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MINIREVIEW

The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on *Saccharomyces cerevisiae* Metabolism[▽]

Lucie A. Hazelwood,[1,2] Jean-Marc Daran,[1,2] Antonius J. A. van Maris,[1,2]
Jack T. Pronk,[1,2] and J. Richard Dickinson[3]*

*Department of Biotechnology, Delft University of Technology,[1] and Kluyver Centre for Genomics of Industrial Fermentation,[2]
2628 BC Delft, The Netherlands, and Cardiff School of Biosciences, Cardiff University, Cardiff CF10 3TL, United Kingdom[3]*

Saccharomyces cerevisiae has been used for at least eight millennia in the production of alcoholic beverages (41). Along with ethanol and carbon dioxide, fermenting cultures of this yeast produce many low-molecular-weight flavor compounds. These alcohols, aldehydes, organic acids, organic sulfides, and carbonyl compounds have a strong impact on product quality. Indeed, the subtle aroma balance of these compounds in fermented foods and beverages is often used as an organoleptic fingerprint for specific products and brands (42). Food fermentation by yeast and lactic acid bacteria is accompanied by the formation of the aliphatic and aromatic alcohols known as fusel alcohols. Fusel oil, which derives its name from the German word *fusel* (bad liquor), is obtained during the distillation of spirits and is enriched with these higher alcohols. While fusel alcohols at high concentrations impart off-flavors, low concentrations of these compounds and their esters make an essential contribution to the flavors and aromas of fermented foods and beverages. Fusel alcohols are derived from amino acid catabolism via a pathway that was first proposed a century ago by Ehrlich (13). Amino acids represent the major source of the assimilable nitrogen in wort and grape must, and these amino acids are taken up by yeast in a sequential manner (23, 32). Amino acids that are assimilated by the Ehrlich pathway (valine, leucine, isoleucine, methionine, and phenylalanine) are taken up slowly throughout the fermentation time (32). After the initial transamination reaction (Fig. 1), the resulting α-keto acid cannot be redirected into central carbon metabolism. Before α-keto acids are excreted into the growth medium, yeast cells convert them into fusel alcohols or acids via the Ehrlich pathway.

Current scientific interest in the Ehrlich pathway is supported by increased demands for natural flavor compounds such as isoamyl alcohol and 2-phenylethanol, which can be produced from amino acids in yeast-based bioconversion processes (14), as well as by the need to control flavor profiles of fermented food products. The goal of this paper is to present a concise centenary overview of the biochemistry, molecular biology, and physiology of this important pathway in *S. cerevisiae*.

EARLY HISTORY OF THE EHRLICH PATHWAY

The turn of the 20th century saw the establishment of many biochemical principles. In 1877 von Ewald and Kühne proposed the notion of enzymes (62), and in 1893 Ostwald proved enzymes to be catalysts (46, 47). The following year (1894), Fischer (15) demonstrated the "lock-and-key" relationship between enzymes and substrates. Alcoholic fermentation in yeast cell extracts was described by Büchner in 1897 (3). In 1902 Fischer (16) and Hofmeister (24) demonstrated that proteins are polypeptides. It was in this background of accomplished chemistry and a growing knowledge of biochemistry that the German biochemist Felix Ehrlich (1877 to 1942) worked.

In 1904, after isolating and characterizing isoleucine, Ehrlich noted the structural similarities between this amino acid and active amyl alcohol and between leucine and isoamyl alcohol. These observations led him to investigate whether these fusel alcohols were derived from the amino acids (13). Indeed, the supplementation of yeast fermentation mixtures with either leucine or isoleucine led to increased production of fusel alcohols. Ehrlich then proposed (13) that the amino acids were split by a "hydrating" enzyme activity to form the corresponding fusel alcohols, along with carbon dioxide and ammonia. Ammonia, which was not detected in these experiments, was assumed to be incorporated into yeast protein. In 1911, Neubauer and Fromherz proposed a modified metabolic scheme (44) that constitutes the Ehrlich pathway as it is still viewed today (Fig. 1). As the first intermediate in the pathway, they proposed an α-keto acid, which can subsequently be decarboxylated into an aldehyde, which is then reduced into the fusel alcohol. The acceptor of the amino group from the starting amino acid was not known and neither were the exact natures of any of the individual enzyme-catalyzed steps. Subsequent work by Lampitt (36), Yamada (67–69), and Thorne (58, 59) confirmed that all fusel alcohols produced by yeast can be derived from amino acid catabolism. An overview of the Ehrlich pathway intermediates and products is presented in Table 1.

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* Corresponding author. Mailing address: Cardiff School of Biosciences, Cardiff University, P.O. Box 915, Cardiff CF10 3TL, United Kingdom. Phone: 442920875762. Fax: 442920874305. E-mail: Dickinson @cardiff.ac.uk.
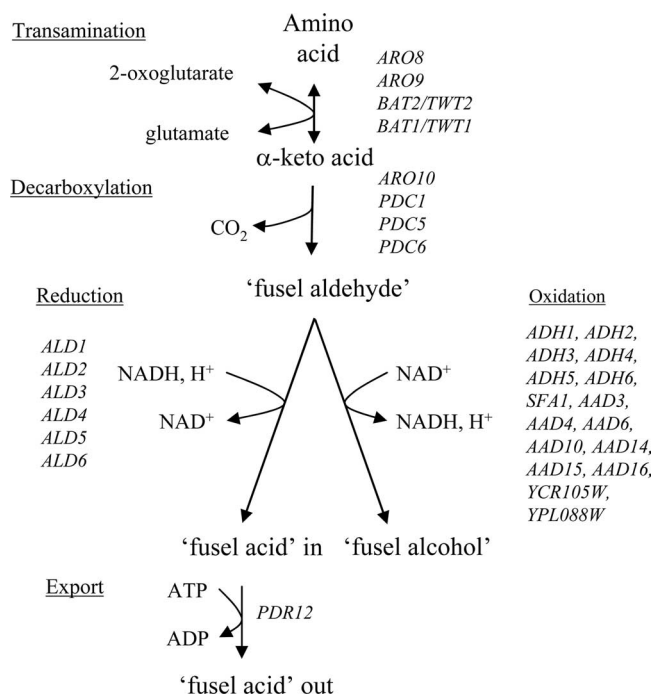
FIG. 1. The Ehrlich pathway. Catabolism of branched-chain amino acids (leucine, valine, and isoleucine), aromatic amino acids (phenylalanine, tyrosine, and trytophan), and the sulfur-containing amino acid (methionine) leads to the formation of fusel acids and fusel alcohols. The genes encoding the enzymes of each step are indicated.

IDENTIFICATION AND CONFIRMATION OF THE KEY ENZYMATIC STEPS IN THE EHRLICH PATHWAY

In the 1950s and 1960s, Sentheshanmuganathan studied the conversion of tyrosine into tyrosol. He demonstrated that cell extracts of *S. cerevisiae* transfer the amino groups from aspartate, isoleucine, leucine, methionine, norleucine, phenylalanine, tryptophan, and tyrosine to α-ketoglutarate and, hence, that the first step of the Ehrlich pathway can be catalyzed by an aminotransferase activity (54, 55). He also demonstrated the decarboxylation of *p*-hydroxy 3-phenylpyruvate into *p*-hydroxy 3-phenylacetaldehyde and the NADH-dependent reduction of the latter compound into tyrosol. This finding established a key reaction sequence for the Ehrlich pathway: transaminase, decarboxylase, and alcohol dehydrogenase.

While, until the late 1990s, most experimental observations were compatible with the existence of the Ehrlich pathway, its in vivo activity was not proven. For example, experiments in which radioactively labeled amino acids were converted into radioactively labeled fusel alcohols did not rule out alternative intermediates and enzymes. Yeast molecular biology raised further questions on the identities of the enzymes involved in the Ehrlich pathway, as many candidate enzymes and genes for each of the reaction steps were identified. This raised the possibility that different isozymes may be involved under different environmental and/or nutritional conditions.

Final experimental verification of the role of the Ehrlich pathway in amino acid catabolism was obtained through ^{13}C labeling studies. After the growth of *S. cerevisiae* under normal physiological conditions in minimal media with individual

TABLE 1. Ehrlich pathway intermediates[a]

Amino acid	α-Keto acid		Fusel aldehyde		Fusel alcohol		Fusel acid	
	Systematic	Traditional	Systematic	Traditional	Systematic	Traditional	Systematic	Traditional
Leu	4-Methyl-2-oxo-pentanoate	α-Ketoisocaproate	3-Methylbutanal	Isoamylaldehyde	3-Methylbutanol	Isoamyl alcohol	3-Methylbutanoate	Isovalerate
Val	3-Methyl-2-oxo-butanoate	α-Ketoisovalerate	2-Methylpropanal	Isobutanal or isovaleraldehyde	2-Methylpropanol	Isobutanol	2-Methylpropanoate	Isobutyrate
Ile	3-Methyl-2-oxo-pentanoate	α-Ketomethylvalerate	2-Methylbutanal	Methylvaleraldehyde	2-Methylbutanol	Active amyl alcohol	2-Methylbutanoate	Methylvalerate
Phe	3-Phenyl-2-oxo-propanoate	Phenylpyruvate	2-Phenylethanal	2-Phenylacetaldehyde	2-Phenylethanol	2-Phenylethanol	2-Phenylethanoate	2-Phenylacetate
Tyr	3-(4-Hydroxyphenyl)-2-oxopropanoate	*p*-Hydroxyphenylpyruvate	2-(4-Hydroxyphenyl)ethanal	*p*-Hydroxyphenylacetaldehyde	2-(4-Hydroxyphenyl)ethanol	*p*-Hydroxyphenylethanol or tyrosol	2-(4-Hydroxyphenyl)ethanoate	*p*-Hydroxy-phenylacetate
Trp	3-(Indol-3-yl)-2-oxopropanoate	3-Indole pyruvate	2-(Indol-3-yl)-ethanal	3-Indole acetaldehyde	2-(Indol-3-yl)ethanol	Tryptophol	2-(Indol-3-yl)ethanoate	2-Phenylacetate
Met	4-Methylthio-2-oxobutanoate	α-Keto-γ-(methylthio)butyrate	3-(Methylthio)propanal	Methional	3-(Methylthio)propanol	Methionol	3-(Methylthio)propanoate	

[a] With the term fusel aldehyde, we indicate the aldehyde that is formed after the decarboxylation of the indicated amino acid-derived α-keto acid. Similarly, the terms fusel alcohol and fusel acid are used to indicate the compound formed by the reduction or oxidation of the fusel aldehyde into the corresponding alcohol or organic acid.

amino acids specifically labeled with ^{13}C as the sole nitrogen source, ^{13}C nuclear magnetic resonance spectroscopy was employed to identify the metabolic sequences involved. Since the fate of individual atoms in each intermediate from the amino acid to the end product could be monitored, the Ehrlich pathway was proved for the first time in the catabolism of leucine, valine, isoleucine, phenylalanine, tryptophan, and methionine into the corresponding fusel alcohols (6–8, 10, 50). Further confirmation of the key in vivo involvement of the Ehrlich pathway and the nonparticipation of other routes was obtained by testing specific mutants in which potential alternative metabolic pathways were blocked (6–8, 10).

During studies of the catabolism of [2-^{13}C]valine into isobutanol labeled at the expected C-1 position, the formation of isoamyl alcohol labeled at C-2 was observed (7). This result was shown to be due to the entry of ^{13}C-labeled α-ketoisovalerate, formed by valine deamination, into the leucine biosynthetic pathway, thus giving rise to labeled isoamyl alcohol (Fig. 1) (7). This finding indicates a mixing of catabolic and biosynthetic pathways for these branched-chain amino acids via the shared intermediate α-ketoisovalerate.

The Ehrlich pathway for methionine catabolism represents a special case. A conventional Ehrlich pathway involves transamination into α-keto-γ-(methylthio)butyrate (α-KMBA), decarboxylation into β-(methylthio)propionaldehyde (often called methional), and then reduction into methionol. In addition, a demethiolase activity acts on both methionine and α-KMBA to produce methanethiol and α-ketobutyrate (50). Whether this reaction occurs spontaneously or enzymatically is still unknown.

INITIAL TRANSAMINATION REACTION

Four *S. cerevisiae* proteins have been implicated in the initial transamination step of the Ehrlich pathway (Fig. 1). Twt1p (also known as Bat1p or Eca39p) is the mitochondrial branched-chain amino acid aminotransferase, and Twt2p (Bat2p or Eca40p) is the cytosolic isozyme. The mitochondrial isozyme is highly expressed in batch cultures during exponential growth and is repressed during stationary phase, while the cytosolic isozyme has the opposite expression pattern. The genes encoding both enzymes were cloned by two independent groups in 1996 (11, 34). Eden et al. (12) showed that an *eca40* (*twt2*) mutation drastically reduces the production of isobutanol. Decreased production of active amyl alcohol and isoamyl alcohol was also seen by others (37, 53, 70), although the results also indicated the involvement of a Twt2p-independent transaminase activity in the formation of these alcohols.

Aro8p and Aro9p were initially characterized as the aromatic amino acid aminotransferases I and II, respectively (31). Urrestarazu et al. (60) demonstrated that Aro8p also exhibits in vitro activity with the amino donors methionine, α-aminoadipate, and leucine and with phenylpyruvate as the amino acceptor and, reversibly, with their α-keto acid analogues as amino acceptors and phenylalanine as the amino donor. Aro9p also has broader substrate specificity than originally described (60). Apparently, Aro8p and Aro9p act as broad-substrate-specificity amino acid transaminases in the Ehrlich pathway. This notion is supported by genome-wide expression profiling; when *S. cerevisiae* is grown in a glucose-limited chemostat with
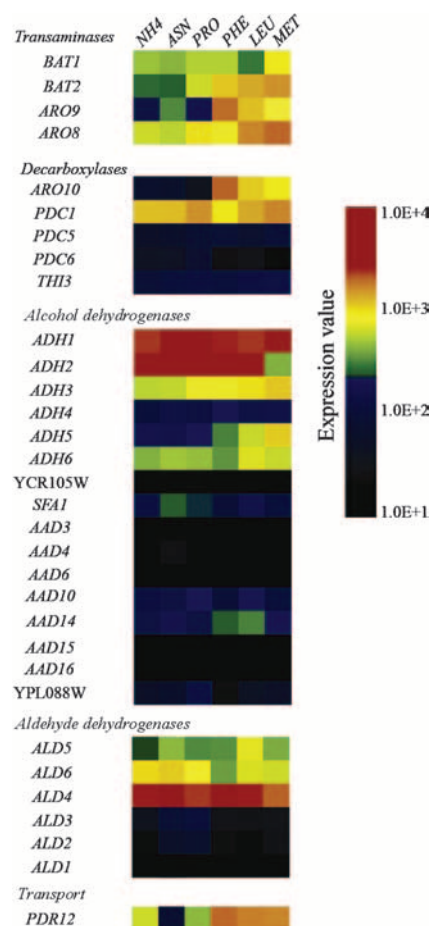

FIG. 2. Heat map and identities of the genes that may encode enzymes involved in the Ehrlich pathway. Genes expressed at high levels are represented in red, and genes expressed at low levels are represented in black. The figure represents the transcript levels of genes in aerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D grown with various nitrogen sources [(NH$_4$)$_2$SO$_4$ (NH4), asparagine (ASN), proline (PRO), phenylalanine (PHE), leucine (LEU), and methionine (MET)] (2). Transcript levels were determined with Affymetrix GeneChips YG-S98 and are the averages of results from three independent experiments.

phenylalanine, methionine, or leucine as the sole nitrogen source, *ARO9* and *TWT2* are consistently upregulated compared to these genes in cultures grown with ammonia, proline, and asparagine, three nitrogen sources whose catabolism does not involve the Ehrlich pathway (2) (Fig. 2).

DECARBOXYLATION STEP

The irreversible decarboxylation step, which commits 2-oxo acids to the Ehrlich pathway, was initially attributed to pyruvate decarboxylase (5, 55, 65). However, the yeast genome was shown to harbor no fewer than five genes that show sequence similarity with thiamine diphosphate (TPP)-dependent decarboxylase genes. Three of these genes (*PDC1*, *PDC5*, and *PDC6*) encode pyruvate decarboxylases (25–28, 52, 57), while *ARO10* and *THI3* are alternative candidate genes for Ehrlich pathway decarboxylases (8, 10, 63).

Null mutants with defects in the five structural genes encod-

ing (putative) TPP-dependent decarboxylases (*pdc1*, *pdc5*, *pdc6*, *aro10*, and *thi3*), both singly and in combination, have been studied to identify whether specific enzymes catalyze the decarboxylation of the individual α-keto acid intermediates. This research led to the initial conclusion that the major decarboxylase involved in leucine catabolism is encoded by *THI3* (8). In valine catabolism, any one of the three isozymes Pdc1p, Pdc5p, and Pdc6p will decarboxylate α-ketoisovalerate (7). However, the conclusions from the leucine and valine experiments were drawn while the decarboxylase encoded by the *ARO10* (YDR380w) gene was not yet known and consequently could not be quantitatively assessed. In isoleucine catabolism, any one of the family of decarboxylases encoded by *PDC1*, *PDC5*, *PDC6*, *ARO10*, and *THI3* was found to be sufficient for the conversion of isoleucine to active amyl alcohol (6). The *THI3* decarboxylase has no role in the catabolism of the aromatic amino acids phenylalanine and tyrosine: in a *thi3* null mutant, the decarboxylation of 3-phenylpyruvate and 3-indolepyruvate is apparently accomplished by any one of the remaining four decarboxylases (10), while in methionine catabolism, the decarboxylation of α-KMBA is effected specifically by Aro10p (50, 63).

In chemostat cultures grown with either leucine, methionine, or phenylalanine as the sole nitrogen source, fusel acids and alcohols derived from amino acids other than the nitrogen source are produced in significant amounts (63). An α-keto acid decarboxylase activity for a broad range of substrates was measured in cell extracts of such cultures and suggested the involvement of a common, broad-specificity decarboxylase activity. *ARO10* was the only decarboxylase gene whose transcript profile correlated strongly with α-keto acid decarboxylase activity in the chemostat cultures (2, 63, 64) (Fig. 2). A comprehensive characterization of Arop10p using a combination of genetic, physiological, and biochemical approaches (63) confirmed that Aro10p is a broad-substrate-specificity decarboxylase. These experiments also demonstrated the existence of an Aro10p-independent α-keto acid decarboxylase activity (64), which requires the functional alleles of both *THI3* and at least one of the pyruvate decarboxylase genes *PDC1*, *PDC5*, and *PDC6*. Transcriptome analyses and decarboxylase activity measurements of an *S. cerevisiae aro10Δ* strain, a double *aro10Δ thi3Δ* deletion strain, and a quadruple *pdc1Δ pdc5Δ pdc6Δ aro10Δ* mutant strain grown in carbon-limited chemostats with phenylalanine as the nitrogen source indicated that (i) *PDC5* is strongly upregulated in an *aro10Δ* background (Fig. 3) and also encodes a broad-substrate α-keto acid decarboxylase (J.-M. Daran, unpublished observation), (ii) *PDC5* expression depends on the presence of *THI3* (Fig. 3), and (iii) in contrast to the expression of *ARO10* only (in a *pdc1Δ pdc5Δ pdc6Δ thi3Δ* strain), the expression of *THI3* only (in a *pdc1Δ pdc5Δ pdc6Δ aro10Δ* strain) does not result in any α-keto acid decarboxylase activity (63, 64). *THI3* has recently been demonstrated to be involved in the regulation of thiamine homeostasis in *S. cerevisiae* (43, 45), which further suggests that its role in the Ehrlich pathway may be regulatory rather than catalytic. While Pdc1, Pdc5, Pdc6, and Aro10 are cytosolic, Thi3 has been observed to be localized in both the cytosol and the nucleus (43, 45), which would support further its role in regulatory function. A systematic investigation of the catalytic properties of all five (putative) TPP-dependent decarboxylases (Aro10p, Thi3p, Pdc1p, Pdc5p, and Pdc6p) is essential for a
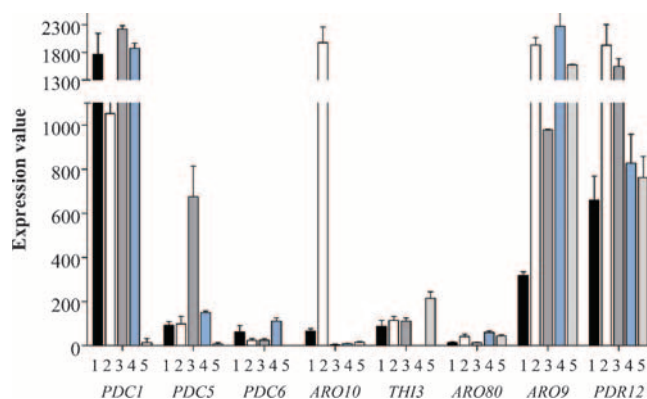

FIG. 3. Expression profiling of the five TPP-dependent decarboxylases genes in various mutant strains grown at a dilution rate of 0.1 h^{-1} in carbon-limited chemostats. Expression values are the means ± standard deviations of data from at least two independent GeneChip (Affymetrix YG-S98) analyses of samples issued from different steady-state chemostat cultivations. 1, CEN.PK113-7D grown in a carbon-limited chemostat with (NH$_4$)$_2$SO$_4$ as the nitrogen source (64); 2, CEN.PK113-7D grown in a carbon-limited chemostat with phenylalanine as the nitrogen source (64); 3, *aro10Δ* strain grown in a carbon-limited chemostat with phenylalanine as the nitrogen source (64); 4, *aro10Δ thi3Δ* strain grown in a carbon-limited chemostat with phenylalanine as the nitrogen source (64); and 5, *pdc1Δ pdc5Δ pdc6Δ aro10Δ* strain grown in a carbon-limited chemostat with phenylalanine as the nitrogen source (64). Corresponding microarray data have been deposited in the Genome Omnibus Expression Database (http://www.ncbi.nlm.nih.gov/geo/) under the series accession number GSE9590 (1).

final resolution of the substrate specificity of these key enzymes in the Ehrlich pathway.

In lactic acid bacteria, the catabolism of branched-chain, aromatic, and sulfur-containing amino acids also involves transamination followed by a decarboxylation reaction. The fate of the aldehyde seems to be species dependent, but in *Lactococcus lactis*, a gene called *kcdA* has been identified and characterized. Similar to the yeast Aro10p gene, *kcdA* encodes a broad-substrate α-keto acid decarboxylase that exhibits the highest level of activity on branched-chain α-keto acids (56).

REDUCTION OR OXIDATION OF FUSEL ALDEHYDES

In its classical description, the final step of the Ehrlich pathway is the reduction of the fusel aldehyde (Table 1) formed in the decarboxylation step by an alcohol dehydrogenase. Indeed, in glucose-grown batch cultures, the amino acids that can be converted via the Ehrlich pathway are almost entirely converted to fusel alcohol and the formation of fusel acid via the oxidation of the aldehydes plays only a minor role (6–8, 10).

Recent studies have shown that the balance between the oxidation and the reduction of the fusel aldehydes depends strongly on cultivation conditions. In aerobic glucose-limited chemostat cultures grown with various amino acids (leucine, methionine, and phenylalanine) as sole nitrogen sources, the amino acids are converted predominantly to fusel acids and only very low concentrations of fusel alcohols are formed (2, 63, 64). In such cultures, the biomass yield on glucose is 40% lower than that of cultures grown on other nitrogen sources, thus implying that a large energy drain is associated with the

formation of fusel acids (2). Given the relatively high levels of hydrophobicity of these weak organic acids, it seems probable that ATP dissipation occurs through classical (29, 49) weak organic acid uncoupling of the plasma membrane pH gradient.

A key role of the cellular redox status in determining the ratio of fusel acid to fusel alcohol production is consistent with the observations discussed above. In glucose-grown batch cultures of *S. cerevisiae*, growth is predominantly fermentative, and when phenylalanine is the sole nitrogen source, it is converted into a mixture of 90% fusel alcohol (phenylethanol) and less than 10% fusel acid (phenylacetate). Furthermore, fully fermentative dissimilation of glucose under anaerobic conditions (in batch or chemostat cultures) results in the almost complete conversion of phenylalanine to phenylethanol (63, 64).

Especially under anaerobic conditions, the reduction step of the Ehrlich pathway may have a relevant impact on the overall cellular redox metabolism. In *S. cerevisiae*, excess NADH formed during anaerobic growth can be oxidized by glycerol-3-phosphate dehydrogenase to yield glycerol (48, 61). However, glycerol formation from glucose requires ATP and is thus energetically unfavorable. Amino acid catabolism via the Ehrlich pathway may provide an alternative, energy-efficient means for NADH regeneration. Although this possible role of the Ehrlich pathway has not been systematically investigated, nitrogen-limited, respirofermentative cultures of *S. cerevisiae* produce less glycerol when valine instead of ammonia is supplied as the nitrogen source (5).

Determining the molecular identities of the oxidoreductases involved in the Ehrlich pathway presents a challenge. The *S. cerevisiae* genome harbors genes for 16 alcohol dehydrogenases, 6 aldehyde dehydrogenases, and at least 2 other broad-spectrum reductases that catalyze the pyridine nucleotide-dependent interconversion of aldehydes and alcohols (Fig. 1). Known roles of the alcohol dehydrogenases are reviewed in reference 10. Using strains containing all possible combinations of mutations affecting the seven *AAD* genes (encoding putative aryl alcohol dehydrogenases), five *ADH* genes, and *SFA1*, Dickinson et al. showed that the final step of the Ehrlich pathway (fusel alcohol formation) can be catalyzed by any one of the ethanol dehydrogenases (Adh1p, Adh2p, Adh3p, Adh4p, and Adh5p) or by Sfa1p (a formaldehyde dehydrogenase) (10). In addition, the NADPH-utilizing aldehyde reductases encoded by *YPR1* and *GRE2* have been shown to have activity toward 2-methylbutyraldehyde and isovaleraldehyde, respectively (17, 20).

Ascribing precise roles to aldo-ketose reductases in many organisms (yeast included) is problematic due to overlapping specificities and the presence of other related enzymes. Transcriptome data for *S. cerevisiae* grown in glucose-limited chemostats with phenylalanine, methionine, or leucine as the sole nitrogen source were not discriminative, as none of the 16 alcohol dehydrogenase and the 6 aldehyde dehydrogenase transcript profiles could correlate the presence of fusel alcohols and that of fusel acids (2) (Fig. 2).

EXPORT OF FUSEL ALCOHOLS AND FUSEL ACIDS

The mechanism by which fusel alcohols are exported from the cells into the culture medium remains unknown. So far, there are no data that link this process to any known membrane transporter in *S. cerevisiae*, but considering the water-octanol partition coefficients (ranging from 0.76 to 1.36), it is conceivable that export occurs by simple passive diffusion across the lipid bilayer (38). Conversely, the export of fusel acids has recently been shown to involve at least one plasma membrane transporter. The expression of *PDR12*, encoding an ATP-dependent transporter which is known to be involved in the export of several weak organic acid food preservatives (e.g., benzoate and sorbate) (29), was strongly increased in chemostat cultures utilizing leucine, methionine, or phenylalanine as the sole nitrogen source (2). The phenotypic analysis of a *pdr12Δ* mutant confirmed the role of Pdr12p in the export of fusel acids formed by the catabolism of leucine, isoleucine, valine, phenylalanine, and tryptophan via the Ehrlich pathway (21). The involvement of an ATP-dependent exporter is fully consistent with the reduced biomass yields observed for chemostat cultures grown on amino acids as the sole nitrogen source that lead to the formation of uncoupling agents (2) (Fig. 2).

REGULATION OF THE EHRLICH PATHWAY

Although the regulation of many of the individual genes discussed above has already been investigated, these studies did not take into account the context of the Ehrlich pathway. Iraqui et al. identified *ARO80* as the transcriptional activator involved in the induction of the *ARO9* and *ARO10* transaminase and decarboxylase genes by the aromatic amino acid tryptophan, phenylalanine, or tyrosine. A 36-bp upstream activating sequence was shown to be necessary and sufficient to promote transcriptional induction by aromatic amino acids (30). Chromatin immunoprecipitation in chip experiments confirmed the binding of Aro80p to a WRCCGWSATTT RCCG motif uniquely present in the *ARO10* and *ARO9* promoters (19, 39). It remains unclear whether the strong induction of *ARO9* and *ARO10* observed in chemostat cultures grown with the Ehrlich pathway precursors leucine and methionine as nitrogen sources (2) is also mediated by Aro80p.

As discussed above, the *PDR12*-encoded plasma membrane transporter has recently been shown to export branched-chain and aromatic fusel acids (21). The transcriptional induction of *PDR12* by the nonphysiological substrates benzoate and sorbate is mediated by War1p, an activator that occupies a *cis*-acting element in the promoter of *PDR12* and becomes active upon phosphorylation (35). A recent genetic screen identified a *WAR1* mutant allele carrying mutations corresponding to three amino acid changes. One of these changes proved to be essential for the phosphorylation and binding of War1p to its target sequence. The absence of the phosphorylation of War1p-42 eliminates *PDR12* induction and leads to hypersensitivity to sorbate (18). It is as yet unclear whether War1p-mediated induction is also the (sole) mechanism for the transcriptional induction of *PDR12* by endogenously produced fusel acids.

In the chemostat-based transcriptome studies by Boer et al. (2), 30 additional genes were shown to follow the transcriptional profile of *ARO9*, *ARO10*, and *PDR12* (i.e., strong transcriptional upregulation during growth with amino acid precursors of the Ehrlich pathway as the nitrogen source).

However, none of the genes implicated in the Ehrlich pathway (Fig. 1), with the exception of the three mentioned above, were found among the set of 30 genes. This coordinate induction suggests that a general regulatory mechanism participates in the regulation of the Ehrlich pathway. As shown by Boer et al. (2), growth on phenylalanine, leucine, and methionine leads to the relief of nitrogen catabolite repression-mediated regulation. We therefore cannot exclude the involvement of GATA factor-mediated transcriptional regulation of the Ehrlich pathway.

Transcriptional control may not be the only level for the regulation of the Ehrlich pathway. The overexpression of the *ARO10* decarboxylase gene from a strong constitutive promoter does not result in increased 3-phenylpyruvate decarboxylase activity during growth on a medium with glucose and ammonium sulfate (63). However, the replacement of either the ammonium sulfate by phenylalanine or of glucose by ethanol leads to a clear increase of 3-phenylpyruvate decarboxylase activity (63). These observations suggest that the functional expression of the *ARO10* gene is regulated at a posttranscriptional level in a carbon and nitrogen source-dependent manner. Further research should address the question of whether this regulation involves posttranslational modification of the Aro10p protein and/or other factors.

FUSEL ALCOHOLS AND ACIDS AS SIGNAL MOLECULES: ROLE OF THE EHRLICH PATHWAY IN THE CONTROL OF CELLULAR MORPHOLOGY

Growth in the presence of fusel alcohols such as isoamyl alcohol (derived from leucine) induces pseudohyphal growth (9, 33). These pronounced morphological modifications are accompanied by an increase in the specific activity of succinate dehydrogenase and an increase of the chitin content (33). These effects are not restricted to isoamyl alcohol, as other reports have shown that for *S. cerevisiae*, the addition of exogenous 2-phenylethanol (4) or 2-indole acetate (also known as the plant hormone auxin) (51) affects morphogenesis and induces invasive growth. These effects indicate a quorum-signaling pathway, which links the environmental sensing of autosignaling molecules and morphogenesis. No common regulatory circuit has been identified so far. While 2-phenylethanol stimulates morphological modifications by inducing the expression of *FLO11* by a Tpk2p-dependent mechanism (4), transcriptional regulation after exposure to 2-indole acetate involves Yap1p as a key mediator of the response (51). Similarly, in the human fungal pathogens *Candida albicans* and *Candida dubliniensis*, phenylethanol and isoamyl alcohol produced during growth stimulate biofilm formation (40).

Based on these reports, it thus seems that one of the functions of the Ehrlich pathway is to form quorum-sensing compounds that induce differentiation and participate in the adaptation of yeast cells to environmental changes. However, knowledge of the thermodynamics of the Ehrlich pathway and, more precisely, of the transaminase reactions may provide more insight and propose a new biological role for the Ehrlich pathway.

THERMODYNAMICS OF THE EHRLICH PATHWAY: A KEY ROLE DURING NITROGEN-LIMITED GROWTH?

The equilibrium constants of the transamination reactions are often close to unity, like, for instance, that of the phenylalanine–3-phenylpyruvate α-ketoglutarate–glutamate aminotransferase reaction (estimated from data from reference 22). Under physiological conditions, such as those studied by Wu et al. (66), the concentrations of the substrates of this reaction, in this example, α-ketoglutarate (36 μmol liter^{-1}) and phenylalanine (52 μmol liter^{-1}), are often much lower than that of the desired product, glutamate (5,850 μmol liter^{-1}). Since this transamination reaction has an essential role under conditions in which nitrogen concentrations are low, simply increasing the substrate concentration is often not feasible. To solve this thermodynamic problem, cells may use active transport to accumulate the amino acids intracellularly, but this method also has its limitation given the often low solubility of these compounds in water. The only remaining solution is then to maintain very low concentrations of the fourth compound involved in this reaction: 3-phenylpyruvate. The transaminating reaction is followed by decarboxylation, which is generally characterized by a strongly negative Gibbs free-energy change (22). Consequently, when branched-chain, aromatic, or sulfur-containing amino acids are the nitrogen source, the decarboxylation reaction contributes to low intracellular α-keto acid concentrations, thereby pulling the transaminating reactions toward complete utilization of the nitrogen-donating amino acids.

OUTLOOK

Branched-chain, aromatic, and sulfur-containing amino acids that are available in malt wort and grape must are important precursors for (off-)flavor formation. In the past decade, many efforts have contributed to a better understanding of the genes involved in this pathway and how their expression is regulated. A desirable trait in beer and wine fermentation is to achieve high levels of branched-chain and aromatic fusel alcohol production in combination with low levels of off-flavors (e.g., methionol). Our growing understanding of the key components of the Ehrlich pathway and their regulation will aid in the design of strains exhibiting specific flavor profiles in foodstuffs as well as in the metabolic engineering of yeast strains for the production of individual Ehrlich pathway products.

REFERENCES

1. **Barrett, T., D. B. Troup, S. E. Wilhite, P. Ledoux, D. Rudnev, C. Evangelista, I. F. Kim, A. Soboleva, M. Tomashevsky, and R. Edgar.** 2007. NCBI GEO: mining tens of millions of expression profiles—database and tools update. Nucleic Acids Res. **35:**D760–D765.
2. **Boer, V. M., S. L. Tai, Z. Vuralhan, Y. Arifin, M. C. Walsh, M. D. Piper, J. H. de Winde, J. T. Pronk, and J. M. Daran.** 2007. Transcriptional responses of *Saccharomyces cerevisiae* to preferred and nonpreferred nitrogen sources in glucose-limited chemostat cultures. FEMS Yeast Res. **7:**604–620.
3. **Büchner, E.** 1897. Alkoholische Gahrung ohne Hefezellen. Ber. Dtsch. Chem. Ges. **30:**117–124.
4. **Chen, H., and G. R. Fink.** 2006. Feedback control of morphogenesis in fungi by aromatic alcohols. Genes Dev. **20:**1150–1161.
5. **Derrick, S., and P. J. Large.** 1993. Activities of the enzymes of the Ehrlich

pathway and formation of branched-chain alcohols in *Saccharomyces cerevisiae* and *Candida utilis* grown in continuous culture on valine or ammonium as sole nitrogen source. J. Gen. Microbiol. **139:**2783–2792.

6. **Dickinson, J. R., S. J. Harrison, J. A. Dickinson, and M. J. Hewlins.** 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae.* J. Biol. Chem. **275:**10937–10942.

7. **Dickinson, J. R., S. J. Harrison, and M. J. Hewlins.** 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae.* J. Biol. Chem. **273:**25751–25756.

8. **Dickinson, J. R., M. M. Lanterman, D. J. Danner, B. M. Pearson, P. Sanz, S. J. Harrison, and M. J. Hewlins.** 1997. A ¹³C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae.* J. Biol. Chem. **272:**26871–26878.

9. **Dickinson, J. R., and V. Norte.** 1993. A study of branched-chain amino acid aminotransferase and isolation of mutations affecting the catabolism of branched-chain amino acids in *Saccharomyces cerevisiae.* FEBS Lett. **326:**29–32.

10. **Dickinson, J. R., L. E. Salgado, and M. J. Hewlins.** 2003. The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae.* J. Biol. Chem. **278:**8028–8034.

11. **Eden, A., G. Simchen, and N. Benvenisty.** 1996. Two yeast homologs of *ECA39*, a target for c-Myc regulation, code for cytosolic and mitochondrial branched-chain amino acid aminotransferases. J. Biol. Chem. **271:**20242–20245.

12. **Eden, A., L. Van Nedervelde, M. Drukker, N. Benvenisty, and A. Debourg.** 2001. Involvement of branched-chain amino acid aminotransferases in the production of fusel alcohols during fermentation in yeast. Appl. Microbiol. Biotechnol. **55:**296–300.

13. **Ehrlich, F.** 1907. Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweissaufbau der Hefe. Ber. Dtsch. Chem. Ges. **40:**1027–1047.

14. **Etschmann, M. M., W. Bluemke, D. Sell, and J. Schrader.** 2002. Biotechnological production of 2-phenylethanol. Appl. Microbiol. Biotechnol. **59:**1–8.

15. **Fischer, E.** 1894. Einfluss der Configuration auf die Wirkung der Enzym. Chem. Ber. **27:**2985–2993.

16. **Fischer, E.** 1902. Uber einige Derivate des Glykocolls, Alanins und Leucins. Ber. Dtsch. Chem. Ges. **35:**1095–1106.

17. **Ford, G., and E. M. Ellis.** 2002. Characterization of Ypr1p from *Saccharomyces cerevisiae* as a 2-methylbutyraldehyde reductase. Yeast **19:**1087–1096.

18. **Gregori, C., B. Bauer, C. Schwartz, A. Kren, C. Schuller, and K. Kuchler.** 2007. A genetic screen identifies mutations in the yeast *WAR1* gene, linking transcription factor phosphorylation to weak-acid stress adaptation. FEBS J. **274:**3094–3107.

19. **Harbison, C. T., D. B. Gordon, T. I. Lee, N. J. Rinaldi, T. W. Danford, N. M. Hannett, J. B. Tagne, D. B. Reynolds, J. Yoo, E. G. Jennings, J. Zeitlinger, D. K. Pokholok, M. Kellis, P. A. Rolfe, K. T. Takusagawa, E. S. Lander, D. K. Gifford, E. Fraenkel, and R. A. Young.** 2004. Transcriptional regulatory code of a eukaryotic genome. Nature **431:**99–104.

20. **Hauser, M., P. Horn, H. Tournu, N. C. Hauser, J. D. Hoheisel, A. J. Brown, and J. R. Dickinson.** 2007. A transcriptome analysis of isoamyl alcohol-induced filamentation in yeast reveals a novel role for Gre2p as isovaleraldehyde reductase. FEMS Yeast Res. **7:**84–92.

21. **Hazelwood, L. A., S. L. Tai, V. M. Boer, J. H. de Winde, J. T. Pronk, and J. M. Daran.** 2006. A new physiological role for Pdr12p in *Saccharomyces cerevisiae*: export of aromatic and branched-chain organic acids produced in amino acid catabolism. FEMS Yeast Res. **6:**937–945.

22. **Henry, C. S., L. J. Broadbelt, and V. Hatzimanikatis.** 2007. Thermodynamics-based metabolic flux analysis. Biophys. J. **92:**1792–1805.

23. **Henschke, P. A., and V. Jiranek.** 1993. Metabolism of nitrogen compounds, p. 77–164. *In* G. H. Fleet (ed.), Wine microbiology and biotechnology. Harwood Academic Publishers, Chur, Switzerland.

24. **Hofmeister, F.** 2007. Über Bau und Gruppierung der Eiweisskorper. Ergeb. Physiol. **1:**759–802.

25. **Hohmann, S.** 1991. Characterization of *PDC6*, a third structural gene for pyruvate decarboxylase in *Saccharomyces cerevisiae*. J. Bacteriol. **173:**7963–7969.

26. **Hohmann, S.** 1991. *PDC6*, a weakly expressed pyruvate decarboxylase gene from yeast, is activated when fused spontaneously under the control of the *PDC1* promoter. Curr. Genet. **20:**373–378.

27. **Hohmann, S.** 1993. Characterisation of *PDC2*, a gene necessary for high level expression of pyruvate decarboxylase structural genes in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **241:**657–666.

28. **Hohmann, S., and H. Cederberg.** 1990. Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. Eur. J. Biochem. **188:**615–621.

29. **Holyoak, C. D., D. Bracey, P. W. Piper, K. Kuchler, and P. J. Coote.** 1999. The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. J. Bacteriol. **181:**4644–4652.

30. **Iraqui, I., S. Vissers, B. Andre, and A. Urrestarazu.** 1999. Transcriptional induction by aromatic amino acids in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19:**3360–3371.

31. **Iraqui, I., S. Vissers, M. Cartiaux, and A. Urrestarazu.** 1998. Characterisation of *Saccharomyces cerevisiae ARO8* and *ARO9* genes encoding aromatic aminotransferases I and II reveals a new aminotransferase subfamily. Mol. Gen. Genet. **257:**238–248.

32. **Jones, M., and J. S. Pierce.** 1964. Absorption of amino acids from wort by yeasts. J. Inst. Brew. **70:**307–315.

33. **Kern, K., C. D. Nunn, A. Pichova, and J. R. Dickinson.** 2004. Isoamyl alcohol-induced morphological change in *Saccharomyces cerevisiae* involves increases in mitochondria and cell wall chitin content. FEMS Yeast Res. **5:**43–49.

34. **Kispal, G., H. Steiner, D. A. Court, B. Rolinski, and R. Lill.** 1996. Mitochondrial and cytosolic branched-chain amino acid transaminases from yeast, homologs of the *myc* oncogene-regulated Eca39 protein. J. Biol. Chem. **271:**24458–24464.

35. **Kren, A., Y. M. Mamnun, B. E. Bauer, C. Schuller, H. Wolfger, K. Hatzixanthis, M. Mollapour, C. Gregori, P. Piper, and K. Kuchler.** 2003. War1p, a novel transcription factor controlling weak acid stress response in yeast. Mol. Cell. Biol. **23:**1775–1785.

36. **Lampitt, L. H.** 1919. Nitrogen metabolism in *Saccharomyces cerevisiae*. Biochem. J. **13:**459–486.

37. **Lilly, M., F. F. Bauer, G. Styger, M. G. Lambrechts, and I. S. Pretorius.** 2006. The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. FEMS Yeast Res. **6:**726–743.

38. **Lipinski, C. A., F. Lombardo, B. W. Dominy, and P. J. Feeney.** 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. **46:**3–26.

39. **MacIsaac, K. D., and E. Fraenkel.** 2006. Practical strategies for discovering regulatory DNA sequence motifs. PLoS Comput. Biol. **2:**e36.

40. **Martins, M., M. Henriques, J. Azeredo, S. M. Rocha, M. A. Coimbra, and R. Oliveira.** 2007. Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signaling molecules produced by planktonic and biofilm cells. Eukaryot. Cell **6:**2429–2436.

41. **McGovern, P. E., J. Zhang, J. Tang, Z. Zhang, G. R. Hall, R. A. Moreau, A. Nunez, E. D. Butrym, M. P. Richards, C. S. Wang, G. Cheng, Z. Zhao, and C. Wang.** 2004. Fermented beverages of pre- and proto-historic China. Proc. Natl. Acad. Sci. USA **101:**17593–17598.

42. **Meilgaard, M. C.** 1975. Flavor chemistry of beer. MBAA Tech. Q. **12:**107–117.

43. **Mojzita, D., and S. Hohmann.** 2006. Pdc2 coordinates expression of the *THI* regulon in the yeast *Saccharomyces cerevisiae*. Mol. Genet. Genomics **276:**147–161.

44. **Neubauer, O., and K. Fromherz.** 1911. Über den Abbau der Aminosäuren bei der Hefegärung. Hoppe-Seyler's Z. Physiol. Chem. **70:**326–350.

45. **Nosaka, K., M. Onozuka, H. Konno, Y. Kawasaki, H. Nishimura, M. Sano, and K. Akaji.** 2005. Genetic regulation mediated by thiamin pyrophosphate-binding motif in *Saccharomyces cerevisiae*. Mol. Microbiol. **58:**467–479.

46. **Ostwald, W.** 1892. Studien zur Energetik I. Z. Phys. Chem. **9:**563–578.

47. **Ostwald, W.** 1892. Studien zur Energetik II. Z. Phys. Chem. **10:**42–63.

48. **Overkamp, K. M., B. M. Bakker, P. Kotter, A. van Tuijl, S. de Vries, J. P. van Dijken, and J. T. Pronk.** 2000. In vivo analysis of the mechanisms for oxidation of cytosolic NADH by *Saccharomyces cerevisiae* mitochondria. J. Bacteriol. **182:**2823–2830.

49. **Pampulha, M. E., and M. C. Loureiro-Dias.** 2000. Energetics of the effect of acetic acid on growth of *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. **184:**69–72.

50. **Perpete, P., O. Duthoit, S. De Maeyer, L. Imray, A. I. Lawton, K. E. Stavropoulos, V. W. Gitonga, M. J. Hewlins, and J. R. Dickinson.** 2006. Methionine catabolism in *Saccharomyces cerevisiae*. FEMS Yeast Res. **6:**48–56.

51. **Prusty, R., P. Grisafi, and G. R. Fink.** 2004. The plant hormone indoleacetic acid induces invasive growth in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **101:**4153–4157.

52. **Schaaff, I., J. B. Green, D. Gozalbo, and S. Hohmann.** 1989. A deletion of the *PDC1* gene for pyruvate decarboxylase of yeast causes a different phenotype than previously isolated point mutations. Curr. Genet. **15:**75–81.

53. **Schoondermark-Stolk, S. A., M. Tabernero, J. Chapman, E. G. Ter Schure, C. T. Verrips, A. J. Verkleij, and J. Boonstra.** 2005. Bat2p is essential in *Saccharomyces cerevisiae* for fusel alcohol production on the non-fermentable carbon source ethanol. FEMS Yeast Res. **5:**757–766.

54. **Sentheshanmuganathan, S.** 1956. The formation of tyrosol (2-*p*-hydroxyethanol) from tyrosine by *Saccharomyces cerevisiae*. Biochem. J. **64:**37P–38P.

55. **Sentheshanmuganathan, S.** 1960. The mechanism of formation of higher alcohols from amino acids by *Saccharomyces cerevisiae*. Biochem. J. **74:**568–576.

56. **Smit, G., B. A. Smit, and W. J. Engels.** 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol. Rev. **29:**591–610.

57. **Ter Schure, E. G., M. T. Flikweert, J. P. van Dijken, J. T. Pronk, and C. T. Verrips.** 1998. Pyruvate decarboxylase catalyzes decarboxylation of

branched-chain 2-oxo acids but is not essential for fusel alcohol production by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **64:**1303–1307.

58. **Thorne, R. S. W.** 1937. The assimilation of nitrogen from amino acids by yeast. J. Inst. Brew. **43:**288–293.

59. **Thorne, R. S. W.** 1941. The growth and fermentation of a strain of *S. cerevisiae* with amino acids as nutrients. J. Inst. Brew. **47:**255–272.

60. **Urrestarazu, A., S. Vissers, I. Iraqui, and M. Grenson.** 1998. Phenylalanine- and tyrosine-auxotrophic mutants of *Saccharomyces cerevisiae* impaired in transamination. Mol. Gen. Genet. **257:**230–237.

61. **van Dijken, J. P., E. van den Bosch, J. J. Hermans, L. R. de Miranda, and W. A. Scheffers.** 1986. Alcoholic fermentation by 'non-fermentative' yeasts. Yeast **2:**123–127.

62. **von Ewald, A., and W. Kühne.** 1877. Über einen neuen Bestandtheil des Nervensystems. Verh. Naturhist.-Med. Ver. Heidelberg **1:**194–198.

63. **Vuralhan, Z., M. A. Luttik, S. L. Tai, V. M. Boer, M. A. Morais, D. Schipper, M. J. Almering, P. Kotter, J. R. Dickinson, J. M. Daran, and J. T. Pronk.** 2005. Physiological characterization of the *ARO10*-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **71:**3276–3284.

64. **Vuralhan, Z., M. A. Morais, S. L. Tai, M. D. Piper, and J. T. Pronk.** 2003.

65. **Watson, T. G., and J. S. Hough.** 1969. Conversion of a-keto-isocaproic acid to iso-amyl alcohol by yeast pyruvate decarboxylase and alcohol dehydrogenase. J. Inst. Brew. **75:**359–363.

66. **Wu, L., M. R. Mashego, A. M. Proell, J. L. Vinke, C. Ras, J. van Dam, W. A. van Winden, W. M. van Gulik, and J. J. Heijnen.** 2006. In vivo kinetics of primary metabolism in *Saccharomyces cerevisiae* studied through prolonged chemostat cultivation. Metab. Eng. **8:**160–171.

67. **Yamada, M.** 1932. Decomposition of amino acids by yeast I. Nippon Nogeikagaku Kaishi **8:**428–432.

68. **Yamada, M.** 1932. Decomposition of amino acids by yeast II. Nippon Nogeikagaku Kaishi **8:**498–505.

69. **Yamada, M.** 1932. Decomposition of amino acids by yeast III. Nippon Nogeikagaku Kaishi **8:**506–508.

70. **Yoshimoto, H., T. Fukushige, T. Yonezawa, and H. Sone.** 2002. Genetic and physiological analysis of branched-chain alcohols and isoamyl acetate production in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **59:**501–508.

Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **69:**4534–4541.