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Effects of Furfural on the Respiratory Metabolism of *Saccharomyces cerevisiae* in Glucose-Limited Chemostats

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Effects of furfural on the aerobic metabolism of the yeast *Saccharomyces cerevisiae* were studied by performing chemostat experiments, and the kinetics of furfural conversion was analyzed by performing dynamic experiments. Furfural, an important inhibitor present in lignocellulosic hydrolysates, was shown to have an inhibitory effect on yeast cells growing respiratively which was much greater than the inhibitory effect previously observed for anaerobically growing yeast cells. The residual furfural concentration in the bioreactor was close to zero at all steady states obtained, and it was found that furfural was exclusively converted to furoic acid during respiratory growth. A metabolic flux analysis showed that furfural affected fluxes involved in energy metabolism. There was a 50% increase in the specific respiratory activity at the highest steady-state furfural conversion rate. Higher furfural conversion rates, obtained during pulse additions of furfural, resulted in respirofermentative metabolism, a decrease in the biomass yield, and formation of furfuryl alcohol in addition to furoic acid. Under anaerobic conditions, reduction of furfural partially replaced glycerol formation as a way to regenerate NAD^+. At concentrations above the inlet concentration of furfural, which resulted in complete replacement of glycerol formation by furfuryl alcohol production, washout occurred. Similarly, when the maximum rate of oxidative conversion of furfural to furoic acid was exceeded aerobically, washout occurred. Thus, during both aerobic growth and anaerobic growth, the ability to tolerate furfural appears to be directly coupled to the ability to convert furfural to less inhibitory compounds.

An important problem in fermentative conversion of lignocellulose to ethanol is the severe inhibitory effects often exerted by lignocellulosic hydrolysates (17, 21). Furfural is a characteristic compound present in dilute acid hydrolysates, particularly in hydrolysates from deciduous woods, in which the furfural concentration can be about 2 to 3 g/liter (37). Furfural has been found to severely inhibit metabolism in the yeast *Saccharomyces cerevisiae* under anaerobic conditions both during batch cultivation (5, 7, 27) and in glucose-limited chemostats (9, 12, 35). Furfural has been reported to have inhibitory effects on the specific growth rate, as well as on the fermentation rate of yeasts (4, 28). However, only few studies have described the effects of furfural under aerobic conditions. These effects are important, since inhibition caused by furfural during respiratory growth has a great impact on yeast propagation in an ethanol production plant based on a lignocellulosic feedstock.

Taherzadeh et al. (39) compared the levels of conversion of furfural in anaerobic and aerobic batch cultures of *S. cerevisiae* growing on glucose. It was found that furfural was mainly converted to furfuryl alcohol by exponentially growing cells under both conditions and that the specific conversion rate was 0.6 g/g · h. Almost the same value for the maximum specific conversion rate of furfural was found in a previous study, in which pulse additions of furfural were made to anaerobic glucose-limited continuous cultures of *S. cerevisiae* (35). To investigate inhibition effects outside glycolysis, *S. cerevisiae* was also grown on the nonfermentable carbon sources ethanol and acetic acid (38). Pulse addition of furfural under these conditions was found to result in strong inhibition of cell growth until complete conversion of furfural occurred. Furthermore, more than 85% of the furfural was converted to furfuryl alcohol, and furoic acid was a minor conversion product when ethanol was used as the carbon and energy source.

The objective of the present work was to study the physiological effects of furfural on the yeast *S. cerevisiae* during respiratory growth on glucose. Complete respiratory sugar metabolism can be achieved only at specific growth rates below a critical value. Therefore, chemostat experiments were performed with a dilution rate of 0.10 h^{-1} and different concentrations of furfural in the inlet medium. The global effects of furfural on metabolism were analyzed by estimating the steady-state metabolic flux distributions by metabolite balancing. Metabolic flux analysis shows the degrees of participation of various pathways in the overall metabolism. Comparisons of metabolic flux maps obtained under different conditions can provide important information regarding substrate utilization, product formation, and energy metabolism (25, 41). Results obtained under steady-state conditions in the present work and steady-state results obtained previously under anaerobic conditions (35) provided the basis for a metabolic flux analysis in which the effects of furfural on aerobic metabolism and anaerobic metabolism of the yeast *S.cerevisiae* were compared. Transient experiments were also performed, in which pulse additions of furfural were made to continuous cultures. In this way,

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the dynamics of furfural conversion could be studied at high furfural concentrations.

MATERIALS AND METHODS

Yeast strain and media. *S.cerevisiae* CBS 8066 obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) was used in this study. Agar plates containing YEPD medium (2% yeast extract, 1% peptone, 2% D-glucose, 2% agar) were used to maintain the strain. Inoculum cultures were grown in 300-ml cotton-plugged conical flasks with a liquid volume of 100 ml on a rotary shaker at 170 rpm and 30°C for 24 h. The growth medium was a defined synthetic medium described by Taherzadeh et al. (40) containing glucose (10 g/liter) as the carbon and energy source.

Cultivation conditions. Aerobic continuous cultivation was performed at 30°C in a Belach BR 0.4 bioreactor (Belach Bioteknik AB, Solna, Sweden) with a stirrer speed of 800 rpm. The working volume of the culture was kept at 0.4 liter, and the pH of the medium was controlled at 5.00 ± 0.05 by addition of 2 M NaOH. The fermentor was flushed with air at a flow rate of 400 ml/min, which was controlled by a mass flow controller (Hi-Tech, Ruurlo, The Netherlands). At all times the dissolved oxygen tension under steady-state conditions was more than 60% of the air saturation value. As previously reported for other strains of *S. cerevisiae*, the CBS 8066 population exhibited spontaneous oscillations during aerobic cultivation. This phenomenon was presumably due to spontaneous cell synchronization (11, 29). The oscillations eventually damped out, and no measurements were obtained until a completely nonoscillatory steady state had been reached.

In the steady-state experiments, no furfural was present in the inlet medium at the first steady state. Subsequently, the concentration of furfural in the feed solution was gradually increased until washout occurred. Between different inlet concentrations the system was allowed to reach a steady state. We assumed that steady-state conditions had been reached when the biomass concentration and the specific rates of carbon dioxide production and oxygen consumption remained constant for at least five residence times. All experiments were performed at a dilution rate of 0.10 h^{-1}.

In the pulse addition experiments, different amounts of furfural (resulting in concentrations of 4, 8, and 12 g/liter in the fermentor) were injected directly into steady-state continuous cultures growing on furfural-free medium. These experiments were also performed at a dilution rate of 0.10 h^{-1}.

Analytical methods. (i) Gas analysis. The carbon dioxide and oxygen contents in the outlet gas were measured continuously with an acoustic gas monitor (model 1308; Bruel & Kjaer, Naerum, Denmark) (8). The gas measurement signals were averaged for 30 s. The instrument was calibrated with gas containing 5% CO_2, 20% O_2, and nitrogen as an inert gas.

(ii) Metabolites and biomass. Samples taken from the broth were centrifuged, and the supernatants were analyzed by high-performance liquid chromatography. The amounts of glucose, ethanol, glycerol, pyruvic acid, succinic acid, furfural, furfuryl alcohol, and furoic acid were determined by using an Aminex ion-exchange column (HPX-87H; Bio-Rad, Richmond, Calif.) (37).

Cell concentrations were determined from measurements of absorbance at 610 nm after samples were diluted to obtain an optical density of less than 0.5. In this range, the absorbance values were found to be linearly related to cell dry weight. The optical density was calibrated with dry weight measurements by using duplicate 3-ml samples, which were centrifuged, washed once with distilled water, and dried at 103°C for 24 h. Duplicate samples differed by no more than 2%.

The total cellular protein content was determined by a modified biuret method by using bovine serum albumin as the standard (44). The total cellular RNA content was determined as described by Benthin et al. (6), except that the concentrations of cold perchloric acid were changed. Thus, samples were washed and deproteinized three times with cold 0.2 M perchloric acid, solubilized in 0.3 M KOH at 37°C, and acidified by addition of cold 1.7 M perchloric acid (13). The precipitate was washed twice with 0.2 M perchloric acid, and after centrifugation the absorbance at 260 nm of the supernatant was determined with 0.2 M perchloric acid as the reference (18).

(iii) Calculation of specific uptake and production rates. Carbon recovery values were calculated from the production of biomass and metabolites and the carbon dioxide evolution rate. The composition of the ash-free biomass was assumed to be $CH_{1.76}O_{0.56}N_{0.17}$ (33).

The respiratory quotient (RQ) was calculated from the measured exhaust gas mole fractions by using the following equation.

$$RQ = \frac{y_{CO_2,out}\dfrac{y_{N_2,in}}{y_{N_2,out}}}{y_{O_2,in} - y_{O_2,out}\dfrac{y_{N_2,in}}{y_{N_2,out}}}$$

where $y_{j,in}$ and $y_{j,out}$ are the mole fractions of j in the inlet and outlet gas, respectively.

Specific uptake and production rates were determined based on the biomass concentrations. Volumetric conversion rates were calculated from mass balances in the bioreactor at steady state. Degrees of reduction balance were calculated by the method of Roels (33).

Stoichiometric network models. The stoichiometric models used to calculate the metabolic flux distributions under aerobic and anaerobic conditions are given in the Appendix.

The metabolic network used for anaerobic conditions comprised 50 compounds in 44 intracellular reactions. The condition number of the stoichiometric matrix was 89, indicating a numerically well-conditioned system. The anaerobic model is presented and discussed in detail elsewhere (14). However, some important points are also mentioned here. In order to obtain as good fit as possible with experimental data under anaerobic conditions, it was assumed that in dehydrogenase reactions $NADP^+$ and NADH were used as cofactors in cases in which the cofactor specificity was uncertain (1, 2). Thus, in the synthesis of amino acids, the homoserine dehydrogenase (Hom6p), D1-pyrroline-5-carboxylate reductase (Pro3p), and saccharopine reductase (Lys9) were assumed to proceed with cytosolic NADH in order to minimize the amount of NADH formed in anabolism (2). For the same reason, 5-methyl tetrahydrofolate was assumed to be formed via the NADPH-specific methylene tetrahydrofolate reductase (Met13p) (32, 34). 5-Formyl tetrahydrofolate formation was assumed to proceed via the NADP-specific Ade3p (46). Glycine formation was assumed to proceed both via the cytosolic serine hydroxymethyltransferase (Shm2p) and via threonine aldolase (Gly1p) (23), in order to balance the formation of 5,10-methylene tetrahydrofolate. Acetyl-coenzyme A (acetyl-CoA) used in the tricarboxylic acid (TCA) cycle was assumed to be formed via pyruvate decarboxylase and a cytosolic $NADP^+$-coupled acetaldehyde dehydrogenase, which was followed by passive transport into the mitochondria (25). Furthermore, it was assumed that furfuryl alcohol was produced via an NADH-coupled alcohol dehydrogenase.

The metabolic network used for aerobic conditions comprised 51 compounds in 45 intracellular reactions. The condition number of the stoichiometric matrix was 88. Under aerobic conditions, acetyl-CoA used in the TCA cycle was assumed to be formed via pyruvate dehydrogenase, whereas cytosolic acetyl-CoA used in biosynthesis was assumed to be formed via pyruvate decarboxylase and NAD^+-coupled acetaldehyde dehydrogenase (25). It was not possible to include both the pyruvate dehydrogenase (PDH) and the PDH bypass for formation of mitochondrial acetyl-CoA (15) since this led to a dramatic increase in the condition number of the stoichiometric matrix. Furoic acid was assumed to be formed via an NAD^+-coupled aldehyde dehydrogenase under aerobic conditions. The P/O ratio was assumed to be 1.1 for both the external and internal NADH dehydrogenases (42).

The amount of ATP used for intracellular pH regulation was estimated on the basis of proton balance by using the plasma membrane H^+-translocating ATPase to account for export of excess protons. An ATP balance was used to estimate the amount of ATP consumed in all unknown processes; hence, this was referred to as nonspecific ATP hydrolysis.

The amino acid composition of cellular protein was obtained from the study of Albers et al. (1), and the nucleotide composition of RNA was obtained from the study of Oura (26). The cellular protein and RNA contents were measured as described above. The biomass was assumed to contain 5% lipids under aerobic conditions and 2% lipids under anaerobic conditions (average lipid compositions) (14, 31). Under anaerobic conditions, unsaturated fatty acids, ergosterol, and inositol were assumed to be taken up from the medium, while under aerobic conditions only inositol was provided in the medium. This was taken into account by modifying the reaction stoichiometry in these cases (see Appendix). The monomer compositions of proteins, RNA, and lipids were assumed to be constant under all the conditions investigated. The remainder of the biomass was assumed to be ash (5%) and carbohydrates (balance up to 100%). The unknown intracellular reaction rates were estimated by weighted, constrained optimization as previously described (14).

RESULTS

Aerobic continuous cultivation. Aerobic continuous cultivation of *S.cerevisiae* was carried out with glucose as the carbon

TABLE 1. Experimentally determined and estimated specific formation rates obtained from aerobic chemostat cultures of *S. cerevisiae* growing on glucose (10 g/liter) as a carbon and energy source with different levels of furfural present in the inlet medium[a]

Metabolite	Approx relative mean SD (%)	Specific rates (C-mmol/g · h) in the presence of:					
		No furfural		1.14 g of furfural liter^{-1}		2.25 g of furfural liter^{-1}	
		Measured value	Estimated value[b]	Measured value	Estimated value[b]	Measured value	Estimated value[b]
Glucose	5	−7.0	−7.2	−8.1	−7.3	−9.1	−9.4
Oxygen	5	−2.8	−2.9	−2.8	−3.0	−5.0	−4.8
Carbon dioxide	5	3.1	3.0	3.2	3.0	4.4	4.6
Pyruvic acid	2	0.0	0.0	0.0	0.0	0.0	0.0
Succinic acid	2	0.1	0.1	0.1	0.1	0.3	0.3
Furoic acid	5	0.0	0.0	1.0	0.9	2.6	2.7
Protein	5	2.2	2.3	2.3	2.3	2.6	2.6
Polysaccharides	10	1.4	1.3	1.3	1.3	1.4	1.4
RNA	5		0.3		0.3		0.3
Lipids	10		0.3		0.3		0.3

[a] For the cultures grown in the presence of 0, 1.14, and 2.25 g of furfural liter^{-1}, the estimated Y_{ATP} values were 16.0, 15.5, and 10.6 g of biomass per mol of ATP, respectively, and the estimated nonspecific ATP consumption values were 0.4, 0.4, and 1.2 mol of ATP per C-mol of biomass, respectively. For the cultures containing no furfural the measured and estimated RQs were 1.1 and 1.0, respectively; for the cultures containing 1.14 g of furfural liter^{-1} the measured and estimated RQs were 1.1 and 1.0, respectively; and for the cultures containing 2.25 g of furfural liter^{-1} the measured and estimated RQs were 0.9 and 1.0, respectively.

[b] Values were estimated from flux calculations.

and energy source at a dilution rate of 0.10 h^{-1}. Under these conditions the glucose metabolism was fully respiratory; i.e., no ethanol or other fermentation products could be detected in the reactor. The measured biomass yield on glucose was 0.52 g/g, and the only other products obtained in furfural-free medium were carbon dioxide and biomass. The specific rates of CO_2 production and O_2 consumption were approximately equal, resulting in an RQ of around 1 (Table 1).

The biomass of and products formed by *S. cerevisiae* were investigated by using different inlet concentrations of furfural (1.14, 2.25, and 3.00 g/liter) at a dilution rate of 0.10 h^{-1}. The results are summarized in Table 1. The glucose metabolism was fully respiratory, and no ethanol or glycerol was formed under any of the steady-state conditions.

Furoic acid was the only furfural-derived product found, and no residual furfural was detected in the bioreactor during the steady states obtained in the presence of 1.14 and 2.25 g of furfural per liter in the feed solution. This is in sharp contrast to fermentative or respirofermentative growth, in which furfuryl alcohol and a recently identified probable acyloin condensation product of furfural and pyruvate, 3-(2-furfuryl)-2-hydroxy-2-methyl-3-oxo-propanoic acid (FHMOPA), were found to be the dominant products (35, 39). Washout occurred when the furfural concentration in the feed solution was increased to 3.00 g/liter, suggesting that the maximum specific conversion rate of furfural is between 0.054 and 0.072 g/g · h under steady-state conditions at a dilution rate of 0.10 h^{-1}.

Flux distributions in aerobic and anaerobic continuous cultures. The systemic effects of furfural were investigated by estimating the metabolic flux distributions for the aerobic steady-state cultures with and without furfural in the medium by using a compartmentalized, stoichiometric network model (Fig. 1). To enable a comparison between the furfural effects on aerobic metabolism and the furfural effects on anaerobic metabolism, anaerobic steady-state results for a dilution rate 0.10 h^{-1} published previously (35) were also analyzed by using the stoichiometric model (Fig. 2). The net production and consumption rates predicted by the model are shown in Tables 1 and 2 for aerobic and anaerobic conditions, respectively. The predicted rates fulfill mass and redox balances and are weighted according to the variances in the measurements (14) (Tables 1 and 2). They are therefore better estimates of the true rates, provided that a correct model has been postulated.

For furfural-free medium, the model describes known metabolic behavior (Fig. 1). This means that during respiratory growth, biomass was the major carbon-containing product. The pentose phosphate pathway was very active, providing NADPH and precursors for biosynthesis; glycolysis provided biosynthetic precursors, as well as pyruvate, and the TCA cycle was highly active.

With furfural present in the medium under aerobic conditions, the specific rates of glycolysis, TCA cycle reactions, and respiration increased by 30, 50, and 50%, respectively, for an inlet furfural concentration of 2.25 g/liter (Fig. 1). The specific rates of the biosynthetic fluxes changed only slightly due to changes in the cellular macromolecular composition. This resulted in a more-than-threefold increase in the nonspecific ATP utilization. The biomass yield on ATP (Y_{ATP}) decreased from 16.0 g of biomass/mol of ATP to 10.6 g/mol, and the nonspecific hydrolysis of ATP increased from 0.4 to 1.2 mol of ATP/C-mol of biomass (i.e., the amount of biomass containing 12 g of carbon) when the medium was changed from furfural-free medium to a medium containing 2.25 g of furfural per liter (Table 1). During respiratory growth the only product of the furfural conversion reaction was furoic acid. In the model this reaction was assumed to proceed with NAD^+ as a cofactor. However, the additional NADH produced in this reaction accounted for only 27% of the additional NADH respired, and the rest originated from the increasing rates of the glyceraldehyde dehydrogenase and TCA cycle reactions.

During anaerobic growth, glycolysis was highly active as an ATP-producing pathway, and ethanol was the main product; only a small fraction of the glucose was converted in the pentose phosphate pathway (Fig. 2). Glycerol formation provided additional reoxidation of cytosolic NADH. The Adh3p provided a redox shuttle for mitochondrial NADH (3), and the TCA cycle operated in a forked mode, providing biosynthetic precursors with minimum net NADH formation (25).

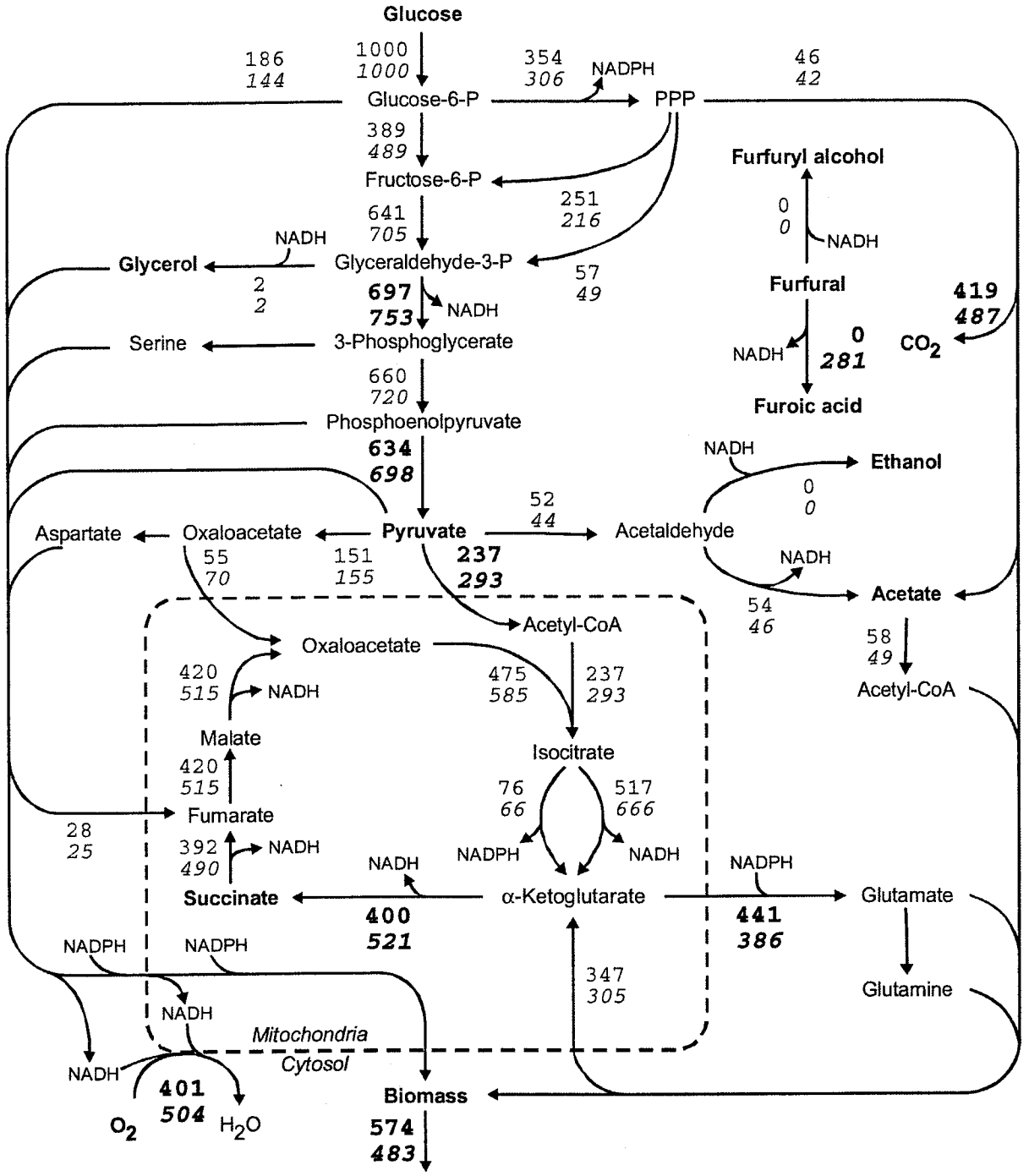

FIG. 1. Flux distribution during respiratory growth of *S. cerevisiae* at a dilution rate of 0.10 h^{-1} calculated on the basis of steady-state data obtained in furfural-free medium (top values) and in the presence of 2.25 g of furfural per liter in the feed solution (bottom values in italics). The data are expressed in C millimoles per C mole of glucose.

Under anaerobic conditions the main product of the furfural conversion reaction was furfuryl alcohol. This compound was assumed to be produced with NADH as a cofactor, possibly by alcohol dehydrogenase I (10, 16, 24). As a consequence, furfuryl alcohol production led to an almost stoichiometric decrease in the flux to glycerol (Fig. 2). In the furfural-free medium, 24 mmol of NADH/C-mol of glucose was converted in glycerol synthesis, whereas 26 mmol of NADH/C-mol of glucose was converted via furfural reduction at the highest furfural concentration. In the furfural-free medium there was a split in the TCA cycle into an oxidative branch and a reductive branch (Fig. 2). When furfural was present in the medium, all the TCA cycle reactions instead appeared to be slightly active in the oxidative direction. This also led to an 85% de-

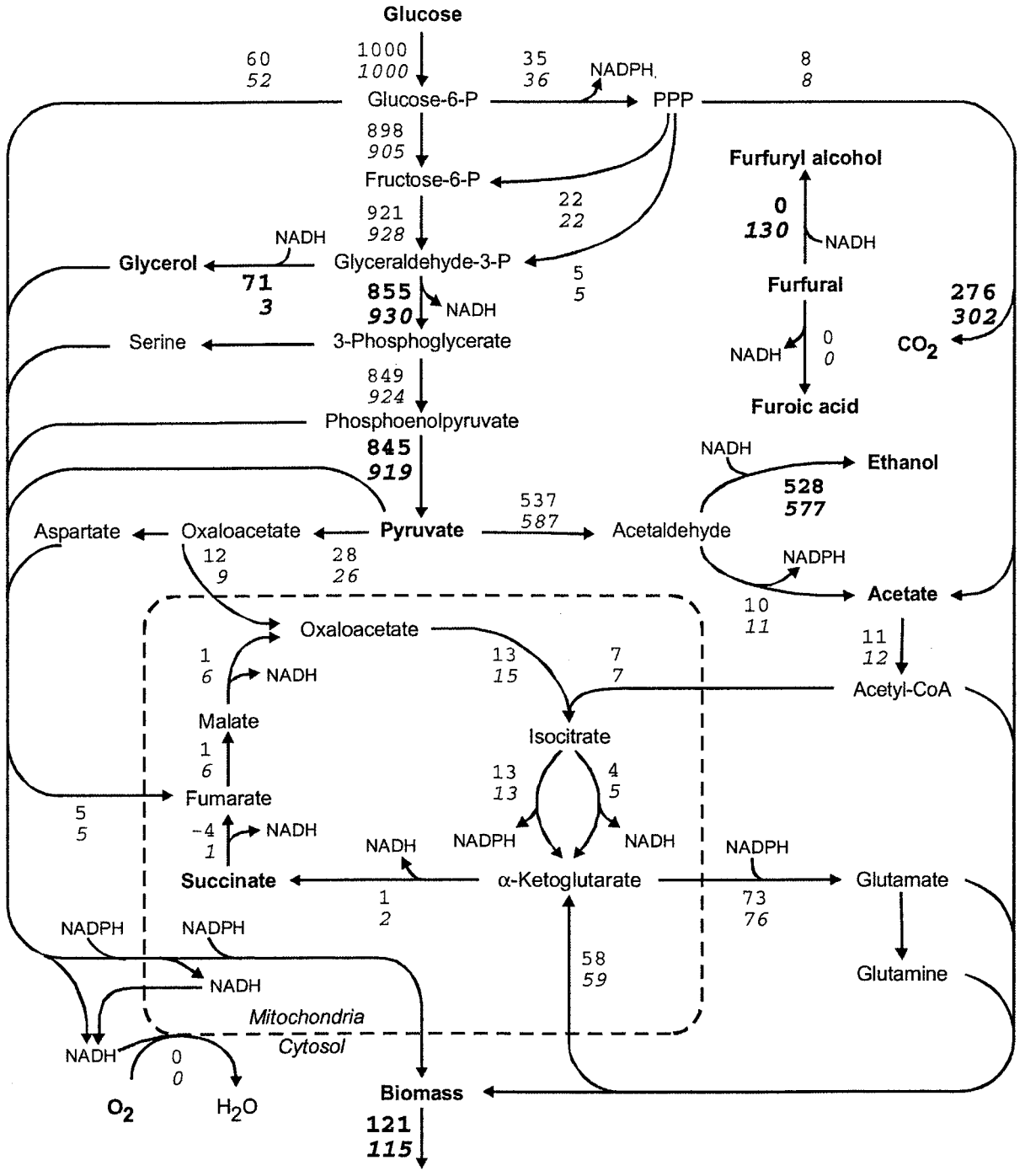

FIG. 2. Flux distribution during anaerobic growth of *S. cerevisiae* at a dilution rate of 0.10 h^{-1} calculated on the basis of steady-state data obtained in furfural-free medium (top values) and in the presence of 5.8 g of furfural per liter in the feed solution (bottom values in italics). The data are expressed in C millimoles per C mole of glucose.

crease in the succinate yield. Furthermore, there was an increase in the movement of reducing equivalents out of the mitochondria via the Adh3p shuttle (25).

At intermediate furfural concentrations in the medium, the biomass yield actually increased by 13%, while the specific glycolytic rate decreased by 11%. This resulted in an increase in the Y_{ATP} from 12.6 to 14.2 g of biomass/mol of ATP and a decrease in the nonspecific ATP consumption from 0.9 to 0.6 mol of ATP/C-mol of biomass (Table 2). However, at the highest furfural concentration, the Y_{ATP} dropped to 10.1 g/mol, and the nonspecific ATP consumption increased by 50% (from 0.9 to 1.3 mol of ATP/C-mol of biomass) compared to the nonspecific ATP consumption in the furfural-free medium (Table 2). This was accompanied by a 12% increase in the

TABLE 2. Experimentally determined and estimated specific formation rates obtained from anaerobic chemostat cultures of *S. cerevisiae* growing on glucose (50 g/liter) as a carbon and energy source with different levels of furfural present in the inlet medium[a]

Metabolite	Approx relative mean SD (%)	Specific rates (C-mol/g · h) in the presence of:									
		No furfural		1.0 g of furfural liter^{-1}		2.0 g of furfural liter^{-1}		3.8 g of furfural liter^{-1}		5.8 g of furfural liter^{-1}	
		Measured value	Estimated value[b]	Measured value	Estimated value[b]	Measured value	Estimated value[b]	Measured value	Estimated value[b]	Measured value	Estimated value[b]
Glucose	5	−35.2	−31.9	−31.0	−29.3	−28.3	−27.5	−29.5	−27.8	−35.2	−32.6
Carbon dioxide	5	8.7	8.8	7.8	8.0	7.9	7.6	8.0	7.9	10.5	9.9
Ethanol	5	15.6	16.8	15.4	15.2	13.8	14.4	13.8	14.9	17.4	18.8
Glycerol	2	2.3	2.3	2.0	2.0	1.7	1.7	1.2	1.2	0.1	0.1
Pyruvic acid	2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Succinic acid	2	0.2	0.2	0.2	0.1	0.1	0.1	0.0	0.0	0.0	0.0
Furfuryl alcohol	5	0.0	0.0	0.6	0.7	1.2	1.2	1.7	1.9	4.1	4.2
Protein	5	1.8	1.6	1.6	1.6	1.7	1.7	1.8	1.8	1.8	1.8
Polysaccharides	10	1.7	1.9	1.9	2.0	1.7	1.7	1.7	1.7	1.7	1.7
RNA	5		0.2		0.2		0.2		0.2		0.2
Lipids	10		0.1		0.1		0.1		0.1		0.1

[a] Some of the experimental data for anaerobic conditions have been published previously (35). For the cultures grown in the presence of 0, 1.0, 2.0, 3.8, and 5.8 g of furfural liter^{-1}, the estimated Y_{ATP} values were 12.6, 13.8, 14.2, 13.4, and 10.1 g of biomass per mol of ATP, respectively, and the estimated nonspecific ATP consumption values were 0.9, 0.7, 0.6, 0.7, and 1.3 mol of ATP per C-mol of biomass, respectively.

[b] Values were estimated from flux calculations.

specific ethanol production rate (Table 2) and a 9% increase in the ethanol yield (Fig. 2). The calculated specific rate of ethanol production was about 8% higher than the measured rate, which can be explained by evaporation of ethanol (36).

Pulse addition of furfural to respiratory continuous cultures. The specific rate of conversion of furfural and the inhibitory effects on cell growth were studied by making pulse additions of furfural (4, 8, and 12 g/liter) to continuous cultures growing at a respiratory steady state. By making direct pulse additions of furfural, it was possible to obtain a high concentration of furfural in the bioreactor that could not be obtained in continuous cultures at a steady state. Furfural significantly affected the specific growth rate. Furthermore, the metabolism immediately switched to mixed respiration and fermentation, as shown by both the amount of ethanol formed and increasing RQ values. Formation of ethanol was measured within 15 min after the pulse additions of furfural (Fig. 3). The RQ immediately increased as a result of furfural addition. The maximum value, 4.5, was reached within 0.5 h, and the RQ subsequently decreased slowly to the initial steady-state value after complete conversion of furfural (data not shown). No transient formation of glycerol was detected.

Pulse addition of furfural resulted in a dramatic decrease in the biomass concentration (Fig. 3). The reason for this could have been the much lower biomass yield as a result of respirofermentative growth or an almost complete cessation of growth in the presence of furfural. Due to the short transition time, it was not possible to distinguish between these two explanations. After all of the furfural was consumed, cell growth resumed, and the same respiratory steady-state conditions that were present before the pulse addition of furfural were obtained.

The residual glucose concentration was not affected by pulse addition of the lowest concentration of furfural (4 g/liter). However, at higher concentrations of furfural (8 and 12 g/liter), the residual glucose concentration transiently increased during furfural conversion (Fig. 3). Also, metabolites, such as succinic acid, acetic acid, and pyruvic acid, were affected. Acetate and pyruvate were transiently excreted into the medium, but they were consumed again when growth was restored. Succinate excretion also increased, but succinate was not consumed again (Fig. 3).

The decrease in the concentration of furfural following a pulse addition was due to both dilution and conversion by the cells. In contrast to the conversion of furfural at a steady state, the main conversion products of furfural in this case were furfuryl alcohol, furoic acid, and FHMOPA. The maximum specific rate of conversion of furfural was estimated to be 0.26 $\pm$ 0.01 g/g · h by using a Michaelis-Menten-type kinetic model (35). Most of the furfural was converted to furfuryl alcohol, for which the maximum specific rate of production was 0.19 $\pm$ 0.01 g/g · h. The maximum specific rate of production of furoic acid was estimated to 0.040 $\pm$ 0.005 g/g · h. The Michaelis-Menten constants for furfural were found to be about 0.1 g/liter for conversion to furfuryl alcohol and almost zero for conversion to furoic acid. This suggests that the affinity for furfural is high, and this suggestion is supported by the fact that the furfural concentrations were below the detection limit under the steady-state conditions.

DISCUSSION

Furfural has previously been found to have profound effects on the growth of and metabolite formation by *S. cerevisiae* during anaerobic growth on glucose (27, 35), respirofermentative growth on glucose (39), and respiratory growth on ethanol (38). Therefore, the strong effect of furfural on respiratory growth on glucose found in the present study was expected. More surprising, however, was the finding that the only conversion product obtained from furfural in chemostat cultures was furoic acid. The main conversion product of furfural in all previously reported studies was furfuryl alcohol. Furfuryl alcohol was the main product of furfural conversion during anaerobic continuous cultivation, and no furoic acid was detected (35). Under transient conditions, after pulse addition of furfural, FHMOPA was found in addition to furfuryl alcohol, but

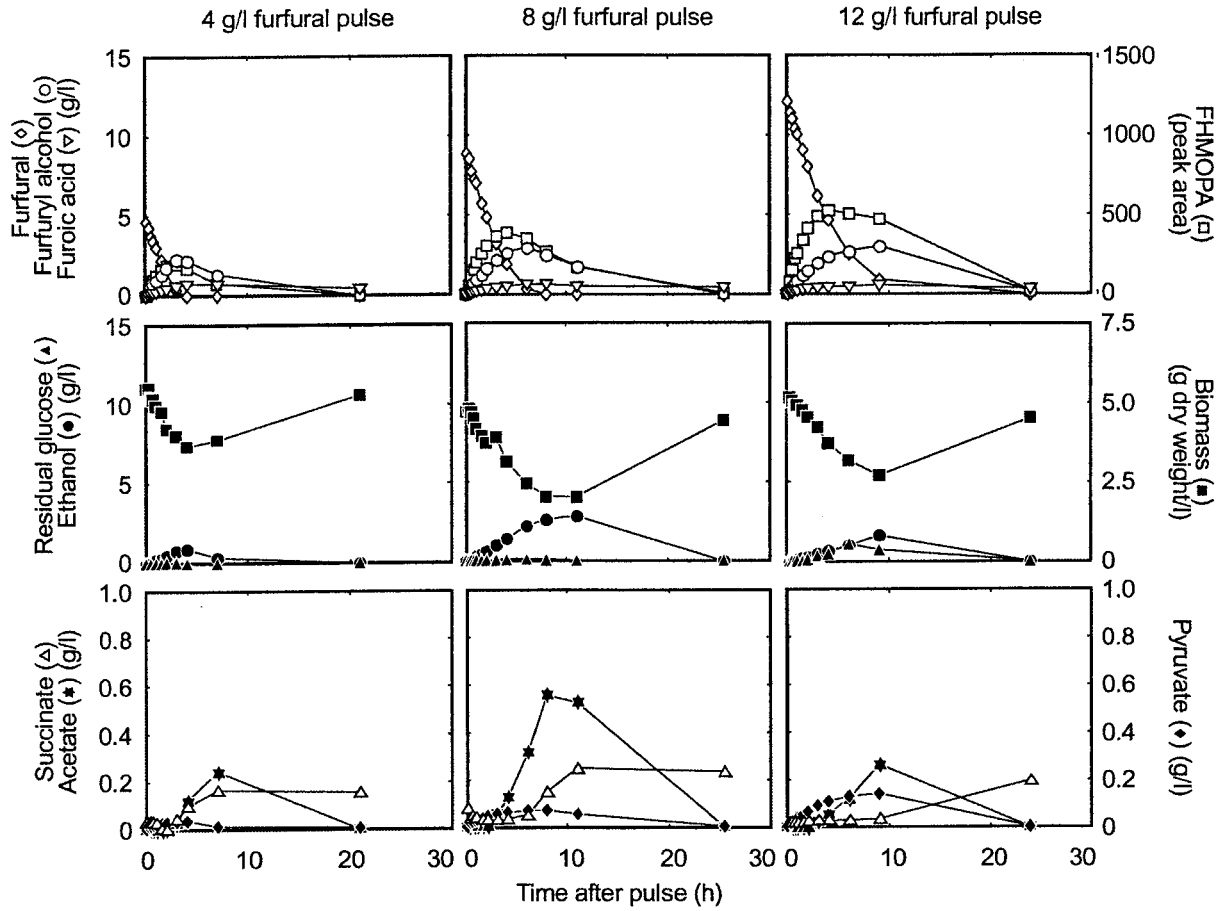

FIG. 3. Metabolite concentrations following pulse additions of furfural (4, 8, and 12 g/liter) to respiratory chemostat cultures of *S. cerevisiae* growing at a dilution rate of 0.10 h^{-1}.

no furoic acid was formed during anaerobic batch cultivation (39) or continuous cultivation on glucose (35). Biotransformation of furfural to furoic acid has been reported only for yeast cells growing on a nonfermentable carbon source, such as ethanol or acetic acid (38). However, furfuryl alcohol was also the dominant conversion product in those cases.

The maximum rate of conversion of furfural to furoic acid under steady-state conditions was between 0.054 and 0.072 g/g · h (at the latter conversion rate washout occurred). On the other hand, at high concentrations of furfural (such as the concentration after a pulse addition), a maximum specific rate of conversion of about 0.26 g/g · h was found. In this case, however, predominantly furfuryl alcohol was obtained (Fig. 3). Consequently, the maximum specific rate of conversion of furfural could be divided into a maximum specific rate of reduction to furfuryl alcohol of 0.19 g/g · h and a maximum specific rate of oxidation to furoic acid of 0.04 g/g · h. The maximum specific rate of furoic acid production was similar to the maximum furfural conversion rate obtained under steady-state conditions during respiratory growth, in which case the only product of the furfural conversion was furoic acid. This may indicate that the oxidation of furfural to furoic acid proceeded at the maximum rate and that, in addition, overflow conversion of furfural to furfuryl alcohol occurred during the pulse addition experiments. Although the maximum value for the aerobic specific rate of conversion of furfural (0.26 g/g · h) is higher than the aerobic chemostat conversion rate, this value is clearly lower than the corresponding maximum conversion rate obtained previously during fermentative metabolism (0.62 g/g · h) both in continuous cultures (35) and in batch cultures (39) of *S. cerevisiae*.

It was found that low concentrations of furfural did not have as strong an effect on cellular metabolism during fermentative growth (Fig. 2) as during respiratory growth (Fig. 1). The main product of the conversion of furfural is furfuryl alcohol under anaerobic conditions. The reduction of furfural can therefore act as an alternative redox sink, redirecting the NADH pool to furfuryl alcohol formation, which results in a substantial decrease in the glycerol yield and a slight increase in biomass formation (27, 35). This was also reflected in an increase in the Y_{ATP} at the intermediate levels of furfural addition (Table 2). Since glycerol formation is accompanied by net ATP consumption, an externally supplied redox sink (e.g., in the form of furfural) could result in more ATP being available for biomass synthesis. Furfural concentrations that are too high, however, result in inhibition of biosynthesis, and growth stops. A limiting factor for furfuryl alcohol production could be that the high

demand for NADH for the reduction of furfural cannot be met during anaerobic growth (35).

The analysis also showed that furfural affects fluxes involved in energy metabolism (Fig. 1). The flux model showed that there was 50% higher specific respiratory activity, including all the reaction steps involved, together with an almost threefold increase in nonspecific ATP consumption when 2.25 g of furfural per liter was present in the inlet medium. This was also reflected in the lower Y_{ATP} (Table 1). The increase in the energy requirement could be a direct effect of the furoic acid formed. In the model used, furoic acid was assumed to be exported via diffusion in its undissociated form. On the other hand, if the dissociated acid is exported via an active anion transporter, additional protons would be liberated both due to the dissociation of the acid and due to the ATP hydrolysis linked to the transport. The export of the anions could tentatively be achieved via the ATP binding cassette transporter Pdr12p, which has been shown to confer resistance to water-soluble, monocarboxylic acids with chains that are one to seven C atoms long, including sorbic acid and benzoic acid (19, 30). In total, 0.8 mol of ATP/C-mol of furoic acid would be used in conjunction with the formation and expulsion of the furoic acid and liberated protons, assuming a stoichiometry of 1 ATP per furoate exported via the Pdr12p transporter. When this information was included in the model, the level of nonspecific ATP hydrolysis was reduced to 1.0 mol of ATP/C-mol of glucose at the highest inlet furfural concentration, a value which is still 2.5-fold higher than the value obtained in the absence of furfural. Furthermore, the low pK_a of furoic acid (pK_a 3.17) makes a futile cycle due to rediffusion of the protonated acid across the plasma membrane unlikely (20, 43). Thus, the increase in the nonspecific energy requirement cannot be explained solely by transport energy requirements.

The variation in the preferred conversion products obtained from furfural may be due to differences in enzyme activities and/or intracellular metabolite levels. Reduction of furfural to furfuryl alcohol is most likely catalyzed by an NADH-coupled alcohol dehydrogenase (5, 28, 38, 45). Assuming that NADPH is a cofactor for furfuryl alcohol production failed to explain the observed metabolite yields and resulted in completely unreasonable metabolic flux distributions. Since the oxidation of furfural to furoic acid is an oxidation of an aldehyde to a carboxylic acid, the reaction is likely to proceed via an aldehyde dehydrogenase. Lower aerobic alcohol dehydrogenase activity, in combination with a lower intracellular NADH level, could well explain the preference for furoic acid formation over furfuryl alcohol formation under aerobic conditions. The cofactor requirement of this reaction has not been determined. However, several facts indicate that NAD^+ is the preferred cofactor in the oxidation of furfural to furoic acid. Modig et al. showed that commercially available NAD^+-dependent aldehyde dehydrogenase was active in converting furfural to furoic acid (22). Furthermore, using NAD^+ as a cofactor for the aldehyde dehydrogenase reaction in our model resulted in a metabolic flux distribution which agrees better with the distribution described by Gombert et al. (15). By using ^{13}C labeling methodology, these authors estimated that the flux through the oxidative part of the pentose phosphate pathway is 44.2% on a molar basis. Importantly, this was done without any use of cofactor balances in the study. When we used NAD^+ in our stoichiometric model, we obtained a value of 35.4% (Fig. 1), while the use of $NADP^+$ resulted in a much lower value, 28.6% (data not shown). Furthermore, if $NADP^+$ was used, the pentose phosphate pathway flux decreased dramatically when furfural was present, whereas with NAD^+ it decreased only in proportion to the decrease in biomass formation (Fig. 1). Moreover, the increased respiratory activity obtained when furfural was present indicates that there is an increased rate of NADH turnover in the respiratory chain. As shown in the metabolic flux analysis, the additional NADH accompanying furoic acid production actually explained only 27% of the increase in the respiratory activity. The rest of the NADH was formed due to an increase in TCA cycle activity. Taken together, these observations support the hypothesis that there is an NAD^+-coupled reaction mechanism.

One possible reason for the lack of a respiratory steady state at an inlet furfural concentration higher than 2.25 g/liter could be a limitation in the oxidative capacity, either in the oxidation of furfural to furoic acid or in the oxidation of NADH to NAD^+. A limitation in the oxidative capacity is in fact suggested by the pulse addition experiments. When the maximum oxidation capacity is reached, ethanol formation is induced, which results in a large decrease in the biomass yield. This in turn results in a decreased overall ability to convert the added furfural. As a consequence, the furfural concentration increases, which leads to inhibition of essential enzymes, most likely including the pyruvate dehydrogenase and aldehyde dehydrogenase enzymes (22). The final result is rapid washout of the culture. Whether the limitation is in the enzymatic conversion of furfural to furoic acid or in the turnover of the NADH produced could not be deduced from the experiments performed in this study.

Based on these observations, we concluded that the ability to survive in the presence of furfural is directly related to the ability to convert furfural to less inhibitory compounds under aerobic conditions, as well as under anaerobic conditions. Under aerobic conditions, a shift to respirofermentative metabolism occurs when the capacity to oxidize furfural is exceeded, which leads to a decrease in the biomass yield. Under anaerobic conditions, the supply of NADH appears to limit the reduction of furfural at conversion rates above rates at which glycerol formation has been totally replaced by furfuryl alcohol production. In both these cases, the result is that the concentration of furfural increases, probably leading to additional direct inhibitory effects on essential enzymatic steps in the central metabolic pathways.

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APPENDIX

Biochemical reactions in the metabolic network model are expressed in C-mol stoichiometry. Water and the cofactors ADP, CoA, NAD^+, $NADP^+$, and tetrahydrofolate are not included. The main product in each reaction is indicated by boldface type; subscript mit and cyt indicate mitochondria and cytoplasm, respectively. Reactions 11a, 14, 17a, 40a, and 46a occur only under anaerobic conditions, and reactions 11b, 17b, 40b, 44, 45, and 46b occur only under aerobic

conditions.

Glycolysis and fermentative products

$$\text{glucose} + 1/6\ \text{ATP} \rightarrow \textbf{glucose 6-phosphate} + 1/6\ \text{H}^+ \quad (1)$$

$$\text{glucose 6-phosphate} \rightarrow \textbf{fructose 6-phosphate} \quad (2)$$

$$\text{fructose 6-phosphate} + 1/6\ \text{ATP} \rightarrow \textbf{glyceraldehyde 3-phosphate} + 1/6\ \text{H}^+ \quad (3)$$

$$\text{glyceraldehyde 3-phosphate} + 1/3\ \text{NADH}_{\text{cyt}} + 1/3\ \text{H}^+ \rightarrow \textbf{glycerol} + 1/3\ \text{P}_i \quad (4)$$

$$\text{glyceraldehyde 3-phosphate} + 1/3\ \text{P}_i \rightarrow \textbf{3-phosphoglycerate} + 1/3\ \text{ATP} + 1/3\ \text{NADH}_{\text{cyt}} + 1/3\ \text{H}^+ \quad (5)$$

$$\text{3-phosphoglycerate} \rightarrow \textbf{phosphoenolpyruvate} \quad (6)$$

$$\text{phosphoenolpyruvate} + 1/3\ \text{H}^+ \rightarrow \textbf{pyruvate} + 1/3\ \text{ATP} \quad (7)$$

$$\text{pyruvate} + 1/3\ \text{H}^+ \rightarrow 2/3\ \textbf{acetaldehyde} + 1/3\ \text{CO}_2 \quad (8)$$

$$3/4\ \text{pyruvate} + 1/4\ \text{CO}_2 + 1/4\ \text{ATP} \rightarrow \textbf{oxaloacetate}_{\textbf{cyt}} + 1/4\ \text{P}_i + 1/2\ \text{H}^+ \quad (9)$$

$$\text{oxaloacetate}_{\text{cyt}} + 1/4\ \text{ATP} \rightarrow \text{oxaloacetate}_{\text{mit}} + 1/4\ \text{P}_i + 1/4\ \text{H}^+ \quad (10)$$

$$\text{acetaldehyde} \rightarrow \textbf{acetate} + 1/2\ \text{NADPH}_{\text{cyt}} + \text{H}^+ \quad (11a)$$

$$\text{acetaldehyde} \rightarrow \textbf{acetate} + 1/2\ \text{NADH}_{\text{cyt}} + \text{H}^+ \quad (11b)$$

$$\text{acetate} + \text{ATP} \rightarrow \textbf{acetyl-CoA} + \text{P}_i + 1/2\ \text{H}^+ \quad (12)$$

$$\text{acetaldehyde} + 1/2\ \text{NADH}_{\text{cyt}} + 1/2\ \text{H}^+ \rightarrow \textbf{ethanol} \quad (13)$$

$$\text{acetaldehyde} + 1/2\ \text{NADH}_{\text{mit}} + 1/2\ \text{H}^+ \rightarrow \textbf{ethanol} \quad (14)$$

$$\text{pyruvate} + 1/3\ \text{H}^+ \rightarrow 2/3\ \textbf{acetoin} + 1/3\ \text{CO}_2 \quad (15)$$

$$\text{acetoin} + 1/4\ \text{NADH}_{\text{cyt}} + 1/4\ \text{H}^+ \rightarrow \textbf{2,3-butanediol} \quad (16)$$

TCA cycle

$$1/3\ \text{acetyl-CoA} + 2/3\ \text{oxaloacetate}_{\text{mit}} \rightarrow \textbf{isocitrate} + 1/6\ \text{CO}_2 + 1/6\ \text{H}^+ \quad (17a)$$

$$1/3\ \text{acetyl-CoA}_{\text{mit}} + 2/3\ \text{oxaloacetate}_{\text{mit}} \rightarrow \textbf{isocitrate} + 1/6\ \text{CO}_2 + 1/6\ \text{H}^+ \quad (17b)$$

$$6/5\ \text{isocitrate} \rightarrow \textbf{2-oxoglutarate} + 1/5\ \text{CO}_2 + 1/5\ \text{NADPH}_{\text{mit}} + 1/5\ \text{H}^+ \quad (18)$$

$$6/5\ \text{isocitrate} \rightarrow \textbf{2-oxoglutarate} + 1/5\ \text{CO}_2 + 1/5\ \text{NADH}_{\text{mit}} + 1/5\ \text{H}^+ \quad (19)$$

$$5/4\ \text{2-oxoglutarate} + 1/4\ \text{P}_i \rightarrow \textbf{succinate} + 1/4\ \text{ATP} + 1/4\ \text{CO}_2 + 1/4\ \text{NADH}_{\text{mit}} \quad (20)$$

$$\text{succinate} \rightarrow \textbf{fumarate} + 1/4\ \text{NADH}_{\text{mit}} + 1/4\ \text{H}^+ \quad (21)$$

$$\text{fumarate} \rightarrow \textbf{malate} \quad (22)$$

$$\text{malate} \rightarrow \textbf{oxaloacetate}_{\textbf{mit}} + 1/4\ \text{NADH}_{\text{mit}} + 1/4\ \text{H}^+ \quad (23)$$

Pentose phosphate pathway

$$6/5\ \text{glucose 6-phosphate} \rightarrow \textbf{ribose 5-phosphate} + 1/5\ \text{CO}_2 + 2/5\ \text{NADPH}_{\text{cyt}} + 2/5\ \text{H}^+ \quad (24)$$

$$5/2\ \text{ribose 5-phosphate} \rightarrow 3/2\ \text{fructose 6-phosphate} + \textbf{erythrose 4-phosphate} \quad (25)$$

$$5/6\ \text{ribose 5-phosphate} + 2/3\ \text{erythrose 4-phosphate} \rightarrow \textbf{fructose 6-phosphate} + 1/2\ \text{glyceraldehyde 3-phosphate} \quad (26)$$

Proton symport of ions

$$\text{NH}_{4,\text{extracellular}} + \text{H}_{\text{extracellular}} \rightarrow \textbf{NH}_4^+ + \text{H}^+ \quad (27)$$

$$\text{PO}_{4,\text{extracellular}} + 2\ \text{H}_{\text{extracellular}} \rightarrow \textbf{P}_\textbf{i} + 2\ \text{H}^+ \quad (28)$$

$$\text{SO}_{4,\text{extracellular}} + 3\ \text{H}_{\text{extracellular}} \rightarrow \textbf{SO}_4^{2-} + 3\ \text{H}^+ \quad (29)$$

Synthesis of amino acids

$$5/4\ \text{glutamate} + \text{oxaloacetate}_{\text{cyt}} \rightarrow \textbf{aspartate} + 5/4\ \text{2-oxoglutarate} \quad (30)$$

$$\text{2-oxoglutarate} + 1/5\ \text{NH}_4^+ + 1/5\ \text{NADPH}_{\text{cyt}} + 1/5\ \text{H}^+ \rightarrow \textbf{Glutamate} \quad (31)$$

$$\text{glutamate} + 1/5\ \text{NH}_4^+ + 1/5\ \text{ATP} \rightarrow \textbf{glutamine} + 1/5\ \text{P}_i + 1/5\ \text{H}^+ \quad (32)$$

$$5/3\ \text{glutamate} + \text{3-phosphoglycerate} \rightarrow \textbf{serine} + 5/3\ \text{2-oxoglutarate} + 1/3\ \text{NADH}_{\text{cyt}} + 1/3\ \text{P}_i + 1/3\ \text{H}^+ \quad (33)$$

$$\text{aspartate} + 1/4\ \text{NADH}_{\text{cyt}} + 1/4\ \text{NADPH}_{\text{cyt}} + 1/2\ \text{ATP} + 1/4\ \text{H}^+ \rightarrow \textbf{threonine} + 1/2\ \text{P}_i \quad (34)$$

$$3/2\ \text{serine} \rightarrow \textbf{glycine} + 1/2\ \text{methylene tetrahydrofolate} \quad (35)$$

$$2\ \text{threonine} \rightarrow \textbf{glycine} + \text{acetaldehyde} \quad (36)$$

Biosynthesis of proteins, RNA, polysaccharides, and lipid

$$\begin{aligned} &0.07162\ \text{acetyl-CoA} + 0.18754\ \text{aspartate} \\ &+ 1.01556\ \text{ATP} + 0.05462\ \text{erythrose 4-phosphate} \\ &+ 0.69153\ \text{glutamate} + 0.13553\ \text{glutamine} \\ &+ 0.03471\ \text{glycine} + 0.00698\ \text{methylene} \\ &\text{tetrahydrofolate} + 0.06816\ \text{NADPH}_{\text{cyt}} \\ &+ 0.04862\ \text{NADPH}_{\text{mit}} + 0.08193 \\ &\text{phosphoenolpyruvate} + 0.27887\ \text{pyruvate} \\ &+ 0.02921\ \text{ribose 5-phosphate} + 0.05502\ \text{serine} \\ &+ 0.00600\ \text{SO}_4^{2-} + 0.04457\ \text{threonine} \rightarrow 0.01199 \\ &\text{acetate} + 0.07322\ \text{CO}_2 + 0.05347\ \text{fumarate} \\ &+ 0.00572\ \text{glyceraldehyde 3-phosphate} + 0.93690\ \text{H}^+ \\ &+ 0.00118\ \text{NADH}_{\text{cyt}} + 0.01489\ \text{NADH}_{\text{mit}} \\ &+ 0.01024\ \text{NH}_4^+ + 0.52965\ \text{2-oxoglutarate} \\ &+ 1.06046\ \text{P}_i + \textbf{protein} \end{aligned} \quad (37)$$

$$\begin{aligned} &0.52102\ \text{aspartate} + 0.95995\ \text{ATP} + 0.04923\ \text{CO}_2 \\ &+ 1.01785\ \text{glutamine} + 0.04923\ \text{methylene} \end{aligned}$$

$$\text{tetrahydrofolate} + 0.52821\ \text{ribose 5-phosphate} + 0.14769\ \text{serine} \rightarrow 0.29537\ \text{fumarate} + 1.01785\ \text{glutamate} + 1.36043\ H^+ + 0.08103\ NADH_{cyt} + 0.04923\ NADPH_{cyt} + 0.95995\ P_i + \textbf{RNA} \quad (38)$$

$$\text{glucose 6-phosphate} + 1/6\ ATP \rightarrow \textbf{polysaccharide} + 1/3\ P_i + 1/6\ H^+ \quad (39)$$

$$0.14098\ \text{acetyl-CoA} + 0.13662\ ATP + 0.05220\ \text{glycerol} + 0.02153\ H^+ + 0.01607\ \text{methylene-tetrahydrofolate} + 0.01607\ NADH_{cyt} + 0.12336\ NADPH_{cyt} + 0.03471\ \text{serine} \rightarrow 0.00890\ CO_2 + \textbf{lipid} + 0.12325\ P_i \quad (40a)$$

$$0.92793\ \text{acetyl-CoA} + 0.58919\ ATP + 0.05405\ \text{glycerol} + 0.04054\ O_2 + 0.16126\ H^+ + 0.00721\ \text{methylene tetrahydrofolate} + 0.00721\ NADH_{cyt} + 0.77027\ NADPH_{cyt} + 0.03446\ \text{serine} \rightarrow 0.03582\ CO_2 + \textbf{lipid} + 0.52703\ P_i \quad (40b)$$

ATP hydrolysis

$$ATP \rightarrow P_i + H_{extracellular} \quad (41)$$

$$ATP \rightarrow P_i + H^+ \quad (42)$$

Respiration

$$1/2\ O_2 + NADH_{cyt} + P/O_{cyt}\ P_i + (1 + P/O_{cyt})\ H^+ \rightarrow P/O_{cyt}\ \textbf{ATP} \quad (43)$$

$$1/2\ O_2 + NADH_{mit} + P/O_{mit}\ P_i + (1 + P/O_{mit})\ H^+ \rightarrow P/O_{mit}\ \textbf{ATP} \quad (44)$$

$$\text{pyruvate} \rightarrow 2/3\ \text{acetyl-CoA}_{mit} + 1/3\ CO_2 + 1/3\ NADH_{mit} \quad (45)$$

Furfural conversion

$$\text{furfural} + 1/5\ NADH_{cyt} + 1/5\ H^+ \rightarrow \textbf{furfuryl alcohol} \quad (46a)$$

$$\text{furfural} \rightarrow \textbf{furoic acid} + 1/5\ NADH_{cyt} + 1/5\ H^+ \quad (46b)$$

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