0273-2289/98/70–72—0845$10.00

Production of L-Malic Acid via Biocatalysis Employing Wild-Type and Respiratory-Deficient Yeasts

XIAOHAI WANG, C. S. GONG, AND GEORGE T. TSAO*

Laboratory of Renewable Resources Engineering, 1295 Potter Engineering Center, Purdue University, West Lafayette, IN 47907

ABSTRACT

The yeast *Saccharomyces cerevisiae* has been used to efficiently produce L-malic acid from fumaric acid. Fumarase is responsible for the reversible conversion of fumaric and L-malic acids in the TCA cycle. To investigate the function of mitochondrial and cytoplasmic fumarase isoenzymes in L-malic acid bioconversion, a wild-type strain and a cytoplasmic respiratory-deficient mutant devoid of functional mitochondria were employed. The mutant strain, which only contained the cytoplasmic fumarase, was still functional in fumaric acid to L-malic acid bioconversion. However, its specific conversion rate was much lower (0.20 g/g·h) than that of the wild-type strain (0.55 g/g·h).

Index Entries: *Saccharomyces cerevisiae*; fumarase; respiratory deficient; L-malic acid; fumaric acid.

INTRODUCTION

L-Malic acid is an intermediate of cell metabolism that is involved in two respiratory metabolic cycles: the tricarboxylic acid cycle and the glyoxylic acid cycle. It is also the predominant acid component in apple and many other fruits. Having a greater acid taste and better taste retention than citric acid, malic acid is commonly used as a food and beverage acidulent *(1)*, and is also used in pharmaceuticals, cosmetics, metal cleaning, coatings, polymers, and resins *(1)*. Extraction from apple juice (0.4–0.7% L-malic acid) was the traditional method for L-malic acid preparation *(2)*. However, the relatively low concentrations of L-malic acid present in natural sources makes its isolation expensive and impractical. Currently, malic acid can be produced by chemical synthesis (D, L-racemate mixture) via

* Author to whom all correspondence and reprint requests should be addressed.

hydration of maleic acid or fumaric acid at elevated temperature and pressure, or by biosynthesis (L-isomer) from fumaric acid *(1–3)*.

Immobilized *Brevibacterium flavum* and *Brevibacterium ammoniagenes* have been employed for the bioconversion of L-malic acid from fumaric acid *(4,5)*. However, succinic acid was found as an undesirable byproduct. Recently, the yeast *Saccharomyces cerevisiae* has been used to efficiently convert fumaric acid to L-malic acid without producing succinic acid *(6)*.

Fumarase catalyzes the interconversion of fumaric acid and L-malic acid in the tricarboxylic acid cycle. Like most tricarboxylic acid cycle enzymes, fumarase is located in the matrix compartment of mitochondria. In the baker's yeast *S. cerevisiae*, two species of fumarase isoenzymes, with mol wt of 48,000 and 53,000, have been isolated from mitochondria and cytoplasm, respectively *(7)*. The enzymatic function of the cytoplasmic fumarase has not been established.

The respiratory deficient mutants, or petite mutants, of *S. cerevisiae* are mutants which are devoid of functional mitochondria. They can grow on standard agar medium containing glucose as energy source, producing ATP by glycolysis. When glucose is depleted from the medium, colonies stop growing, and their sizes are thus very much smaller than those of normal colonies at stationary phase. Respiratory-deficient cells are unable to grow on nonfermentable medium containing, for example, ethanol as the sole carbon and energy source *(8)*.

As a means to study the in vivo bioconversion function of mitochondrial and cytoplasmic fumarase, we have employed a wild-type *S. cerevisiae* strain that contains both isoenzymes and a corresponding cytoplasmic respiratory-deficient petite strain, which only contains the cytoplasmic fumarase in this study. Their growth patterns and capabilities in bioconversion have been examined.

MATERIALS AND METHODS

Strains

S. cerevisiae strain 43 (ATCC 42510) and its corresponding cytoplasmic respiratory-deficient mutant strain 311 (ATCC 42511) *(9)* were employed in this study.

Triphenyltetrazolium Staining Assay

The test medium consists of 2,3,5-triphenyltetrazolium chloride (1 g/L) and Bacto-agar (15 g/L, Difco, Detroit, MI) in 0.067 *M* phosphate buffer at pH 7.0. 2,3,5-Triphenyltetrazolium chloride is not sterilized, since it is reduced chemically by autoclaving with the agar. About 50 yeast colonies per plate were grown for 3 d on a conventional agar medium containing yeast extract (10 g/L, Difco), peptone (20 g/L, Difco), Bacto-agar (20 g/L, Difco), and glucose (10 g/L). The assay was performed by pouring 20 mL

test medium at 50°C over the colony-bearing plates. Red or white colonies were scored at 3 h after overlay *(10,11)*.

Media and Cultivation

YPD medium *(12)*, used for cell growth, contains yeast extract (10 g/L, Difco), peptone (20 g/L, Difco), and glucose (20 g/L). SD medium contains yeast nitrogen base without amino acids (6.7 g/L, Difco), and glucose (3 g/L). SE medium contains yeast nitrogen base without amino acids (6.7 g/L, Difco), and ethanol (3 g/L). The SD and SE media were buffered at pH 5.0 with 0.05 *M* citrate buffer. YPF medium, used for bioconversion, contains yeast extract (10 g/L, Difco), peptone (20 g/L, Difco), Triton X-100 (1 g/L), Tween-80 (1 g/L), and fumaric acid (95 g/L) neutralized with sodium hydroxide to pH 7.0.

Batch cultures for cell growth and aerobic bioconversion were conducted in 250-mL Erlenmeyer flasks with silicone sponge closures (Sigma, St. Louis, MO) containing 20 or 30 mL medium. All flasks were incubated in an incubator shaker (model G24, New Brunswick Scientific, Edison, NJ) at 30°C and 250 rpm. Batch cultures for anaerobic bioconversion were conducted in 50-mL Erlenmeyer flasks sealed with rubber plugs containing 25 mL medium, and were incubated in the incubator shaker at 30°C and 80 rpm.

Analytical Methods

Biomass concentrations were determined from turbidimetric measurements at 600 nm and a correlation between biomass and OD 600 nm. Fumaric acid, L-malic acid, succinic acid, glucose, and ethanol were determined and quantified by HPLC, with an Intelligent Pump (Hitachi Instrument, L-6200A), an Intelligent Auto Sampler (Hitachi Instrument, AS-4000), a Bio-Rad (Hercules, CA) Aminex HPX-87H ion-exclusion column (300 × 7.8 mm), a refractive index detector (Hitachi Instrument, L-3350 RI), and a Chromato-Integrator (Hitachi Instrument, D-2500). The column temperature was maintained at 60°C, and the column was eluted with 5 m*M* sulfuric acid at a flow rate of 0.8 mL/min.

RESULTS AND DISCUSSION

Characterization of Respiratory—Deficient Mutant by Tetrazolium Overlay Assay

Actively respiring yeast cells can rapidly reduce 2,3,5-triphenyltetrazolium chloride to 2,3,5-triphenylformazan, which is an insoluble red pigment. The vital staining by overlaying yeast colonies with 2,3,5-triphenyltetrazolium agar can be used to discriminate respiration-competent wild-type cells and respiration-deficient mutants. The respiration-competent cells turn red, and the respiration-deficient mutants stay white *(10,11)*.

To confirm the respiration capability or deficiency of the strains employed in the study, about 100 colonies each from strains 43 and 311 were tested by the triphenyltetrazolium staining assay as described above. All strain 43 colonies turned red, showing respiration competency; all strain 311 colonies remained white during the test period, indicating their respiration deficiency.

Growth Behavior

The growth of the wild-type strain 43 and the respiratory-deficient mutant strain 311 was studied. Two distinct media were employed: a minimal defined glucose-containing medium SD, and a minimal defined ethanol-containing medium SE. The growth was monitored by measuring OD of the cultures. Glucose and ethanol concentrations were also measured. The strains employed were first grown overnight in the SD medium inoculated from frozen stocks at −70°C. All batch cultures were started with 0.012 g/L biomass inoculated from the overnight SD cultures. The results are shown in Figure 1 and Figure 2 for growth in the SD and SE medium, respectively.

In the minimal glucose-containing SD medium (Fig. 1), strain 43 grew much faster than strain 311, as expected. The specific growth rates of strains 43 and 311 were 0.345/h and 0.196/h, respectively. Final biomass concentration of strain 43 reached 0.82 g/L, and was 4.8 × that of strain 311 (0.17 g/L). Glucose concentrations decreased to zero for strain 43 and to 0.13 g/L for strain 311 at 15 h. Ethanol concentrations accumulated to 0.67 g/L and 1.21 g/L at 15 h, and then dropped to zero at 25 h and to 0.97 g/L at 32.5 h for strains 43 and 311, respectively.

When the minimal medium SE, which contained ethanol as the sole carbon and energy source, was employed (Fig. 2), the growth profile of strain 43 showed a distinctive 25-h-long exponential phase that ended with the depletion of ethanol. The specific growth rate was 0.16/h and the maximal biomass concentration achieved 0.74 g/L. The strain 311, however, did not grow at all. This is consistent with the respiratory-deficient phenotype of petite mutants, and further confirms that strain 311 is deficient in respiration. The ethanol profile of strain 311, however, did not stay at the initial level. It decreased to 1.1 g/L at the end of the culture. Ethanol might be converted to other metabolites inside the cell, but it could not be used as the carbon and the energy source to support the growth of strain 311 in the SE medium.

Aerobic Conversion of Fumaric Acid to L-Malic Acid

Strains 43 and 311 were grown in the YPD medium overnight. Cells were precipitated by centrifugation. An equal amount of cell mass (3.62 g/L) of each strain was inoculated into the culture flasks containing YPF medium for the bioconversion of fumaric acid to L-malic acid. The results of aerobic bioconversion by the wild-type strain 43 and the petite strain 311

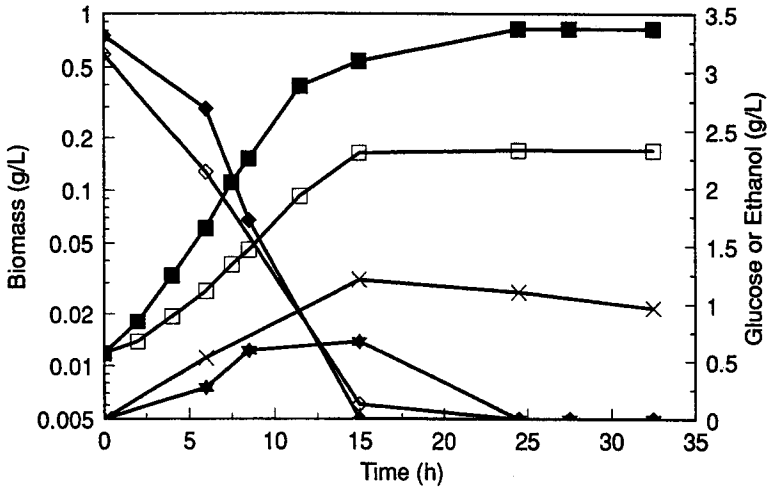

Fig. 1. Growth curves, glucose and ethanol profiles of the wild-type (wt) strain 43 and the respiratory-deficient (rd) mutant strain 311 in the minimal glucose-containing SD medium: Biomass (wt) (-■-), Biomass (rd) (-□-), Glucose (wt) (-◆-), Glucose (rd) (-◇-), Ethanol (wt) (-★-), and Ethanol (rd) (-×-).

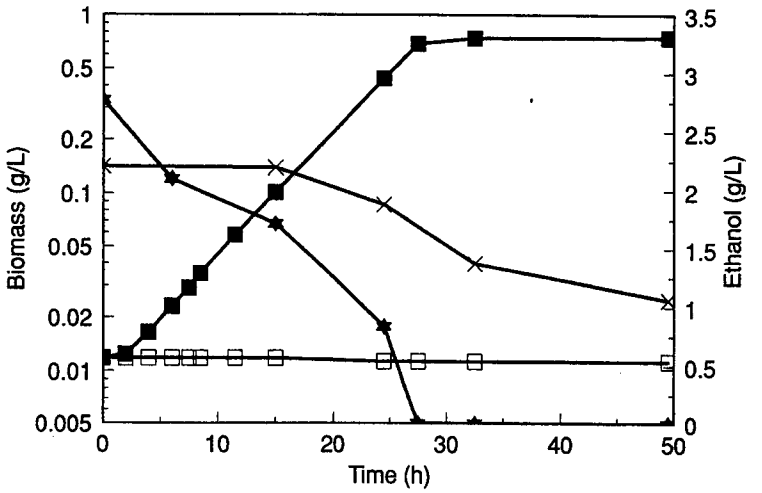

Fig. 2. Growth curves and ethanol profiles of the wild-type (wt) strain 43 and the respiratory-deficient (rd) mutant strain 311 in the minimal ethanol-containing SE medium: Biomass (wt) (-■-), Biomass (rd) (-□-), Ethanol (wt) (-★-), and Ethanol (rd) (-×-).

are shown in Fig. 3. The production of L-malic acid by the wild-type strain 43 was much faster than that by the respiratory-deficient strain 311. Final L-malic acid concentration reached 92.5 g/L in the strain 43 culture, compared to 37.8 g/L in the strain 311 culture at 60 h. Fumaric acid consumption was closely correlated to the production of L-malic acid for both strains.

Table 1
Specific and Volumetric L-Malic Acid Production Rates

	Strain 43		Strain 311	
	SP[a] (g/g·h)	VP[b] (g/L·h)	SP[a] (g/g·h)	VP[b] (g/L·h)
Aerobic[c]	0.55	2.24	0.20	0.72
Anaerobic[d]	0.29	1.13	0.11	0.33

[a] SP, Average specific production rate of L-malic acid (g L-malic acid/g cells·h).
[b] VP, Average volumetric production rate of L-malic acid (g L-malic acid/L·h).
[c] Average rates under aerobic condition, incubation time of 34 h.
[d] Average rates under anaerobic condition, incubation time of 25 h.

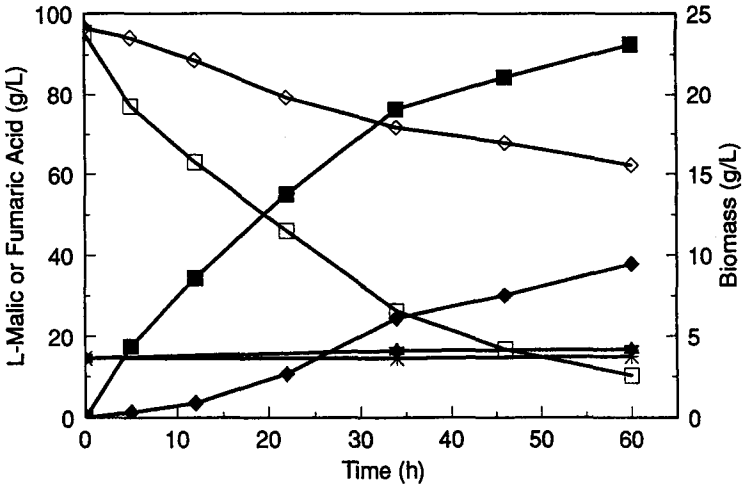

Fig. 3. Kinetic profiles of aerobic L-malic acid production, fumaric acid consumption, and biomass variation employing the wild-type (wt) strain 43 and the respiratory deficient (rd) mutant strain 311: Malic (wt) (-■-), Malic (rd) (-◆-), Fumaric (wt) (-□-), Fumaric (rd) (-◇-), Biomass (wt) (-★-), and Biomass (rd) (-*-).

Slight variations in biomass were also observed. The specific and volumetric production rates of L-malic acid are listed in Table 1. A substantial difference in fumarase activity was implied in the rate data. Hiraga et al. *(13)* also reported a higher enzymatic activity for the mitochondrial fumarase. The exact reason for the observed difference is not clear at this point. It is possible that cytoplasmic fumarase represents a premature version of the enzyme, therefore less active, and needs to be further processed in functional mitochondria to yield the mature fumarase. The specific L-malic acid production rate achieved by the wild-type strain 43 is comparable with our previous results employing the laboratory *S. cerevisiae* strain SHY2 *(6)*. It is also better than, or similar to, most of the specific production rates achieved by *B. ammoniagenes* treated with various detergents, except

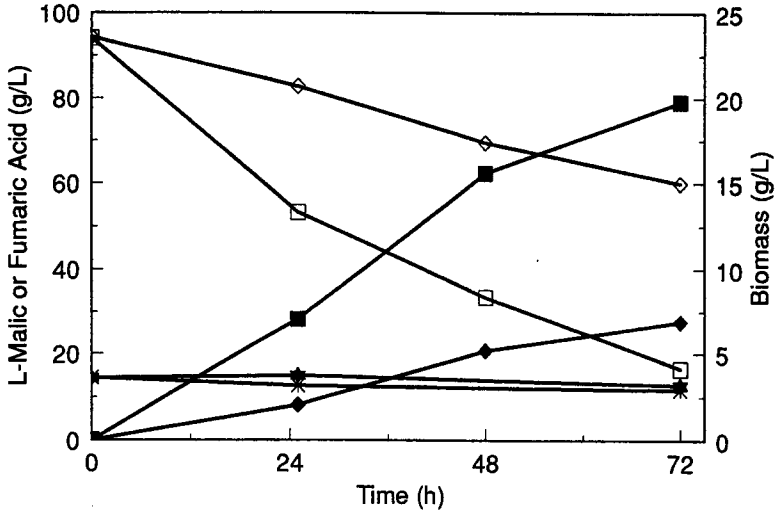

Fig. 4. Kinetic profiles of anaerobic L-malic acid production, fumaric acid consumption, and biomass variation employing the wild-type (wt) strain 43 and the respiratory deficient (rd) mutant strain 311: Malic (wt) (-■-), Malic (rd) (-◆-), Fumaric (wt) (-□-), Fumaric (rd) (-◇-), Biomass (wt) (-★-), and Biomass (rd) (-*-).

bile extract *(4)*. No byproduct, such as succinic acid, was detected in our experiments.

Anaerobic Conversion of Fumaric Acid to L-Malic Acid

Under anaerobic conditions, the respiratory activity of the wild-type strain 43 is also repressed. To investigate any possible effect of respiration on fumarase activity and to place those two strains on the same energetic stage for fair comparison, the authors further conducted the experiment under anaerobic conditions. Strains 43 and 311 were grown and harvested as described above. The same initial biomass concentration (3.62 g/L) was also employed for each strain. The results of anaerobic bioconversion by the wild-type strain and the petite strain are shown in Figure 4. Final L-malic acid concentration achieved at 72 h by strain 43 was much higher, 79.1 g/L, than that achieved by strain 311, 27.6 g/L. Fumaric acid consumption was also closely correlated to the production of L-malic acid for both strains. A slight decrease in final biomass was observed. The specific and volumetric production rates are shown in Table 1. Compared to aerobic bioconversion, anaerobic bioconversion of fumaric acid to L-malic acid was less efficient in both cases employing wild-type and petite mutant strains. Consistent with the data from aerobic bioconversion, the production of L-malic acid by the wild-type strain 43 was also much faster than that by the respiratory-deficient strain 311 under anaerobic conditions.

CONCLUSION

The results indicate that both cytoplasmic and mitochondrial fumarase are functional in the in vivo conversion of fumarate to L-malate. The respiratory-deficient mutant strain containing only the cytoplasmic fumarase, however, appeared to be less active in the bioconversion than the wild-type strain under both aerobic and anaerobic conditions.

ACKNOWLEDGMENTS

This work was supported in part by the National Science Foundation (Grant BES-9412582), and the US Department of Agriculture (Grant 96-35500-3192).

REFERENCES

1. Blair, G. T. and DeFraties, J. J. (1995), in *Kirk-Othmer Encyclopedia of Chemical Technology*, vol. 13, Kroschwitz, J. I. and Howe-Grant, M., eds., John Wiley, New York, pp. 1063–1081.
2. Peleg, Y., Stieglitz, B., and Goldberg, I. (1988), *Appl. Microbiol. Biotechnol.* **28,** 69–75.
3. Battat, E., Peleg, Y., Bercovitz, A., Rokem, J. S., and Goldberg, I. (1991), *Biotechnol. Bioeng.* **37,** 1108–1116.
4. Yamamoto, K., Tosa, T., Yamashita, K., and Chibata, I. (1976), *Eur. J. Appl. Microbiol.* **3,** 169–183.
5. Takata, I., Yamamoto, K., Tosa, T., and Chibata, I. (1980), *Enzyme Microb. Technol.* **2,** 30–36.
6. Wang, X., Gong, C. S., and Tsao, G. T. (1996), *Biotechnol. Lett.* **18,** 1441–1446.
7. Boonyarat, D. and Doonan, S. (1988), *Int. J. Biochem.* **20,** 1125–1132.
8. Wilkie, D. (1983), in *Yeast Genetics: Fundamental and Applied Aspects*, Spencer, J. F. T., Spencer, D. M., and Smith, A. R. W., eds., Springer-Verlag, New York, pp. 255–267.
9. Heerde, E. and Radler, F. (1978), *Arch. Microbiol.* **117,** 269–276.
10. Ogur, M., St. John, R., and Nagai, S. (1957), *Science* **125,** 928–929.
11. Boker-Schmitt, E., Francisci, S., and Schweyen, R. J. (1982), *J. Bacteriol.* **151,** 303–310.
12. Sherman, F., Fink, G. R., and Hicks, J. (1986), *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
13. Hiraga, K., Inoue, I., Manaka, H., and Tuboi, S. (1984), *Biochem. Int.* **9,** 455–461.