Stellingen

1. Uit het feit dat de oxidatiecapaciteit van *Zygosaccharomyces rouxii* niet geremd wordt door glucose, concluderen Brown and Edgley (1980) ten onrechte dat *Z. rouxii* niet Crabtree positief is.
   (Hoofstuk 2 en 4 van dit proefschrift).

2. Hoewel meestal alginaat wordt gebruikt voor het immobiliseren van sojasaus gisten, is alginaat niet geschikt voor toepassing in (langdurige) sojasaus processen.
   (Hoofstuk 6 en 7 van dit proefschrift).

3. Het uitblijven van economisch succes in landen als Indonesië en Suriname toont aan, dat dit succes niet alleen bepaald wordt door de aanwezigheid van natuurlijke rijkdommen.

4. Het 'messageboxje' van de mail doet één van de grote voordelen van de e-mail, dat je de mail leest wanneer het je goed uitkomt, teniet.

5. Dat een samenvatting in gewone mensentaal nodig is om wetenschappelijk onderzoek begrijpbaar te maken voor de gewone mens, geeft bijzonder goed de geïsoleerde positie van de wetenschapper weer.
   (Naar aanleiding van proefschrift van L.P. Ooijkaas).

6. De objectiviteit van het reviewen van artikelen zou verbeterd kunnen worden door de namen van de auteurs onbekend te laten voor de reviewers.

7. Door industrie gefinancierde aio's moeten niet alleen het wetenschappelijk belang van hun onderzoek kunnen aangeven, maar tevens het industriële belang kunnen verdoezelen voor de buitenwereld.

8. Voedingsmiddelen zijn per definitie functioneel en daarom zou de term 'functional foods' beter veranderd kunnen worden in 'extrafunctional foods'.

Stellingen behorende bij het proefschrift:
Production of Japanese Soy-Sauce Flavours.

Catrinus van der Sluis
Wageningen, 3 april 2001

Production of Japanese Soy-Sauce Flavours

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Production of Japanese Soy-Sauce Flavours

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman,
in het openbaar te verdedigen
op dinsdag 3 april 2001
des namiddags te vier uur in de Aula.

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Cover: *Zygosaccharomyces rouxii* cells stained with fluorescein
(photo by Arjan de Leeuw van Weenen/design by Tjibbe Chris Kuipers)

Foar ús soarchsume en leave heit

Contents

Chapter 1

General introduction

Soy sauce

Since ancient times soy sauce has been very popular as a food-flavouring agent in the Orient. Nowadays soy sauce is being used all over the world. Soy sauce is a dark brown liquid with an aroma suggestive of meat extracts. This liquid is prepared by hydrolysis of plant materials.

For this hydrolysis a fermentation or chemical process can be used. In the fermentation process the plant materials are hydrolysed very slowly under mild conditions, whereas the chemical hydrolysis under more extreme conditions is very fast. Therefore the chemical soy sauce is less expensive than the fermented one, but the latter is superior in flavour (Luh, 1995).

Production of fermented soy sauce

The conventional process for production of fermented soy sauce starts with mixing the raw plant materials, which are soybeans and wheat (Figure 1) (Yong and Wood, 1974; Beuchat, 1983; Sugiyama, 1984; Fukushima, 1985; Yokotsuka, 1986; Röling, 1995). The ratio of soybeans to wheat used is dependent on the type of soy sauce. Basically two types of fermented soy sauce can be distinguished, a Japanese and a Chinese type (Röling, 1995). For the Japanese type approximately equal amounts of soybeans and wheat are used, whereas wheat is hardly or not used for the Chinese type.

After mixing, *Aspergillus* species are added to the raw materials in order to start a solid-state fermentation. During the solid-state fermentation, *Aspergillus* grows and produces extracellular enzymes, such as proteases and amylases (Yong and Wood, 1977), which hydrolyze the protein and starch of the soybeans and wheat. In about two days *Aspergillus* is grown throughout the mixture of soybeans and wheat.

In the following step of the production, the moulded soybeans and wheat are mixed with a brine solution of 17% (w/v) salt. In the brine solution the enzymes from *Aspergillus* go

on with hydrolyzing the protein and starch of the soybeans and wheat, and consequently many amino acids and sugars are formed. These amino acids and sugars are used for the growth of salt-tolerant lactic-acid bacteria (*Tetragenococcus halophila*) and yeasts (*Zygosaccharomyces rouxii* and *Candida versatilis*) during the so-called brine fermentation. Unlike the growth of these desirable micro-organisms, that of undesirable ones is effectively inhibited by the high salt content of the brine solution (Sugiyama, 1984).

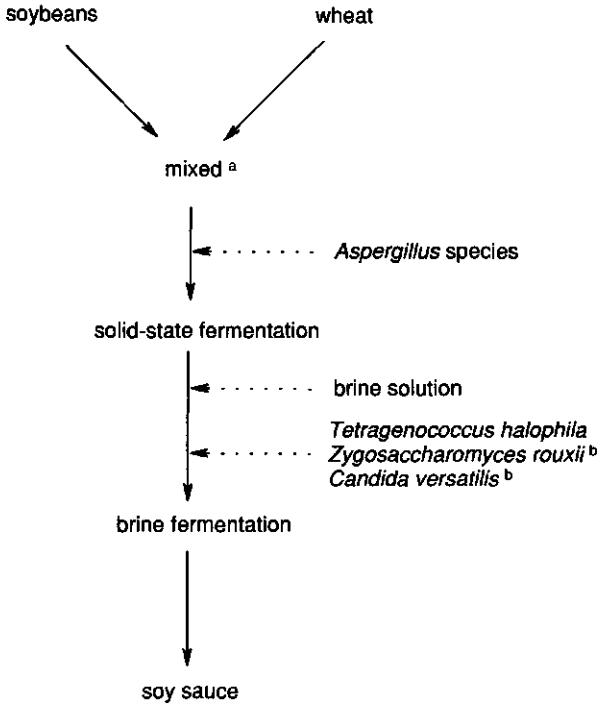

Figure 1. Process for production of fermented soy sauce. Superscript indicates: a: ratio of soybeans to wheat is dependent on type of soy sauce; b: fermentation by *Zygosaccharomyces rouxii* and *Candida versatilis* hardly occurs in the Chinese type of soy-sauce production, but is very obvious in the Japanese type of soy-sauce production.

In the beginning of the brine fermentation the pH is around 7. At this pH *T. halophila* grows very fast and produces lactic acid, which decreases the pH. As the pH approaches 5, the growth of *T. halophila* ceases and fermentation with the yeasts *Z. rouxii* and

C. versatilis starts. During this yeast fermentation many flavour components like ethanol, higher alcohols, 4-hydroxyfuranones and 4-ethylguaiacol are produced.

The extent of the yeast fermentation is dependent on the type of soy-sauce production (Röling, 1995). In Japanese type of soy-sauce production the yeast fermentation is clearly present and has a great impact on the flavour of the final product, while it hardly or not takes place during the Chinese type of soy-sauce production. Instead, in the Chinese type spices like garlic, ginger and nutmeg are added to give the final flavour.

The difference between the yeast fermentation for both types is caused by the fact that in the Japanese type a greater ratio of wheat to soybeans is used than in the Chinese type (Röling, 1995). Wheat is poorer in protein but much richer in starch than soybeans. Consequently, the brine solution of the Japanese type contains much more sugars arisen from the wheat starch than that of the Chinese type. For that reason, after the growth of *T. halophila*, in Japanese type plenty of sugars are left for the growth of yeasts and their flavour production, while at that time no sugars are remaining for the yeast fermentation in the Chinese type.

Yeast flavour formation in Japanese soy sauce

Because the flavour components formed by the salt-tolerant yeasts give a characteristic flavour to Japanese soy sauce, much research has been done to understand the metabolism of especially *Z. rouxii* (Sugiyama, 1984; Aoki et al., 1991; Hecquet et al., 1996). Nevertheless, there is still not so much known about the yeast flavour formation, which makes it difficult to control.

In addition the flavour formation by the yeasts is very slow and therefore usually more than 6 months are required to complete the yeast fermentation. The reason for this long fermentation time is the fact that the metabolic activity of the yeast is low due to the high salt content of the brine solution (17% (w/v)) (Yong and Wood, 1974). In order to shorten the fermentation time, a high concentration of yeast cells is needed. For that reason, much effort has been put into the application of immobilized salt-tolerant yeasts

(Osaki et al., 1985; Horitsu et al., 1990; Hamada et al., 1991). This resulted in the development of new continuous immobilized-cell process, which appeared to be 10 times faster than the conventional process (Iwasaki et al., 1991). In this way the process time was decreased from 6 months to a few weeks (Osaki et al., 1985).

In the new process the salt-tolerant yeasts were mostly entrapped in alginate gel beads. However, alginate gel is mechanically very weak (Horitsu et al., 1990; Muscat et al., 1996) and also chemically unstable towards high salt concentrations (Martinsen et al., 1989; Horitsu et al., 1990). Because of the latter, it is expected that the high salt content of the brine solution will have a negative effect on the chemical stability of alginate. Therefore, a long-term operation with salt-tolerant yeasts immobilized in alginate gel is not feasible (Horitsu et al., 1990).

Outline of this thesis

In this thesis, research about the flavour formation by salt-tolerant yeasts in Japanese soy-sauce processes is described. The aim of this research was to obtain more knowledge about the metabolism of the salt-tolerant yeasts in order to enhance the control of the flavour formation. By this, the formation of desired flavour components can be promoted, which offers the possibility to influence the flavour composition of the soy sauce or to produce specific natural flavour components instead. Another aim of this thesis was to replace alginate with a chemically crosslinked polyethylene-oxide gel for immobilizing the salt-tolerant yeasts. In this way, the long-term operation of the continuous immobilized-cell process with an accelerated flavour formation is feasible.

Important yeast-derived flavour components of Japanese soy sauce are the higher alcohols produced by *Z. rouxii*. The formation of higher alcohols in *Z. rouxii* is closely related to its amino-acid metabolism, in which α-keto acids are key intermediates. Therefore, the separate effects of the amino acids threonine, cystathionine and the branched-chain amino acids on the metabolism of *Z. rouxii* were investigated (Chapter 2 of this thesis). In this chapter, the regulation of the metabolism around α-ketobutyrate, an α-keto acid, is described as well. The regulation of the aspartate-derived amino-acid

metabolism in *Z. rouxii* compared to that in *Saccharomyces cerevisiae*, which was used as reference yeast all through the thesis, is discussed in Chapter 3. Chapter 4 deals with the steady-state culture characteristics of *Z. rouxii*, which were determined with the acceleration-stat (A-stat) cultivation method that was recently developed by Paalme and Vilu (1992). The A-stat cultivation can be much less time-consuming than the usually used chemostat cultivation, especially when high accelaration rates are applied. The determination of the highest acceleration rate for estimation of the steady-state culture characteristics during A-stats with yeasts is reported as well in Chapter 4. The A-stat cultivation was also used in Chapter 5 to study the concomitant extracellular accumulation of α-keto acids and higher alcohols by *Z. rouxii*. In the following two chapters, the immobilization of the salt-tolerant yeasts *Z. rouxii* and *C. versatilis* in a new polyethylene-oxide gel is described. First the polyethylene-oxide gel was developed and characterized (Chapter 6), and after that the newly developed gel was applied in a continuous stirred-tank reactor for flavour production (Chapter 7). Finally, in the last chapter of this thesis, recent advances in the research about the yeast flavour formation in Japanese soy-sauce processes are reviewed.

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Chapter 2

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

Abstract

Zygosaccharomyces rouxii is an important yeast in the formation of flavour 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-alcohols production. It also appeared 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 in order 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.

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Introduction

Zygosaccharomyces rouxii, a salt-tolerant yeast, is important for the flavour 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 flavour (Nunomura et al., 1976). Much research has been done to understand the biosynthesis of these flavours by *Z. rouxii* (Aoki and Uchida, 1991a; Aoki and Uchida, 1991b; Yoshikawa et al., 1995). 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* in order 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* (Figure 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* (Shaw and Berg, 1980; Primerano and Burns, 1982) and *Corynebacterium glutamicum* (Eggeling et al., 1987). 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 Figure 1 (Jones and Fink, 1982). In the isoleucine biosynthesis, the first enzyme is threonine deaminase (L-threonine hydro-lyase [deaminating]; EC 4.2.1.16) which catalyzes the deamination of threonine to α-ketobutyrate and ammonia. 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-

α-hydroxybutyrate. The same enzyme also catalyzes the flow of two molecules pyruvate towards valine and leucine by forming α-acetolactate.

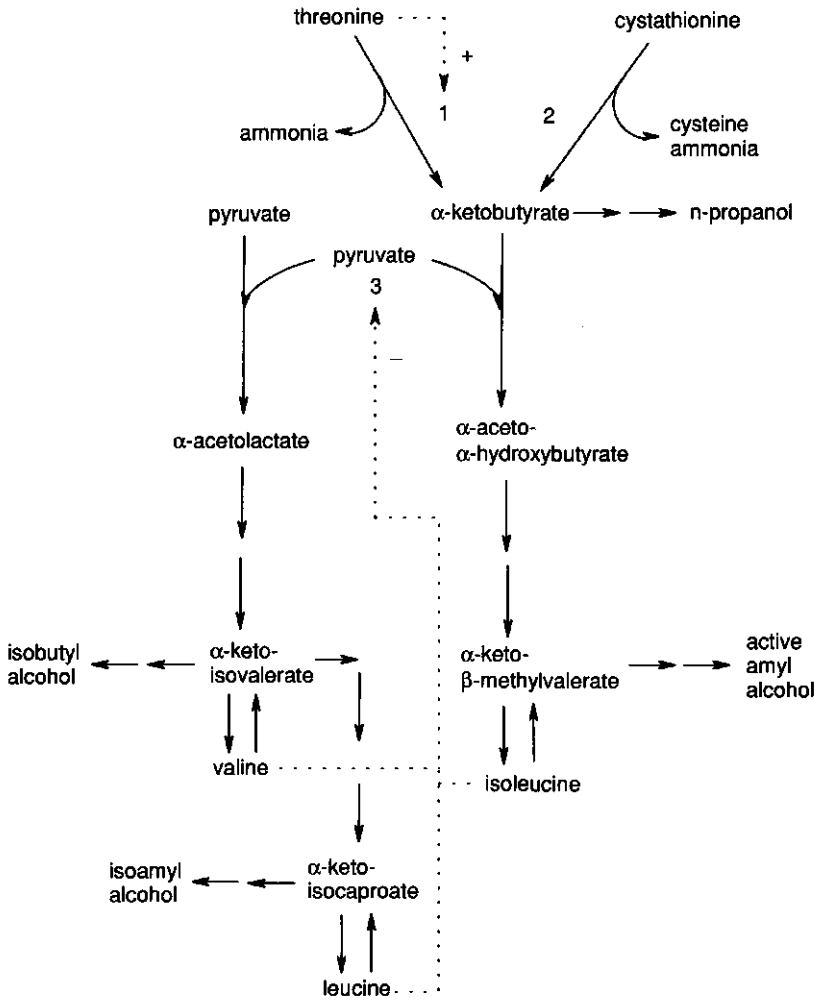

Figure 1. Biosynthetic pathways of isoleucine, valine, leucine and cysteine in *S. cerevisiae* (solid lines). Enzymes: 1: threonine deaminase; 2: cystathionine γ-lyase; 3: acetohydroxy acid synthase. Regulation at the enzyme level (dotted lines): +: induction by threonine; -: repression by isoleucine, valine and leucine together.

In Figure 1, the formation of the higher alcohols n-propanol, isobutyl alcohol, active amyl alcohol and isoamyl alcohol is also shown (Dellweg and Kettler, 1975). 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 respectively α-keto-β-methylvalerate, α-keto-isovalerate and α-keto-isocaproate which are converted into respectively active amyl alcohol, isobutyl alcohol and isoamyl alcohol. 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) (Dickinson and Dawes, 1992; Derrick and Large, 1993).

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 (Figure 1). The specific activity of threonine deaminase in *S. cerevisiae* can be increased by threonine, which induces the synthesis of threonine deaminase (Ramos and Wiame, 1982). 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* (Jones and Fink, 1982). 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 which are all three necessary to repress the synthesis of acetohydroxy acid synthase (Bussey and Umbarger, 1969; Bollon, 1975).

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, growth and production of ethanol, glycerol and higher alcohols were monitored. Additionally, in order 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.

Materials and methods

Yeast strain

Z. rouxii CBS 4021, supplied by the 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.

Inoculum cultures

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

Bioreactor cultures

Batch cultures were carried out in a bench-scale autoclavable bioreactor with a maximum volume of 2 liter. The height and the diameter of the bioreactor were respectively 0.20 and 0.12 m. The bioreactor was stirred 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 glucose.$1H_2O$, 7.65 g NH_4Cl, 2.81 g KH_2PO_4, 0.59 g $MgSO_4.7H_2O$, 10 ml trace metal solution, 2 ml vitamin solution. The trace metal solution contained per liter demineralized water: 5.5 g $CaCl_2.2H_2O$, 3.75 g $FeSO_4.7H_2O$, 1.4 g $MnSO_4.1H_2O$, 1.35 g $ZnSO_4.7H_2O$, 0.4 g $CuSO_4.5H_2O$, 0.82 g $CoCl_2.6H_2O$, 0.29 g $Na_2MoO_4.2H_2O$, 0.4 g H_3BO_3, 0.25 g KI and 33.21 g $C_{10}H_{14}N_2Na_2O_8.2H_2O$. 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 D-biotin, 5.00 g thiamine hydrochloride,

47 g m-inositol, 1.2 g pyridoxine, 23 g hemi-calcium 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* (Bollon, 1975). The glucose, salts and trace metals were separately autoclaved at 120°C for 20 minutes. 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 rpm. 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 dioxide in the ingoing air was measured with this analyser before the cultivation started. From these measurements, respiratory quotients were calculated. The foam level was controlled by automatically adding a diluted (50 times) 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 rpm and 5°C for 10 minutes. 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 hours.

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, 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 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 done 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) (Verdouw et al., 1978).

Amino acids were analysed by reversed-phase HPLC after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters Chromatography, Millipore Corp.) (Cohen et al., 1993).

α-Ketobutyrate concentrations were determined by reversed-phase HPLC (Chromspher 5 C8, Chrompack) after derivatization with 2,4-dinitrophenylhydrazine (Buslig, 1982). 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 hour. The supernatant was used for analysis after filtering (0.2 μm

filters). Intracellular α-ketobutyrate concentrations were determined after a mixture of 1 ml sample from the -80°C freezer and 1 ml of 80% (v/v) ethanol had been boiled for 30 minutes. 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 minutes. After incubation, a head-space sample was taken and collected in a cold trap (liquid nitrogen at -110°C). 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.

Enzyme assays

Samples (1 ml) from the -80°C freezer were centrifuged for 10 minutes at 13,000 rpm 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 using glass beads with a size ranging from 0.25-0.50 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 five times for 45 seconds, alternating with 45 seconds 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 (1971). However, here threonine was used as substrate instead of serine.

The method of Flavin and Slaughter (1971) was used for determining the activity of cystathionine γ-lyase.

The activity of acetohydroxy acid synthase was determined according to the method of Eggeling et al. (1987).

For determining the protein content of the cells, samples (1 ml) from -80°C freezer were centrifuged for 5 minutes at 13,000 rpm 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 0.1M NaOH. This suspension was boiled for 30 minutes and subsequently cooled. Afterwards, the samples were neutralized by adding 0.2 ml 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) (Smith et al., 1985). The incubation procedure used was 30 minutes at 60°C.

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

Results and discussion

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 Figure 2A, the consumption of glucose in the different batches is shown. Figure 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 (Figure 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.

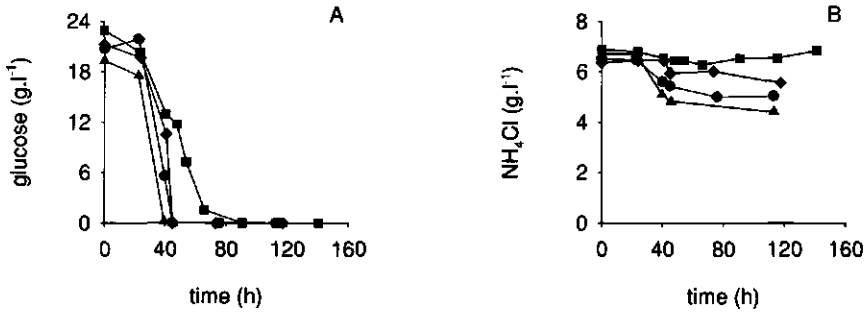

Figure 2. Effect of amino acids on substrate consumption by *Z. rouxii* in batch cultures (●: no additions; ■: threonine (5 mM); ▲: cystathionine (5 mM); ◆: branched-chain amino acids (each 5 mM)). A: glucose; B: NH_4Cl.

It appears from the amino-acid analyses (Figure 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. Figure 3 shows that threonine was only slightly consumed while the branched-chain amino acids were completely consumed at almost the same rate.

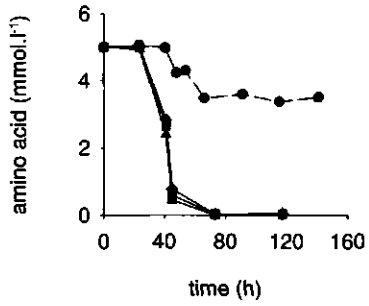

Figure 3. Consumption of amino acids by *Z. rouxii* in batch culture with threonine (broken line) or branched-chain amino acids (solid line) (●: threonine; ■: isoleucine; ▲: valine; ◆: leucine).

The growth was followed by measuring biomass dry weight, optical density and cell number. In Figure 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. Figure 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.

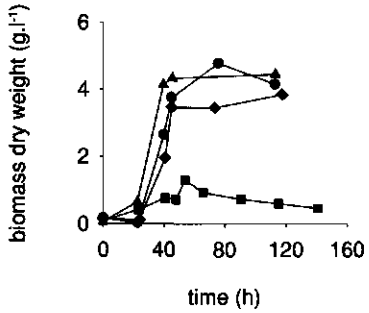

Figure 4. Effect of amino acids on growth of *Z. rouxii* in batch cultures (●: no additions; ■: threonine (5 mM); ▲: cystathionine (5 mM); ◆: branched-chain amino acids (each 5 mM)).

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 (Ramos and Calderon, 1992). This regulation by threonine might have inhibited the growth of *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 (Primerano and Burns, 1982). However, this growth inhibition seems not very likely because pantothenate was present in the medium used (see materials and methods). Another possibility is that intracellularly accumulated α-ketobutyrate inhibited the synthesis of α-acetolactate catalyzed by acetohydroxy acid synthase (Figure 1). This inhibition results in a shortage of valine, as shown in *S. typhimurium* (Shaw and Berg, 1980) and *C. glutamicum* (Eggeling et al., 1987). However, accumulation of α-ketobutyrate could not be detected (with a threshold level of about 5 $mg.l^{-1}$) in the cells from the threonine batch, just like in the cells from the other batches (data not shown).

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 in order 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 (Gancedo and Serrano, 1989). Furthermore, glycerol is the primary osmoregulatory solute of *Z. rouxii* (Edgley and Brown, 1978; van Zyl and Prior, 1990). 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 (Figure 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 Figure 5A. These measurements were comparable with the HPLC measurements (data not shown). It appears from Figure 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 (Figure 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 (Wang et al., 1977).

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 (Sonnleitner and Käppeli, 1986). In *S. cerevisiae*, this limited oxidation capacity is partly due to repression of respiration by glucose (Gancedo and Serrano, 1989), which does not occur in *Saccharomyces rouxii* (Brown, 1975), which is a synonym for *Z. rouxii* (Kreger-van Rij, 1984). Probably for this reason, Brown and Edgley concluded that *S. rouxii* does not show a Crabtree effect (Brown and

Edgley, 1980). However, based on our results, this conclusion seems not to be justified. Figure 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 (Wang et al., 1977).

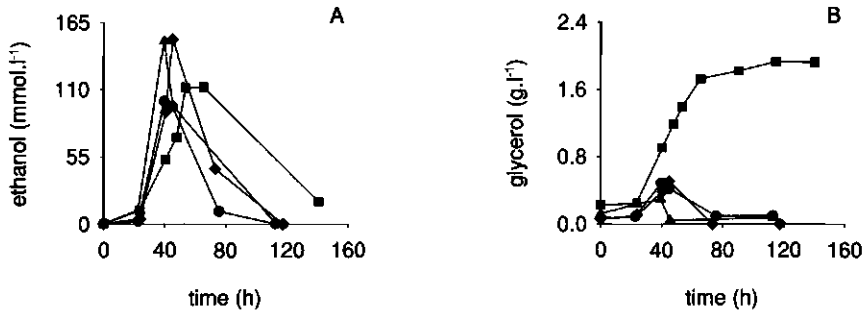

Figure 5. Effect of amino acids on metabolites production by *Z. rouxii* in batch cultures (●: no additions; ■: threonine (5 mM); ▲: cystathionine (5 mM); ◆: branched-chain amino acids (each 5 mM)). A: ethanol; B: glycerol.

Figure 5B shows the effect of the amino acids on the glycerol production. It appears 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 (Edgley and Brown, 1978). 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 in order to keep the intracellular NADPH/NADP ratio in balance (Gancedo and Serrano, 1989). 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 (Brown and Edgley, 1980).

The production of the higher alcohols n-propanol, isobutyl alcohol and active amyl alcohol plus isoamyl alcohol during the different batches is shown in Figure 6. From these

figures, it appears 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-acids batches the production of the higher alcohols was significantly different than in the batch with no additions.

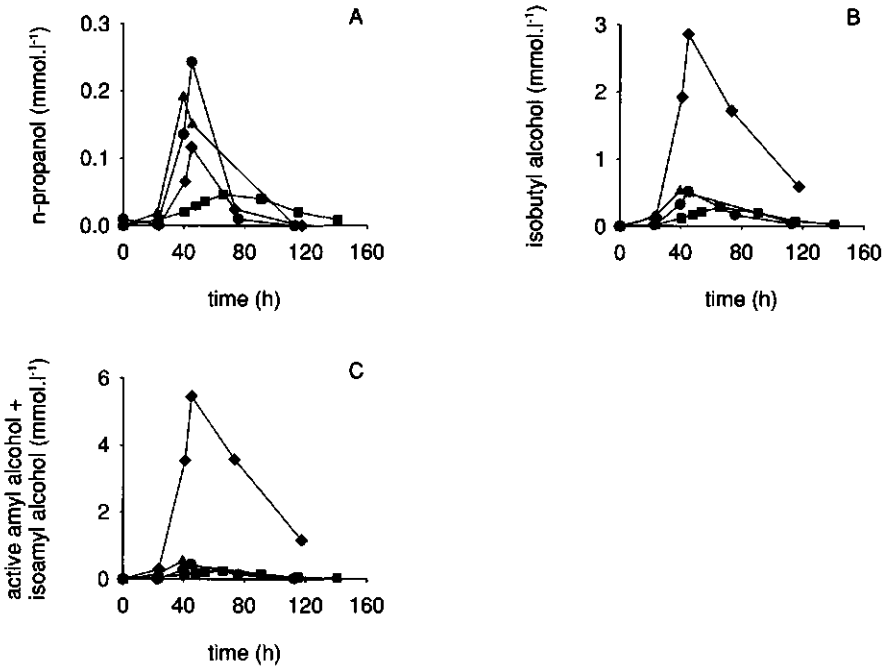

Figure 6. Effect of amino acids on higher-alcohols production by *Z. rouxii* in batch cultures (●: no additions; ■: threonine (5 mM); ▲: cystathionine (5 mM); ◆: branched-chain amino acids (each 5 mM)). A: n-propanol; B: isobutyl alcohol; C: active amyl alcohol plus isoamyl alcohol (With the analysis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

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 alcohol plus isoamyl alcohol was considerably increased. These results show that, like in soy sauce (Aoki and Uchida, 1991b), 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 (Figure 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.

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 in order 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 respectively threonine deaminase and cystathionine γ-lyase 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 Figure 7, the effect of the amino acids on the specific activity of threonine deaminase, cystathionine γ-lyase and 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 (Figure 1), was expected. Furthermore, during the growth phase, which lasted till about 60 hours of cultivation time (Figure 4), the consumption of amino acids also occurred (Figure 3).

It appears from Figure 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.

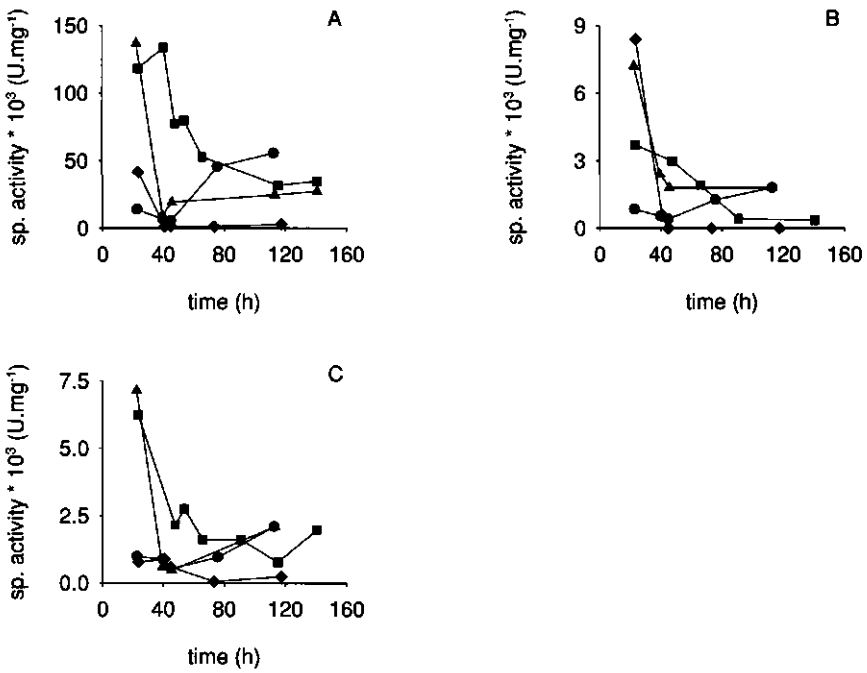

Figure 7. Effect of amino acids on specific enzyme activities of *Z. rouxii* in batch cultures (●: no additions; ■: threonine (5 mM); ▲: cystathionine (5 mM); ◆: branched-chain amino acids (each 5 mM)). A: threonine deaminase; B: cystathionine γ-lyase; C: acetohydroxy acid synthase.

These measured enzyme activities show that threonine and cystathionine increased both the formation and conversion rate of α-ketobutyrate while 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* (Shaw and Berg, 1980).

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 $mg.l^{-1}$) in final samples of both supernatant and cells. In the batch with no additions, we already knew from a preliminaty 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 (Figure 1), in these batches compared to the batch with no additions.

Conclusions

The metabolism of *Z. rouxii* was investigated by separately adding the amino acids threonine, cystathionine and the branched-chain amino acids. It appeared 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 alcohol 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 appeared 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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Chapter 3

Regulation of aspartate-derived amino-acid metabolism in *Zygosaccharomyces rouxii* compared to *Saccharomyces cerevisiae*

Abstract

In order to elucidate the growth inhibitory effect of threonine, the regulation of the aspartate-derived amino-acid metabolism in *Zygosaccharomyces rouxii*, an important yeast for the flavour development in soy sauce, was investigated. It was shown that threonine inhibited the growth of *Z. rouxii* by blocking the methionine synthesis. It appeared that threonine blocked this synthesis by inhibiting the conversion of aspartate. In addition, it was shown that the growth of *Z. rouxii*, unlike that of *Saccharomyces cerevisiae*, was not inhibited by the herbicide sulfometuron methyl (SMM). From enzyme assays, it was concluded that the acetohydroxy acid synthase in *Z. rouxii*, unlike that in *S. cerevisiae*, was not sensitive to SMM. Furthermore, the enzyme assays demonstrated that the activity of threonine deaminase in *Z. rouxii*, like in *S. cerevisiae*, was strongly inhibited by isoleucine and stimulated by valine. From this work, it is clear that the aspartate-derived amino-acid metabolism in *Z. rouxii* only partly resembles that in *S. cerevisiae*.

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Introduction

In our research, we are investigating the metabolism of the salt-tolerant yeast *Zygosaccharomyces rouxii*, which is a well-known flavour producer in soy-sauce fermentations (Sugiyama, 1984). In the amino-acid metabolism of this yeast, α-ketobutyrate is a key intermediate; some α-ketobutyrate is needed to synthesize isoleucine, but intracellularly accumulated α-ketobutyrate may inhibit the growth as found in *Salmonella typhimurium* (Shaw and Berg, 1980; Primerano and Burns, 1982; LaRossa et al., 1987) and *Corynebacterium glutamicum* (Eggeling et al., 1987).

In *Saccharomyces cerevisiae*, which we use as our reference yeast, the isoleucine synthesis starts with the deamination of threonine (Figure 1) (Jones and Fink, 1982). The deamination of threonine is catalyzed by threonine deaminase (L-threonine hydro-lyase [deaminating]; EC 4.2.1.16) and results in the formation of α-ketobutyrate and ammonia. α-Ketobutyrate is converted further towards isoleucine by acetohydroxy acid synthase (acetolactate pyruvate-lyase [carboxylase]; E.C. 4.1.3.18]. This enzyme catalyzes the conversion of α-ketobutyrate and pyruvate into α-aceto-α-hydroxybutyrate and also the conversion of two molecules of pyruvate into α-acetolactate, which is converted via α-keto-isovalerate to valine and leucine.

In previous studies, aiming at α-ketobutyrate accumulation, we added threonine (5 mM) to batch cultures of *Z. rouxii* (van der Sluis et al., 2000). Although no α-ketobutyrate accumulation was observed, growth of *Z. rouxii* was severely inhibited due to the addition of threonine. In *S. cerevisiae*, threonine is the key compound that regulates the flow to a common metabolic pathway for biosynthesis of threonine and methionine (Figure 1) (Jones and Fink, 1982; Ramos and Calderon, 1992). The growth inhibition of *Z. rouxii* due to threonine addition could be caused by threonine interfering with the biosynthesis of other amino acids.

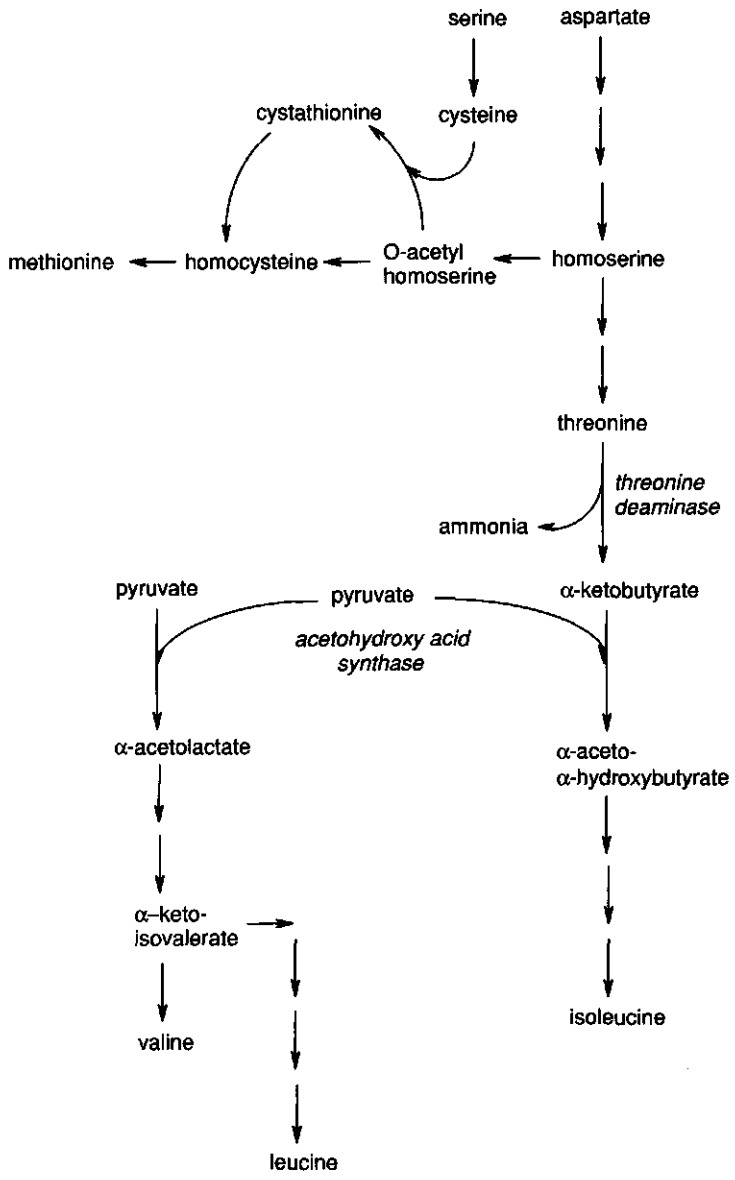

Figure 1. Biosynthetic pathways of isoleucine, valine, leucine and methionine in *S. cerevisiae*.

The pathway for synthesizing threonine and methionine in *S. cerevisiae* starts from aspartate and leads to homoserine in three steps (Figure 1). Homoserine is the branching point from which the pathways for threonine and methionine diverge. Threonine is made in two steps from homoserine while the next step in the synthesis of methionine is the acetylation of homoserine yielding O-acetylhomoserine. O-acetylhomoserine can be converted to homocysteine in two ways. The first pathway is a direct sulfhydrylation of O-acetylhomoserine to homocysteine, the precursor of methionine. The second pathway involves condensation of O-acetylhomoserine with cysteine that can be directly synthesized from serine. This condensation yields cystathionine, which is further converted to homocysteine. Methylation of homocysteine results in methionine formation.

In this work, we have studied the regulation of the aspartate-derived amino-acid metabolism in *Z. rouxii*. Batch growth experiments were executed in which we determined whether the addition of aspartate and aspartate-derived amino acids could restore the growth of *Z. rouxii* in the presence of threonine. Furthermore, we determined the effect of ammonium on the growth of *Z. rouxii*. In addition, we investigated the effect of some nitrogen compounds on the activity of the enzymes threonine deaminase and acetohydroxy acid synthase in *Z. rouxii* and we studied the effect of the herbicide SMM, which is an inhibitor of acetohydroxy acid synthase in various plants, bacteria and yeasts (LaRossa and Falco, 1984; Falco and Dumas, 1985).

Materials and methods

Yeast strains

Z. rouxii CBS 4021 and *S. cerevisiae* CBS 6978, supplied by the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and maintained on a mixture of glycerol and skimmed milk at -80°C, were used in this work.

Growth experiments

The yeasts were grown in 300 ml Erlenmeyer flasks, containing 100 ml defined medium, on a rotary shaker (Gallenkamp, Orbital Incubator) at 28°C and 200 rpm. The defined medium had the following composition per liter demineralized water: 22 g glucose.$1H_2O$ (Merck), 21 g citric acid.$1H_2O$ (Merck) and 6.7 g Bacto Yeast Nitrogen Base without amino acids (Difco). The pH of this medium was adjusted to a value of 5 using NaOH and the medium was filter-sterilized (Nalgene, 0.2 µm filters).

During the experiments, the effects of the following amino acids on the growth of *Z. rouxii* were determined: L-threonine (Sigma), L-methionine (Sigma), L-homocysteine (Sigma), L-homoserine (Sigma), L-aspartate (Sigma), L-cystathionine (Fluka), L-cysteine (Sigma) and L-serine (Sigma). In addition, the effect of SMM (6 µg/ml), that was kindly provided by Du Pont Agricultural Products (Wilmington), on the growth of *Z. rouxii* and *S. cerevisiae* was determined. SMM was, before addition to the medium, dissolved in acetone at a concentration of 2 mg/ml. The addition of acetone appeared to have no effect on the growth of *Z. rouxii* and *S. cerevisiae*. The effect of ammonium on the growth of *Z. rouxii* was determined by using the Bacto Yeast Nitrogen Base without amino acids and ammonium sulfate (Difco) instead of the Bacto Yeast Nitrogen Base without only amino acids.

The growth of both yeasts was followed, by measuring the optical density at 610 nm (Pharmacia Biotech, Ultraspec). For this, samples were taken and diluted with demineralized water to obtain absorbance values less than 0.7. Below 0.7, the absorbance values were linearly related to biomass dry weight.

Enzyme assays

The yeast was grown as above and harvested when the mid-exponential growth phase was reached. For this, the cells were centrifuged for 10 minutes at 9,500 g and 4°C (Beckman J2-MC centrifuge, Beckman Instruments Inc., Palo Alto, California, USA). After centrifugation the supernatant was removed and the cells were suspended in a 50 mM

potassium-phosphate buffer (pH 7). The cell suspension was stored at -80°C until used for the enzyme assays.

After thawing at room temperature the cell suspensions from -80°C were washed three times with the potassium-phosphate buffer. Then, the cells were broken using glass beads (Emergo, Landsmeer, The Netherlands) with a size ranging from 0.25 to 0.50 mm while vortexing (TM01, Labotech) at maximum speed six times for 1 minute, alternating with 1 minute of cooling on ice. The crude cell extract obtained was used immediately for determining enzyme activities.

The activity of threonine deaminase was measured by using the method for serine dehydratase of Suda and Nakagawa (1971), except that threonine was used as substrate instead of serine. The α-ketobutyrate formed was determined colorimetrically by converting it to a hydrazone in the presence of 2,4-dinitrophenylhydrazine (Aldrich, Zwijndrecht, The Netherlands)

The activity of acetohydroxy acid synthase was determined by measuring the acetolactate formed (Eggeling et al., 1987). For this, the acetolactate was decarboxylated to acetoin which was determined colorimetrically (Westerveld, 1945).

Results and discussion

Growth experiments

In order to investigate the growth inhibition of *Z. rouxii* by threonine, batch growth experiments were carried out. In these experiments, we determined whether aspartate and aspartate-derived amino acids could counteract the growth inhibitory effect of threonine on *Z. rouxii*. Furthermore, we also determined the effect of ammonium on the growth of *Z. rouxii*. During these experiments, the growth was followed by measuring the optical density.

In Figure 2, the effect of some aspartate-derived amino acids and ammonium on the growth of *Z. rouxii* in batch cultures, containing excess nitrogen, is shown. This figure confirms that the growth of *Z. rouxii* in cultures with excess ammonium (75 mM) is inhibited by addition of threonine (5 mM) (van der Sluis et al., 2000). This figure shows further that addition of methionine (1 mM) to the medium with ammonium (75 mM) and threonine (5 mM) could fully oppose this growth inhibitory effect of threonine. In addition, it can be seen from this figure that ammonium was favourable for the growth of *Z. rouxii*; the growth of *Z. rouxii* was very poor in the cultures in which ammonium was replaced by threonine (75 mM) or by threonine (75 mM) and methionine (7.5 mM) together. Therefore, neither threonine alone nor together with methionine can be regarded as adequate nitrogen sources for the growth of *Z. rouxii*. The growth in the medium with threonine and methionine together was better than that in the medium with threonine as sole nitrogen source. This confirmed that addition of methionine can improve the growth of *Z. rouxii* in the presence of threonine.

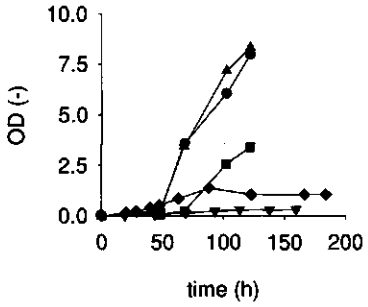

Figure 2. Effect of aspartate-derived amino acids and ammonium on the growth of *Z. rouxii* in batch cultures (●: ammonium (75 mM); ■: ammonium (75 mM) plus threonine (5 mM); ▲: ammonium (75 mM) plus threonine (5 mM) plus methionine (1 mM); ◆: threonine (75 mM) plus methionine (7.5 mM); ▼: threonine (75 mM)).

Figure 3 shows the effect of aspartate (1 mM) and some other aspartate-derived amino acids (1 mM) on the growth of *Z. rouxii* in batch cultures with excess ammonium (75 mM) and threonine (5 mM). It can be seen from this figure and Figure 2 that aspartate, serine and cysteine could not counteract the inhibitory effect of threonine on the growth of *Z. rouxii*. The same holds for the amino acids valine and isoleucine (data not shown).

When all these amino acids (1 mM) were added to batch cultures of *Z. rouxii* with only ammonium (75 mM) as nitrogen source, no inhibitory effect on the growth was observed (data not shown). This demonstrates that these amino acids were not growth inhibitors themselves. On the other hand, it can be seen from Figure 2 and 3, that cystathionine and homocysteine clearly improved the growth of *Z. rouxii* in the presence of threonine while homoserine, like methionine, could fully oppose the inhibitory effect of threonine.

Furthermore, it appeared that the addition of methionine (5 mM) to batch cultures of *Z. rouxii* with ammonium (75 mM) did, unlike the addition of threonine (5 mM), not have any growth inhibitory effect on *Z. rouxii* (data not shown). The results obtained with these growth experiments show that in *Z. rouxii*, like in *S. cerevisiae*, threonine seems to be the key compound that regulates the flow to the common biosynthetic pathway for threonine and methionine (Figure 1). The results show further that this strong regulation of threonine in *Z. rouxii* resulted in a shortage of homoserine and, as a consequence, methionine. Due to this homoserine shortage, the amino acids aspartate, serine and cysteine could not counteract the growth inhibitory effect of threonine while the amino acids cystathionine, homocysteine and especially, homoserine and methionine could. From these observations, it can be concluded that threonine inhibited one of the three conversion steps between aspartate and homoserine.

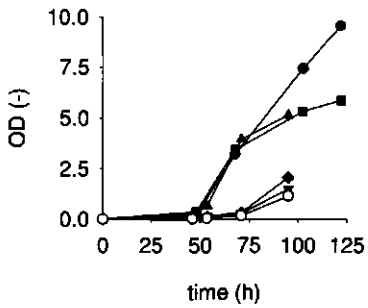

Figure 3. Effect of aspartate and aspartate-derived amino acids on growth of *Z. rouxii* in batch cultures (●: homoserine (1 mM); ■: homocysteine (1 mM); ▲: cystathionine (1 mM); ◆: serine (1 mM); ▼: aspartate (1 mM); ○: cysteine (1 mM)). All cultures contained ammonium (75 mM) plus threonine (5 mM).

In *S. cerevisiae*, the first conversion step between aspartate and homoserine, that is catalyzed by the enzyme aspartate kinase, is strongly feed-back inhibited and repressed by threonine but not so strong by methionine (Jones and Fink, 1982; Ramos et al., 1991). For this reason, it is most probable that threonine inhibited the growth of *Z. rouxii* by adversely affecting the activity of aspartate kinase. The measurement of the aspartate kinase activity in crude cell extracts of *Z. rouxii*, using the methods of Black (1962) and Thèze et al. (1974), gave no clear results. For both methods, the background activity of the cell extract seemed too high to be able to measure the aspartate kinase activity. Therefore, it was impossible to determine whether the inhibition was due to repression of enzyme synthesis or caused by inhibition of aspartate kinase activity.

The regulation of the aspartate-derived amino-acid metabolism was studied further with growth experiments in which we tested the effect of the herbicide SMM, a well-known inhibitor of the enzyme acetohydroxy acid synthase in *S. cerevisiae*. As can be seen in Figure 4, SMM (6 µg/ml) inhibited the growth of *S. cerevisiae*, as was reported before (Falco and Dumas, 1985), but it did not affect the growth of *Z. rouxii*. This resistance of *Z. rouxii* to SMM may be due to just a single amino-acid change in the acetohydroxy acid synthase, as found in SMM-resistant mutants of *S. typhimurium* and *S. cerevisiae* (LaRossa and Falco, 1984).

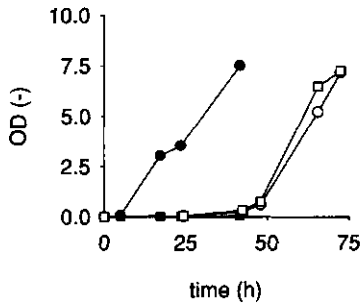

Figure 4. Effect of SMM on growth of *Z. rouxii* (open symbol) and *S. cerevisiae* (solid symbol) in batch cultures (circle: no additions; square: SMM (6 µg/ml)).

Enzyme assays

As acetohydroxy acid synthase is the target for SMM, the effect of SMM on the activity of the acetohydroxy acid synthase in crude cell extracts of *Z. rouxii* and *S. cerevisiae* was investigated. In Figure 5, the effect of SMM on the acetohydroxy acid synthase in both yeasts is shown. Figure 5 shows that SMM, al least at high concentrations (around 500 ng/ml), inhibited the activity of acetohydroxy acid synthase in *S. cerevisiae*. The SMM concentrations needed for inhibition were higher than the ones reported by Falco and Dumas (1985), which might be caused by the fact that we measured acetohydroxy acid synthase in crude cell extracts while they used permeabilized cells. In crude cell extracts, acetohydroxy acid synthase is less sensitive than in permeabilized cells (Magee and de Robichon-Szulmajster, 1968). Figure 5 also shows that SMM did not affect at all the activity of acetohydroxy acid synthase in *Z. rouxii*. This insensitivity of acetohydroxy acid synthase in *Z. rouxii* to SMM is apparently the reason that the growth of *Z. rouxii* was also not affected by SMM.

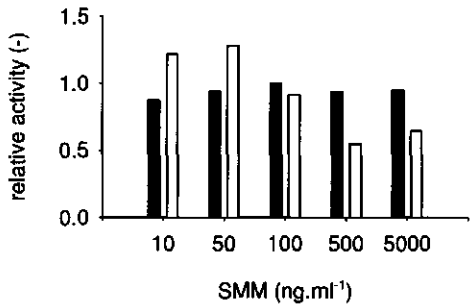

Figure 5. Effect of SMM on relative activity of acetohydroxy acid synthase from *Z. rouxii* (solid bar) and *S. cerevisiae* (open bar). Activity without SMM is one.

The regulation of the acetohydroxy acid synthase in *Z. rouxii* was investigated further by determining the effect of the amino acids isoleucine, valine and leucine separately (20 mM) or mixtures of them (each 5 mM) on the activity of acetohydroxy acid synthase. The concentrations we used were quite high in order to compensate for the fact that we measured in crude cell extracts. However, we did not observe any clear effects of neither

valine nor the other amino acids on the activity of acetohydroxy acid synthase from *Z. rouxii* (data not shown), while in *S. cerevisiae*, the activity of this enzyme is inhibited by valine (Magee and de Robichon-Szulmajster, 1968).

Finally, we also investigated the regulation of the enzyme threonine deaminase from *Z. rouxii*. It is well-known that, in *S. cerevisiae*, the activity of this enzyme is strongly inhibited by isoleucine (Holzer et al., 1963; de Robichon-Szulmajster and Magee, 1968) and stimulated by valine (Holzer et al., 1963; Cennamo et al., 1964; de Robichon-Szulmajster and Magee, 1968). In addition, it is also reported that ammonium stimulates the activity of this enzyme in *S. cerevisiae* (Holzer et al., 1964) whereas leucine inhibits (Cennamo et al., 1964), but the biological significance of this inhibition and stimulation is not understood.

In Figure 6, the effect of the amino acids isoleucine, valine and leucine, and of ammonium on the activity of threonine deaminase in crude cell extracts of *Z. rouxii* is shown. This figure shows that, like in *S. cerevisiae*, the activity of threonine deaminase in *Z. rouxii* was strongly inhibited by isoleucine and stimulated by valine. In addition, it can be seen from this figure that the effects of leucine and ammonium on the activity of threonine deaminase in *Z. rouxii* were different from those found in *S. cerevisiae*; ammonium did not have much effect while leucine stimulated the activity slightly.

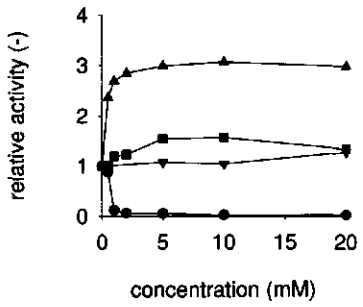

Figure 6. Effect of amino acids on relative activity of threonine deaminase from *Z. rouxii* (●: isoleucine; ■: leucine; ▲: valine; ▼: ammonium).

Biologically, it seems reasonable that ammonium does not stimulate the activity of threonine deaminase because ammonium is known to regulate this enzyme, dependent on the ammonium need of the cell, by repressing its synthesis (Holzer et al., 1963). In addition, it also seems reasonable that leucine, like valine, stimulates the activity of threonine deaminase in order to balance the synthesis of the amino acids isoleucine, valine and leucine (Figure 1), dependent on how much these amino acids are needed for the protein synthesis. If there is an excess leucine and a shortage of isoleucine for the protein synthesis, leucine will stimulate threonine deaminase to synthesize more isoleucine.

Conclusions

The main purpose of our studies was to investigate the regulation of the aspartate-derived amino-acid metabolism in *Z. rouxii*. In this investigation, it appeared that threonine strongly regulated the flow of the aspartate-derived amino acid metabolism by inhibiting the conversion of aspartate to homoserine. When threonine was added to batch cultures of *Z. rouxii*, this inhibition resulted in a lack of methionine and by that poor growth of *Z. rouxii*. This poor growth of *Z. rouxii* in the presence of threonine could be improved by adding the amino acids cystathionine, homocysteine and homoserine but not by aspartate, serine and cysteine.

In addition, it was shown that growth of *Z. rouxii* was stimulated by ammonium and, in contrast to that of *S. cerevisiae*, not inhibited by the herbicide sulfometuron methyl because of the insensitivity of the enzyme acetohydroxy acid synthase in *Z. rouxii*. This enzyme was also not sensitive to the amino acids valine, leucine and isoleucine in crude cell extracts of *Z. rouxii*. On the other hand, the enzyme threonine deaminase in *Z. rouxii* was sensitive to these amino acids but not to ammonium; the activity of this enzyme was inhibited by isoleucine and stimulated by valine and leucine, while ammonium did not affect its activity. From these observations, it was concluded that aspartate-derived amino-acid metabolism in *Z. rouxii* is only partly similar to that in *S. cerevisiae*.

Acknowledgements

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Chapter 4

Estimation of steady-state culture characteristics during acceleration-stats with yeasts

Abstract

Steady-state culture characteristics are usually determined in chemostat cultivations, which are very time-consuming. In contrast, acceleration-stat (A-stat) cultivations in which the dilution rate is continuously changed with a constant acceleration rate are not so time-consuming, especially at high acceleration rates. Therefore, the A-stat could be advantageous to use instead of the chemostat. However, the highest acceleration rate, meaning the fastest A-stat that can be applied for estimating steady-state culture characteristics, is not known yet.

Experimental results obtained with *Zygosaccharomyces rouxii*, an important yeast in soy-sauce processes, showed that the culture characteristics during the A-stat with an acceleration rate of 0.001 h^{-2} were roughly comparable to those of the chemostat. For higher acceleration rates the deviation between the culture characteristics in the A-stat and those in the chemostat obtained at the same dilution rate generally started to increase. The source of these deviations was examined by simulation for *Saccharomyces cerevisiae*. The simulations demonstrated that this deviation was not only dependent on the metabolic adaptation rate of the yeast, but also on the rate of change in environmental substrate concentrations during A-stats. From this work, it was concluded that an A-stat with an acceleration rate of 0.001 h^{-2} is attractive to be used instead of chemostat, whenever a rough estimation of steady-state culture characteristics is acceptable.

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Introduction

In our research we are studying the metabolism of *Zygosaccharomyces rouxii*, which is an important flavour producer in soy-sauce processes (Sugiyama, 1984). Especially we are focussing on the steady-state culture characteristics of *Z. rouxii*. For determining steady-state characteristics the conventional chemostat is widely used.

In chemostat cultivation a sequence of step-wise changes in dilution rate or medium composition is applied. After each change the cultivation needs to be stabilized for at least three residence times in order to obtain a new steady state. This makes chemostat cultivation very time-consuming, especially when a large number of steady-state points are needed. Less time-consuming and much more informative than the chemostat, is the acceleration-stat (A-stat) cultivation that was recently developed by Paalme and Vilu (1992).

The A-stat was developed for a fast microbial characterization at a practically unlimited number of dilution rate values (Paalme and Vilu, 1992; Paalme et al., 1995; Paalme et al., 1997a; Paalme et al., 1997b). This was done by applying a constant smooth change of dilution rate (known as the acceleration rate), after starting the experiment as a chemostat. The higher the acceleration rate applied, the faster the A-stat cultivation becomes. Paalme and co-workers used acceleration rates varying between 0.01-0.05 h^{-2}. At these high acceleration rates the A-stat is very fast, but does not provide steady-state culture characteristics (Paalme et al., 1995). For this, in theory an indefinitely low acceleration rate should be applied. However, to reduce experimental times, it might in practice be possible to use higher acceleration rates to provide a satisfactory approximation of steady-state conditions. Therefore it is meaningful to find the highest acceleration rate (i.e. the fastest A-stat) that can be used for an estimation of the steady-state characteristics, and to quantify at this acceleration rate the advantage of the A-stat compared to the chemostat.

In the study described here, we determined the highest acceleration rate at which the behaviour of *Z. rouxii* culture characteristics in the A-stat was comparable to that in the chemostat. For this, A-stat cultivations with different acceleration rates were carried out

and compared with chemostat cultivations. From these cultivations it appeared that the acceleration rate applied during the A-stats had a significant effect on the culture characteristics at a certain dilution rate. To explain this, simulations were done using *Saccharomyces cerevisiae* as a reference yeast. For this, we used the model of Sweere et al. (1988) which was developed in order to describe the growth of *S. cerevisiae* under dynamic conditions. The model showed that the effect of the acceleration rate on the culture characteristics at a certain dilution rate was not only a function of the metabolic adaptation rate of the yeast but also of the rate of change in environmental substrate concentrations during the A-stats.

Materials and methods

Yeast strain

Z. rouxii CBS 4021, supplied by the 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.

Media

The inoculum cultures were grown on GPY medium which has the following composition per liter of demineralized water: 40 g glucose.$1H_2O$, 5 g peptone and 5 g yeast extract. This medium was sterilized by separately autoclaving the components at 120°C for 20 minutes.

The chemostat and A-stat cultivations were carried out in a defined medium, in which the growth was limited by glucose. This medium had the following composition per liter demineralized water: 10 g glucose.$1H_2O$, 7.5 g NH_4Cl, 4.0 g KH_2PO_4, 1.0 g NaCl, 0.5 g $MgSO_4.7H_2O$, 10 ml trace-metal solution and 10 ml vitamin solution. The trace-metal solution contained per liter demineralized water: 5.5 g $CaCl_2.2H_2O$, 3.8 g $FeSO_4.7H_2O$, 1.4 g $MnSO_4.1H_2O$, 2.2 g $ZnSO_4.7H_2O$, 0.4 g $CuSO_4.5H_2O$, 0.5 g $CoCl_2.6H_2O$, 0.3 g

$Na_2MoO_4.2H_2O$, 0.4 g H_3BO_3, 0.3 g KI and 30.0 g $C_{10}H_{14}N_2Na_2O_8.2H_2O$. The pH of this solution was adjusted to 4 with 4 M NaOH. The composition of the vitamin solution was per liter demineralized water: 0.1 g D-biotin, 5.0 g thiamine hydrochloride, 47.0 g m-inositol, 1.2 g pyridoxine and 23.0 g hemi-calcium pantothenate. The glucose and salts of this medium were separately autoclaved at 120°C for 20 minutes, while the trace metals and vitamins were filter-sterilized (0.2 μm filters).

Cultivations

Starter cultures were made in 300 ml Erlenmeyer flasks containing 100 ml GPY medium, on a rotary shaker at 28°C and 200 rpm. The cells were used for the chemostat and A-stat cultivations when they were in the exponential growth phase (between 20 and 40 hours cultivation).

The chemostat and A-stat cultivations were carried out in a bench-scale autoclavable bioreactor with a height and diameter of respectively 0.20 and 0.12 m. This bioreactor contained 0.75 liter cultivation medium, which was inoculated with 100 ml of a starter culture. After the cells had started to grow, the medium flow rate, controlled by a Bioexpert (Applikon), was started.

The chemostat cultivations were conducted at the following dilution rates: 0.05, 0.075, 0.1, 0.122, 0.150 and 0.173 h^{-1}. At these dilution rates the cultivations were allowed to stabilize for at least three residence times before samples were taken. Steady-state conditions were ascertained by constant carbon-dioxide production of the culture, which was continuously measured all the way through the cultivations.

All A-stat cultivations were started at a dilution rate of 0.1 h^{-1}. At this dilution rate the culture was stabilized for at least 50 hours and subsequently a smooth increase in dilution rate was started according to the following equation:

$$D = D_0 + a \cdot t$$

Depending on the cultivation the following acceleration rates (a) were applied: 0.001, 0.01 and 0.1 h^{-2}. The A-stat cultivations with the acceleration rate of 0.01 and 0.1 h^{-2} were done in respectively triplicate and duplicate. These replicates gave comparable results.

During all cultivations 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, medium flow rate and concentration of oxygen and carbon dioxide in the bioreactor 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 48.6 $l.h^{-1}$. The oxygen tension in the broth was kept above 30% of air saturation by controlling the stirrer speed, which was at least 100 rpm. The stirrer used was a six-blade Rushton turbine with a diameter of 45 mm. The concentration of oxygen in the ingoing and outgoing air was measured on-line with a Servomex Xentra 4100 Gas Purity Analyser. The carbon dioxide from the ingoing air was kept constant at zero by removing it with soda-lime pellets, and the concentration of carbon dioxide in the outgoing air was measured on-line with a Servomex 1440 Gas Analyser. From these measurements respiratory quotients were calculated. The foam level was controlled by automatically adding a diluted (50 times) Antifoam B Silicone emulsion (J.T. Baker). During the chemostat and A-stat cultivations samples for off-line analyses were withdrawn from the bioreactor. The samples were used for measuring optical density, glucose and ethanol.

Analyses

The optical density was measured at 610 nm using a Pharmacia Biotech, Ultraspec 2000 spectrophotometer. Samples were diluted to obtain absorbance values less than 0.7. In this range, the absorbance values were linearly related to the biomass concentration.

Glucose was measured using a GM7 analyser (Analox Instruments). This analyser measures the decrease in oxygen concentration during a reaction between glucose and oxygen catalyzed by glucose oxidase.

Ethanol was determined by GC. The samples were incubated at 60°C for 10 minutes. After incubation a head-space sample was taken and collected in a cold trap (liquid nitrogen at -110°C). 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).

Calculations

For calculating the specific growth rate and the yield of biomass on glucose and oxygen, the concentrations of biomass, glucose, ethanol and oxygen are needed. During A-stat cultivations these concentrations change with time and therefore differential equations are needed (Paalme et al., 1992). To be able to differentiate, the measured discrete data points for the concentrations of biomass, glucose and ethanol were interpolated by using TableCurve™ (Jandel Scientific).

Simulations

Simulations of *S. cerevisiae* culture characteristics during A-stats were carried out in Simulink and Matlab (The MathWorks, Inc.) using the model of Sweere et al. (1988) (see Appendix I).

Results and discussion

Experimental study with *Z. rouxii*

In order to determine the highest acceleration rate for estimating steady-state culture characteristics with the A-stat, A-stats with acceleration rates of 0.001, 0.01 and 0.1 h^{-2} as well as chemostats at fixed dilution rates were conducted. During these cultivations the concentrations of glucose, biomass and ethanol were measured and the specific growth

rate, yield of biomass on glucose and oxygen, and respiratory coefficients determined. In Figure 1 the behaviour of some *Z. rouxii* culture characteristics during representative single cultivations is shown.

All these culture characteristics except for the specific growth rate were standardised in order to have the same starting point for comparison of the behaviour during the different cultivations. For this a characteristic at a certain dilution rate was divided by that characteristic at the dilution rate of 0.1 h^{-1}, which was the starting point of the A-stats. This standardisation was needed because the steady-state characteristics for the different experiments at the dilution rate of 0.1 h^{-1} were not exactly the same (Table 1). The deviation seemed to be caused by experimental errors in analyses and errors in the control of the cultivations. Especially the analysis of glucose at the dilution rate of 0.1 h^{-1} appeared to be difficult, because the concentration of glucose was then very low. Some control errors occurred during the long stabilisation time that was needed to ascertain steady-state conditions at the dilution rate of 0.1 h^{-1}. Although finally always a steady-state condition was reached the cultivation procedure and for that reason, the history of the cells was never exactly the same, which might have caused a deviation in steady-state characteristics as well. However, it was not the intention of this work to provide the exact steady-state characteristics at the dilution rate of 0.1 h^{-1}, but to show the evolution of the culture characteristics.

Figure 1 shows that that the culture characteristics in the A-stat were largely influenced by the applied acceleration rate; an increased acceleration rate generally resulted in an increased deviation between the culture characteristics in the A-stat and those in the chemostat obtained at the same dilution rate. Furthermore, Figure 1 shows that the culture characteristics in the A-stat with an acceleration rate of 0.001 h^{-2} were roughly comparable to those in the chemostat. This was not observed for the ethanol concentrations (Figure 1C), a fact we could not explain.

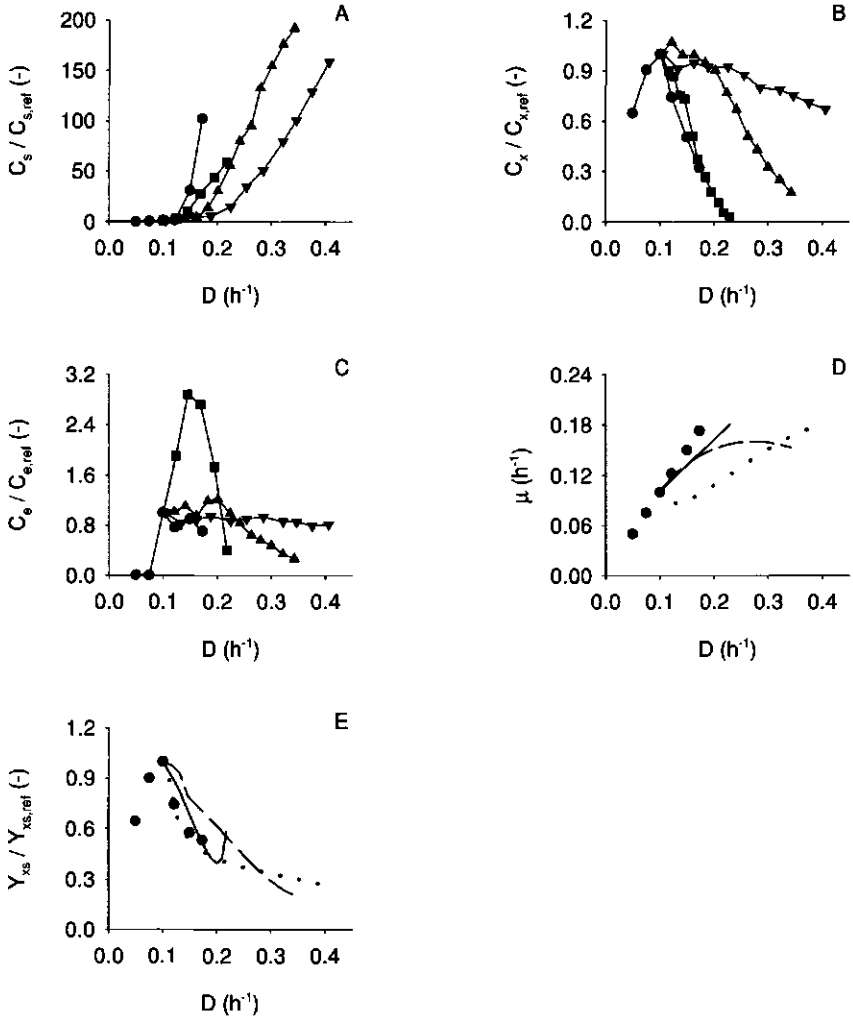

Figure 1. Comparison between the measured characteristics of *Z. rouxii* in chemostat culture (●) and A-stat cultivations with acceleration rates of 0.001 h^{-2} (■ in A-C, solid line in D, E), 0.01 h^{-2} (▲ in A-C, dashed line in D, E) and 0.1 h^{-2} (▼ in A-C, dotted line in D, E). All culture characteristics except for the specific growth rate were standardised with respect to their reference value at the dilution rate of 0.1 h^{-1} (starting point of the A-stats). A: glucose concentration (C_s); B: biomass concentration (C_x); C: ethanol concentration (C_e); D: specific growth rate (μ); E: yield of biomass on glucose (Y_{xs}). The reference values for the different cultures are shown in Table 1.

Table 1. Measured steady-state characteristics ($g.l^{-1}$) of *Z. rouxii* at a dilution rate of 0.1 h^{-1} from different experiments as used to standardise the data in Figure 1.

Characteristic	Chemostat [a]	A-stat with acceleration rate [b]		
		0.001 h^{-2}	0.01 h^{-2}	0.1 h^{-2}
Glucose	0.04	0.14	0.04	0.01
Biomass	3.34	3.77	1.49	2.92
Ethanol	2.53	0.53	2.54	1.52

[a] Chemostat data are from a single experiment in which steady-states were established over a range of dilution rates.

[b] A-stat data are the initial conditions for experiments at acceleration rates of 0.001, 0.01 and 0.1 h^{-2}, each of which was started from a chemostat at a dilution rate of 0.1 h^{-1}.

On the other hand, Figure 1C clearly shows that *Z. rouxii* produced ethanol during all cultivations, which were executed with excess oxygen. This ethanol production under aerobic conditions confirmed that *Z. rouxii*, like *S. cerevisiae*, displays the Crabtree effect (van der Sluis et al., 2000). This Crabtree effect means that the yeast shifts from a purely oxidative to an oxidoreductive metabolism (Sonnleitner and Käppeli, 1986). *Z. rouxii* showed this metabolic shift already at a dilution rate of 0.1 h^{-1} (Figure 1C) compared to 0.3 h^{-1} for *S. cerevisiae* (Sonnleitner and Käppeli, 1986). The metabolic shift was accompanied for *Z. rouxii*, as for *S. cerevisiae*, by a decrease in biomass yield on glucose (Figure 1E), increase in biomass yield on oxygen (data not shown) and respiratory coefficients greater than 1.0 (data not shown). In contrast to our results, van Zyl and Prior (1990) did not observe any ethanol production by *Z. rouxii* under aerobic conditions. They assumed, in common with Brown and Edgley (1980), that *Z. rouxii* does not display the Crabtree effect. However, based on the results presented here and our earlier results (van der Sluis et al., 2000), their assumption seems not to be justified.

It appears from Figure 1D that the maximum specific growth rate observed during the different cultivations varied between 0.15-0.18 h^{-1}. These maximums were slightly lower than the maximum of 0.19 h^{-1} which we previously determined for *Z. rouxii* in batch culture (unpublished work) and significantly lower than the maximum of 0.45 h^{-1} for *S. cerevisiae* (Sonnleitner and Käppeli, 1986).

Simulation study for *S. cerevisiae*

In order to explain the observed influence of the acceleration rate on the culture characteristics of *Z. rouxii* in A-stats, we sought to simulate the effect using the model of Sweere et al. (1988) (Appendix I). This model was developed to describe the growth of *S. cerevisiae* in a changing environment and is based on a hypothesis of Sonnleitner and Käppeli (1986) that the growth of *S. cerevisiae* is controlled by its limited oxidation capacity, which seems to be valid for *Z. rouxii* as well (van der Sluis et al., 2000). However, the simulations could not be done for *Z. rouxii* because its model parameters are not known in the literature and they could also not be clearly estimated from our experimental study (data not shown). Therefore, the simulations were done for *S. cerevisiae* instead. Because the model has not been used before for simulating *S. cerevisiae* culture characteristics during A-stats, the model was first validated for this.

Model validation

The validity of the model, which was programmed in Matlab, for simulating *S. cerevisiae* culture characteristics during an A-stat with an acceleration rate of 0.01 h^{-1} was tested by comparing the simulated characteristics with the ones measured by Paalme et al. (1997b). The model parameters and the starting values used for this simulation can be found respectively in Tables 2 and 3.

Table 2. Values of model parameters for *S. cerevisiae* (Sweere et al., 1988).

Parameter	Value	Parameter	Value
K_m	$1.7*10^{-4}$ mol.l^{-1}	$Y_{xs,ox}$ [a]	3.65 C-mol.mol^{-1}
K_n	$3.6*10^{-4}$ mol.l^{-1}	$Y_{xs,red}$ [a]	0.36 C-mol.mol^{-1}
K_o	$3.0*10^{-6}$ mol.l^{-1}	Y_{es}	1.88 mol.mol^{-1}
K_s	$5.0*10^{-5}$ mol.l^{-1}	Y_{os}	2.17 mol.mol^{-1}
τ_s	2.5 h	$Q_{s,max,p}$ [a]	0.50 mol.C-mol^{-1}.h^{-1}
τ_o	1.6 h	$Q_{o,max,p}$ [a]	0.20 mol.C-mol^{-1}.h^{-1}

[a] 1 C-mol of biomass has the composition $CH_{1.83}N_{0.17}O_{0.56}$.

Table 3. Values of conditions used for the simulations of A-stat cultivations with *S. cerevisiae* adapted from Paalme et al. (1997b).

Condition	Value	Condition	Value
D_0	0.1 h^{-1}	$C_{e,0}$	0 $mol.l^{-1}$
$C_{s,in}$	$6.67*10^{-2}$ $mol.l^{-1}$	$C_{o,0}$ [b]	$5.4*10^{-5}$ $mol.l^{-1}$
$C_{s,0}$ [a]	$4.64*10^{-5}$ $mol.l^{-1}$	$Q_{s,max,0}$ [a]	$5.7*10^{-2}$ $mol.mol^{-1}.h^{-1}$
$C_{x,0}$ [a]	0.24 $mol.l^{-1}$	$Q_{o,max,0}$ [a]	$6.7*10^{-2}$ $mol.mol^{-1}.h^{-1}$

[a] Values were calculated using the model equations of Sweere et al. (1988) under steady-state conditions.

[b] In the simulations the dissolved oxygen concentration was assumed to be 40% of air saturation all the time, while in practice (Paalme et al., 1997b) it was maintained within the range of 20-80% of air saturation.

In Figure 2 this model validation is shown. This figure shows that the behaviour of the simulated results, which were the biomass and ethanol concentration (Figure 2A), and glucose concentration and specific growth rate (Figure 2B), corresponded very well with the experimental ones.

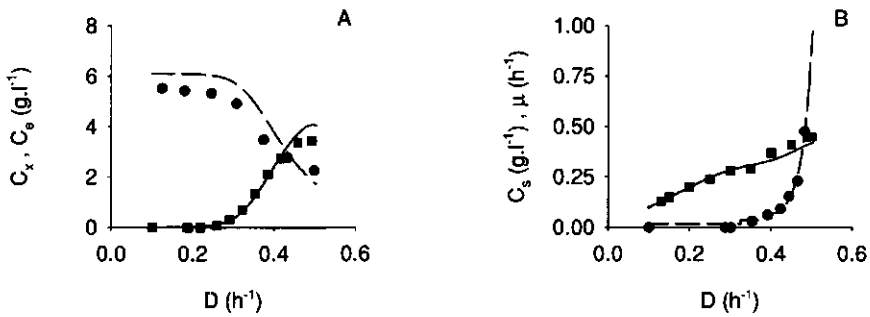

Figure 2. Comparison between the simulated and measured (Paalme et al., 1997b) culture characteristics of *S. cerevisiae* during an A-stat cultivation with an acceleration rate of 0.01 h^{-2}. The symbols give the measured characteristics and the lines the simulated ones. A: ●, dashed line: biomass concentration (C_x) and ■, solid line: ethanol concentration (C_e); B: ●, dashed line: glucose concentration (C_s) and ■, solid line: specific growth rate (μ).

In agreement with the A-stat experiments, the model predicted that the specific growth rate increased proportionally to the dilution rate up to the dilution rate of 0.3 h^{-1}. At this dilution rate ethanol production starts and the increase in specific growth rate temporarily

declines (Paalme et al., 1997b), due to the shift from pure oxidative to oxidoreductive metabolism. During such a metabolic shift steady-state conditions are usually not approached during A-stat cultivations (Paalme et al., 1995). From this validation we concluded that the model of Sweere et al. (1988) simulated the behaviour of *S. cerevisiae* culture characteristics during A-stats well.

Effect of acceleration rate on culture characteristics during A-stats

The verified model of Sweere et al. (1988) was then used to simulate the effect of the acceleration rate on the behaviour of *S. cerevisiae* culture characteristics in A-stats. In Figure 3 the simulated culture characteristics, which were the concentrations of glucose, biomass and ethanol, specific growth rate and yield of biomass on glucose, in A-stats with acceleration rates of 0.001, 0.01 and 0.1 h^{-2} and chemostats are compared. These simulations were carried out until the maximum specific growth rate of *S. cerevisiae* (0.45 h^{-1}) was reached. It can be seen from this figure that also for *S. cerevisiae* the A-stat with an acceleration rate of 0.001 h^{-2} gave more or less the same results as the chemostat, while A-stats with higher acceleration rates showed an increased deviation from the chemostat at a given dilution rate. This demonstrated that the simulated behaviour of *S. cerevisiae* was affected in the same way by the applied acceleration rate as the measured behaviour of *Z. rouxii* (Figure 1).

Figure 3 shows also that the culture characteristics during the A-stats were not only shifted to higher dilution rates than during the chemostat, but also the characteristics themselves were changed by the applied acceleration rate. For example, the applied acceleration rate not only affected the dilution rate at which the maximum ethanol concentration was reached, but also the maximum ethanol concentration itself (Figure 3C). On the other hand, Figure 3 shows that the initial and final culture characteristics in the A-stat with the different acceleration rates were the same as those in the chemostat. This is caused by the fact that the A-stat cultivation starts as a chemostat and, if the cultivation lasts long enough (until wash-out), will end as a chemostat. For this reason the applied acceleration rates did not affect for example the simulated maximum specific growth rate (Figure 3D).

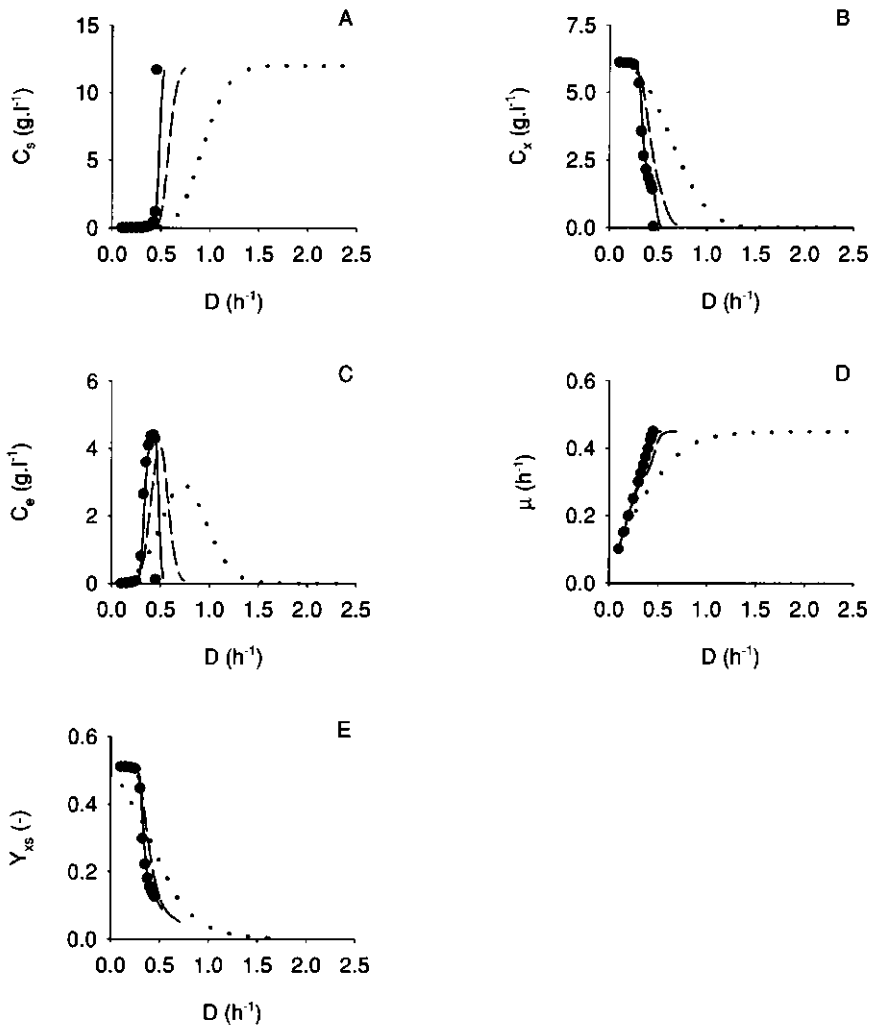

Figure 3. Comparison between the simulated behaviour of the culture characteristics of *S. cerevisiae* in chemostat and A-stat cultivations with different acceleration rates (●: chemostat characteristics; solid line, dashed line and dotted line: A-stat characteristics for acceleration rates of respectively 0.001, 0.01 and 0.1 h^{-2}). A: glucose concentration (C_s); B: biomass concentration (C_x); C: ethanol concentration (C_e); D: specific growth rate (μ); E: overall yield of biomass on glucose (Y_{xs}).

Deviation between A-stats and chemostats

The deviation between A-stat and chemostat culture characteristics at a given dilution rate is expected to be caused by the fact that the yeast can not adapt fast enough to its changing environment during A-stats (Paalme et al., 1997b). This could be the explanation for the observation that higher acceleration rates, which means a faster changing environment, resulted in larger deviations (Figures 1 and 3). According to this, the deviation from steady-state conditions during A-stats should decrease if the yeast is able to adapt faster. In the model of Sweere et al. (1988) (see Appendix I) the yeast adapts faster if the induction times for the synthesis of glucose and oxygen consumption capacity are smaller. The effect of these induction times, which are determined to be about 2 hours (Sweere et al., 1988) (see Table 2), on the deviation from steady-state conditions during A-stats was simulated. For this an arbitrary tenfold change in induction times was made and the deviation of the specific growth rate of *S. cerevisiae* from steady-state conditions was determined by subtracting the dilution rate from the specific growth rate following the biomass balance during A-stats (Paalme and Vilu, 1992):

$$\frac{dC_x}{dt} = C_x \cdot (\mu - D)$$

The expression μ - D is zero under steady-state conditions but is negative during the non-steady-state conditions in the A-stat because the specific growth rate is then smaller than the dilution rate. Therefore, an increased deviation from steady-state conditions during the A-stat is shown by larger negative values for μ - D.

In Table 4 the maximum deviation from steady-state conditions observed during A-stat simulations with different acceleration rates is shown. Larger induction times resulted in an increased deviation from steady state for all acceleration rates. This effect became more pronounced at higher acceleration rates. At a high acceleration rate, like 0.1 h^{-2}, the yeast metabolic adaptation rate is too slow to follow its fast changing environment.

Furthermore, Table 4 shows that some deviation from steady state, even at a low acceleration rate of 0.001 h^{-2}, still existed at extremely low induction times of around 0.2

hours, corresponding to a very rapidly adapting yeast. From this it was clear that the limiting metabolic adaptation rate contributes to the deviation between A-stat and chemostat culture characteristics obtained at the same dilution rate, but there should be another cause for this deviation as well.

Table 4. Simulated effect of variations in the induction times for the synthesis of glucose (τ_s) and oxygen consumption capacity (τ_o) on the maximum deviation (h^{-1}) of the specific growth rate of *S. cerevisiae* from steady-state conditions during A-stats with different acceleration rates.

Induction times	A-stat with acceleration rate		
τ_s; τ_o (h)	0.001 h^{-2}	0.01 h^{-2}	0.1 h^{-2}
0.25; 0.16	-0.02	-0.10	-0.45
2.5;1.6 [a]	-0.02	-0.13	-0.96
25; 16	-0.04	-0.74	-8.84

[a] These are the induction times according to Sweere et al. (1988).

Another cause could be the rate of environmental change during the A-stats in comparison with chemostats. As an example for this, the change in the glucose concentration in the bioreactor during A-stats was calculated by assuming the absence of yeast (see Appendix II). For this, an ingoing glucose concentration of 12 $g.l^{-1}$ and a starting dilution rate of 0.1 h^{-1} was chosen (Paalme et al., 1997b). At this starting dilution rate, the initial glucose concentration in the bioreactor was calculated to be 0.008 $g.l^{-1}$. For this calculation, the model equations of Sweere et al. (1988) were used under steady-state conditions, as is the case at the start of the A-stat.

In Figure 4 the calculated glucose concentration in the absence of yeast during chemostat and A-stat cultivations with different acceleration rates are compared. It can be seen from this figure that the glucose concentration in the A-stats was delayed in dilution rate compared to that concentration in the chemostat. This delay was increased at an increased acceleration rate during the A-stats. The delay of glucose concentration, which also applies for the other medium components in the yeast environment and the yeast itself (data not shown), might also contribute to the deviation between A-stat and chemostat culture characteristics obtained at the same dilution rate.

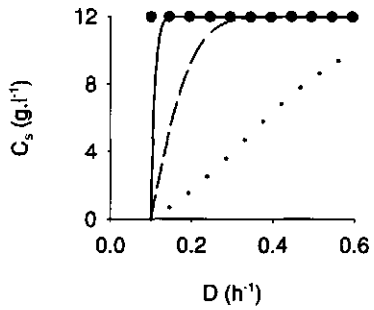

Figure 4. Comparison between the calculated glucose concentration (C_s) in the absence of yeast during chemostat and A-stat cultivations with different acceleration rates (●: chemostat; solid line, dashed line and dotted line: A-stat with acceleration rates of respectively 0.001, 0.01 and 0.1 h^{-2}).

The influence of the delay of the concentrations of the medium components on this deviation can be eliminated by plotting the culture characteristics against the concentration of one of the medium components instead of the dilution rate. This was done with the simulated results for *S. cerevisiae* from Figure 3. As an example, Figure 5A shows a plot of the simulated specific growth rate of *S. cerevisiae* (Figure 3D) against the simulated glucose concentration (Figure 3A). It appears from Figure 5A that for acceleration rates of 0.01 and 0.1 h^{-2} there was still some deviation between the specific growth rate of *S. cerevisiae* in A-stats and that in the chemostat at the same substrate concentration. This shows that eliminating the delay of the concentrations of the medium components could not fully take away the deviation between the A-stat and chemostat. According to the above, the residual deviation should disappear if *S. cerevisiae* is able to adapt faster.

In order to demonstrate this a rapidly adapting *S. cerevisiae* was simulated by using induction times of around 0.2 hours (Figure 5B) instead of the normal 2 hours (Figure 5A). Figure 5B shows that in this case for all acceleration rates the deviation between the A-stat and chemostat completely disappeared. This proves that the deviation between the A-stat and chemostat culture characteristics obtained at the same dilution rate is dependent on both the delay of the concentrations of the medium components in the A-

stat compared to the chemostat, and the limitations in the metabolic adaptation rate of *S. cerevisiae*.

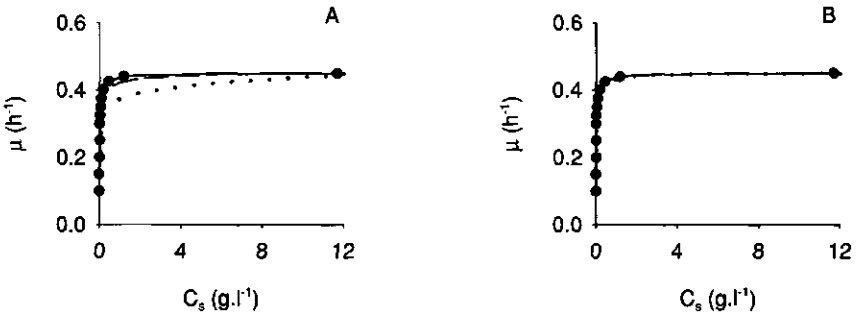

Figure 5. Comparison between the simulated behaviour of *S. cerevisiae* specific growth rate (μ) as a function of the simulated glucose concentration (C_s) in chemostat and A-stat cultivations with different acceleration rates (●: chemostat; solid line, dashed line and dotted line: A-stat characteristics for acceleration rates of respectively 0.001, 0.01 and 0.1 h^{-2}). The yeast induction times for the synthesis of glucose (τ_s) and oxygen consumption capacity (τ_o) respectively used for the simulation were in A: 2.5 and 1.6 h; B: 0.25 and 0.16 h.

Estimation of steady-state culture characteristics during A-stats with yeasts

From the measurements with *Z. rouxii* (Figure 1) and simulations for *S. cerevisiae* (Figure 3) it was clear that in order to approach chemostat conditions in the A-stat, the choice of the acceleration rate is very important. It was shown that there was a significant difference between the A-stat with an acceleration rate of 0.01 h^{-2}, which was applied for *S. cerevisiae* (Paalme and Vilu, 1992; Paalme et al., 1997b), and chemostat culture. This difference increased when the acceleration rate was increased to 0.1 h^{-2}, while it was smaller at an acceleration rate of 0.001 h^{-2}. At such a slow acceleration rate, the metabolic adaptation rate of the yeast is not limiting (Table 4) and there is hardly any delay of environmental substrate concentrations (Figure 4). Therefore, the acceleration rate of 0.001 h^{-2} is the fastest rate, which can be used for approaching steady-state culture characteristics during A-stats with yeasts. In our experimental study with *Z. rouxii*, more or less the same time was needed for running the A-stat with an acceleration rate of 0.001 h^{-2} as for the chemostats. In this time, a practically unlimited number of data points (all data are not

shown) were obtained with this A-stat while the chemostats only delivered six data points. From this it appears that it is interesting to use the A-stat instead of the chemostat, whenever a rough estimation of steady-state culture characteristics is acceptable.

Conclusions

The main purpose of the study presented here was to determine the highest acceleration rate, meaning the fastest A-stat, at which steady-state culture characteristics can be estimated. Measurements for *Z. rouxii* showed that steady-state culture characteristics could be roughly estimated in an A-stat with an acceleration rate of 0.001 h^{-2}. Higher acceleration rates generally resulted in an increased difference between the A-stat and chemostat culture characteristics at a given dilution rate. It appeared that the A-stat with an acceleration rate of 0.001 h^{-2} provided in the same time much more information than the chemostat. Therefore, it is clearly attractive to use the A-stat instead of the chemostat for roughly estimating steady-state culture characteristics.

The measurements with *Z. rouxii* were confirmed with simulations for *S. cerevisiae* using the model of Sweere et al. (1998). This model also showed that if A-stats are to be used to replicate chemostat conditions, both the metabolic adaptation rate of the yeast and the rate of change of environmental substrate concentrations should be taken into account.

Furthermore, the measurements confirmed that *Z. rouxii*, like *S. cerevisiae*, produced ethanol under aerobic conditions (Crabtree effect). In contrast to *S. cerevisiae*, *Z. rouxii* started to produce ethanol at a dilution rate of 0.1 h^{-1} (versus 0.3 h^{-1}) and had a maximum specific growth rate around 0.17 h^{-1} (versus 0.45 h^{-1}).

Acknowledgements

We would like to thank Jan Cozijnsen for his help concerning the GC analysis. This research was funded by SENTER project BIO95028.

Appendix I

The model presented below, which we used to describe the growth of *S. cerevisiae* during A-stat and chemostat cultivations, is according to Sweere et al. (1988). Sweere et al. based their model on the hypothesis of Sonnleitner and Käppeli (1986) that the growth of *S cerevisiae* is controlled by its limited respiratory capacity. Here the consumption of ethanol, which was assumed not to occur during the cultivations, was omitted.

Glucose consumption follows Monod kinetics:

$$Q_s = Q_{s,\max} \cdot \frac{C_s}{C_s + K_s}$$

The limited oxidation capacity is dependent on the availability of dissolved oxygen:

$$Q_{o,\lim} = Q_{o,\max} \cdot \frac{C_o}{C_o + K_o}$$

The changes in maximum consumption rates of glucose and oxygen are described by first-order transfer functions:

$$\frac{dQ_{s,\max}}{dt} = \frac{1}{\tau_s} \cdot (Q_{s,\max,p} \cdot \frac{C_s}{K_n + C_s} - Q_{s,\max})$$

$$\frac{dQ_{o,\max}}{dt} = \frac{1}{\tau_o} \cdot (Q_{o,\max,p} \cdot \frac{C_o}{K_o + C_o} \cdot \frac{2 \cdot C_s + C_e}{K_m + 2 \cdot C_s + C_e} - Q_{o,\max})$$

The limited oxidation capacity determines the amount of glucose that can be oxidized:

$$Q_{s,ox} \leq \frac{Q_{o,\lim}}{Y_{os}}$$

If not all glucose consumed can be oxidized, then the excess glucose will be reduced:

$$Q_{s,red} = Q_s - Q_{s,ox}$$

The reduction of glucose results in ethanol production:

$$Q_{e,pr} = Y_{es} \cdot Q_{s,red}$$

The specific growth rate is dependent on the oxidative and reductive glucose metabolism:

$$\mu = Y_{xs,ox} \cdot Q_{s,ox} + Y_{xs,red} \cdot Q_{s,red}$$

The balances for glucose, biomass and ethanol during the A-stat are respectively:

$$\frac{dC_s}{dt} = D \cdot C_{s,in} - D \cdot C_s - Q_s \cdot C_x$$

$$\frac{dC_x}{dt} = -D \cdot C_x + \mu \cdot C_x$$

$$\frac{dC_e}{dt} = -D \cdot C_e + Q_{e,pr} \cdot C_x$$

The dissolved oxygen concentration was assumed to be 40% of air saturation all the time.

Appendix II

As an example of the effect of the rate of change of the yeast environment during A-stats, the glucose concentration in the bioreactor was calculated by assuming the absence of yeast. The glucose balance during A-stats is:

$$\frac{dC_s}{dt} = D \cdot C_{s,in} - D \cdot C_s$$

The dilution rate is changed according to the following equation:

$$D = D_0 + a \cdot t$$

Combining the above equations results in the differential equation:

$$\frac{dC_s}{dt} = (D_o + a \cdot t) \cdot (C_{s,in} - C_s)$$

The solution of this differential equation is given by:

$$C_s = C_{s,in} - (C_{s,in} - C_{s,0}) \cdot e^{-D_0 t - \frac{1}{2} a t^2}$$

Nomenclature

a	acceleration rate	h^{-2}
C	concentration	$mol.l^{-1}$
D	dilution rate	h^{-1}
K	saturation constant	$mol.l^{-1}$
K_m	substrate saturation constant for the induction of the synthesis of oxidation capacity	$mol.l^{-1}$
K_n	glucose saturation constant for the induction of the synthesis of glucose consumption capacity	$mol.l^{-1}$
Q	specific consumption rate	$mol.mol^{-1}.h^{-1}$
t	time	h
Y_{ij}	yield of component i on j	$mol.mol^{-1}$
τ	time constants with respect to the rate of change of the maximal consumption rates	h
μ	specific growth rate	h^{-1}

Subscripts:

e: ethanol; in: ingoing; lim: limited capacity; max: maximum; o: oxygen; ox: oxidation; p: plateau; pr: production; red: reduction; ref: reference value; s: glucose; x: biomass; 0: time zero.

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Chapter 5

Concomitant extracellular accumulation of α-keto acids and higher alcohols by *Zygosaccharomyces rouxii*

Abstract

α-Keto acids are key intermediates in the formation of higher alcohols, important flavour components in soy sauce, by the salt-tolerant yeast *Zygosaccharomyces rouxii*. Unlike most of the higher alcohols, the α-keto acids are usually not extracellularly accumulated. In order to accumulate the α-keto acids from the aspartate-derived amino-acid metabolism, the amino acids valine, leucine, threonine and methionine were exogenous supplied during batch and A-stat cultivations with (mutants of) *Z. rouxii*. It was shown that all α-keto acids from the aspartate-derived amino-acid metabolism, except α-ketobutyrate, could be extracellularly accumulated. In addition, it appeared from the concomitant extracellular accumulation of α-keto acids and higher alcohols that in *Z. rouxii*, valine, leucine and methionine were converted via similar Ehrlich pathways as in *Saccharomyces cerevisiae*. Unlike these amino acids, threonine was converted via both the Ehrlich and amino-acid biosynthetic pathways in *Z. rouxii*.

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Introduction

Zygosaccharomyces rouxii, a salt-tolerant yeast, is responsible for the higher-alcohols formation in soy sauce. The higher alcohols are important components of the soy-sauce flavour (Aoki and Uchida, 1991a). In order to control the production of the higher alcohols by *Z. rouxii*, much research has been done in the last decade (Aoki and Uchida, 1990; Aoki and Uchida, 1991a; Aoki and Uchida, 1991b; Yoshikawa et al., 1995). In most of these investigations, the biosynthetic pathways for the higher alcohols in *Z. rouxii* were assumed to be the same as those in *Saccharomyces cerevisiae* because the pathways in *Z. rouxii* have hardly been reported so far.

In *S. cerevisiae*, the biosynthesis of several higher alcohols is closely related to the aspartate-derived amino-acid metabolism. In this metabolism, aspartate is converted via homoserine into threonine or methionine (Figure 1) (Jones and Fink, 1982). Consequently, the threonine formed can be used for the isoleucine synthesis. For this, threonine is first deaminated by threonine deaminase (L-threonine hydro-lyase [deaminating]; EC 4.2.1.16) to the α-keto acid α-ketobutyrate, the precursor of the higher alcohol n-propanol (Webb and Ingraham, 1963). Hereafter, α-ketobutyrate is converted with pyruvate further towards isoleucine. This conversion is catalyzed by acetohydroxy acid synthase (acetolactate pyruvate-lyase [carboxylase]; EC 4.1.3.18). In a similar pathway, this enzyme also converts two molecules of pyruvate towards valine and leucine. The amino acids isoleucine, valine and leucine are finally formed by transamination of the α-keto acids respectively α-keto-β-methylvalerate, α-keto-isovalerate and α-keto-isocaproate, which can be converted into the higher alcohols, respectively active amyl alcohol, isobutyl alcohol and isoamyl alcohol as well. The higher alcohols n-propanol, active amyl alcohol, isobutyl alcohol and isoamyl alcohol are not only derived from the amino-acid biosynthetic pathways but also from the deamination or transamination of the extracellular amino acids (Ehrlich pathway) (Webb and Ingraham, 1963).

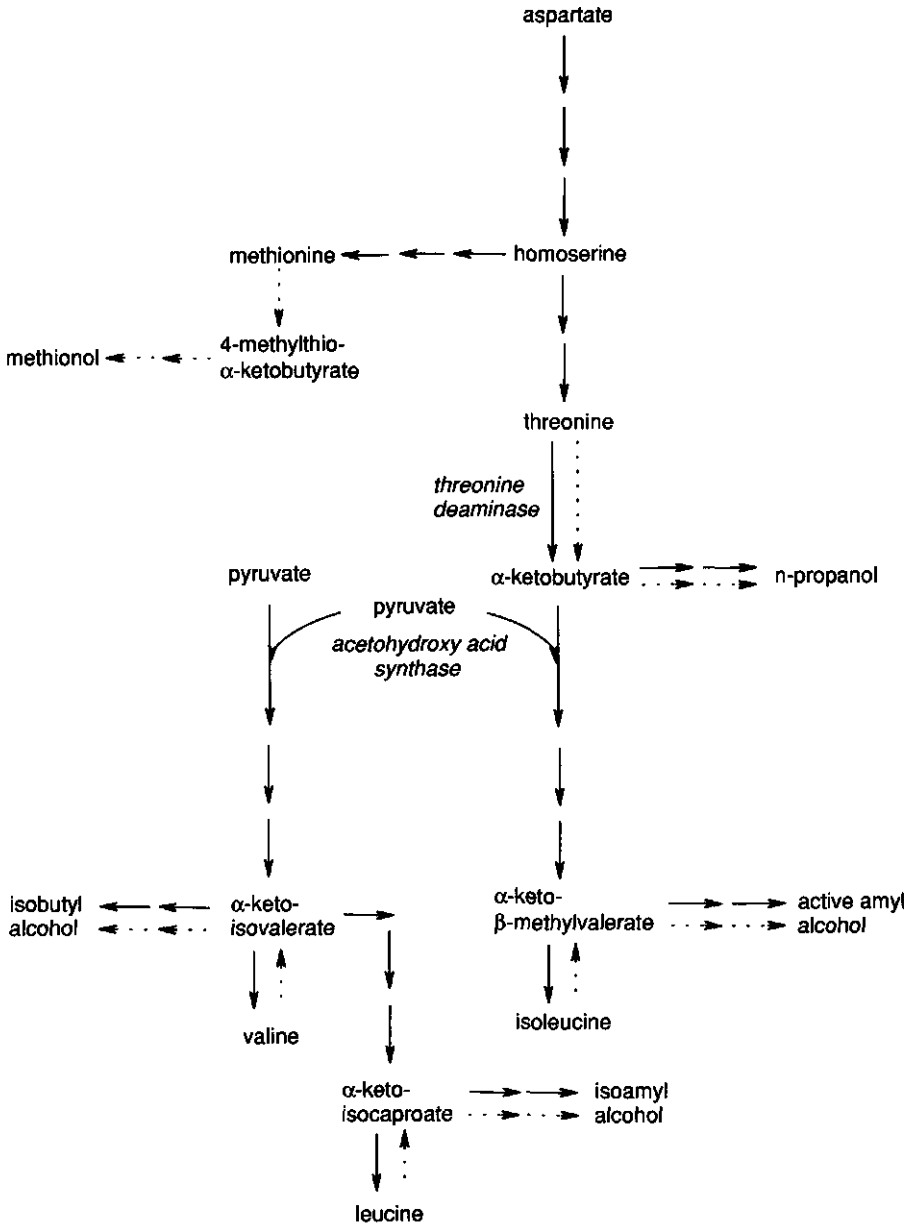

Figure 1. Aspartate-derived amino-acid metabolism in *S. cerevisiae*. Solid lines: amino-acid biosynthetic pathways; dotted lines: Ehrlich pathways.

The Ehrlich pathway is assumed to be the only pathway for producing the higher alcohol methionol (Aoki and Uchida, 1991b). In this pathway, methionol is produced from the α-keto acid 4-methylthio-α-ketobutyrate, which results from the uptake and transamination of methionine. In *S. cerevisiae*, also other pathways for the catabolism of valine, leucine and isoleucine exist (not shown) but in these pathways higher alcohols are not formed (Dickinson and Dawes, 1992; Derrick and Large, 1993).

From the above-described pathways it is clear that for the formation of higher alcohols in the aspartate-derived amino-acid metabolism of *S. cerevisiae*, the α-keto acids are key intermediates. These α-keto acids, unlike most of the higher alcohols, are usually not extracellularly accumulated. In previous studies, the extracellular accumulation of α-ketobutyrate with *Z. rouxii* failed because its formation and conversion rate was balanced all the time (van der Sluis et al., 2000a). In this work, we attempted to accumulate the other α-keto acids with *Z. rouxii* as well. To accomplish the accumulation of these α-keto acids their formation in *Z. rouxii* was increased by increasing the flow through the Ehrlich pathways or the amino-acid biosynthetic pathways. The flow through the Ehrlich pathways of *Z. rouxii* increases at increased concentrations of extracellular amino acids (Aoki and Uchida, 1991a; van der Sluis et al., 2000a). The flow through the isoleucine-biosynthetic pathway of the aspartate-derived amino-acid metabolism is increased in mutants of *S. cerevisiae* with resistance to thia-isoleucine, an isoleucine analogue (Fukuda et al., 1993).

In the research presented here, the accumulation of α-keto acids by *Z. rouxii* was studied. In addition, the concomitant accumulation of higher alcohols was determined in order to know more about the biosynthetic pathways for the higher alcohols in *Z. rouxii*. For this, batch cultivations were executed with the separate addition of valine and leucine. Furthermore, mutants of *Z. rouxii* were isolated with resistance to the growth-inhibitors threonine (van der Sluis et al., 2000a) and thia-isoleucine. These mutants were cultivated batch-wise on a medium with a mixture of threonine and methionine as only nitrogen sources. One of these mutants was also cultivated in an acceleration-stat (A-stat) in which the ratio of threonine to methionine in the medium was continuously changed.

Materials and methods

Yeast culture

Z. rouxii CBS 4021 cells, supplied by the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and maintained on a mixture of glycerol and skimmed milk at -80°C, were cultivated in 300 ml Erlenmeyer flasks, containing 100 ml defined medium, on a rotary shaker at 28°C and 200 rpm.

Medium

The medium used for *Z. rouxii* had the following composition per liter of demineralized water: 22 g glucose.$1H_2O$, 21 g citric acid.$1H_2O$, 5 g ammonium sulphate and 6.7 g Bacto Yeast Nitrogen Base without amino acids and ammonium sulphate (Difco). The pH of this medium was adjusted to a value of 5 using NaOH and the medium was filter-sterilized (0.2 μm filters).

Mutagenesis

Z. rouxii cells were used for mutagenesis, when they were in the exponential growth phase of the cultivation. Then the cells were harvested and concentrated by centrifugation for 10 minutes at 9,500 g and 5°C. After centrifugation, the cells were washed with a sterilized potassium-phosphate buffer (50 mM, pH 7) and centrifuged again. Hereafter, the concentrated yeast suspension obtained was treated with a 1-methyl-3-nitro-1-nitrosoguanidine solution (2 mg/ml) for 5 or 15 minutes, resulting in survival rates of about 15 or 3%, respectively.

Isolation of mutants

After the nitrosoguanidine treatment, *Z. rouxii* cells with resistance to threonine and thia-isoleucine were isolated on agar plates, which were supplemented with threonine (10 mM)

or thia-isoleucine (1 mM). For this, the plates were incubated at 25°C. The agar plates were made by adding 3.3 g glucose.1H_2O, 5 g ammonium sulphate, 1.1 g Bacto Yeast Nitrogen Base without amino acids and ammonium sulphate (Difco) and 15.6 g agar to 1 liter of demineralized water. Before this, the agar was autoclaved for 20 minutes at 120°C and all other components were filter-sterilized (0.2 μm).

Characterization of mutants

The isolated mutants were characterized by comparing the specific maximum growth rate with that of the wild-type. For this, the cells were cultivated in Erlenmeyer flasks as described above. The effect of additional threonine (10 mM) or thia-isoleucine (1 mM) on the specific maximum growth rate of both mutants and the wild-type was determined as well.

Accumulation of α-keto acids and higher alcohols during batch cultivations

The effect of amino acids on the accumulation of α-keto acids and higher alcohols by *Z. rouxii* was measured during batch cultivations in Erlenmeyer flasks, as described above. For this, 5,10 and 20 mM valine and leucine were separately added to the medium with ammonium sulphate. In addition, threonine (75 mM) or a mixture of threonine (75 mM) and methionine (7.5 mM) were used as nitrogen source instead of ammonium sulphate. During all these batches, the growth was measured as well.

Accumulation of α-keto acids and higher alcohols during A-stat cultivation

The effect of continuously changing ratio of threonine to methionine in the medium on the accumulation of α-keto acids and higher alcohols by *Z. rouxii* was measured during an A-stat cultivation. For the A-stat cultivation a starter culture of exponentially growing cells was made in Erlenmeyer flasks in which a mixture of threonine (45 mM) and methionine (5 mM) was used as nitrogen source instead of ammonium sulphate (see above). The starter culture of 100 ml was added to a bench-scale autoclavable bioreactor (height and

diameter of respectively 0.2 and 0.12 m) containing 0.75 liter of the medium with the same composition, except that citric acid was omitted.

The cultivation in the bioreactor was controlled by a Bio Controller (Applikon) and a Bioexpert (Applikon) acquired the on-line data, which were the temperature, pH, oxygen tension in the broth, foam level, stirrer speed, medium flow rate 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 48.6 l/h. The oxygen tension in the broth was kept above 30% of air saturation by controlling the stirrer speed, which was at least 350 rpm. The stirrer used was a six-blade Rushton turbine stirrer with a diameter of 45 mm. The concentration of oxygen and carbon dioxide in the outgoing air was measured on-line with respectively the Xentra 4100 Gas Purity Analyser and 1440 Gas Analyser (both from Servomex). The foam level was controlled by automatically adding a diluted (50 times) Antifoam B Silicone emulsion (J.T. Baker).

In the bioreactor, a continuous cultivation was started with a medium flow rate (F) of 0.022 $l.h^{-1}$, which was composed of two flows (F_{thr} and F_{met}) from different medium vessels (Figure 2). The two medium vessels contained both a medium without ammonium sulphate but one contained 50 mM threonine ($C_{thr,ves}$) and the other 50 mM methionine ($C_{met,ves}$) as sole nitrogen source instead. During this continuous cultivation, the flow of the medium with threonine was 0.02 l/h ($F_{thr,0}$) and that of the medium with methionine 0.002 l/h ($F_{met,0}$), resulting in a ratio of threonine to methionine of 45:5 (mM/mM) in the ingoing medium. On this medium, the culture was allowed to stabilize in order to obtain steady-state conditions, which were ascertained by constant carbon-dioxide production of the culture.

After this, the cultivation was continued with the A-stat cultivation. During the A-stat cultivation, the same ingoing medium flow rate was applied (0.022 $l.h^{-1}$) but the flow rates from the two medium vessels were changed in time in order to change the ratio of threonine to methionine in the ingoing medium from 45:5 to 0:50 (mM/mM). This was

done by adjusting the flow rate of the medium with threonine (F_{thr}) and that of the medium with methionine (F_{met}) according to the following equations, respectively:

$$F_{thr} = F_{thr,o} - a \cdot t$$

$$F_{met} = F_{met,o} + a \cdot t$$

For this, a constant acceleration rate (a) of 0.00075 l/h^2 was applied. This corresponds to the acceleration rate of 0.001 h^{-2}, which was previously shown to give a good estimation of steady-state yeast culture characteristics (van der Sluis et al., 2000b). During the A-stat cultivation the growth, the consumption of amino acids and the accumulation of α-keto acids and higher alcohols was followed.

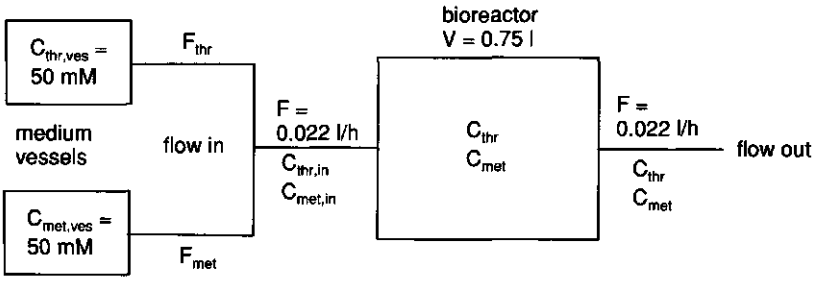

Figure 2. Set-up of the A-stat cultivation. Symbols are explained in nomenclature section.

Analyses

The growth of *Z. rouxii* was followed, by measuring the optical density at 610 nm (Pharmacia Biotech, Ultraspec). For this, samples were taken and diluted with demineralized water to obtain absorbance values less than 0.7. Below 0.7, the absorbance values were linearly related to biomass dry weight.

The accumulation of the α-keto acids α-ketobutyrate, α-keto-β-methylvalerate, α-keto-isovalerate, α-keto-isocaproate and 4-methylthio-α-ketobutyrate was determined by reversed-phase HPLC (Chromspher 5 C8, Chrompack) after derivatization with 2,4-dinitrophenylhydrazine (Buslig, 1982). The flow rate of the eluens, a mixture of demineralized water and acetonitrile (60:40), was 1.5 ml/min. For derivatization, 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 acetonitrile and after 1 hour, centrifuged. The supernatant was used for analysis after filtering (0.2 μm). This HPLC method could not separate α-ketobutyrate from 4-methylthio-α-ketobutyrate and α-keto-β-methylvalerate from α-keto-isocaproate. α-Ketobutyrate appeared to be separated from 4-methylthio-α-ketobutyrate, when both the ratio of demineralized water to acetonitrile in the eluens and the eluens flow rate were changed to 40:60 and 2.0 ml/min, respectively.

The accumulation of the higher alcohols n-propanol, active amyl alcohol, isobutyl alcohol and isoamyl alcohol was determined by GC. The samples were incubated at 60°C for 10 minutes. After incubation, a head-space sample was taken and collected in a cold trap (liquid nitrogen at -110°C). 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.

The consumption of the amino acids threonine and methionine was determined with the Biochrom 20 Amino Acid Analyser (Pharmacia). With this analyser, the amino acids were separated on a column of cation-exchange resin and after that, the amino acids reacted with a ninhydrin reagent to form coloured compounds. The coloured compounds were detected at two wavelengths, 440 and 570 nm.

Results and discussion

Separate addition of valine and leucine to batch cultures

In order to increase the flow through the Ehrlich pathways of the aspartate-derived amino-acid metabolism valine and leucine were used as additional nitrogen sources (Figure 1). This was done by separately adding 5, 10 and 20 mM valine and leucine to batch cultures of *Z. rouxii*, which contained ammonium sulphate (5 g/l). The additions of these amino acids appeared to have no effect on *Z. rouxii* growth (data not shown). The effect of valine and leucine on the accumulation of α-keto acids and higher alcohols is shown in respectively Figure 3 and 4.

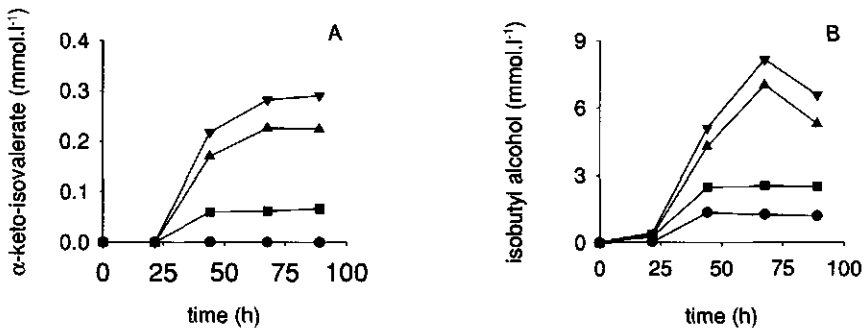

Figure 3. Effect of valine on the accumulation of α-keto acids and higher alcohols with *Z. rouxii* in batch cultures with ammonium sulphate (●: no addition; ■: 5 mM; ▲: 10 mM; ▼: 20 mM). A: α-keto acids; B: higher alcohols.

Figure 3 and 4 show that addition of valine and leucine (5 mM) triggered the accumulation of respectively α-keto-isovalerate and α-keto-β-methylvalerate plus α-keto-isocaproate and at the same time, increased the accumulation of respectively isobutyl alcohol and active amyl alcohol plus isoamyl alcohol. The addition of more valine and leucine (10 and 20 mM) resulted in more accumulation of these α-keto acids and higher alcohols. On the other hand, the accumulation of the other α-keto acids and higher alcohols was neither initiated nor affected by valine and leucine (data not shown).

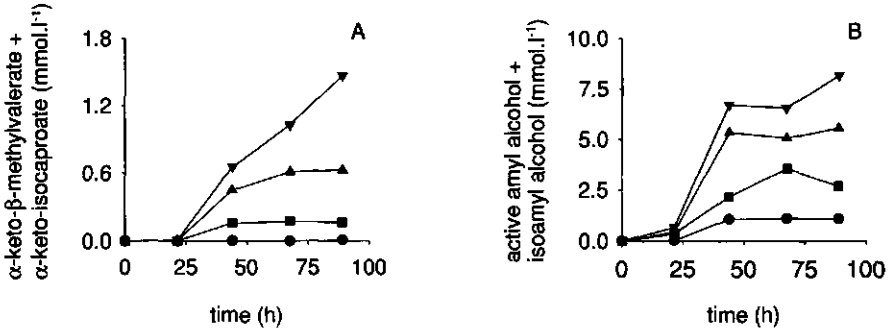

Figure 4. Effect of leucine on the accumulation of α-keto acids and higher alcohols with *Z. rouxii* in batch cultures with ammonium sulphate (●: no addition; ■: 5 mM; ▲: 10 mM; ▼: 20 mM). A: α-keto acids; B: higher alcohols.

Although we could not separate α-keto-β-methylvalerate from α-keto-isocaproate and active amyl alcohol from isoamyl alcohol, it seemed that in *Z. rouxii*, like in *S. cerevisiae* (Figure 1), the exogenous supplied valine and leucine were transaminated to respectively α-keto-isovalerate and α-keto-isocaproate, which were converted into the isobutyl alcohol and isoamyl alcohol, respectively. The extracellular accumulation of the transaminated derivatives from valine and leucine by *Z. rouxii* was also observed for nitrogen-starved cells of *S. cerevisiae* (Woodward and Cirillo, 1977).

Use of threonine in batch cultures

Another way to increase the flow through the Ehrlich pathways of the aspartate-derived amino-acid metabolism is by using threonine as additional nitrogen source (Figure 1). However, the addition of threonine (5 mM) severely inhibits *Z. rouxii* growth (van der Sluis et al., 2000a) by inhibiting the conversion of aspartate to homoserine (van der Sluis et al., 2000c). Therefore, we attempted to obtain mutants of *Z. rouxii* with resistance to threonine. For this, the wild-type was treated with the mutagent nitrosoguanidine for 5 or 15 minutes. Both treatment-times gave mutants, which could grow on agar plates in the presence of threonine (10 mM). Six of them were isolated and further characterized.

The effect of threonine on the growth of these mutants and the wild-type was determined during batch cultures on a medium with ammonium sulphate (5 g/l) with no additions or additional threonine (10 mM). In Table 1, the maximum specific growth rates of the mutants and wild-type during the different batches are compared. Table 1 shows that the specific growth rate of the mutants and the wild-type were comparable high on the medium without threonine, while the addition of threonine hardly affected the specific growth rate of the mutants but reduced that of the wild-type with 50%. This demonstrated that the mutants, unlike the wild-type, were resistant to the growth inhibition by threonine.

Table 1. Effect of addition of threonine on the maximum specific growth rate (h^{-1}) of *Z. rouxii* strains in a medium containing ammonium sulphate (5 g/l).

Additions	Strain						
	WT[a]	THR1[b]	THR2[b]	THR3[b]	THR4[b]	THR5[b]	THR6[b]
No additions	0.13	0.12	0.10	0.12	0.12	0.11	0.10
Threonine (10 mM)	0.06	0.10	0.12	0.12	0.11	0.11	0.10

[a] WT: wild-type.

[b] THR: mutant with resistance to threonine.

The effect of threonine on the accumulation of α-keto acids and higher alcohols by these mutants and the wild-type was determined as well. For this, batch growth experiments were done on a medium with ammonium sulphate (5 g/l) or threonine (75 mM) as sole nitrogen source. In Figure 5, the concentrations of the α-keto acids and higher alcohols in the stationary growth phase of the different batches are shown.

Figure 5A shows that the mutants and the wild-type did not accumulate any α-keto acids in the presence of ammonium and accumulated about the same amounts of n-propanol, isobutyl alcohol and active amyl alcohol plus isoamyl alcohol. Furthermore, the growth of the mutants and the wild-type on this medium were also comparable (data not shown). Therefore, it seemed that the mutants had more or less the same flow through the aspartate-derived amino-acid metabolism as the wild-type, otherwise an altered higher-

alcohols accumulation would have been expected as well. This means that the flow through the amino-acid biosynthetic pathways of the mutants was the same as that of the wild-type because, on this medium without amino acids, there is assumed to be no flow through the Ehrlich pathways.

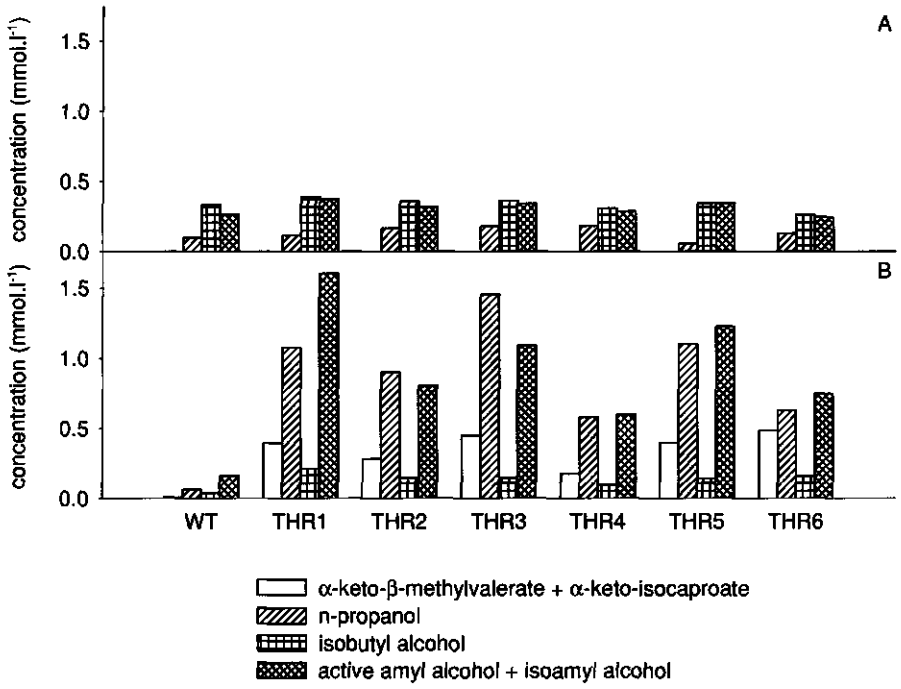

Figure 5. Effect of nitrogen source on the accumulation of α-keto acids and higher alcohols with threonine-resistant mutants of *Z. rouxii* in batch cultures. A: ammonium sulphate (5 g/l); B: threonine (75 mM). WT: wild-type; THR1..THR6: threonine-resistant mutants.

In addition, Figure 5 shows that the replacement of ammonium by threonine had an opposite effect on the mutants than on the wild-type. The mutants started to accumulate significant amounts of α-keto-β-methylvalerate plus α-keto-isocaproate and accumulated many times more of n-propanol and active amyl alcohol plus isoamyl alcohol, while the wild-type did not accumulate these α-keto acids and accumulated less of these higher alcohols. For both the mutants and the wild-type, the use of threonine did not result in

accumulation of α-ketobutyrate (data not shown) and resulted in a decreased accumulation of isobutyl alcohol. These results suggest that in *Z. rouxii* threonine was converted not only via the Ehrlich pathway to n-propanol but also via the isoleucine-biosynthetic pathway to α-keto-β-methylvalerate and active amyl alcohol (Figure 1). Most probably both pathways were used for this conversion in order to prevent that a toxic endogenous level of α-ketobutyrate is reached, as found in *Salmonella typhimurium* (LaRossa et al., 1987) and *Corynebacterium glutamicum* (Eggeling et al., 1987). This means that the flow through the isoleucine-biosynthetic pathway of *Z. rouxii* was increased in the presence of threonine, which is in agreement with the fact that the activity of acetohydroxy acid synthase increases by threonine (van der Sluis et al., 2000a).

In addition, the use of threonine instead of ammonium decreased the growth for the mutants and the wild-type (data not shown), which probably is the reason for the decreased accumulation of isobutyl alcohol. The decrease in *Z. rouxii* growth confirmed that ammonium is favourable (van der Sluis et al., 2000c). However, the growth of the mutants was less decreased by threonine than that of the wild-type (data not shown) because of the higher resistance of the mutants towards threonine. The difference in growth between the mutants and wild-type in the presence of threonine explains the large difference in accumulated α-keto acids and higher alcohols.

Thia-isoleucine-resistant mutants in batch cultures

Thia-isoleucine-resistant mutants of *S. cerevisiae* have an increased flow through the isoleucine-biosynthetic pathway of the aspartate-derived amino-acid metabolism because of a threonine deaminase with a decreased feed-back sensitivity to isoleucine (Figure 1) (Fukuda et al., 1993). This increased flow causes that these mutants accumulate three to four times more active amyl alcohol and a little bit more n-propanol than the wild-type. In order to obtain thia-isoleucine-resistant mutants of *Z. rouxii*, the wild-type was treated with nitrosoguanidine for 5 and 15 minutes resulting in mutants, which could grow on agar plates in the presence of 1 mM thia-isoleucine.

The growth of six of these mutants and the wild-type was followed in batch cultures, containing ammonium sulphate (5 g/l) with no additions or additional thia-isoleucine (1 mM). In Table 2, the effect of thia-isoleucine on the maximum specific growth rates of the mutants and the wild-type is shown. It appears from Table 2 that the mutants had more or less the same specific growth rate as the wild-type on the medium with no additions. As expected the addition of thia-isoleucine dramatically decreased the specific growth rate of the wild-type, while that of the mutants was only slightly decreased. This clearly showed that the mutants were much more resistant to thia-isoleucine than the wild-type.

Table 2. Effect of addition of thia-isoleucine on the maximum specific growth rate (h^{-1}) of *Z. rouxii* strains in a medium containing ammonium sulphate (5 g/l).

Additions	Strain						
	WT[a]	TI1[b]	TI2[b]	TI3[b]	TI4[b]	TI5[b]	TI6[b]
No additions	0.13	0.14	0.10	0.11	0.10	0.10	0.11
Thia-isoleucine (1 mM)	0.01	0.11	0.09	0.08	0.08	0.09	0.11

[a] WT: wild-type.

[b] TI: mutant with resistance to thia-isoleucine.

The accumulation of α-keto acids and higher alcohols by these mutants was also compared with that of the wild-type during batch cultures with ammonium sulphate (5 g/l) as sole nitrogen source. In Figure 6A, the concentrations of the α-keto acids and higher alcohols, measured in the stationary phase of these batches, are shown. It can be seen in Figure 6A that both *Z. rouxii* mutants and wild-type did not accumulate any α-keto acids and accumulated more or less the same amounts of n-propanol and active amyl alcohol plus isoamyl alcohol. From this it appeared that the mutants were not really different from the wild-type, although some of the mutants showed an increased isobutyl-alcohol accumulation, which we could not explain. The absence of an increased accumulation of n-propanol and active amyl alcohol demonstrated that our thia-isoleucine-resistant mutants of *Z. rouxii* reacted different from those of *S. cerevisiae* (Fukuda et al., 1993). Based on this and because the *Z. rouxii* mutants did not have a decreased

feed-back sensitivity to isoleucine as well (data not shown), it seemed that the flow through the isoleucine-biosynthetic pathway of the *Z. rouxii* mutants was not increased.

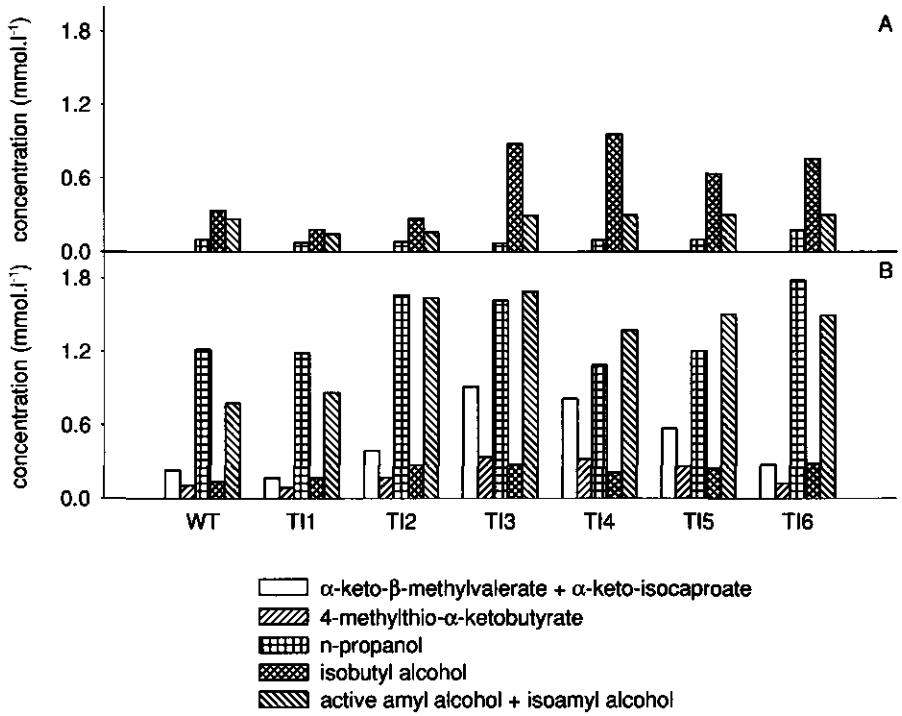

Figure 6. Effect of nitrogen source on the accumulation of α-keto acids and higher alcohols with thia-isoleucine-resistant mutants of *Z. rouxii* in batch cultures. A: ammonium sulphate (5 g/l); B: threonine (75 mM) plus methionine (7.5 mM). WT: wild-type; TI1..TI6: thia-isoleucine-resistant mutants.

To increase the flow through the Ehrlich pathways of these *Z. rouxii* mutants and wild-type, threonine (75 mM) and methionine (7.5 mM) were used as nitrogen source instead of ammonium during batch cultivations (Figure 1). The addition of methionine also improves the growth of *Z. rouxii* in the presence of threonine (van der Sluis et al., 2000c). In Figure 6B the α-keto acids and higher alcohols accumulated in the stationary growth phase of these batches, are shown. Figure 6 shows that the replacement of ammonium by the mixture of threonine and methionine triggered the accumulation of α-keto-β-

methylvalerate plus α-keto-isocaproate and 4-methylthio-α–ketobutyrate, increased the accumulation of n-propanol and active amyl alcohol plus isoamyl alcohol and decreased the accumulation of isobutyl alcohol for both the mutants and the wild-type. From these results and the earlier results, it seemed that threonine was converted into n-propanol, α-keto-β-methylvalerate and active amyl alcohol, while methionine was transaminated via the Ehrlich pathway into 4-methylthio-α-ketobutyrate (Figure 1). In *S. cerevisiae*, the transaminated derivatives from methionine are also extracellularly accumulated (Woodward and Cirillo, 1977). Based on the Ehrlich pathway for methionine in *S. cerevisiae* (Figure 1), we expected methionol accumulation in these batches as well, but this did not happen.

Furthermore, Figure 6B shows that in the presence of the mixture of threonine and methionine, most of the mutants accumulated significant more of the α-keto acids and higher alcohols than the wild-type. This might have been caused by the fact that the growth of the mutants was slightly better than that of the wild-type on this medium (data not shown), but the reason for this remains unclear.

Thia-isoleucine-resistant mutant in A-stat culture

In order to investigate the Ehrlich pathway towards methionol in *Z. rouxii* further, the flow through this pathway was increased by adding more methionine to the medium (Figure 1) (Aoki and Uchida, 1991b). This was done by continuously changing the ratio of threonine to methionine in the ingoing medium (without ammonium), from 45:5 to 0:50 (mM/mM) during an A-stat cultivation with mutant TI3 (Figure 7A). For this the A-stat cultivation was used because this cultivation method is much more informative than other methods (Paalme et al., 1995; van der Sluis et al., 2000b). In addition, mutant TI3 was chosen because it showed the highest accumulation of 4-methylthio-α-ketobutyrate (Figure 6B), the precursor of methionol (Figure 1).

The effect of changing the concentration of threonine and methionine in the ingoing medium on their concentrations in the bioreactor was calculated by assuming absence of consumption of these amino acids by the yeast (see Appendix) (Figure 7A). Figure 7A

shows that for reaching a threonine to methionine ratio of 25:25 (mM/mM) in the bioreactor without consumption by the yeast more than 30 hours is needed, while that ratio was already reached in the ingoing medium within 12 hours. This shows that the change in threonine and methionine concentration in the bioreactor was delayed compared to that in the ingoing medium.

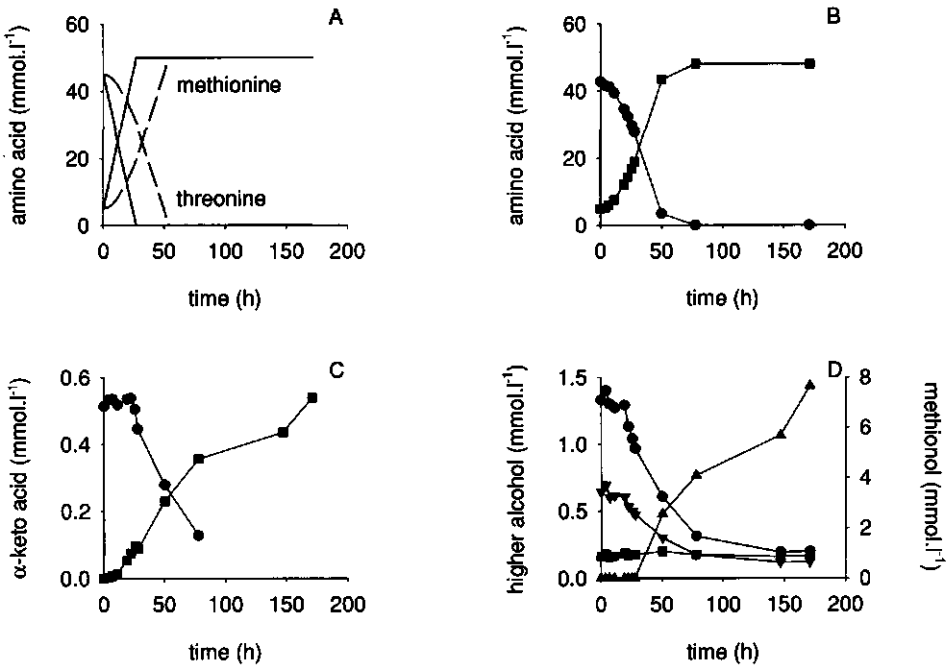

Figure 7. Effect of changing ratio threonine to methionine on the accumulation of α-keto acids and higher alcohols by a thia-isoleucine-resistant mutant of *Z. rouxii* during A-stat cultivation. A: calculated amino-acid concentrations (solid line: ingoing medium; dashed line: bioreactor without consumption by yeast). B: calculated amino-acid consumption (●: threonine; ■: methionine). C: α–keto acids (●: α-keto-β-methylvalerate plus α-keto-isocaproate; ■: 4-methylthio-α-ketobutyrate). D: higher alcohols (●: n-propanol; ■: isobutyl alcohol; ▼: active amyl alcohol plus isoamyl alcohol; ▲: methionol).

The consumption of threonine and methionine during the A-stat cultivation was determined by subtracting the measured concentrations from the ones calculated by assuming absence of consumption by the yeast (Figure 7B). From Figure 7B it appears

that the amino-acid consumption by this mutant TI3 during this A-stat cultivation followed the change from threonine to methionine in the bioreactor very well.

The effect of the change in threonine to methionine ratio on the accumulation of α-keto acids and higher alcohols by this mutant was measured as well (respectively Figure 7C and 7D). These figures clearly show that the accumulation of some of these compounds, but not isobutyl alcohol, was affected by the changing ratio threonine to methionine. At a decreasing threonine to methionine ratio, the mutant gave a decreasing accumulation of α-keto-β-methylvalerate plus α-keto-isocaproate, n-propanol and active amyl alcohol plus isoamyl alcohol, and started to increasingly accumulate 4-methylthio-α-ketobutyrate and methionol. From this, it appeared once again, that threonine was converted into n-propanol, α-keto-β-methylvalerate and active amyl alcohol, while 4-methylthio-α-ketobutyrate and methionol were derived from methionine (Figure 1). The fact that methionol was only accumulated when methionine was added, confirmed that methionol is formed via the Ehrlich pathway only (Aoki and Uchida, 1991b).

Conclusions

In this study the concomitant extracellular accumulation of α-keto acids and higher alcohols by *Z. rouxii* was investigated. From this, it appeared that in *Z. rouxii*, like in *S. cerevisiae*, exogenous supplied valine, leucine and methionine were converted via Ehrlich pathways to respectively α-keto-isovalerate and isobutyl alcohol, α-keto-isocaproate and isoamyl alcohol, and 4-methylthio-α-ketobutyrate and methionol. It was confirmed that methionol is formed only via the Ehrlich pathway in *Z. rouxii*, while the other higher alcohols are formed via the amino-acid biosynthetic pathways as well. In addition, it appeared that exogenous supplied threonine was converted via the Ehrlich pathway in *Z. rouxii* to propanol and via the isoleucine-biosynthetic pathway to α-keto-β-methylvalerate and active amyl alcohol. Unlike the above-mentioned α-keto acids, it was not possible to extracellularly accumulate α-ketobutyrate, an intermediate of these threonine conversion pathways.

Appendix

The calculation of the threonine and methionine concentrations in the bioreactor during the A-stat with a changing ratio of these amino acids in the ingoing medium is shown below. For this calculation, the A-stat set-up as given in Figure 2 was used and absence of consumption of these amino acids by the yeast was assumed. The amino-acid balance during the A-stat without consumption by yeast is:

$$\frac{dC_{ami}}{dt} = \frac{F}{V} \cdot (C_{ami,in} - C_{ami})$$

The medium flow rate during the A-stat is composed of a flow from a medium vessel with threonine and one from a medium vessel with methionine as nitrogen source:

$$F = F_{thr} + F_{met}$$

The threonine and methionine flow rates are changed according to the following equations, respectively:

$$F_{thr} = F_{thr,0} - a \cdot t$$

$$F_{met} = F_{met,0} + a \cdot t$$

For the concentration of respectively threonine and methionine in the ingoing medium holds:

$$C_{thr,in} = \frac{(F_{thr,0} - a \cdot t)}{F_{in}} \cdot C_{thr,ves}$$

$$C_{met,in} = \frac{(F_{met,0} + a \cdot t)}{F_{in}} \cdot C_{met,ves}$$

Combining the above equations and applying the Laplace transformation gives the following equations for threonine and methionine, respectively:

$$C_{thr}(s) = \frac{1}{\tau \cdot s + 1} \cdot \frac{\frac{F_{thr,0} \cdot C_{thr,ves}}{F}}{s} - \frac{1}{\tau \cdot s + 1} \cdot \frac{\frac{a \cdot t \cdot C_{thr,ves}}{F}}{s^2} + \frac{\tau}{\tau \cdot s + 1} \cdot C_{thr,0}$$

$$C_{met}(s) = \frac{1}{\tau \cdot s + 1} \cdot \frac{\frac{F_{met,0} \cdot C_{met,ves}}{F}}{s} + \frac{1}{\tau \cdot s + 1} \cdot \frac{\frac{a \cdot t \cdot C_{met,ves}}{F}}{s^2} + \frac{\tau}{\tau \cdot s + 1} \cdot C_{met,0}$$

Based on these Laplace equations, the concentrations of respectively threonine and methionine in the bioreactor during the A-stat without yeast can be calculated as follows:

$$C_{thr} = \frac{F_{thr,0} \cdot C_{thr,ves}}{F} \cdot (1 - e^{\frac{-t}{\tau}}) - \frac{a \cdot t \cdot C_{thr,ves}}{F} \cdot (t + \tau \cdot e^{\frac{-t}{\tau}} - \tau) + e^{\frac{-t}{\tau}} \cdot C_{thr,0}$$

$$C_{met} = \frac{F_{met,0} \cdot C_{met,ves}}{F} \cdot (1 - e^{\frac{-t}{\tau}}) + \frac{a \cdot t \cdot C_{met,ves}}{F} \cdot (t + \tau \cdot e^{\frac{-t}{\tau}} - \tau) + e^{\frac{-t}{\tau}} \cdot C_{met,0}$$

Nomenclature

a	acceleration rate	$l.h^{-2}$
C	concentration	$mol.l^{-1}$
F	flow rate	$l.h^{-1}$
s	Laplace variable	
t	time	h
V	volume	l
τ	residence time	h

Subscripts:

ami: amino acid; in: ingoing; thr: threonine; met: methionine; ves: vessel with medium; 0: time zero.

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Chapter 6

Immobilized soy-sauce yeasts: development and characterization of a new polyethylene-oxide support

Abstract

Entrapment of cells in alginate gel is a widely used mild immobilization procedure. However, alginate gel is not very suitable for use in long-term continuous soy-sauce processes because alginate is sensitive to abrasion and chemically unstable towards the high salt content of soy-sauce medium. Therefore, a chemically crosslinked polyethylene-oxide gel was used instead. The disadvantage of this gel was that due to the crosslinking reaction the viability of the cells after immobilization was poor.

For this reason, a new mild procedure for immobilizing soy-sauce yeasts in polyethylene-oxide gel was developed, resulting in high survival percentages of the soy-sauce yeasts *Zygosaccharomyces rouxii* and *Candida versatilis*. This newly developed polyethylene-oxide gel, unlike alginate gel, appeared not to be sensitive to abrasion, even in the presence of high salt concentrations. Therefore, we concluded that this newly developed polyethylene-oxide gel is more suitable than alginate gel for use as immobilization material in long-term processes with a high salt content, like soy-sauce processes.

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Introduction

The soy-sauce yeasts, *Zygosaccharomyces rouxii* and *Candida versatilis*, are important flavour producers in soy-sauce processes. In these processes, *Z. rouxii* produces ethanol and other flavour components like 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (Nunomura et al., 1976), while *C. versatilis* produces phenolic compounds, like 4-ethylguaiacol (Röling, 1995). These phenolic compounds give the characteristic flavour to soy sauce.

In order to shorten the process time, to increase the production efficiency and to make continuous operation of soy-sauce processes easier, much attention has been paid in the last decade to the application of immobilized soy-sauce yeasts (Osaki et al., 1985; Hamada et al., 1989; Hamada et al., 1990a; Hamada et al., 1990b; Horitsu et al., 1990; Horitsu et al., 1991; Iwasaki et al., 1991; Hamada et al., 1992; Motai et al., 1993). It was shown that the immobilization of soy-sauce yeasts considerably decreased the total time required for the soy-sauce process. In most of these investigations, alginate gel was used as immobilization material because immobilization in this gel is a mild and convenient method that can be scaled up.

However, alginate gel has the disadvantage of mechanical weakness (Horitsu et al., 1990; Muscat et al., 1996) and furthermore, it is chemically unstable towards high salt concentrations (Martinsen et al., 1989; Horitsu et al., 1990). The latter is also expected for alginate gel beads in soy-sauce medium in which the salt content is high (Horitsu et al., 1990). Because of these disadvantages, a continuous long-term process with soy-sauce yeasts immobilized in alginate gel is not feasible (Horitsu et al., 1990). For this reason, they developed a ceramic carrier to be used as support material instead. In this work, we investigated the possibility of replacing alginate with a chemically crosslinked polyethylene-oxide gel, that we used before for other purposes (Leenen et al., 1996).

Leenen et al. (1996) made a polyethylene-oxide gel by adding a crosslinker to a mixture of a prepolymer solution and a cell suspension. This polyethylene-oxide-based gel has better characteristics than alginate gel. The gel does not dissolve in the presence of salt and is

insensitive to abrasion; therefore, the durability of this polyethylene-oxide gel is expected to be high (Leenen et al., 1996). However, the crosslinking reaction during the immobilization process is toxic which results in low survival of the cells. Leenen et al. (1996) estimated that 0.5% of their *Nitrosomonas europaea* cells survived during the immobilization process. Similarly low survival percentages were measured by Tanaka et al. (1996) for nitrifying sludge immobilized in a polyethylene-glycol gel. In a preliminary experiment, we did not even observe any survival of the soy-sauce yeast *Z. rouxii* at all, after immobilizing in the polyethylene-oxide gel according to the procedure as described by Leenen et al. (1996).

Therefore, in the research described here, we directed our efforts to improving this immobilization procedure for *Z. rouxii* cells. We determined the effect of the concentration of the prepolymer solution and the crosslinker on the activity of *Z. rouxii*. The effect of the contact time with the crosslinker on the activity of *Z. rouxii* was determined as well. Subsequently, we developed a new, mild immobilization procedure for the soy-sauce yeasts in polyethylene-oxide gel, which resulted in high survival percentages of *Z. rouxii* and *C. versatilis*.

This new gel was investigated further in rheological studies. The formation of the polyethylene-oxide gel network was followed in time by measuring the storage modulus. This modulus is a measure for the elastic energy stored in the material (Whorlow, 1992); it will increase during gelation and level off when the gelation is completed (Verheul et al., 1998). Furthermore, the sensitivity of the new polyethylene-oxide and alginate gel to abrasion was studied with oscillation experiments as was done before by Martins dos Santos et al. (1997). Oscillation experiments show the fatigue of gel materials, which is likely to be related to the abrasion sensitivity of these materials in bioreactors (Martins dos Santos et al., 1997). In addition, the salt effect on the abrasion sensitivity for both gels was determined. Finally, the absence of fracture during a large-deformation experiment showed the flexibility of the new polyethylene-oxide gel.

Materials and methods

Yeast cultures

Z. rouxii CBS 4021 and *C. versatilis* CBS 4019 were cultivated in 300 ml Erlenmeyer flasks, containing 100 ml medium, on a rotary shaker (Gallenkamp, Orbital Incubator) at 28°C and 200 rpm.

Media

The medium used for *Z. rouxii* had the following composition per liter of demineralized water: 40 g glucose.$1H_2O$, 5 g peptone and 5 g yeast extract. The components were separately autoclaved for 20 minutes at 120°C. For *C. versatilis,* a defined medium with the following composition per liter of demineralized water was used: 22 g glucose.$1H_2O$, 21 g citric acid.$1H_2O$ and 6.7 g Bacto Yeast Nitrogen Base without amino acids (Difco). The pH of this medium was adjusted to 5 using NaOH and the medium was filter-sterilized (Nalgene, 0.2 μm filters).

Preparation of yeast suspension

The yeast cells were concentrated by centrifugation for 15 minutes at 9,500 g and 4°C, when they were in the early stationary growth phase. After centrifugation, the concentrated cell suspension was washed with sterilized PBS-buffer (pH 7.4) of the following composition per liter of demineralized water: 8.2 g NaCl, 1.9 g $Na_2HPO_4.2H_2O$ and 0.3 g $NaH_2PO_4.2H_2O$. Hereafter, the yeast suspension was centrifuged again. The pellet obtained was used for the determination of the toxicity of the prepolymer solution and crosslinker or for immobilization, after appropriate dilution in PBS-buffer.

Determination of the toxicity of the prepolymer solution and crosslinker

The toxicity of the prepolymer solution, containing carboxy-methyl-cellulose and polyethylene-oxide, and crosslinker (carbodiamide) was determined by separately incubating them for 1 hour with a *Z. rouxii* suspension and hereafter, estimating the survival percentage with the respiration-activity assay. The toxicity of the crosslinker for *Z. rouxii* was determined further as a function of the contact time with the yeast and the crosslinker yeast ratio. For this, both respiration-activity assay and colony count on agar plates were used.

Immobilization procedure

The procedure for making a polyethylene-oxide gel (Holland Biomaterials Group, Enschede, The Netherlands) as described by Leenen et al. (1996) was adapted. In this work, the carbodiamide (crosslinker) was added to only a carboxy-methyl-cellulose solution and not to a mixture of prepolymer solution, containing both carboxy-methyl-cellulose and polyethylene-oxide, and yeast suspension. For this, 0.125 ml crosslinker solution (0.1 mg/µl) and 4.5 ml carboxy-methyl-cellulose solution (5 mg/ml) were mixed and allowed to react for 5 minutes. Then, a mixture of 0.4 ml yeast suspension and 0.475 ml polyethylene-oxide solution (100 mg/ml) was added and thoroughly mixed. After 1 hour of gelation, the cylindrical gel was washed with PBS-buffer in order to remove a possible excess of crosslinker.

Alginate gel was made by mixing 5 ml of a 6% (w/v) alginate solution (Protanal LF 10/60, high guluronic acid content, Pronova Biopolymers, Norway) with 1 ml yeast suspension. Hereafter, 4 ml of a 12.5% (w/v) $CaCl_2$ solution was added to make a cylindrical gel.

To determine the survival percentage with the respiration-activity assay after immobilization, both polyethylene-oxide and alginate gels were cut into small pieces (about 1 mm) in order to avoid that the respiration-activity assay was controlled by diffusion (van Ginkel et al., 1983). For the rheological tests, the gels were made without yeast.

Alginate gel beads with a diameter of about 1 mm were made by dropwise extrusion of a 3% (w/v) alginate solution through a hollow needle using air pressure. The droplets were collected in a stirred 5% (w/v) $CaCl_2$ solution and left in there for at least 24 hours for hardening. The resulting gel beads were used to evaluate their chemical stability towards a soy-sauce medium. For this, 100 ml gel beads were added to 500 ml soy-sauce medium, containing 13% (w/v) NaCl. The medium with beads was stored at room temperature for three days and during that time, the force to fracture these beads was measured.

Respiration-activity assay

The respiration-activity assay was used in order to determine the survival percentage after incubating the cells with prepolymer solution and crosslinker or after immobilizing them. For this, the oxygen consumption rate of the incubated or immobilized cells and that of untreated cells was measured. From this, survival percentages were calculated. The oxygen consumption rate was measured at 30°C in a Biological Oxygen Monitor (BOM, Yellow Springs Instruments, Ohio, USA). To a 24 cm^3 vessel, containing PBS-buffer, a known amount of cells (free or immobilized) was added and aerated for 10 minutes. After aeration, the vessel was sealed with an oxygen electrode (model 5331, Yellow Springs Instruments, Ohio, USA) and the decrease in oxygen concentration was recorded as a function of time. The initial oxygen consumption rate was used to calculate the respiration activity.

Colony count on agar plates

The colony count on agar plates was used to determine the survival percentage after incubating the cells with crosslinker. For this, the colony count from a yeast suspension incubated with crosslinker was divided by that from an untreated yeast suspension. For this, an appropriate dilution of the yeast suspension in sterilized PBS-buffer was made and added to agar plates. After the yeast was grown on the plates for two weeks at 25°C, the number of colonies was counted. The agar plates were made by adding 4.0 g

glucose.1H_2O, 1.0 g Bacto Yeast Nitrogen Base without amino acids (Difco), 20 g agar to 1 l demineralized water. Before this the agar was autoclaved for 20 minutes at 120°C and the glucose and Bacto Yeast Nitrogen Base were filter-sterilized (Nalgene, 0.2 μm filters).

Rheological tests

The force to fracture beads was measured in order to evaluate the chemical stability of alginate gel beads towards a soy-sauce medium. For this, at different points of time during the incubation, a sample was taken and 12 gel beads from this sample were separately compressed until fracturing. This compression was done at room temperature with a tension-compression device (Overload Dynamics Table model S100) fitted with a 50 N load cell. On the fixed bar of this device, a single gel bead was placed on a wet filter paper in order to prevent dehydration. The gel bead was compressed using the moving bar at a fixed compression speed of 60 mm/min. The force needed for fracturing the bead was recorded.

The storage modulus during formation of the polyethylene-oxide gel network was measured with a CVO Rheometer System (Bohlin Instruments). This system was used with a concentric-cylinder measuring geometry, which consists of a rotating inner cylinder located in a fixed outer cylinder. In the annular gap between the two cylinders, the polyethylene-oxide gel was made according to the procedure described above. During gel formation, a sinusoidal oscillation was applied to the inner cylinder. The frequency of the oscillation applied was 0.1 Hz. Through the oscillation of the driving system, the inner cylinder will also oscillate sinusoidal but at a smaller amplitude and with a phase difference, if the gel has a linear visco-elastic behaviour. Linear visco-elastic behaviour means that the storage modulus is independent of the magnitude of deformation and deformation rate applied (Whorlow, 1992). This will be the case when the amplitude of the oscillation is small enough (Roefs et al., 1990; Whorlow, 1992). In our case, the amplitude was kept low enough to ensure linear behaviour. From the amplitude and phase difference, some rheological properties like the storage modulus can be calculated (Roefs et al., 1990; Whorlow, 1992). The storage modulus was measured at 20°C, and during the

measurement the gelling solution was covered with paraffin oil in order to prevent drying out.

The CVO Rheometer System was also used for a large-deformation experiment, after the gelation was complete. During this experiment the storage modulus was measured as a function of the relative shear deformation in order to determine the maximum relative deformation at fracture.

Oscillation compression tests, as described by Martins dos Santos et al. (1997), were applied to gel cylinders in order to evaluate the fatigue of the immobilization materials. For this, polyethylene-oxide and alginate gel samples were made according to the procedures as described above. After gelation the samples were cut into gel cylinders of 20 by 20 mm. At least two cylinders of each sample were tested. The oscillation compression tests were done at room temperature with the same tension-compression device as we used to measure the force to fracture gel beads. For the compression tests this device was fitted with a 2000 N load cell. The gel cylinders, which were placed in a beaker containing PBS-buffer in order to avoid dehydration, were 6 mm compressed with the moving bar at a compression speed of 50 mm/min. The applied relative deformation was small (0.3) in order to stay within the linear region for both gels. After compression the moving bar returned to its original position. This oscillation was repeated 1000 times for one gel cylinder. During these oscillations the resistance to compression in time was determined. Furthermore, the salt effect on the fatigue of both polyethylene-oxide and alginate gel was determined by incubating the gel cylinders for at least 24 hours in a modified PBS-buffer containing 12.5% (w/v) NaCl and doing the compression tests with the cylinders placed in the same modified buffer.

Results and discussion

Chemical stability of alginate gel beads towards soy-sauce medium

Horitsu et al. (1990) expected that the high salt content of the soy-sauce medium would adversely affect the chemical stability of alginate gel beads. Therefore, we determined the chemical stability of alginate gel beads towards soy-sauce medium by following the force to fracture the alginate beads during incubation in this medium. If the stability of the alginate gel is adversely affected by the salt, the gel will start to dissolve and the force to fracture will decrease because of a lowered gel concentration (Martinsen et al., 1989). For this experiment alginate with a high guluronic acid content was used because this kind of alginate gel has the highest tolerance to salt (Martinsen et al., 1989; Smidsrød and Skjåk-Bræk, 1990).

In Figure 1 the effect of the incubation time in soy-sauce medium on the mean force at fracture of alginate gel beads is shown. It appears from this figure that, after about 24 hours of incubation, this mean force was reduced by almost 50% and became more or less constant. This reduction in force was accompanied by some white colouring in the medium.

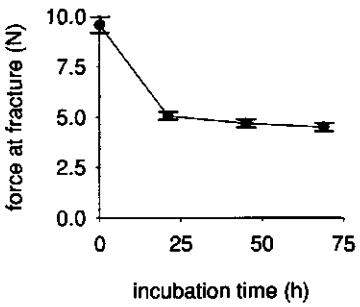

Figure 1. Effect of the incubation time in soy-sauce medium on the mean force at fracture of alginate gel beads. The soy-sauce medium contained 13% (w/v) NaCl. To 500 ml of this medium, 100 ml gel beads were added. Error bars show the 95% confidence interval of the measurements.

These observations show that alginate gel was partly dissolved in the soy-sauce medium. Because the soy-sauce medium we used had a lower salt content (13%) than the conventional one (16-18%) (Osaki et al., 1985; Röling et al., 1995), it is likely that alginate gel will dissolve even more in the latter. From this, we concluded that alginate is chemically unstable towards soy-sauce medium.

Toxicity of prepolymer solution and crosslinker

In a preliminary experiment, we did not observe any survival of the soy-sauce yeast *Z. rouxii* after immobilizing in the chemically crosslinked polyethylene-oxide gel that we used before (Leenen et al., 1996). For this reason, we studied the toxicity of the crosslinking reaction of this gel. The polyethylene-oxide gel was made by mixing a carbodiamide (crosslinker) with a mixture of a prepolymer solution, containing carboxy-methyl-cellulose and polyethylene-oxide, and a cell suspension (see materials and methods section). We separately determined the effect of the prepolymer solution and crosslinker on the activity of *Z. rouxii*. This was done by incubating *Z. rouxii* cells with the prepolymer solution or crosslinker for 1 hour. This incubation time was chosen because the contact time of the yeast with prepolymer solution or crosslinker during the immobilization process needs to be 1 hour. After incubating the survival was determined using respiration-activity assays. From this (data not shown), it appeared that the prepolymer solution was not toxic at all for *Z. rouxii* while the crosslinker was very toxic. This toxic effect of the crosslinker was not found by Leenen et al. (1996) for *N. europaea* cells.

The toxicity of the crosslinker was studied further by determining the effect of the contact time with crosslinker and crosslinker yeast ratio on the activity of *Z. rouxii*. For this, both the respiration-activity assay and the colony count on agar plates were used. The contact time and crosslinker yeast ratio applied had the same order of magnitude as during the preliminary experiment (respectively 1 hour and 2 $g.g^{-1}$).

The effect of the contact time and crosslinker yeast ratio on the survival of *Z. rouxii*, as determined with the respiration-activity assay, can be seen in Figure 2. This figure shows that the survival of *Z. rouxii* cells strongly decreased with an increase in contact time and

crosslinker yeast ratio. Similar results were obtained with the colony count on agar plates (data not shown). It can be seen in Figure 2A that, after about 1 hour, less than 20% of the *Z. rouxii* cells did survive at a crosslinker yeast ratio of 0.4 $g.g^{-1}$. Figure 2B shows that less than 30% of the *Z. rouxii* cells was still alive after incubating for 50 minutes at a crosslinker yeast ratio of 2 $g.g^{-1}$. Hence, an even lower survival percentage can be expected when *Z. rouxii* cells are incubated for about 1 hour at a crosslinker yeast ratio of 2 $g.g^{-1}$ like in the preliminary immobilization experiment. For this reason, the severe toxic effect of the crosslinker seems to be a good explanation for the absence of survival of *Z. rouxii* cells after immobilization in the polyethylene-oxide gel.

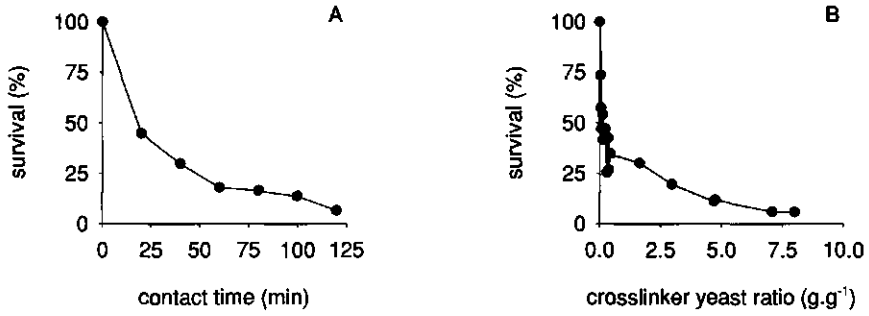

Figure 2. Effect of the contact time with crosslinker (A) and the crosslinker yeast ratio (B) on the survival of *Z. rouxii*. The applied conditions were a crosslinker yeast ratio of 0.4 $g.g^{-1}$ (A) and a contact time of 50 minutes (B).

In order to reduce the severe toxic effect of the crosslinker during the immobilization process, it was tried to avoid the direct contact between the crosslinker and yeast. For this, the immobilization process was started without yeast by using the crosslinker to activate carboxy-methyl-cellulose. After this, the activated carboxy-methyl-cellulose was added to a mixture of polyethylene-oxide and yeast suspension, as described in the materials and methods section.

Survival of soy-sauce yeasts in new polyethylene-oxide gel

The survival of the soy-sauce yeasts *Z. rouxii* and *C. versatilis* after immobilization in the new polyethylene-oxide gel was determined with the respiration-activity assay in order to examine the toxicity of the new immobilization process. The same was done for alginate. In Table 1 the survival percentages of the yeasts in both gels can be found.

Table 1. Survival percentages of the soy-sauce yeasts *Z. rouxii* and *C. versatilis* after immobilization in the new polyethylene-oxide and alginate gel.

Yeast	New polyethylene-oxide gel	Alginate gel
Z. rouxii	86	74
C. versatilis	90, 84 [a]	90, 96 [a]

[a] The survival of *C. versatilis* in both gels was determined in duplicate.

It can be seen in this table that the survival of both yeasts in the new polyethylene-oxide gel was excellent (more than 80%). This clearly demonstrates that the new immobilization process was much milder than the original process. It can also be seen in Table 1 that there was not a big difference between the survival percentages in both gels. This shows that conditions for immobilizing the soy-sauce yeasts in the new polyethylene-oxide gel are comparable to the conditions in alginate gel which are very mild (Smidsrød and Skjåk-Bræk, 1990; Leenen et al., 1996).

Rheological tests with new polyethylene-oxide gel

Mechanical properties of the new polyethylene-oxide gel were investigated by using rheological tests. The gelation was followed and the sensitivity to abrasion and the maximum relative deformation at fracture were determined.

Gelation

The gelation of polyethylene-oxide was followed in time by measuring the storage modulus, which is a measure for the elastic energy stored in the material (Whorlow, 1992). An increase in the storage modulus shows the formation of the gel network while the storage modulus becomes constant when the gelation is completed (Verheul *et al.*, 1998). In Figure 3 the gelation of polyethylene-oxide is shown.

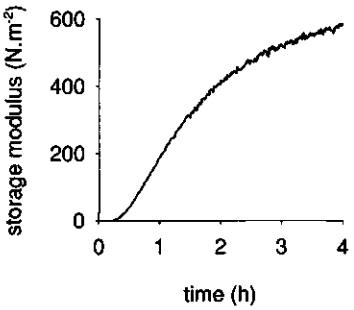

Figure 3. The formation of the polyethylene-oxide gel network, as shown by the storage modulus, in time. The timer was started after all the gel components were thoroughly mixed.

In this figure it can be seen from the increase in storage modulus that the formation of the polyethylene-oxide gel network started about 20 minutes after all the gel components were mixed. It can also be seen in this figure that the gelation is largely completed within about 4 hours. At that time, the storage modulus became more or less constant. During immobilization experiments we already started to wash the gel with PBS-buffer after 1 hour of gelation (see materials and methods section). This was done to remove a possible excess of the toxic crosslinker and appeared to have no effect on the formation of the gel network.

Abrasion sensitivity

The sensitivity to abrasion of the polyethylene-oxide gel was determined by oscillation experiments as described by Martins dos Santos et al. (1997). They found that the

sensitivity to abrasion of gel materials in bioreactors is likely to be related to the fatigue of the gel materials. The fatigue of gel materials can be determined with oscillation compression experiments. In these experiments, the gel material is exposed to repetitive compressions and the change in resistance to compression is measured. If the resistance changes only slightly, the gel material will be less liable to fatigue and for that reason, less sensitive to abrasion than when the resistance alters considerably (Martins dos Santos et al., 1997).

The oscillation compression experiments were done with both the new polyethylene-oxide and alginate gel. These experiments were also done with both gels in the presence of salt in order to predict their abrasion sensitivity in soy-sauce-like processes. For alginate again a gel with a high guluronic acid content was used, because this kind of alginate gel has not only the highest tolerance to salt, but also the highest mechanical stability (Smidsrød and Skjåk-Bræk, 1990).

In Figure 4, the representative results of the oscillation compression experiments with the polyethylene-oxide and alginate gels can be found. This figure shows that the resistance to compression of the alginate gels at the start of the experiment was higher than that of the polyethylene-oxide gels, which reflects the difference in rheological behaviour of the two gels. Alginate gels are more viscous than polyethylene-oxide gels (Leenen et al., 1996). For this reason, alginate gels resist more against a relatively small compression, like we applied during these experiments, than polyethylene-oxide gels. However, a higher resistance does not give any information on the mechanical stability of the gel material to abrasion because the magnitude of the resistance measured in these experiments is much higher than the maximum shear stress gel materials may encounter in bioreactors (Martins dos Santos et al., 1997). For the information about abrasion sensitivity, the change in resistance during the oscillation experiment is important.

Figure 4A shows that there was hardly any change in the resistance to compression of the new polyethylene-oxide gels, like the polyethylene-oxide gels made by Leenen et al. (1996). There was also no effect of salt on the change in resistance. Therefore, the

polyethylene-oxide gel will not be sensitive to abrasion in bioreactors, even in the presence of salt.

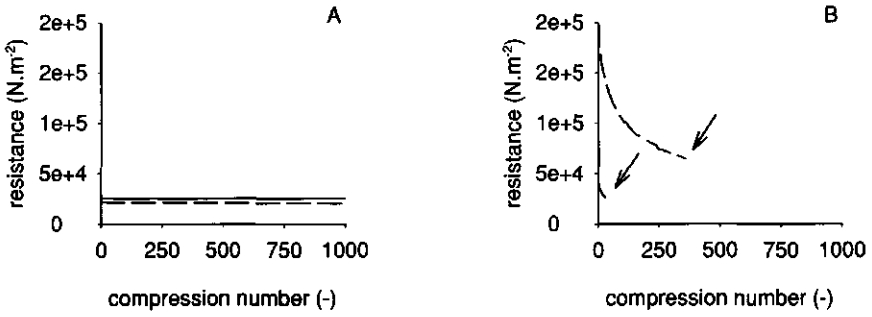

Figure 4. The effect of salt and gel material on the evolution of the resistance to compression (dashed line: without salt; solid line: with 12.5% (w/v) salt). A: polyethylene-oxide gel; B: alginate gel. Arrows indicate that the alginate gel was too much deformed and for this reason, the repetitive compression had to be stopped.

On the other hand, Figure 4B shows that the resistance to compression of the alginate gels clearly decreased with the number of oscillations, like found by Vogelsang et al. (2000). The oscillations for the alginate gels had even to be stopped before the end of the experiment (1000 oscillations) was reached because the gels were too much deformed, which confirmed the fatigue. In the presence of salt, this already happened after 30 oscillations while without salt around 350 oscillations could be done. These results confirm that alginate gel is sensitive to abrasion in bioreactors (Muscat et al., 1996), especially in the presence of salt. In addition, Figure 4B shows that salt also caused a lower resistance to compression of the alginate gel confirming the lowered strength of the gel at high salt concentrations, like in the soy-sauce medium.

Our results of the oscillation compression experiments were in agreement with the conclusion of Leenen et al. (1996) that 'elastic' gels like polyethylene-oxide will accommodate stresses better and for that reason, be relatively insensitive to abrasion than more 'viscous' gels like alginate and carrageenan. For this reason, the new polyethylene-oxide gel will be more suitable than alginate for use in a continuous process for a long period of time, especially in the presence of high salt concentrations. In our laboratory,

the new polyethylene-oxide gel has already been successfully applied for the continuous processes with immobilized soy-sauce yeasts in the presence of high salt concentrations, which will be the subject of the next chapter.

Maximum relative deformation at fracture

Finally, a large-deformation experiment was done with the new polyethylene-oxide gel in order to determine the maximum relative deformation at fracture. This was done by measuring the storage modulus as a function of the relative shear deformation. The storage modulus, which is a measure of the elastic energy stored in the material (Whorlow, 1992), will increase during a large deformation until fracture occurs. After that, the storage modulus will decrease.

In Figure 5 the effect of deformation on the storage modulus of polyethylene-oxide gel can be seen. This figure shows that the storage modulus kept increasing until a large relative deformation of 1.0. This means that fracture did not occur and that the new polyethylene-oxide gel, like rubber and gelatine but unlike alginate gel, is very flexible. In a flexible gel colonies of growing cells can expand more easily without (locally) breaking the gel than in a not so flexible gel (Hüsken et al., 1996). Therefore, we expect that the polyethylene-oxide gel will suffer less from the growth of immobilized cells than alginate (Nussinovitch et al., 1994).

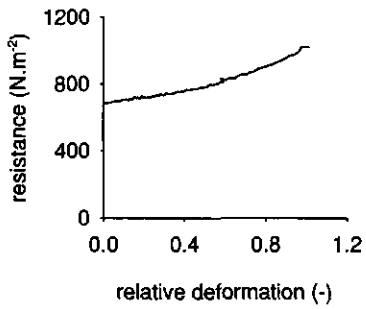

Figure 5. The effect of deformation on the storage modulus of polyethylene-oxide gel.

Conclusions

The high toxicity during the immobilization process in a chemically crosslinked polyethylene-oxide gel as was used by Leenen et al. (1996) resulted in no survival of the soy-sauce yeast *Z. rouxii*. This toxicity was caused by the crosslinker that we used for making this gel. The toxicity of this crosslinker was dependent on the contact time with the yeast and the crosslinker yeast ratio. A newly developed immobilization process for polyethylene-oxide gel, in which direct contact between the crosslinker and yeast was circumvented, appeared to be mild for the soy-sauce yeasts *Z. rouxii* and *C. versatilis*; the survival percentages of these yeasts in the polyethylene-oxide gel were high (more than 80%) and comparable to those in alginate.

The formation of polyethylene-oxide gel network appeared to be largely completed within four hours. The resulting very flexible gel was not sensitive to abrasion, even in the presence of salt. On the other hand, we confirmed that alginate gel is very sensitive to abrasion, especially in the presence of salt. Furthermore, alginate gel appeared to be chemically unstable towards soy-sauce medium. For this reason we concluded that the new polyethylene-oxide gel is better applicable than alginate gel for immobilization of yeast in long-term processes with a high salt content, like soy-sauce processes.

Acknowledgements

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Chapter 7

Immobilized salt-tolerant yeasts: application of a new polyethylene-oxide support in a continuous stirred-tank reactor for flavour production

Abstract

Immobilization of salt-tolerant yeasts considerably decreases the total time required for the flavour development in soy-sauce processes. For immobilization of cells, alginate gel is mostly used as support material. However, alginate is not very suitable for use in soy-sauce processes because alginate is sensitive to abrasion and chemically unstable towards the high salt content of the soy-sauce medium. In contrast, a newly developed polyethylene-oxide gel seems to be more suitable, but this gel has not been used so far for flavour production in a bioreactor with a high salt content.

Therefore, this gel was applied with immobilized salt-tolerant yeasts in a continuous stirred-tank reactor, containing more than 12.5% (w/v) salt. In this reactor the polyethylene-oxide gel particles did not show any abrasion for several days, while alginate gel beads were already destroyed within one day. In addition, the polyethylene-oxide gel particles with immobilized salt-tolerant yeasts *Candida versatilis* and *Zygosaccharomyces rouxii* showed a good flavour production. From this work, it was concluded that the application of polyethylene-oxide gel in long-term soy-sauce processes is attractive in the case the sticking together of polyethylene-oxide gel particles can be controlled.

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Introduction

The salt-tolerant yeasts, *Candida versatilis* and *Zygosaccharomyces rouxii*, are important for the flavour development in soy-sauce processes. In these processes, *C. versatilis* produces phenolic compounds, like 4-ethylguaiacol (Yokotsuka, 1986; Hamada et al., 1990a; Röling, 1995), while *Z. rouxii* produces ethanol and higher alcohols, like methionol (Aoki and Uchida, 1991a). 4-Ethylguaiacol and methionol give, amongst other compounds, the characteristic flavour to soy sauce (Yokotsuka, 1986).

Although there is not so much literature available on the 4-ethylguaiacol production by *C. versatilis*, it is known that *C. versatilis* synthesizes 4-ethylguaiacol from ferulic acid, which arises from the raw materials in the soy-sauce process (Yokotsuka, 1986; Röling, 1995). This synthesis involves a decarboxylation and reduction, as shown for *Brettanomyces* species (Chatonnet et al., 1992; Edlin et al., 1995) (Figure 1).

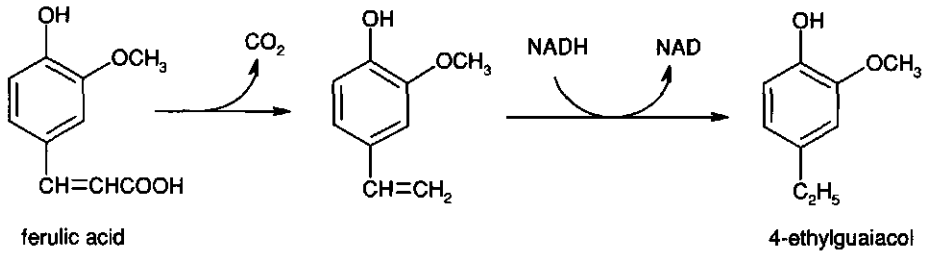

Figure 1. Biosynthesis of 4-ethylguaiacol by *C. versatilis*.

Ethanol is produced from glucose by *Z. rouxii* both under anaerobic and aerobic conditions (Hamada et al., 1989). For aerobic ethanol production, an excess glucose is necessary (van der Sluis et al., 2000a; van der Sluis et al., 2000b). The higher alcohols like n-propanol, isobutyl alcohol, active amyl alcohol and isoamyl alcohol are derived from the amino-acid biosynthetic pathways in *Z. rouxii*, but also from the deamination or transamination of extracellular amino acids (Ehrlich pathway) (Aoki and Uchida, 1991b; van der Sluis et al., 2000c). The Ehrlich pathway is the only pathway in *Z. rouxii* for producing the higher alcohol methionol (Aoki and Uchida, 1991a; van der Sluis et al., 2000c). In this pathway, methionol is synthesized from methionine by transamination and

subsequently, decarboxylation and reduction (Figure 2). In the transamination reaction the amino group is transferred from methionine to α-ketoglutarate in order to form glutamate.

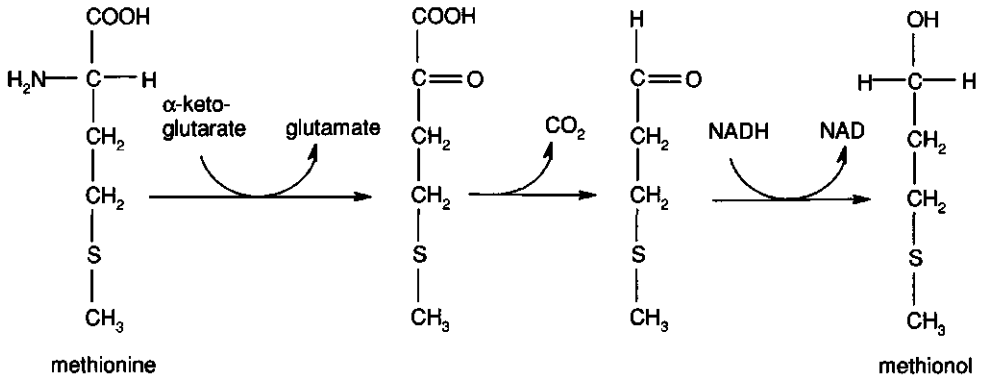

Figure 2. Biosynthesis of methionol by *Z. rouxii*.

The flavour development in the soy-sauce process normally takes a long time because the metabolic activity of the salt-tolerant yeasts is low due to the high salt content of the soy-sauce medium (about 17% (w/v)) (Yong and Wood, 1974). Furthermore, the slurry state of the soy-sauce medium makes the substrates poorly available for the yeasts. For this reason, many investigations were done in the last decade to decrease the process time by decreasing the salt content of the medium (Yong et al., 1978; Muramatsu et al., 1993), applying a higher process temperature (Yong et al., 1978) or using immobilized salt-tolerant yeasts (Osaki et al., 1985; Hamada et al., 1989; Hamada et al., 1990a; Hamada et al., 1990b; Horitsu et al., 1990; Horitsu et al., 1991; Iwasaki et al., 1991; Hamada et al., 1991; Hamada et al., 1992; Motai et al., 1993). Especially, the application of immobilized salt-tolerant yeasts in a continuous bioreactor showed to be very effective by reducing the process time with about 90%. In most of these investigations with immobilized salt-tolerant yeasts, alginate gel was used as immobilization material because immobilization in this gel is a mild and convenient method that can be scaled up easily.

However, alginate is not very suitable for use in soy-sauce processes because alginate is sensitive to abrasion and chemically unstable towards the high salt content of the soy-sauce medium (Horitsu et al., 1990; van der Sluis et al., 2000d). Therefore, Horitsu et al. (1990, 1991) and Iwasaki et al. (1991) used a ceramic carrier and we developed a new chemically crosslinked polyethylene-oxide gel to be used as support material instead of alginate (van der Sluis et al., 2000d).

This newly developed polyethylene-oxide gel appeared to be very mild for immobilizing the salt-tolerant yeasts (van der Sluis et al., 2000d). Furthermore, it appeared from rheological studies that this polyethylene-oxide gel, unlike alginate gel, is not sensitive to abrasion, even in the presence of high salt concentrations. However, the stability of the polyethylene-oxide gel in a bioreactor with a high salt content and the flavour producing ability of the salt-tolerant yeast immobilized in this gel have not been tested so far.

In the research presented here, batch and continuous cultivations with *C. versatilis* and *Z. rouxii*, immobilized in polyethylene-oxide gel, were done in a stirred-tank reactor in the presence of high salt concentrations. During these cultivations, the stability of the polyethylene-oxide gel in the stirred-tank reactor was determined, like the production of 4-ethylguaiacol, ethanol and higher alcohols by the immobilized *C. versatilis* and *Z. rouxii* cells. The stability of the polyethylene-oxide gel was compared with that of alginate gel. In addition, the metabolism of free *C. versatilis* cells was investigated in more detail.

Materials and methods

Yeast strains

C. versatilis CBS 4019 and *Z. rouxii* CBS 4021, supplied by the Centraalbureau voor Schimmelcultures (Delft, The Netherlands), were used in this work.

Media

Depending on the cultivation a defined or GPY medium was used. The defined medium had the following composition per liter of demineralized water: 22 g glucose.$1H_2O$, 21 g citric acid.$1H_2O$, 5 g ammonium sulphate and 6.7 g Bacto Yeast Nitrogen Base without amino acids and ammonium sulphate (Difco). The GPY medium contained per liter of demineralized water: 40 g glucose.$1H_2O$, 5 g peptone, 5 g yeast extract. The pH of the defined and GPY medium was adjusted to respectively 5 and 4.5. Both media were sterilized by filtration (0.2 μm).

Cultivation in Erlenmeyer flasks

The yeast strains were cultivated in 300 ml Erlenmeyer flasks, containing 100 ml medium, on a rotary shaker at 28°C and 200 rpm. For *C. versatilis*, the defined medium was used and for *Z. rouxii*, the GPY medium with additional 170 gram NaCl/l. This cultivation was used to obtain yeast for immobilization and to investigate the metabolism of free *C. versatilis* cells. For immobilization the yeast cells were harvested in the early stationary growth phase and subsequently, a concentrated yeast suspension was prepared. The metabolism of free *C. versatilis* cells was investigated by determining the effect of salt (0-15% (w/v)) on the maximum specific growth rate and the yield of 4-ethylguaiacol on ferulic acid (initial concentration of 20 $mg.l^{-1}$). The effect of the initial ferulic acid concentration (20-200 $mg.l^{-1}$) on that yield in the presence of 12.5% (w/v) salt was determined as well.

Preparation of a concentrated yeast suspension

The yeast cells were concentrated by centrifugation for 15 minutes at 9,500 g and 4°C. After centrifugation, the concentrated cell suspension was washed with sterilized PBS-buffer (pH 7.4) of the following composition per liter of demineralized water: 8.2 g NaCl, 1.9 g $Na_2HPO_4.2H_2O$ and 0.3 g $NaH_2PO_4.2H_2O$. Hereafter, the yeast suspension was centrifuged again. The concentrated suspension obtained was used for immobilization, after appropriate dilution in PBS-buffer.

Immobilization procedure

For making 5.5 ml polyethylene-oxide gel (Holland Biomaterials Group, Enschede, The Netherlands) (van der Sluis et al., 2000d), 0.125 ml crosslinker solution (0.1 mg carbodiamide/µl) and 4.5 ml carboxy-methyl-cellulose solution (5 $mg.ml^{-1}$) were mixed and allowed to react for 5 minutes. Then, a mixture of 0.4 ml concentrated yeast suspension and 0.475 ml polyethylene-oxide solution (100 $mg.ml^{-1}$) was added and thoroughly mixed. This mixture starts to gelate after about 20 minutes (van der Sluis et al., 2000d). Gels with different volumes were made by proportionally adjusting the amount of each component.

Polyethylene-oxide gel particles with a size of approximately 0.2 mm were obtained from the gelling mixture in two ways. The first way was by emulsification. For this, 200 ml gelling mixture and 600 ml n-decane solution were poured into a cylindrical reactor (height and diameter of 15.5 and 9.5 cm, respectively), which was equipped with baffels and a four-blade Rushton turbine stirrer (diameter of 4.0 cm). A stirrer speed of around 1000-1500 rpm was used to obtain small gelling droplets surrounded by n-decane. After 1 hour of gelation, gel particles were removed from the n-decane solution. The second way was by cutting the gel after 1 hour of gelation into pieces and their size was reduced further by chopping with a food mixer (Braun vario 300 W). In both cases the gel particles obtained were washed with PBS-buffer and left in PBS-buffer to complete the gelation, which takes about 4 hours (van der Sluis et al., 2000d).

Alginate gel beads with a diameter of about 1 mm were made by dropwise extrusion of a 3% (w/v) alginate solution (Protanal LF 10/60, high guluronic acid content, Pronova Biopolymers, Norway), containing 9% (v/v) yeast suspension, through a hollow needle using air pressure. The droplets were collected in a stirred 5% (w/v) $CaCl_2$ solution and left in there for at least 24 hours for hardening.

Cultivations with immobilized salt-tolerant yeasts in a stirred-tank reactor

Batch and continuous cultivations with salt-tolerant yeasts, immobilized in polyethylene-oxide gel, were done in a stirred-tank reactor. The characteristics of these cultivations, which were dependent on the flavours to produce, can be found in Table 1. The media used for these cultivations were sterilized by filtration (0.2 μm). During these cultivations, the concentration of the flavours 4-ethylguaiacol, ethanol and higher alcohols, and the substrates ferulic acid and glucose in the stirred-tank reactor were determined. In order to evaluate the stability of polyethylene-oxide gel, the batch cultivation was done with alginate gel as well. Furthermore, the size of a sample of the polyethylene-oxide gel particles was measured before and after a long-term continuous cultivation.

Table 1. Characteristics of batch and continuous cultivations with salt-tolerant yeasts, immobilized in polyethylene-oxide gel, in a stirred-tank reactor.

Characteristic	Batch	Continuous	Continuous	Continuous
Yeast	*C. versatilis*	*C. versatilis*	*Z. rouxii*	*Z. rouxii*
Flavours	4-Ethylguaiacol	4-Ethylguaiacol	Ethanol Higher alcohols	Ethanol Higher alcohols
Medium [a]	Defined	Defined [b]	GPY	Defined [b,c]
Additions:				
Ferulic acid ($mg.l^{-1}$)	100	200	-	-
NaCl ($g.l^{-1}$)	125	125	170	170
Methionine ($g.l^{-1}$)	-	-	-	0.78
Gel volume (ml)	75	200	135	74
Liquid volume (ml)	750	800	715	330
Air flow ($l.min^{-1}$)	0.066	0.08	0.81	0.81
pH	5	4.5-5	4.5	4.5
Temperature (°C)	30	30	28	28
Dilution rate (h^{-1})	-	0.05	0.1 0.01	0 0.1

[a] Composition of the medium can be found above.

[b] Citric acid was omitted.

[c] Ammonium sulphate concentration was reduced from 5 to 3.3 $g.l^{-1}$.

All the cultivations were done in bench-scale autoclavable bioreactors, which were stirred with a six-blade Rushton turbine stirrer. The stirrer speed was at least 350 rpm. During the continuous cultivations, the polyethylene-oxide gel particles were kept in the bioreactor by using a spinner filter (75 μm) or a sample screen (105 μm) at the flow outlet.

Analyses

The maximum specific growth rate of *C. versatilis* was calculated from optical density measurements at 610 nm (Pharmacia Biotech, Ultraspec). For this, samples were taken and diluted with demineralized water to obtain absorbance values less than 0.7. Below 0.7, the absorbance values were linearly related to biomass dry weight.

Glucose was measured with a Haemo-Glukotest (Boehringer Mannheim).

Ferulic acid and 4-ethylguaiacol were determined by HPLC. For this, samples were diluted with an equal volume of trichloro-acetic acid solution 2% (w/w). The resulting sample (10 μl) was injected on an Inertsil 5 ODS-2 column (Chrompack), which was kept at room temperature. An eluens flow profile of pure methanol and acetic acid solution (2 ml acetic acid dissolved in 100 ml milli-Q water) was used to separate the components. After separation, the components were detected with an UV detector at 280 nm. From this determination, the yield of 4-ethylguaiacol on ferulic acid was calculated by dividing the produced 4-ethylguaiacol by the consumed ferulic acid.

The higher alcohols n-propanol, isobutyl alcohol, active amyl alcohol, isoamyl alcohol and methionol were analyzed by GC. For this, the samples were incubated at 60°C for 10 minutes. After incubation, a head-space sample was taken and collected in a cold trap (liquid nitrogen at -110°C). 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 each other.

The size of polyethylene-oxide gel particles was measured with Magiscan using General Image Analysis Software. For this, a sample of around 50 gel particles was put on a Petri dish, containing a thin layer of PBS-buffer, to which blue dextran (1 $mg.ml^{-1}$) was added in order to obtain sufficient contrast. This measurement was done in fivefold.

Results and discussion

Batch cultivation with free *C. versatilis*

The metabolism of free *C. versatilis* cells was investigated in order to determine which process conditions could be used for production of 4-ethylguaiacol from ferulic acid (Figure 1) with immobilized *C. versatilis*. For this, batch cultivations were done with a defined medium. During these cultivations, the effect of salt on the maximum specific growth rate and the yield of 4-ethylguaiacol on ferulic acid (initial concentration of 20 $mg.l^{-1}$) (Figure 3A) was determined. The effect of the initial ferulic acid concentration on that yield in the presence of 12.5% (w/v) salt was determined as well (Figure 3B).

It can be seen from Figure 3A that the salt content did affect the maximum specific growth rate of *C. versatilis*. The maximum specific growth rate reached its maximum (0.16 h^{-1}) at a salt content of 5% (w/v). This confirmed that *C. versatilis* prefers some salt, unlike *Z. rouxii*, which is only tolerant to salt (Fukushima, 1985).

Figure 3A further shows that the salt content did affect the yield of 4-ethylguaiacol on ferulic acid as well. No 4-ethylguaiacol was produced when there was no salt, while the yield was constant around 30% (w/w) when the salt content was 5% (w/v) or higher. In addition, Figure 3B shows that the initial ferulic acid concentration did not have much effect on the yield of 4-ethylguaiacol on ferulic acid in the presence of 12.5% (w/v) salt; all concentrations of ferulic acid added resulted in a yield of about 35% (w/w).

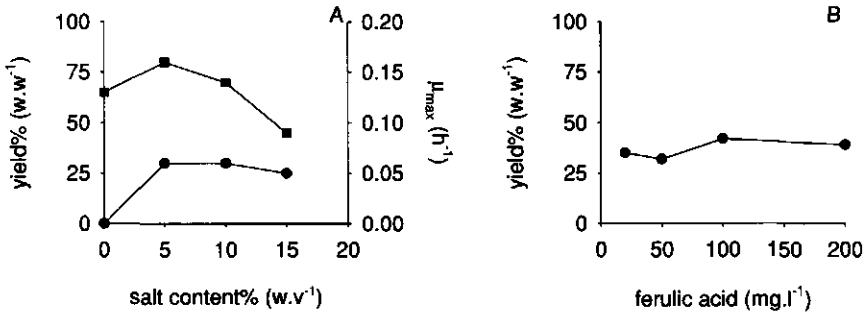

Figure 3. Batch cultivations with free *C. versatilis* cells. A: Effect of salt on the yield of 4-ethylguaiacol on ferulic acid (●) and the maximum specific growth rate (■); B: Effect of initial ferulic acid concentration on the yield of 4-ethylguaiacol on ferulic acid (●).

Batch cultivation with immobilized *C. versatilis*

In order to investigate the production of 4-ethylguaiacol by *C. versatilis*, immobilized in polyethylene-oxide gel, batch cultivation was done with the defined medium. Based on the previous experiments, ferulic acid (100 mg.l^{-1}) and salt (12.5% (w/v)) were added to the medium in order to produce 4-ethylguaiacol. For this cultivation, polyethylene-oxide particles with a size of approximately 0.2 mm, made by emulsification, were used. This cultivation was done in a stirred-tank reactor in order to apply high shear forces (van Ginkel et al., 1983) for testing the stability of polyethylene-oxide gel at the same time. A stirrer-speed of at least 350 rpm was used for this. To compare the stability of polyethylene-oxide gel with alginate gel, the same batch cultivation was done with alginate gel beads (diameter of about 1 mm), which were made by drop-wise extrusion. For alginate, a gel with a high guluronic acid content was used, because this kind of alginate gel has a high tolerance to salt and a high mechanical stability (Smidsrød and Skjåk-Bræk, 1990).

In Figure 4, the concentrations of glucose, ferulic acid and 4-ethylguaiacol during this cultivation with *C. versatilis*, immobilized in polyethylene-oxide gel, are shown. It is shown

that the immobilized cells were able to produce 4-ethylguaiacol (almost 25 $mg.l^{-1}$), for which they completely consumed the added ferulic acid (100 $mg.l^{-1}$) and glucose at the same time. Based on the expectation that glucose was consumed for growth, this shows that the conversion of ferulic acid to 4-ethylguaiacol is related to that.

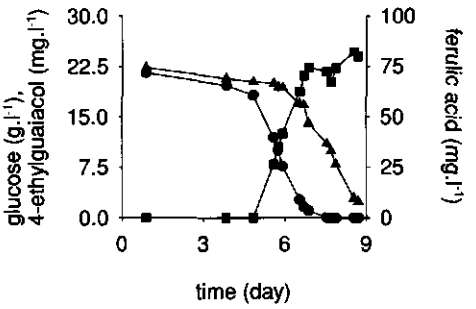

Figure 4. Batch production of 4-ethylguaiacol by *C. versatilis* immobilized in polyethylene-oxide gel (▲: glucose; ●: ferulic acid; ■: 4-ethylguaiacol).

The above results with immobilized *C. versatilis* cells demonstrated that the yield of 4-ethylguaiacol on ferulic acid was 25% (w/w), which was quite similar to the yield of 30-35% that we obtained with the free cells. From this it was concluded that the immobilization in the polyethylene-oxide gel did not have a clear effect on the 4-ethylguaiacol production by the *C. versatilis* cells. In literature it is reported that the immobilization in alginate gel has no effect on the 4-ethylguaiacol production by the *C. versatilis* cells as well (Hamada et al., 1990b). However, the *C. versatilis* cells immobilized in alginate showed a much higher yield (about 60%) (Hamada et al., 1990a) than the one in this case (25%). This difference might be due to the fact that we used a lot of citric acid to buffer the defined medium (see materials and methods section) and normally soy-sauce medium, as used by Hamada et al. (1990a), does not contain that much citric acid. Citric acid might, like other acids, inhibit the growth of salt-tolerant yeasts (Röling, 1995).

During this cultivation for almost 9 days in the stirred-tank reactor with 12.5% (w/v) salt, we did not observe any clear abrasion of the polyethylene-oxide gel particles. In contrast, under the same conditions, the alginate gel beads started to abrade right from the start of

the cultivation, as shown by the appearance of small gel particles and white colouring of the medium. Within one day these beads were completely destroyed. The high abrasion rate of alginate gel beads in this case was not only caused by their mechanical weakness and low tolerance to salt (van der Sluis et al., 2000d), but also by their instability towards citric acid (Smidsrød and Skjåk-Bræk, 1990), which was the buffer in the medium. Nevertheless, the destabilizing effect of citric acid in this medium was by itself not large enough to destroy the alginate gel beads completely (data not shown). Therefore, these batches confirmed that polyethylene-oxide gel is more stable towards high shear forces and salt content than alginate gel (van der Sluis et al., 2000d).

Continuous cultivation with immobilized *C. versatilis*

The polyethylene-oxide gel particles with immobilized *C. versatilis* were also used for production of 4-ethylguaiacol in a continuous cultivation with the stirred-tank reactor. For this, the defined medium with 12.5% (w/v) salt was used again but this time more ferulic acid (200 $mg.l^{-1}$) was added and citric acid was omitted from the medium. During the continuous cultivation, a dilution rate of 0.05 h^{-1} was applied and the concentrations of ferulic acid and 4-ethylguaiacol were followed in the stirred-tank reactor (Figure 5).

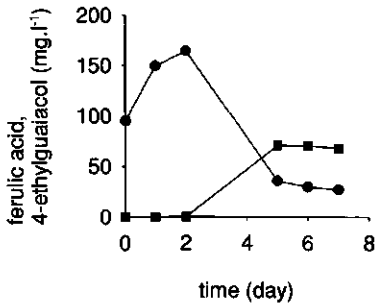

Figure 5. Continuous accumulation of 4-ethylguaiacol by *C. versatilis* immobilized in polyethylene-oxide gel (●: ferulic acid; ■: 4-ethylguaiacol).

In the beginning the cultivation was rather unstable, resulting in fluctuating concentrations, but after about 5 days more or less constant concentrations of ferulic acid

(30 mg.l^{-1}) and 4-ethylguaiacol (70 mg.l^{-1}) were reached. This demonstrated that the yield of 4-ethylguaiacol on ferulic acid was around 40% (w/w). This yield was higher than the one of 25% (w/w) obtained with the batch cultivation. The higher yield might have been caused by the fact that citric acid was omitted this time, but the yield was still lower than the one obtained by Hamada et al. (1990a).

Continuous cultivation with immobilized *Z. rouxii*

The ability of *Z. rouxii* immobilized in polyethylene-oxide gel to produce the flavours ethanol and higher alcohols during a continuous cultivation in a stirred-tank reactor was investigated as well. The polyethylene-oxide gel consisted of chopped gel particles with a size of about 0.2 mm. For the flavour production, an undefined rich medium (GPY), containing a lot of glucose and amino acids, was used and aerobic conditions were applied (van der Sluis et al., 2000a). In addition, salt (170 g.l^{-1}) was added to the medium. In Figure 6, the accumulation of ethanol and higher alcohols in the reactor during this cultivation is shown.

It appears from Figure 6 that the immobilized *Z. rouxii* could clearly produce the flavours ethanol, isobutyl alcohol and isoamyl alcohol plus active amyl alcohol during the continuous cultivation with a dilution rate of 0.1 h^{-1}. However, no accumulation of n-propanol and methionol was observed under these conditions. n-Propanol appeared to be accumulated as well when the dilution rate was decreased to 0.01 h^{-1} after 2 days cultivation. From about 3.5 days till the end of the cultivation, rather constant accumulation of ethanol, n-propanol, isobutyl alcohol and isoamyl alcohol plus active amyl alcohol was reached in the stirred-tank reactor.

In order to investigate whether the immobilized *Z. rouxii* cells could accumulate methionol as well, the same polyethylene-oxide gel particles were used under similar conditions but with a different medium. This time a defined medium was used, to which methionine (0.78 g.l^{-1}) and ammonium sulphate (3.3 g.l^{-1}) were added in order to produce methionol (Figure 2) (van der Sluis et al., 2000c) and support the growth (van der Sluis et al., 2000e), respectively.

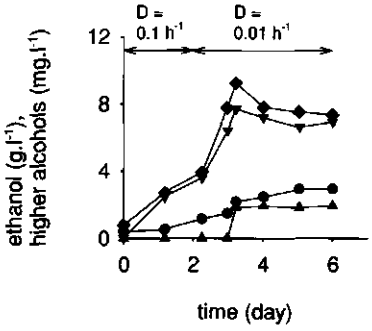

Figure 6. Continuous accumulation of ethanol and higher alcohols by *Z. rouxii*, immobilized in polyethylene-oxide gel, on GPY medium (●: ethanol; ▲: n-propanol; ▼: isobutyl alcohol; ◆: isoamyl alcohol plus active amyl alcohol). The applied dilution rates (D) are shown in the figure.

During this continuous cultivation with a dilution rate of 0.1 h^{-1}, which was started after a batch-period of 1 day, it was confirmed that the immobilized *Z. rouxii* cells could produce methionol as well (Figure 7). High concentrations of methionol (800 $mg.l^{-1}$) were accumulated by *Z. rouxii*, which accumulated ethanol, n-propanol, isobutyl alcohol and isoamyl alcohol plus active amyl alcohol at the same time.

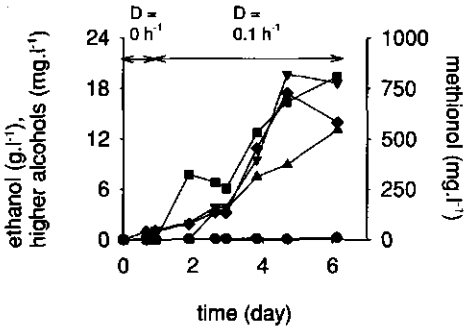

Figure 7. Continuous accumulation of ethanol and higher alcohols by *Z. rouxii*, immobilized in polyethylene-oxide gel, on a defined medium with additional methionine (●: ethanol; ▲: n-propanol; ▼: isobutyl alcohol; ◆: isoamyl alcohol plus active amyl alcohol; ■: methionol). The applied dilution rates (D) are shown in the figure.

Practical limitations of polyethylene-oxide gel

From the above cultivations it was clear that the polyethylene-oxide gel shows no abrasion and gives flavour production with immobilized salt-tolerant yeasts. However, it also appeared that it was rather difficult to reach and keep steady-state conditions during the continuous cultivations with the polyethylene-oxide gel particles, which is clearly shown for example in Figure 7. This difficulty was caused by the fact that the polyethylene-oxide particles disturbed the outgoing flow by blocking-up the filter at the flow outlet. In addition, the polyethylene-oxide particles were sticking together on the sensors (e.g. pH sensor), which influenced the measurements and by that the process control.

The stability of the polyethylene-oxide gel was evaluated by measuring the size of the particles before and after a continuous cultivation in a stirred-tank reactor (Table 2). It appears from Table 2 that the mean size of the gel particles increased from 1.4 till 3.5 mm^2 during the cultivation for 14 days. This increase of particle size confirmed the stickiness of the particles, which should be investigated further in order to make long-term continuous processing feasible.

Table 2. Analysed characteristics of the size of polyethylene-oxide particles before and after a continuous fermentation for 14 days in a stirred-tank reactor.

Characteristic	Before fermentation	After fermentation
Mean size (mm^2)	1.37	3.51
Standard deviation (mm^2)	0.07	0.14

Conclusions

The main purpose of the study presented here was to test the stability of a newly developed polyethylene-oxide gel in a bioreactor with a high salt content and the flavour producing ability of the salt-tolerant yeasts immobilized in this gel. During batch and

continuous cultivations in a stirred-tank reactor with 12.5-17% (w/v) salt, no abrasion of the polyethylene-oxide gel particles was observed for several days, while alginate gel beads were completely destroyed within one day. These results confirmed that the polyethylene-oxide gel, unlike alginate gel, has an excellent mechanical stability and tolerance towards salt. Despite this, long-term continuous processing with the polyethylene-oxide gel particles was difficult because the particles stick together.

It also appeared during these cultivations that the salt-tolerant yeasts *C. versatilis* and *Z. rouxii* immobilized in the polyethylene-oxide gel were able to produce the flavours 4-ethylguaiacol, ethanol and higher alcohols, like methionol. The immobilized *C. versatilis* cells showed a similar yield of 4-ethylguaiacol on ferulic acid as free *C. versatilis* cells.

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Chapter 8

Enhancing and accelerating flavour formation by salt-tolerant yeasts in Japanese soy-sauce processes

Abstract

In soy-sauce processes salt-tolerant yeasts are very important for the flavour formation. This flavour formation is, however, slow and poorly understood. In the last decades a concerted research effort has increased the understanding and resulted in the derivation of mutants with an enhanced flavour formation. In addition, a new process using immobilized salt-tolerant yeasts has been developed and shown to be very effective for accelerating the flavour formation. From this study it was, however, concluded that immobilizing the salt-tolerant yeasts gives only small productivity benefits to this new process. For increasing the productivity the continuous microfiltration membrane reactor seems to be a good alternative.

This chapter is going to be submitted for publication as: van der Sluis C, Tramper J, Wijffels RH. 2000. Enhancing and accelerating flavour formation by salt-tolerant yeasts in soy-sauce processes.

Introduction

Japanese soy sauce is a traditional oriental all-purpose seasoning with a salty taste and sharp flavour, which is becoming more and more popular all over the world. The conventional batch process of brewing soy sauce starts with a solid-state fermentation of *Aspergillus* species on a mixture of soybeans and wheat with a ratio of 1 to 1 (Figure 1) (Yong and Wood, 1974; Beuchat, 1983; Sugiyama, 1984; Fukushima, 1985; Yokotsuka, 1986; Röling, 1995). During an aerobic fermentation for 2 days at 30°C, *Aspergillus* produces extracellular enzymes, such as proteases and amylases (Yong and Wood, 1977a), which start to hydrolyze the proteins and polysaccharides of the soybeans and wheat.

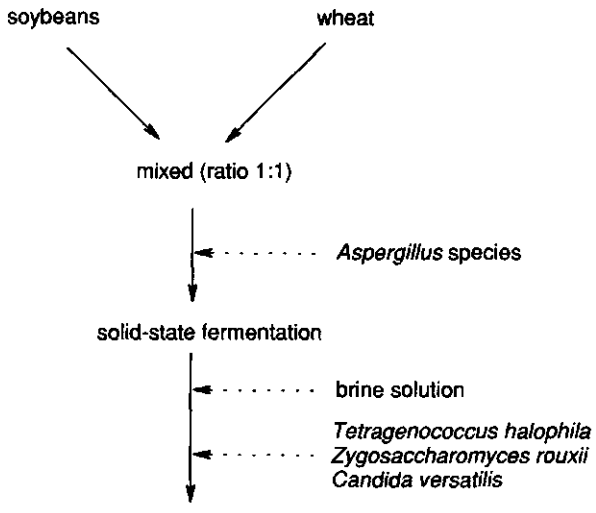

Figure 1. Process of brewing Japanese soy sauce.

After that, the moulded soybeans and wheat are mixed with a brine solution of 17% salt. This brine solution is kept around 30°C and semi-anaerobic conditions are applied by

occasional brief aeration. In the brine solution the *Aspergillus* enzymes continue to hydrolyze the soybeans and wheat and as a result, a surplus of different kinds of sugars and amino acids arise (Yong and Wood, 1977b). These sugars and amino acids are consumed by salt-tolerant lactic-acid bacteria (*Tetragenococcus halophila*) and yeasts (*Zygosaccharomyces rouxii* and *Candida versatilis*) during the so-called brine fermentation.

In the first stage of this brine fermentation, *T. halophila* decreases the initial pH of about 7.0 by producing lactic acid. After the pH has dropped below 5.0, *T. halophila* is unable to grow and an alcoholic fermentation by *Z. rouxii* starts. As a result, 2% ethanol and many other flavour components like higher alcohols and 4-hydroxyfuranones are produced. At the last stage of the brine fermentation, when *Z. rouxii* is not active anymore, *C. versatilis* starts to grow and to produce phenolic flavour components, like 4-ethylguaiacol and 4-ethylphenol. The flavour components formed by *Z. rouxii* and *C. versatilis* give, amongst other components, the characteristic flavour to soy sauce.

Despite the fact that the yeast brine fermentation (Table 1) is known to have a great impact on the flavour of soy sauce, the flavour formation by the yeast is poorly understood and for that reason difficult to control. Furthermore, this flavour formation is very slow and as a result normally more than 6 months are needed to complete the entire yeast fermentation plus ageing. Such a long time is needed because the metabolic activity of the yeast is low due to the high salt content of the soy-sauce medium (about 17% (w/v)) (Yong and Wood, 1974). Additionally the slurry state of the medium makes the substrates poorly available for the yeast.

Therefore, in the last decades many investigations have been done in order to enhance and accelerate the flavour formation. For this, mutants were derived and a new process with immobilized cells was developed. In this paper, recent advances in the research about the flavour formation during the yeast brine fermentation are described and discussed.

Table 1. Characteristics of the conventional and immobilized-cell process for the yeast brine fermentation.

Characteristic	Conventional process	Immobilized-cell process [a]
Reactor concept	Batch	Continuous
Medium composition	Slurry	Solid-free solution
Temperature (°C)	30	30
pH	5	5
Aeration rate (vvm)	Low	0.002-0.01
NaCl (% (w/v))	17	13
Process time	6 months	Few weeks

[a] Osaki et al. (1985).

Flavour formation by *Z. rouxii*

Ethanol

Ethanol is synthesized by *Z. rouxii* from the sugars, which are ample and in wide variety present during the brine fermentation. However, the kinds of sugars that can be fermented to ethanol by *Z. rouxii* is limited because of the high salt content of the brine solution (Table 1) (Röling, 1995). For example, in the brine solution maltose can not be fermented by *Z. rouxii*, while *Z. rouxii* is able to ferment maltose in a salt-free medium (Onishi, 1963). Without sugar fermentation *Z. rouxii* and also *C. versatilis* would not be able to survive during the brine fermentation because of the low availability of oxygen, which is caused by the low aeration rate (Table 1) and poor solubility of oxygen in the brine solution. Some traces of oxygen are, however, required for this fermentation (Visser et al., 1990). In addition, this fermentation is only possible when the pH is lower than about 5 (Table 1). At a higher pH, it seems that *Z. rouxii* can not maintain a proton gradient that is necessary for its salt tolerance (Watanabe and Tamai, 1992).

Higher alcohols

In the formation of higher alcohols, such as isobutyl alcohol, isoamyl alcohol, methionol and 2-phenylethanol, in *Z. rouxii* the α-keto acids are key intermediates (Figure 2) (van der Sluis et al., 2000a). Higher alcohols are formed through decarboxylation and subsequently reduction of the corresponding α-keto acids. The α-keto acids themselves normally arise in two pathways.

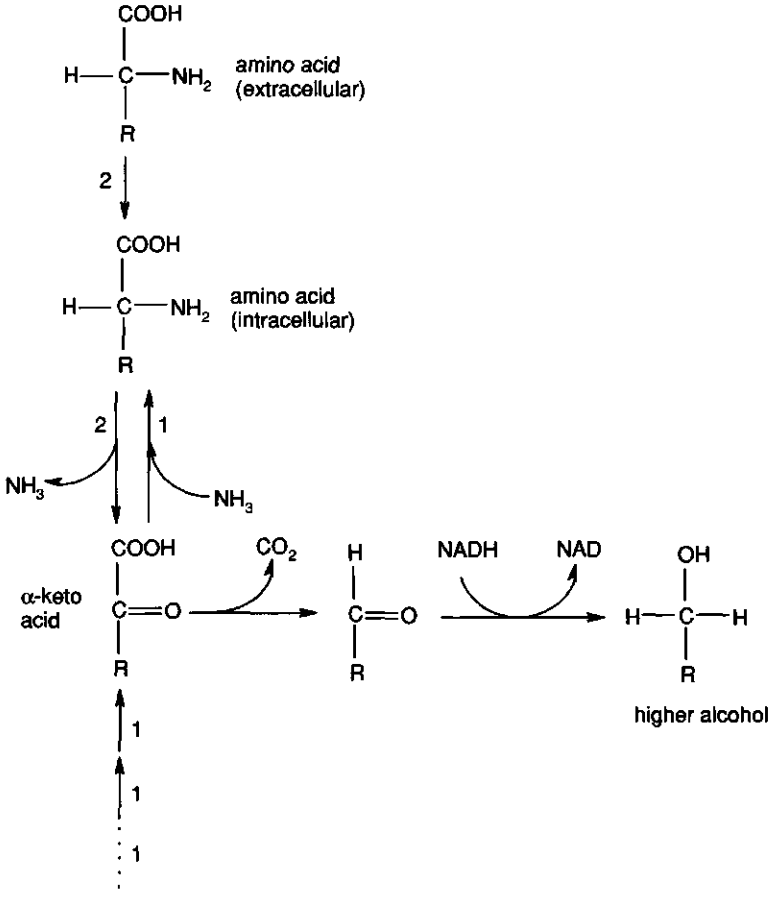

Figure 2. Formation of higher alcohols by Z. rouxii. Metabolic pathways: 1: amino-acid biosynthetic pathway; 2: Ehrlich pathway.

One pathway is the amino-acid biosynthetic pathway and the other pathway is the so-called Ehrlich pathway (Webb and Ingraham, 1963). In the Ehrlich pathway the α-keto acids are formed through consumption and subsequently deamination or transamination of extracellular amino acids. Since very high concentration of extracellular amino acids are present during the brine fermentation, it is expected that most of the higher alcohols are formed by *Z. rouxii* via the Ehrlich pathway (Aoki and Uchida, 1991a; van der Sluis et al., 2000b). The Ehrlich pathway is shown to be the only pathway for the formation of methionol (Aoki and Uchida, 1991b; van der Sluis et al., 2000a).

4-Hydroxyfuranones

Two 4-hydroxyfuranones derivatives, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF) (Sasaski et al., 1991) and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) (Hecquet et al., 1996), have been reported to be synthesized by *Z. rouxii*. Both HEMF and HDMF give rise to a sweet and caramel type of flavour. Although many investigations have been done to elucidate *Z. rouxii* 4-hydroxyfuranone biosynthesis (Sasaki et al., 1991; Sugawara et al., 1994; Sasaki, 1996; Hecquet et al., 1996; Hayashida et al., 1997; Hayashida et al., 1998; Sugawara and Sakurai, 1999; Hayashida et al., 1999) this biosynthesis is still poorly understood. The reason for this is that 4-hydroxyfuranones usually arise in foods like soy sauce by spontaneous reactions between sugars and amino acids (Maillard reactions) while only in some situations they are produced by yeast as well. For this production the yeast uses Maillard intermediates as precursors, which complicates the elucidation of the biosynthesis (Figure 3) (Hayashida et al., 1999). Until now the precursors and the yeast enzymes, which are involved in the furanones biosynthesis, are not known (Hayashida et al., 1999).

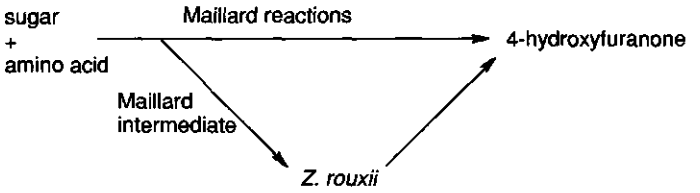

Figure 3. Formation of 4-hydroxyfuranones in soy sauce.

Flavour formation by *C. versatilis*

C. versatilis produces the phenolic compounds 4-ethylguaiacol and 4-ethylphenol, which give respectively a spicy clove-like and woody, smoky flavour (Edlin et al., 1995) to soy sauce. 4-Ethylguaiacol and 4-ethylphenol are derived by *C. versatilis* from respectively ferulic acid and p-coumaric acid (Figure 4), which arise mainly from the wheat fraction of the raw materials in the soy-sauce process (Yokotsuka, 1986). This derivation involves a decarboxylation and reduction, as shown for *Brettanomyces* species (Chatonnet et al., 1992; Edlin et al., 1995). *Z. rouxii* is unable to accomplish this derivation (Yokotsuka, 1986).

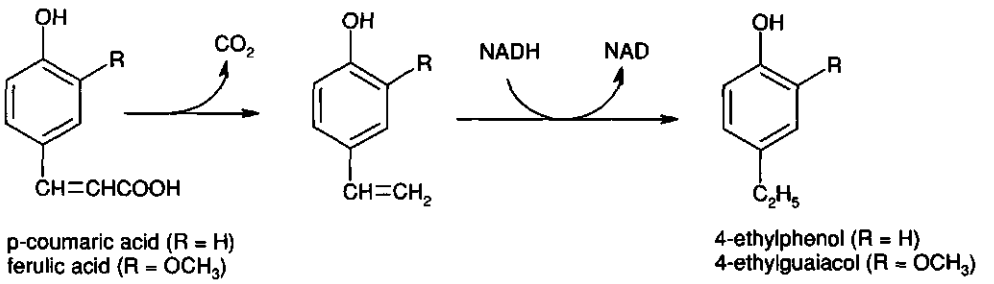

Figure 4. Formation of phenolic flavour compounds by *C. versatilis*.

Enhancing and accelerating flavour formation

Mutants

In order to enhance the formation of higher alcohols via the amino-acid biosynthetic pathway (Figure 2), mutants of *Z. rouxii* with an increased activity of the amino-acid biosynthetic enzymes were derived. A mutant was found to produce about 38 times as much 2-phenylethanol as the wild-type (Aoki and Uchida, 1990), while another mutant produced a 3-fold higher level of isoamyl alcohol (Yoshikawa et al., 1995). Also mutants producing about 60-fold as much of methionol as the parental strain were found (Aoki and Uchida, 1991b). It appeared that these mutants were able to use the amino-acid biosynthetic pathway to produce methionol, which is normally only formed via the Ehrlich pathway (Figure 2).

In addition, mutants of *Z. rouxii* with a restricted consumption of amino acids were derived in order to control the production of higher alcohols via the Ehrlich pathway (Figure 2) (Aoki and Uchida, 1991a). A number of these mutants showed a remarkable decrease in leucine and phenylalanine consumption and as a result, their production of isoamyl alcohol and 2-phenylethanol was reduced to about 34 and 9% compared to that of the wild-type, respectively.

Moreover a novel hybrid from *Z. rouxii* and *C. versatilis* was obtained (Kim, 1993). This hybrid produces the characteristic flavour components of soy sauce, which are normally produced by *Z. rouxii* and *C. versatilis* separately in different stages of the brine fermentation. Therefore, by using the hybrid instead of the two strains, it is possible to easily control the production of flavour components and to shorten the fermentation time.

Immobilized cells

In order to accomplish an acceleration of the flavour formation during the brine fermentation, a high concentration of yeast cells is indispensable. For that reason, much

attention has been paid in the last decades to the application of immobilized salt-tolerant yeasts, which resulted in the development of a new continuous immobilized-cell process (Table 1) (Osaki et al., 1985; Akao et al., 1986; Hamada et al., 1989; Hamada et al., 1990a; Hamada et al., 1990b; Hamada et al., 1991; Hamada et al., 1992; Motai et al., 1993). Apart from using immobilized yeasts in a continuous mode, the new process also differed from the conventional batch process by applying a lower salt content in order to stimulate the metabolic activity of the yeasts (Yong et al., 1978). Furthermore a solid-free soy-sauce medium was used instead of the slurry medium to make the substrates better available for the yeasts.

In the new process alginate gel beads were mostly used for immobilizing the salt-tolerant yeasts because immobilization in alginate gel is a mild and convenient method. However, alginate is very sensitive to abrasion and chemically unstable towards the high salt content of the soy-sauce medium, which makes it not very suitable for use in long-term soy-sauce processes (Horitsu et al., 1990; van der Sluis et al., 2000c). Therefore, more stable immobilization materials like ceramic carriers (Horitsu et al., 1990; Horitsu et al., 1991; Iwasaki et al., 1991) and polyethylene-oxide gel particles (van der Sluis et al., 2000c; van der Sluis et al., 2000d) were used instead of alginate as well.

The immobilized-cell process appeared to be very effective for the flavour production in a short period. Its ethanol productivity, an index of fermentation, was about 5 to 10 times higher than that of the conventional process (Iwasaki et al., 1991) and as a consequence, the process time was decreased from about 6 months to a few weeks (Osaki et al., 1985) (Table 1). This decrease seemed to be caused by the 10 to 100-fold higher concentration of yeast cells in the immobilized-cell process compared to the conventional process (Hamada et al., 1991).

Although the continuous immobilized-cell process is shown to be much faster than the conventional batch process (Table 1), the productivity benefits of applying immobilized instead of free yeast cells in the continuous process are not clearly shown in literature. Therefore, using the data from the work of Hamada et al. (1990a) we made a comparison between an immobilized-cell and free-cell process for the continuous yeast brine

fermentation (Table 2). In this comparison, we took into account that also free cells are present in the immobilized-cell process and that both free and immobilized cells contribute to the productivity of the immobilized-cell process (Hamada et al., 1990a).

Table 2. Comparison between the productivity of an immobilized-cell and free-cell process for the continuous yeast brine fermentation with *Z. rouxii* and *C.versatilis* [a].

Productivity	*Z. rouxii*		*C. versatilis*	
	Immobilized-cell process	Free-cell process	Immobilized-cell process	Free-cell process
Cell concentration (cells.l^{-1}) [b]				
Immobilized	$1.1*10^{11}$	-	$2.0*10^{11}$	-
Free	$2.9*10^{10}$	$2.9*10^{10}$	$3.0*10^{10}$	$3.0*10^{10}$
Total	$5.3*10^{10}$	$2.9*10^{10}$	$8.1*10^{10}$	$3.0*10^{10}$
	(1.8) [c]		(2.7) [c]	
Flavour production rate (g.l^{-1}.h^{-1}) [b]				
Ethanol	1.29	0.86	0.76	0.68
	(1.5) [c]		(1.1) [c]	
4-Ethylguaiacol	-	-	$1.43*10^{-3}$	$0.32*10^{-3}$
			(4.5) [c]	

[a] Calculated from the data of Hamada et al. (1990a). They used an airlift reactor with a volume of 1 liter. For the immobilized-cell process the reactor contained 300 ml alginate gel beads with immobilized yeasts.

[b] The cell concentration and flavour production rate are the averages from measurements at three different dilution rates.

[c] Values in parentheses indicate the ratio between the immobilized-cell and free-cell process.

From Table 2 it appears that the total concentration of *Z. rouxii* and *C. versatilis* cells in the immobilized-cell process was respectively, 1.8 and 2.7 times higher than in the free-cell process. However, the ethanol productivity of the immobilized-cell process with *Z. rouxii* and *C. versatilis* was only respectively, 1.5 and 1.1 higher compared to that of the free-cell process. This demonstrated that the immobilized cells of *Z. rouxii* and, especially those of *C. versatilis* have a lower specific ethanol productivity (ethanol production per hour per cell) than the free cells (Hamada et al., 1990a). This lower ethanol productivity of the immobilized cells seems to be caused by the fact that the immobilized cells are exposed to

higher ethanol concentrations than the free cells. Increasing ethanol concentrations increasingly decrease the fermentative activity of yeast cells and by that, the ethanol production (Hamada et al., 1990a).

On the other hand, the 4-ethylguaiacol productivity of the immobilized-cell process with *C. versatilis* was 4.5 times higher than that of the free-cell process, showing that the immobilized cells of *C. versatilis* have a higher specific 4-ethylguaiacol productivity than the free cells. The reason for this higher specific 4-ethylguaiacol productivity remains unclear but seems to indicate that the production of ethanol and 4-ethylguaiacol are independent of each other (Hamada et al., 1990a).

From this comparison, it can be concluded that the application of immobilized yeasts instead of free yeasts for the continuous production of soy sauce gives only small productivity benefits because of the low fermentative activity of the immobilized yeasts. Therefore, the fact that the continuous immobilized-cell process is much faster than the conventional batch process (Table 1) is not caused by the benefits of applying immobilized cells, but seems to be caused by the benefits of applying a lower salt content and better available substrates in the immobilized-cell process.

An immobilized-cell process will be more beneficial if the immobilized yeasts become less sensitive towards ethanol, or if they are less exposed to ethanol. The latter has already been successfully applied by using a continuous microfiltration membrane reactor (Iwasaki et al., 1991). In this reactor a high cell concentration and fermentative activity are maintained because of the membrane, which keeps the cells in the reactor and removes the harmful ethanol from the reactor. The ethanol productivity of this reactor appeared to be several times higher than that of the conventional and immobilized-cell process.

Concluding remarks

From this study, it is clear that in the last decades a considerable progress has been made in the understanding of the flavour formation by salt-tolerant yeasts during soy-sauce

processes. This understanding has resulted in a better control of the flavour formation and derivation of mutants with an enhanced flavour formation. In this way, it is possible to promote the formation of desired flavour components and by that, to influence the flavour composition of the soy sauce or to produce specific natural flavour components instead.

In addition, the flavour formation was accelerated by means of using a continuous process with immobilized salt-tolerant yeasts. This continuous immobilized-cell process showed to be ten times faster than the conventional batch process with free cells. However, this study clearly demonstrated that using immobilized instead of free yeasts only slightly increases the productivity of the continuous process for soy-sauce production. For productivity reasons the continuous microfiltration membrane reactor seems to be the most promising alternative.

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Summary

The salt-tolerant yeasts *Zygosaccharomyces rouxii* and *Candida versatilis* are important for the formation of flavour in Japanese soy-sauce processes. In these processes *Z. rouxii* produces the flavour components ethanol, higher alcohols and 4-hydroxyfuranones, while *C. versatilis* is responsible for the production of phenolic flavour components, such as 4-ethylguaiacol and 4-ethylphenol. These yeast-derived flavour components give, amongst other components, the characteristic flavour to Japanese soy sauce.

Little is known, however, about the flavour formation by the salt-tolerant yeasts, making the process difficult to control. Therefore, in this study, the metabolism of the salt-tolerant yeasts was investigated. Especially, much attention has been paid to the formation of higher alcohols by *Z. rouxii*. The higher-alcohols formation in *Z. rouxii* is strongly related to its amino-acid metabolism, wherein α-keto acids are key intermediates.

The separate effects of the amino acids threonine, cystathionine, and the branched-chain amino acids (isoleucine, valine and leucine) on the metabolism of *Z. rouxii* were determined. The exogenous addition of these amino acids appeared to have large effects on the higher-alcohols production by *Z. rouxii*. For the production of the higher alcohols isobutyl alcohol, active amyl alcohol and isoamyl alcohol the Ehrlich pathway appeared to be very important. In this pathway, uptake and transamination of amino acids results in the higher-alcohols formation. The added amino acids also clearly influenced the formation and conversion rate of the α-keto acid α-ketobutyrate, as appeared from measured enzyme activities. This influence did not result in the accumulation of α-ketobutyrate, because the α-ketobutyrate pool size in *Z. rouxii* appeared to be tightly regulated. Furthermore, independent of the amino acids added, *Z. rouxii* produced ethanol under aerobic conditions (Crabtree effect), which is in contrast to what is described in the literature. In addition, the supply of threonine severely inhibited the growth of *Z. rouxii*.

In order to clarify the growth inhibitory effect of threonine, the regulation of the aspartate-derived amino-acid metabolism in *Z. rouxii* was investigated. It was shown that

the poor growth of *Z. rouxii* in the presence of threonine was due to a lack of methionine, which was caused by a blocked methionine synthesis. Threonine seemed to block this synthesis in *Z. rouxii* by inhibiting the conversion of aspartate. In addition, it was shown that the growth of *Z. rouxii* was not inhibited by the herbicide sulfometuron methyl (SMM) that is a well-known growth inhibitor of various plants, bacteria and yeasts like *Saccharomyces cerevisiae*. The insensitivity of *Z. rouxii* growth to SMM appeared to be caused by the fact that the activity of the enzyme acetohydroxy acid synthase in *Z. rouxii*, unlike that in *S. cerevisiae*, was not affected by SMM. On the other hand, the activity of the enzyme threonine deaminase in *Z. rouxii* was similarly regulated as that in *S. cerevisiae*. Based on these observations it was concluded that the aspartate-derived amino-acid metabolism in *Z. rouxii* is only partly like that in *S. cerevisiae*.

The steady-state culture characteristics of *Z. rouxii* were investigated in this study as well. It was confirmed that *Z. rouxii*, like *S. cerevisiae*, showed the Crabtree effect. It appeared that *Z. rouxii* started to produce ethanol at a lower dilution rate than *S. cerevisiae* (0.1 versus 0.3 h^{-1}), while also the maximum specific growth rate of *Z. rouxii* was lower than that of *S. cerevisiae* (0.17 versus 0.45 h^{-1}). For this investigation, the acceleration-stat (A-stat) cultivation method in which the dilution rate is continuously changed with a constant acceleration rate was used. The A-stat cultivation can be much less time-consuming than the widely used chemostat cultivation, especially at high acceleration rates. However, at very high acceleration rates the A-stat does not provide steady-state culture characteristics. The highest acceleration rate for estimating the steady-state culture characteristics of *Z. rouxii* was determined to be 0.001 h^{-2}. For higher acceleration rates, an increased difference between A-stat and chemostat culture at a given dilution rate was observed. This observation for *Z. rouxii* was confirmed with simulations for *S. cerevisiae*. Moreover these simulations showed that, for estimating the steady-state culture characteristics with the A-stat, both the metabolic adaptation rate of the yeast and the rate at which the environmental substrate concentrations change should be taken into consideration. At an acceleration rate of 0.001 h^{-2}, the A-stat proved to be advantageous to the chemostat, because the A-stat provided much more data in the same time than the chemostat.

The A-stat cultivation was also used to study the concomitant extracellular accumulation of α-keto acids and higher alcohols by *Z. rouxii*. All α-keto acids from the aspartate-derived amino-acid metabolism, except α-ketobutyrate, could be extracellularly accumulated by exogenous supplying the amino acids valine, leucine, threonine and methionine. From this study it was concluded that in *Z. rouxii* valine, leucine and methionine were converted via similar Ehrlich pathways as in *S. cerevisiae*, while for the conversion of threonine both the Ehrlich and amino-acid biosynthetic pathways in *Z. rouxii* were used. Additionally the Ehrlich pathway appeared to be the only pathway for the formation of the higher alcohol methionol in *Z. rouxii*.

Another problem associated with the yeast flavour formation in Japanese soy-sauce processes is that the flavour formation is normally very slow. For this reason the conventional batch process takes about 6 months. In literature the development of a new continuous process using immobilized salt-tolerant yeasts resulted in a 90% reduction of the process time. However, the new immobilized-cell process seemed not very suitable for long-term operation, because alginate was used as immobilization material. The reason for this is that alginate is sensitive to abrasion and chemically unstable towards the high salt concentration in the soy-sauce medium (about 17% (w/v)). To replace alginate, a chemically crosslinked polyethylene-oxide gel was investigated in this study.

The problem of the chemically crosslinked polyethylene-oxide gel was that *Z. rouxii* cells did not survive the immobilization procedure. The absence of survival appeared to be caused by the toxic effect of the crosslinker used for making this gel. Therefore, a new immobilization procedure, in which direct contact between the crosslinker and yeast was circumvented, was developed. For both *Z. rouxii* and *C. versatilis* the survival percentages in the newly developed polyethylene-oxide gel were high and comparable to those in alginate. Unlike alginate gel, the new polyethylene-oxide gel showed, during rheological studies, to be insensitive to abrasion, even in the presence of high salt concentrations.

The insensitivity to abrasion of the new polyethylene-oxide gel was confirmed during cultivations in a stirred-tank reactor with varying high salt concentrations (12.5-17% (w/v)). In this reactor no abrasion of polyethylene-oxide gel particles was observed for

several days, while alginate gel beads were completely destroyed within one day. However, the polyethylene-oxide particles appeared to stick together, making long-term processing difficult. It also appeared during these cultivations that *Z. rouxii* and *C. versatilis* immobilized in the polyethylene-oxide particles were capable of producing characteristic soy-sauce flavours. Therefore, it was concluded that the application of polyethylene-oxide gel in long-term soy-sauce processes is attractive provided that the stickiness of the particles can be controlled.

However, from a comparison of literature data it was also concluded that the application of immobilized salt-tolerant yeasts instead of free yeasts cells in the continuous process hardly accelerates the flavour formation. For accelerating the flavour formation the continuous microfiltration membrane reactor seems to be more promising. Finally, it was concluded that this study and other recent research has enhanced the understanding of the yeast flavour formation during Japanese soy-sauce processes, which facilitates process control.

Samenvatting

De zouttolerante gisten *Zygosaccharomyces rouxii* en *Candida versatilis* zijn belangrijk voor de aromavorming tijdens de productie van Japanse sojasaus. Hierin produceert *Z. rouxii* de aromastoffen ethanol, hogere alcoholen en 4-hydroxyfuranonen, terwijl *C. versatilis* verantwoordelijk is voor de productie van fenolachtige aromastoffen zoals 4-ethylguaiacol and 4-ethylfenol. Deze door de gisten geproduceerde aromastoffen dragen bij aan het karakteristieke aroma van Japanse sojasaus.

Er is echter weinig bekend over de aromavorming door de zouttolerante gisten en dit maakt het proces moeilijk beheersbaar. Daarom is in deze studie het metabolisme van de zouttolerante gisten bestudeerd. In het bijzonder is hierbij veel aandacht besteed aan de vorming van de hogere alcoholen door *Z. rouxii*. De vorming van hogere alcoholen in *Z. rouxii* is nauw verwant aan het aminozuur metabolisme en hierin zijn de α-ketozuren essentiële metabolieten.

De afzonderlijke effecten van de aminozuren threonine en cystathionine, en de aminozuren met vertakte ketens (isoleucine, valine en leucine) op het metabolisme van *Z. rouxii* is onderzocht. Toevoeging van deze aminozuren bleek grote invloed te hebben op de productie van hogere alcoholen door *Z. rouxii*. Voor de productie van de hogere alcoholen isobutyl alcohol, actief amyl alcohol en isoamyl alcohol bleek de Ehrlich route erg belangrijk. In deze metabole route vormt de gist hogere alcoholen door consumptie en daaropvolgend transaminatie van exogene aminozuren. De toegevoegde aminozuren hadden ook een grote invloed op de vorming- en afbraaksnelheid van het α-ketozuur α-ketobutyraat, wat viel af te leiden uit de meting van enzymactiviteiten. Deze invloed leidde niet tot de ophoping van α-ketobutyraat, omdat de α-ketobutyraatconcentratie in de cel nauwkeurig gereguleerd werd. Verder bleek *Z. rouxii* ethanol te produceren onder aërobe omstandigheden (Crabtree effect). Dit was onafhankelijk van de toevoeging van aminozuren en in strijd met wat in de literatuur wordt beschreven. Tenslotte werd vastgesteld dat de toevoeging van threonine tot een drastische remming van de groei van *Z. rouxii* leidde.

Om de oorzaak van de groeiremming door threonine te achterhalen werd de regulatie van het van aspartaat afgeleide aminozuur metabolisme in *Z. rouxii* onderzocht. Hierbij werd aangetoond dat de slechte groei van *Z. rouxii* door threonine wordt veroorzaakt door een tekort aan methionine, hetgeen te wijten leek aan een geremde synthese van methionine. Threonine leek deze synthese in *Z. rouxii* te remmen door het remmen van de omzetting van aspartaat. Ook werd aangetoond dat de groei van *Z. rouxii* niet geremd wordt door het herbicide sulfometuron methyl (SMM). Dit herbicide is een bekende groeiremmer van verscheidene planten, bacteriën en gisten zoals *Saccharomyces cerevisiae*. Dat *Z. rouxii* niet gevoelig is voor SMM leek veroorzaakt te zijn door het feit dat de activiteit van het enzym acetohydroxy acid synthase in *Z. rouxii*, in tegenstelling met dat enzym in *S. cerevisiae*, niet beïnvloed werd door SMM. Daarentegen bleek de activiteit van het enzym threonine deaminase in *Z. rouxii* op dezelfde manier gereguleerd te worden als in *S. cerevisiae*. Gebaseerd op deze waarnemingen werd geconcludeerd dat het van aspartaat afgeleide aminozuur metabolisme in *Z. rouxii* slechts gedeeltelijk overeenstemt met dat in *S. cerevisiae*.

De steady-state cultuurkarakteristieken van *Z. rouxii* werden ook onderzocht in deze studie. Hierbij werd bevestigd dat *Z. rouxii* evenals *S. cerevisiae* het Crabtree effect vertoont. Het bleek dat *Z. rouxii* ethanol begint te produceren bij een lagere verdunningssnelheid dan *S. cerevisiae* (0.1 versus 0.3 uur^{-1}), terwijl de maximale specifieke groeisnelheid van *Z. rouxii* ook lager was dan die van *S. cerevisiae* (0.17 versus 0.45 uur^{-1}). Voor dit onderzoek werd gebruik gemaakt van een acceleratie-stat (A-stat) cultivatiemethode. Deze methode houdt in dat de verdunningssnelheid continu wordt veranderd met een constante acceleratiesnelheid. Vooral bij hoge acceleratiesnelheden kan de A-stat aanzienlijk minder tijd vergen dan de wijd en zijd gebruikte chemostaat cultivatie. Echter bij erg hoge acceleratiesnelheden zal er geen sprake zijn van steady-state condities in de A-stat. De hoogste acceleratiesnelheid voor het benaderen van de steady-state cultuurkarakteristieken van *Z. rouxii* werd vastgesteld op 0.001 uur^{-2}. In het geval van hogere acceleratiesnelheden werd een grotere afwijking tussen de A-stat en chemostaat cultuur bij een bepaalde verdunningssnelheid waargenomen. Deze waarneming voor *Z. rouxii* werd bevestigd met simulaties voor *S. cerevisiae*. De simulaties toonden bovendien aan dat voor het schatten

van de steady-state cultuurkarakteristieken met de A-stat er rekening dient te worden gehouden met zowel de metabolische aanpassingssnelheid van de gist als de veranderingssnelheid van de substraatconcentraties in de omgeving. Bij een acceleratiesnelheid van 0.001 uur^{-2} bleek het voordelig te zijn om de A-stat te gebruiken in plaats van de chemostaat, omdat met de A-stat veel meer data in dezelfde tijd werden verkregen dan met de chemostaat.

De A-stat werd ook gebruikt voor het bestuderen van de samengaande uitscheiding en ophoping van α-ketozuren en hogere alcoholen door *Z. rouxii*. Alle α-ketozuren uit het van aspartaat afgeleide aminozuur metabolisme, met uitzondering van α-ketobutyraat, konden buiten de gistcel worden opgehoopt door het exogeen toevoegen van de aminozuren valine, leucine, threonine en methionine. Uit dit onderzoek werd geconcludeerd dat in *Z. rouxii* valine, leucine en methionine worden omgezet via vergelijkbare Ehrlich routes als in *S. cerevisiae*, terwijl voor de omzetting van threonine zowel de Ehrlich als de aminozuur biosynthese routes worden gebruikt. De Ehrlich route bleek bovendien de enige route te zijn voor de vorming van het hogere alcohol methionol in *Z. rouxii*.

Een ander probleem met de vorming van aromastoffen door gisten tijdens de productie van Japanse sojasaus is dat de aromavorming gewoonlijk erg langzaam gaat. Daarom duurt het conventionele batch proces ongeveer 6 maanden. Uit de literatuur blijkt dat de ontwikkeling van een nieuw continu proces, waarin gebruikt wordt gemaakt van geïmmobiliseerde gistcellen, heeft geleid tot een reductie van de procestijd met 90%. Echter het nieuwe proces met geïmmobiliseerde cellen bleek niet erg geschikt voor langdurende toepassingen, omdat alginaat werd gebruikt als immobilisatiemateriaal. De verklaring hiervoor is dat alginaat gevoelig is voor slijtage en chemisch niet stabiel is bij het hoge zoutgehalte van het sojasaus medium (ongeveer 17% (w/v)). Om alginaat te vervangen is in deze studie een synthetisch polyethyleenoxide gel onderzocht.

Het probleem van het synthetische polyethyleenoxide gel was dat *Z. rouxii* cellen de immobilisatieprocedure niet overleefden. Dit bleek te worden veroorzaakt door het toxisch effect van de crosslinker die wordt gebruikt voor het maken van dit gel. Daarom

werd een nieuwe immobilisatieprocedure, waarbij direct contact tussen de crosslinker en de gist werd vermeden, ontwikkeld. Voor zowel *Z. rouxii* en *C. versatilis* waren de overlevingspercentages in het nieuw ontwikkelde gel hoog en vergelijkbaar met die in alginaat. In tegenstelling tot alginaat, bleek het nieuwe polyethyleenoxide gel tijdens reologische metingen niet gevoelig te zijn voor slijtage, zelfs niet bij hoge zoutconcentraties.

De slijtageongevoeligheid van het nieuwe polyethyleenoxide gel werd bevestigd tijdens cultivaties in een geroerde tank reactor met variërende hoge zoutconcentraties (12.5-17% (w/v)). In deze reactor werd geen slijtage van polyethyleenoxide geldeeltjes waargenomen gedurende verscheidene dagen, terwijl alginaat gelbolletjes al binnen een dag helemaal stuk waren. De polyethyleenoxide geldeeltjes bleken echter samen te plakken wat de langdurige toepassing bemoeilijkte. Wel bleek tijdens de cultivaties dat *Z. rouxii* en *C. versatilis*, geïmmobiliseerd in de polyethyleenoxide geldeeltjes, in staat waren om de voor sojasaus karakteristieke aromastoffen te produceren. Daarom werd geconcludeerd dat de toepassing van het polyethyleenoxide gel in langdurige sojasaus productieprocessen aantrekkelijk kan zijn, mits het samen plakken van de deeltjes beheerst kan worden.

Uit een vergelijking van literatuurdata werd echter ook geconcludeerd dat het gebruik van geïmmobiliseerde zouttolerante gisten in plaats van vrije gistcellen in het nieuwe continue proces nauwelijks bijdraagt aan het versnellen van de aromavorming. Voor een werkelijk versnelde aromavorming lijkt de continue microfiltratie membraanreactor een beter alternatief. Tenslotte werd geconcludeerd dat deze studie en ander recent onderzoek heeft geleid tot een verbetering van het begrip van de aromavorming door de zouttolerante gisten tijdens de productie van Japanse sojasaus, waardoor het proces makkelijker beheersbaar wordt.

Nawoord

Hoe staat het met je proefschrift, werd me de afgelopen tijd veelvuldig gevraagd. Bijna af antwoordde ik dan maar aan dat bijna af leek maar geen einde te komen. Gelukkig hoef ik nu alleen nog maar dit nawoord te schrijven en is het proefschrift dus meer bijna af dan ooit tevoren. Nu vraag ik me zelf af waarom ik ooit aan het schrijven van een proefschrift over de smaakvorming door gisten tijdens het maken van Japanse sojasaus ben begonnen.

De interesse voor de productie van levensmiddelen met behulp van micro-organismen is ontstaan tijdens mijn HBO studie levensmiddelentechnologie in Bolsward. Daar raakte ik danig onder de indruk van het boek 'Basic Bioreactor Design' van de professoren Tramper en van 't Riet uit het verre Wageningen. Van dit boek wou ik meer weten, hetgeen me er toe bracht Friesland te verlaten om een vervolgstudie en uiteindelijk dit promotie-onderzoek in Wageningen te gaan doen. Hierbij heb ik zowel met Hans en Klaas mogen samenwerken; Hans is mijn promotor en Klaas heb ik geassisteerd bij het geven van college uit het hierboven genoemde boek. Deze samenwerking was bijzonder leerzaam. Hans leerde me tijdens het schrijven kop en staart te onderscheiden, maar slaagde er jammer genoeg niet in mij het gevoel voor komma's en streepjes bij te brengen. Klaas gooide mij voor een volle collegezaal, wat me nog steeds 's nachts doet ontwaken.

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Catrinus

Curriculum vitae

Catrinus van der Sluis werd geboren op 15 oktober 1969 in Gorredijk (Friesland). Hij behaalde in 1988 het VWO diploma aan het Drachtster Lyceum. In dat zelfde jaar startte hij zijn studie levensmiddelentechnologie aan de Agrarische Hogeschool Friesland te Bolsward. Tijdens deze opleiding liep hij stages bij onder andere Heineken (Den Bosch) en Aviko (Steenderen). In 1992 rondde hij deze opleiding af met een afstudeerscriptie over bakkersgistfermentaties dat werd uitgevoerd in opdracht van Unilever Research (Vlaardingen). De studie levensmiddelentechnologie zette hij hierna voort aan de Landbouwuniversiteit Wageningen. Aan deze universiteit deed hij een afstudeervak in de richting bioprocestechnologie en liep stage bij de University of Strathclyde in Glasgow (Schotland). In 1995 werd de studie met lof afgerond en begon hij bij de Landbouwuniversiteit Wageningen (sectie Proceskunde) aan zijn promotieonderzoek dat beschreven staat in dit proefschrift.