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Immobilization of *Leuconostoc oenos* Cells for Wine Deacidification

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포도주의 신맛 조절을 위한 *Leuconostoc oenos* 세포의 고정화

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Abstract

By using whole cells of *Leuconostoc oenos* ML-34 immobilized in polyacrylamide gel, deacidification of grape juice and wine was attempted. The immobilization did not destroy the original malo-lactic fermentation ability of the cells. However, the speed of malic acid decomposition by the immobilized cells was slow due to the slow transportation of the substrate through the gel layer. By reducing malic acid content in grape juice to a desired degree one may control the level of acid taste in wine fermented with the treated grape juice.

Introduction

Moderate acidity is of importance among several factors influencing wine taste. The acid taste of wine comes from acid components of grapes, mainly malic acid and tartaric acid. When high acid grapes are used, several practices are employed to deacidify grape musts and wines. Much work[15~17,22] has been devoted to the development of effective procedures of adjusting acidity, which include amelioration, blending, neutralization with carbonate salts, precipitation of double salts of malate and tartarate with calcium carbonate, the use of anion-exchange resins, and biological degradation of L-malic acid with so-called malo-lactic bacteria[7~10,19,21] and *Schizosaccharomyces pombe* [1,3,4,13,18,24]. Although wine deacidification was successful, such treated wines have usually been found to be atypical in aroma and taste. Wines which underwent malo-lactic fermentation (bacterial conversion of L-malate to L-lactate and carbon dioxide) have been preferred organoleptically[19] and thus have received much interest. However, few wineries today have active programs of malo-lactic fermentation. In fact, it is not easy to have a right degree of deacidification during the stage of fermentation or aging. Since malo-lactic organisms are fastidious in nutritional requirement, it is hard to initiate malo-lactic fermentation in the tanks of fermentation or aging. Once the initiation is achieved, however, there is no way to terminate the malo-lactic fermentation at proper time without damaging wine quality. In the present study, by using the whole cells of a malo-lactic strain immobilized in polyacrylamide gel, a cont-

rolable deacidification in wine was attempted.

Materials and Methods

Chemicals

N, N'-Methylene-bis-acrylamide (BIS), β-dimethylamino propionitrile (DMAP), and malic dehydrogenase were obtained from Sigma Co. Acrylamide monomer and potassium persulfate were purchased from Wako Pure Chemical Co. and Mallinckrodt Inc., respectively.

Preparation of the immobilized cells

Leuconostoc oenos ML-34 used was a gift from Dr. G. J. Pilone, The Christian Brothers, Mont La Salle Vineyards, Napa, CA., USA. This strain was grown statically in Tomato Juice Glucose Broth (TGB)[20] enriched with 0.3% DL-malate for 5 days at pH 4.5. The cells were collected by centrifugation and washed with 0.9% cold saline solution. The cells (2 *g* wet weight) were suspended in 5 *ml* of saline solution. To the cell suspension, 0.5 *ml* of acrylamide monomer solution (1.5 *g/ml*), 1 *ml* of BIS solution (0.04 *g/ml*), 0.5 *ml* of DMAP solution (5%, v/v) and 0.5 *ml* of potassium persulfate solution (2.5%, w/v) were added. The polymerization was completed in a chamber which was consisted of two parallel glass plates (20×20 *cm*) kept 1.5 *mm* apart by Tygon tubing[11].

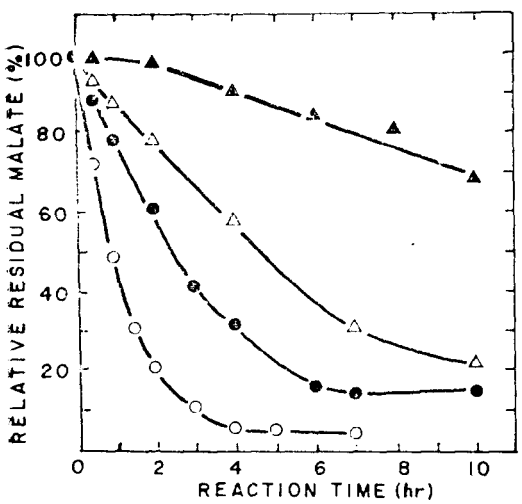

Fig. 1. Effect of immobilization on L-malic acid decomposition by *L. oenos* ML-34 cells
In 30 *ml* of L-malic acid solution (15 *mM*) 2 *g* (wet weight) of free(○) and immobilized cells were added and incubated at 33°C. The average particle sizes (diameter) of cell-imbedded gel were; 0.5 *mm* (●), 1.0 *mm*(△), and 1.5 *mm*(▲).

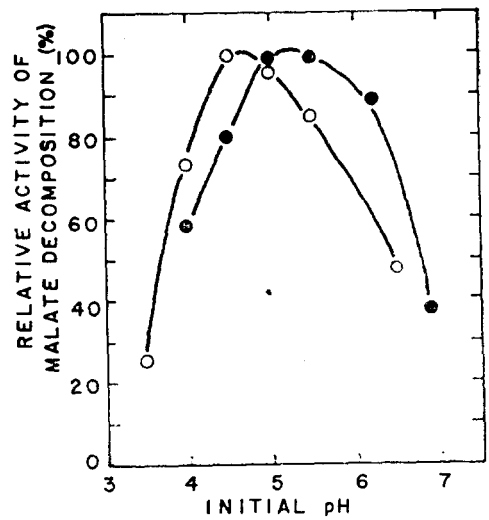

Fig. 2. Effect of initial pH on the ability of L-malic acid decomposition by free (○) and immobilized (●) cells of *L. oenos* ML-34
Used 0.1*M* acetate buffer for pH 3.5~5.5 and 0.1 *M* phosphate buffer for pH 6.0~7.0. Other conditions as shown in Fig. 1.

During polymerization, the chamber was kept cold in an ice bath. The gel sheets thus obtained were subdivided by forcing through a sieve having apertures of certain size to get gel particles. The particles were washed with 0.9% saline solution until the decanted fluid showed absorbance less than 0.05 at 280 *nm*. The immobilized cells were activated by incubation in 15 *mM* malate solution at 50°C for 30 min.

Assay of malate-decomposing activity

The malte-decomposing activity of ML-34 cells was determined by measuring the change of malate concentration in reaction mixture. A reaction mixture of 30 *ml* L-malate solution (15 *mM*) containing gel particles embedded with 2 *g* wet cells was incubated at 33°C with shaking (200 rpm). After the incubation the immobilized cells were filter-

ed off and the amount of L-malate remained in the filtrate was determined quantitatively with malic dehydrogenase[12]. The activity was expressed as *mmoles* of L-malic acid decomposed per hr under the conditions described.

Results

Possibility of immobilization

The original ability of L-malic acid decomposition of *Leuconstoc oenos* ML-34 cells was found to be retained substantially after the cells were immobilized (Fig. 1). The efficiency of L-malic acid decomposition, however, was dependent on the size of the gel particle which embeded cells. Apparently, the time for the substrate to diffuse through the gel layer to reach the cells governs the decomposition efficiency.

L-malate decomposition character

Using the ML-34 cells immobilized in 0.5 *mm* gel particles the mode of L-malic acid decomposition under different reaction conditions was observed comparing with that of free cells. The immobilization changed slightly the pH profile with the shift of optimum value from 4.5 for free cells to 5.5 for immobilized cells (Fig. 2). The similar change was also observed in temperature profile (Fig. 3); optimum temperature of 28°C for intact cells shifted to 35°C for immobilzed cells. The maximum reaction veloicity for the free cells, $V_{max}=10.53$, reduced to one half, $V_{max}=4.63$, when the cells were immobilized (Fig. 4-A). The K_m values, however, showed little change; 5.52 for free cells and 4.65 for immobilized cells (Fig. 4-B). These facts suggest that the drastic reduction in reaction velocity associated with the immobilization is entirely due to the physical barrier of the gel layer and not to any alteration in microbial enzyme itself.

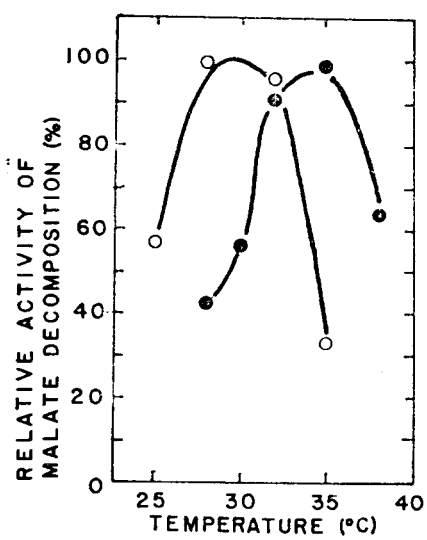

Fig. 3. Effect of temperature on the ability of L-malic acid decomposition by free (○) and immobilized (●) cells of *L. oenos* ML-34

Reaction condition as shown in Fig. 1.

Table 1. Stability of immobilized *L. oenos* ML-34 cells during repeated uses*

Number of uses	Malate decomposing activity**	Relative activity (%)
0	120	100
5	122	102
10	118	98
12	104	87
14	90	75
16	77	64
18	52	43

* For procedures see text.

** μM malate decomposed/hr/*ml* reaction mixture.

Stability

The stability on the L-malic acid decomposing activity of the immobilized cells was tested by using them repeatedly. Fifteen *mmole* of L-malic acid in 30 *ml* reaction mixture was treated with 2 *g* (wet weight) of ML-34 cells entrapped in polyacrylamide gel (0.5 *mm* diameter) for 10 hr under continuous shaking (200 rpm) at 33°C. After the treatment the gel particles were recovered by filtration and washed thoroughly with saline solution. The recovered immobilized cells were reused to treat fresh substrate solutions. At each treatment the activity of cells was determined by assaying the amount of L-malic acid decomposed during the first 30 min of the reaction. As shown in Table 1 the activity of the cells decreased with repeated uses. However, the loss of activity was not significant until 10 sequential uses after which the

Table 2. Reactivation of malate decomposing activity of the immobilized *L. oenos* ML-34 cells*

Incubation media	Malate decomposing activity	Relative activity(%)
None	56	100
TGB**	58	104
TGB+Malate(0.3%)	72	129
TGB+Malate(0.03%)+Mn^{++}(1 *mM*)	81	145
Glucose(0.2%)+Malate(0.3%)	65	116
Glucose(0.2%)+Malate(0.3%)+Mn^{++}(1 *m M*)	78	139

* Immobilized cells having poor activity after repeated uses were reactivated by incubating in the listed media for 24 hr at 28°C.

** Tomato juice glucose broth used for cell culture as described in text.

cells were inactivated rapidly. The activity lost during the repeated uses could be restored substantially by incubating the immobilized cells in culture medium or glucose solution having L-malic acid (Table 2). Addition of Mn^{2+} to the incubation solutions allowed better reactivation of the spent immobilized cells.

Practical trials

Freshly brewed white wine was treated with free and immobilized ML-34 cells to reduce L-malic acid content. As shown in Fig. 5, the immobilized cells showed little effect in deacidification during the 16 hr treatment especially when the sulfite content in the wine was high. However, when the cells were applied at the stage of grape juice prior to fermentation and sulfite additon, the effect was high and more than 50% of the original L-malic acid was removed within 12 hr even with the immobilized cells (Fig. 6).

Discussion

L. oenos ML-34 cells, which have been used to reduce acidity in wines through malo-lactic fermentation, a conversion of dicarboxilic malic acid to monocarboxilic lactic acid, could be immobilized in polyacrylamide gel without any signficant alterations in the characleristics of their enzymes involved. Furthermore, the immobilized cells were quite stable during the 10 repeated uses. Hence,

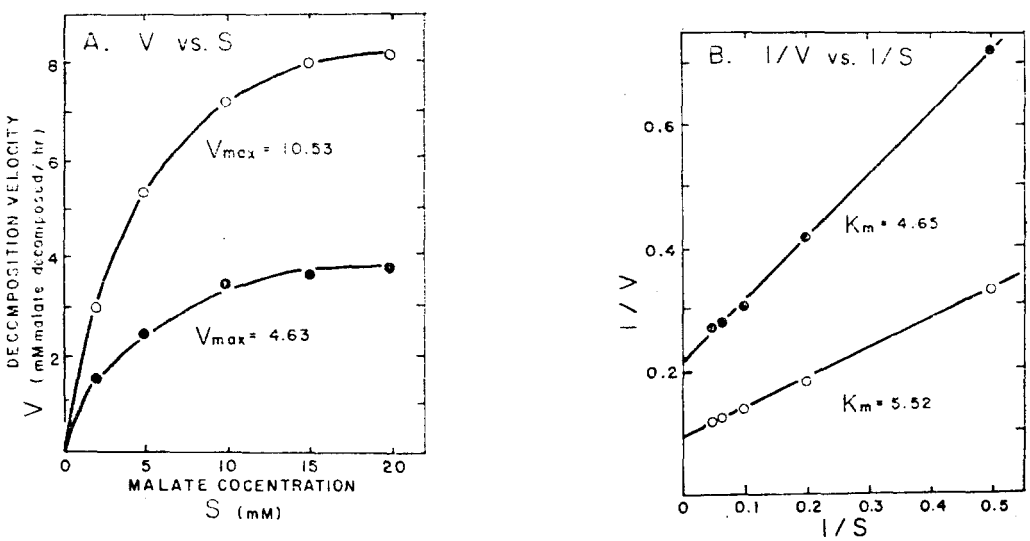

Fig. 4-A, B. Effect of substrate concentration on the initial rate of L-malic acid decomposition by free (○) and immobilized (●) cells of *L. oenos* ML-34
Reaction conditions as shown in Fig. 1.

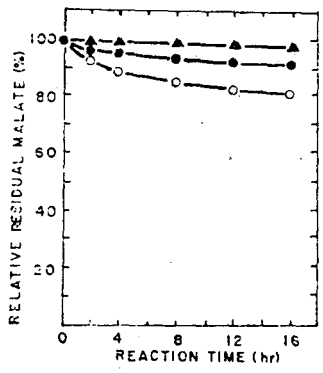

Fig. 5. Treatment of wine with *L. oenos* ML-34 cells

Fresh white wines (30 *ml* each) containing 12% ethanol, 50 ppm sulfite (○, ●) and 100 ppm sulfite (△, ▲) were treated with 2 *g* each of free cells (○, △) and immobilized cells (●, ▲).

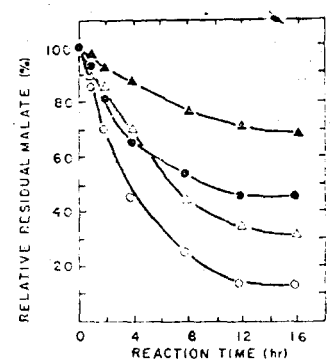

Fig. 6. Treatment of grape juice with *L. oenos* ML-34 cells

Fresh white grape juices (30 *ml* each) containing 0 ppm sulfite (○, ●) and 50 ppm sulfite (△, ▲) were treated with 2 *g* each of free cells (○, △) and immobilized cellls (●, ▲).

this repeated use may save production cost substantially and further savings may be expected by reactivating the spent cells simply by incubating in proper solution (Table 2). The largest advantage for using the immobilized cells, however, would be the possibility of controlling the degree of malo-lactic fermentation in grape juice and/or wine. Simply by adjusting the amount of immobilized cells or the length of incubation time one may obtain desired levels of acid taste in wine.

Embedding the active cells within gel particles, however, reduced the velocity of malate decomposition drastically. Reduction in particle size allowing faster reach of substrate to the cells may have certain limit, because particle sizes affect flow rates when immobilized cells are packed in a colum and through which wine or grape juice is passed to remove malic acid. The suitable gel particle size for the most effective deacidification should be decided by combined studies on malate decomposition velocity along with column efficiency.

For a practical use of the immobilized ML-34 cells in controlling wine flavor the stage of fresh grape juice should be chosen. The high alcohol content together with the added sulfite in the fermented wine prolong the malate removal time and consequently disqualify the immobilized cells, because they cannot be inoculated in fermentors or aging tanks and be remained for a long period of time as the free cells.

요 약

Leuconostoc oenos ML-34균의 세포를 polyacrylamide gel 속에 고정시키고, 이것을 이용하여 포도즙과 포도주의 신맛을 감소시켜 보았다. 세포가 가졌던 malo-lactic 발효능은 고정화 시킴으로서 감소되지는 않았다. 그러나 고정화 세포에 의한 사과산 분해의 속도는 느려졌는데, 이것은 기질이 gel층을 통과하는데 시간이 걸리기 때문이었다. 고정화 세포로서 포도즙 속의 사과산의 양을 적절한 수준까지 감소시킴으로서 포도주의 신맛을 조절할 수 있을 것 같다.

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