Biosynthesis of 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone by *Zygosaccharomyces rouxii*

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4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) production by *Zygosaccharomyces rouxii* was studied under various culture conditions. Among the precursors added, D-fructose 1,6-bisphosphate was the best. Aerobic conditions led to a higher HDMF level, and the concentration of the added D-fructose 1,6-bisphosphate up to 10% increased HDMF production; HDMF biosynthesis then decreased and was strongly inhibited by 20% of D-fructose 1,6-bisphosphate. HDMF was produced by *Z. rouxii* during the growth phase and the stationary phase. This microbiological method enabled the biosynthesis of "natural" HDMF at 100 ppm.

Keywords: *4-Hydroxy-2,5-dimethyl-3(2H)-furanone; Zygosaccharomyces rouxii*

INTRODUCTION

Among flavor compounds exhibiting caramel-like odors, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) is of special interest because of its relatively low flavor thresholds of 0.03 mg (taste) and 0.1 mg per liter of water (odor) (Pittet et al., 1970). The odorant was reported for the first time in pineapples (Rodin et al., 1965) and strawberries (Mayerl et al., 1989). Recently, HDMF was identified as a natural insect product, in *Eurycotis floridana*, male gland extracts revealed a characteristic odor of caramel which is attractive at a distance for the females (Farine et al., 1993). HDMF has also been detected in several heat-processed foods, e.g. beef broth (Tonsbeek et al., 1974), roasted almonds (Takei and Yamanishi, 1974), and popcorn (Tressl et al., 1978), and was recently identified as an important odorant in wheat bread crust (Schieberle, 1992). HDMF has also been isolated from shoyu (shoyu is the Japanese name for soy sauce) (Osaki et al., 1985). The content of HDMF in shoyu is very low (10 ppm). In processed foods, HDMF is probably formed in the course of cooking; model experiments have revealed that thermal degradation of fructose (Shaw and Berry, 1977) or 6-deoxyhexoses such as rhamnose (Hodge, 1953) in the presence of amines or amino acids produces HDMF by Maillard reactions (Pischetsrieder et al., 1994). However, in natural raw products, the formation pathway of HDMF is unknown. In fruits, a glucoside of the odorant has been identified (Mayerl et al., 1989); it seems likely that a biogenic pathway is involved in its formation. No evidence that HDMF can be biosynthesized by microorganisms has been produced so far. This problem is of obvious importance; synthetic HDMF is largely used as an additive in food industries, and its production by fermentation, providing a natural labeled compound, would be of great economic interest.

Recently, Sasaki et al. (1991) studied the formation of a homolog of HDMF, 4-hydroxy-2-ethyl-3(2*H*)-furanone (HEMF) and concluded that D-sedoheptulose 7-phosphate is the precursor of HEMF. We considered that HDMF could be produced by the same microorganism from six-carbon unit sugars. We had already studied the enzymatic synthesis of the natural precursor of HDMF (Delest and Demuynck, 1991). This paper describes the production of HDMF by shoyu yeast *Zygosaccharomyces rouxii*.

MATERIALS AND METHODS

Chemicals. All chemical reagents were purchased from Sigma and Aldrich. The organic solvents were obtained from Carlo-Erba. The products utilized for microbiological media were obtained from Difco Laboratories.

Microorganisms. Koji was made with a mixture of *Aspergillus oryzae* and *Aspergillus sojae* spores utilized in Japan for shoyu preparation and called koji starter. The yeast strain used in this investigation was *Z. rouxii*, ATCC 13356.

Media for *Z. rouxii*. *Shoyu Koji Preparation.* A mixture of cooked soybeans (50 g) and roasted crushed wheat kernels (50 g) was mixed, and this powder was suspended in 100 mL of distilled water. The mixed materials were cultured in tanks with 0.1−0.2% (w/w) of the starter mold, *A. oryzae* and *A. sojae*, for 72 h at 30 °C.

Koji-Based Medium. One hundred grams of shoyu koji was dissolved in 900 mL of distilled water and incubated in a water bath at 58 °C for 6 h. The mixture was boiled for 15 min. The hot liquid was vacuum filtered through filter paper and sterilized under a pressure of 15 psi at 120 °C for 15 min. The pH of the filtrate was 6.5. The koji-based medium was prepared by addition of 17% NaCl (w/v) and 5% glucose (w/v) to the above koji-based medium, and the pH was then adjusted to 4.8 with dilute lactic acid. Each of the sugars or the phosphate sugars was added to the koji-based medium. The medium was sterilized by passage through a 0.22 μm membrane filter.

Yeast/Peptone/Dextrose (YPD) Medium. This medium contained yeast extract (5 g/L), peptone (5 g/L), KH_2PO_4 (5 g/L), $MgSO_4 \cdot 7H_2O$ (5 g/L), glucose (50 g/L), and NaCl (170 g/L). Each of the sugars and the phosphate sugars was added, and the medium was sterilized by filtration.

Cultivation. *Starter Culture.* Five milliliters of koji-based medium or YPD medium was dispensed into 10 mL screw-cap vials. Each vial was inoculated with 100 μL (2%, v/v) of the cell suspension. This starter culture was incubated for 10 days (koji-based medium) or 4 days (YPD medium) at 30 °C in (i) aerobic (the vials are loosely capped) and (ii) anaerobic conditions.

Culture. One hundred microliter aliquots from these two starter cultures were dispensed into 5 mL of koji-based medium or YPD medium, grown 12 days at 30 °C on a rotary shaker, similary in (i) aerobic and (ii) anaerobic conditions.

Experiment with *Z. rouxii* Resting Cells. *Z. rouxii* was cultured in YPD medium in aerobic conditions. After 4 days, the cells were harvested by centrifugation at 1500*g* for 15 min and washed three times with NaCl (1 M). After the last centrifugation, the cells were kept at −30 °C or utilized directly. The cells (1 g) were resuspended in 50 mL of distilled water containing D-fructose 1,6-bisphosphate (10% w/v). A

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Table 1. Amount of HDMF and HEMF with Different Precursor Sugars[a]

precursor sugar		HDMF (ppm) aerobic conditions	HDMF (ppm) anaerobic conditions	HEMF (ppm) aerobic conditions
control 1: koji for 3 days at 30 °C		0	–	–
control 2: koji after sterilization		1.8	–	–
control 3: *Z. rouxii* without precursor sugars		1.6–1.8	1.6	–
control 4: precursors sugars without *Z. rouxii*		1.8	1.6	1.5
D-fructose	5%	1.8	–	–
6-deoxy-L-mannose	5%	5.0	–	–
6-deoxyhexulose	5%	18.0	–	–
D-ribose 5-phosphate, sodium salt, and D-xylulose 5-phosphate, barium salt	5%	2.0	–	210
D-glucose 6-phosphate, sodium salt	5%	1.8	–	2
D-fructose 6-phosphate, sodium salt	5%	3.8	1.8	4.2
D-fructose 1-phosphate, sodium salt	5%	1.9	1.6	3
D-fructose 1,6-bisphosphate, sodium salt	5%	52.4	18.3	6

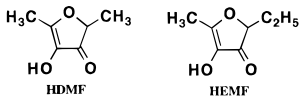
[a] Koji-based medium; starter culture 10 days; culture 12 days. Glucose (5%) and NaCl (17%) were added to all cultures.

control without D-fructose 1,6-bisphosphate was prepared. The media were incubated for 12 days at 30 °C on a rotary shaker. Each experiment was repeated three times with very similar results.

Determination of Volatiles. A sample (1 mL) of the medium culture was centrifuged at 5000*g* for 10 min, filtered on a 0.45 μm membrane filter, and directly analyzed by high-performance liquid chromatography (HPLC). Quantitative HPLC analysis was carried out on a Waters 600E 4 apparatus. Separation was performed on a column (250 mm × LiChrosorb RP 18-7 μm) protected with a guard cartridge (25 mm × LiChroCart RP 18-7 μm). The eluents used were orthophosphoric acid (10^{-2} M) (A) and methanol (B) with a gradient over 0–20 min of 85% A/15% B to 75% A/25% B and a flow rate of 1 mL/min. The substances were detected with UV light (Waters 484) at 290 nm. Quantitative analyses were obtained with a standard curve of commercial HDMF and HEMF. The retention times of HEMF and HDMF were 620 and 750 s, respectively.

RESULTS

First, we tested the influence of some possible precursors on the formation of HEMF and HDMF in *Z. rouxii* broth, and second, we optimized the conditions of HDMF production.



Two fermentation media were used: (i) one employed in Japan in soy sauce for *Z. rouxii* based on koji (Sasaki et al., 1991) and (ii) a medium commonly employed for yeasts in general (YPD). Glucose (5%, w/v) and NaCl (17%, w/v) were added to all the preparations. The presence of HDMF and HEMF was detected by HPLC.

Conditions of HDMF Production. The results in Table 1 show the amount of HDMF and HEMF with different precursor sugars. No HDMF was present in koji (control 1), and about 2 ppm was formed in the sterilization process (control 2), probably by the Maillard reaction. This amount did not increase during the culture with *Z. rouxii* if no precursor was added (control 3). We also verified that no HDMF was formed in the presence of precursor sugars without yeast, in the culture conditions: with koji-based medium or YPD medium, 5% glucose, pH 4.8, and incubation for 22 days with koji-based medium or 16 days with YPD medium without yeast (control 4).

HDMF was not formed from D-glucose (control 3) or D-fructose. When 6-deoxy-L-mannose (6-deoxyaldose) was introduced, the production of HDMF increased to 5 ppm. The increase was more significant with 6-deoxyhexulose (18 ppm), which was a mixture of 6-deoxy-L-sorbose and 6-deoxy-D-fructose obtained chemoenzymatically (Hecquet et al., 1994). Monophosphate sugars (D-glucose 6-phosphate, D-fructose 1-phosphate, and D-fructose 6-phosphate) formed small amounts of HDMF and HEMF (2–4.2 ppm), whereas the bisphosphate sugar tested, D-fructose 1,6-bisphosphate, produced 52 ppm.

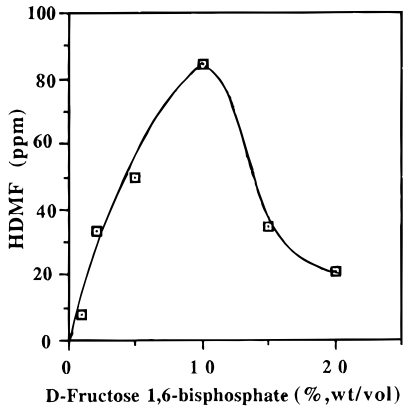


Figure 1. Influence of D-fructose 1,6-bisphosphate concentration on HDMF production. *Z. rouxii* cultured with koji-based medium; starter culture 10 days; culture 12 days; aerobic conditions.

We also incubated cultures under aerobic and anaerobic conditions. The results showed that with D-fructose 1,6-bisphosphate the HDMF production was 3 times greater in aerobic conditions.

Study of Optimal Conditions of HDMF Formation. *Influence of D-Fructose 1,6-Bisphosphate Concentration*. HDMF production was found to increase in proportion to the concentration of the added D-fructose 1,6-bisphosphate up to 10% (w/v). Beyond this level, HDMF production decreased and was strongly inhibited by 20% of D-fructose 1,6-bisphosphate (Figure 1).

Influence of Culture Times. This study was carried out with two culture media: koji-based medium and yeast/peptone/dextrose medium (YPD). Biomass (2×10^8 cells per milliliter of culture medium) was the same for the two media. With koji-based medium (Figure 2A), HDMF production increased between days 1 and 11 (110 ppm) during the growth phase. Next, we observed a slow decrease. With YPD medium (Figure 2B), the results were practically the same. The amount of HDMF was also optimal after 11 days (96 ppm), but with this medium, HDMF was produced during the

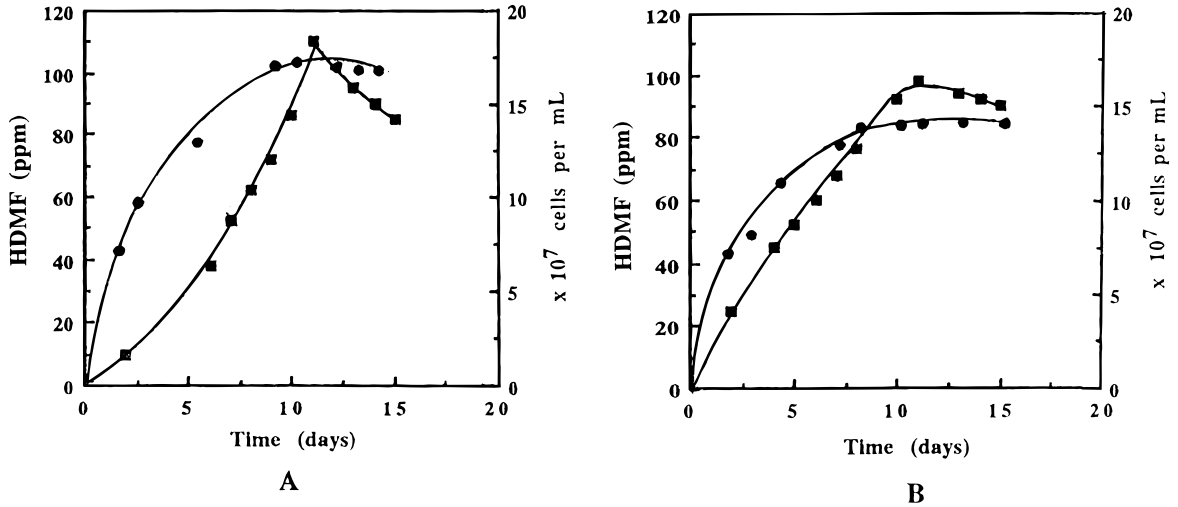


Figure 2. (A) Influence of culture times on HDMF production: (●) growth of *Z. rouxii* and (■) HDMF production. *Z. rouxii* cultured with koji-based medium; starter culture 10 days; culture 12 days; aerobic conditions. (B) Influence of culture times on HDMF production: (●) growth of *Z. rouxii* and (■) HDMF production. *Z. rouxii* cultured with YPD medium; starter culture 10 days; aerobic conditions.

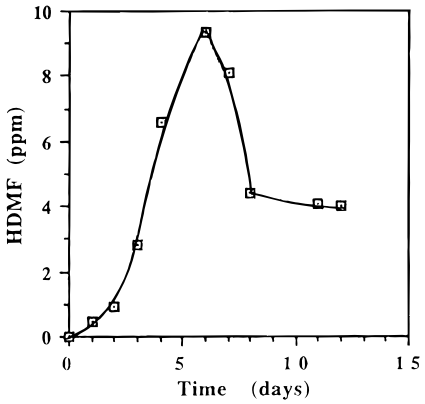


Figure 3. HDMF production by resting cells.

growth phase and the beginning of the stationary phase.

Study of HDMF Production with Z. rouxii Resting Cells. The resting cells (cultures made with YPD medium) were incubated with D-fructose 1,6-bisphosphate (10%). Figure 3 shows that HDMF production increased until day 6, decreased up to day 11, and then leveled out. HDMF was not detected in the controls where D-fructose 1,6-bisphosphate was omitted. Under these conditions, the HDMF value was lower than the value obtained with growing cells. In the resting cells, the cofactors necessary for the enzyme activity were not regenerated and the biosynthesis stopped.

DISCUSSION

This study demonstrates that HDMF is a secondary metabolite of *Z. rouxii* that is produced when the medium is supplemented with six-carbon ketoses. Aerobic conditions are more favorable to its production than anaerobic conditions. Two of the tested compounds, 6-deoxyketose and fructose 1,6-bisphosphate, can act as precursors or inducers of the biosynthesis (the latter, an intermediate in the glycolysis, is present in cells in fermentation, but at very low concentrations). Inhibition of 20% fructose 1,6-bisphosphate could be due to the very high ionic strength of the medium in these conditions. No other compound is needed for HDMF production. The complex koji medium, most often used in this and other studies, gave nearly the same results as YPD medium. The concentration of HDMF in broth can attain 110 ppm. This is appreciable although probably insufficient for industrial applications. At this concentration, loss of HDMF, which is a volatile and unstable compound, may become important.

Our results can be compared with the study of Sasaki et al. (1991), who showed that sedoheptulose 1-phosphate or precursors of this ketose by the pentose phosphate cycle are the precursors of HEMF. HDMF may be produced by the same biosynthetic pathway from fructose 1,6-bisphosphate. In this case, 6-deoxyfructose or 6-deoxysorbose could be intermediates. This possibility requires further study with labeled precursors.

CONCLUSION

These findings demonstrate that HDMF is produced by *Z. rouxii* grown aerobically with D-fructose 1,6-bisphosphate (10%) as precursor. We have also shown that koji-based medium used in a HEMF biosynthesis study by Sasaki et al. (1991) can be replaced by YPD medium with the same results. The advantage is that this medium is easier to prepare and scale up later. These conditions gave the most HDMF (100 ppm; 100 mg/L). The production of HDMF by this microbiological method was low with a rather high level of D-fructose 1,6-bisphosphate, but the amount of HDMF was 50 times greater than that obtained without precursor sugar and 10 times greater than that detected in shoyu (Osaki et al., 1985). The reproducibility of these experiments has shown that D-fructose 1,6-bisphosphate was a real precursor.

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