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# 4-Hydroxy-2,5-dimethyl-3(2H)-furanone Formation by *Zygosaccharomyces rouxii*: Effect of the Medium

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The formation of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) by *Zygosaccharomyces rouxii* was studied in yeast−peptone−dextrose medium containing D-fructose 1,6-diphosphate under various culture conditions. Cell growth and HDMF production was heavily dependent on medium pH and sodium chloride concentration. Higher pH values of the nutrient medium had a positive effect on HDMF formation but retarded cell growth resulting in an optimal pH value of 5.1 with regard to the yield of HDMF. Salt stress stimulated HDMF formation by *Z. rouxii* as increasing sodium chloride concentration led to higher amounts of HDMF. The HDMF concentration in the culture supernatant and HDMF formation per yeast cell peaked at 20% sodium chloride in the nutrient medium. The nonutilizable carbohydrate D-xylose displayed a weak effect on HDMF formation, and the addition of glycerol to salt-stressed cells had no effect on the production of HDMF.

**KEYWORDS:** ***Zygosaccharomyces*; furanone; flavor; sodium chloride; salt stress**

## INTRODUCTION

4-Hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF, Furaneol), a potent aroma compound found in many fruits such as strawberry, mango, pineapple, and raspberry, is extensively used as food flavoring due to its low odor threshold and flavor-enhancing properties (*1*). Aside from fruits, HDMF has been isolated from yeasts, bacteria, and insects, and it is formed chemically during the so-called Maillard reaction (*1*, *2*). Enzymatical formation of HDMF is expected in fruits and microorganisms although no enzyme has been characterized until now.

In 1991, the formation of a homologue of HDMF, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone, by shoyu yeasts was studied and D-xylulose-5-phosphate as well as D-ribulose-5-phosphate were proposed as precursors of the furanone (*3*). Some years later, the production of HDMF by the shoyu yeast *Zygosaccharomyces rouxii* was demonstrated in nutrient solutions containing D-fructose 1,6-diphosphate and D-glucose (*4*). *Z. rouxii* belongs to a small number of yeasts that can grow well in media of high osmolarity, and it is a constituent of the shoyu microflora. This yeast can propagate over a wide range of salt concentrations and accumulates high levels of glycerol in response to increases in the concentration of sodium chloride in the growth medium (*5*). Although *Z. rouxii* cells can grow in media with D-glucose as the sole carbon source, HDMF is only produced when media are supplemented with D-fructose 1,6-diphosphate (*6*). As only the singly labeled furanone was formed after the addition of 1-$^{13}$C-D-fructose 1,6-diphosphate to the medium and unlabeled HDMF was formed in the presence of $^{13}C_6$-D-glucose, it was concluded that the carbons in HDMF originate exclusively from exogenously supplied D-fructose 1,6-diphosphate (*6*). The effect of various environmental factors on the production of furanones by *Z. rouxii* has already been investigated in shoyu koji medium (*7*−*9*) but not in the less complex yeast−peptone−dextrose (YPD) medium as proposed by Hecquet et al. (*4*). Therefore, we investigated the effect of salt stress and pH value on the formation of HDMF in a YPD nutrient medium and determined the effect of externally added glycerol on HDMF concentration.

## RESULTS AND DISCUSSION

**Effect of Sodium Chloride.** The effect of increasing sodium chloride concentration on cell growth and HDMF formation of *Z. rouxii* was investigated in nutrient media consisting of YPD media containing 1% D-glucose and 4.5% D-fructose 1,6-diphosphate. Sodium chloride concentrations ranging from 0 to 26% were chosen. *Z. rouxii* did not grow in the presence of 23 and 26% sodium chloride. In the high salt media, the growth rate was retarded and the maximum cell number decreased with increasing sodium chloride content (**Figure 1**). However, HDMF concentration increased with increasing sodium chloride content, although cell numbers decreased (**Figure 1**). Thus, salt stress stimulated HDMF formation by *Z. rouxii* resulting in a productivity increase of the individual cells. In control experiments, without the addition of yeast cells, HDMF was not formed (data not shown). In a second experiment, media containing 7, 10, 13, and 17% NaCl were inoculated with *Z.*

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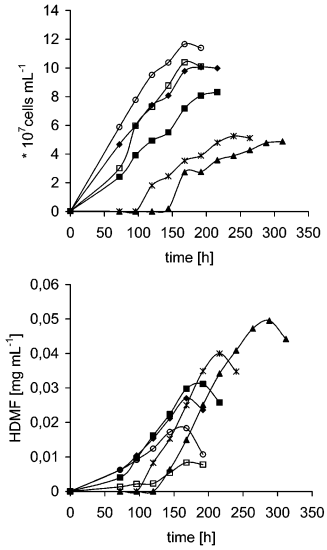


**Figure 1.** *Z. rouxii* growth (top) and HDMF formation (bottom) in media containing 0 (□), 5 (○), 9 (♦), 13 (■), 17 (*), and 20% (▲) sodium chloride.

*rouxii* cells. Cell numbers peaked at 7% NaCl, and the highest HDMF concentration was determined at 17% NaCl. These values confirmed the already obtained data.

The influence of sodium chloride on the levels of flavor compounds produced by the shoyu yeast *Z. rouxii* has already been investigated (*7*). However, in this former experiment, a complex shoyu koji culture medium, containing 375 g of defatted soybean powder per liter, was used without additional carbohydrates. It was shown that the production of 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone, a homologue of HDMF biosynthesized through the pentose−phosphate cycle, reached a maximum at a sodium chloride concentration of 16%. In contrast, HDMF concentration did not correlate with sodium chloride concentration. The authors concluded that HDMF was nonenzymatically formed during the preparation of the fermentation broth.

We can exclude a nonenzymatic production of HDMF as the formation of HDMF from D-fructose 1,6-diphosphate was demonstrated in YPD medium only when *Z. rouxii* cells were added (*4*). Externally added D-fructose 1,6-diphosphate constitutes the sole carbon source for HDMF as shown recently by the application of precursors labeled with stable isotopes (*6*).

*Z. rouxii* is a salt tolerant yeast accumulating glycerol in the cells as the primary osmoregulatory compound (*10*). The intracellular amount of glycerol in *Z. rouxii* was shown to be proportional to the concentration of sodium chloride in the growth medium. The mechanism of glycerol accumulation is thought to depend not on the increase in glycerol production but rather on the change of plasma membrane function in retaining intracellular glycerol (*10*). We assume that changes occurring in the cell wall and membrane of *Z. rouxii* during adaptation to salt stress promote biosynthesis of HDMF from D-fructose 1,6-diphosphate. The cell wall and membrane of *Z. rouxii* have recently been identified as sites where at least the first step of HDMF formation takes place (*6*).

**Effect of Medium pH Value on the Formation of HDMF.** It is assumed that at least one step of the D-fructose 1,6-diphosphate biotransformation to HDMF is an enzymatically catalyzed process localized in the cell wall or the membrane of the yeast cells (*6*). Thus, the effect of the pH value of the nutrient medium on the formation of HDMF was investigated. Studies on the effect of the pH value on the growth rate of *Z. rouxii* have already been performed. At high sugar concentrations

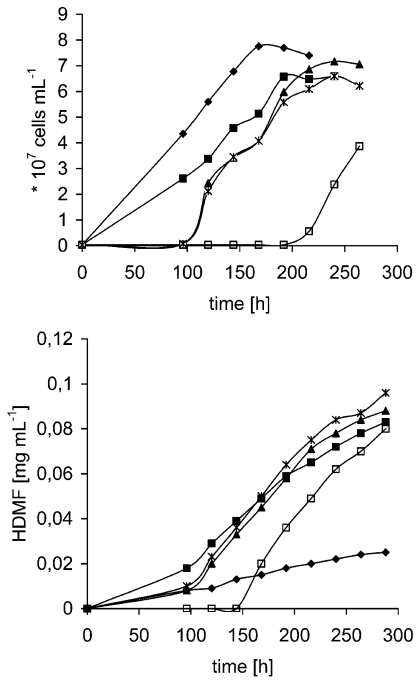


**Figure 2.** *Z. rouxii* growth (top) and HDMF formation (bottom) in media exhibiting a pH value of 4.1 (♦), 4.5 (■), 4.8 (▲), 5.1 (*), and 5.5 (□).

(12%), *Z. rouxii* is able to grow over a wide pH range from 1.5 to 10.5 (*11*) with an optimum range from pH 3.5 to 5 (*12*). The pH tolerance of *Z. rouxii* is broader in high glucose (1.8−8.0) than in high salt (3.0−6.6) medium, and the pH tolerance range narrows as the salt concentration increases (*13*). Thus, pH values from pH 4.1 to 5.5 were chosen. Nutrient solutions consisting of YPD media containing 1% D-glucose and 5% D-fructose 1,6-diphosphate were prepared, pH values were adjusted, and the solutions were inoculated with yeast cells, respectively. During the incubation period of 12 days, 0.5 mL samples were withdrawn daily, cell numbers were determined, and the HDMF concentration was quantified in the supernatant after centrifugation by high-performance liquid chromatography (HPLC) with UV detection. **Figure 2** shows the cell number and calculated HDMF concentration obtained at different pH values. HDMF concentration peaked at pH 5.1 while the growth of *Z. rouxii* was retarded at higher pH values. Cell numbers reached in the end almost the same values except for the sample with pH 5.5. Consequently, at pH 5.5, HDMF formation per yeast cell was increased by a factor of 4 as compared with the HDMF concentration produced at pH 4.1. Former studies on the biosynthesis of HDMF in *Z. rouxii* obtained only approximately 50 ppm after 12 days of incubation with the same concentration of D-fructose 1,6-diphosphate and D-glucose (*4*). In a similar experiment using media with pH values of 3.88, 4.2, 4.7, 5.2, and 5.7, the HDMF concentration peaked at pH 5.2 and comparable growth curves were determined confirming the previously obtained data. Thus, the pH value plays an important role for HDMF formation.

**Effect of Glycerol.** During osmoregulation of *Z. rouxii* following osmotic upshock, a wide variety of steps are taken by the cells to survive the stress imposed on them by the loss of cellular water. These include the accumulation of glycerol by active transport and by endogenous production of glycerol (*14*). The sodium chloride study already showed that HDMF formation correlates with the osmotic stress experienced by *Z. rouxii*. Therefore, we studied the effect of externally added glycerol on HDMF formation to find a correlation between glycerol and HDMF production during osmotic stress. We

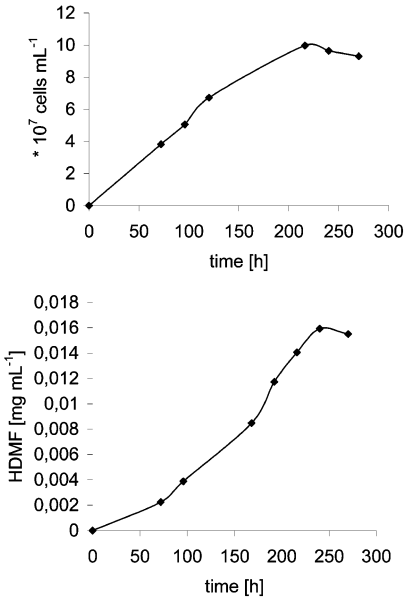


**Figure 3.** *Z. rouxii* growth (top) and HDMF formation (bottom) in media containing 17% D-xylose instead of sodium chloride.

further increased the osmotic stress already caused by sodium chloride by gradually increasing the glycerol concentration. Nutrient solutions consisting of YPD media, containing 17% NaCl, supplemented with 5% D-fructose 1,6-diphosphate and 0−5% glycerol, were prepared and inoculated with yeast cells. To determine the ability of *Z. rouxii* to grow on glycerol as a single carbon source, one sample containing 5% glycerol was prepared and inoculated as described above but without D-glucose. Samples were withdrawn daily over an incubation time of 10 days, and cell numbers and HDMF concentration in the media were determined. No significant influence of the various growth conditions on the cell growth and the HDMF yields was observed. Thus, *Z. rouxii* is able to grow on glycerol as a single carbon source, whereby the HDMF formation is not influenced. Consequently, HDMF production does not appear to correlate with glycerol formation during osmotic stress.

**Effect of D-Xylose.** Instead of sodium chloride, the nonutilizable carbohydrate D-xylose was added to nutrient medium containing 1% D-glucose and 4.5% D-fructose 1,6-diphosphate (*15*) to examine the influence of the chemical nature of the solute causing the osmotic stress on yeast cell growth and HDMF formation. The addition of 17% D-xylose (1.1 M) to the nutrient medium had a similar effect on cell growth like the addition of 9% sodium chloride (1.5 M) (**Figure 3**). HDMF formation correlated with cell growth, but the maximum HDMF yield (0.016 mg/mL) was lower than the yield obtained by the addition of 9% sodium chloride (0.025 mg/mL). Thus, addition of D-xylose displays a similar effect on HDMF formation by *Z. rouxii*, but it seems that HDMF productivity per yeast cell is higher in sodium chloride supplemented medium than in D-xylose supplemented medium under similar osmotic conditions.

## EXPERIMENTAL SECTION

**Chemicals.** Chemicals, salts, and solvents of high purity were obtained from Fluka (Deisenhofen, Germany), Sigma (Deisenhofen, Germany), and Aldrich (Deisenhofen, Germany). Solvents were redistilled prior to use.

**Strain, Growth Media, and Culture Conditions.** The yeast strain used in this investigation was *Z. rouxii* ATCC 13356. The YPD medium (pH 4.6) consisted of 5 g $L^{-1}$ yeast extract, 5 g $L^{-1}$ peptone, 4 g $L^{-1}$ $KH_2PO_4$, 5 g $L^{-1}$ $MgSO_4 \cdot 7H_2O$, 10 g $L^{-1}$ D-glucose, and 170 g $L^{-1}$ NaCl. The solutions were autoclaved at 120 °C, at 2 bar for 16 min, and stored at 7 °C. The D-fructose 1,6-diphosphate stock solution (200 g $L^{-1}$) was filtered (0.2 $\mu$m pore filter).

For a standard incubation experiment, 45 or 50 g $L^{-1}$ D-fructose 1,6-diphosphate was added to YPD medium and the medium was poured into Erlenmeyer flasks. The media were inoculated with 0.02 mL of a *Z. rouxii* culture (approximately $2 \times 10^6$ cells) and incubated at 30 °C in a GFL 3033 shaker (GFL, Burgwedel, Germany) at 150 rpm for the time period indicated in the text. During the incubation period, 0.5 mL samples were withdrawn daily, and the cell count and HDMF concentrations were determined.

**Effect of Sodium Chloride Concentration on HDMF Formation.** Ten milliliter portions of YPD media, 45 g $L^{-1}$ D-fructose 1,6-diphosphate, and 0−260 g $L^{-1}$ sodium chloride were inoculated with *Z. rouxii* cells ($1.9 \times 10^6$ cells) and incubated at 30 °C. The *Z. rouxii* cell count and HDMF concentration were determined daily.

**Effect of Medium pH Value on HDMF Formation.** Ten milliliter portions of YPD medium containing 50 g $L^{-1}$ D-fructose 1,6-diphosphate and 170 g $L^{-1}$ sodium chloride were adjusted to pH values of 4.1, 4.5, 4.8, 5.1, and 5.5. Media were inoculated with $3 \times 10^6$ yeast cells and incubated at 30 °C. The *Z. rouxii* cell count and HDMF concentration were determined daily.

**Effect of Glycerol on HDMF Formation.** Nutrient solutions (10 mL) containing YP medium, 0−5 g $L^{-1}$ D-glucose, 50 g $L^{-1}$ D-fructose 1,6-diphosphate, 170 g $L^{-1}$ sodium chloride, and 0−5 g $L^{-1}$ glycerol were inoculated with $3 \times 10^6$ yeast cells. The yeast cell count and HDMF concentration were determined daily.

**Effect of D-Xylose on HDMF Formation.** Nutrient solutions (10 mL) containing YP medium, 0−5 g $L^{-1}$ D-glucose, 4.5 g $L^{-1}$ D-fructose 1,6-diphosphate, and 170 g $L^{-1}$ D-xylose were inoculated with $1.9 \times 10^6$ yeast cells. The yeast cell count and HDMF concentration were determined daily.

**Estimation of Cell Counts.** After the cells in a *Z. rouxii* cell suspension were counted using a Thoma-Neubauer counting chamber, a calibration curve was plotted by measuring the absorbance at 600 nm (turbidity) (Spectronic Genesys 2PC, Spectronic Instruments Inc., Rochester NY) of individual dilutions of the cell suspension against water. The equation $Y = 5 \times 10^{-8} \times X + 0.0512$, where $X$ represents the number of cells and $Y$ represents the absorbance, was obtained; $R^2 = 0.9914$. The cell counts of *Z. rouxii* cultures were calculated using the presented equation after turbidity measurements.

**HPLC Analysis.** Samples withdrawn from the media were centrifuged at 5000*g* for 5 min, and the supernatant was directly analyzed using an HPLC system equipped with a Spark Holland Basic Marathon autosampler (Spark Holland, Emmen, The Netherlands) connected to a Knauer Maxistar pump and Knauer variable wavelength monitor (Knauer, Berlin, Germany). Knauer Eurochrom 2000 software was used for data acquisition. A Eurospher 100-C18 column (length, 240 mm; inner diameter, 4 mm; and particle size, 5 $\mu$m) (Knauer) was employed. A gradient starting from 95% (A: 0.05% formic acid in water) and 5% (B: acetonitrile) to 80% A within 10 min then to 0% A in 30 min was used at a flow rate of 1 mL $min^{-1}$. A wavelength of 285 nm was recorded. Under these conditions, HDMF was eluted at 9.1 min. Quantitative analysis was performed using a standard curve of commercial HDMF.

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