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## D-saccharic acid-1,4-lactone – Producing Acetic Acid Bacteria in Kombucha Fermentation Process

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### ABSTRACT

Kombucha is a refreshing beverage with a slightly sour taste, obtained by the fermentation of probiotic microorganisms. The main compositions include acetic acid, lactic acid, gluconic acid, glucuronic acid as well as D-saccharic acid-1,4-lactone (DSL). D-Saccharic acid-1,4-lactone (DSL) is a crucial substance involved in liver detoxification. DSL has ability to inhibits the activity of  $\beta$ -glucuronidase, an enzyme related with cancers. The objective of this study was to isolate acetic acid bacteria which produced DSL from fruit, fermented fruit juices and kombucha. One hundred and fifty-three isolates of acetic acid bacteria were obtained from pineapple, 7 fermented fruit juices and 3 samples of kombucha. Sixty-eight isolates had ability to produced acid from green tea broth. They were selected for DSL production and compared with *Acetobacter tropicalis* P6. It was found that fourteen isolates, PM20, PM30, PM31, PM35, PM36, PM56, PM60, PM70, PM99, PM103, PM123, PM131, PM135, PM136 and *A. tropicalis* P6 gave the yield of DSL between 0.02 to 0.33 mg/ml.

**Keywords:** Acetic acid bacteria, Kombucha, DSL

### INTRODUCTION

Kombucha is a traditional fermented tea beverage which is widely consumed because of its beneficial properties (Chakravotry et al., 2016). It contains many compounds such as antioxidants, organic acids, tea polyphenols

and minerals (Jayabalan et al., 2007). D-Saccharic acid-1,4-lactone (DSL) is one of valuable organic acid in kombucha. It inhibits the activity of  $\beta$ -glucuronidase, an enzyme that generates toxic and carcinogenic substances in the lumen of the gut. In human, fecal  $\beta$ -glucuronidase activity was shown to be higher in colorectal cancer patients as compared to healthy controls. DSL at the concentration from 0.03 to 0.15 mg/ml can inhibit  $\beta$ -glucuronidase activity (Wang et al., 2010).

In many previous studies, acetic acid bacteria had ability to produced DSL (Yang et al., 2010; Wang et al., 2013; Nguyen et al., 2015). Acetic acid bacteria (AAB) are Gram-negative, rod shape cells, obligate aerobic bacteria belong to the family *Acetobactaceae* of *Alphaproteobacteria* (Saichana et al., 2015). These bacteria can be isolated from fruits, fermented fruit juices, alcoholic beverage, vinegar, flowers, honey (Klawpiyapamornkun et al., 2015) Thus, acetic acid bacteria will be isolated to promote DSL production in kombucha and increase the benefit of kombucha

## MATERIAL AND METHODS

### Materials

Fermented fruit juices were collected from Chanthaburi province, Thailand in December 2017. Twelve samples of fermented fruit juices includes indian gooseberry, longan, lychee, lime, lime & kaffir lime, mangosteen, mulberry, myrobalan wood, passion fruit, pineapple, santol and star fruit. Pineapple and 3 samples of kombucha were purchased from Chiang Mai province, Thailand in February 2018.

### Isolation of acetic acid bacteria

Fermented fruit juices and Kombucha were incubated in 10% sugared water in the ratio of 1:1 at 30°C for 7 days. Samples were dilute and spread on bromocresol purple ethanol agar and GYC agar supplemented with 100 ppm of cyclohexamide. Plates were incubated at 30°C for 4 days. The colonies which produce yellow zone on bromocresol purple ethanol agar and clear zone on GYC agar were selected and purified.

### Identification of acetic acid bacteria

Gram negative bacterial cells were selected for further biochemical tests compare with *Acetobacter aceti* TISTR354 and *Acetobacter tropicalis* P6. Catalase test was determined (Nguyen et al., 2015). The overoxidation of ethanol was determined on Carr medium (Karin and Kain, 2006). Ketogenesis

from glycerol, cellulose production, water-soluble brown pigment, acid production from glucose and growth in ethanol were tested according to Klawpiyapamornkun et al. (2015).

### **Acid production from green tea broth**

Green tea broth was prepared by using 1% of dry tea leaves in boiled water and steeped for 10 minutes, and then leaves were separated by a sieve. After that, 10% sucrose was added and boiled for 5 minutes.

The isolates were transferred to green tea broth and incubated at  $30\pm 2^{\circ}\text{C}$  for 15 days. pH of green tea broth were determined by pH indicator strips. Isolates which produced acid from green tea broth were selected for DSL production. Ten percent of isolates were inoculated into 250 ml flask containing 100 ml green tea broth and incubated at  $30\pm 2^{\circ}\text{C}$  for 15 days.

### **Determination of organic acid by High-performance liquid chromatography (HPLC)**

Samples were used to determine the organic acid content (glucuronic, gluconic, DSL, ascorbic and acetic) using reversed phase chromatography, with Agilent 1200 series HPLC. Samples were filtrated through a  $0.45\mu\text{m}$  nylon membrane filter. Analysis was performed in isocratic mode with 3% methanol in 20mM  $\text{KH}_2\text{PO}_4$ , pH 2.4. A 20  $\mu\text{l}$  of filtrate sample was injected onto column (Inertsil ODS-3 C18  $4.6\times 150$  mm, 5  $\mu\text{m}$ ) at a flow rate of 0.8 ml/min with column temperature of  $28^{\circ}\text{C}$  (Chakravorty et al., 2016). The analyses were monitored at wavelength of 210 nm by comparing the retention time of the standard compounds.

## **RESULTS**

### **Isolation of acetic acid bacteria**

One hundred and fifty three isolates of Acetic acid bacteria were isolated from 7 fermented fruit juices, pineapple and 3 kombucha samples (Table 1).

All of isolates were gram negative, rod shape, overoxidation and catalase positive. Sixty-eight isolates had ability to produced acid from green tea broth. After that, 68 isolates were tested for their ability to produced DSL. Isolates PM20, PM30, PM31, PM35, PM36, PM56, PM60, PM70, PM99, PM103, PM123, PM131, PM135, PM136 and *A. tropicalis* P6 showed DSL production (Table 2).

**Table 1.** Acetic acid bacteria isolated from fermented fruit juices, fruit and kombucha

fermented fruit juice		fruit		kombucha	
samples	No. of isolates	samples	No. of isolates	samples	No. of isolates
mangosteen	60	pineapple	1	kombucha1	10
indian gooseberry	17			kombucha2	4
star fruit	22			kombucha3	1
lychee	15				
mulberry	10				
chebulic myrobalans	12				
lime+kaffir lime	1				
<b>Total</b>	<b>137</b>		<b>1</b>		<b>15</b>
<b>153 isolates</b>					

**Table 2.** Biochemical characteristics of acetic acid bacteria

isolates	Acid from Glucose	Brown pigment	Cellulose	Hoyer's medium	Ketogenesis from glycerol	Acid from tea broth	DSL production
<i>A. tropicalis</i> P6	+	-	-	+	-	+	+
<i>A. aceti</i> TISTR354	+	-	-	+	-	-	-
PM20	+	-	-	+	+	+	+
PM30	+	-	+	+	+	+	+
PM31	+	-	+	+	+	+	+
PM35	+	-	+	+	+	+	+
PM36	+	-	+	+	+	+	+
PM56	+	-	+	+	+	+	+
PM60	+	-	+	+	+	+	+
PM70	+	-	-	+	+	+	+
PM99	+	-	-	+	+	+	+
PM103	+	-	-	+	+	+	+

**Table 2.** Biochemical characteristics of acetic acid bacteria (Cont.)

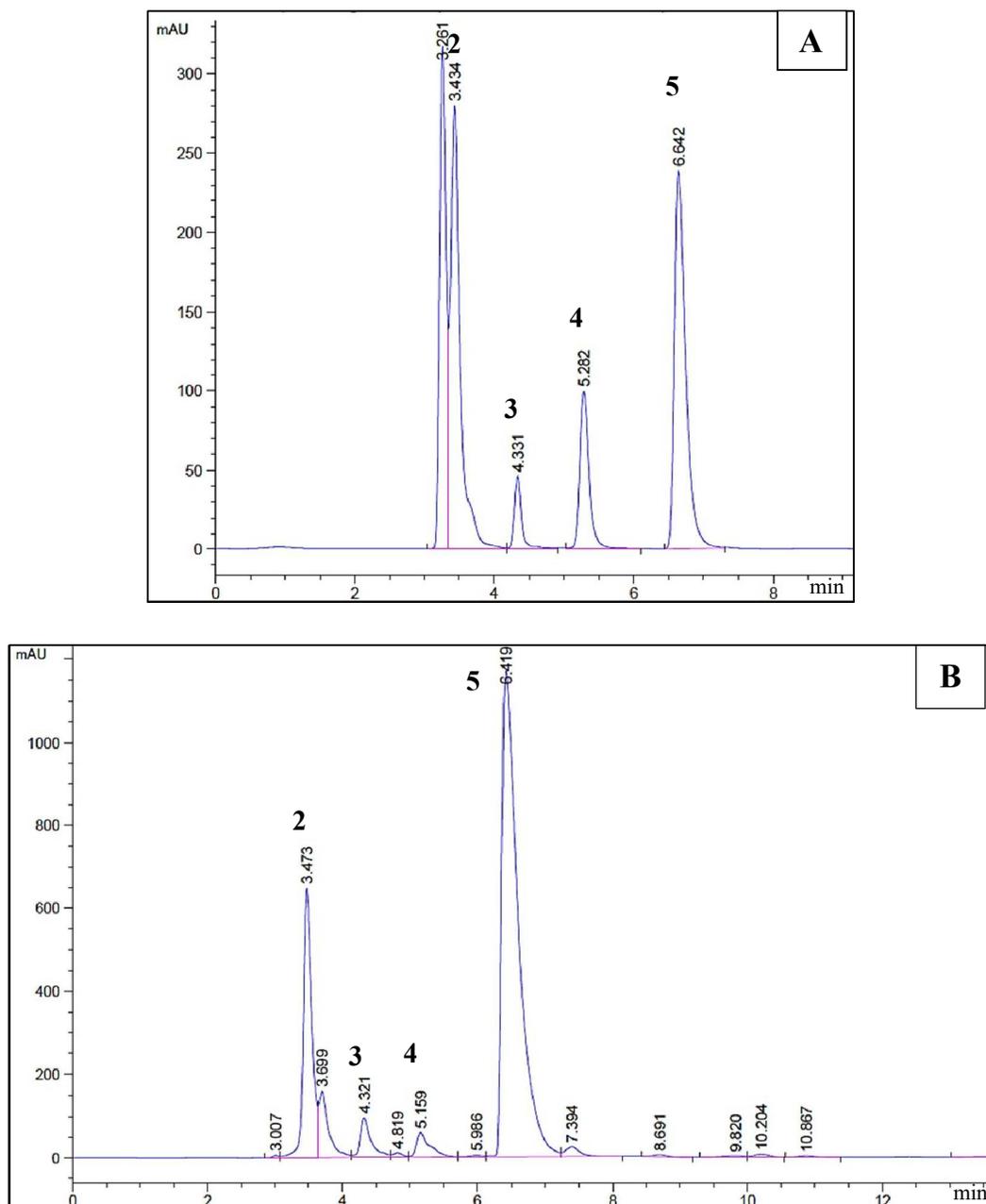
isolates	Acid from Glucose	Brown pigment	Cellulose	Hoyer's medium	Ketogenesis from glycerol	Acid from tea broth	DSL production
PM123	+	-	+	+	+	+	+
PM131	+	-	+	+	+	+	+
PM135	+	-	-	+	+	+	+
PM136	+	-	-	+	+	+	+

Fourteen isolates; PM20, PM30, PM31, PM35, PM36, PM56, PM60, PM70, PM99, PM103, PM123, PM131, PM135, PM136 and *A. tropicalis* P6 showed DSL production between 0.02 to 0.33 mg/ml. Moreover, they can produced gluconic acid and ascorbic acid. Isolates PM30, PM35, PM36, PM60, PM135 and *A. tropicalis* P6 were able to produced acetic acid (Table 3). The HPLC chromatograms are shown in Figure 1.

**Table 3.** Organic acid contents of acetic acid bacteria isolates

isolates	organic acid (mg/ml)				
	glucuronic	gluconic	DSL	ascorbic	acetic
<i>A. tropicalis</i> P6	ND	1.93	0.21	0.08	1.95
PM20	ND	1.88	0.06	0.06	ND
PM30	ND	1.98	0.24	0.09	1.53
PM31	ND	0.92	0.15	0.09	ND
PM35	ND	2.40	0.02	0.14	1.74
PM36	ND	2.36	0.11	0.15	1.92
PM56	ND	3.53	0.08	0.14	ND
PM60	ND	2.43	0.26	0.10	1.56
PM70	ND	1.92	0.17	0.12	ND
PM99	ND	2.11	0.28	0.09	ND
PM103	ND	1.96	0.19	0.14	ND
PM123	ND	3.19	0.10	0.14	ND
PM131	ND	2.38	0.14	0.14	ND
PM135	ND	2.13	0.33	0.14	1.87
PM136	ND	1.99	0.19	0.14	ND

ND: not detected



**Figure 1.** HPLC chromatogram of standards (A) and sample (B). Peak (1) glucuronic acid; Peak (2) gluconic acid; Peak (3) DSL; Peak (4) ascorbic acid and Peak (5) acetic acid respectively.

## DISCUSSION

Acetic acid bacteria (AAB) were found in a variety of sources (Sengun and Karabiyikli, 2011; Klawpiyapamornkun et al., 2015). *Gluconacetobacter* sp. A4 is an AAB which had ability to produce D- Saccharic acid 1,4- lactone (DSL) from black tea broth (Yang et al. 2010). This compound is reported to be the hepatoprotective and the inhibitor of  $\beta$ -glucuronidase (Wang et al., 2010; Chakravorty et al., 2016). The concentration of DSL between 0.03 to 0.15 mg/ml can inhibit  $\beta$ -glucuronidase activity (Wang et al., 2010) Moreover, AAB play a major role in organic acids production in kombucha (Jayabalan et al., 2014). In this study we found AAB that can produce DSL at the concentration from 0.02 to 0.33 mg/ml. which can inhibited  $\beta$ -glucuronidase activity.

## CONCLUSION

AAB have the important roles in production of DSL in kombucha. The concentration of DSL produced has an inhibitory potential against  $\beta$ -glucuronidase activity. However, the optimized conditions for DSL production are under investigation.

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## REFERENCES

- Chakravotry, S., Bhattacharya, S., Chatzinotas, A., Chakraborty, W., Bhattacharya, D. and Gachhui, R. 2016. Kombucha tea fermentation: microbial and biochemical dynamic. *International Journal of Food Microbiology*, 220: 63-72.
- Jayabalan, R., Marimuthu, S. and Swaminathan, K. 2007. Changes in content of organic acids and tea polyphenols during Kombucha Tea fermentation. *Food Chemistry*, 102: 392-398.
- Jayabalan, R., Malbasa, R. V., Loncar, E. S., Vitas, J. S. and Sathishkumar, M. 2014. A Review on kombucha tea- microbiology, composition, fermentation, beneficial effects, toxicity and tea fungus.

- Comprehensive Reviews in Food Science and Food Safety, 13: 538-550.
- Karin, S. and Kain, M. 2006. *Acetobacter tropicalis* in spontaneously fermented wines with vinegar fermentation in Austria. *Mitteilungen Klosterneuburg*. 56: 102–107.
- Klawpiyapamornkun, T., Bovonsombut, S. and Bovonsombut, S. 2015. Isolation and characterization of acetic acid bacteria from fruits and fermented fruit juices for Vinegar Production. *Food and Applied Bioscience Journal*, 3: 30-38.
- Nguyen, N.K., Nguyen, P.B., Nguyen, H.T. and Le, P.H. 2015. Screening the optimal ratio of symbiosis between isolated yeast and acetic acid bacteria strain from traditional kombucha for high-level production of glucuronic acid. *LWT-Food Science and Technology*. 64: 1149-1155.
- Saichana, N., Matsushita, K., Adachi, O., Frebort, I. and Frebortova, J. 2015. Acetic acid bacteria: A group of bacteria with versatile biotechnological applications. *Biotechnology Advances*. 33: 1260-1271.
- Sengun, I. Y. and Karabiyikli, S. 2011. Importance of acetic acid bacteria in food industry. *Food Control*. 22: 647-656.
- Wang, K., Gan, X., Tang, X., Wang, S. and Tan, H. 2010. Determination of D-Saccharic acid-1,4-lactone from brewed kombucha broth by High-Performance Capillary Electrophoresis. *Journal of Chromatography B*, 878: 371-374.
- Wang, Y., Ji, B., Wu, w., Wang, R., Yang, Z., Zhang, D. and Tian, W. 2013. Hepatoprotective effects of kombucha tea: identification of functional strains and quantification of functional components. *Journal of the Science of Food and Agriculture*. 94(2): 265-272.
- Yang, Z., Zhou, F., Ji, B., Li, B., Luo, Y., Yang, L. and Li, T. 2010. Symbiosis between microorganisms from kombucha and kefir: potential significance to the enhancement of kombucha function. *Applied Biochemistry and Biotechnology*, 160: 446-455.