

# Manganese Ion Increases LAB-yeast Mixed-species Biofilm Formation

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Remarkable LAB-yeast mixed-species biofilm was formed by lactic acid bacteria (LAB) *Lactobacillus plantarum* ML11-11 isolated from Fukuyama pot vinegar and *Saccharomyces cerevisiae*. This mixed-species biofilm formation increased in proportion to the YPD medium concentration but decreased in proportion to the MRS medium concentration. The effect of MRS components on mixed-species biofilm formation was investigated in a YPD medium environment, and it was clarified that beef extract (one of the MRS medium components) decreased mixed-species biofilm formation. On the other hand, manganese sulfate (another component in MRS) remarkably increased both LAB single- and LAB-yeast mixed-species biofilm formation. LAB single- and mixed-species biofilm formation were increased in proportion to the manganese sulfate concentration up to 1 mM and 100  $\mu$ M, respectively. The growth of *L. plantarum* ML11-11 was increased significantly by the addition of 10  $\mu$ M manganese sulfate and was resistant to higher concentration of up to 100 mM, but growth of *S. cerevisiae* was sensitive to manganese ion above 100  $\mu$ M. These results suggested that mixed-species biofilm formation could be controlled artificially by controlling the manganese ion level.

**Key words:** manganese ion, mixed-species biofilm, *Lactobacillus plantarum*, *Saccharomyces cerevisiae*

## INTRODUCTION

Formation of a biofilm, a microbial community on the interface [1–3], is important in traditional fermentations that start from solid biomaterials [4, 5].

Previously, we reported that a mixed-species biofilm is formed by lactic acid bacteria (LAB) *Lactobacillus plantarum* ML11-11 isolated from Fukuyama pot vinegar, a traditional Japanese and domestic type of rice vinegar [6–8], and yeast *Saccharomyces cerevisiae* with direct cell-cell contact [7] and also reported co-aggregation properties between *L. plantarum* ML11-11 and *S. cerevisiae* [6].

LAB and yeast mixed-species biofilm can be applied as immobilized cells, and our experiment showed that it would be a promising tool for ethanol production [9]. Immobilization of yeast cells in mixed culture with LAB cells does not require any artificial immobilizing agents and cumbersome immobilizing manipulation, and therefore is promising for a low-cost continuous fermentation.

In this study, we examined the effect of medium

composition on mixed-species biofilm formation between *L. plantarum* ML11-11 and *S. cerevisiae* in order to optimize medium conditions for immobilizing yeast cells in mixed culture with LAB cells. In particular, we extensively investigated the effect of manganese ion on the growth and the single- and mixed-species biofilm formation of the LAB *L. plantarum* ML11-11 and the yeast *S. cerevisiae*.

## MATERIALS AND METHODS

### *Microorganisms and culture conditions*

The LAB strain used in this study was *Lactobacillus plantarum* ML11-11, which was isolated from brewing samples of Fukuyama pot vinegar [7]. To prepare seed cultures, LAB were grown in DeMan, Rogosa, Sharpe broth (MRS; Becton, Dickinson and Company, Sparks, MD, USA) at 28°C for 24 hr. Yeast was grown in YPD broth (YPD; Becton, Dickinson and Company) at 28°C for 24 hr. The yeast strain used in this study was *S. cerevisiae* BY4741 (*MATa leu2 $\Delta$ 0 ura3 $\Delta$ 0 his3- $\Delta$ 1 met15 $\Delta$ 0*).

Unless otherwise stated, all the reagents used in this study were of reagent grade and were purchased from Kanto Chemical (Tokyo), Wako Pure Chemical Industries (Osaka, Japan) or Sigma-Aldrich (St. Louis, MO).

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### *Biofilm formation assay*

Biofilm formation and the assay protocol were almost the same as in our previous studies [6, 7]. To assay biofilm formation, stationary phase LAB and yeast cultures were inoculated into fresh YPD or MRS at a dilution rate of 1:100 in monoculture, while for co-culture, both stationary phase cultures of LAB and yeast were inoculated into fresh YPD or MRS at a dilution rate of 1:200. A 96-well polystyrene microtiter plate was used for biofilm formation. After inoculation, both mono- and co-culture samples were incubated at 30°C for 24 hr. Quantification of biofilm formation was done by the conventional titer plate method with minor modifications [10–12].

Components of MRS broth, beef extract (10 g/l), polysorbate 80 (1.0 g/l), ammonium citrate (2.0 g/l), sodium acetate (5.0 g/l), magnesium sulfate (0.1 g/l), manganese sulfate (0.05 g/l) and dipotassium phosphate (2.0 g/l) (all equivalent to the concentration in MRS broth), were added to YPD broth, and single- and mixed-species biofilm formation of *L. plantarum* ML11-11 and *S. cerevisiae* were assayed.

Manganese sulfate at various concentrations was added to YPD broth, and single- and mixed-species biofilm formation of *L. plantarum* ML11-11 and *S. cerevisiae* were assayed. The assays were performed at least 3 times.

### *Measurement of growth*

Manganese sulfate at various concentrations was added to YPD broth, and single- and mixed-species growth of *L. plantarum* ML11-11 and *S. cerevisiae* were estimated. LAB and yeasts cultures were inoculated into fresh YPD at a dilution rate of 1:100 in monoculture, and after inoculation, culture samples were incubated at 30°C for 24 hr. Incubated cultures were diluted 10 times with physiological saline. Diluted cultures were transferred into an ultrasonic apparatus, Bioruptor (Cosmo Bio, Tokyo), and gently treated to break down the biofilm. The number of viable cells in the culture was determined by the viable plate count method by using LAB selective agar medium, MRS medium with 10 mg/ml cycloheximide and yeast selective agar medium, YPD with 10 µg/ml of streptomycin and 100 units/ml of penicillin. Plates were incubated at 30°C for 48 hr. The assays were performed 3 times.

### *Observation of biofilm by phase contrast microscopy*

Mixed-species biofilms were formed on a cover glass by culturing at 30°C for 6 hr in 2 mL of YPD medium in a petri dish. After 6 hr cultivation, the cover glasses were washed twice and dried at room temperature for 30 min

(some samples were Gram-stained), observed with the Olympus BX60 phase contrast microscope equipped with a UPlanFl oil immersion lens (Olympus, Tokyo) and photographed with a DP90 digital camera (Olympus).

## RESULTS

### *Effect of initial pH on biofilm formation*

Effect of initial pH on single- and mixed-species biofilm formation of *L. plantarum* ML11-11 and *S. cerevisiae* BY4741 was investigated in YPD medium. LAB and yeast could form mixed-species biofilm under the initial pH between 5.0 and 9.0, and significant mixed-species biofilm was formed under a pH of between 6.0 and 8.5 (Fig. 1). These results showed that pH significantly affects mixed-species biofilm formation.

### *Effect of YPD medium concentration on biofilm formation*

Effect of YPD medium concentration on single- and mixed-species biofilm formation of *L. plantarum* ML11-11 and *S. cerevisiae* BY4741 was investigated. Mixed-species biofilm formation increased in proportion to the YPD medium concentration (Fig. 2). This suggested that there is an essential factor for mixed-species biofilm formation in YPD medium.

### *Effect of MRS medium concentration on biofilm formation*

Effect of MRS medium concentration on single- and mixed-species biofilm formation of *L. plantarum* ML11-11 and *S. cerevisiae* BY4741 was also investigated. In contrast to YPD medium, mixed-species biofilm formation decreased in proportion to the MRS medium concentration (Fig. 3). This suggested that there is an inhibitor of mixed-species biofilm formation in MRS medium. This was an unexpected result, and we tried to identify the inhibitor.

### *Effect of MRS medium components on biofilm formation*

The effect of addition of MRS broth components to YPD medium on *L. plantarum* ML11-11 single- and mixed-species biofilm formation was investigated. The tested components were beef extract, polysorbate 80, ammonium citrate, sodium acetate, magnesium sulfate, manganese sulfate and dipotassium phosphate (equivalent to the concentration in MRS broth). From these experiments, it was clarified that beef extract inhibited mixed-species biofilm formation. In these experiments, we incidentally found out that manganese sulfate, one of MRS components, remarkably stimulated both single- and mixed-species biofilm formation (Fig. 4). This was also an unexpected result, and we therefore

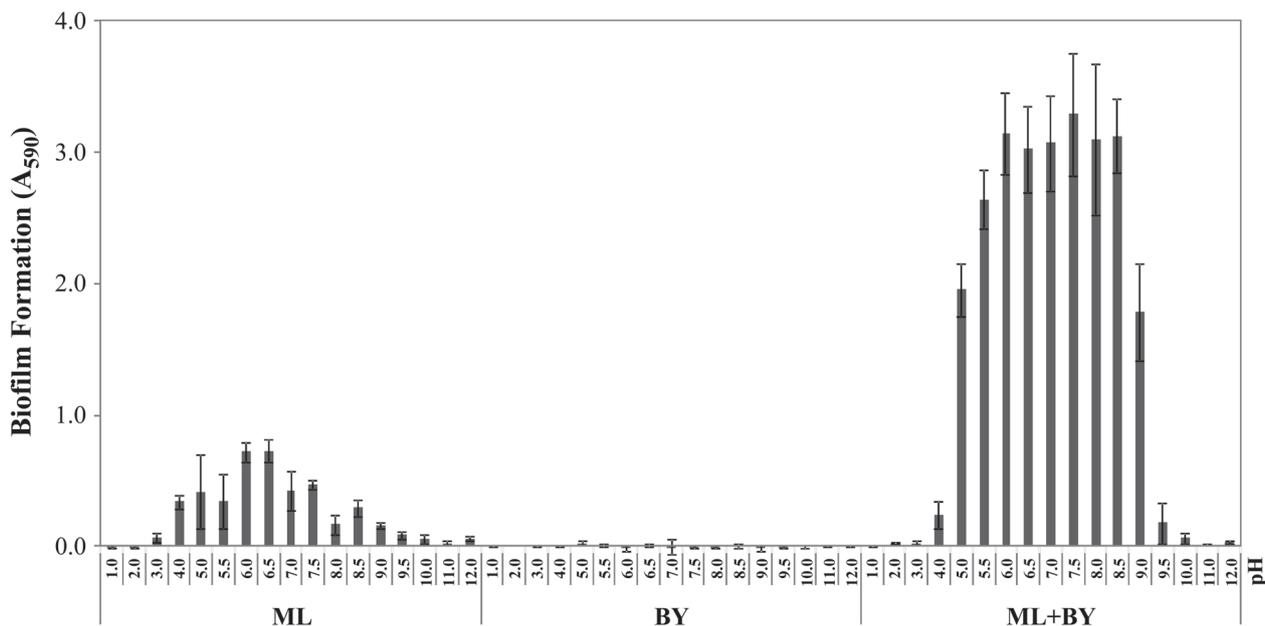


Fig. 1. Effect of initial pH of YPD medium on single- and mixed-species biofilm formation of *L. plantarum* ML11-11 with *S. cerevisiae* BY4741.

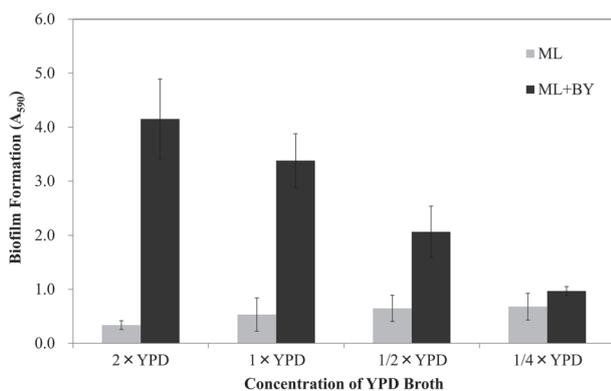


Fig. 2. Effect of YPD medium concentration on single- and mixed-species biofilm formation of *L. plantarum* ML11-11 with *S. cerevisiae* BY4741.

tried to investigate the effect of manganese sulfate concentration on biofilm formation. Manganese chloride also showed almost the same effects (data not shown), indicating that manganese ion was the effective factor.

#### Effect of manganese sulfate concentration on biofilm formation

As shown in Fig. 5, single biofilm formation of *L. plantarum* ML11-11 increased significantly in proportion to the manganese sulfate concentration up to 1 mM, and mixed-species biofilm formation increased up to 100  $\mu$ M

(Fig. 5). The increase in mixed-species biofilm formation would mostly depend on the increase in *L. plantarum* ML11-11 single biofilm formation. There was a tenfold difference in the optimum manganese ion concentration between LAB single- and mixed-species biofilm formation. We considered that this difference came from the difference in growth rate of both strains at various manganese ion concentrations. Then we investigated the effect of manganese ion concentration on growth.

#### Effect of manganese sulfate concentration on growth

The effect of manganese sulfate concentration on growth of *L. plantarum* ML11-11 and *S. cerevisiae* BY4741 in single- and mixed-species culture were investigated (Fig. 6). When 10  $\mu$ M manganese sulfate was added to YPD medium, an approximately 100-fold increase in growth of *L. plantarum* ML11-11 was observed compared with that without addition of manganese sulfate. Furthermore, the growth of *L. plantarum* ML11-11 was resistant to up to 100 mM of manganese sulfate. These results indicated that *L. plantarum* ML11-11 requires some manganese ions for optimum growth and also has higher resistance to manganese ion. On the other hand, the growth of *S. cerevisiae* was not stimulated by manganese sulfate addition but was sensitive to a manganese sulfate concentration above 100  $\mu$ M.

From these results, the relatively low growth rate of *S. cerevisiae* in a high manganese ion environment

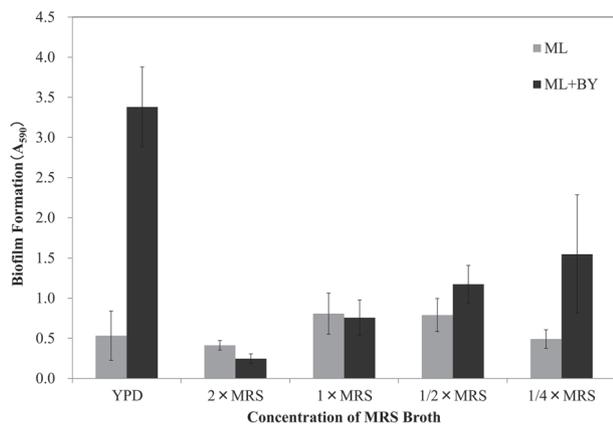


Fig. 3. Effect of MRS medium concentration on single- and mixed-species biofilm formation of *L. plantarum* ML11-11 with *S. cerevisiae* BY4741.

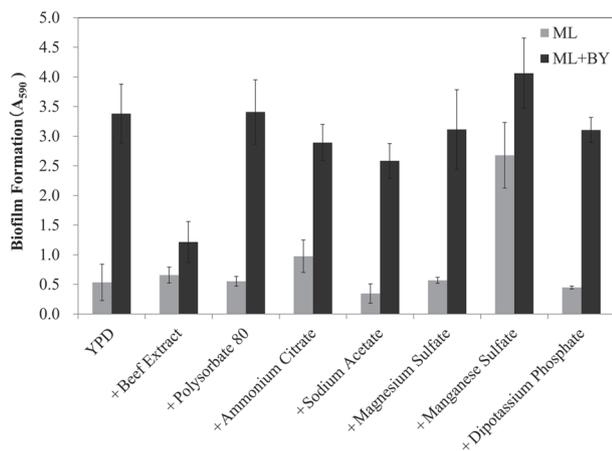


Fig. 4. Effect of MRS medium components on single- and mixed-species biofilm formation of *L. plantarum* ML11-11 with *S. cerevisiae* BY4741.

was considered to be a reason for the difference in the optimum manganese concentration between single biofilm formation by *L. plantarum* ML11-11 and mixed-species biofilm formation by *L. plantarum* ML11-11 with *S. cerevisiae*.

#### Phase contrast microscopic observation of mixed-species biofilm

Phase contrast microscopic observation of biofilm formed in mono- and co-culture of *L. plantarum* ML11-11 and *S. cerevisiae* BY4741 with manganese sulfate added to the environment was conducted. Manganese sulfate increased biofilm formation in co-culture as well as LAB monoculture and showed no effect on yeast monoculture

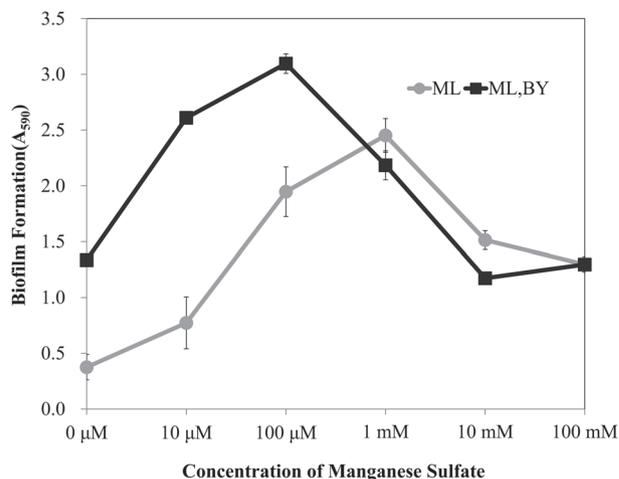


Fig. 5. Effect of manganese sulfate concentration on single- and mixed-species biofilm formation of *L. plantarum* ML11-11 with *S. cerevisiae* BY4741.

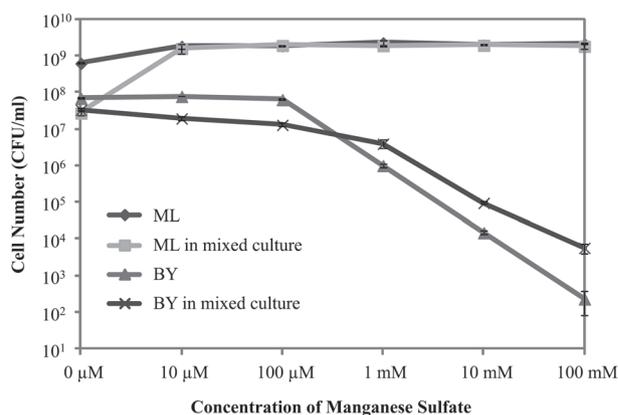


Fig. 6. Effect of manganese sulfate concentration on the growth of *L. plantarum* ML11-11 and *S. cerevisiae* BY4741 in single- and mixed-species culture.

(Fig. 7). These results corresponded to the results of the biofilm formation assay.

## DISCUSSION

It is generally recognized that a suboptimal nutrient concentration is better for biofilm formation [13]. However, as shown in this study, the proportional relationships between mixed-species biofilm formation and YPD medium concentration (Fig. 2) indicated that abundant cell growth under nutrient-rich conditions was favorable in the case of LAB-yeast mixed-species biofilm formation. This mixed-species biofilm was known to be formed by direct cell-cell adhesion [7], and formation

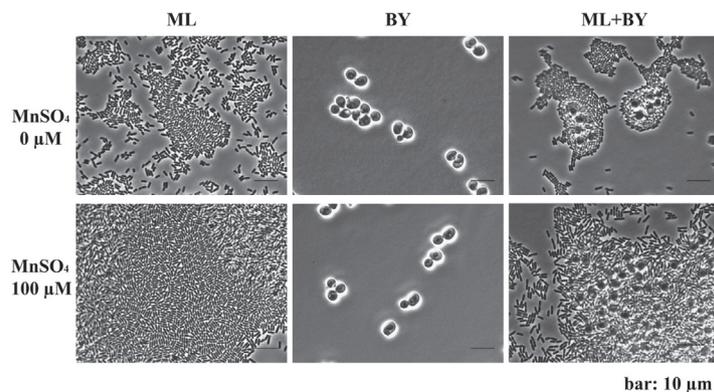


Fig. 7. Phase-contrast microscopic observation of mixed-species biofilm formed by *L. plantarum* ML11-11 and *S. cerevisiae* BY4741 with manganese sulfate added to the environment.

would be enhanced in proportion to cell density in culture. This would be a unique feature of this mixed-species biofilm different from general biofilm.

On the other hand, mixed-species biofilm formation decreased in proportion to the MRS medium concentration (Fig. 3), suggesting that there was some inhibitor of mixed-species biofilm formation in MRS medium. Among the MRS medium components tested, beef extract inhibited mixed-species biofilm formation (Fig. 4). The precise mechanism of this inhibition and the inhibitive component in beef extract have not been elucidated to date. Since beef extract in MRS medium is favorable for LAB growth, some component in beef extract would seem to inhibit yeast growth or cell-cell adhesion between LAB and yeast.

The remarkable stimulation of the LAB single- and LAB-yeast mixed-species biofilm formation by manganese ion was an unexpected result (Figs. 4, 5). LAB single- and LAB-yeast mixed-species biofilm formation increased in proportion to the manganese sulfate concentration up to 1 mM and 100 μM, respectively (Fig. 5). Stimulation of LAB growth in mixed-species biofilm by addition of manganese sulfate (Fig. 6) resulted in an increase in mixed-species biofilm formation (Fig. 5). These results indicated that LAB growth was limited by manganese ion, especially in biofilm in which nutrients were scantily supplied. It is therefore considered that regulation of the manganese ion concentration is important for not only LAB single biofilm formation but also LAB-yeast mixed-species biofilm formation. Growth of LAB reached the maximum at 10 μM and was resistant to up to 100 mM, suggesting that LAB requires manganese ion for optimum growth and also has some system for higher resistance to manganese ion (Fig. 6). The positive effect of manganese ion on LAB growth was previously known. Manganese

ion is known to have an anti-oxidative effect, acting as coenzyme for some important enzymes such as lactate dehydrogenase [14], manganese type SOD [15], catalase [16], RNA polymerase [17] and xylose isomerase [18].

In contrast to LAB, growth of yeast was sensitive to manganese sulfate above a concentration of 100 μM. There have been some studies on the sensitivity of *S. cerevisiae* to manganese ion, and it was indicated that above 1 mM, manganese ion was poisonous the yeast cells [19].

Thus, manganese ion affects differently the growth of LAB and yeast and affects deeply the formation of LAB biofilm and LAB-yeast mixed-species biofilm. Our preliminary result on the effect of manganese sulfate on ethanol fermentation using LAB-yeast mixed-species biofilm showed that a concentration above 100 μM was rather inhibitive for ethanol production (data not shown). The decreased ratio of yeast cells compared with LAB cells in the mixed-species biofilm formed with addition of 100 μM manganese sulfate (Fig. 6) seemed to cause this reduction in ethanol production. Manganese ion did not inhibit co-aggregation of LAB and yeast at 100 μM (data not shown). From these results, it was considered that the domination of LAB under manganese ion-rich conditions suppressed ethanol fermentation by yeast, and therefore optimization of the manganese ion level to form a mixed-species biofilm containing the optimum yeast-LAB cell ratio would be necessary for efficient ethanol fermentation.

Our previous experiments showed that LAB-yeast mixed-species biofilm formed by *L. plantarum* and *S. cerevisiae* can be applied as immobilized cells for ethanol production and had a potential to produce ethanol in repetitive culture and continuous culture [9]. Artificial cell immobilization requires expensive

immobilizing agents, and therefore, fermentation using immobilized cells has not been applied commercially in large-scale continuous fermentation in spite of its potential benefit. On the other hand, cell immobilization technology that makes use of LAB-yeast mixed-species biofilm formation does not require any artificial agents or any artificial handling, and therefore is promising for low-cost continuous fermentation. We hope that this cell immobilization technology, born out of one of the most primitive types of fermentation, namely Fukuyama pot vinegar fermentation, can contribute to the yeast industry, including biofuel production, in the future. As shown in this report, the formation of LAB-yeast mixed-species biofilm can be controlled artificially through control of the manganese ion level, and optimization of the manganese ion level would be one of the important factors controlling this fermentation system.

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