# Amino Acid and Ammonium Utilization by *Saccharomyces cerevisiae* Wine Yeasts From a Chemically Defined Medium

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The utilization of amino acids and ammonium by nine wine strains of *Saccharomyces cerevisiae* was systematically studied in a chemically defined medium resembling the composition of grape juice. Both quantitative and qualitative differences in the utilization of assimilable nitrogen were observed. The mean concentration of total nitrogen utilized during the catabolism of 1.11 *M* (200 g/L) glucose when all amino acids were in excess was 400 mg N/L with a range of 140 mg N/L. Nitrogen utilization was influenced by the presence of air in the fermentation headspace, ammonium supplementation and initial glucose concentration. The kinetics of utilization of individual amino acids varied between strains. However, arginine, serine, glutamate, threonine, aspartate and lysine typically comprised the bulk of the nitrogen consumed. Addition of ammonium delayed and reduced the extent of accumulation of most amino acids but increased the total nitrogen consumed.

KEY WORDS: amino acid, ammonium, *Saccharomyces cerevisiae*

The intrinsic importance of assimilable nitrogen to yeast growth and metabolism is well known by the alcoholic beverage industries. Imbalances and, in particular, deficiencies in the supply of assimilable nitrogen compounds remain as the most common causes of fermentation faults (1,5,9,22,35,42,45). In the wine industry, deficiencies can be addressed in two ways. In an effort to prevent their occurrence, much research has targeted the vineyard, where increases in the assimilable nitrogen content of grapes have been attempted through a variety of viticultural practices and fertilization regimes (1,3,4,11,12,17,38). Most commonly, however, nitrogen deficient fermentations are corrected by supplementation with nitrogenous compounds (6,9,22, 31,33,42,46), usually ammonium salts (22,24,31). Without knowledge of the nitrogen content of grape juice or the requirement for nitrogen by yeast, such additions are being made empirically. Given that a high rate of addition of inorganic nitrogen is often regulated against and that a high content of residual nitrogen may encourage microbial instability and ethyl carbamate (34,36) accumulation in the wine, these approaches may not always be appropriate. For this reason, attempts to quantifying the requirement of wine yeasts for nitrogen (1,5,25,37) are extremely useful and may allow the more efficient employment of yeast strains and render the use of nitrogen supplements as optional rather than routine. A reduced use of supplements would clearly be in keeping with the desire of the wine industry to minimize its use of additives while producing wines of optimal quality.

Previous studies have used either the total or the minimal amount of nitrogen consumed by a yeast as a measure of its nitrogen requirement. Total requirement is based on the actual or the predicted amount of nitrogen utilized to achieve maximal growth and/or sugar catabolic rates (1,22,45). Minimum nitrogen requirement, on the other hand, corresponds to the concentration of assimilable nitrogen below which the rate or time for completion of fermentation is unsatisfactory. A minimum requirement for nitrogen of 120 to 140 mg N/L has been widely reported (1,5,6,7,40). Although average amino acid-nitrogen concentrations of grape juices from different viticultural regions (see reviews by 2,19) exceed this figure, fermentations initiated with approximately twice this concentration of nitrogen may nonetheless produce hydrogen sulfide (27). In fact, complete suppression of this common fermentation fault was only achieved when excess nitrogen was present throughout fermentation. Therefore, in practical terms, an estimate of nitrogen consumption made under conditions of nitrogen excess and sugar limitation may prove to be a more useful measure of yeast nitrogen requirements.

At the time of the preliminary report of this work (25), there were two principal shortcomings of earlier studies of this type. The test media used, generally grape juice, were often only partially defined in not only nutritional and chemical composition but also in the presence of other nitrogenous compounds that may influence amino acid uptake and cast doubt on the interpretation of results. Also, estimates of amino acid requirements may be distorted in such media where amino acid species are under-represented, since depletion of any amino acid species would result in greater utilization of others (8,13). In addition, the contribution

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of the genetic variability of yeast strains in terms of both quantitative and qualitative nitrogen requirements was seldom recognized. In fact, as has already been reported (26) and is implicit in the work of others (37), the existence between strains of significant quantitative differences in nitrogen requirements may provide a novel approach to dealing with fermentation problems arising from nitrogen deficiencies.

This report is aimed at defining the specific amino acid nitrogen requirements of a number of wine strains of *Saccharomyces cerevisiae*. The relative importance of some common enological factors which influence nitrogen demand are also considered.

## Materials and Methods

**Organisms:** The strains of *Saccharomyces cerevisiae* used in this study were all isolates of commercial wine yeasts, except for 59, and were obtained as freeze-dried cultures from the Australian Wine Research Institute culture collection. Yeast cultures were maintained at 4°C on slopes of MYPG medium (malt extract, 3 g/L; yeast extract, 3 g/L; peptone, 5 g/L; glucose, 10 g/L; agar, 20 g/L).

**Culture media:** A chemically defined grape juice media (GJM) approximating the composition of typical grape juice has been reported previously (19). Nitrogen was supplied as indicated either as ammonium ($NH_4Cl$) or by the inclusion of the stated amount of an amino acid stock solution. The concentration of each amino acid was chosen so that the fermentations were carbon-limited. The amino acid stock solution contained per liter (mg): arginine (750), glutamate (500), proline (500), serine (400), aspartate (350), threonine (350), leucine (300), lysine (250), glutamine (200), isoleucine (200), valine (200), asparagine (150), histidine (150), methionine (150), phenylalanine (150), alanine (100), tryptophan (100), glycine (50), and tyrosine (20). Chemically defined grape juice starter medium (GJSM) used for the propagation of yeast starter cultures differed from GJM only in containing glucose at 555 m*M* and being supplemented to 25 m*M* ergosterol and 0.05% (v/v) monoleate (Tween 80®).

**Experimental cultures:** Yeast cultures were initiated in 25 mL of MYPG medium in 50-mL flasks and incubated overnight with shaking (180 rpm) at 25°C. Organisms were subcultured to $2 \times 10^6$ cells/mL into 25 mL of GJSM in 250-mL baffled Erlenmeyer flasks plugged with cotton wool and incubated as above until early stationary phase (*ca.* 24 hr). These cultures were then used to inoculate fermentation trials to a cell density of $5 \times 10^6$ cells/mL. Fermentations were conducted in 250-mL screw-cap Erlenmeyer flasks fitted with a side-arm port sealed with a rubber septum (Subaseal) for sampling and supplementation. A glass fermentation lock was fitted to the neck of the flask using a compression cap and O-ring (Corning). The inoculated fermentation medium (150 mL) was poured into autoclaved flasks, fermentation locks were fitted ,and when required, an anaerobic headspace was established by introducing 25 L of oxygen-stripped nitrogen over 5 minutes via an 18-G needle inserted into the septum. Fermentations were incubated as above. Fermentation progress was monitored by determining the refractive index of the cell-free fermentation medium. Fermentations were considered 'dry' at glucose concentrations of ≤ 2.5 g/L as estimated from reaction with $CuSO_4$/NaOH Clinitest tablets (Ames).

**Analysis:** Residual ammonium and glucose were quantified using enzymic test kits (Boehringer Mannheim) according to the manufacturers instructions. Amino acid contents of fermentation samples were determined by separation and quantitation of the *o*-phthaldialdehyde derivatives by high performance liquid chromatography (HPLC) on a C18 reverse-phase column with fluorometric detection based on a previously published method (28). Proline was not studied since its metabolism does not occur under the conditions used here (23,29). Derivatization reagent was made by dissolving 25 mg of *o*-phthaldialdehyde in 2 mL of 0.4 *M* potassium borate buffer (pH 10.4), to which was added 0.5 mL of methanol, 25 µL of *Brij* 35 (30%, Pierce) and 13 µL of 2-mercapto-ethanol. This reagent was stored at 4°C and was diluted 1:3 with 0.4 *M* potassium borate buffer (pH 10.4) before use. Fermentation samples (1 mL) were clarified by centrifugation in a microcentrifuge at 12 000 rpm for three minutes. A 400-µL aliquot of the supernatant was transferred to a fresh microcentrifuge tube, diluted with an equal volume of ethanolamine internal standard (*ca.* 3 m*M* in 0.1 *M* HCl) and 240 µL of 30% sulfosalicylic acid, vortexed and centrifuged as before. The resultant supernatant was diluted with an equal volume of 0.4 *M* potassium borate buffer (pH 10.4), and filtered through a 0.45-µm membrane into a fresh microcentrifuge tube and stored at 4°C until analyzed. External standard was prepared as above from uninoculated GJM.

The HPLC system consisted of an ETP-Kortec K45 gradient controller, K25M pump, K35M pump, K65 autosampler, a Waters CX4-2 column heater and 420-AC fluorescence detector and Delta Chromatography Data System software (Digital Solutions, Brisbane) run on a Clarke PC-88-3 computer. Separation of amino acid derivatives was effected on a Techsphere 3 mm ODS column (4.6 mm × 100 mm, HPLC Technologies) pre-

Table 1. Gradient program for the HPLC quantitation of amino acids in fermentation samples.

| Time (min) | Buffer composition (B as percent of A) |
|---|---|
| 0 | 3 |
| 5 | 18 |
| 25 | 18 |
| 30 | 47 |
| 47 | 100 |
| 55 | 100 |
| 58 | 3 |
| 62 | 3 |

ceded by a RP18 guard column (Brownlee). The column chamber was maintained at 30°C. Buffer flow rate was 1 mL/min. Immediately prior to analysis, diluted derivatization reagent (25 $\mu$L) was mixed with 5 $\mu$L of prepared sample, incubated at ambient temperature for one minute, and 10 $\mu$L injected onto the column. Elution of the sample then proceeded according to the gradient program shown in Table 1. Buffer A contained 25 m*M* sodium acetate (pH 6.8), 9% methanol, 1% tetrahydrofuran, and 0.1 m*M* EDTA in reagent grade water. Buffer B contained 95% methanol. Both buffers were filtered (0.45 $\mu$m), degassed by sonication under vacuum and kept under a headspace of helium.

## Results

Yeasts were cultivated in GJM containing a mixture of 18 assimilable amino acids. Analysis at the end of fermentation typically showed various degrees of removal from the medium of all amino acid species except glycine (Table 2, Fig. 1). The provision of all amino acids in excess of yeast requirements enabled the maximum requirement for both individual amino acids and total nitrogen to be calculated for each strain. Marked differences were evident in the degree to which individual yeast strains utilized each amino acid during the fermentation of 1.11 *M* (200 g/L) glucose from air-saturated GJM initiated with an anaerobic headspace (Table 2). Values for total nitrogen requirements ranged from 328 to 467 mg N/L for eight strains with a mean of 395 mg N/L. Increasing the initial concentration of glucose by 25% to 1.39 *M* (250 g/L) resulted in an increase in the total nitrogen utilized by two strains and a decrease for two strains. Nevertheless, the mean nitrogen requirement of these four strains was increased by 5% from 405 to 427 mg N/L.

Qualitative aspects of this data are most evident in Figure 1. The contribution by individual amino acids to the total requirement for nitrogen was relatively uniform for the eight yeast strains. Irrespective of the strain, arginine, serine, glutamate, threonine, aspartate and lysine were generally most heavily utilized, contributing between 70.6% and 79.0% of the total nitrogen requirement. Arginine alone contributed between 32.5% and 46.5%. By comparison, glycine was often excreted during fermentation (Table 2). Differences in total requirement for nitrogen did not result from a disproportionate variation in the utilization of a single or limited number of amino acids but rather reflected general trends for all amino acids.

In addition to a dependence on yeast strain and the initial glucose concentration of the medium, the requirement for nitrogen was also influenced by the presence of air during the early stages of the fermentation. Thus while strains 59, 65, 72, 73, and 77 cultured in GJM containing 1.11 *M* glucose displayed a mean

Table 2. Amino acid utilization (mg N/L) by yeast strains during growth under a nitrogen headspace at two glucose concentrations.

| | 1.11 *M* initial glucose | | | | | | | 1.39 *M* initial glucose | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | **59** | **65** | **72** | **73** | **77** | **81** | **92F** | **59** | **72** | **73** | **77** | **92** |
| **Amino acid** | | | | | | | | | | | | |
| Asp | 25.8± 1.1 | 20.1±0.3 | 25.2±0.6 | 21.5± 2.8 | 21.5±1.8 | 23.3±0.9 | 22.4± 0.5 | 28.2± 1.7 | 17.7± 0.9 | 21.8± 0.4 | 18.6± 0.9 | 22.3±0.2 |
| Glu | 27.9± 2.6 | 24.5±0.7 | 29.1± 1.4 | 20.3± 4.2 | 21.5± 2.7 | 28.1± 0.7 | 25.9± 1.3 | 33.0± 2.0 | 18.0± 1.3 | 22.0± 1.5 | 20.2± 1.1 | 25.6±0.2 |
| Asn | 18.3± 0.3 | 13.3±0.5 | 15.6± 0.7 | 13.8± 1.2 | 15.3± 0.9 | 17.5± 0.6 | 14.9± 0.41 | 8.6± 0.8 | 17.4± 0.6 | 18.4± 0.0 | 18.2± 0.4 | 17.2±1.2 |
| Ser | 36.6± 0.7 | 28.4±0.8 | 36.7± 0.9 | 29.6± 2.3 | 30.2± 1.5 | 35.3± 1.1 | 33.5± 0.6 | 38.9± 1.4 | 30.9± 0.9 | 34.5± 0.3 | 32.6± 0.9 | 35.7±0.2 |
| Gln | 18.3± 2.3 | 14.8±1.2 | 19.2± 1.0 | 10.4± 0.9 | 8.2± 1.9 | 16.0± 0.3 | 16.0± 0.6 | 8.6± 0.8 | 18.5± 1.0 | 19.5± 0.2 | 19.9± 1.2 | 21.1±0.3 |
| His | 11.9± 1.4 | 10.1±0.9 | 8.5± 0.6 | 13.9± 1.3 | 7.3± 1.3 | 9.0± 0.5 | 10.2± 0.6 | 9.6± 1.8 | 15.1± 0.8 | 15.0± 0.7 | 11.5± 0.3 | 10.8±0.2 |
| Gly | -1.2± 0.4 | -0.1±0.0 | -2.3± 0.1 | -0.8± 0.5 | -1.5± 0.0 | -0.4± 0.1 | -0.6± 0.1 | -0.7± 0.1 | 1.7± 0.2 | 1.1± 0.4 | 1.9± 0.1 | 2.8±0.2 |
| Thr | 29.3± 2.6 | 20.1±0.8 | 29.5± 1.02 | 7.5± 1.6 | 25.9± 1.6 | 25.6± 0.8 | 26.1± 0.6 | 31.8± 1.2 | 24.8± 0.6 | 30.6± 0.1 | 25.9± 0.7 | 27.9±0.1 |
| Arg | 165 ± 3 | 132 ±5 | 128 ± 4 | 182 ± 8 | 143 ± 7 | 212 ± 4 | 137 ± 4 | 138 ± 4 | 171 ± 3 | 199 ± 1 | 162 ± 3 | 137 ±3 |
| Ala | 5.7± 0.5 | 0.7±0.5 | 2.0± 0.5 | -1.2± 0.9 | 5.1± 0.9 | 2.9± 0.7 | 3.6± 0.5 | 8.8± 0.5 | -2.4± 0.7 | -0.3± 0.2 | 5.3± 0.5 | 4.9±0.6 |
| Tyr | 1.0± 0.1 | 0.2±0.0 | 0.2± 0. | 10.7± 0.0 | 0.6± 0.3 | 0.2± 0.1 | 0.3± 0.1 | 0.9± 0.1 | 0.7± 0.0 | 0.5± 0.1 | 0.5± 0.0 | 0.6±0.0 |
| Met | 8.7± 0.8 | 5.4±0.3 | 6.7± 0.2 | 6.0± 0.1 | 6.7± 0.2 | 6.9± 0.2 | 6.2± 0.2 | 8.5± 0.3 | 8.5± 0.2 | 7.5± 0.1 | 8.2± 0.2 | 8.7±0.1 |
| Val | 12.5± 1.2 | 4.3±0.3 | 6.5± 6.5 | 6.4± 0.8 | 9.0± 1.2 | 7.3± 0.6 | 6.2± 0.6 | 13.4± 0.5 | 10.8± 0.4 | 9.0± 0.5 | 10.3± 0.4 | 12.0±0.4 |
| Trp | 6.4± 2.2 | 3.5±0.2 | 4.6± 0.1 | 1.6± 0.9 | 2.2± 0.9 | 4.5± 0.1 | 3.4± 0.2 | 2.8± 0.6 | 7.6± 0.1 | 6.1± 0.1 | 8.1± 0.3 | 7.7±0.3 |
| Phe | 7.6± 0.4 | 4.2±0.2 | 5.7± 0.4 | 5.4± 0.3 | 4.7± 1.6 | 5.8± 0.3 | 5.4± 0.3 | 8.6± 0.5 | 8.0± 0.2 | 6.6± 0.3 | 6.9± 0.1 | 7.9±0.1 |
| Ile | 15.5± 0.5 | 9.2±0.1 | 12.4± 0.3 | 10.5± 0.3 | 12.9± 0.3 | 13.1± 0.3 | 11.6± 0.3 | 16.5± 0.6 | 11.9± 0.4 | 9.6± 0.2 | 12.0± 0.2 | 13.0±0.3 |
| Leu | 26.1± 0.7 | 15.1±0.2 | 20.5± 0.6 | 15.9± 1.2 | 19.7± 0.8 | 21.1± 0.6 | 19.4± 0.5 | 25.6± 1.1 | 22.6± 0.8 | 17.6± 0.6 | 21.0± 0.3 | 22.6±0.7 |
| Lys | 30.5± 1.6 | 22.4±1.8 | 30.7± 1.6 | 27.8± 2.2 | 25.9± 4.7 | 39.3± 1.7 | 28.6± 1.9 | 33.7± 3.4 | 37.6± 0.8 | 35.6± 0.4 | 34.0± 1.4 | 36.4±0.8 |
| Total | 446 ±12 | 328 ±12 | 379 ±11 | 391 ±22 | 358 ±12 | 467 ±10 | 369 ±12 | 424 ±11 | 420 ±11 | 454 ± 5 | 417 ± 8 | 415 ±2 |

Fermentations were initiated in air-saturated GJM under a headspace of nitrogen (medium:headspace ratio, 1:1). The medium contained 1.11 *M* or 1.39 *M* glucose and excess amino acids (20 and 25 mL amino acid stock/L, respectively). Reported values are the difference between the initial and the residual amino acid concentrations ± S.E.M.

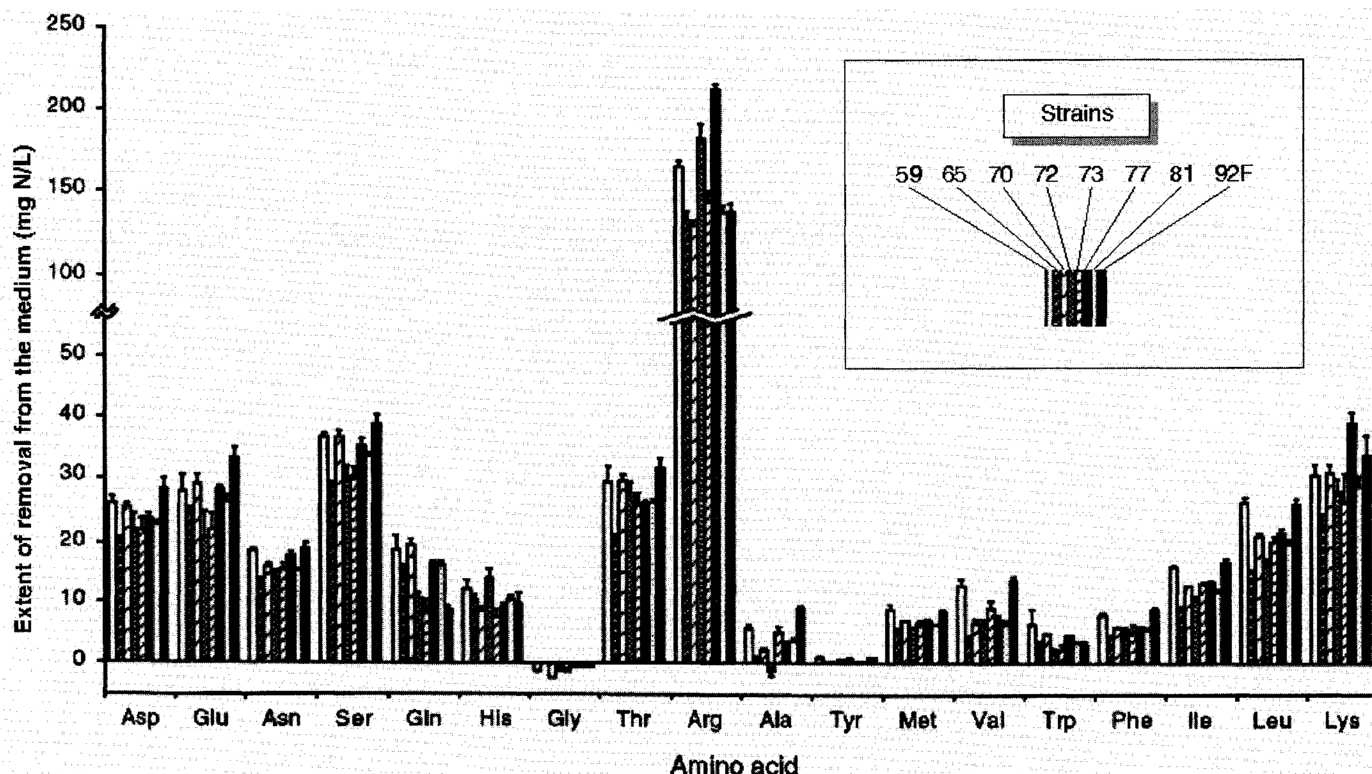


Fig. 1. Amino acid utilization by *Saccharomyces cerevisiae* yeast strains during growth under a nitrogen headspace. Strains 59, 65, 70, 72, 73, 77, 81, and 92F were grown in GJM containing 1.11 *M* glucose under the fermentation conditions described for Table 2. Values quoted are the mean of four determinations ± S.E.M.

Table 3. Effect of initial access to oxygen on amino acid utilization (mg N/L) by yeast strains during growth at two glucose concentrations.

| | 1.11 *M* initial glucose | | | | | | 1.39 *M* initial glucose | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | **59** | **65**[a] | **72** | **73** | **77** | **108** | **59** | **72** | **73** | **77** |
| **Amino acid** | | | | | | | | | | |
| Asp | 40.4 | 28.5 | 44.7 | 34.8 | 33.2 | 32.4 | 50.1 | 42.0 | 40.4 | 40.4 |
| Glu | 51.4 | 28.7 | 53.0 | 40.1 | 34.0 | 35.4 | 58.7 | 40.3 | 38.7 | 36.1 |
| Asn | 29.2 | 18.6 | 24.3 | 25.1 | 23.2 | 25.1 | 36.7 | 30.2 | 30.4 | 33.1 |
| Ser | 51.5 | 38.5 | 58.8 | 48.7 | 47.3 | 47.9 | 72.2 | 63.3 | 52.0 | 62.9 |
| Gln | 30.8 | 18.2 | 31.8 | 26.1 | 26.1 | 22.0 | 32.3 | 28.5 | 29.1 | 37.5 |
| His | 20.4 | 11.7 | 20.4 | 16.8 | 17.7 | 20.1 | 27.8 | 20.2 | 14.3 | 21.0 |
| Gly | -2.0 | 0.1 | -1.8 | -1.4 | -0.7 | 0.4 | -1.5 | -0.9 | -6.3 | 0.2 |
| Thr | 37.3 | 28.9 | 41.1 | 33.9 | 33.7 | 32.4 | 48.3 | 40.5 | 42.9 | 42.4 |
| Arg | 287.0 | 169.0 | 315.0 | 276.0 | 302.0 | 193.0 | 350.0 | 312.0 | 331.0 | 348.0 |
| Ala | 9.7 | 0.1 | 1.2 | 8.8 | 5.3 | 9.9 | 9.3 | 10.2 | -3.3 | 8.8 |
| Tyr | 1.1 | 0.4 | 1.5 | 0.8 | 0.5 | 1.4 | 1.6 | 0.9 | -0.5 | 1.9 |
| Met | 12.1 | 9.1 | 11.7 | 11.0 | 10.9 | 11.4 | 15.4 | 13.3 | 12.7 | 13.6 |
| Val | 22.0 | 9.1 | 20.5 | 16.6 | 14.3 | 19.6 | 25.9 | 18.4 | 18.9 | 17.9 |
| Trp | 5.1 | 2.9 | 3.6 | 3.6 | 3.9 | 2.9 | 5.8 | 3.4 | 5.8 | 5.8 |
| Phe | 13.4 | 7.0 | 12.9 | 9.4 | 9.0 | 12.5 | 16.0 | 10.5 | 11.8 | 10.6 |
| Ile | 22.4 | 22.4 | 21.8 | 18.9 | 17.6 | 20.0 | 27.2 | 22.3 | 21.4 | 21.1 |
| Leu | 40.8 | 23.8 | 38.9 | 33.1 | 32.2 | 37.3 | 52.3 | 39.9 | 41.8 | 40.4 |
| Lys | 85.1 | 38.4 | 80.5 | 66.1 | 78.8 | 61.0 | 90.5 | 68.4 | 65.5 | 84.9 |
| Total | 758 | 455 | 780 | 668 | 689 | 585 | 918 | 763 | 746 | 827 |

Fermentations were initiated in air-saturated GJM under a headspace of air (medium:headspace ratio, 1:1). The medium contained 1.11 M or 1.39 *M* glucose and excess amino acids (40 and 50 mL amino acid stock per liter, respectively).

[a]Values extrapolated from incomplete fermentation. Reported values are the difference between the initial and the residual amino acid concentrations.

Table 4. Influence of initial access to oxygen on fermentation duration (hr).

| Yeast strain | Nitrogen headspace | Air headspace |
|---|---|---|
| 59 | 89.0 | 55.5 |
| 72 | 91.0 | 38.5 |
| 73 | 75.5 | 40.0 |
| 77 | 74.0 | 44.0 |

Fermentations were conducted in GJM containing 1.11 *M* glucose under the conditions described for Tables 2 and 3.

utilization of nitrogen of 398 mg N/L for fermentations initiated with an anaerobic headspace (Table 2), this value was increased by 68% to 670 mg N/L when cultures were initiated with a headspace of air (Table 3). Furthermore, a headspace of air reduced the time required to complete the catabolism of glucose by as much as 58% (Table 4). Increasing the initial content of glucose by 25% to 1.39 *M* resulted in a 12% increase in mean utilization of nitrogen to 814 mg N/L (Table 3). In all cases, arginine remained the single greatest contributor to the yeasts requirement for nitrogen. Ammonium supplementation of fermentations initiated with a headspace of air reduced the degree of amino acid removal but increased net removal of assimilable nitrogen by ca. 20% in the case of strain 72 (data not shown).

Kinetic studies revealed that the removal of individual amino acids from a nitrogen sufficient medium by strains 77 and 72 occurred in an ordered manner (Fig. 2, 3). The main difference between the two strains was a lower rate of removal of histidine, glycine and alanine by strain 77. The inclusion of ammonium in the medium brought about the delayed and/or reduced removal of several amino acid species. Glutamate, arginine, glycine, valine, and phenylalanine were especially affected. Based on mean data for strains 72 and 77, nitrogen compounds were classified into three accumulation groups according to their kinetics and extent of removal from the medium and sensitivity of these to additions of ammonium (Table 5). Under the conditions used here, Group A components displayed a rapid onset and rate of removal, typically achieving 50% depletion within 20 hours and close to complete depletion in the absence of ammonium and at least 75% in its presence. The group B components showed reduced rates and extents of removal and/or a greater inhibition of their removal by ammonium. Characteristics of the third group included either a distinct lag preceding removal,

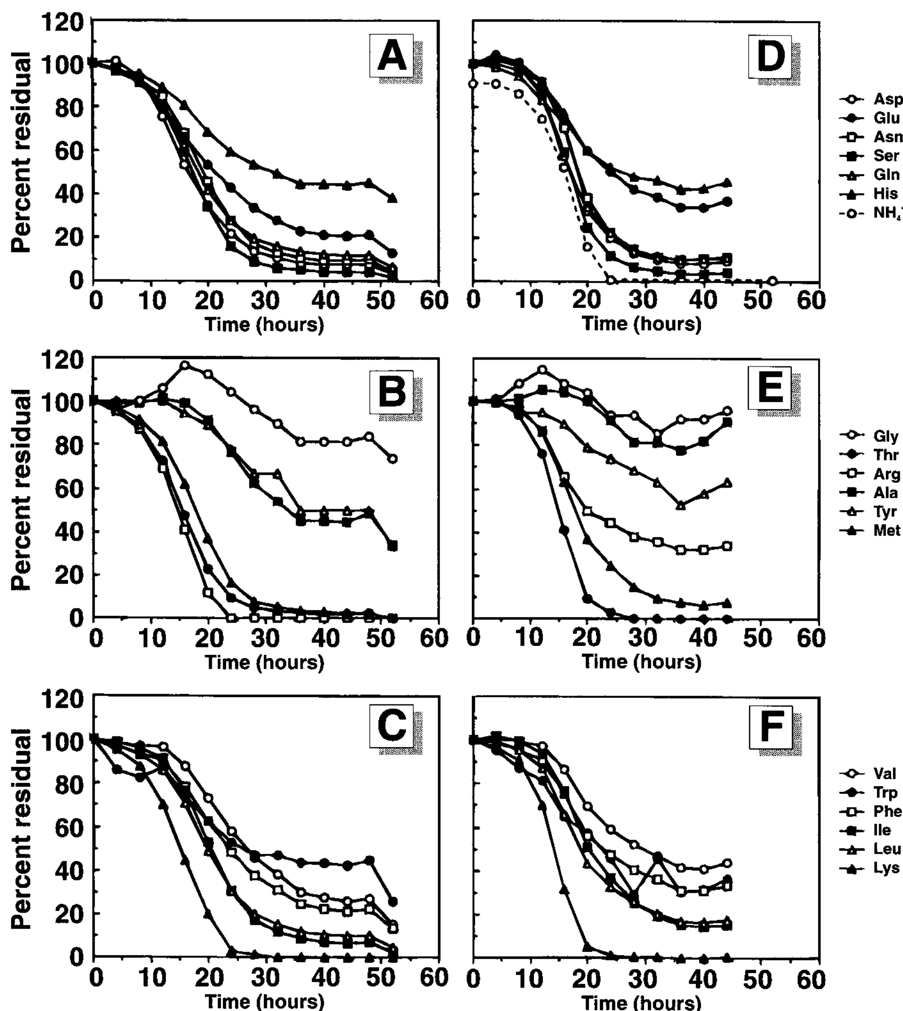


Fig. 2. Removal of amino acids and ammonium ions during fermentation of grape juice medium by *Saccharomyces cerevisiae* strain 72. Fermentations were conducted in GJM containing 1.11 *M* glucose and limited nitrogen (12.5 mL amino acid stock/L) alone (**A, B, C**) or together with ammonium ions (155 m*M*; **D, E, F**). Values quoted are the mean of four determinations.

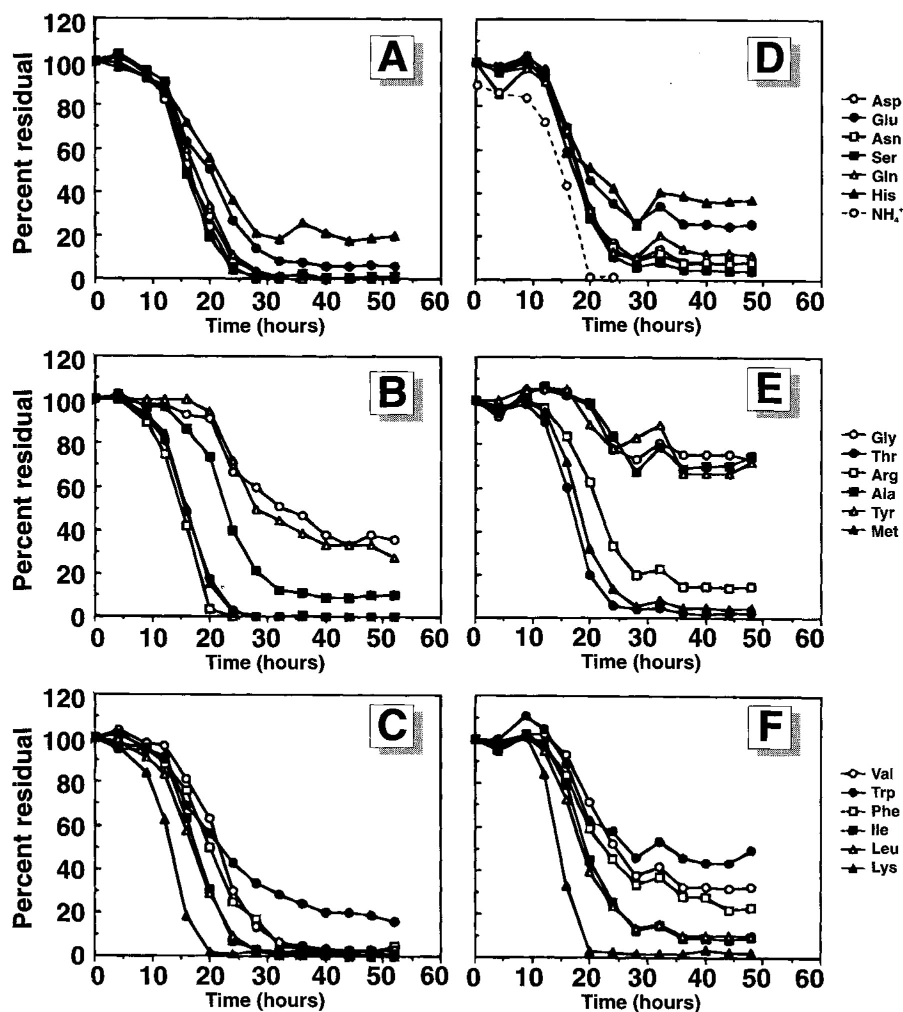


Fig. 3. Removal of amino acids and ammonium ions during fermentation of synthetic grape juice medium by *Saccharomyces cerevisiae* strain 77. Fermentations were conducted in GJM containing 1.11 *M* glucose and limited nitrogen (12.5 mL amino acid stock/L) alone (**A, B, C**) or together with ammonium ions (155 m*M*; **D, E, F**). Values quoted are the mean of four determinations.

a failure to attain 50% removal in ammonium-free media or a dramatic decline in removal in the presence of ammonium.

## Discussion

By cultivating yeasts in a medium of unrestricted amino acid availability, it has been possible to determine both the total requirement and the relative preference for amino acids by the wine yeasts studied. The mean total requirement for nitrogen of 400 mg N/L reported for GJM containing 1.11 *M* glucose agrees well with two recent estimates of 300 mg FAN/L (5,6) and 429 mg N/L (calculated from 37) obtained under similarly defined conditions in grape juice fermentations. The significance of these values is that they generally exceed the average amino acid-nitrogen content of grape juices (2,19), suggesting that many juices lack sufficient nitrogen for optimal or satisfactory fermentation. Commercial experience supports this empirically through the routine need for nitrogen supplements to reduce the incidence of incomplete fermentation and the evolution of hydrogen sulfide, which is known to develop upon nitrogen limitation (18,27,44).

Knowing the nitrogen requirement of specific yeast strains will provide a better guide for controlling the nitrogen content of fermentation. Practical exploitation of this strategy will, however, require a convenient protocol for the routine determination of the assimilable nitrogen rather than chemical nitrogen content of grape juice (see 19 for discussion). A bioassay for assimilable nitrogen (32) may be useful in achieving this aim. Until such an assay has been proven reliable, meeting the nitrogen requirement of yeast more accurately will not be possible. The fermentation of low nitrogen musts can however be improved by using nitrogen efficient strains as demonstrated previously (26).

For the eight strains examined here in GJM containing 1.11 *M* glucose, we observed a range in nitrogen demands of the order of 140 mg N/L. Although the genetic variability between strains in this phenotype has been recognized (29,37), the concept of using this property to reduce fermentation problems, such as sluggish or stuck fermentations has not been considered. Interestingly, similar differences between strains can be calculated from published data (19) such as that obtained for four Californian wine yeasts in a panel of 11 grape juices (37). Total nitrogen demands were found to range by 81 mg N/L between 392 and 473 mg N/L. When considered as a ratio of nitrogen consumed versus sugar fermented, the variations reported here represent a

Table 5. Mean kinetics of removal of nitrogen compounds during fermentation by *Saccharomyces cerevisiae* strains 72 and 77.

| | Hours to 50% depletion | | Final utilization (%) | | Accumulation group |
|---|---|---|---|---|---|
| Ammonium | - | + | - | + | |
| **Nitrogen compound** | | | | | |
| Alanine | 32 | >50 | 79 | 19 | B |
| Ammonium | - | 16 | - | 100 | A |
| Arginine | 15 | 21 | 100 | 76 | A |
| Asparagine | 19 | 18 | 98 | 90 | A |
| Aspartate | 17 | 17 | 99 | 90 | A |
| Glutamate | 21 | 23 | 96 | 68 | B |
| Glutamine | 18 | 19 | 99 | 89 | A |
| Glycine | >50 | >50 | 50 | 16 | C |
| Histidine | 29 | 25 | 87 | 60 | B |
| Ileucine | 20 | 20 | 99 | 88 | A |
| Leucine | 20 | 19 | 98 | 86 | A |
| Lysine | 15 | 15 | 100 | 99 | A |
| Methionine | 18 | 18 | 100 | 89 | A |
| Phenylalanine | 23 | 23 | 91 | 73 | B |
| Serine | 17 | 17 | 97 | 95 | A |
| Threonine | 16 | 16 | 100 | 99 | A |
| Tryptophan | 25 | 24 | 78 | 57 | C |
| Tyrosine | 35 | >50 | 61 | 34 | C |
| Valine | 25 | 9 | 90 | 62 | B |

Fermentations conditions were as described for Figures 2 and 3.

useful range of nitrogen 'efficiencies'. In fact, the choice between the two strains at the extremes of nitrogen demand is equivalent to a large proportion of the amount of nitrogen additive legally permitted in several countries. These being 64, 200, and potentially 360 mg N/L as dibasic ammonium phosphate (DAP) in Europe, the USA, and Australia, respectively. Clearly, there exists considerable scope for reducing the use of additives simply through appropriate selection of wine strains.

It is noteworthy that nitrogen demand under the conditions of nitrogen excess does not necessarily predict other fermentation characteristics. For example, strains identified here as having a low nitrogen demand do not necessarily ferment more efficiently during nitrogen starvation (26). Similarly, nitrogen demand does not correlate with the empirical labeling or actual laboratory performance (27, unpublished results) of these strains as high-, low- or non-hydrogen sulfide producers. Instead, it is suggested that nitrogen demand merely determines how soon in a nitrogen-limited fermentation nitrogen becomes depleted resulting in the development of some or all of the associated fermentation problems.

In addition to strain effects, three other factors, potentially under the control of the winemaker, influenced the requirement for nitrogen. The increase in nitrogen utilization which accompanies the fermentation of a higher initial concentration of sugar has been frequently observed (1,5,37). The mean increase of 5% (22 mg N/L) reported here for the anaerobic fermentation of an additional 25% (0.28 *M*) glucose compares with a 30 mg N/L increase described by Agenbach (1) for a similar increase in sugar concentration. Greater nitrogen consumption most likely reflects the extended maintenance of carbon catabolic machinery. The current report of oxygen related increases of 68% in nitrogen consumption, falls within the range of 34% and 193% reported by Ingledew and Kunkee (22). Although initial access to oxygen dramatically reduces fermentation time, this advantage may be of reduced benefit given the concomitant increase in yeast biomass (data not shown) and a greater consumption of nitrogen. Supplementation of the medium with ammonium also increased the utilization of nitrogen by yeast. The Group B and Group C amino acids were especially susceptible to delayed/reduced uptake in response to ammonium supplementation, a finding probably related to the lower relative efficiencies of these groups as nitrogen sources (13) (see below). The preferential utilization of ammonium appears to stimulate yeast growth which leads to an overall increase in nitrogen utilization. This apparent decrease in nitrogen efficiency of the strain may arise from the greater tendency of nitrogen added in the early stages of fermentation to be diverted to biomass formation (1,5,8). These influential factors need to be investigated more fully in terms of their implications for the fermentation of a must of limited nitrogen content.

The temporal investigation of amino acid utilization was partly undertaken to identify metabolically important amino acids which may be involved in fermentation problems seen in the wine industry. Key amino acids were expected to become apparent by their rapid and/or preferential utilization by yeast. However, the existence of such amino acids is disputed by the recent finding from this laboratory (27) that most amino acids are equally effective as suppressants of nitrogen deficiency-induced hydrogen sulfide formation. Thus, in this regard at least, individual amino acids have no special importance beyond their role as nitrogen sources, acting mainly to fuel transamination reactions as observed for brewing yeasts (30).

The ordered accumulation of amino acids is suggested to reflect the ease by which they are transported and degraded to meet cellular nitrogen requirements. In agreement, rapidly accumulated amino acids are largely classed as good nitrogen sources (13). In fact, the culture doubling times reported by Cooper (13) for growth on single amino acid nitrogen sources capable of sustaining growth correlates well ($r = 0.69$; $p \leq 0.01$) with the times seen here for the corresponding amino acid to become depleted by 50%. Thus, it is concluded that more efficient nitrogen sources are generally utilized early in fermentation. The differences in kinetics may stem from the relative economy of amino acid transport (14,15,43), the physiological basis for which is

transporter expression and activity (8,21,41). The similarities in the kinetics of amino acid absorption seen between strains 72 and 77 can be extended to accumulation sequences quoted in or derived from earlier brewing and enological studies (10,16,29). Such similarities despite differences in strain and nutritional and chemical composition of the medium, suggest that these absorption kinetics are a trait common to several yeasts rather than a consequence of the specific conditions used. Areas of future interest would include a determination of the role of these accumulation kinetics in the kinetics or strain dependence of the formation of aroma compounds which have amino acids as biosynthetic precursors or regulators (see review by 39). In light of the ability of ammonium addition to delay arginine utilization and to affect urea excretion and reabsorption (20,33), this common method of nitrogen supplementation needs to be further examined as a means of controlling urea accumulation in the medium and hence the potential for the formation of ethyl carbamate (34,36).

In this study, we have begun to characterize the specific nitrogen requirements of *Saccharomyces* wine strains and so provided information to help optimize the use of nitrogen supplements in the wine fermentation. The identification of strain variations highlights the fact that estimates of yeast nitrogen demands cannot be generalized across strains and, in fact, suggests the usage of selected strains as an alternative approach to overcoming the problems associated with nitrogen deficient fermentations. The practicality of this approach should increase upon expansion of the current survey of commercial strains. The potential benefits of such a characterization argues in favor of it forming a useful additional descriptor of currently available commercial yeast strains. Further investigation is needed to uncover the best means of balancing the effects of aeration and the timing and extent of supplementation to maximize the efficiency of nitrogen utilization and possibly influence the generation of metabolic by-products of health importance and those contributing to the aroma profile of wine.

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