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L-Malic Acid Production Using Immobilized *Saccharomyces cerevisiae*

Z. M. B. FIGUEIREDO AND L. B. CARVALHO, JR.*

Laboratorio de Enzimologia, Departamento de Bioquimica, Centro de Ciencias Biologicas, Universidade Federal de Pernambuco, Cidade Universitaria, 50,000, Recife, Brazil

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ABSTRACT

L-Malate was produced from fumarate by using immobilized *Saccharomyces cerevisiae* cells entrapped in polyacrylamide. This preparation performed better when pretreated with malonate. Under the experimental conditions described here, succinate was not detected as a by-product of the reaction, as had been reported for other microorganisms.

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

INTRODUCTION

Fumarase (EC: 4.2.1.2) is an enzyme found in many biological systems, such as bacteria (*1*), molds (*2*), yeasts (*3*), higher plants (*3*), and animals (*4*), as part of the enzymes of the tricarboxylic acid cycle (*5*) catalyzing the following reaction:

$$\text{Fumarate} + H_2O \rightarrow \text{L-malate}$$

The product of this reaction, L-malate, is widely employed in pharmaceutical industries (*6,7*), and in the food industry it is used as an acidulant (*8,9*). Therefore, fumarase has become industrially important. L-Malate was first obtained in large quantities from fumarate using *Lactobacillus*

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

Table 1
Preparations of Immobilized Cells and Organells Producing L-Malic Acid from Fumarate

Preparation	Matrices	Reference
Brevibacterium	carrageenan	10
	polyacrylamide	11
B.ammoniagenes	polyacrylamide	12,13
	acryloylamide	14
	calcium alginate	15
B.flavum	carrageenan	16
	carrageenan plus polyethyleneimine	17
	k-carrageenan	18-20
	k-carrageenan modified	21
	k-carrageenan plus Chinese gallotannin	22
Candida rugosa	polyacrylamide	23
B.flavum;	amine derivatives of k-carrageenan	24
B.ammoniagenes	k-carrageenan plus polyethyleneimine	25

brevis (*5*). In 1974, immobilized *Brevibacterium ammoniagenes* was used instead of this method (*7*). Since then, several aspects of the process have been modified to create a system that is more efficient than the previous one (Table 1).

Although there are many advantages of immobilizing microorganisms, rather than enzymes, to produce certain chemicals (*26*), an undesirable outcome can occur, such as the release of other compounds as side effects of the presence of multienzymatic complexes. For instance, Yamamoto et al. (*7*) have found succinic acid as a byproduct of the action of immobilized *B. ammoniagenes* on fumaric acid to produce L-malic acid. They proposed several procedures to overcome the formation of this other tricarboxylic compound: autolysis, freeze-thawing, heat treatment, acetone, detergents, and bile-extract treatment (shown to be the most efficient way to decrease succinic acid production).

In this paper is described L-malic acid production by the action of immobilized *Saccharomyces cerevisiae* on fumaric acid; no succinic acid was detected under the experimental conditions used, which were similar to those described by Yamamoto et al. (*7*).

MATERIAL AND METHODS

S. cerevisiae was obtained from Fleischmann Royal Produtos Alimenticios Ltda., Brazil. Sodium fumarate, L-malic acid, and sodium cholate were purchased from The British Drug Houses Ltd., England; Wako Pure Chemical Industries, Japan; and Nutritional Biochemicals Corporation,

USA, respectively. All other reagents were analytical grade, obtained from Merck, Brazil.

The cells (3 g wet wt) were suspended in 0.9% w/v NaCl (12 mL). Then acrylamide (2.25 g), *N,N'*-methylenebisacrylamide (0.12 g). *N,N,N',N'*-tetrametylethylenediamine (1.5 mL), and 1% w/v ammonium persulfate (1.5 mL) were added to the suspension. This mixture was incubated in a Petri dish containing a grid that allowed disks of gel measuring 4 mm in diameter and 3 mm thick to be obtained after polymerization (ca. 15 min). These disks were washed with the NaCl solution and kept in 0.1*M* phosphate buffer, pH 7.5, at 0–4°C, until use.

A suspension of free *S. cerevisiae* in 0.9% w/v NaCl (625 mg wet wt of cells of 2.5 mL) was incubated with 50 m*M* sodium fumarate prepared in 0.1*M* phosphate buffer, pH 7.5, containing detergents (0.2% v/v Triton X-100; 0.3% w/v sodium cholate; 0.2% w/v sodium deoxycholate, and 0.02% w/v sodium dodecyl sulfate) at 37°C for 20 h with constant stirring. Afterwards, these cells were centrifuged at 1085*g*, washed with the buffer solution, and used for the determination of fumarase activity. Identical procedures were carried out with the other sample of *S. cerevisiae*, except that 6 m*M* malonate replaced sodium fumarate and the concentration of the detergents was lower (0.01% v/v Triton X-100; 0.002% w/v sodium deoxycholate, and 0.0003% w/v sodium dodecylsulfate), as recommended by Godbole et al. (*27*). Controls were established by incubating the cell suspension with sodium fumarate and 6 m*M* malonate prepared in phosphate buffer.

Immobilized *S. cerevisiase* (ca. 0.7 g) was also incubated in either 50 m*M* sodium fumarate or 6 m*M* malonate (5 mL) prepared in phosphate buffer, both containing the detergents described above at the same concentrations and in the absence of those detergents (controls). After these treatments, the disks were washed with 0.9% w/v NaCl and used for the determination of the fumarase activity.

The action of the free and immobilized *S. cerevisiae* on fumarate (enzymatic activity) was carried out by incubating either the cells or their immobilized derivatives with 50 m*M* sodium fumarate (5 mL) prepared in 0.1M phosphate buffer, pH 7.5, with stirring, at 37°C. Samples were removed at appropriate time intervals, and centrifuged to obtain the free cells (1085*g*) or filtered to obtain the immobilized cells. Then 0.2 mL of aqueous phase was 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 $mM^{-1} \cdot cm^{-1}$ was used. One International Unit (IU) was defined as the amount of enzyme capable of consuming one micromole of fumarate per minute under the experimental conditions. The samples (2 mL) obtained after 1 h of incubation were also used for the detection of succinic acid, according to the method of Yamamoto et al. (*7*).

RESULTS AND DISCUSSION

As described above, one of the disadvantages of using immobilized organelles or cells instead of an enzyme is the possible formation of by-products, since multienzymatic systems will participate in the process. This is the problem involved in the production of L-malic acid using immobilized microorganisms like *Brevibacterium ammoniagenes* (*7*), in which succinic acid is released from another step of the tricarboxylic acid cycle. Tung-Fu (*11*), working with immobilized microorganisms, showed the effect on the cell wall of cholic acid, which alters the permeability and consequently increases the fumarase activity. Yamamoto et al. (*7*) explained this possible action of detergents as follows:

1. The stability of fumarase and succinic dehydrogenase are different with respect to the detergents.
2. Either cofactors essential to the formation of succinic acid are released from the cells, or their regeneration is stopped under detergent action.
3. In addition to the decrease of succinic acid formation, substrate and product can access the fumarase easily because of the membrane alterations, and the rate of the reaction increases.

Godbole et al. (*27*) added malonate to the detergent to inhibit the succinic dehydrogenase.

The action of detergents on the fumarase activity of free *S. cerevisiae* is presented in Table 2. The specific activity was found to be 11.90 IU/g of cells without treatment. An increase in specific activity was observed when a previous treatment of the cells was preceded with 50 m*M* fumarate (17.50 or ca. 50%). However, treatment with 1*M* fumarate reduced the activity to 9.94 IU/g of cell. Inhibition resulting from an excess of substrate probably caused this decrease, since this phenomenom has been reported for the free enzyme (*28*). It is worthwhile to note that treatment with fumarate plus detergents decreased the specific activity of *S. cerevisiae*, except when Triton X-100 was used. These findings contradict those reported in the literature (*7*). However, malonate preincubation increased the specific activity, although the addition of detergents did not change the value considerably from that calculated for the untreated *S. cerevisiae*.

The effect of detergents on the immobilized *S. cerevisiae* is shown in Table 3. This preparation showed a specific activity of 5.81 IU/g of cells, i.e., 48.8% of that observed for the free cell. The activity increased when previously treated with fumarate and fumarate plus Triton X-100, whereas all other treatments (fumarate plus other detergents) reduced it. These results are similar to those observed for the free cells. A substantial increase in activity was found with pretreatment with either malonate or malonate plus detergents, particularly Triton X-100. Again, this behavior followed a pattern similar to that noted for the free cells.

Table 2
Action of Detergents on Free *S. cerevisiae*[a]

Treatment	Cells (g)	Activity (IU)	Specific activity (IU/g)	Observed activity (%)[b]
no treatment	0.20	2.38	11.90	100.0
50 mM fumarate	0.14	2.45	17.50	147.0
1 M fumarate	0.21	2.14	9.94	83.5
fumarate + sodium cholate	0.12	0.97	8.08	67.9
fumarate + sodium deoxycholate	0.14	1.33	9.50	79.8
fumarate + sodium dodecyl sulphate	0.12	1.02	8.50	71.4
fumarate + triton X-100	0.10	1.92	19.20	161.3
malonate	0.18	1.80	9.75	81.9
malonate + sodium cholate	0.16	1.96	11.91	100.0
malonate + sodium dodecyl sulphate	0.13	1.87	13.84	116.3
malonate + triton X-100	0.11	1.73	15.08	126.7

[a]A suspension of *S. cerevisiae* (2.5 mL of a preparation containing 250 mg/mL) in 0.9% w/v NaCl was incubated with 50 m*M* sodium fumarate (5 mL), prepared in 0.1 *M* phosphate buffer, pH 7.5, containing the listed detergents, for 20 h at 37°C, with stirring. Afterward, the cells were centrifuged, washed with the buffer, and incubated with 50 m*M* sodium fumarate, prepared in the same buffer, at 37°C, with stirring. Samples were removed at appropriate time intervals and centrifuged, and fumarate was spectrophotometrically (280 nm) determined in the supernatant. Treatments of free cells were also carried out, in which 50 m*M* fumarate was replaced by 1 *M* fumarate, 6 m*M* malonate, and 6 m*M* malonate plus detergents as recommended by Godbole et al. (*27*). Free *S. cerevisiae* not submitted to any treatment was used as a control.

[b]100% was considered the specific activity of the free *S. cerevisiae* not submitted to treatment.

A comparison between the specific activities found for the free and the immobilized cells shows that they are reduced for the latter, except when pretreated with malonate (alone and plus detergents). These results suggest that concentration of detergent can play an important role as a tool for altering the properties of the cells, since lower concentrations of detergents were used in the pretreatment with malonate. Furthermore, they showed that immobilized *S. cerevisiae* on polyacrylamide, pretreated with malonate, can be an alternative to synthesize L-malic acid from fumaric acid.

The presence of succinic acid was not detected using the paper chromatography procedure recommended by Yamamoto et al. (*7*). Both the supernatants obtained from the incubation mixture of the free and immobilized *S. cerevisiae* were tested. Godbole et al. (*27*) reported that, for 1.5 mol of L-malic acid produced by rat liver mitochondria, 0.5 mol of succinic acid is formed (3:1). Yamamoto et al. (*7*), working with immobilized *Brevibacterium ammoniagenes* on polyacrylamide acting on fumarate, detected 1–5% of the L-malic acid produced in terms of succinic acid. Therefore, the absence of succinic acid in this work cannot be attributable to a limitation of the assay method employed. Thus, provided that the experimental

Table 3
Action of Detergents on Immobilized *S. cerevisiae*[a]

Treatment	Cells (g)	Activity (IU)	Specific activity (IU/g)	Observed activity (%)*
No treatment	0.17	0.97	5.81	48.8
fumarate	0.17	1.11	6.33	53.1
fumarate + sodium cholate	0.18	0.67	3.74	31.4
fumarate + sodium deoxycholate	0.17	0.66	3.78	31.7
fumarate + sodium dodecyl sulphate	0.18	0.71	3.86	32.4
fumarate + triton X-100	0.18	1.36	7.63	64.1
malonate	0.19	3.11	15.77	132.4
malonate + sodium deoxycholate	0.19	2.08	10.54	88.6
malonate + sodium dodecyl sulphate	0.19	1.94	9.77	82.0
malonate + triton X-100	0.19	3.06	15.37	129.1

[a]Immobilized cells on polyacryamide (ca 0.7 g) were incubated in either 50 m*M* sodium fumarate (5 mL), or 6 m*M* malonate (5 mL) prepared in 0.1 *M* phosphate buffer, pH 7.5, containing the listed detergents, for 20 h at 37°C, with stirring. After these treatments, the immobilized cells were washed with 0.9% w/v NaCl and incubated with 50 m*M* sodium fumarate, prepared in the same buffer, at 37°C, with stirring. Samples were removed at appropriate time intervals, filtered, and fumarate was spectrophotometrically (280 nm) determined in the aqueous phase. Disks not submitted to any treatment were used as controls, as well as immobilized cells treated only with fumarate or malonate.

[b]100% was considered the specific activity of the free *S. cerevisiae* not submitted to treatment.

Table 4
Production of L-Malic Acid from Fumaric Acid by the Action of Immobilized *S. cerevisiae* on Polyacrylamide

Treatment	L-malic acid produced (mmol/h/g cell)	L-malic acid produced (mmol/h/g gel)
no treatment	0.34	0.09
fumarate	0.38	0.10
fumarate + sodium cholate	0.22	0.06
fumarate + sodium deoxycholate	0.25	0.07
fumarate + sodium dodecyl sulphate	0.23	0.06
fumarate + triton X-100	0.45	0.12
malonate	0.94	0.25
malonate + sodium dodecylsulphate	0.63	0.16
malonate + sodium deoxycholate	0.60	0.15
malonate + triton X-100	0.91	0.24

conditions described here are followed, succinic acid contamination will be negligible.

Finally, production of L-malic acid by the methods proposed here is expressed in terms of mmol/h/g of cell and mmol/h/g of gel in Table 4. Comparing these figures with those presented by Yamamoto et al. (*7*), the best performance found in this work (0.94 mmol of L-malic acid/h/g of cells—immobilized cells pretreated with malonate) is still about eight times less efficient than that described for immobilized *B. ammoniagenes* (7.48 mol of L-malic acid/h/g of cell preparation pretreated with bilis extract). Improvements in the present study are worthwhile, since this procedure has the advantage of not producing succinic acid as a byproduct of the process. Using immobilized *S. cerevisiae* on polyacrylamide, pretreated with malonate, to synthesize L-malic acid from fumaric acid can be an attractive industrial proposal.

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