0273-2289/94/4701—0065$03.00

L-Malic Acid Production by Entrapped *Saccharomyces cerevisiae* into Polyacrylamide Gel Beads

ELZABETH A. OLIVEIRA, AURELIO A. R. COSTA, ZILDA M. B. FIGUEIREDO, AND LUIZ B. CARVALHO, JR.*

Laboratorio de Enzimologia, Departamento de Bioquimica and Laboratorio de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Cidade Universitaria, 50.760, 901, Recife, PE, Brazil

Received September 28, 1993; Accepted September 29, 1993

ABSTRACT

The yeast *Saccharomyces cerevisiae* was entrapped within polyacrylamide gel beads by employing a procedure that uses sodium dodecylsulfate as a detergent to improve the spherical configuration of the beads. The resulting preparation showed a rate of fumarate bioconversion to L-malic acid about 60 times higher than that found for the free cells. Almost all fumarate was converted in 30 min of incubation. The thermal stability of the immobilized cells did not significantly differ from the free cells. An optimal pH of 5.7 was found for the immobilized preparation and no succinic acid was detected as a byproduct in the incubation mixture.

Index Entries: L-Malic acid; *Saccharomyces cerevisiae;* polyacrylamide gel beads.

INTRODUCTION

L-Malic acid was first industrially produced from fumarate by using cells of *Lactobacillus brevis* (*1*). In 1974, Chibata et al. (2) proposed a technique using entrapped cells of *Brevibacterium ammoniagenes* into polyacrylamide gel. Succinic acid was detected as an undesired byproduct and

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

several procedures were used to overcome it, such as, autolysis, freeze-thawing, thermal treatment associated with detergents, and acetone. An alternative method was reported by Tung-Fu (3) using gamma irradiation to induce gel formation and cholic acid as the detergent. Immobilized *B. ammoniagenes* on acrylamide and calcium alginate was also used to produce L-malic from fumarate (4–6). Takata et al. (7) working with four different genera (*Brevibacterium, Proteus, Pseudomonas,* and *Sarcina*) selected *Brevibacterium flavum* as the best source. Those cells were entrapped onto carragenan and a previous treatment with sodium fumarate containing bile extract increased about 2.3 times the L-malic acid production, compared with the conventional procedures. This method has been proposed in industrial scale. Entrapped *Candida rugosa* into polyacrylamide gel has also been described to produce L-malic acid (8).

In our lab, *Saccharomyces cerevisiae* has been entrapped into polyacrylamide gel discs and the action of detergents, fumarate and malonate on its performance were studied. No succinic acid was detected as a byproduct either working with the free or the immobilized cells (9).

Peleg et al. (10) described a strain of *S. cerevisiae* with a very high conversion rate of fumaric to L-malic acid with no formation of succinic acid by cloning the fumarase gene of this yeast into an expression vector. Afterward, Neufeld et al. (11) studied the kinetics of the bioconversion with this free and immobilized transformed *S. cerevisiae*. The cells were immobilized within agarose beads of varying diameter to examine the effect of diffusion on the measured rates of conversion.

Here, *S. cerevisiae* was entrapped into polyacrylamide gel beads and the optimization of the process was investigated by studying some of the preparation properties.

MATERIAL AND METHODS

S. cerevisiae was obtained from Fleischmann Royal Produtos Alimenticios Ltda., Rio de Janeiro, Brazil. Sodium fumarate and L-malic acid were purchased from The British Drug Houses LTD., Poole, England, and Wako Pure Chemical Industries, Osaka, Japan, respectively. All other reagents were analytical grade obtained from Merck, Brazil.

Treatment of the Free Cells with Malonate

A suspension of the free cells of *S. cerevisiae* (25 mL of a preparation containing 250 mg/mL prepared in 0.9% w/v NaCl) was incubated with 6 m*M* malonate (5 mL) prepared in 0.05 M phosphate buffer, pH 7.5, for 20 h at 37°C with stirring. The cells were centrifuged and washed with the phosphate buffer.

Immobilization of *Saccharomyces cerevisiae* in Polyacrylamide Gel Beads

A solution (2.5 mL) prepared by dissolving 30g of acrylamide and 1.0 g of N,N'-methylenebisacrylamide in 123 mL of water was maintained at 4°C. After addition of the catalyst reagents, consisting of N,N,N'N'-tetramethylethylenediamine 0.28% v/v (1.25 mL) and ammonium persulfate 0.14% w/v (5.0 mL); *S. cerevisiae* (4.8 g), treated with malonate, suspended in 0.1*M* phosphate buffer pH 7.5 (5 mL) and sodium dodecylsulfate (10 mg) were introduced into the mixture. Then this material (ca. 13 mL) was incubated in a solvent mixture (90 mL) composed by chloroform/toluene 2:1 (v/v) under N_2, at 25°C with continuous stirring (about 200 rpm). After approx 20 min of incubation, the gel, now in the form of beads, was thoroughly washed with deionized water followed by 0.01*M* phosphate buffer, pH 7.5, in order to remove unpolymerized toxic monomers, the catalyst reagents, detergent, and organic solvents. The granules were washed with the buffer solution until no protein was present in the effluent as determined by the method described by Lowry et al. (*12*).

Determination of Activity

Free and immobilized cells (0.7 and 0.085 g, respectively) of *S. cerevisiae* were incubated with 30 m*M* fumarate (5 mL) under stirring at 37°C (batch reactor). Aliquots of 0.2 mL were removed from the supernatant at appropriate time intervals and added to 2.8 mL of phosphate buffer for the fumarate determination at 280 nm (Cary spectrophotometer, model 118, USA). A molar extinction coefficient of 0.278 m*M*/cm was used.

Study of Properties

The influence of fumarate concentration on the L-malic acid production by either free or immobilized *S. cerevisiae* was investigated by incubating the cells (0.7 and 0.085 g, respectively) with different concentrations of the substrate, prepared in 0.1*M* phosphate buffer, pH 7.5. Other experimental conditions were as described for the determination of activity. The thermal stability of the free and immobilized *S. cerevisiae* was determined by a previous incubation of the cells with 0.9% w/v NaCl (5 mL) for 10 min at temperatures varying from 37 to 80°C. The effect of pH on the preparations was studied by estimating their activities at pH ranging from 3.7–8.0 using 0.1*M* acetate (3.7–5.6) and 0.1*M* phosphate (5.7–8.0) buffers. Other experimental conditions were similar to those described in the determination of activity.

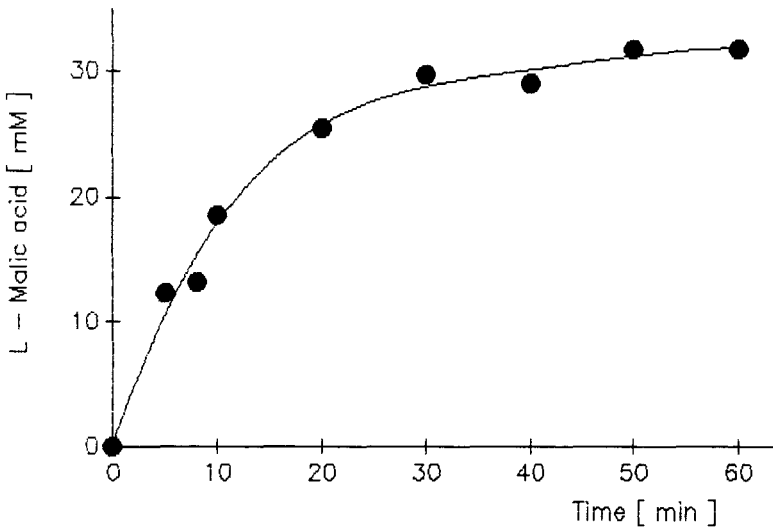

Fig. 1. Time curve of fumarate conversion to malic acid by immobilized *S. cerevisiae* within polyacrylamide gel beads. Immobilized cells (0.085 g) were incubated with 30 m*M* sodium fumarate (5 mL), prepared in 0.1*M* phosphate buffer, pH 7.5, at 37°C, with stirring. Aliquots (0.2 mL) were removed from the incubation mixture at appropriate intervals and their absorbances at 280 nm were established.

RESULTS AND DISCUSSION

The production of L-malic acid from fumarate by using immobilized *S. cerevisiae* into polyacrylamide cells was previously reported by Figueiredo and Carvalho (*9*). The preparation performed better when pretreated with malonate (0.94 mmol of L-malic acid/h/g of immobilized cells). Owing to the feature of no detection of succinic acid as a byproduct, as had been reported for other microorganisms, immobilized *S. cerevisiae* on polyacrylamide pretreated with malonate to synthesize L-malic acid from fumarate showed to be an attractive industrial proposal, although this preparation is about eight times less efficient than that described for immobilized *B. ammoniagenes* (*2*).

In this work, some properties of the entrapped *S. cerevisiae* cells in polyacrylamide was then investigated in order to improve the yield of L-malic acid from fumarate.

First of all, the gel was synthesized as beads instead of discs. A typical time curve of fumarate conversion to malic acid by immobilized *S. cerevisiae* within polyacrylamide gel beads is shown in Fig. 1. From this result it can be seen that all fumarate was nearly converted to L-malic acid in 30 min.

Figure 2 shows the relationship between L-malic acid production and fumarate concentration. The values of maximal rate of the free and immobilized cells were 1.218 mmol/h/g and 60.6 mmol/h/g of cells, respectively.

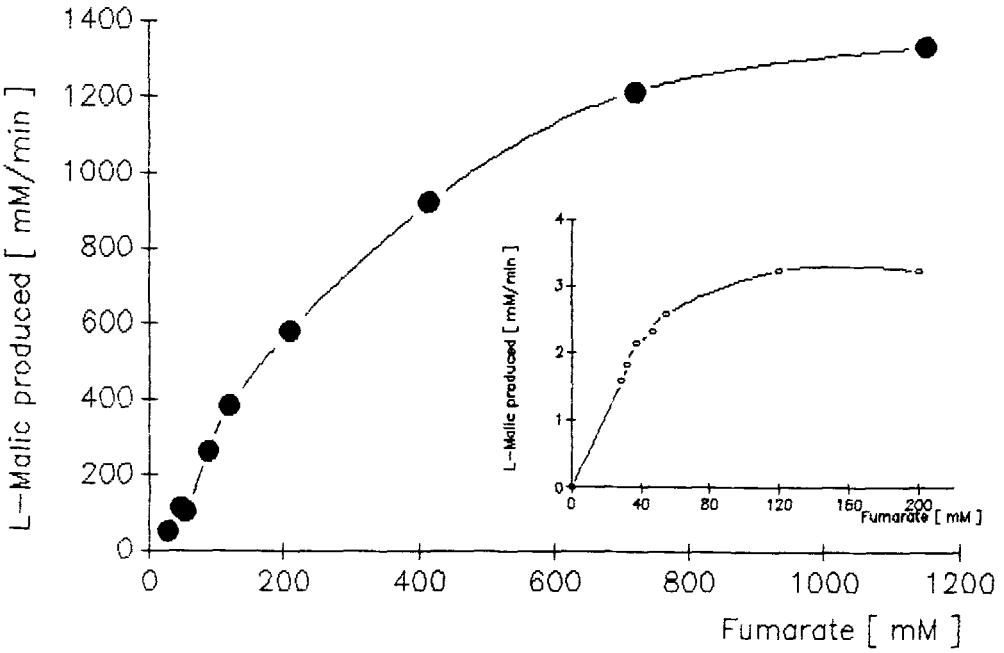

Fig. 2. Relationship between fumarate concentration and L-malic acid produced by entrapped *S. cerevisiae* within polyacrylamide gel beads. Immobilized cells (0.085 g) were incubated with different concentrations of sodium fumarate (5 mL), prepared in 0.1*M* phosphate buffer, pH 7.5. Aliquots (0.2 mL) were removed from the incubation mixture at appropriate intervals and from their absorbances at 280 nm initial velocities were calculated. The results for the free cells are also presented in the insert.

Furthermore, the concentration of fumarate that produces half of the maximal rate was equal to 38.3 and 531.6 m*M* for the free and immobilized cells, respectively. The higher value for the immobilized cells is probably owing to diffusion limitations of the substrate to the fumarase, mainly through the cell wall.

It is important to notice that the rate of L-malic acid produced by the immobilized preparation (60.6 mmol/h/g of cells) is about sixty times higher than that reported by Figueiredo and Carvalho (*9*): 0.94 mmol of L-malic acid/h/g of immobilized cells; and even higher than that described by Yamamoto et al. (*6*): 7.48 mmol of L-malic acid/h/g of immobilized *B. ammoniagenes*. Probably, the difference between the procedures to synthesize the polyacrylamide plays an important role on these results. Here, sodium dodecylsulfate was added to the monomers and catalysts during the polyacrylamide gel formation to "stabilize" the spherical shape of the beads. Provided that the cells are also included in this mixture they are preincubated with sodium dodecyl sulfate. Detergents as sodium dodecylsulfate have been described to alter the cell wall permeability and thus increasing the fumarase activity (*3,6*).

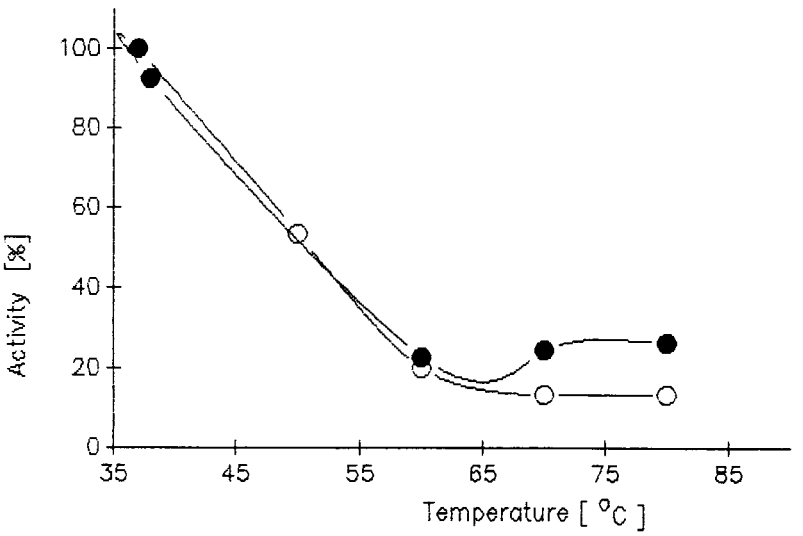

Fig. 3. Thermal stability of entrapped *S. cerevisiae* within polyacrylamide gel beads. Free (0.7 g)—○—and immobilized cells (0.085 g)—●—were incubated at indicated temperatures for 10 min and after room temperature equilibration (about 30 min) their fumarase activities were established.

Furthermore, it is also important to compare the data reported by Neufeld et al. (*11*) with the present results. They described the transformed *S. cerevisiae* as presenting activities of 65 m*M*/h/g (1.95 mmol of L-malic acid/h/g in 30 mL of fumaric acid 1*M*) and 70 m*M*/h/g (2.1 mmol of L-malic acid/h/g in 30 mL of fumaric acid 1*M*) for the free and immobilized cells, respectively. A rate of 0.7 m*M*/h/g (0.21 mmol of L-malic acid/h/g) was found for the wild-type strain. This rate is lower than the one shown in this communication.

Figure 3 shows the thermal stability of the immobilized *S. cerevisiae* that did not significantly differ from the pattern of the free cell. The rate of L-malic acid production decreased in both preparations at temperatures higher than used throughout the present study, namely, 37°C.

The pH profile of the L-malic acid production by immobilized *S. cerevisiae* is depicted in Fig. 4. The optimal pH for the immobilized preparation was found to be 5.7, which is lower than that reported for the soluble fumarase (pH 7.5). From this result one can conclude that an increase of 4.5 times would occur if the pH of L-malic acid production was set up to this pH instead of 7.5.

The presence of succinic acid was not detected as one of the products of the incubation of fumarate with the free and immobilized *S. cerevisiae*. L-malic acid, fumarate, and succinic acid were analyzed by paper chromatography as recommended by Yamamoto et al. (*6*). Thus, succinic acid contamination will be negligible provided experimental conditions described are followed.

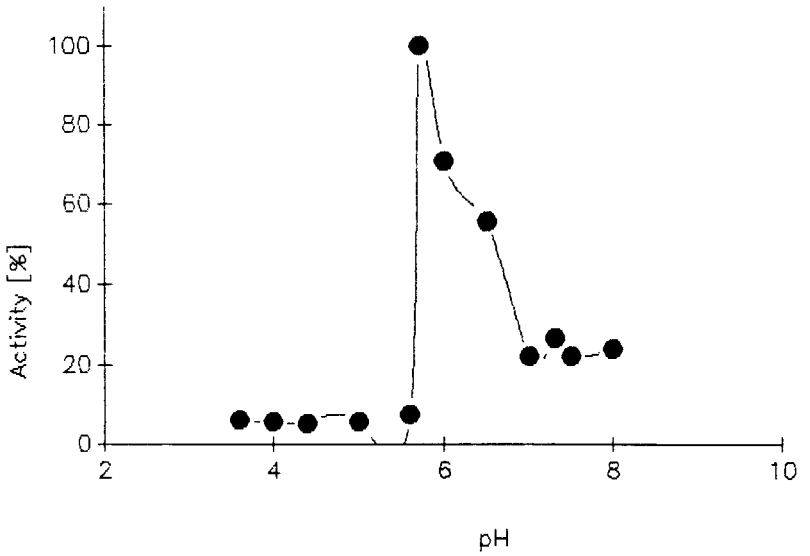

Fig. 4. pH Profile for the fumarate conversion to L-malic acid by entrapped *S. cerevisiae* within polyacrylamide gel beads. Immobilized cells (0.085 g) were incubated with 30 m*M* sodium fumarate (5 mL) prepared in either acetate buffer (pH 3.7–5.6) or phosphate buffer (pH 5.7–8.0) and their fumarase activities were established.

The results obtained in the present study suggest as the best operational conditions of L-malic acid production from fumarate by entrapped *S. cerevisiae* cells into polyacrylamide gel beads: 1*M* fumarate prepared in 0.1*M* sodium phosphate buffer, pH 5.7, at 37°C, using pretreated cells with 6 m*M* malonate prepared in 0.05*M* sodium phosphate buffer, pH 5.7. Under these conditions an enhanced L-malic acid products would be achieved.

ACKNOWLEDGMENTS

This work was financially supported by CNPq, FACEPE, FINEP (Brazilian agencies), and Japan International Cooperation Agency. The authors are deeply grateful to Howard H. Weetall for his helpful comments and Otaviano T. Costa for his skillful technical assistance.

REFERENCES

1. Kitahara, K., Fukui, S., and Misawa, M. (1960), *J. Gen. Appl. Microbiol.* **6(2),** 108–116.
2. Chibata, I., Tosa, T., and Sato, T. (1974), *Appl. Microbiol.* **27,** 878–885.

3. Tung-Fu, L. (1982), *Radiat. Phys. Chem.* **20(3),** 209–214.
4. Ger Offen. 2,450,137 (Cl. co7cg). 24 Apr. 1975. Japan. Appl. 73 118,832. 22 Oct. 1973. 30 p.
5. Yamamoto, K., Tosa, T., Yamashita, K., and Chibata, I. (1977), *Biotechnol. Bioeng.* **19(8),** 1101–1114.
6. Yamamoto, K., Tosa, T., Yamashita, K., and Chibata, I. (1976), *Eur. J. Appl. Microbiol. Biotechnol.* **3,** 169–183.
7. Takata, I., Yamamoto, K., Tosa, T., and Chibata, I. (1979), *Eur. J. Appl. Microbiol. Biotechnol.* **7,** 161–172.
8. Yang, L. and Zhong, L. (1980), *Wei Shing Wusuch PaO* **20(3),** 296–302.
9. Figueiredo, Z. M. B. and Carvalho, L. B., Jr. (1991), *Appl. Biochem. Biotechnol.* **30,** 217–224.
10. Peleg, Y., Rokem, J. S., Goldberg, I., and Pines, O. (1990), *Appl. Environ. Microbiol.* **56,** 2777–2783.
11. Neufeld, R. J., Peleg, Y., Okem, J. S., Pines, O., and Goldberg, I. (1991), *Enzyme Microb. Technol.* **13,** 991–996.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.