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Exopolysaccharide in *Lactobacillus* Rhamnosus Pn04 After Co-Culture with *Leuconostoc mesenteroides* Vtcc-B-643

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ABSTRACT

Exopolysaccharides (EPS) from lactic acid bacteria (LAB) have many applications in food industry, and pharmaceutical field. To improve EPS production in *Lactobacillus rhamnosus* PN04 and *Leuconostoc mesenteroides* VTCC-B-643, these bacteria were co-cultured together. There were shape changes in *Lactobacillus rhamnosus* into curved rod. Their EPS were estimated after co-cultured. After ethanol precipitation method, the colorimetric method were done using phenol-sulfuric acid assay, the EPS from *L. rhamnosus* PN04 increased from 2.446 mg/l to 4.228 mg/l while *Leuconostoc mesenteroides* increased from 3.217 mg/l to 4.712 mg/l. By SDS-PAGE analysis in both straight and curved *Lactobacillus rhamnosus*, there were the expressed proteins with the estimated molecular weight of 14, 60, 66 kDa while the curved *Lactobacillus rhamnosus* showed another proteins with molecular weight of 14, 20, 31, 45, 60, 66, 95 kDa. The results suggested that there were the interaction between two these strains. This is the first report showed there were differences in EPS production and protein expression after co-culture of *Lactobacillus rhamnosus* PN04 and *Leuconostoc mesenteroides* VTCC-B-643.

INTRODUCTION

Currently, most of exopolysaccharides are used for pharmaceutical and food industries. Generally, three groups of microbial polymers include cell wall polysaccharides, intercellular polysaccharides and extracellular polysaccharides. Especially, the extracellular polysaccharides, which are also called exopolysaccharides (EPS), diffuse into cell culture medium and easily isolate from the culture media from protein and cell debris. These exopolysaccharides have functional effects on beneficial health effects, so they are economically important (Cerning, 1990; Zhang *et al*., 2010).

Exopolysaccharides are also used as an emulsifier, thickeners, stabilizers, and gelling agents (Gamar *et al*., 1997; Duboc and Mollet, 2001). The use of EPS-producing lactic acid bacteria (LAB) could be a source to supply the safe exopolysaccharide (Frengova *et al*., 2002). Moreover, the exopolysaccharides of LAB have antitumoral, antiulcer, immunomodulating or cholesterol–lowering activity (Welman *et al*., 2003). Lactic acid bacteria (LAB) are gram - positive, non - respiring cocci or rods. They produce lactic acid as their major end productand through fermentation of carbohydrates. They have short sequences of 16S rDNA and their growth is optimum at pH 5.5-5.8. The organisms also require complex nutrition such as amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates (Khalisanni, 2011).

Lactobacillus could produced minicells for drug delivery (Doan and Nguyen, 2013). There are three important groups of EPS produced by LAB including glucans, fructans, and heteropolysaccharides (Pham *et al*., 2000). The EPS produced by *Lactobacillus rhamnosus* belong to the third group (Taeto *et al*., 2007).

Themesophilic LAB such as *Leuconostoc mesenteroides* also produces heteropolysaccharides (Welman and Maddox, 2003). Basing on the wide use of EPS, the trial to obtain more EPS should be required. Especially, *Lactobacillus rhamnosus* PN04 was firstly isolated from Vietnamese plant called *Houttuynia cordata*.Thunb (Nguyen *et al*., 2013) that was not studied on exopolysaccharide production.

Therefore, the study tried to find out the cause for exopolysaccharides producing from *Lactobacillus rhamnosus* PN04 (*L. rhamnosus*) by co-culture with *Leuconostoc mesenteroides* VTCC - B – 643 (*L. mesenterodes*).

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MATERIALS AND METHODS

Microorganisms and growth condition

Lactobacillus rhamnosus PN04 isolated from *Houttuynia cordata*.Thunb (Nguyen THK *et al*., 2013) was used in this study. *Leuconostoc mesenteroides* VTCC - B - 643 was purchased from Vietnam type culture collection. These strains were maintained in MRS agar and MRS broth. The MRS medium contains peptone, glucose, yeast extract, tween 80, dipotassium phosphate, sodium acetate, ammonium citrate, magnesium sulphate and manganese sulphate (De Man *et al*., 1960).

Co-culture procedure

Each colony of *L. mesenteroide* and *L. rhamnosus* was inoculated in MRS broth and incubated in aerobic conditions at 30^{o}C. After 48 hours for incubation, 100 µl of each culture was mixed together in one tube containing MRS broth. Then, the mixture was shaken for 10 minutes to homogenize. After 3 days, the bacteria mixture was spreaded on MRS agar and incubated in aerobic conditions at 30^{o}C for 48 hours.

The single colonies were taken randomly for screening the *L. mesenteroides* and *L. rhamnosus*. Then, the isolated colonies were selected for *Lactobacilli* and *Leuconostoc* by microscopic examination. The co-cultured *Lactobacillus* and co-cultured *Leuconostoc* were selected and inoculated individually in 5ml MRS broth and incubated in aerobic conditions at 30^{o}C for 48 hours.

Exopolysaccharide (EPS) isolation

EPS detection and purification were carried out by ethanol precipitation with a few modifications (Cerning J, 1990). The cell free supernatants were collected by centrifugation at 12.000rpm for 15 min at 4^{o}C. The EPS was precipitated from the supernatant with 3 volumes of 95% ethanol at 4^{o}C overnight, and collected by centrifugation at 12.000 rpm for 20 min. The EPS pellets were dissolved in distilled water. Quantitative analysis of EPS yield was performed by the phenol sulfuric acid method (Dubois M *et al*., 1991). Firstly, the 10-ml test tubes were washed with distilled water, and a blank solution was prepared with 500µl of distilled water, 500 µl of 4% phenol and 2.5 ml of 96% sulfuric acid. Next, the EPS standards were calibrated by 5 different EPS standard solutions. Then, 500µl of analyzed samples were added with 500 µl of 4% phenol followed by 2.5 ml of 96% sulfuric acid to all the tubes. All the solutions were measured at the wavelength of 490 nm. If the absorbance values of the measured samples were higher than 1.0 or higher thanthe values of the standards, the EPS from tested samples was diluted with distilled water. Final, the concentrations of EPS presented in the samples were calculated based on a graph plotting of the absorbance versus EPS concentration (mg/ml) and the EPS calibration standards.

SDS-PAGE analysis

In order to identify the effects of co-culture process on the EPS production of *L. rhamnosus*, the detection of protein expression was analyzed on SDS-PAGE. The protein marker was purchased from Bio-rad.

RESULTS AND DISCUSSION

Morphology differentiation

After co-cultured, the colonies of *Lactobacillus* and *Leuconostoc* were picked up and checked for the shape changes. Interestingly, there were the changes of the shape in *Lactobacillus* (Fig. 1). The morphology of *L. rhamnosus* after co-cultured was straight or curved rods while *L. mesenteroides* shapes before and after co-culture were not distinguished. As such result, the components in cell wall of *L. rhamnosus* could be affected after co-cultured. This phenomenon also implies that there were many changes in one strain if it was existed with the other strains. From this point of view, the probiotic preparation including more than one lactic acid strains should be considered. However, *Lactobacillus rhamnosus* PN04 isolated in *Houttuynia cordata*. Thunb (Nguyen *et al*., 2013) will not have properties as intestinal *Lactobacillus rhamnosus*.

Therefore, optimization of conditions to improve this strain is necessary. The morphological differentiation in *Lactobacillus rhamnosus* PN04 studies showed that its EPS could be produced and increased after co-cultured with *Leuconostoc mesenteroides*.

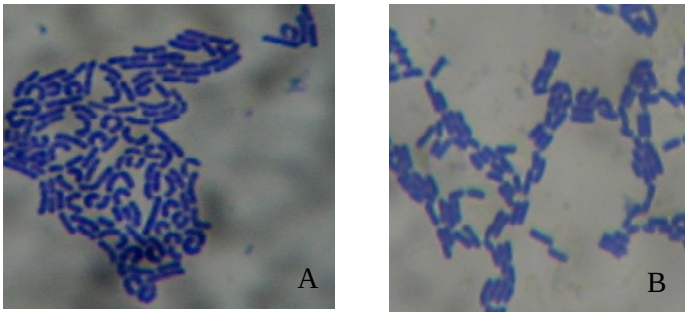

Fig. 1: Photomicrograph of *Lactobacillus rhamnosus* after co-culture. A: the curved rod shape of *Lactobacillus rhamnosus* after co-culture. B: the straight rod shape of *Lactobacillus rhamnosus* after co-culture.

Quantitative analysis

After analysis the morphological differentiation of *Lactobacillus rhamnosus* PN04 after co-cultured with *Leuconostoc mesenteroides*, the EPS was thought to have the effects on the morphological differentiation.

Therefore, the EPS is the subject to analyze. Based on the phenol sulfuric acid assay, the experiments were done in three replicates. After measuring the absorbance of the EPS standard with different concentrations, the equation of linear regression was given:

$$OD = -0.013 + 0.184 \times \text{concentration of EPS } (r^2 = 0.997)$$

Depending on this above equation, the exopolysaccharide contents of *L. rhamnosus* and *L. mesenteroides* before and after co-culture were calculated (Figure 2 and table 1).

Table. 1: EPS production before and after co-cultured.

	Before co-cultured (mg EPS/ml)	After co-cultured (mg EPS/ml)
Lactobacillus rhamnosus	2.446 ± 0.41895	4.228 ± 0.56533
Leuconostoc mesenteroides	3.217 ± 0.45400	4.712 ± 0.00567

(Mean ± SD, n = 3).There was a statistically significant difference between groups asdetermined by one-way ANOVA (F (3,8) = 11.812, p = 0.003).

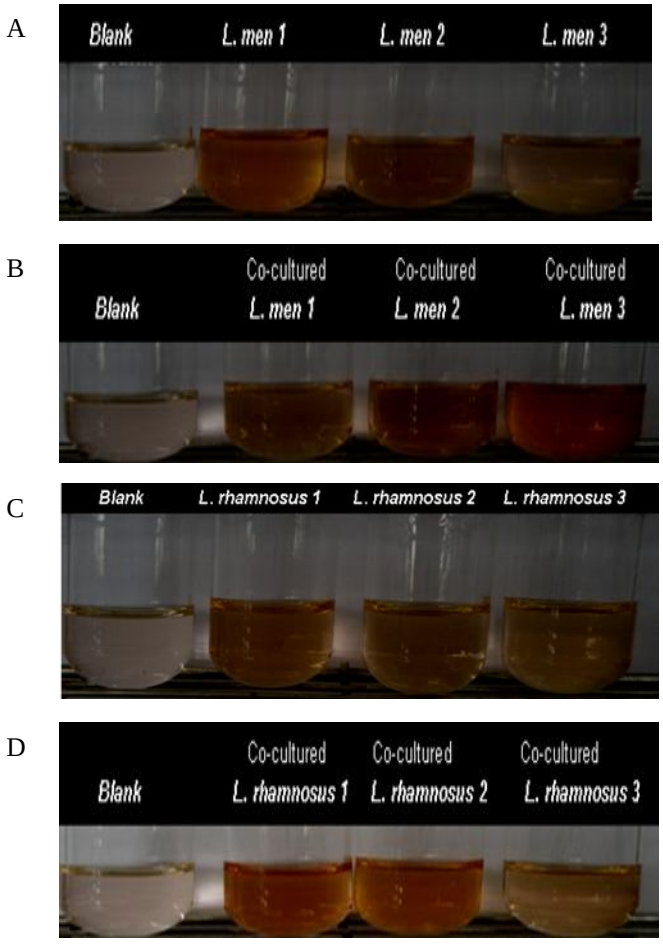

Fig. 2: Exopolysaccharide identification and quantification. Each experiment was performed in three replicates. A: Test for EPS yield of *Leuconostoc mesenteroides* before co-culture; A: Test for EPS yield of *Leuconostoc mesenteroides* after co-culture; C: Test for EPS yield of *Lactobacillus rhamnosus* before co-culture; D: Test for EPS yield of *Lactobacillusrhamnosus* after co-culture.

The EPS yields determined in *L.rhamnosus* and *L.mesenteroides* before and after co-cultured were statistically analyzed. There was a significant difference between these strains by one-way ANOVA (F (3, 8) =11.812, p = 0.003). A Tukey post-hoc test revealed that there was no statistically significant difference between *Leuconostoc* before and after co-culture (P = 0.066). However, the EPS of *Leuconostoc* also increased incredibly from 3.217 mg/ml to 4.712 mg/ml. In the co-cultured *Lactobacillus*, the EPS was statistically significant difference before and after co-cultured (P = 0.011). The concentration of EPS in co-cultured *Lactobacillus* was shown 4.228 mg/ml and higher than before cultured. The study also pointed that *Lactobacillus rhamnosus* and *Leuconostoc mesenteroides* could be packaged together without any inhibition.

SDS-PAGE analysis

After testing for EPS yields and morphological differentiation, SDS-PAGE analysis of proteins expressed in *Lactobacillus rhamnosus* and *Leuconostoc mesenteroides* after co-cultured. As presented in Figure 3, the protein bands at 14, 60, 66kDa appeared in *L.rhamnosus* with the straight rods while 14, 20, 31, 45, 60, 66, 95 kDa appeared in *L. rhamnosus* with the curved rods. Consequently, the expressed bands with 20, 31, 45, 96 kDa might affect on the morphology differentiation. It was thought that these proteins might play a role in cell shape and EPS expression. One of these protein may be ABC transporter permeases coding for part of a new pathway for synthesis of EPS such as a study in Mycoplasma pulmonis (Daubenspeck *et al*., 2009). Further study will be done to determine which proteins will relate to EPS production.

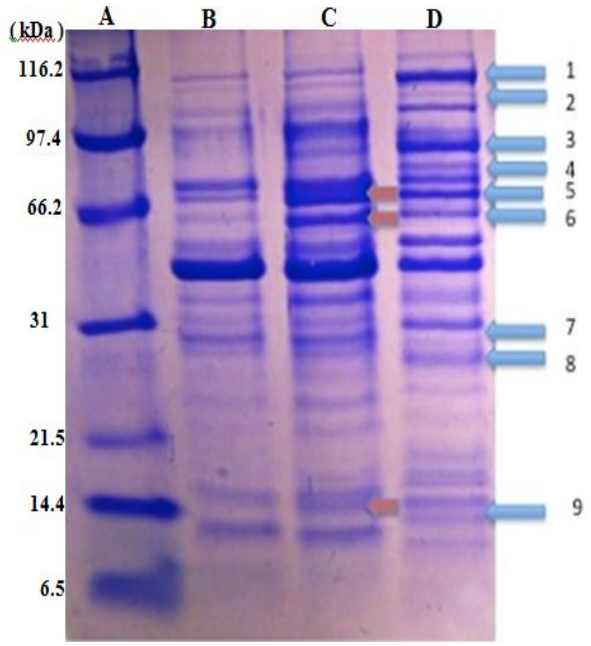

Fig. 3: SDS-PAGE analysis of *Lactobacillusrhamnosus*. A: Protein marker; B: *Lactobacillusrhamnosus* PN04 before co-culture; C: *Lactobacillusrhamnosus* PN04 had straight rod after co-culture; D: *Lactobacillusrhamnosus* PN04 had curved rod after co-culture.

CONCLUSIONS

Exopolysaccharides have good applications in industry, such as dairy industry, cosmetic. More recently, polysaccharides have been applied in drug delivery and delayed drug released formulation.

EPS serves as prebiotics as a nondigestible food ingredients that stimulate the growth and activity of a limited numbers of bacteria in colon, thus EPS is used to improve the host health. EPS also has effect on cancer cells.The study was successful in co-culturing of *Lactobacillus rhamnosus* and *Leuconostoc mesenteroides*. These strains did not interfere together. The morphological changes and EPS yield improvement might be due to the expression of proteins expressed on SDS-PAGE. It meant that *Leuconostoc* might produce some substances

that affected on *Lactobacillus rhamnosus*. From the expressed proteins, the pathway for EPS biosynthesis will be discovered so far.

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