# Bacteriocin Production by Lactic Acid Bacteria Encapsulated in Calcium Alginate Beads

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## Abstract

Thirty two strains of lactic acid bacteria were isolated form traditional fermented foods and bio-extract for their abilities to produce bacteriocins. One strain, LAB7, which isolated from bio-extract were found to be able to inhibit *Escherichia coli* TISTR 780 and *Staphylococcus aureus* TISTR 1466. Its properties were heat tolerance at 121 °C for 15 min and stable at wide pH range of 2-9. The encapsulation experiments were conducted with LAB7 strain entrapped in calcium alginate beads which contained $10^6$ CFU/bead. The encapsulated cells were inoculated into MRS broth at 10% inoculums and grew in batch condition at 30°C for 24 h. Free cells with and without agitation were used to compare the effect of encapsulation on the bacteriocin production. Substrate consumption, biomass and lactic acid production were also investigated. The results showed that the encapsulated cells produced the highest lactic acid at 2.92 g/l/h. The bacteriocin activity against *E. coli* TISTR 780 was 3200 AU/ml after cultured for 10 h and the activity against *S. aureus* TISTR 1466 was 800 AU/ml after cultured for 8 h. Whereas, free cells without agitation condition gave bacteriocin activity against *E. coli* TISTR 780 and *S. aureus* TISTR 1466 only 1600 AU/ml and 400 AU/ml, respectively. It was indicated that LAB7 not only produced bacteriocin against both Gram positive and Gram negative bacteria, but also strongly present high bacteriocin activity when encapsulated cell in calcium alginate beads.

**Keywords:** Bacteriocin, Calcium alginate beads, Lactic acid bacteria

## Introduction

Lactic acid bacteria (LAB) occur naturally in several fermented foods such as fermented vegetable, fermented fish, fermented meat and fermented milk (Rattanachaikunsopon and Phumkhachorn, 2000: Tserovska et al., 2000-2002: Tanasupawat et al., 2002: Salminen et al., 2004: Urso et al., 2006). They are used as natural or selection starters in fermented food in which they perform acidification due to production lactic acid flavor. LAB play an important role in food fermentation as the products obtains with there aid which are characterized by hygienic safety, storage stability and attractive sensory properties (Savadogo et

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al., 2006). They are Gram positive, nonspore forming and microaerophilic bacteria. LAB can produce many substances such as organic acids, diacetyl and so on contributing to smell, taste and texture of the fermented foods. Moreover, several antimicrobial substances including organic acids, hydrogen peroxide and bacteriocins against many food spoilage bacteria and food borne pathogens are found. In addition, LAB are also found to have probiotic potential. Several LAB were also isolated from animal gastrointestinal tract (Hove et al., 1999: Pilasombut et al., 2005: Khunajakr et al., 2008). Bacteriocins are antimicrobial substances produced by many strains of bacteria, especially lactic acid bacteria. Because of their antagonistic activity against specific sensitive bacteria, more bacteriocins have become substances of interest to be developed to use as the biopreservatives (Settanni and Corsetti, 2008). In this work, a number of lactic acid bacteria isolated from Thai traditional fermented food available in local market were screened for the bacteriocin production. Batch fermentation by encapsulated LAB producing bacteriocin and free cell cultivation was also studied.

## Materials and Methods

*1. Isolation and Screening of LAB for antibacterial activity*

LAB strains were isolated from bio-extract, a liquid derived from the fermentation of vegetable and fruits with sugar, obtained from Program Environmental science, Faculty of Science and Technology, Valaya-Alongkorn Rajabhat University,Thailand and Thai traditional fermented food such as Pla-ra, Pla-jom, Pak-sian dong, Hom dong, Goong-jom and Nam which purchased from local market at Pathumtani, Prachinburi and Sakaeo provinces. Ten grams of each sample were homogenized in 90 ml sterile distilled water and subsequently ten fold serials dilution were carried out. The dilutions were then spread on Glucose-Yeast-Peptone (GYP) agar plates containing 0.5% $CaCO_3$. The plates were incubated at 37°C for 24-48 h. Only clear zone producing colonies were selected and transferred to a new MRS agar plate containing 0.5% $CaCO_3$. The agar well diffusion method according to Muangsombat (2007) was then used for pre-screening antimicrobial activity. Briefly, cell-free supernatants obtained by centrifugation of culture at 1000 rpm for 10 min were adjusted to pH 7 by 1M NaOH, filtered through 0.45 µm pore size membrane filter. The supernatant (100 µl) was placed in wells (7 mm in diameter) cut in MRS agar plates (20ml) seeded (0.1% (v/v) with each of the indicator strains, *Escherichia coli* TISTR 780 and *Staphylococcus aureus* TISTR 1466. The plates were incubated at 37°C for 24 h. Positive results were recorded when the inhibition zone of at least 1 mm around the well was observed.

*2. Effect of pH and heat on antibacterial activity*

To determine the effect of temperature and pH on the stability of the bacteriocin, the supernatant was adjusted to various pH level of 2-9 and incubated at 30°C for 2 h according to Pilasombut et al. (2005). Heat stability was determined by heating the neutralized cell free supernatant at 100°C for 10, 20, 30 min and at 121 °C for 15 min. The remaining of antibacterial activity was examined by swab-paper disc technique modified from Ngendaung (2001). Briefly, the overnight culture of the indicator bacteria were diluted to a turbidity equivalent to that of a 3.0 McFarland standard (bioMerieux, France) with a sterilized 0.85% saline solution and spread with a cotton swab over the surface of Meuller Hinton Agar plate. Sterile filter paper discs 6 mm in diameter were placed on the surface of the agar containing the indicator strain. Ten microlitter of cell free supernatant was dropped

on the paper disc. After overnight incubation at 37°C, the plates were checked for inhibition zones around the paper discs. Positive results were recorded when the inhibition zone of at least 1 mm around disc was observed.

*3. Encapsulation*

Cells of isolated strain were obtained from culture grown in MRS medium at 30 °C, for 18 h. Cells were then harvested by centrifugation at 5000 rpm, 4°C for 15 min and then washed thoroughly with 0.85% saline solution. The cells were encapsulated by mixing with 2% (w/v) sodium alginate. The mixture then dropped by syringe into 0.1M $CaCl_2$ solution with agitation at 200 rpm for 30 min and transferred to fresh $CaCl_2$ then incubated again with agitation for 30 min. The Ca-alginate beads were then washed twice with sterilized distilled water and selected for further studies.

*4. Batch fermentation*

The encapsulated cells were then seeded into 1 l fermentor (Biostat Q, B.Braun Biotech Inc., Germany) working volume 750 ml of MRS broth with 10% inoculums and without pH control. The temperature was held at 30 °C and cultured for 24 h without agitation. The fermentations of free cell with agitation at 120 rpm and without agitation were performed under the same conditions as the encapsulated cells. Samples were removed at different time interval for determination of biomass, glucose consumption, lactic acid production and antibacterial activity.

*5. Determination of biomass, glucose consumption and Lactic acid production*

Cell biomass concentration was monitored spectophotometrically by measuring the optical density at 600 nm and correlating the optical density with cell dry weight measurements (Anastasiadou et.al.,2008). Cell-free supernatant was then measured for glucose consumption, lactic acid production and bacteriocin activity. The glucose consumption was determined using phenol-sulfuric method (Dubois et al., 1956). Lactic acid production was determined by colorimetric method according to Barker and Summerson (1941).

*6. Determination of Bacteriocin activity*

Bacteriocin activity in the fermentation broth was examined by swab-paper disc against *E. coli* TISTR 780 and *S. aureus* TISTR 1466 according to modified method of Ngendaung (2001) explained previously. The inhibitory activity was expressed in an arbitrary unit (AU/ml) by testing serial two-fold dilutions. The arbitrary unit (AU) was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the indicator strain. AU was calculated as (1000/10)D, where D was the dilution factor (Parente et al., 1995).

*7. Determination of cell concentration in beads*

One ml of calcium alginate beads were suspended in 0.1M phosphate buffer followed by gentle shaking for 30 min for destruction of the beads. The number of obtained cells was determined by plate counting method using MRS agar.

## Results and Discussion

*1. Isolation screening and mode of action of bacteriocin producing LAB*

LAB were isolated from bio-extract obtained from Program environmental science, Faculty of Science and Technology, Valaya Alongkorn Rajabhat University and 6 types of traditional fermented foods including Pla-ra, Pla-jom, Pak-sian dong, Hom dong, Goong-jom and Nam purchased from local market at Pathumtani, Prachinburi and Sakaeo provinces. The

experiment was performed by using GYP medium containing 0.5% $CaCO_3$ cultured in aerobic condition and incubated at 37°C for 48 h. Thirty two clear zone producing colonies were selected and screened for the production of bacteriocin using *E. coli* TISTR 780 and *S. aureus* TISTR 1466 as the Gram-negative and Gram-positive bacteria indicator.

Three of Thirty two isolates designated as LAB7, LAP12 and LAG1 were shown to be able to inhibit the growth of the indicator strains. Cell free supernatants of the selected strains were examined for the effect of pH and heat on antibacterial activity (Table 1). The bacterial strain, LAB7, isolated from bio-extract was found to tolerance heat at 100°C for 10, 20, 30 min and at 121 °C for 15 min. Its activity was also stable at wide pH range of 2-9 in both bacteria indicators. Therefore, LAB7 strain was selected for further studies.

**Table 1.** Antimicrobial effects of the supernatant of lactic acid bacteria isolated from traditional fermented food product and bio-extract

| Isolated | Sample | *E. coli* TISTR 780 | | | | | *S. aureus* TISTR 1466 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pH 2-9 | 100 °C 10min | 100 °C 20min | 100 °C 30min | 121 °C 15min | pH 2-9 | 100 °C 10min | 100 °C 20min | 100 °C 30min | 121 °C 15min |
| LAP12 | Pak-sain dong | + | + | + | + | - | + | + | + | - | - |
| LAG1 | Goong-jom | + | + | - | - | - | + | - | - | - | - |
| LAB7 | Bio-extract | + | + | + | + | + | + | + | + | + | + |

Positive results were recorded when the zone of inhibition of at least 1 mm around disc was observed.

*2. Kinetics of growth in batch fermentation*

To compare growth and antimicrobial activity between free cell and encapsulated cell, batch fermentation of LAB7 strain was performed using 1 l fermentor (Biostat Q, B.Braun Biotech Inc.,Germany) with 750 ml working volume. The fermentation conditions were free cell with 120 rpm agitation, free cell without agitation and encapsulated cell without agitation condition. Cells were seeded in fermentor all condition with 10% inoculums. For free cell fermentation, 18 h grown inoculums having approximately $10^8$ CFU/ml was used. In parallel for encapsulated cells, the 18 h grown inoculums was encapsulated by mixing with 2% (w/v) sodium alginate. The mixture then dropped by syringe into 0.1M $CaCl_2$ solution with agitation at 200 rpm for 30 min. The Ca-alginate beads have diameter 2 mm and cell concentration of approximately $10^6$ CFU per bead. Figure 1-3, show the fermentation plots of free cell with agitation, free cell without agitation and encapsulated cell without agitation, respectively. The results revealed that the exponential phase of this strain was during 2-8 h of cultivation. The glucose consumption rate of each condition was non-significantly different whereas the lactic acid production rate of encapsulated cell showed the highest value. The lactic acid production of Ca-alginate fermentation was 2.92 g/l/h whereas lactic acid production rate of free cell without agitation and free cell with agitation were 1.22 and 1.01 g/l/h, respectively. Similar results have also been reported by various researchers. For example, Rao et al. (2008) immobilized *Lactobacillus delbrueckii* cells in Ca-alginate beads for lactic acid production. Their result showed that the immobilized cell can increased lactic acid production yield when compared to the free cells.

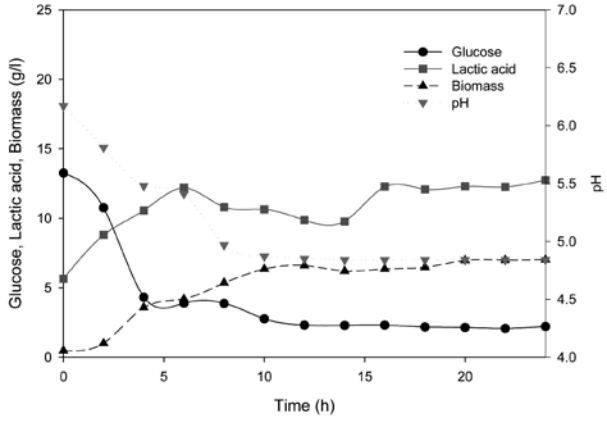


**Figure 1.** Cultivation of Free cell of LAB7 in a fermentor with 120 rpm agitation. Time courses of biomass, lactic acid, residual glucose and pH of the culture broth

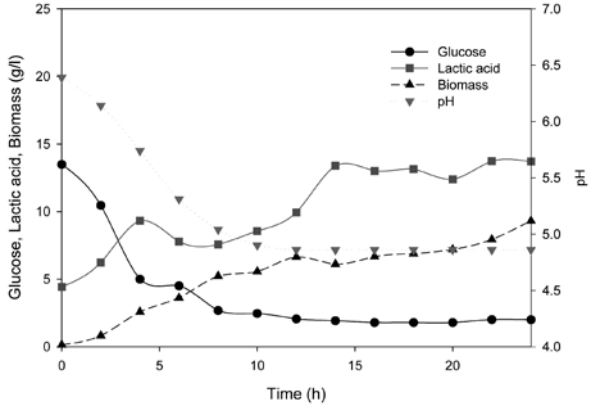


**Figure 2.** Cultivation of Free cell of LAB7 in a fermentor without agitation. Time courses of biomass, lactic acid, residual glucose and pH of the culture broth

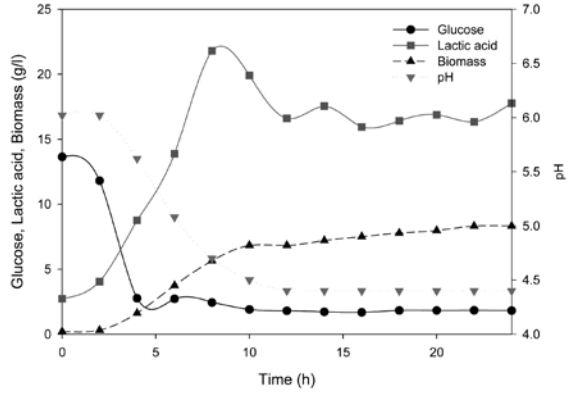


**Figure 3.** Cultivation of Ca-alginate encapsulated of LAB7 in a fermentor without agitation. Time courses of biomass, lactic acid, residual glucose and pH of the culture broth

*3. Bacteriocin production*

The comparison of the antibacterial activity of each fermentation condition against *E. coli* TISTR780 and *S. aureus* TISTR 1466 are shown in Figure 4. Under the encapsulation without agitation condition, the antibacterial activity against *E. coli* TISTR780 and *S. aureus* TISTR 1466 reached a maximum of 3200 AU/ml and 800 AU/ml respectively at 8-10 h cultivation. Comparing to free cell cultivation with and without agitation, the inhibitory activity gave less activity than encapsulated cell in both bacterial indicators. Free cells cultivation without agitation gave bacteriocin activity against *E. coli* TISTR 780 only 1600 AU/ml and 400 AU/ml, respectively. Free cells cultivation with agitation had the least bacteriocin activity than other fermentation conditions.

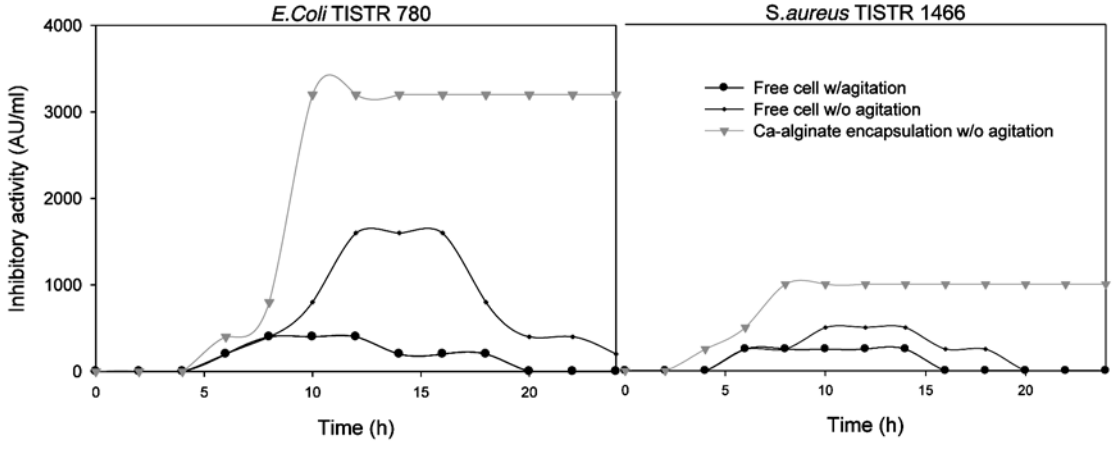


**Figure 4.** Antimicrobial activity of LAB7 in different culture condition against *E. coli* TISTR 780 and *S. aureus* TISTR 1466

In free cell fermentation system, bacteriocin activity was decreased, probably due to the digestion by proteolytic enzyme systems of the cells after cell death. This result similar to Ivanova et al., (2000-2002) immobilized lactic acid bacteria *Enterococcus faecium* A2000 in Ca-alginate beads. They reported that the bacteriocin concentration was increased by 50%, compared with the fermentation with free cells. It could be explained that the alginate beads had a protective role separating the bacteriocin in the medium from the proteolytic enzymes in the beads. This culture system advantages for long term bacteriocin production and encapsulated cell could be reused more than 2 times after filtration and resuspension in a new medium (Ivanova et al., 2000-2002: Rao et al., 2008).

## Conclusion

In this work, the results clearly showed that bacteriocin from LAB7 isolated from bio-extract not only have antibacterial activity against both Gram-positive and Gram-negative bacteria, but also strongly present high bacteriocin activity when encapsulated cell in calcium alginate bead. Further studies are required regarding to identification LAB7 strain, characterization and purification of its bacteriocin.

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