Formation of 4-Hydroxy-2,5-Dimethyl-3[2H]-Furanone by Zygosaccharomyces rouxii: Identification of an Intermediate

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The formation of the important flavor compound 4-hydroxy-2,5-dimethyl-3[2H]-furanone (HDMF; Furaneol) from D-fructose-1,6-bisphosphate by the yeast Zygosaccharomyces rouxii was studied with regard to the identification of intermediates present in the culture medium. Addition of α-phenylenediamine, a trapping reagent for α-dicarbonyls, to the culture medium and subsequent analysis by high-pressure liquid chromatography with diode array detection revealed the formation of three quinoxaline derivatives derived from D-fructose-1,6-bisphosphate under the applied growth conditions (30°C; pH 4 to 5). Isolation and characterization of these compounds by tandem mass spectrometry and nuclear magnetic resonance spectroscopy led to the identification of phosphoric acid mono-[2,3,4-(trihydroxy-4-quinoxaline-2-yl-butyl)] ester (Q1), phosphoric acid mono-[2,3-dihydroxy-3-(3-methyl-quinoxaline-2-yl)-propyl] ester (Q2), and phosphoric acid mono-[2-hydroxy-3-(3-methyl-quinoxaline-2-yl)-propyl] ester (Q3). Q1 and Q2 were formed independently of Z. rouxii cells, whereas Q3 was detected only in incubation systems containing the yeast. Identification of Q2 demonstrated for the first time the chemical formation of 1-deoxy-2,3-hexodiulose-6-phosphate in the culture medium, a generally expected but never identified intermediate in the formation pathway of HDMF. Since HDMF was detected only in the presence of Z. rouxii cells, additional enzymatic steps were presumed. Incubation of periplasmic and cytosolic protein extracts obtained from yeast cells with D-fructose-1,6-bisphosphate led to the formation of HDMF, implying the presence of the required enzymes in both extracts.

4-Hydroxy-3[2H]-furanones are exceptional aroma compounds due to their attractive flavor and low odor thresholds (34). They are biosynthesized by plants, microorganisms, and insects but are also formed during the thermal treatment of food in the so-called Maillard reaction. In the 1970s 4-hydroxy-3[2H]-furanones were disclosed as secondary metabolites in plants and microorganisms. However, the biosynthesis of an intermediate is still unclear since no enzyme or intermediate involved in HDMF formation has been identified so far. Recently, the chemical formation of HDMF from FBP in the presence of NAD(P)H was reported (11). The authors proposed the dicarbonyl compound 1-deoxy-2,3-hexodiulose-6-phosphate as a possible intermediate which is subsequently reduced by NAD(P)H to the final product, HDMF. A similar biosynthetic pathway is anticipated in plants and microorganisms.

Prior to the usage of Z. rouxii for the biotechnological production of the economically important flavor compound HDMF, it is of utmost significance to know the biosynthetic pathway of HDMF. The objective of this study was to obtain detailed information about HDMF formation from FBP by the yeast Z. rouxii. Here, we report on potential intermediates, such as α-dicarbonyls in the culture medium. Employment of the α-dicarbonyl-trapping reagent α-phenylenediamine revealed three highly reactive α-dicarbonyl structures derived from FBP in the nutrient medium. Structure elucidation of the corresponding quinoxaline derivatives demonstrated the chemical formation of the often postulated but never identified intermediate

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1-deoxy-2,3-hexulose-6-phosphate, and incubation of periplasmic and cytosolic protein extracts obtained from Z. rouxii confirmed the existence of oxidoreductases involved in the bioformation of HDMF.

MATERIALS AND METHODS

General methods. High-pressure liquid chromatography (HPLC) analysis with UV detection of the quinoxalines and HDMF, a Hewlett-Packard (Waldbonn, Germany) 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector were used including Hewlett-Packard Chemstation software for data acquisition and evaluation. A Eurosep 100-C, column (250-mm length, 4-mm inside diameter [i.d.], 5-μm particle size) (Knauer) was employed for the quinoxalines and HDMF analysis. A binary gradient starting from 95% A (0.05% [vol/vol] formic acid in water) and 5% B (acetonitrile) to 80% A and 20% B was used at a flow rate of 1 ml min⁻¹. The injection volume was 20 μl. An anion-exchange HPLC column (Nucleosil 100-10/8; Macherey-Nagel, Duren, Germany) (length, 240 mm; diameter, 4 mm) was employed at a flow rate of 1 ml min⁻¹ for the separation of phosphorylated carbohydrates. A binary gradient starting from 95% A (water) and 5% B (1 M NH₄HCOO) to 100% B within 20 min was used. HPLC radiodeTECTION was performed with a Canberra-Packard ( Dreieich, Germany) A100 on-line radio-detector applying a solid scintillation cell. Thin-layer chromatography radiodeTECTION was conducted with a Berthold (Bad Wildbad, Germany) linear analyzer with Ar·CH₄ (90:10) as the counting gas at 1,381 V. HPLC-tandem mass spec- trometry (HPLC-MS/MS) was performed with a TSQ 7000 tandem mass spectrometer system equipped with an electrospray ionization (ESI) interface (Finnig- nan MAT, Bremen, Germany) and an Applied Biosystems (BAI, Bensheim, Germany) 140b pump. Data acquisition and evaluation were conducted on a DEC 5000/33 (Digital Equipment, Unterfohring, Germany) computer with Finnigan MAT ICIS 8.1 software. HPLC separation with MS detection of the quinoxalines and HDMF was carried out on a Knauer Eurosep 100-C α column (100-mm length, 2-mm i.d., 5-μm particle size) with a binary gradient. Solvent A was 0.05% (vol/vol) trifluoroacetic acid (TFA) in water, and solvent B was acetonitrile. HPLC was programmed as described above except for the use of a flow rate of 200 μl min⁻¹. The injection volume was 5 μl, and mass spectra were acquired in the positive ion mode. For pneumatically assisted ESI, the spray voltage was set to 3.5 kV, and the temperature of the heated capillary was 210°C. Nitrogen served both as sheath (70 lb/in²) as and as auxiliary gas (10 U). Product ion scanning was performed at a collision gas pressure of 0.5 mtorr and a collision energy of 25 eV with a total scan duration of 1.5 s for a single spectrum.

The aqueous extract was applied to a Lichroprep C₁₈ column (33 by 3.3 cm) (40 to 63-μm particle size; Merck) equilibrated with water-formic acid solution (100:0.05, vol/vol). The column was eluted with 25 liters of water-acetonitrile-formic acid solution (95:5.5:0.05, vol/vol) and then with 1 liter of water-acetonitrile-formic acid (90:10:0.05, vol/vol). Fractions of approximately 10 ml were collected, measured by UV spectroscopy at 313 nm against water, and analyzed by RP18-HPLC with UV detection at 313 nm. Fractions containing Q1, Q2, and Q3 were pooled respectively, and the solvents were removed by rotary evaporation at 40°C and lyophilized. The three samples were separately applied onto two glass column (12 by 2.5 cm) filled with Lichroprep RP18 material equilibrated with water-TFA solution (100:0.05, vol/vol). Separation was carried out by employing a gradient composed of mobile phase A (water-TFA [100:0.05, vol/vol]) and B (acetonitrile). The portion of solvent B was increased from 0 to 24% B in 4% steps with 50-ml fractions. Fractions (approximately 10 ml) were collected and analyzed by HPLC with UV detection at 313 nm. Fractrions containing the target compounds were combined and freeze-dried after rotary evaporation at 40°C. The procedures described yielded the following compounds as pale yellow amorphous solids: Q1, ESI-MS, [M + H]⁺ m/z 331; ESI-MS/MS (precursor ion m/z 331, 25 eV) m/z 331, 235, 215; UV λₘₐₓ (H₂O-TFA-acetonitrile, 84.05:0.16, vol/vol), 318 nm, 383 nm; [H NMR (600 MHz, CD₃OD) δ 9.14 (s, 1H, H-3), 8.08 (d, 2H, H-6 and H-7), 7.82 (m, 2H, H-5 and H-8), 5.34 (s, 3H, H-4), 4.29 (m, 1H, H-1), 4.16 (m, 1H, H-6), 4.06 (m, 1H, H-2), 3.97 (d, 1H, H-3). 13C NMR (150 MHz, CD₃OD) δ 159.8 (C-2), 159.8 (C-8a), 156.1 (C-2), 152.7 (C-4a). 142.1 (C-8a), 141.6 (C-5), 131.2 (C-5), 128.7 (C-6), 129.8 (C-7), 130.4 (C-8), 129.7 (C-6), 129.7 (C-5), 128.7 (C-8), 128.7 (C-5). 156.1 (C-2), 156.1 (C-8a), 142.1 (C-8a), 141.6 (C-5), 131.2 (C-5), 128.7 (C-6), 129.8 (C-7), 130.4 (C-8), 129.7 (C-6), 129.7 (C-5). 156.1 (C-2), 156.1 (C-8a), 142.1 (C-8a), 141.6 (C-5), 131.2 (C-5), 128.7 (C-6), 129.8 (C-7), 130.4 (C-8), 129.7 (C-6), 129.7 (C-5). 156.1 (C-2), 156.1 (C-8a), 142.1 (C-8a), 141.6 (C-5), 131.2 (C-5), 128.7 (C-6), 129.8 (C-7), 130.4 (C-8), 129.7 (C-6), 129.7 (C-5). 156.1 (C-2), 156.1 (C-8a), 142.1 (C-8a), 141.6 (C-5), 131.2 (C-5), 128.7 (C-6), 129.8 (C-7), 130.4 (C-8), 129.7 (C-6), 129.7 (C-5).
Preparation of Z. rouxii cytosolic protein extract. Z. rouxii cells were grown at 30°C in YPD medium (240 ml). During the mid-logarithmic phase the yeast cells were harvested by centrifugation at 5,000 × g for 10 min, washed twice in cooled (4°C) buffer (20 mM Tris-Cl at pH 7.5, 17% NaCl), and resuspended in 4 ml of cooled (4°C) lysis buffer (50 mM Tris-Cl at pH 7.5; 150 mM NaCl; 30 μg each of leupeptin, pepstatin, and antipain per ml). The cell suspension was mixed with an equal volume of cold glass beads (diameter, 0.5 mm; Roth, Karlsruhe, Germany), and cells were broken by vortexing the mixture six times for 1 min each with 1-min cooling intervals on ice as described previously (19, 44). Unbroken cells and cell wall debris were removed by centrifugation at 1,000 × g, and the supernatant was centrifuged at 27,000 × g for 1 h at 4°C. The supernatant (soluble cytosolic proteins) was removed and subjected to dialysis against 0.1 M potassium phosphate buffer (pH 7.0) at 4°C.

Preparation of the periplasmic fraction. Z. rouxii cells were grown to mid-logarithmic phase (4 days) in YPD medium and harvested by centrifugation for 15 min at 5,000 × g and 4°C. Cells were washed three times with 25 ml of 20 mM Tris-Cl buffer (pH 7.5) containing 17% NaCl. After incubation with 20 ml of 20 mM Tris-Cl buffer (pH 7.5) containing 17% NaCl, 1 mM dithiothreitol, and 10 U of zymolyase (yeast lytic enzyme, obtained from ICN) ml⁻¹ at 30°C for 2.5 h, the released periplasmic fraction was separated from the spheroplasts by centrifugation at 8,420 × g for 10 min. The supernatant (containing the periplasmic proteins) was removed and subjected to dialysis against 0.1 M potassium phosphate buffer (pH 7.0) at 4°C.

FIG. 1. HPLC-DAD analysis of an extract obtained by incubation of YPD medium supplemented with FBP and Z. rouxii cells with o-phenylenediamine overnight (top), of YPD medium supplemented with FBP with o-phenylenediamine overnight (middle), and of YPD medium with o-phenylenediamine overnight (bottom). Columns effluent was monitored at 318 nm. Unlabeled peaks did not show absorption maxima at 238 and 318 nm.

RESULTS

Detection of o-dicarbonyl compounds derived from FBP. Three nutrient solutions were prepared according to the method of Hecquet et al. (13). Two media were supplemented with FBP, and one of the phosphohexose-containing media was inoculated with Z. rouxii cells. After 7 days of incubation each of the three solutions was supplemented with o-phenylenediamine, a widely used trapping reagent for highly reactive o-dicarbonyl compounds (10). After overnight incubation yeast cells were removed by centrifugation, and the supernatant as well as the incubations without yeast cells was analyzed by HPLC with DAD. In the sample containing yeast cells and FBP three compounds (Q1, Q2, and Q3) exhibiting the characteristic quinoxaline UV spectrum (35) with two absorption maxima at 238 and 318 nm were detected (Fig. 1) in addition to HDMF. Q1 and Q2 were also present in the sample supplemented with FBP but devoid of yeast cells (Fig. 1). Since none of these compounds was detected in the nutrient solution without FBP and yeast cells (Fig. 1), it was deduced that Q1 and Q2 are formed by the reaction of o-phenylenediamine with compounds formed nonenzymatically from FBP under moderate conditions. However, formation of Q3 and HDMF was observed only in the presence of FBP and yeast cells, implying a decisive participation of the yeast cells. Since the other compounds shown in Fig. 1 did not show quinoxaline or furanone characteristic UV spectra, they were not further examined. Solid-phase extraction on XAD-2 according to the method of Beuerle et al. (2) and subsequent analysis of the diethyl ether extracts as well as methanol extracts by reversed-phase HPLC with UV detection at 318 nm revealed that Q1, Q2, and Q3 are exclusively eluted with methanol while HDMF was detected.
only in the diethyl ether extract. After further purification on RP18 material, pseudomolecular ions at m/z 331 [M + H]⁺ for Q1, m/z 315 [M + H]⁺ for Q2, and m/z 299 [M + H]⁺ for Q3 were determined by HPLC-ESI-MS analysis. The product ion spectra of all compounds under study as obtained by low-energy collision-induced dissociation were dominated by the loss of 98 AMU presumably due to the abstraction of phosphoric acid. Thus, the combined UV, MS, and MS/MS data indicated the formation of phosphorylated quinoxalines by the reaction of o-phenylenediamine and phosphorylated α-dicarboxyl compounds derived from FBP under the applied conditions. Due to their different molecular masses it appeared that the three compounds differed only in their hydroxylation status.

**Identification of Q1, Q2, and Q3 by NMR spectroscopy.** Q1, Q2, and Q3 were isolated from a culture medium containing FBP and *Z. rouxii* cells for structure elucidation. Identification was achieved by NMR spectroscopy including ¹H, ¹³C, distortionless enhancement by polarization transfer, homonuclear correlation spectroscopy, heteronuclear multiple-bond connectivity, and ¹H-detected heteronuclear multiple-quantum coherence experiments. All three substances showed similar signals in the ¹H and ¹³C NMR spectra arising from the protons H-5’ and H-8’ and from the carbons C-4a’, C-5’, C-6’, C-7’, C-8’, and C-8a’ of the quinoxaline skeletal structure (9, 16, 20, 42). In contrast, Q1 to Q3 displayed clear differences in the carbohydrate backbone at C-2’ and C-3’. The NMR data for Q1 exhibited a proton bound to C-3’ due to the chemical shift of C-3a’ (δc = 22.5 ppm for Q2 and Q3) and the heteronuclear multiple-bond connectivity correlation between H-3’ and C-3’. The NMR data for Q2 and Q3 revealed the presence of a methyl group at C-3’ due to the chemical shift of C-3a’ (δc = 22.5 ppm for Q2) (36). The presence of phosphorus-carbon coupling and the corresponding coupling constants (J C1-P = 5.2 Hz and J C2-P = 7.5 Hz for Q1, J C1-P = 5.5 Hz and J C2-P = 7.5 Hz for Q2) (36). The presence of phosphorus was additionally confirmed by ³¹P NMR (data not shown). Thus, Q1 and Q2 were unequivocally identified as phosphoric acid mono-(2,3,4-trihydroxy-4-quinoxaline-2-yl-buty)-ester and phosphoric acid mono-[2,3-dihydroxy-3-(3-methyl-quinoxaline-2-yl)-propyl] ester, respectively (Fig. 2). For Q3 the NMR spectra confirmed the attachment of a 1-phospho-2,3-dihydroxypropyl moiety to C-2’ of the quinoxaline skeleton. The position of the methylene group at C-3 was evidenced by the chemical shift (δc = 39 ppm) and the negative distortionless enhancement by polarization transfer signal. Analogously to Q1 and Q2 the presence of the phosphate group was confirmed by the split signals in the ¹³C spectra (J C1-P = 5.7 Hz and J C2-P = 8.3 Hz), and therewith the formation of phosphoric acid mono-[2-hydroxy-3-(3-methyl-quinoxaline-2-yl)-propyl] ester (Fig. 2) was unambiguously demonstrated. Consequently, the α-dicarboxyl structures D1, D2, and D3 are formed in the nutrient solutions as can be deduced from their quinoxaline derivatives Q1, Q2, and Q3, respectively (Fig. 2).

**Incubation with protein extracts obtained from *Z. rouxii*.** HDMF was formed only in media containing FBP and yeast cells, but the predicted precursor 1-deoxy-2,3-hexodiulose-6-phosphate (D2) was formed independently of the yeast cells. As a reduction is required to transform the intermediate into HDMF, we concluded that there was an additional enzymatic reduction catalyzed by a nonspecific yeast ketoreductase. In order to localize this reductase activity, periplasmic protein extracts were prepared by zymolysate (yeast lytic enzyme, lytic-case) digestion of the cell wall according to the method of Yoda et al. (45), and cytosolic protein extracts were prepared by vortexing *Z. rouxii* cells with glass beads followed by high-speed centrifugation (19, 44). Both extracts were dialyzed to remove low-molecular-mass components (<12 kDa) and were subsequently incubated at 30°C for 24 h with an excess of FBP to ensure the in situ formation of the required precursor. After solid-phase extraction on RP18 cartridges samples were analyzed by HPLC with UV detection at 285 nm. In Fig. 3A the HDMF amounts obtained from incubations of an active periplasmic extract and a heat-inactivated periplasmic extract and of a control incubation including the zymolysate solution are displayed. HDMF was exclusively formed in the active sample. Addition of reducing agents such as NADH or NADPH was not required, and they did not increase the yield of HDMF. For further characterization of HDMF formation in the periplasmic extract, a time course experiment was performed revealing the liberation of the HDMF-forming enzymatic activity during cell wall digestion. Aliquots were withdrawn periodically from a zymolysate digestion and treated as described above. The HDMF-forming enzymatic activity was gradually liberated from the cells and reached a maximum level. In parallel, the efficiency of the cell wall digestion was monitored by measurement of the absorbance at 800 nm.

In the case of the cytosolic extracts significant amounts of HDMF were detected only after the addition of NADPH or NADH and FBP (Fig. 3B). When the enzyme extract was inactivated by heat treatment prior to the incubation or when the cofactor was omitted, HDMF formation was not observed. Due to the recently noted chemical formation of HDMF from FBP in the presence of NADs (11), control experimental mixtures including the appropriate buffer instead of the extract were treated similarly. The HDMF formation in the control incubations was well below the formation in the active extracts (Fig. 3B).

**Regeneration of NAD(P)H.** It was assumed that the bound NAD(P)H accounts for only a single turnover, which does not account for the amount of reduced product that was formed in the incubation. Thus, an NAD(P)H regeneration system was proposed for the periplasmic extract. Such a system could consist of FBP aldolase and glyceraldehyde-3-phosphate dehydrogenase. Enzymatically active glyceraldehyde-3-phosphate dehydrogenase has already been described as a constituent of the cell wall of *Candida albicans*, *Streptococcus pyogenes*, and *Schistosoma mansoni* (3, 28, 37). Aldolase activity in the periplasmic extract was demonstrated by radiochemical analysis of the products formed after the incubation with [U-¹⁴C]fructose-1,6-diphosphate as well as GC-MS analysis of the aldito acetate derivatives of the products generated after the incubation with unlabeled FBP. The experiments clearly proved the formation of dihydroxyacetone phosphate and glyceraldehyde-
3-phosphate, products formed by aldolase activity. Final evidence was provided by an aldolase enzyme assay. Addition of EDTA inhibited the enzymatic reaction as expected for a yeast aldolase. Thus, the combined action of glyceraldehyde-3-phosphate dehydrogenase and FBP aldolase on the very high concentration of FBP would account for the formation of NADH, which could serve as the reductant, allowing multiple turnovers. We assume that aldolase is present in the yeast cell wall like other glycolytic enzymes described previously (1, 8), but we cannot rule out that it is released from the cytoplasm during the extraction procedure (3).

**DISCUSSION**

We demonstrated for the first time the formation of highly reactive α-dicarbonyl compounds from FBP. This transformation occurred under moderate growth conditions in the Z. rouxii culture medium (30°C; pH 4 to 5). The structures of...
2-hexosulose-6-phosphate (D1), 1-deoxy-2,3-hexodiulose-6-phosphate (D2), and 1,4-dideoxy-2,3-hexodiulose-6-phosphate (D3) were deduced from the quinoxalines Q1, Q2, and Q3. The formation of \( \text{H}_2\text{dicarbonyls} \) from carbohydrates has been intensively studied by the use of trapping reagents, especially \( o\)-phenylenediamine derivatives and aminoguanidines, but carbohydrate phosphates have rarely been used as precursors (10, 15, 16, 17, 21, 22). Since \( \text{H}_2\text{dicarbonyls} \) represent a major group of reactive intermediates formed during the so-called Maillard reaction, most of the researches studied their formation in heated mixtures of pentoses or hexoses with amino acids (10, 17, 21) and determined their in vivo generation during the advanced Maillard reaction (26). Only a few examples are available of investigations of the formation of \( \text{H}_2\text{dicarbonyls} \) derived from carbohydrate phosphates in consequence of a phosphate elimination (27). Larimer et al. (18) trapped for the first time a phosphorylated \( \text{H}_2\text{dicarbonyl} \) derived from D-ribulose-1,5-diphosphate with \( o\)-phenylenediamine. However, Q1 to Q3 have not been detected until now. This is the first report on the formation of \( \alpha\)-dicarbonyls derived from a hexose phosphate under moderate conditions.

We assume that D1 is formed by oxidation of D-fructose-6-phosphate generated from FBP by hydrolysis due to the slightly acid pH of the nutrient medium (pH 4 to 5). This assumption was substantiated by the detection of Q1 by means of HPLC-ESI-MS/MS analysis in a phosphate buffer containing D-fructose-6-phosphate and \( o\)-phenylenediamine (data not shown). According to the report of Glomb and Tschirnich (10) formation of dicarbonyls is effected by the trapping reagent itself due to its oxidative potency. Incubation of the Amadori product of N\(^{\text{N}}\)-tert-butoxycarbonyl-lysine and glucose in the presence of \( o\)-phenylenediamine led to a significant formation of glucosone, the nonphosphorylated analog of the observed 2-hexosulose-6-phosphate.

1-Deoxy-2,3-hexodiulose-6-phosphate (D2) is formed by \( \beta\)-elimination of the phosphate group at C-1 in consequence of an initial 2,3-enolization. The formation of Q2 confirms results obtained by incubation of D-ribulose-1,5-diphosphate with \( o\)-phenylenediamine for 15 h in a phosphate buffer leading to the quantitative conversion of the trapping reagent to a quinoxaline derivative. ESI-MS analysis revealed a molecular ion at \( m/z \) 285[M + H]\(^+\), in accordance with the structure proposed by Larimer et al. (18).

The formation of D3 can be explained by dehydration of D2 followed by a reduction step. The exclusive generation in the presence of \( Z. \) rouxii cells indicates that the reduction is obviously performed by the yeast cells.

The \( \alpha\)-dicarboxyl compounds formed either chemically or by yeast biocatalysis represent possible HDMF precursors. Recently, the chemical formation of HDMF in solutions composed of FBP and reducing agents was demonstrated, and D2 was postulated as a precursor of HDMF formed from FBP (11). Since a very large amount of FBP is required in the nutrient medium of \( Z. \) rouxii (up to 8%) to obtain a reasonable concentration of HDMF (6, 13), we expected a chemical transformation rather than a biotransformation as the initial step of...
HDMF formation. Generally, a phosphate elimination via 2,3-enolization was assumed, but this mechanism of formation of D2 (11, 32) was never confirmed. However, in a similar reaction sequence dihydroxyacetone phosphate (29) is transformed to methylglyoxal and D-ribulose-1,5-diphosphate forms the corresponding phosphorylated 1-desoxypentose (27).

We trapped and characterized D2 for the first time as a quinoxaline derivative. Our results indicate that the first step of HDMF formation by the yeast Z. rouxii is the chemical formation of D2 in the culture medium, since only exogenously supplied FBP is transformed to HDMF, as shown by Dahlen et al. (6), and D2 is formed in the culture medium independently of the yeasts. The formation of D2 might be the limiting step in the bioformation of HDMF because very large amounts of the sugar phosphate are necessary (up to 8% in the culture medium) to yield reasonable amounts of the target molecule. We conclude that a combination of chemical reactions leading to the elimination of at least one phosphate group and an enzymatic reduction mediated by an oxidoreductase of the yeast cells is responsible for HDMF formation in the culture media (Fig. 4). Indeed, we detected the generation of HDMF in cell periplasmic and cytosolic protein extracts. Thus, we assume the existence of nonspecific ketoreductases in the yeast cells participating in HDMF formation. Yeast-mediated reductions have been intensively investigated (5, 7, 38). Tadashi et al. (40, 41) purified a carbonyl reductase from baker’s yeast, exhibiting high enantioselectivity and broad substrate specificity. Costello et al. (4) isolated and characterized a ketoreductase from cytosolic protein extracts obtained from Z. rouxii, able to reduce methylketones, α-ketolactones, and diketones effectively and enantioselectively.

Since the periplasmic extract shows activity without the addition of a cofactor, the presence of enzyme-bound NAD(P)H is anticipated. The well-characterized periplasmic NAD-containing glucose-fructose oxidoreductase detected in the gram-negative bacterium Zymomonas mobilis represents such a candidate (43).

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