized water: 5.5 g of $CaCl_2 \cdot 2H_2O$, 3.75 g of $FeSO_4 \cdot 7H_2O$, 1.4 g of $MnSO_4 \cdot 1H_2O$, 1.35 g of $ZnSO_4 \cdot 7H_2O$, 0.4 g of $CuSO_4 \cdot 5H_2O$, 0.82 g of $CoCl_2 \cdot 6H_2O$, 0.29 g of $Na_2MoO_4 \cdot 2H_2O$, 0.4 g of H_3BO_3, 0.25 g of KI, and 33.21 g of $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$. The pH of this solution was adjusted to 4.0 with 4 M NaOH. The composition of the vitamin solution was per liter demineralized water: 0.05 g of D-biotin, 5.00 g of thiamine hydrochloride, 47 g of m-inositol, 1.2 g of pyridoxine, and 23 g of hemicalcium pantothenate. Depending on the batch, the following amino acids were added: (1) no additions; (2) L-threonine; (3) L-cystathionine; and (4) L-isoleucine, L-valine, and L-leucine. The initial concentration of each amino acid in the bioreactor was 5 mM. This concentration was chosen because 5 mM of each of the branched-chain amino acids was necessary for the repression of acetohydroxy acid synthase in *S. cerevisiae* [14]. The glucose, salts, and trace metals were separately autoclaved at 120°C for 20 min. The vitamins and amino acids were filter-sterilized (0.2 μm filters).

During the batch cultures, a Bio Controller (Applikon) controlled the cultivations and a Bioexpert (Applikon) acquired the on-line data, which were the temperature, pH, oxygen tension in the broth, foam level, stirrer speed, and concentration of oxygen and carbon dioxide in the outgoing air. The temperature was controlled at 28°C and the pH at 4.5 by automatic addition of 1 M HCl or 1 M NaOH. The cells were aerobically grown with an air flow rate of 0.8 l/min. The oxygen tension in the broth was kept above 30% of air saturation by controlling the stirrer speed. The stirrer speed was at least 250 rev./min. The concentration of oxygen and carbon dioxide in the outgoing air was measured on-line by a Servomex 1400 O_2/CO_2 Analyser. The concentration of oxygen and carbon in the ingoing air was measured with this analyzer before the cultivation started. From these measurements, respiratory quotients were calculated. The foam level was controlled by automatically adding a diluted (50×) Antifoam B Silicone emulsion (J.T. Baker). During the cultivation, samples for off-line analyses were taken from the bioreactor. A part of a fresh sample was immediately used for measuring optical density and cell number or stored at −80°C. The other part was centrifuged (Labofuge 1, Heraeus Christ) at 3700 rev./min and 5°C for 10 min. The supernatant was used for analysis of extracellular substrates (glucose, ammonia, and amino acids) and metabolites (α-ketobutyrate, ethanol, glycerol, and higher alcohols) and the remaining pellet was used for determining the biomass dry weight. The cultivations were stopped after about 140 h.

2.4. Analyses

For the biomass dry weight determination, the pellet obtained as described above was used. This pellet was washed with demineralized water and centrifuged again, and after this, was dried overnight at 80°C in an oven.

The optical density was measured at 610 nm (Pharmacia Biotech, Ultraspec 2000) after dilution of samples to obtain absorbance values less than 0.7. In this range, the absorbance values were linearly related to biomass dry weight.

The cell number was measured by using a cell counter (Schärfe System, CASY 1) after appropriate dilution in an isotonic solution (Isoton, Schärfe System).

Glucose, ethanol, and glycerol concentrations were determined by HPLC on an Aminex HPX-87H Ion Exclusion column (300∗7.8 mm, BioRad) heated to 60°C. Ultrapure water (Milli Q, Millipore), adjusted with H_2SO_4 to pH 2.0, served as the mobile phase. The flow rate was 0.8 ml/min. Detection was conducted by using a refractive index detector (LKB differential refractometer, Pharmacia).

Ammonia concentrations were spectrophotometrically

measured by means of a modified Berthelot reaction with an auto analysis system (Skalar) [15].

Amino acids were analyzed by reversed-phase HPLC after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Waters Chromatography, Millipore Corp.) [16].

α-Ketobutyrate concentrations were determined by reversed-phase HPLC (Chromspher 5 C8, Chrompack) after derivatization with 2,4-dinitrophenylhydrazine [17]. For derivatization, a 1-ml sample was mixed with a 0.5-ml solution of dinitrophenylhydrazine (0.40 g dinitrophenylhydrazine dissolved in 60 ml 2N HCl) and allowed to stand overnight at room temperature. After this, the sample was mixed with 5 ml of acetonitrile and centrifuged after 1 h. The supernatant was used for analysis after filtering (0.2-μm filters). Intracellular α-ketobutyrate concentrations were determined after a mixture of a 1-ml sample from the −80°C freezer and 1 ml of 80% (v/v) ethanol had been boiled for 30 min. Hereafter, the α-ketobutyrate concentration in the mixture was determined as described above.

Ethanol and higher alcohols were determined by GC. The samples were incubated at 60°C for 10 min. After incubation, a head-space sample was taken and collected in a cold trap (−110°C liquid nitrogen). Through subsequent heating of the cold trap (240°C), the compounds were injected on a DB-wax column (30 m∗0.542 mm, film 1.0 μm). A temperature profile was used from 30 to 110°C at a rate of 2.5°C/min. Helium was used as carrier gas (about 15 ml/min, 30 kPA) and the compounds were detected with a flame ionization detector (EL 980, Fison Instruments). With this method, active amyl alcohol and isoamyl alcohol could not be separated from another.

2.5. Enzyme assays

Samples (1 ml) from the −80°C freezer were centrifuged for 10 min at 13 000 rev./min in an Eppendorf centrifuge (MicroCen 13, Herolab). After the supernatant had been removed, the cells were washed with demineralized water and centrifuged again. The remaining cells were broken by using glass beads with a size ranging from 0.25 to 0.50 mm in a potassium phosphate buffer of which the composition was dependent on the enzyme assay used. For this, the samples were vortexed (TM01, Labotech) at maximum speed 5× for 45 s, alternating with 45 s cooling on ice. The crude cell extract obtained was immediately used for determining enzyme activities.

The activity of threonine deaminase was determined according to the method for serine dehydratase of Suda and Nakagawa [18]. However, here threonine was used as substrate instead of serine. The method of Flavin and Slaughter was used for determining the activity of cystathionine γ-lyase [19]. The activity of acetohydroxy acid synthase was determined according to the method of Eggeling et al [7].

For determining the protein content of the cells, samples (1 ml) from a −80°C freezer were centrifuged for 5 min at 13 000 rev./min in an Eppendorf centrifuge (MicroCen 13, Herolab). After the supernatant had been removed, the cells were washed with demineralized water and centrifuged again. Then, the remaining cells were suspended in 0.8 ml of 0.1M NaOH. This suspension was boiled for 30 min and subsequently cooled. Afterwards, the samples were neutralized by adding 0.2 ml of 0.4 M HCl. The protein content of the samples obtained was determined by using the BCA protein assay with bovine serum albumin as standard (Pierce) [20]. The incubation procedure used was 30 min at 60°C.

Specific enzyme activities were expressed in μmol product formed per minute per mg protein (U/mg).

3. Results and discussion

3.1. Substrate consumption and growth

In the different batches for studying the metabolism of *Z. rouxii*, the separate effect of threonine, cystathionine, and the branched-chain amino acids on the consumption of substrates (glucose, ammonia, and different amino acids) and growth was determined. In Fig. 2A, the consumption of glucose in the different batches is shown. Fig. 2A shows that the glucose consumption was more or less the same in all batches except for the threonine batch. In the threonine batch, glucose was consumed more slowly.

The ammonia measurements (Fig. 2B) clearly show that some ammonia was consumed in all batches except for the threonine batch, but that most of the added ammonia was remaining at the end of all batches. It appears from the amino acid analyses (Fig. 3) that, threonine and the branch-chain amino acids were consumed in their batches, despite the excess ammonia, which is preferred as nitrogen source. The consumption of cystathionine could not be confirmed because the results of the analyses were not clear. Fig. 3 shows that threonine was only slightly consumed, whereas the branched-chain amino acids were completely consumed at almost the same rate.

The growth was followed by measuring biomass dry weight, optical density, and cell number. In Fig. 4, the biomass dry weight measurements are shown. The optical density and cell number measurements (data not shown) were comparable to the biomass dry weight measurements. Fig. 4 shows that the growth, like the glucose consumption, was comparable in all batches except for the batch with added threonine. In the threonine batch, the growth was severely inhibited.

This growth inhibition could have been caused by threonine itself and/or a product originating from threonine like α-ketobutyrate. In *S. cerevisiae*, threonine seems to be the key compound that regulates the flow through a metabolic pathway that leads to threonine and methionine [21]. This regulation by threonine might have inhibited the growth of

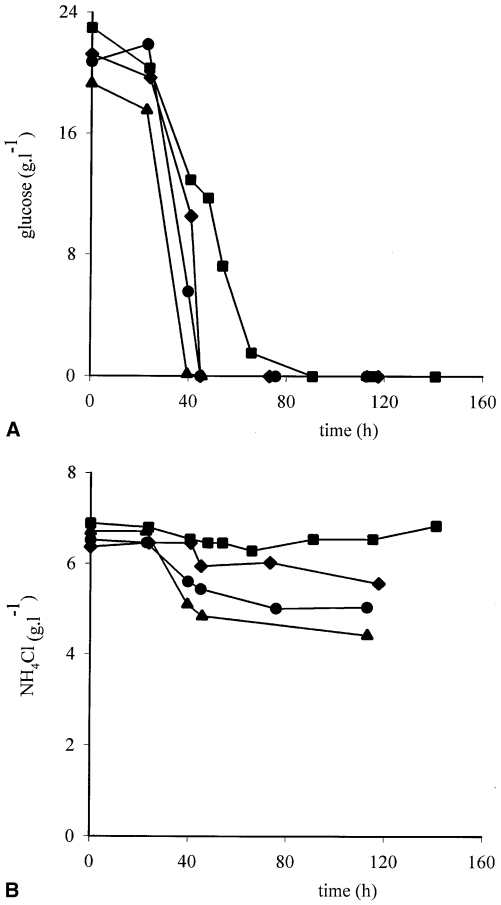

Fig. 2. Effect of amino acids on substrate consumption by *Z. rouxii* in batch cultures: ●, no additions; ■, threonine (5 mM); ▲, cystathionine (5 mM); and ◆, branched-chain amino acids (each 5 mM). A, glucose; B, NH_4Cl.

Z. rouxii by causing a shortage of methionine. α-Ketobutyrate can also inhibit the growth by causing a methionine requirement. In *S. typhimurium*, intracellularly accumulated α-ketobutyrate causes a requirement for methionine by limiting the formation of pantothenate [5]. However, this growth inhibition seems not very likely because pantothenate was present in the medium used (see section 2). Another possibility is that intracellularly accumulated α-ketobutyrate inhibited the synthesis of α-acetolactate catalyzed by acetohydroxy acid synthase (Fig. 1). This inhibition results in a shortage of valine, as shown in *S. typhimurium* [6] and *C. glutamicum* [7]. However, accumulation of α-ketobutyrate could not be detected (with a threshold level of about 5 mg/l) in the cells from the threonine batch, just like in the cells from the other batches (data not shown).

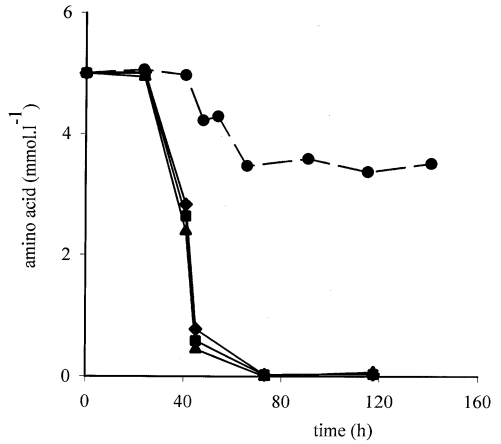

Fig. 3. Consumption of amino acids by *Z. rouxii* in batch culture with threonine (broken line) or branched-chain amino acids (solid line): ●, threonine; ■, isoleucine; ▲, valine; and ◆, leucine.

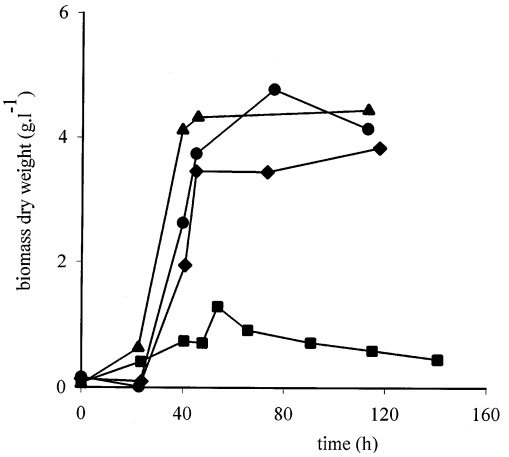

Fig. 4. Effect of amino acids on growth of *Z. rouxii* in batch cultures: ●, no additions; ■, threonine (5 mM); ▲, cystathionine (5 mM); and ◆, branched-chain amino acids (each 5 mM).

3.2. Production of ethanol, glycerol, and higher alcohols

The separate effect of the amino acids, which were used in the different batches, on the production of ethanol, glycerol, and higher alcohols was also determined to gain more insight into the metabolism of *Z. rouxii*. Ethanol and glycerol were monitored because their production is important for yeasts to keep the intracellular NAD(P)H/NAD(P) ratio in balance [22]. Furthermore, glycerol is the primary osmoregulatory solute of *Z. rouxii* [23,24]. The production of higher alcohols by *Z. rouxii* was studied because the production of some of them is closely related to the part of the amino acid metabolism in which we are interested (Fig. 1). Furthermore, α-ketobutyrate is the precursor of *n*-propanol.

The ethanol production during the different batches, which was measured by GC, is shown in Fig. 5A. These measurements were comparable with the HPLC measurements (data not shown). It seems from Fig. 5A that the amino acids had no large effect on the ethanol production. Also in the threonine batch, much ethanol was produced despite the poor growth. In the beginning of all batches, ethanol was produced when glucose was still ample present (Fig. 2A). In this phase of the fermentation, the respiratory quotients determined (data not shown) were greater than 1.0, confirming a metabolism with ethanol production [25].

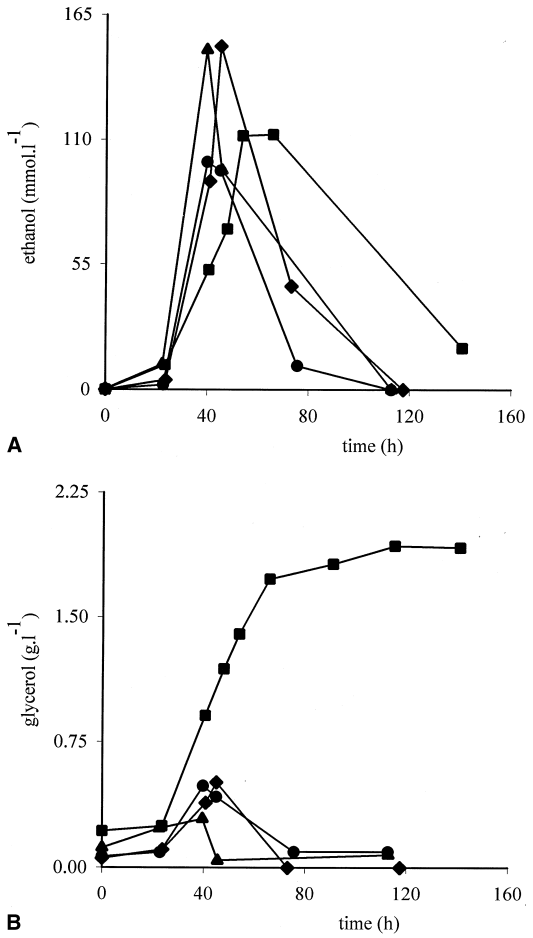

Fig. 5. Effect of amino acids on metabolites production by *Z. rouxii* in batch cultures: ●, no additions; ■, threonine (5 mM); ▲, cystathionine (5 mM); and ◆, branched-chain amino acids (each 5 mM). (A), ethanol; (B), glycerol.

The ethanol production under glucose and oxygen excess demonstrates that *Z. rouxii* showed, like *S. cerevisiae*, the Crabtree effect. This effect means that glucose is simultaneously oxidized and, to keep the intracellular NADH/NAD ratio in balance, reduced to ethanol at high glucose consumption rates under aerobic conditions and seems to be caused by a limited oxidation capacity of the yeast [26]. In *S. cerevisiae*, this limited oxidation capacity is partly due to repression of respiration by glucose [22], which does not occur in *S. rouxii* [27], which is a synonym for *Z. rouxii* [28]. Probably for this reason, Brown and Edgley concluded that *S. rouxii* does not show a Crabtree effect [29]. However, based on our results, this conclusion seems not to be justified. Fig. 2A and 5A also show that the ethanol concentration decreased in all batches, after glucose had completely been consumed. In this phase, the respiratory quotients determined were lower than 0.6, demonstrating ethanol consumption [25].

Figure 5B shows the effect of the amino acids on the glycerol production. It seems that glycerol was only slightly produced in all batches, except for the threonine batch. In this batch, much glycerol was produced. It is known that *S. rouxii* generally produces glycerol and intracellularly retains a proportion of it, dependent on the water activity [23]. However, the water activity in the threonine batch was very likely the same as in the other batches. For this reason, the high glycerol production in the threonine batch could not be explained by osmoregulation. In the threonine batch, glycerol was probably produced to keep the intracellular NADPH/NADP ratio in balance [22]. Due to the poor growth in this batch, NADP could probably not be regenerated by biosynthesis but by glycerol synthesis instead. *S. rouxii* depends, unlike *S. cerevisiae*, for glycerol synthesis mainly on NADPH generated by the pentose phosphate cycle [29].

The production of the higher alcohols, *n*-propanol, isobutyl alcohol, and active amyl plus isoamyl alcohol during the different batches is shown in Fig. 6. From these figures, it seems that the production of the higher alcohols in the cystathionine batch was more or less the same as in the batch with no additions. On the other hand, in the threonine and branched-chain amino acid batches the production of the higher alcohols was significantly different than in the batch with no additions. In the threonine batch, the production of all higher alcohols measured was decreased. In the branched-chain amino acids batch, the *n*-propanol production was slightly decreased but the production of isobutyl alcohol and active amyl plus isoamyl alcohol was considerably increased. These results show that, like in soy sauce [3], isobutyl alcohol, isoamyl alcohol, and active amyl alcohol are probably produced by *Z. rouxii* via the Ehrlich pathway; the uptake and transamination of isoleucine, valine, and leucine resulted in an increased production of respectively active amyl alcohol, isobutyl alcohol, and isoamyl alcohol (Fig. 1). If these alcohols had been directly produced from amino-acid biosynthetic pathways, an increased *n*-propanol production in the branched-chain amino acids batch would have been expected as well.

3.3. Enzyme activities and accumulation of α-ketobutyrate

In this work, we studied the regulation of α-ketobutyrate metabolism in *Z. rouxii* at the enzyme level as well. For this, threonine, cystathionine, and the branched-chain amino acids were separately used in batch cultures to increase the formation rate of α-ketobutyrate or to decrease its conversion rate in *Z. rouxii*. Threonine and cystathionine were added to stimulate the formation rate of α-ketobutyrate by threonine deaminase and cystathionine γ-lyase, respectively, and the branched-chain amino acids were added to inhibit the conversion rate of α-ketobutyrate by acetohydroxy acid synthase. The effect of these amino acids on the specific activities of threonine deaminase, cystathionine γ-lyase, and acetohydroxy acid synthase in *Z. rouxii* was determined.

In Fig. 7, the effect of the amino acids on the specific activity of threonine deaminase, cystathionine γ-lyase, and

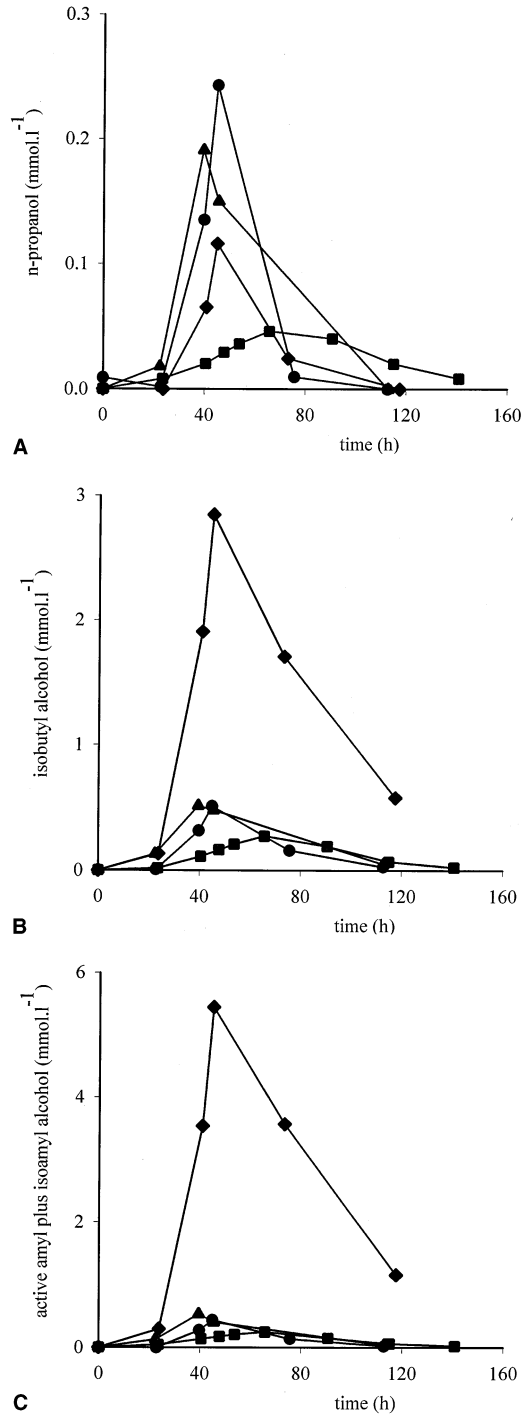

Fig. 6. Effect of amino acids on higher alcohols production by *Z. rouxii* in batch cultures: ●, no additions; ■, threonine (5 mM); ▲, cystathionine (5 mM); and ◆, branched-chain amino acids (each 5 mM). (A), *n*-propanol; (B), isobutyl alcohol; (C), active amyl plus isoamyl alcohol (with the analysis used, active amyl alcohol and isoamyl alcohol could not be separated from another).

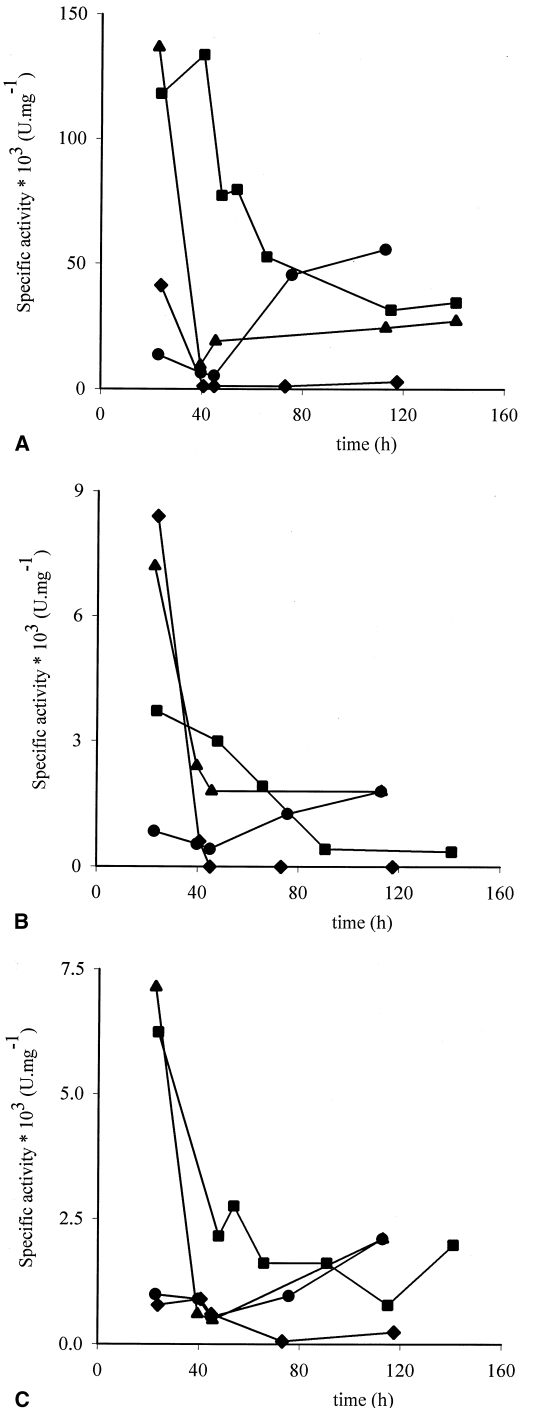

Fig. 7. Effect of amino acids on enzyme activities of *Z. rouxii* in batch cultures: ●, no additions; ■, threonine (5 mM); ▲, cystathionine (5 mM); and ◆, branched-chain amino acids (each 5 mM). (A), threonine deaminase; (B), cystathionine γ-lyase; (C), acetohydroxy acid synthase.

acetohydroxy acid synthase is shown. Although the specific enzyme activities were determined at different points of time during the whole batch cultivation, the activities during the growth phase were considered to be the most important ones because, in that phase, formation of α-ketobutyrate, which is an intermediate of biosynthetic pathways for amino acids (Fig. 1), was expected. Furthermore, during the growth phase, which lasted till about 60 h of cultivation time (Fig. 4), the consumption of amino acids also occurred (Fig. 3).

It seems from Fig. 7 that, especially during the growth phase, the amino acids had much effect on the enzyme activities. During the growth phase, threonine not only increased the specific activity of threonine deaminase but also that of cystathionine γ-lyase and acetohydroxy acid synthase. More or less, the same holds for cystathionine; cystathionine not only increased the specific activity of cystathionine γ-lyase but also that of threonine deaminase and acetohydroxy acid synthase. Furthermore, the branched-chain amino acids not only decreased the specific activity of acetohydroxy acid synthase but also that of threonine deaminase and cystathionine γ-lyase. This decrease in specific enzyme activities by the branched-chain amino acids appeared just to happen towards the end of the growth phase. These measured enzyme activities show that threonine and cystathionine increased both the formation and conversion rate of α-ketobutyrate whereas the branched-chain amino acids decreased both rates. For this reason, it seems that the α-ketobutyrate pool size in *Z. rouxii* was tightly regulated; its formation and conversion rate were balanced all the time. This conclusion about the tightly regulated α-ketobutyrate pool in *Z. rouxii* is in agreement with that found in *S. typhimurium* [6].

The effect of the different amino acids on the accumulation of α-ketobutyrate by *Z. rouxii* was also determined. However, no α-ketobutyrate was detected (with a threshold level of about 5 mg/l) in final samples of both supernatant and cells. In the batch with no additions, we already knew from a preliminary experiment that α-ketobutyrate would not be accumulated. The absence of α-ketobutyrate accumulation in the batches with addition of amino acids agrees well with the measured enzyme activities and with the lower productivity of *n*-propanol, of which α-ketobutyrate is the precursor (Fig. 1), in these batches compared to the batch with no additions.

4. Conclusions

The metabolism of *Z. rouxii* was investigated by separately adding the amino acids threonine, cystathionine, and the branched-chain amino acids. It seemed that the addition of threonine severely inhibited the growth of *Z. rouxii*, which resulted in the accumulation of significant amounts of glycerol and only small amounts of higher alcohols. On the other hand, the addition of the branched-chain amino acids increased the production of the higher alcohols isobutyl alcohol and active amyl plus isoamyl alcohol via the Ehrlich pathway. Furthermore, *Z. rouxii* showed the Crabtree effect but this was not dependent of the amino acids added. In addition, the added amino acids also influenced the specific activities of the enzymes catalyzing the formation or conversion of α-ketobutyrate in *Z. rouxii*. Despite this, it seemed that the α-ketobutyrate pool size in *Z. rouxii* was tightly regulated all the time, resulting in no accumulation of α-ketobutyrate in both the supernatant and cells.

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