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Detection of uronic acid of *Lactobacillus rhamnosus* PN04

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ABSTRACT

Lactobacillus rhamnosus is known as species that had many benefits in pharmaceutical, dairy food industry. To supply more applications for life, the detection of uronic production of *Lactobacillus rhamnosus* PN04 was done. After the extracellular components of *Lactobacillus rhamnosus* PN04 culture were precipitated with 1% cetyl pyridium chloride, the precipitants were used to check the uronic acid production capacity by carbazole assay according to BP98. As a result, the highest uronic acid yield was produced at 96 h cultivation. The maximum of uronic acid was about 74mg/L. The study was the first report the uronic acid existing in *Lactobacillus rhamnosus* PN04.

INTRODUCTION

Lactobacillus rhamnosus is a bacterium that was originally considered to be a subspecies of *Lactobacillus casei*, but the later genetic research found it to be a species of its own. Some strains of *Lactobacillus rhamnosus* are being used as probiotics (Coeuret *et al.,* 2007). The species are sometimes presented in yogurt and other dairy products (Coeuret et al, 2003). The most common used *Lactobacillus rhamnosus* strain is *Lactobacillus rhamnosus GG* ATCC 3103.

That was isolated in 1983 from the intestinal tract of healthy human being; filed for patent 17 April 1985, by Sherwood Gorbach and Barry Goldin (Gorbach *et al.,* 1989*). Lactobacillus rhamnosus GG* ATCC 3103 can be used to prevent and treat diarrhea both in children and adults (Canaani *et al.,* 2007; Guandalini *et al.,* 2000). *Lactobacillus rhamnosus* can be used to treat gastrointestinal disorders caused by vancomycin-resistant enterococcus in renal patients (Manley *et al.,* 2007*).* Additionally, *Lactobacillus rhamnosus* was found to have antimicrobial effects to pathogens such as *Streptococcus sobrinus* (Meurman *et al.,* 1995), *Salmonella typhimurium* (De Keersmaecker *et al.,* 2006).

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In order to obtain many benefits of *Lactobacillus rhamnosus*, the study had detected the uronic acid. This study focused on *Lactobacillus rhamnosus* PN04 isolated in the plant named *Hottuynia cordata Thunb*. (Nguyen *et al.,* 2013). Because the source of this strain is from plant, not from intestine or milk found before, it has thought to supply the different applications for life because of the symbiotics.

Uronic acids are constituents of hyaluronic acid that composes of D-glucuronic acid and D-N-acetylglucosamine. It is one of the most potential biological materials and widely used in pharmaceutical and cosmetics. Hyaluronic acid plays an important role in cancer metastasis (Bharadwaj *et al.,* 2007). Uronic acid is also component of glycosaminoglycans used for therapeutic cancer (George *et al.,* 2006). Hyaluronic acid production by *Streptococcus zooepidemicus* (Vázquez *et al.,* 2010). As the above stated statements, it is necessary to detect uronic acid from the safe source. The study on *Lactobacillus rhamnosus* PN04 isolated in the plant named *Hottuynia cordata Thunb*. to bring out many applications so far.

MATERIAL AND METHOD

Bacteria strains

L. rhamnosus PN04 was isolated from *Hottuynica cordata* Thunb (Nguyen *et al.,* 2013).

Uronic acid isolation

L. rhamnosus PN04 was cultured in MRS broth (de Man et al, 1960). The starting cell numbers equal to 10^6 CFU/ml. The cultivation was optimized in difference times at 24h, 48h, 72h, 96h and 120h. The supernatant was collected and precipitated with cetyl pyridinium chloride according to the method of Rosa (Rosa *et al.*, 2007). The 1% cetyl pyridinium chloride (CPC) (Merck) was added into the supernatant. The reaction was left at room temperature for 24h. Then, the mixture was centrifuged at 10 000 rpm at 4°C for 5 minutes. The pellet was obtained and dissolved in 0.5ml of 0.9M sodium chloride (NaCl). Then 1ml of absolute ethanol was added and the mixture was left for 24h at 20°C. After centrifugation, the supernatant was discarded and the pellet was washed once with 3ml of 80% ethanol. The pellet was dried by freeze-dried.

Carbazole assay

Uronic acid was determined using carbazole calorimetric reagent (Dische, 1946). 500 µl of sample was added in the test tube. The tube was cooled in ice-bath, then carefully added with 3ml of ice-cold sulfuric acid containing borate. The mixture was mixed well and incubated at 100°C for 10 min. After incubation, the mixture was cooled in ice bath and 100µl of 0.1% carbazole (Merck) was added. The mixture was mixed well and incubated at 100°C for 15 min. Then, the tube was cooled at room temperature and measured the absorbance at 525nm. The standard used in the study was D-galacturonic acid (Merck).

High performance liquid chromatography (HPLC) analysis

Freeze-dried sample was re-suspended in the solution of 1M sodium chloride, centrifuged and passed through 0.22 µm filter membrane. The solutions were analyzed by the HPLC, with C18 column, refractive index detector along with D-galacturonic acid as standard. The mobile phase was the solution of 0.5M KH_2PO_4, pH 2.5 with current speed of 1ml/minute. Column temperature was maintained at 30°C.

Statistic analysis

All of samples were performed triplicate for collect data. And all of datas were analyzed by SPSS 16.0.

RESULTS AND DISCUSSION

Uronic acid qualification

After precipitation with cetyl pyridium chloride (CPC), the precipitant was collected after centrifugation and freeze-dried. The weighed biomasses in different times were shown in figure 1. The highest biomass was produced approximately 2.6 mg for 96h. The lowest was produced approximately 1.4 mg for 24h. The biomass amounts increased from 24h to 96h and then reduced at 120h (figure 1). The evaluated biomass were used to qualify the uronic acid. All samples were detected by the reaction to carbazole. The red-purple color appeared in the detected sample (figure 2). The darkest color appeared in the 96h sample and the lightest color was appeared in the 24h sample. The uronic acid produced in 120h was higher than in 24h even the biomass was lower. There was the other products precipitated with CPC. More studies should be done.

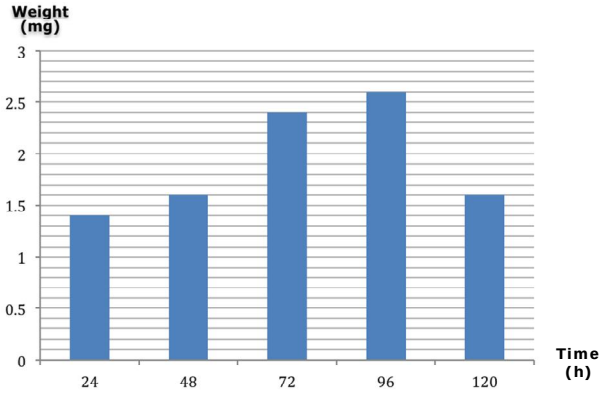

Fig. 1: Biomass collection according to different incubation time.

Uronic acid quantification

To determine the uronic acid yield, the reaction mixtures were quantitative in different periods (table 1). By carbazole assay, the uronic acid yields in the examined periods were significantly different by statistic analysis. The highest yield was in 96h culture (figure 2). The maximum production was 7.4 mg/L. The lowest yield was 0.17 mg/L in 24h culture. The yieds of uronic acid in 48h, 72h, 120h were 0.22 mg/L, 0.24mg/L and 0.37mg/L, respectively. However, there were no significant differences in the uronic acid yields after 48h, 72h, 120h incubation.

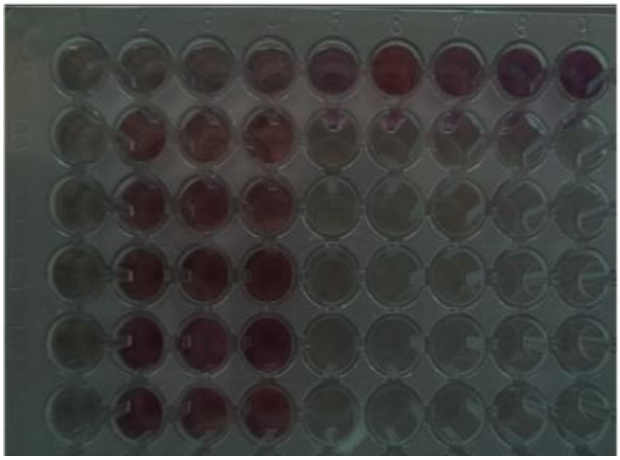

Fig. 2: Carbazole assay results. Wells of A1, B1, C1, D1, E1, F1: negative control. Wells of A2, A3, A4, A5, A6, A8, A9: positive control. Wells of B2, B3, B4: Samples of 24h. Wells of C2, C3, C4: Samples of 24h. Wells of D2, D3, D4: Samples of 48h. Wells of E2, E3, E4: Samples of 96h. Wells of F2, F3, F4: Samples of 120h.

Table. 1: The uronic acid measurement in different time.

Sample	Uronic acid yields (mg)
24 h	0.17 ±0.02
48 h	0.22±0.05
72 h	0.24±0.05
96 h	0.74±0.1
120 h	0.37±0.07

Mean ±Stdev

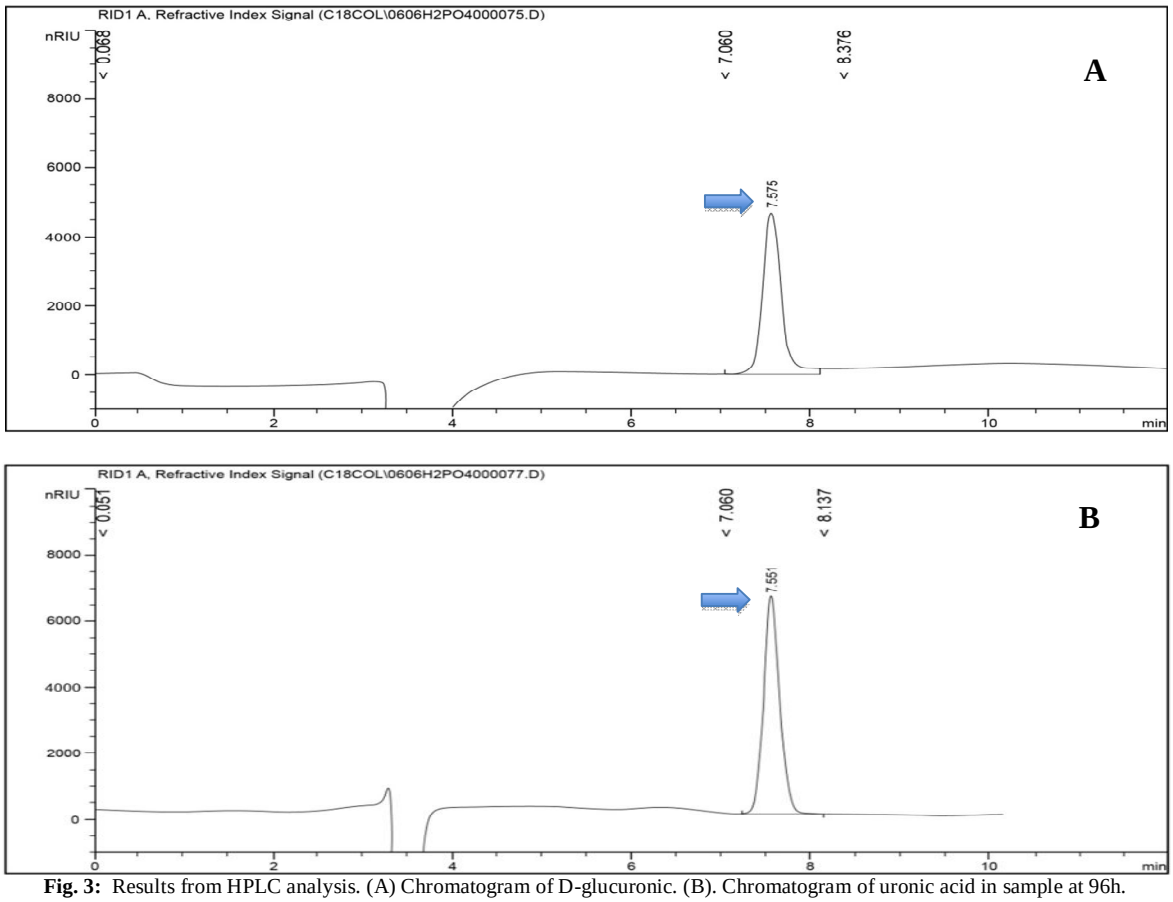

Fig. 3: Results from HPLC analysis. (A) Chromatogram of D-glucuronic. (B). Chromatogram of uronic acid in sample at 96h.

High performance liquid chromatography analysis

To confirm the uronic acid produced by *L. rhamnosus* PN04, the supernatant of sample 96h was analyzed by HPLC. In those chromatograms, there were the peaks existed at the similar retention time of 7.575 and 7.561 min corresponding the standard glucuronic acid and tested uronic acid, respectively (figure 3). Therefore, the uronic acid existed in the culture of *L. rhamnosus* PN04.

CONCLUSION

In conclusion, the result from this study was showed that uronic acid was detected by growing *L. rhamnosus* PN04. Detected uronic acid of *L. rhamnosus* PN04 showed that *L. rhamnosus* PN04 might be a potent source for hyaluronic acid and other extrapolysaccharides supplying for pharmaceutical field. To study these compounds in *L. rhamnosus*, more study should be done.

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