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Growth of the salt-tolerant yeast *Zygosaccharomyces rouxii* in microtiter plates: effects of NaCl, pH and temperature on growth and fusel alcohol production from branched-chain amino acids

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Abstract

Zygosaccharomyces rouxii, a salt-tolerant yeast isolated from the soy sauce process, produces fusel alcohols (isoamyl alcohol, active amyl alcohol and isobutyl alcohol) from branched-chain amino acids (leucine, isoleucine and valine, respectively) via the Ehrlich pathway. Using a high-throughput screening approach in microtiter plates, we have studied the effects of pH, temperature and salt concentration on growth of *Z. rouxii* and formation of fusel alcohols from branched-chain amino acids. Application of minor variations in pH (range 3–7) and NaCl concentrations (range 0–20%) per microtiter plate well allowed a rapid and detailed evaluation of fermentation conditions for optimal growth and metabolite production. Conditions yielding the highest cell densities were not optimal for fusel alcohol production. Maximal fusel alcohol production occurred at low pH (3.0–4.0) and low NaCl concentrations (0–4%) at 25°C. At pH 4.0–6.0 and 0–18% NaCl, considerable amounts of α -keto acids, the deaminated products from the branched-chain amino acids, accumulated extracellularly. The highest cell densities were obtained in plates incubated at 30°C. The results obtained under various incubation conditions with (deep-well) microtiter plates were validated in Erlenmeyer shake-flask cultures.

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Keywords: Fermentation; Screening; Metabolites; Microtiter plates; Soy sauce; *Zygosaccharomyces rouxii*

1. Introduction

Zygosaccharomyces rouxii, a salt-tolerant yeast, is important for aroma development in the soy sauce fermentation process [1,2]. It produces isoamyl alcohol (3-methyl-1-butanol), active amyl alcohol (2-methyl-1-butanol) and isobutyl alcohol (2-methyl-1-propanol). These so-called fusel alcohols are important flavor compounds in the soy sauce. In *Saccharomyces cerevisiae* fusel alcohols can be produced from the corresponding branched-chain amino acids via the Ehrlich pathway, involving an aminotransferase reaction (yielding the corresponding α -keto acids), a decarboxylation reaction (resulting in aldehyde formation), followed by reduction of aldehydes to fusel alcohols (Fig. 1) [3,4]. Alternatively, the α -keto acids, synthesized

as key intermediates in the respective amino acid biosynthetic pathways, may be directly converted into fusel alcohols. Other pathways for fusel alcohol formation in *S. cerevisiae* remain possible [5–7]. *Z. rouxii* and *S. cerevisiae* appear to employ similar fusel alcohol biosynthetic pathways [1,8,9]. Addition of branched-chain amino acids to *Z. rouxii* [9] and *S. cerevisiae* [10,11] cultures resulted in both cases in increased production of the corresponding fusel alcohols.

Sasaki [12] has studied the effects of variations in salt concentrations in the soy sauce process, but other important parameters such as pH and temperature have not been studied. Evaluation of different fermentation conditions on yeast strain performance is often performed in bioreactors, which is generally a time-consuming and costly process. Alternative procedures for screening fermentation conditions may include the use of multi-well microtiter plates. Here we report the results of a rapid and detailed analysis of the effects of (variations in) pH, temperature and salt on growth and formation of fusel

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alcohols in *Z. rouxii*, using a high-throughput screening approach, with minor variations in growth conditions per microtiter plate well.

2. Materials and methods

2.1. Strain, media and cultivation procedures

Z. rouxii strain AS 2.1521, isolated from the soy sauce process, supplied by Unilever Research Laboratories, Vlaardingen, The Netherlands, was used. Cultures were maintained on skimmed milk and glycerol at -80°C . Pre-culturing of *Z. rouxii* was done at 30°C and 150 rpm on a rotary shaker (Unitron AJ200 shaker, Infors AG, Bottmingen, Switzerland), in 500-ml Erlenmeyer flasks containing 100 ml medium. The medium used was yeast nitrogen base (without ammonium sulfate and amino acids, Difco Laboratories, Detroit, MI, USA), supplemented with 6% glucose (autoclaved), $(\text{NH}_4)_2\text{SO}_4$ (0.75 g l^{-1}) and citrate buffer (0.2 M; also containing 0.1 M KH_2PO_4) at pH 5.0. The complete medium was filter-sterilized ($0.2\text{ }\mu\text{m}$). Cultures were grown until exhausted of the nitrogen source and cells were stored at 4°C . Such cultures were also immediately tested for growth and isoamyl alcohol formation from leucine (10 mM), using the same medium with $(\text{NH}_4)_2\text{SO}_4$ replaced by leucine. No differences were observed in isoamyl alcohol production and growth of *Z. rouxii* in cultures inoculated with cells stored at 4°C for up to three weeks, compared with cells transferred directly to fresh medium containing leucine. Adaptation to high NaCl concentrations was achieved by transferring cultures (1% v/v) to fresh medium (with 0.75 g l^{-1} $(\text{NH}_4)_2\text{SO}_4$) containing 5% NaCl and grown until exhausted of $(\text{NH}_4)_2\text{SO}_4$. This procedure was repeated three times, increasing NaCl concentration with 5% per step, until reaching 20% NaCl. Media used in further experiments had the same composition as described above, with $(\text{NH}_4)_2\text{SO}_4$ (0.75 g l^{-1}) or leucine (10 mM) as nitrogen source, and citrate buffer with the desired pH. Experiments in deep-well (96-well) microtiter plates with a pH gradient (in one direction at eight different values of pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0) and an NaCl gradient (in the other direction with 11 different concentrations of 0 to 20% NaCl with a step size of 2% NaCl; wells with 0% NaCl were done in duplicate) were inoculated to an OD_{600} of 1.0, and incubated at temperatures as indicated in the text. This high initial OD_{600} value was chosen in order to reduce differences in lag phase between different wells. The inocula for wells with medium containing 0% NaCl, 2 and 4% NaCl/6, 8 and 10% NaCl/12 and 14% NaCl/16, 18 and 20% NaCl were pre-cultured on medium containing 0%/5%/10%/15%/20% NaCl, respectively. Erlenmeyer flasks (500 ml, containing 100 ml medium) were incubated at 150 rpm on a rotary shaker, and (deep-well) microtiter plates at 900 rpm (total orbit of 1.5 mm) on a Titramax

1000 rotary shaker (Heidolph Instruments, Schwabach, Germany). After rapid cooling of microtiter plates, or samples, at 4°C for at least 30 min, metabolites were measured in supernatants obtained after centrifugation using a centrifuge with a rotor for (deep-well) microtiter plates (Sigma 4-15C, Sigma Laboratory Centrifuges, Osterode, Germany).

2.2. Analytical methods

Isoamyl alcohol, isobutyl alcohol, active amyl alcohol, 2-methylbutyraldehyde, and 3-methylbutyraldehyde concentrations were determined by gas chromatography with flame ionization detector (GC 8000 Top equipped with two autosamplers in order to double the number of samples that can be handled at the same time; CE Instruments, Thermoquest, Milan, Italy), using on-column injection and He as carrier gas. Columns used were Zebron ZB-1 (30 m, ID 0.32 mm, 0.50 μm film, Phenomenex, Torrance, CA, USA; to separate active amyl alcohol and isoamyl alcohol), and DB-WAX (30 m, ID 0.53 mm, 1.00 μm film, J and W Scientific, Folsom, CA, USA; all other situations). Samples were extracted with methyl formate (1:3), containing 1-hexanol or 1-pentanol as internal standards. Methods were optimized for fast separation by using short temperature gradients to increase the throughput of samples (retention times of 5 min or less). Leucine, isoleucine and valine were measured using HPLC after derivatization with 30 μl *o*-phthalaldehyde/3-mercaptopropionate mixture (25 mM *o*-phthalaldehyde and 25 mM 3-mercaptopropionate in 50% methanol) and 200 μl 0.3 M borate buffer (pH 9.3). The system was equipped with a Luna 5 μ C18 column (30 \times 4.60 mm, Phenomenex) and a UV6000LP diode array detector (Thermo Separation Products, San Jose, CA, USA); the eluate was monitored at 335 nm. α -Keto- β -methylvalerate, α -ketoisovalerate, α -ketoisocaproate were derivatized with *o*-phenylenediamine as fluorogenic agent with a modified method as described by Pailla et al. [13]: 240 μl *o*-phenylenediamine solution (133 mg in 100 ml 3 M HCl) was added to 20 μl supernatant (of the appropriate dilution). This was heated for 20 min at 80°C . After cooling to room temperature, 240 μl of 3 M NaOH was added to neutralize the samples. Samples were measured using HPLC with a Luna 5 μ C18 column (150 \times 4.60 mm, Phenomenex). Elution (1 ml min^{-1}) was done with a linear gradient from 1% acetic acid (pH 5.0) to 100% methanol in 10 min. Monitoring of the eluate was done at 410 nm (emission) and at 350 nm (excitation) with an FL3000 fluorescence detector (Thermo Separation Products).

Glucose was determined with the enzymatic kit of Roche diagnostics (Mannheim, Germany). $(\text{NH}_4)_2\text{SO}_4$ was determined with the Berthelot reaction [14]. Growth was measured in a polycarbonate microtiter plate at 600 nm after appropriate dilution in water, using a spectrophotometer (SPECTRAMax PLUS384 Microplate Spec-

trophotometer, Molecular Devices, Sunnyvale, CA, USA). Above-mentioned assays were routinely done using automated robotized equipment for pipetting and plate handling: the Plato-7 pipetting robot (Rosys-Anthos, Hombrechtikon, Switzerland), the Zymate robot for plate handling, and the RapidPlate-96 pipetting robot (Zymark, MA, USA). Contour plots were made using MATLAB version 6.1.0.450 Release 12.1 (MathWorks, Natick, MA, USA).

3. Results and discussion

3.1. Growth of *Z. rouxii* AS 2.1521 in deep-well microtiter plates

Growth of *Z. rouxii* was tested in different types of deep-well and microtiter plates, closed with various types of seals, tape, lids or mats (to prevent evaporation of volatile compounds, e.g. alcohols), in media (at pH 5.0 and 0% NaCl, as described in [Materials and methods](#)) containing 6% glucose, and 10 mM leucine incubated at 30°C. Growth in a deep-well microtiter plate (0.3 ml medium in a round deep-well microtiter plate with a total volume of 1.2 ml) closed with a polypropylene mat showed most reproducible results ([Fig. 2](#); each point represents an average of 16 wells). During the incubations the pH remained constant. Overnight incubations of the plates resulted in the depletion of oxygen (demonstrated with 0.5 mg l⁻¹ resazurin in the medium). The doubling time in a deep-well microtiter plate was 5.6 h (calculated from two separate experiments), compared to 4.4 h in an Erlenmeyer flask incubated under similar conditions. Variation in growth and doubling time between identical wells was 15–20%, not uncommon for cultivation in (deep-well) microtiter plates [15]. Growth stopped upon exhaustion of the N-source (leucine). The consumption of leucine was much faster than isoamyl alcohol formation, which continued in time after leucine depletion from the medium.

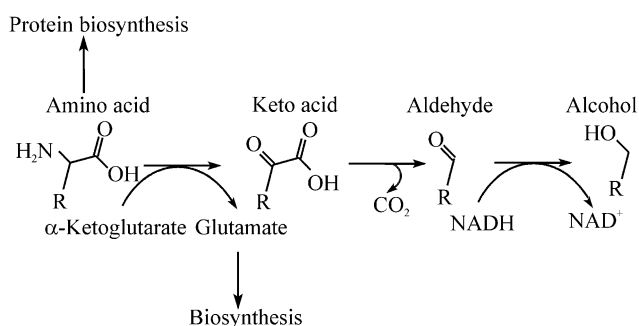


Fig. 1. The Ehrlich pathway [3,4]. A schematic representation of the conversion of branched-chain amino acids to fusel alcohols; leucine via α -keto isocaproate and 3-methylbutyraldehyde to isoamyl alcohol; isoleucine via α -keto- β -methylvalerate and 2-methylbutyraldehyde to active amyl alcohol; valine via α -ketoisovalerate and isobutyraldehyde to isobutyl alcohol (valine: R = C₃H₇, leucine and isoleucine: R = C₄H₇).

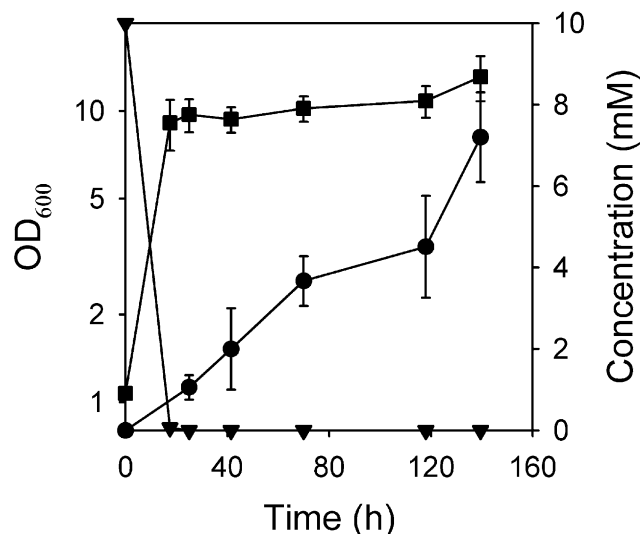


Fig. 2. Growth of *Z. rouxii* in deep-well microtiter plates closed with a polypropylene mat in medium (pH 5.0 and 0% NaCl) with 6% glucose and 10 mM leucine at 30°C. Each point represents an average of 16 wells with the standard deviation indicated with the error bars. Symbols: ●, isoamyl alcohol (mM); ▼, leucine (mM); ■, OD₆₀₀ (left axis, exponential scale).

Conversion of leucine (the only N-source for growth) to isoamyl alcohol was not stoichiometric: consumption of 10.0 mM leucine resulted in 7.2 mM isoamyl alcohol after 140 h of incubation. No increase in isoamyl alcohol concentration was observed after 140 h ([Fig. 2](#)).

3.2. Substrate consumption and formation of extracellular metabolites in deep-well microtiter plates

Z. rouxii was cultivated under 88 different conditions (pH and NaCl) in deep-well microtiter plates. The incubations were done at different temperatures (from 10–40°C with 5°C increase in temperature) with leucine, isoleucine, valine and all possible combinations thereof. [Fig. 3](#) shows a typical example of results obtained with *Z. rouxii* with leucine as N-source, when grown for 7 d (168 h) at 25°C in a deep-well microtiter plate with pH and NaCl gradients as described in [Materials and methods](#) (experiments done in triplicate). Obviously, this strain is very salt-tolerant: high cell densities are observed at salt concentrations of 0–18% NaCl over a broad pH range. Earlier observations with a *Z. rouxii* strain isolated from the soy sauce process showed that the pH range for the growth without NaCl is very broad (pH 3–7), while in a medium containing 18% NaCl the pH range for growth is narrow (pH 4–5) [1]. There is a good correlation between growth ([Fig. 3A](#)) and consumption of leucine ([Fig. 3C](#)), the only nitrogen source present. The formation of isoamyl alcohol does not follow the growth pattern at all ([Fig. 3B](#)). Isoamyl alcohol concentrations are highest at low pH (pH 3.0–4.0), at low NaCl concentrations (0–4% NaCl) and at 25°C. 3-Methylbutyraldehyde, the aldehyde that can be formed from leucine via the Ehrlich pathway ([Fig. 1](#)), was not detected

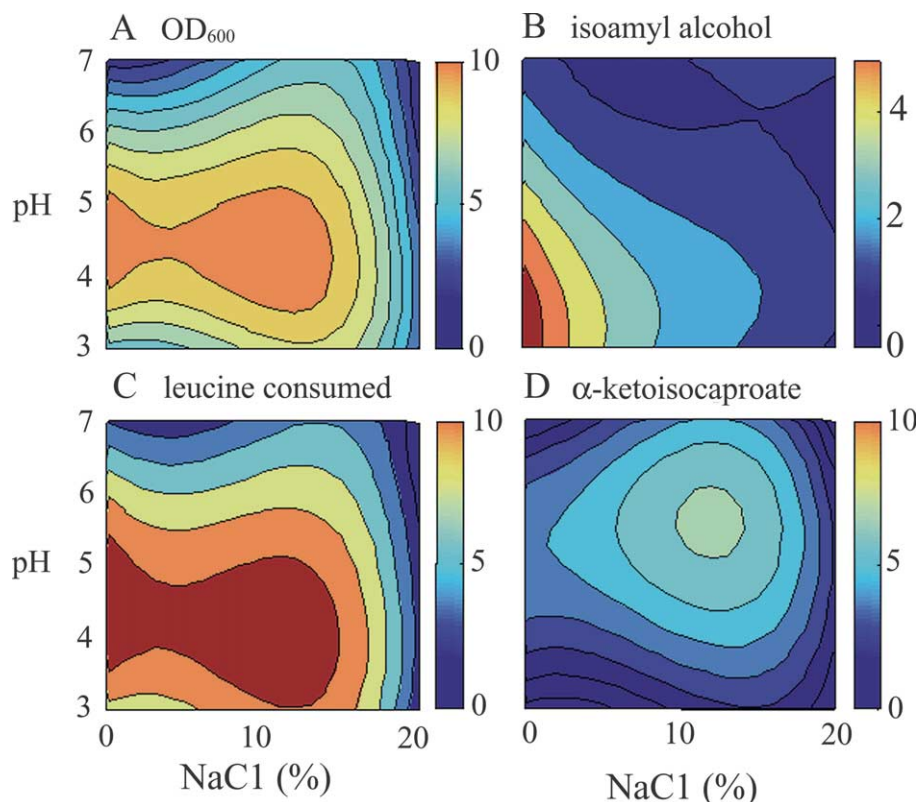


Fig. 3. Contour plots of *Z. rouxii* grown in deep-well microtiter plates in medium with 6% glucose and 10 mM leucine at 25°C with final values of OD₆₀₀ (A), isoamyl alcohol produced (B, mM), leucine consumption (C, mM), and α -ketoisocaproate produced (D, mM). Vertical bars indicate OD₆₀₀ value or concentrations.

in culture supernatants. Above pH 4 substantial amounts of extracellular α -ketoisocaproate accumulated, reaching peak concentrations of 7.5 mM at pH 5.5 and 12% NaCl. α -Ketoisocaproate was also produced in the absence of NaCl in the medium. Recently, Van der Sluis et al. [16] reported the extracellular accumulation of α -keto acids from the corresponding branched-chain amino acids studying another *Z. rouxii* strain, using Erlenmeyer flasks containing a medium at pH 5.0 and no NaCl. In our experiments with leucine, not only isoamyl alcohol and α -ketoisocaproate but also isobutyl alcohol (up to 0.3 mM) and the corresponding α -keto acid, α -ketoisovalerate (up to 1.5 mM) accumulated extracellularly. α -Keto- β -methylvalerate (0.5 mM at the maximum) was detected extracellularly, but not the corresponding fusel alcohol, active amyl alcohol (Fig. 1). α -Ketoisovalerate and α -keto- β -methylvalerate show similar patterns towards NaCl and pH as α -ketoisocaproate. Cross talk between catabolic and anabolic reactions of the branched-chain amino acid metabolism may explain the formation of the different keto acids and fusel alcohols, as described by Dickinson et al. [6,7]. Van der Sluis [16] has not reported the accumulation of these α -keto acids, which are not derived from leucine. Possible explanations are differences in strains or growth conditions used in both studies. Under various incubation conditions we observed that the substrate/product(s) ratios were not stoichiometric.

We measured concentrations of the metabolites in supernatants and it is very likely that certain metabolites are present intracellularly.

Incubations of *Z. rouxii* in deep-well microtiter plates with leucine, isoleucine and valine in all possible combinations, resulted in similar growth and product formation patterns with respect to pH and NaCl concentrations as described for leucine only at incubation temperatures of 20–35°C (Fig. 3). None of the incubations resulted in aldehyde formation in culture supernatants. No growth was observed with incubations at 10 and 40°C, and no conversion of branched-chain amino acids into fusel alcohols occurred. At 15 and 35°C, fusel alcohol and intermediate concentrations formed were much lower. The highest cell densities were obtained in deep-well microtiter plates incubated at 30°C.

3.3. Validation of growth in deep-well microtiter plates with Erlenmeyer shake flasks

Three examples of growth and product formation of *Z. rouxii* inoculated in Erlenmeyer flasks with leucine (12 mM) are shown in Fig. 4 (experiments done in duplicate; one experiment shown). The following conditions were tested at 25°C in a medium containing 0% NaCl at pH 5.0 (Fig. 4A), 14% NaCl at pH 5.0 (Fig. 4B) and 0% NaCl at pH 3.0 (Fig. 4C). Supernatants of *Z. rouxii* cultures

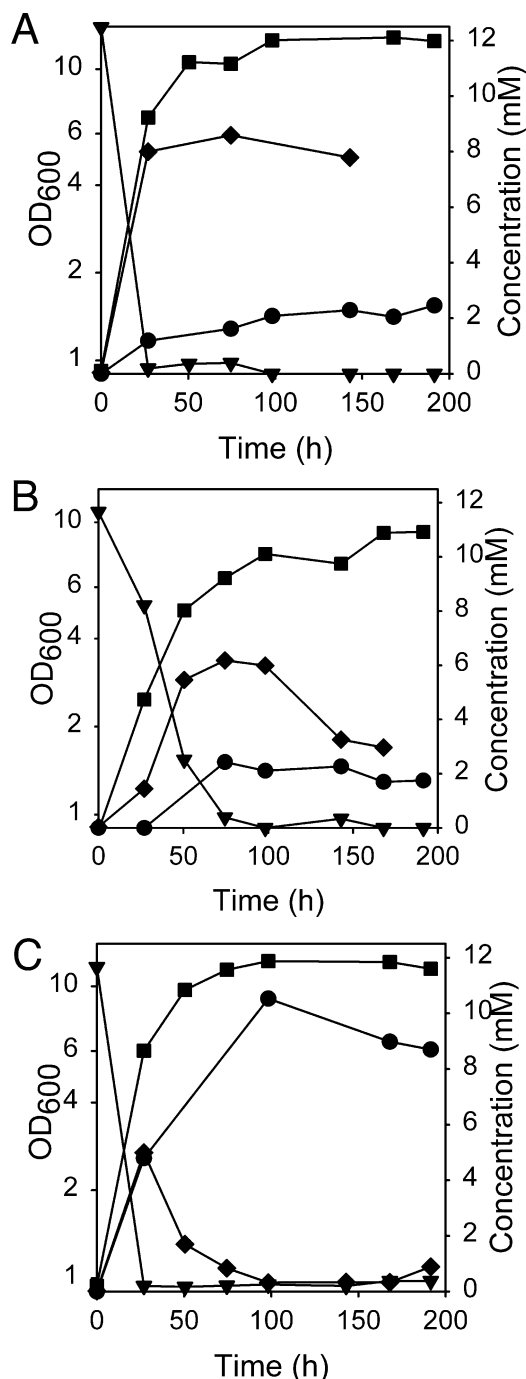


Fig. 4. Growth curves of *Z. rouxii* in Erlenmeyer flasks in medium with 6% glucose and 12 mM leucine at 25°C. Growth conditions are pH 5.0 and 0% NaCl (A), pH 5.0 and 14% NaCl (B), pH 3.0 and 0% NaCl (C). Symbols: ■, OD₆₀₀ (left axis); ●, isoamyl alcohol concentration (mM); ▼, leucine concentration (mM); ◆, α -ketoisocaproate (mM).

grown at pH 5.0 and 0% NaCl (after 27 h of incubation) contained up to 8.6 mM α -ketoisocaproate (Fig. 4A). The highest isoamyl alcohol concentration reached in these cultures was 2.5 mM. Incubations at the same pH with 14% NaCl resulted in slower growth and leucine consumption. Maximal isoamyl alcohol concentrations were 2.3

mM, and α -ketoisocaproate concentrations reached 6.2 mM (Fig. 4B). At pH 3.0 and 0% NaCl, α -ketoisocaproate initially accumulated extracellularly (concentrations up to 5.0 mM) and was consumed again upon prolonged incubation. After 7 d of incubation 9.0 mM isoamyl alcohol was present in the supernatant. Part of the isoamyl alcohol formed in these Erlenmeyer experiments evaporated from the culture medium, as can be seen in Fig. 4C. In the experiments described in Fig. 4A and B, the isoamyl alcohol concentration remained fairly constant, most likely due to conversion of keto acid into fusel alcohol. A comparison of the results obtained with Erlenmeyer flasks and deep-well microtiter plates clearly shows that the observations made in microtiter plates experiments are reliable; the highest isoamyl alcohol formation occurs in incubations at pH 3.0 and 0% NaCl, whereas at pH 5.0 α -ketoisocaproate concentrations are higher than the isoamyl alcohol concentrations.

4. Conclusions

Growth of *Z. rouxii* in deep-well microtiter plates is a useful scale-down from larger volume systems such as Erlenmeyer flasks (and probably also bioreactors). This provides a high-throughput screening cultivation method to optimize conditions for yeast growth and/or desired product formation. Using this approach we have shown that the cultivation of *Z. rouxii* to the highest cell densities (pH 4.0–6.0, with NaCl concentrations of 0–18% at 30°C) did not result in the highest fusel alcohol concentrations. Highest fusel alcohol concentrations were observed with incubations at low pH and salt concentration. These results are in agreement with the observations of Sasaki [12] with *Z. rouxii* ATCC13356 that the concentrations of flavor compounds (such as isobutyl alcohol, active amyl alcohol, isoamyl alcohol) decreased with higher sodium chloride concentrations. This may stimulate the development of a new type of soy sauce with a relatively low salt concentration. For health reasons consumers tend to prefer food products with low salt concentrations. Moreover, in the traditional soy sauce process, yeast displays a relatively low metabolite activity at the high salt concentration used {approximately 17% (w/v)} [17]. At low salt concentrations and higher metabolic activities, the soy sauce production time may even be reduced.

The microtiter plate cultivation method described in this manuscript can also be used for the optimization of other microbial fermentation processes, such as media composition or conditions for product formation, characterization of mutagenized organisms—basically all applications that require the evaluation of large numbers of cultures. In these situations there is an enormous advantage (saving time and materials) to cultivating on a small scale, suitable for automated robotized equipment for microtiter plate handling and reading, as described in this manuscript.

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