# ISOLATION OF A NEW LYSINIBACILLUS STRAIN PRODUCING ANTIBACTERIAL PROTEINS FROM ROTTEN CITRUS

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**Abstract** - To investigate a reusable method of citrus peels and low-value citrus fruits, microbes from rotten citrus were analyzed. A bacterium with antibacterial activity was isolated and further analyzed. Phylogenetic analysis with 16S rDNA from the bacterium was reveled to new Lysinibacillus strain. The Lysinibacillus strain has strong antibacterial activity against Gram(+) bacteria such as *Staphylococcus aureus, Lysinibacillus sphaericus* and *Pichia scaptomyzae* but not Gram(-) bacteria such as *Escherichia coli* and *Enterobacter aerogenes.* The antibacterial activity was most increased on the culture conditions of 24□, 7 days in the YPD broth and the activity was disappeared by treatment of heat or trypsin. These results indicated that the new Lysinibacillus strain produce antibacterial proteins against Gram(+) bacteria.

**Keywords** - Lysinibacillus, Antibacterial Proteins, Rotten Citrus

## I. INTRODUCTION

There are many pathogenic Gram-positive(+) bacteria in animals and plants such as Staphylococcus, Streptococcus, Pneumoniae, Diphtheria, Tetanus, Anthrax, Actinomycetes etc. In particular, *Staphylococcus aureus* is the major harmful food-born pathogen causing severe illness such as vomiting, nausea, stomach cramps, diarrhea, headache, etc. Antibiotics against microbes have been discovered from penicillin by Fleming. Microorganisms are shown the response to different types of antibiotics which has an antimicrobial activity on bacteria to the cell membrane production-inhibiting (penicillin, cephalosporin), or inhibition of protein synthesis (aminoglycosides, tetracycline, chloramphenicol, etc.). There are needs to the constant development of new antimicrobial agents due to antibiotics resistance. Recent antibiotics overuse causes a problem of antibiotic-resistant strains generating super bacteria to kill humans and animals. There are some reports that Lysinibacillus isolated from waste of vegetable and fruits produces bacteriocin. But the mode of antimicrobial activity is not well understood. This research is performed to isolate of a new Lysinibacillus strain producing antibacterial proteins from rotten citrus and to safely and effectively control the harmful bacteria.

## II. MATERIALS AND METHODS

### 2.1. Isolation of a bacterial strain

To identify new bacteria harboring antibacterial activity from rotten citrus, serial diluted bacterial mixture were incubated in YPD agar until achieving several single colonies to form. A single colony was separated and examined the antimicrobial activity against *Lysinibacillus sphaericus* using a paper disc method.

### 2.2. Molecular identification of the bacteria

To investigate the molecular features for microbial identification, DNA from isolated bacteria was extracted and 16S rRNA gene was amplified by PCR using 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) primers under normal conditions. The amplified 16s rRNA gene fragment is confirmed of its length using the gel electrophoresis and further perform to sequencing using 337F, 518F, 785F, 518R, 800R, as well as the sequencing primers 27F and 1492R. In addition, the nucleotide BLAST (NCBI, NIH, USA) program compared the homology of the nucleotide sequence in related bacteria.

### 2.3. Antibacterial assay of *Lysinibacillus spp.* Y-4B

The antimicrobial activity in *Lysinibacillus spp.* Y-4B was tested for the paper disc (according to the CLSI standard method) method. The novel strain was cultured in YPD broth (SIGMA-AKDRICH, Y1375), The culture solution put the paper disc on the culture medium against the several bacterial lawns such as *Staphylococcus aureus, Lysinibacillus sphaericus, Pichia scaptomyzae, Escherichia coli* and *Enterobacter aerogenes.* The antibiotic ampicillin (Amp) and kanamycin (Kan) were used as controls.

### 2.4. Treatment of Heat and Trypsin

The antimicrobial activity in the supernatant of the bacterial culture was examined after heat treatment (100□) or trypsin digestion.

## III. RESULTS AND DISCUSSION

### 3.1. Isolation and Molecular identification of the bacteria

The eight microbial colonies were separated and a bacterial colony showed an excellent antibacterial activity using a paper disc method. The sequencing of 16S rDNA and phylogenetic analysis showed that the strain is novel bacteria and belong to *Lysinibacillus spp.* in phylogenetic classification (Fig

1). As a result, the novel strain had highest homology to *Lysinibacillus macroides* in 99.6% (Table 1). The novel strain was named "*Lysinibacillus spp.* Y-4B". Recent reports revealed that *Lysinibacillus macroides* is genomically distinct which is determined by 16S rRNA sequencing from its close relatives *Lysinibacillus boronitolerans* and *Lysinibacillus xylanilyticus.* Our results of the phylogenetic analysis confirmed again the previous conclusions.

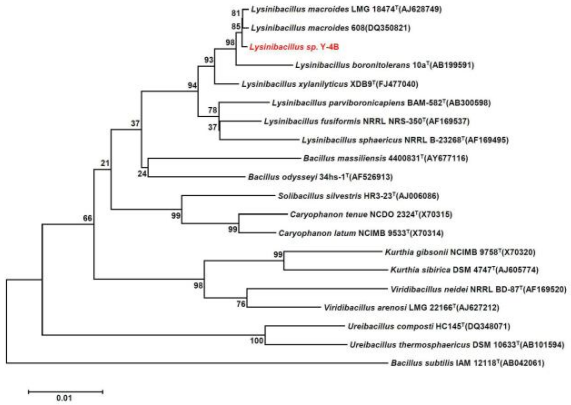



**Fig.1. Phylogenetic anaylsis of a novel strain *Lysinibacillus sp.* Y-4B.**

| Rank | Bacteria | Type strain | Accession No | Similarity (%) |
|---|---|---|---|---|
| 1 | *L. macroides* | LMG 18474(T) | AJ628749 | 99.60 |
| 2 | *L. boronitolerans* | T-10a(T) | AB199591 | 99.18 |
| 3 | *L. xylanilyticus* | XDB9(T) | FJ477040 | 99.04 |
| 4 | *L. fusiformis* | NBRC5717(T) | AB271743 | 98.51 |
| 5 | *L. sphaericus* | C3-41 | CP000817 | 98.48 |

**Table 1: Homology among sequences of 16S rDNAs**

### 3.2. Antibacterial assay of *Lysinibacillus spp.* Y-4B

The novel strain, *Lysinibacillus spp.* Y-4B, showed the antibacterial ability for a variety of microorganisms
against *Lysinibacillus sphaericus, Pichia scaptomyzae* or *Staphylococcus aureus* using a paper disc method (Table 2).

| | Strain | Gram | Inhibition zone (mm) | Effective time (hour) |
|---|---|---|---|---|
| KCTC 1185 | *L. sphaericus* | + | 17 | 24 |
| KCTC 17376 | *P. scaptomyzae* | + | 12 | 12 |
| KCTC 1916 | *S. aureus* | + | 14 | 12 |
| KCTC 3065 | *S. mutans* | + | 0 | NA |
| KCTC 2190 | *E. aerogenes* | - | 0 | NA |
| KCTC 1682 | *E. coli* | - | 0 | NA |

**Table 2: Antibacterial activity of the *Lysinibacillus spp.* Y-4B**

### 3.3. Antibacterial active substance of *Lysinibacillus spp.* Y-4B

The following experiment was performed to determine if the antimicrobial active substance is protein-nature or not. First, after the culture supernatant of the strains treated by heat (100 □) and analyzed for antimicrobial activity. It was confirmed that its activity is lost in the high temperature. This result was expected that the protein would be inactivated by heat, in order to further determine whether the antibacterial substance is any of the chemical or protein, the trypsin was treated with the supernatant. The protease trypsin is characterized by cutting a lysine and arginine in proteins specifically, if trypsin-treated when its activity is reduced or lost, the antimicrobial active substance is protein. The culture supernatant treated with trypsin was found that antibacterial activity is disappeared. Therefore, it was found that the novel bacteria produce proteins with antibacterial activity from these results.

## CONCLUSIONS

Isolation and characterization of a new Lysinibacillus was studied and major conclusions are as follows:

1. The isolated new Lysinibacillus spp. Y-4B showed antibacterial activity against some Gram(+) bacteria including *Staphylococcus aureus.*
2. The substance showing antibacterial activity is protein because the activity was not shown after treatment of heat or trypsin.

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