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# Inactivation of Gram-Positive Bacteria Using Lytic Enzyme Mixture

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The mass of air breathed by a human per day is equivalent to 10 times the mass of food consumed in that time. However, fundamental safety measures for atmospheric bacterial control have not yet been implemented. The purpose of our research is to prevent wound infections via a cell wall lytic enzyme, which can inactivate bacteria in air that cause infectious diseases by decomposing their cell envelopes. On the basis of the structure of the cell walls of bacteria, we proposed the use of a lytic enzyme mixture including glycosidase, protease and lipase. In this study, the use of a lytic enzyme mixture derived from *Pseudomonas* sp. and *Rhizopus niveus* was examined, the performance of the lytic enzyme mixture was evaluated using lysozyme, a typical lytic enzyme, as a control. The substrate used was *Micrococcus luteus*, a gram-positive bacterium. The experimental results showed that the use of the lytic enzyme mixture exhibited a lytic rate per hour that was 13–39% greater than that of the control. Furthermore, although there are different phases during bacterial multiplication, the lytic rate per hour improved for all phases when the lytic enzyme mixture was used.

## 1. Introduction

Humans breathe 15 times per minute and inhale 0.5 liters of air in each breath, resulting in the inhalation of 10,800 liters of air every day. If the specific gravity of the air is defined as 1.2 kg/m$^3$, the mass of air inhaled per day is 13 kg. This is equivalent to 10 times the mass of our daily food and drink intake. Foods containing substances that are known to cause allergies have already been regulated by law[(1)] to define specific substances for safety

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reasons. Unfortunately, fundamental environmental safety measures to limit bacteria levels in the atmosphere have not yet been implemented. The authors have been studying "bioremediation", a recovery process for the environment using biotechnological advances, to improve the quality of life (QOL), including improvements to the in-house air environment.[2,3] Bioremediation using this enzyme is advantageous due to low energy consumption and low production of industrial waste.[4]

Hospital-acquired infections caused by exposure to antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa,* are becoming a problem.[5] The increasing prevalence of pathogenic bacteria is associated with the high morbidity of wound infections. The purpose of our research is to prevent wound infections via a cell wall lytic enzyme (lytic enzyme) that can destroy the cell walls of bacteria to inactivate them in the atmosphere. Various bacteria cause hospital-acquired infections, so cell wall lytic filters should cover a wide range of the lytic spectrum.[6,7] Bacterial cell walls consist of glucose, protein and lipid. Each component has a specific hydrolytic enzyme, such as glycosidase for glucose, protease for protein, and lipase for lipid.

In this experiment, these three kinds of lytic enzyme derived from *Pseudomonas* sp. and *Rhizopus niveus* were mixed to improve lytic efficiency. The results were compared with lysozyme (EC3.2.1.17), a typical lytic enzyme. We used *Micrococcus luteus*, a gram-positive bacterium, as the substrate. We report the evaluation of the lytic rate per hour obtained from changes in the optical density of a suspension of bacterial cells.

## 2. Materials and Methods

### 2.1 *Lytic enzymes and bacterial cells evaluated*

Lysozyme (EC3.2.1.17) was used as a lytic enzyme control. Lysozyme hydrolyzes the $\beta$-1, 4-bonds of N-acetylglucosamine and N-acetylmuramic acid present in the peptidoglycan layer constituting the cell envelope of bacteria (Fig. 1). In addition, this lysozyme is a glycosidase and a well-known typical lytic enzyme.[8,9]

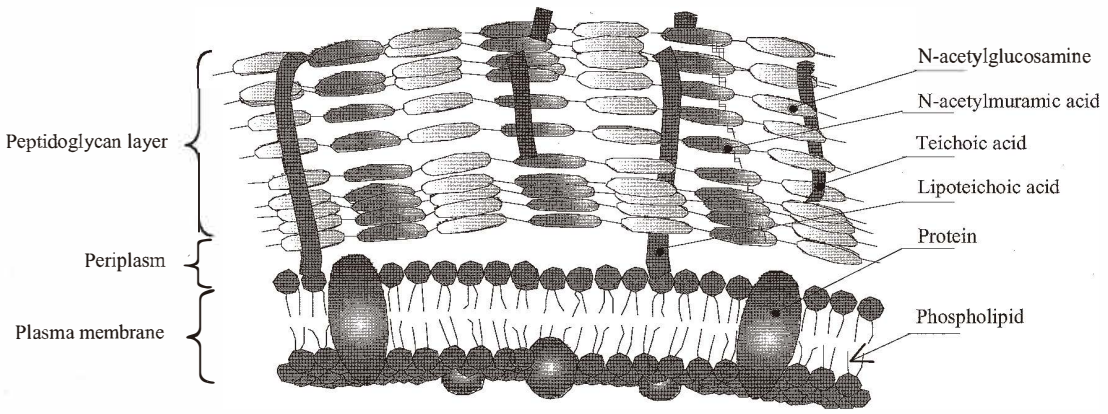


Fig. 1. Architecture of cell envelope of gram-positive bacterium.

Enzyme-1 and Enzyme-2 were newly selected for this experiment (Table 1). Enzyme-1, a mixture of enzymes, was derived from *Pseudomonas* sp. (formerly *Achromobacter lunatus*), and its major component enzyme is endo-1,3(4)-$\beta$-glucanase (a glycosidase) that randomly cuts $\beta$-1,3-glucan, which is a major component of the bacterial cell envelope; it also contains both neutral and alkaline proteases. Its enzyme activity was defined as 1 Unit (U) according to the volume of enzyme required to reduce the $OD_{660}$ of a solution by 0.01 in one minute of reaction time at pH 7.0 using a suspension of *Candida utilis* (obtained from the Institute for Fermentation, IFO5686) as the substrate. Enzyme-1 acted effectively over a wide pH range (pH 5.0–to pH 8.0), with optimal activity at pH 7.0.

Enzyme-2, a mixture of enzymes, was derived from *Rhizopus niveus,* and its major component enzyme (triacylglycerol lipase) hydrolyzes triacylglycerol into glycerol and fatty acids as follows:

$$\begin{array}{l} CH_2OCOR \\ | \\ CHOCOR \\ | \\ CH_2OCOR \end{array} + 3\,H_2O \xrightarrow{\text{Enzyme-2}} \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array} + 3\,RCOO^- \qquad (1)$$

Enzyme-2 contained protease in addition to lipase. The protease activity was defined as follows: after the enzyme reacted with milk-casein at pH 3.0, the volume of trichloroacetic acid-soluble amino acid was calculated by tyrosine conversion. The enzyme activity that produced a volume of amino acid equivalent to 100 $\mu$g of tyrosine in the solution after 60 min of reaction was defined as 1 Unit. Enzyme-2 was stable in the acid pH range and reacted effectively between pH 3.5–pH 5.0.

*Micrococcus luteus* was selected to evaluate the lytic rate per hour of a lytic enzyme mixture. This is a gram-positive bacterium and also a lysozyme substrate.[10] Various phases of activity take place during the multiplication of bacteria. It was suggested that the characteristics and forms of the cell envelopes could be different according to the phase involved. Therefore, both lyophilized (freeze dried, Sigma-Aldrich Co., M3770, ATCC No. 4698) and viable cells (obtained from Japan Collection of Microorganisms, JCM No.

Table 1
Characteristics of lytic enzymes used for mixed lytic enzymes.

| Name | Kinds of enzyme | Substrates of enzyme | Organism |
|---|---|---|---|
| Enzyme-1 | Glycosidase, Protease | Endo-1,3(4)$\beta$-glucanase, Acid and neutral protease | *Rhizopus niveus* |
| Enzyme-2 | Lipase, Protease | Triacylglycelol lipase, Acid protease | *Pseudomonas* sp. |

1464) of *Micrococcus luteus* were used. The lyophilized cells were in either in the lag phase or stationary phase. The viable cells were prepared as a logarithmic-growth phase.

### 2.2 *Evaluation of a single enzyme using lyophilized cells*

A pH 7.0 phosphoric acid buffer solution was applied to the lyophilized cells of *Micrococcus luteus* until $OD_{540}$=1.0. Then, 100 U (100 $\mu$L) of lysozyme was added to a 2.5 mL suspension of bacterial cells to use as a control. Enzyme-1 (2.8 mg/mL, 50 U) was dissolved in 2.5 mL of the suspension of bacterial cells and 100 $\mu$L of phosphoric acid buffer solution was added. Enzyme-2 (1.36 mg/mL, lipase 50 U, protease 11.6 U) was dissolved in 2.5 mL of the bacterial cell suspension and 100 $\mu$L of phosphoric acid buffer solution (pH 7) was added (Table 2). These suspensions of bacterial cells were incubated at 45°C for 30 min and the reduction of the optical density in $OD_{540}$ was measured every 10 min.

### 2.3 *Evaluation of mixed lytic enzymes using lyophilized cells*

A pH 7.0 phosphoric acid solution was applied to the lyophilized cells of *Micrococcus luteus* until $OD_{540}$=1.0. Both 2.8 mg/mL (50 U) of Enzyme-1 and 1.36 mg/mL (lipase 50 U, protease 11.6 U) of Enzyme-2 were dissolved in 2.5 mL of the bacterial cell suspension and 100 $\mu$L of phosphoric acid buffer solution was added (referred to hereafter as the basic mixture). In addition, this suspension of bacterial cells was also prepared with 100 U (100 $\mu$L) of lysozyme (referred to hereafter as the lytic enzyme mixture) (Table 2). These suspensions of bacterial cells were incubated at 45°C for 30 min and $OD_{540}$ was measured every 10 min.

Table 2
Protocol of the combination of the enzyme and its substrate.

| Paragraph | Enzyme | Substrate |
|---|---|---|
| 2.2 | Single enzyme<br>a) Enzyme-1<br>b) Enzyme-2 | Lyophilized cells of *Micrococcus luteus* |
| 2.3 | Mixed enzyme<br>a) Basic mixture<br>(consist from Enzyme-1 and Enzyme-2)<br>b) Lytic enzyme micture (consist from Enzyme-1, Enzyme-2 and lysozyme) | Lyophilized cells of *Micrococcus luteus* |
| 2.4 | Mixed enzyme<br>a) Basic mixture<br>(consist from Enzyme-1 and Enzyme-2)<br>b) Lytic enzyme mixture (consist from Enzyme-1, Enzyme-2 and lysozyme) | Viable cells of *Micrococcus luteus* |

### 2.4 *Evaluation of mixed lytic enzymes using viable cells*

The viable cells of *Micrococcus luteus* were incubated (Fig. 2) then shake-cultured until $OD_{540}$=1.0 before washing with phosphoric acid buffer solution. Phosphoric acid buffer solution was added until $OD_{540}$=1.0, which is equivalent to the initial state of the logarithmic-growth phase. Both 1.4 mg/mL (25 U) of Enzyme-1 and 0.68 mg/mL (lipase 25 U, protease 5.8 U) of Enzyme-2 were dissolved in the suspension of bacterial cells and 100 $\mu$L of phosphoric acid buffer solution was added (the basic mixture). In addition, this suspension of bacterial cells was also prepared with 100 U (100 $\mu$L) of lysozyme (the lytic enzyme mixture) (Table 2). These suspensions of bacterial cells were incubated at 45°C for 30 min and $OD_{540}$ was measured every 10 min.

## 3. Results and Discussion

### 3.1 *Evaluation of a single enzyme using lyophilized cells*

Each single enzyme was evaluated using lyophilized cells of *Micrococcus luteus* by comparing the optical density of a suspension of bacterial cells with that of the control (Fig. 3). The optical densities after 30 min reaction decreased by 52% for lysozyme (control), 10% for Enzyme-1 and 3% for Enzyme-2. The results indicated that, compared to the control, a clear decrease in optical density did not occur using either Enzyme-1 or Enzyme-2 as a single enzyme.

### 3.2 *Evaluation of mixed lytic enzymes using lyophilized cells*

The decreases in the *OD* that were exhibited by the various mixed enzyme combinations were as follows: the lysozyme alone was 52%, the basic mixture (Enzyme-1 and Enzyme-2) was 10%, and the lytic enzyme mixture (Enzyme-1, Enzyme-2 and lysozyme) was 71% (Fig. 4). This indicated that a mixture of Enzyme-1 and Enzyme-2 did not improve lytic efficiency. However, by adding Enzyme-1 and Enzyme-2 to lysozyme (the lytic enzyme mixture), it was confirmed that the lytic rate per hour increased 1.39-fold over that of lysozyme alone.

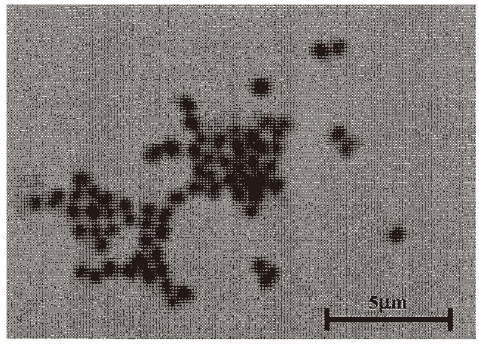


Fig. 2. Photomicrograph of *Micrococcus luteus* stained by the basic Gram method (×1000).

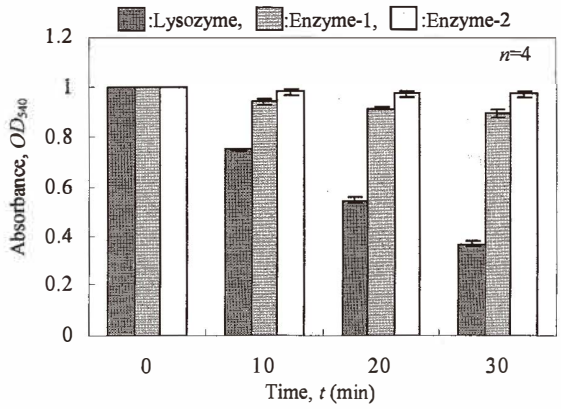


Fig. 3. Evaluation of a single enzyme using lyophilized cells. Lysozyme: 100 U, Enzyme-1: 50 U (in total), Enzyme-2: 50 U of lipase and 11.6 U of protease.

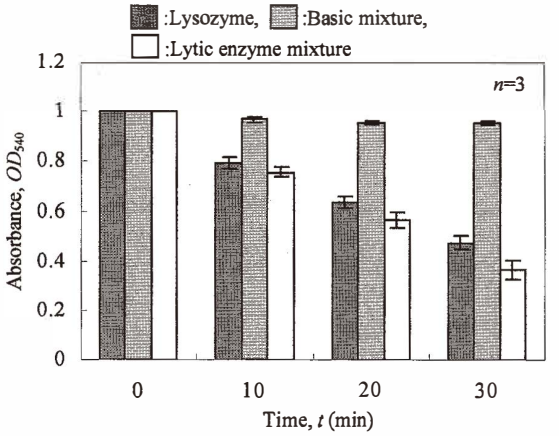


Fig. 4. Evalution of mixed lytic enzymes using lyophilized cells. Lysozyme: 100 U, Enzyme-1: 50 U (in total), Enzyme-2: 50 U of lipase and 11.6 U of protease.

### 3.3 *Evaluation of mixed lytic enzymes using viable cells*

The experimental results using viable cells showed that the decrease in the *OD* for lysozyme alone was 54%, the basic mixture (Enzyme-1 and Enzyme-2) was 8%, and the lytic enzyme mixture (Enzyme-1, Enzyme-2 and lysozyme) was 61% (Fig. 5). This indicated that, when using viable cells, adding both Enzyme-1 and Enzyme-2 to lysozyme caused the lytic rate per hour to increase 1.13-fold over that of lysozyme alone. Because

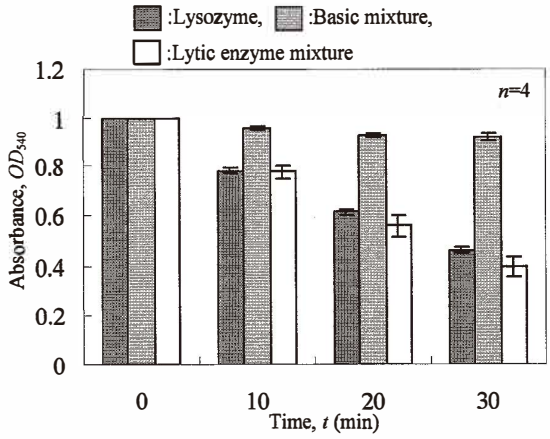


Fig. 5. Evaluation of mixed lytic enzymes using viable cells. Lysozyme: 50 U, Enzyme-1: 25 U (in total), Enzyme-2: 25 U of lipase and 5.8 U of protease.

the cell envelope of gram-positive bacteria contains a thick peptidoglycan layer, lysis of the bacteria was dependent on the destruction of the envelope. It was suggested that, compared with the use of a single enzyme, a mixture of protease and lipase showed greater lytic ability against the peptidoglycan layer.

## 4. Conclusions

In a previous study, lysozyme was used as a high performance lytic enzyme without a mixture. On the basis of the structure of the cell walls of bacteria, we proposed the use of a lytic enzyme mixture derived from *Pseudomonas* sp. and *Rhizopus niveus*. The lytic rate per hour of mixed lytic enzymes was evaluated using gram-positive bacteria. The experimental results indicate that the use of a lytic enzyme mixture, such as glycosidase, protease and lipase, improved the lytic rate per hour by 13–39%. As there are different amplification phases, such as the lag phase, the stationary phase and the logarithmic-growth phase for bacteria, the characteristics of the cell envelope vary depending on the phases that are present. The lytic enzyme mixture that we suggested may be appropriate for all of these phases.

The cell envelope structures differ from bacterium to bacterium. However, the use of this lytic enzyme mixture creates the possibility of realizing a cell wall lytic filter that may be effective across a wide range of the lytic spectrum.

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