Improvement of Nitrogen Assimilation and Fermentation Kinetics under Enological Conditions by Derepression of Alternative Nitrogen-Assimilatory Pathways in an Industrial Saccharomyces cerevisiae Strain

JEAN-MICHEL SALMON* AND PIERRE BARRE

Laboratoire de Microbiologie et de Technologie des Fermentations, Institut des Produits de la Vigne, Institut National de la Recherche Agronomique, 34060 Montpellier Cedex 1, France

Received 1 December 1997/Accepted 17 July 1998

Metabolism of nitrogen compounds by yeasts affects the efficiency of wine fermentation. Ammonium ions, normally present in grape musts, reduce catabolic enzyme levels and transport activities for nonpreferred nitrogen sources. This nitrogen catabolite repression severely impairs the utilization of proline and arginine, both common nitrogen sources in grape juice that require the proline utilization pathway for their assimilation. We attempted to improve fermentation performance by genetic alteration of the regulation of nitrogen-assimilatory pathways in Saccharomyces cerevisiae. One mutant carrying a recessive allele of ure2 was isolated from an industrial S. cerevisiae strain. This mutation strongly deregulated the proline utilization pathway. Fermentation kinetics of this mutant were studied under enological conditions on simulated standard grape juices with various nitrogen levels. Mutant strains produced more biomass and exhibited a higher maximum CO2 production rate than the wild type. These differences were primarily due to the derepression of amino acid utilization pathways. When low amounts of dissolved oxygen were added, the mutants could assimilate proline. Biomass yield and fermentation rate were consequently increased, and the duration of the fermentation was substantially shortened. S. cerevisiae strains lacking URE2 function could improve alcoholic fermentation of natural media where proline and other poorly assimilated amino acids are the major potential nitrogen source, as is the case for most fruit juices and grape musts.

A wide variety of nitrogen-containing compounds are present in grape juice, depending upon the grape variety and time of harvest. During fermentation, these compounds are taken up during the first part of the Saccharomyces cerevisiae growth phase. Biosynthetic pools of amino acids are filled and the remaining nitrogenous compounds are utilized as nitrogen sources (17). Once pools are filled and growth begins, nitrogenous compounds are taken up and degraded in a specific order depending on environmental, physiological, and strain-specific factors (30, 32). Ammonium ions, which may constitute up to 10% of the total assimilable nitrogen in the must (26), reduce catabolic enzyme levels and transport activity for nonpreferred nitrogen sources through a phenomenon known as nitrogen catabolite repression (18). Nitrogen catabolite repression is attributed to the action of three proteins, GLN3, URE2, and GAP1 (36). The GLN3 and URE2 gene products are required for the transcription of many genes involved in alternative nitrogen-assimilatory pathways (22). GLN3 activates their transcription when preferred nitrogen sources are not available (38, 39), and URE2 represses their transcription when alternative nitrogen sources are not needed (20). GAP1, the general amino acid permease that transports all biological amino acids across the plasma membrane (28), is regulated at the transcriptional level by GLN3 and URE2 and is inactivated by dephosphorylation in the presence of glutamate and glutamine (48).

Alternative nitrogen-assimilatory pathways are not expressed when ammonium is present. In grape juice, ammonium is the preferred nitrogen source. As ammonium is consumed, amino acids are taken up in a pattern determined by their concentration relative to cell needs for biosynthesis and to total nitrogen availability (40–42). Two exceptions are known: (i) proline is not taken up from grape juice under anaerobic fermentative conditions (27) and proline metabolism requires oxygen and a functioning electron transport chain to cleave the proline ring (51) and (ii) arginine and γ-aminobutyrate are usually taken up during the latter stages of fermentation under enological conditions and are always detectable in the final wine (9). Proline and arginine are the most common nitrogenous compounds in grape juice and represent 30 to 65% of the total amino acid content of grape juices (26). Both amino acids require the proline utilization pathway for conversion to glutamate and ammonia (12). Proline is transported into S. cerevisiae by the general amino acid permease and a proline-specific permease (product of \textit{PUT4} [36]). Proline is converted to glutamate in the mitochondria by proline oxidase (product of the \textit{PUT1} gene [51]) and \textit{Δ}-pyrroline-5-carboxylate dehydrogenase (product of \textit{PUT2} [33]). The expression of the \textit{PUT} genes is regulated by the PUT3 activator protein. This protein responds to the presence of proline in the medium and increases transcription of \textit{PUT1} and \textit{PUT2} genes (10, 13). URE2 represses transcription of the \textit{PUT} genes and proline transporters under nitrogen-repressing conditions; the GLN3 protein has no effect on these genes (13, 53).

The objective of our work was to isolate mutants of an industrial strain of \textit{S. cerevisiae} that were no longer subject to nitrogen catabolite repression, while studying the fermentation kinetics of these mutants on simulated standard grape juice under enological conditions. The ultimate goal of this research...
is to enhance the degradation of proline and other poorly assimilated amino acids during the growth phase and evaluate the potential impact of these physiological changes on yeast metabolism and fermentation kinetics.

MATERIALS AND METHODS

Strains, vectors, and culture conditions. Yeast strains. S. cerevisiae strains used in this study were V5 (MATa ura3) and A5 (MATα). These two strains were derived from the same diploid industrial wine strain. Both strains exhibited identical fermentation kinetics under enological conditions. The V5 strain was preserved in our laboratory collection (Institut National de la Recherche Agronomique, Montpellier, France). Isogenic laboratory S. cerevisiae strains MYC1 (MATα ade2Δ1) and MYC2 (MATα ade2Δ2) were used as mating-type tester strains (J. Conde, Seville, Spain).

Culture media. All media were heat sterilized (110°C, 30 min). The standard nutrient medium used for the general cultivation of yeast strains contained 1% yeast extract (Difco), 2% Bacto Peptone (Difco), and 2% glucose (YPD). Glucose-glutamate, glucose-proline, and glucose-ammonia liquid media contained 0.17% yeast nitrogen base (YNB) without amino acids and ammonium sulfate (Difco), 2% glucose, 0.002% uracil, and 0.1% glutamine, 0.1% proline, or 0.2% NH4Cl, respectively. The synthetic fermentation media used in this study were prepared by mixing 20% glucose, 0.17% yeast nitrogen base (YNB) without amino acids and ammonium sulfate (J. Conde, Sevilla, Spain).

MAT and MYC2) and MYC2 (Montpellier, France). Isogenic laboratory S. cerevisiae strains MYC1 (MATα ade2Δ1) and MYC2 (MATα ade2Δ2) were used as mating-type tester strains (J. Conde, Seville, Spain).

Oxygen diffusion in the medium was prevented by using bubbling CO2 outlets. Ammonium was provided at a low initial level to initiate cell growth. Conditions for YPD medium and glucose-ammonia and glucose-proline liquid media, yeasts were inoculated at 10^6 cells ml\(^{-1}\) in 25-mL Erlenmeyer flasks containing 5 ml of liquid medium and incubated at 28°C on a rotary shaker. For MSr fermentation media, yeasts were precultured at 28°C in small fermentors (250 ml) with fermentation locks under discontinuous magnetic stirring (30 s, 10 W) with an electronic Coulter Counter (model ZBI; Coulter Coultronics, Margency, France) fitted with a 100-μm probe.

(ii) Cellular dry weight. Cellular dry weight was obtained by filtering 10 ml of culture medium through membrane filters (pore size, 1.2 μm). Filters were rinsed with the same amount of distilled water, and cells were dessicated at 108°C until a constant weight was obtained (24 h).

(iii) Total cell protein. Total cell proteins were extracted as described by Jaworski et al. (29).

(iv) Protein determination. The protein concentration was determined with the bicinchoninic acid protein assay reagent (Pierce Chemicals, Rockford, Ill.), with crystalline bovine serum albumin as standard.

(v) Determination of assimilable nitrogen in fermentation media. Ammonium and α-amino acids concentrations were measured by enzymatic assay (8) and the TNBS (2,4,6-trinitrobenzenesulfonilic acid) method (23), respectively. Proline concentrations in fermentation media were determined by the method of Yemm and Cocking (54).

(vi) Determination of amino acid profiles in fermentation media. An aliquot of each fermentation medium (10 ml) was mixed with 50 ml of 96% (vol/vol) ethanol and allowed to stand for 48 h at 20°C to precipitate proteins and polysaccharides. After centrifugation (20,000 × g, 20 min), the supernatant was dried under vacuum and resuspended in 0.2 N lithium citrate buffer (pH 2.2).

Amino acids were separated by ion-exchange chromatography on an anionic Ultrasphere-5 lithium form resin (Pharmacia) with a Chromatop 400 analyzer (Kontron) and detected after reaction with ninhydrin (6, 7).

(vii) Proline uptake experiments. We estimated high-affinity proline uptake by using the proline-specific permease (PUT4 gene product) and low-affinity proline uptake by using both the general amino acid and the proline-specific permeases (GAP1 and PUT4 gene products, respectively). The methodology described by Brandriss and Magasanik (11) was used. Since the affinities of these permeases for proline differ, the uptake of proline was estimated using radioactive proline (1 Ci mmol\(^{-1}\)).

(iv) Tetrad dissection. The asc sac was digested with Helix pomatia gut juice (SHP; IBF-Sepracor) at 28°C for 20 min according to the method described by Johnston and Mortimer (31), and spores were separated with a micromanipulator.

(v) Plasmid. Centromeric plasmid p13-CS contained the URE2 gene inserted into the ClaI/Usal site of the Ycp50 plasmid (20). For this plasmid, V5 strain transformation was carried out on yeast spheroplasts (14).

(iii) Sporulation. Approximately 10^6 cells were grown for 24 h on a plate of presporulation medium (1% yeast extract, 0.5% Bacto Peptone, 2% agar, and 10% glucose) and then spread on a plate of sporulation medium (1% yeast extract, 2% Bacto Peptone, 2% agar, and 1% potassium acetate) and incubated at 28°C. Sporulation efficiency was expressed as the ratio of ase to vegetative cells in a total population of at least 10^6 cells.

(iv) Mutation. Mating type was determined by observing zygote formation after mixed inoculation of cells of both tester strains MYC1 (MATα) and MYC2 (MATa) on 2% agar-YPD plates.

(vi) Postgrowth. V5 cells were spread on a plate of sporulation medium containing 0.17% YNB without amino acids and ammonium sulfate, 20% glucose, 0.002% uracil, 0.25% proline (0.3 g of N liter\(^{-1}\)), and 0.009% (NH4)2SO4 (20 mg of N liter\(^{-1}\)). Ammonium was provided at a low initial level to initiate cell growth.

(iv) Determination of ammonium uptake by using both the general amino acid and the proline-specific permeases (GAP1 and PUT4 gene products, respectively). The methodology described by Brandriss and Magasanik (11) was used. Since the affinities of these permeases for proline differ, the uptake of proline was estimated using radioactive proline (1 Ci mmol\(^{-1}\)).

(v) Plasmid. Centromeric plasmid p13-CS contained the URE2 gene inserted into the ClaI/Usal site of the Ycp50 plasmid (20). For this plasmid, V5 strain transformation was carried out on yeast spheroplasts (14).

(iii) Sporulation. Approximately 10^6 cells were grown for 24 h on a plate of presporulation medium (1% yeast extract, 0.5% Bacto Peptone, 2% agar, and 10% glucose) and then spread on a plate of sporulation medium (1% yeast extract, 2% Bacto Peptone, 2% agar, and 1% potassium acetate) and incubated at 28°C. Sporulation efficiency was expressed as the ratio of ase to vegetative cells in a total population of at least 10^6 cells.

(ii) Mating type. Mating type was determined by observing zygote formation after mixed inoculation of cells of both tester strains MYC1 (MATα) and MYC2 (MATa) on 2% agar-YPD plates.

(iii) Tetrad dissection. The asc sac was digested with Helix pomatia gut juice (SHP; IBF-Sepracor) at 28°C for 20 min according to the method described by Johnston and Mortimer (31), and spores were separated with a micromanipulator.

(v) Plasmid. Centromeric plasmid p13-CS contained the URE2 gene inserted into the ClaI/Usal site of the Ycp50 plasmid (20). For this plasmid, V5 strain transformation was carried out on yeast spheroplasts (14).

(iii) Sporulation. Approximately 10^6 cells were grown for 24 h on a plate of presporulation medium (1% yeast extract, 0.5% Bacto Peptone, 2% agar, and 10% glucose) and then spread on a plate of sporulation medium (1% yeast extract, 2% Bacto Peptone, 2% agar, and 1% potassium acetate) and incubated at 28°C. Sporulation efficiency was expressed as the ratio of ase to vegetative cells in a total population of at least 10^6 cells.

(iv) Mutation. Mating type was determined by observing zygote formation after mixed inoculation of cells of both tester strains MYC1 (MATα) and MYC2 (MATa) on 2% agar-YPD plates.
TABLE 1. Proline assimilation capacities of mutant strains under oxygen-limiting conditions

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>Final cell number* (10^7 ml^-1)</th>
<th>Residual proline concentration* (g liter^-1)</th>
<th>Nitrogen assimilation efficiency* (mg of N10^-12 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V5</td>
<td>3.0</td>
<td>2.5</td>
<td>7.0</td>
</tr>
<tr>
<td>UV9</td>
<td>5.5</td>
<td>2.3</td>
<td>8.0</td>
</tr>
<tr>
<td>Other mutant strains</td>
<td>3.0-4.1</td>
<td>2.4</td>
<td>7.1-7.3</td>
</tr>
</tbody>
</table>

* V5, wild type; UV9, mutant strain. A total of five mutant strains were tested for data presented for other mutant strains.

RESULTS

Isolation and characterization of an S. cerevisiae mutant strain V5 derepressed for proline utilization. Derepressed mutants were obtained after UV mutagenesis by selecting for resistance to the ammonium analog methylamine. Mutant strains were screened for their ability to grow on plates containing proline as the sole nitrogen source and methylamine at a repressive concentration. Except for one, all 40 isolated mutants exhibited clear derepression of amino acid utilization under nitrogen-repressing conditions (data not shown). Most of these strains could not grow on plates with proline as the sole nitrogen source under oxygen-limiting conditions (Table 1). Only one mutant (UV9) could degrade proline under such conditions and increase biomass. UV9 cells had better nitrogen assimilation efficiency and therefore higher nitrogen contents than the other tested strains. This result suggests that UV9 was altered in its ability to regulate proline utilization. Proline uptake requires either the general amino acid permease or a specific proline permease. Since both systems are subject to nitrogen repression, we estimated the capacities of wild-type and UV9 mutant strains for proline uptake by both permeases under different culture conditions (Table 2). The UV9 strain had a higher capacity for high-affinity proline uptake (PUT4 function) than the wild type under nitrogen-repressing conditions (MS300 medium). Control experiments performed under derepressing conditions (glucose-proline medium) revealed a significant increase in proline-specific permease activity in the UV9 mutant.

We also studied amino acid utilization by UV9 cells on simulated standard grape juice. Mutant and wild-type strains were inoculated at the same cell density on MS80 and MS300 synthetic media and harvested at the end of the growth phase. The amino acid composition was determined in both fermentation media (Fig. 1 and 2). In both cases, UV9 utilized more amino acids than the V5 strain, except for histidine, leucine, lysine, methionine, and threonine. Glutamate, which is one of the two end products of nitrogen catabolic pathways of yeasts (34), is excreted at low concentration into the fermentation medium by wild-type cells during the growth phase (52) but is not excreted by UV9. This result may indicate the presence of a strongly derepressed catabolic NAD-linked glutamate dehydrogenase (NAD-GDH) favoring the interconversion of glutamate into ammonia within UV9 cells (21, 37). We tested this hypothesis by measuring the level of NAD-GDH in both strains under strongly repressing conditions (glucose-glutamine medium). We observed that the mutant strain exhibited higher NAD-GDH specific activity than the wild type under such conditions (490 ± 8 nmol min^-1 mg of protein^-1 versus 59 ± 6 nmol min^-1 mg of protein^-1, three determinations). In the absence of oxygen on these two fermentation media, the UV9 mutant always reached a higher final biomass than V5 cells (2.5 versus 2.3 g [dry weight] liter^-1 and 5.2 versus 3.4 g [dry weight] liter^-1 on MS80 and MS300 media, respectively).

Characterization of the mutated URE2 allele. We crossed UV9 with the wild-type A45 strain. The diploids could not
grow on plates containing proline as the sole nitrogen source and methylamine at a repressive concentration. We characterized these cells for their ability to use proline as the nitrogen source for growth under oxygen-limiting conditions (Table 3). Heterozygous diploids (initial cross A45 × UV9; Z6 and Z14) reached the same biomass as the wild-type strain, indicating that the mutation is recessive. All 25 tetrads from this cross segregated 2+2− for this character, indicating that a single mutation is responsible for this phenotype. Feedback crosses with the parental UV9 strain or direct crossing of haploids allowed us to construct homozygous diploids for the corresponding mutation (Table 3).

Since ure2 mutant alleles (also known as usu and gdhCR) have been isolated in a number of screens designed to isolate mutants with increased amino acid permease activity (22) or genetic derepression of NAD-linked glutamate dehydrogenase (24), we also checked the identity of the isolated mutation as a recessive mutation in the URE2 gene. Strains with recessive URE2 gene mutations were previously characterized as possessing nitrogen catabolic enzymes insensitive to nitrogen catabolite repression (16), mainly in the pathways involved in glutamate, glutamine, arginine, allantoin, urea, γ-aminobutyrate, and proline assimilation (13). As previously described for ure2 mutants (53), the UV9 strain grew aerobically more slowly than the wild-type strain V5 on a glucose-ammonia medium (doubling time of 3.5 versus 2.6 h) or on a glucose-proline medium (doubling time of 8.9 versus 5.5 h). Similarly, exposure of the mutant strain to heat shock (45°C, 3 h) resulted in reduced recovery at 30°C compared with the V5 wild-type strain on YPD plates: survival rates were 13 and 49%, respectively.

All diploids homozygous for the mutation failed to sporulate (Table 3). Very low sporulation efficiency is a characteristic of homozygous ure2/ure2 diploids (53). To confirm that the isolated mutation had occurred in ure2, the UV9 mutant was transformed with the centromeric plasmid p1C-CS containing URE2 (20). The resulting strain UV9/p1C-CS exhibited a phenotype analogous to that of the wild-type strain V5. Finally, in the wild-type strain V5, we disrupted URE2, leading to a URE2 null allele (mutant strain V5/ure2::kan). This mutant behaved like UV9 for most of its growth phenotypes (Table 3).

### TABLE 3. Genetic analysis of the mutation carried by the UV9 strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mating type</th>
<th>Phenotype</th>
<th>Sporulation</th>
<th>Final cell population on medium containing proline* (10⁶ cells ml⁻¹)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>V5</td>
<td>a</td>
<td>Ura⁻</td>
<td>–</td>
<td>2.5 ± 0.5 (5)</td>
<td>Wild type</td>
</tr>
<tr>
<td>A45</td>
<td>α</td>
<td>Ura⁺</td>
<td>–</td>
<td>1.0 ± 0.1 (3)</td>
<td>Wild type</td>
</tr>
<tr>
<td>UV9</td>
<td>a</td>
<td>Ura⁻</td>
<td>+</td>
<td>4.9 ± 0.1 (5)</td>
<td>Mutant of V5</td>
</tr>
<tr>
<td>Z6</td>
<td>a/α</td>
<td>Ura⁺</td>
<td>+</td>
<td>2.6 (1)</td>
<td>Initial cross of A45 and UV0</td>
</tr>
<tr>
<td>Z14</td>
<td>a/α</td>
<td>Ura⁺</td>
<td>+</td>
<td>3.0 ± 0.2 (2)</td>
<td>Initial cross of A45 and UV0</td>
</tr>
<tr>
<td>Z6-5A</td>
<td>α</td>
<td>Ura⁺</td>
<td>–</td>
<td>1.8 (1)</td>
<td>Segregant of Z6</td>
</tr>
<tr>
<td>Z6-5B</td>
<td>α</td>
<td>Ura⁺</td>
<td>–</td>
<td>3.6 (1)</td>
<td>Segregant of Z6</td>
</tr>
<tr>
<td>Z6-5C</td>
<td>a</td>
<td>Ura⁺</td>
<td>–</td>
<td>4.1 (1)</td>
<td>Segregant of Z6</td>
</tr>
<tr>
<td>Z6-6D</td>
<td>a</td>
<td>Ura⁻</td>
<td>–</td>
<td>1.1 (1)</td>
<td>Segregant of Z6</td>
</tr>
<tr>
<td>Z6-11A</td>
<td>a</td>
<td>Ura⁺</td>
<td>–</td>
<td>4.4 (1)</td>
<td>Segregant of Z6</td>
</tr>
<tr>
<td>Z6-11B</td>
<td>a</td>
<td>Ura⁺</td>
<td>–</td>
<td>1.0 (1)</td>
<td>Segregant of Z6</td>
</tr>
<tr>
<td>Z6-11C</td>
<td>a</td>
<td>Ura⁻</td>
<td>–</td>
<td>1.3 (1)</td>
<td>Segregant of Z6</td>
</tr>
<tr>
<td>Z6-11D</td>
<td>α</td>
<td>Ura⁺</td>
<td>–</td>
<td>4.1 (1)</td>
<td>Segregant of Z6</td>
</tr>
<tr>
<td>Z14-9A</td>
<td>α</td>
<td>Ura⁻</td>
<td>–</td>
<td>1.2 (1)</td>
<td>Segregant of Z14</td>
</tr>
<tr>
<td>Z14-9B</td>
<td>a</td>
<td>Ura⁺</td>
<td>–</td>
<td>1.9 (1)</td>
<td>Segregant of Z14</td>
</tr>
<tr>
<td>Z14-9C</td>
<td>a</td>
<td>Ura⁻</td>
<td>–</td>
<td>4.1 (1)</td>
<td>Segregant of Z14</td>
</tr>
<tr>
<td>Z14-9D</td>
<td>α</td>
<td>Ura⁻</td>
<td>–</td>
<td>4.4 (1)</td>
<td>Segregant of Z14</td>
</tr>
<tr>
<td>UV9/Z14-9D</td>
<td>a/α</td>
<td>Ura⁻</td>
<td>–</td>
<td>3.8 (1)</td>
<td>Cross of UV9 and Z14-9D</td>
</tr>
<tr>
<td>Z6-5D/Z14-9A</td>
<td>a/α</td>
<td>Ura⁻</td>
<td>+</td>
<td>2.4 (1)</td>
<td>Cross of Z6-5D and Z14-9A</td>
</tr>
<tr>
<td>Z14-9C/Z6-11A</td>
<td>a/α</td>
<td>Ura⁻</td>
<td>–</td>
<td>3.3 (1)</td>
<td>Cross of Z14-9C and Z6-11A</td>
</tr>
<tr>
<td>Z14-9D/Z6-11B</td>
<td>a/α</td>
<td>Ura⁻</td>
<td>+</td>
<td>2.4 (1)</td>
<td>Cross of Z14-9D and Z6-11B</td>
</tr>
<tr>
<td>UV9/p1C-CS</td>
<td>a</td>
<td>Ura⁻</td>
<td>–</td>
<td>2.4 ± 0.2 (5)</td>
<td>UV9 strain carrying plasmid p1C-CS</td>
</tr>
<tr>
<td>V5/ure2::kan</td>
<td>a</td>
<td>Ura⁻</td>
<td>–</td>
<td>4.2 ± 0.2 (2)</td>
<td>V5 strain carrying disruption of URE2</td>
</tr>
</tbody>
</table>

* Cells were counted after a 48-h incubation at 28°C without agitation on argon-deaerated liquid medium containing 0.17% YNB without amino acids and ammonium sulfate, 20% glucose, 0.002% uracil, 0.25% proline (0.3 g of N liter⁻¹), and 0.009% (NH₄)₂SO₄ (20 mg of N liter⁻¹). Numbers in parentheses indicate the number of determinations for each strain.
Absence (A and B) or in the presence (C and D) of 6 mg of dissolved oxygen liter\(^{-1}\). The CO\(_2\) production rate patterns are represented as a function of fermentation progress (panels A and C) or of fermentation time (panels B and D). Final cell populations were 236 \(\times 10^6\), 286 \(\times 10^6\), and 293 \(\times 10^6\) cells ml\(^{-1}\) in the absence of oxygen and 235 \(\times 10^6\), 340 \(\times 10^6\), and 367 \(\times 10^6\) cells ml\(^{-1}\) in the presence of oxygen for V5, UV9, and V5/ure2::kan strains, respectively.

Potential technological application of ure2 mutant strains in wine fermentations. Typical concentrations of total available nitrogen in real grape juices ranged from 50 to 800 mg of N liter\(^{-1}\), although assimilable nitrogen represents only 30 to 500 mg of N liter\(^{-1}\) (4, 5). An assimilable nitrogen concentration of 80 mg of N liter\(^{-1}\) is considered limiting for both growth and fermentation of industrial \(S\). \(cerevisiae\) strains under enological conditions (5). In the absence of oxygen, both ure2 strains had a higher maximum CO\(_2\) production rate and final biomass than V5 on two simulated standard grape juices, leading to quicker fermentation (about 100 instead of 115 h) (Fig. 3A and B). Under such conditions, regardless of initial must fermentation (about 100 instead of 115 h) (Fig. 3A and B). Under such conditions, regardless of initial must fermentation, assimilation of all nitrogen substrates was better in ure2 strains than in the wild type (Table 4). The increased level of amino acid permeases and derepression of amino acid utilization under ammoniacal nitrogen repression in ure2 strains may be solely responsible for these effects.

We also tested the ability of ure2 mutants to degrade proline in simulated standard grape juices in the presence of oxygen. Air or oxygen diffusion during wine fermentation is a legal practice (23a); some authors have shown that oxygen addition improves the synthesis of anaerobic growth factors (ergosterol and unsaturated fatty acids [2, 3]) at the end of the cell growth phase (43, 44). Nevertheless, in enological conditions, this oxygen requirement is low and is estimated at 5 to 10 mg liter\(^{-1}\) (43). We tested growth with increased initial dissolved oxygen concentrations in a nitrogen-limited simulated grape juice (MS80) containing anaerobic growth factors. Under these conditions, at oxygen concentrations equal to or above 6 mg liter\(^{-1}\), both ure2 strains utilized proline more efficiently than wild type, produced more biomass, and exhibited a higher maximum CO\(_2\) production rate (Table 4).

We also checked the effect of dissolved oxygen addition at the end of the cell growth phase on MS80 and MS300 media. On MS80 medium, oxygen addition had a slight effect on the maximum fermentation rate of UV9 and no effect on V5; this effect was not sufficient to reduce the fermentation duration (Fig. 4). This effect was amplified on MS300 medium, where there were higher levels of assimilable nitrogen compounds and proline. V5 was weakly affected by oxygen addition (Fig. 3C and D), but ure2 strains produced more biomass and maintained a higher CO\(_2\) production rate than V5 throughout the fermentation. Consequently, the fermentation duration decreased from 100 to only about 85 h. This effect of oxygen addition could be attributed primarily to deregulation of the proline utilization pathway in ure2 mutants.

### Table 4. Effect of initial oxygen addition on fermentation characteristics of wild-type V5 and UV9 and UV9/ure2::kan mutant strains in MS80 synthetic medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Initial oxygen concentration (mg liter(^{-1}))</th>
<th>Maximum CO(_2) production rate (g of CO(_2) liter(^{-1}) h(^{-1}))</th>
<th>Final cell population (10(^8) cells ml(^{-1}))</th>
<th>Assimilated nitrogen(^a) (mg of N liter(^{-1}))</th>
<th>Assimilated proline(^b) (mg of N liter(^{-1}))</th>
<th>Nitrogen assimilation efficiency(^c) (mg of N/10(^8) cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V5</td>
<td>0</td>
<td>1.1</td>
<td>1.1</td>
<td>71</td>
<td>5.6</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.1</td>
<td>1.2</td>
<td>73</td>
<td>5.5</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.1</td>
<td>1.2</td>
<td>75</td>
<td>5.4</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.1</td>
<td>1.2</td>
<td>79</td>
<td>6.6</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.1</td>
<td>1.2</td>
<td>79</td>
<td>9.0</td>
<td>7.2</td>
</tr>
<tr>
<td>UV9</td>
<td>0</td>
<td>1.1</td>
<td>1.1</td>
<td>78</td>
<td>7.3</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.1</td>
<td>1.2</td>
<td>79</td>
<td>7.6</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.2</td>
<td>1.2</td>
<td>79</td>
<td>13</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.2</td>
<td>1.2</td>
<td>80</td>
<td>20</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.2</td>
<td>1.4</td>
<td>80</td>
<td>20</td>
<td>7.1</td>
</tr>
<tr>
<td>V5/ure2::kan</td>
<td>0</td>
<td>1.1</td>
<td>1.1</td>
<td>79</td>
<td>7.0</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.1</td>
<td>1.2</td>
<td>79</td>
<td>7.8</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.1</td>
<td>1.2</td>
<td>79</td>
<td>12</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.2</td>
<td>1.2</td>
<td>80</td>
<td>20</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.2</td>
<td>1.4</td>
<td>80</td>
<td>20</td>
<td>7.0</td>
</tr>
</tbody>
</table>

\(^a\) Ammonium salts and \(\alpha\)-amino acids (all amino acids except proline) in the medium were considered residual assimilable nitrogen (initial values: ammonium salts, 32 mg of N liter\(^{-1}\); \(\alpha\)-amino acids, 48 mg of N liter\(^{-1}\)).

\(^b\) The residual proline concentration was determined by spectrophotometry at 460 nm after reaction with ninhydrin (initial value, 19.9 mg of N liter\(^{-1}\)).

\(^c\) Nitrogen assimilation efficiency was calculated by dividing the total amount of degraded nitrogen by the final cell population.
DISCUSSION

Metabolism of nitrogen compounds by *S. cerevisiae* may govern the efficiency of alcoholic fermentation and affect the final product quality. Proline and arginine are the most abundant amino acids in fruit juices, but *S. cerevisiae* is not able to completely utilize these two amino acids during alcoholic fermentation. Derepression for the assimilation of amino acids in *ure2* mutant strains of *S. cerevisiae* leads to better amino acid assimilation during alcoholic fermentation. Moreover, these strains can assimilate a significant amount of proline, especially following incorporation of small amounts of oxygen in the fermentation medium (as low as 6 mg of dissolved oxygen liter$^{-1}$) at the end of the yeast growth phase. Cleavage of the proline ring requires oxygen and a functioning electron transport chain (51). As high hexose concentrations inhibit respiration by first closing mitochondrial voltage-dependent anion-selective channels (1) and then repressing key enzymes in the respiratory chain (19), this observed proline degradation in *ure2* strains under strong glucose-repressive conditions indicates that mitochondria retain their full potential for this degradation. Further research is needed to clarify the specific role of mitochondria under such conditions.

The more efficient use of amino acids allowed *ure2* strains to reach a higher final biomass and consequently to ferment natural media faster than wild-type cells. Thus, natural and industrial yeasts might be expected to lose *URE2* repressor function during evolution. The much longer generation time of *ure2* mutants on glucose-containing media could explain why selection for a selective and electroneutral $K^+/	ext{H}^+$ exchanger is required in a defined medium. J. Cell. Comp. Physiol. 43:271–281.

ACKNOWLEDGMENTS

We thank M. J. Biron for technical assistance, especially with the genetic methods, E. Baptista for the construction of *ure2* disruptants, and M. Pradal for assistance with the amino acid analyses. The plasmid p1C-CS was kindly provided by D. Rowen (Massachusetts Institute of Technology, Cambridge, Mass.).

REFERENCES


