PAPER

INFLUENCE OF EXOPOLYSACCHARIDE ON THE GROWTH OF LACTIC ACID BACTERIA

H. TSUDA*[1,2], S. OKUDA[2], T. HARAGUCHI[2] and K. KODAMA[2]

[1]Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki city, Aomori 036-8561, Japan

[2]Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, 562, Nanatsuka, Shobara City, Hiroshima 727-0023, Japan

*Corresponding author: Tel.: +89172362111; Fax: +81172393750

E-mail address: tsudah@hirosaki-u.ac.jp

ABSTRACT

The highest production of exopolysaccharides (EPSs) by *Lactobacillus buchneri* GM3701 and *Lb. plantarum* RB-3 was 216 and 79.0 mg/L, when incubated in 10% glucose media at 25°C for 6 d and 5% glucose media at 25°C for 4 d, respectively. The EPSs consisted of mainly glucose. Bacterial growth in the media supplemented with the EPSs was investigated using various bacteria, including *Lactobacillus, Staphylococcus* and *Escherichia* strains. The EPS enhanced the growth of *Lb. farciminis* HM2001. This result suggests that the growth of some lactic acid bacteria can be enhanced by the supplementation with an EPS produced by *Lactobacillus* strains.

Keywords: exopolysaccharide, growth enhancement, Lactobacillus, yeast extract

1. INTRODUCTION

Exopolysaccharides (EPSs) are long-chain carbohydrate polymers that are released by a wide range of microorganisms, including fungi and bacteria (DONOT *et al*., 2012). EPSs are present outside of the cell wall, and they exhibit great diversity, not only in their sugar composition but also in their linkage, branching, and substitution (CHAPOT-CHARTIER and KULAKAUSKAS, 2014). EPSs can be bound or unbound to the cell wall, and cell-bound EPSs are distinguished into capsular polysaccharides (CPS) (CAGGIANIELLO *et al*., 2016). The physiological role of bacterial EPSs is not yet completely understood. EPSs may be associated with cell protection against unfavourable environmental conditions, like desiccation, the presence of oxygen or toxic compounds, low temperatures, high osmotic pressures, and bacteriophage attack, and they may contribute to the uptake of metal ions, biofilm formation, and cell adhesion mechanisms (CAGGIANIELLO *et al*., 2016; CERNING, 1990; SANCHEZ *et al*., 2006). On the other hand, LIU *et al*. (2017) reported that EPSs produced by *Lactobacillus plantarum* inhibited the biofilm formation of *Pseudomonas, Escherichia, Salmonella*, and *Staphylococcus*.

Generally, it is thought to be very unlikely that bacteria can use EPSs as an energy source; however, there are some studies that have reported that EPSs are degraded by lactic acid bacteria (LAB). PHAM *et al*. (2000) reported that EPSs produced by *Lactobacillus rhamnosus* were degraded by the enzymes of this strain and that some reducing sugars were liberated. Additionally, some *Bifidobacterium* strains can breakdown plant cell wall polysaccharides (VAN DEN BROEK *et al*., 2008). Furthermore, the growth of LAB and *Bifidobacterium* strains were enhanced by supplementing the cultures with the EPS produced by lactobacilli (HONGPATTARAKERE *et al*., 2012; KORAKLI *et al*., 2002; RUIJSSENAARS *et al*., 2000; TSUDA and MIYAMOTO, 2010). It is unclear if this enhancement was because of the utilization of monosaccharides degraded from the EPSs. To begin with, there are only a few reports about the influence of EPSs on the growth of LAB, and more studies are necessary to better understand the influence of EPSs on the growth of LAB. We should point out that crudely purified EPSs may contain mannan from the yeast extract in media, and the mannan may be used by some bacteria.

In this study, EPSs, produced by a *Lactobacillus* strain, were investigated for their yields and monosaccharide components. Furthermore, the influence of EPSs on growth was evaluated using *Lactobacillus, Streptococcus, Staphylococcus, Aerococcus,* and *Escherichia* strains.

2. MATERIAL AND METHODS

2.1. Bacterial strains

In the present study, 22 bacterial strains, including *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Staphylococcus, Aerococcus, Paenibacillus,* and *Escherichia coli*, were used (Table 1). The strains were isolated from Wagyu milk, Japanese pickles and fermented sushi at our laboratory unless otherwise stated (TSUDA *et al*., 2012; TSUDA, 2015). The lactic acid bacteria were incubated in TYG broth (10 g/L tryptone, 5.0 g/L yeast extract, 5.0 g/L glucose, 1.0 g/L Tween 80, and 0.1 g/L L-cysteine HCl monohydrate, pH 6.8 ± 0.2). The other strains were also incubated in TYG broth to compare the growth rate. All strains were stored in 10% reconstituted skim milk at -20°C. An inoculum of 1% was used for all tests.

Table 1. Strains used in the present study.

Species	Strain No.	Source
Lactobacillus reuteri	PUHM1004	Wagyu milk
Lb. coryniformis	SAB01	Japanese pickles
Lb. sakei subsp. *sakei*	SAB04	Japanese pickles
Lb. delbruckii subsp. *bulgaricus*	NBRC 13953	*
Lb. alimentarius	EM2001	Fermented sushi
Lb. casei	HM3701	Fermented sushi
Lb. buchneri	GM3701	Fermented sushi
Lb. farciminis	HM2001	Fermented sushi
Lb. acidipiscis	JAM3706	Fermented sushi
Lb. plantarum	JAB2001	Fermented sushi
Lb. plantarum	RB3	Japanese pickles
Lb. plantarum	PUHM1023	Wagyu milk
Enterococcus faecalis	PUHM1006	Wagyu milk
Lactococcus lactis	PUHM1014	Wagyu milk
Streptococcus thermophilus	NBRC 13957	*
Str. salivarius	AB3002	Fermented sushi
Str. pluranimalium	PUHM1022	Wagyu milk
Aerococcus viridans	PUHM5301	Wagyu milk
Staphylococcus auricularis	PUHM5201	Wagyu milk
Sta. aurigulas	PUHM5203	Wagyu milk
Paenibacillus turicensis	PUHM5101	Wagyu milk
Escherichia coli	NBRC 102203	*

*: NBRC: NITE Biological Resource Center.

2.2. Effects of the incubation conditions on exopolysaccharide (EPS) production

Lb. buchneri GM3701 and *Lb. plantarum* RB-3 were used as EPS-producing LAB. The EPS productivities were tested using the 22 strains in Table 1, and the cultures of the two strains showed ropiness. Therefore, strains GM3701 and RB-3 were selected as EPS producers. The ropiness was confirmed by inserting a sterile wire loop and pulling ropes from the media. A clear zone area using the Indian ink method was used as a simplified indicator of the EPS yield. Twenty microliter of LAB culture was put on a glass slide, and a few drops of Indian ink were added and mixed. A cover slip was placed over the mixture, and then, the prepared slide was observed microscopically with immersion oil. A clear zone area for each individual cell was obtained as follows: the cell area was subtracted from the clear zone area, and then, the obtained figures were divided by the cell numbers. This assay was performed with at least 10 clumps.
The effect of the incubation temperature on EPS production was investigated at 25, 30, and 37°C. Glucose, fructose, sucrose, and lactose were supplemented in TY broth (10 g/L tryptone, 5.0 g/L yeast extract, 1.0 g/L Tween 80, and 0.1 g/L L-cysteine HCl monohydrate, pH 6.8 ± 0.2) as carbon sources at 25, 50, and 100 g/L.
All assays were performed at least three times.

2.3. Preparation of the EPSs

A modified version of the method from LINDSAY *et al*. (2003) was used to prepare EPSs from bacterial culture. EPSs in the bacterial culture were precipitated with two volumes of cold ethanol, followed by stirring for 1 h at 4°C. The precipitated EPSs were collected by suction filtration, and the collected EPSs were dissolved in deionized water. The EPSs were again precipitated with two volumes of cold ethanol and subsequently lyophilized. Furthermore, EPSs from the TYG broth were prepared using the same method.
The lyophilized EPSs were analysed for their carbohydrate and protein content. The total amount of carbohydrates in the lyophilized EPSs was determined with the phenol-sulphuric acid method using glucose as the standard (DUBOIS *et al*., 1956). The protein content was determined using the protein-dye binding method with bovine serum albumin as the standard (BRADFORD, 1976).
All assays were performed at least three times.

2.4. Monosaccharide analysis of the EPSs

The lyophilized EPSs were hydrolysed in 2 M trifluoroacetic acid (TFA) at 120°C for 2 h. After hydrolysis, the water and TFA were removed with a centrifugal concentrator (CC-181, TOMY, Tokyo, Japan), and the dry sample was dissolved in water. The hydrolysed EPS solution was spotted onto silica gel thin layer chromatography plate (Merck, Tokyo, Japan), along with standard solutions for glucose, galactose, mannose, arabinose, xylose, and rhamnose. The plate was developed in 1-butanol:2-propanol:H_2O (3:12:4), dried, sprayed with a phenol-sulfuric solution, and then heated at 110°C for 15 min to visualize brown spots (Adachi, 1965; Huebner *et al*., 2007). The monosaccharides of the EPSs were further analysed as follows. The sugar composition was determined by high pressure liquid chromatography with refractive-index detection (column: Sugar-D (Nacalai tesque, Kyoto, Japan); mobile phase: 85% acetonitrile; flow rate: 1.0 mL/min; temperature: 30°C). Glucose, mannose, N-acetylglucosamine, arabinose, xylose, and rhamnose were used as the standards.

2.5. Influence of the EPSs on bacterial growth

Growth in media with EPSs as the sole carbon source was tested with the 22 strains. Glucose medium was used as a control. The growth rate was determined using a modified version of the method from Tsuda and Miyamoto (2010). Briefly, the tested strains were inoculated into TY broth (10 g/L tryptone, 5.0 g/L yeast extract, 1.0 g/L Tween 80, and 0.1 g/L L-cysteine HCl monohydrate, pH 6.8 ± 0.2) containing 0.2% (w/v) glucose and the lyophilized EPSs, and the cultures were incubated for 24 h at 37°C. The optical density at 660 nm (OD_{660}) of the culture was measured at 0 and 24 h. All assays were performed at least three times. The growth rate against glucose was determined using the following equation:

$$\text{Growth rate} = (\text{Log } OD_{660} \text{ of TYE at 24 h} - \text{Log } OD_{660} \text{ of TYE at 0 h}) / (\text{Log } OD_{660} \text{ of TYG at 24 h} - \text{Log } OD_{660} \text{ of TYG at 0 h})$$

TYE: TY broth containing the EPSs produced by strains GM3701 or RB-3
TYG: TY broth containing glucose

2.6. Statistical analysis

To identify differences, a one-way analysis of variance (ANOVA) was applied to the means, and the Student-Newman-Keuls test ($P<0.05$) was applied using Statview 5.0 software (SAS Institute, Cary, NC, USA).

3. RESULTS AND CONCLUSIONS

3.1. Effects of the incubation conditions on EPS production

Lb. buchneri GM3701 and *Lb. plantarum* RB-3 are EPS-producing LAB strains. Ropiness was confirmed with a loop, and the clear zone surrounding the cell was confirmed by the Indian ink method. The effects of the incubation temperature and carbon source, which included glucose, fructose, sucrose, and lactose (100 g/L), on EPS production were investigated with *Lb. buchneri* GM3701 and *Lb. plantarum* RB-3 (Fig. 1). Concerning strain GM3701, the EPS yield was higher at 25 and 30°C than at 37°C for all of the sugars ($P<0.05$). There were no differences among the four tested sugars at 25°C, and the EPS yield in the glucose media was higher at 30°C than in the sucrose or lactose media ($P<0.05$). Concerning strain RB-3, the EPS yield was higher at 25 and 30°C than at 37°C for all of the sugars ($P<0.05$). The EPS yield in the glucose media was higher than in the sucrose media, and there were no differences among the four tested sugars at 30°C ($P<0.05$). From these results, it was presumed that a suitable incubation temperature and sugar for EPS production by strains GM3701 and RB-3 were 25°C and glucose, respectively. Therefore, these incubation conditions were applied in the series of tests.
Subsequently, the effect of the carbon concentration (25, 50, or 100 g/L) on EPS production was investigated with glucose at 25°C (Fig. 2). EPS production by GM3701 was higher at 100 g/L glucose after 5, 6, and 7 days than at 25 or 50 g/L ($P<0.05$), and the production by RB-3 was higher at 50 and 100 g/L glucose after 4 days than at 25 g/L ($P<0.05$).
The EPS yields are likely to decrease after reaching a maximum, as many studies have reported, and this is caused by enzymes, such as glycohydrolase, that are produced by bacteria (Pham *et al*., 2000). However, it is unclear whether the degraded EPSs were used for growth in that paper.

3.2. Characteristics of the EPSs

The EPS-producing strain was incubated at the above condition, and then, the EPSs were purified by ethanol precipitation and lyophilized. Similarly, the EPSs from the TYG broth were lyophilized. The polysaccharide (PS) yield from the TYG broth was 50.8 mg/L, and the EPS yields from strains GM3701 and RB-3 were 340 and 146 mg/L, respectively (Table 2). The carbohydrate and protein contents in these lyophilized EPSs are shown in Table 2. All of the EPSs contained more than 76% carbohydrates and less than 9.6% protein. These results confirmed that the lyophilized EPSs were not proteinaceous slime. The monosaccharide analysis of the EPSs was done using seven monosaccharides that are known as constituents of EPSs (glucose, galactose, mannose, N-acetylglucosamine, arabinose, xylose, and rhamnose) as standards. The PSs from the TYG broth consisted mainly of mannose.

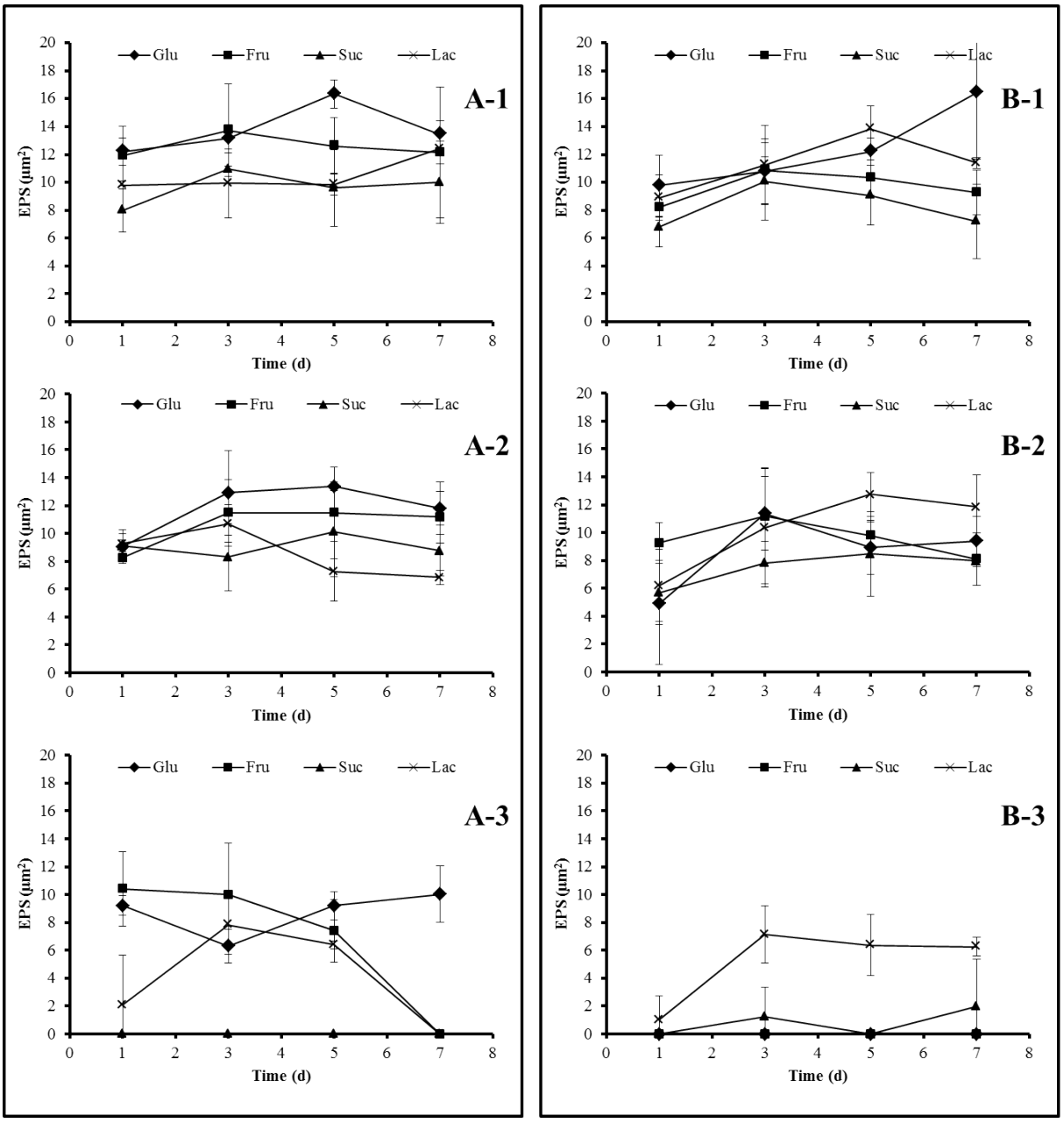

Figure 1. Effects of the incubation temperature (1: 25°C, 2: 30°C, 3: 37°C) and carbon source on EPS production by *Lb. buchneri* GM3701 (A) and *Lb. plantarum* RB-3 (B). Bars represent the standard deviation from the mean (n=3). ♦: glucose, ■: fructose, ▲: sucrose, ×: lactose.

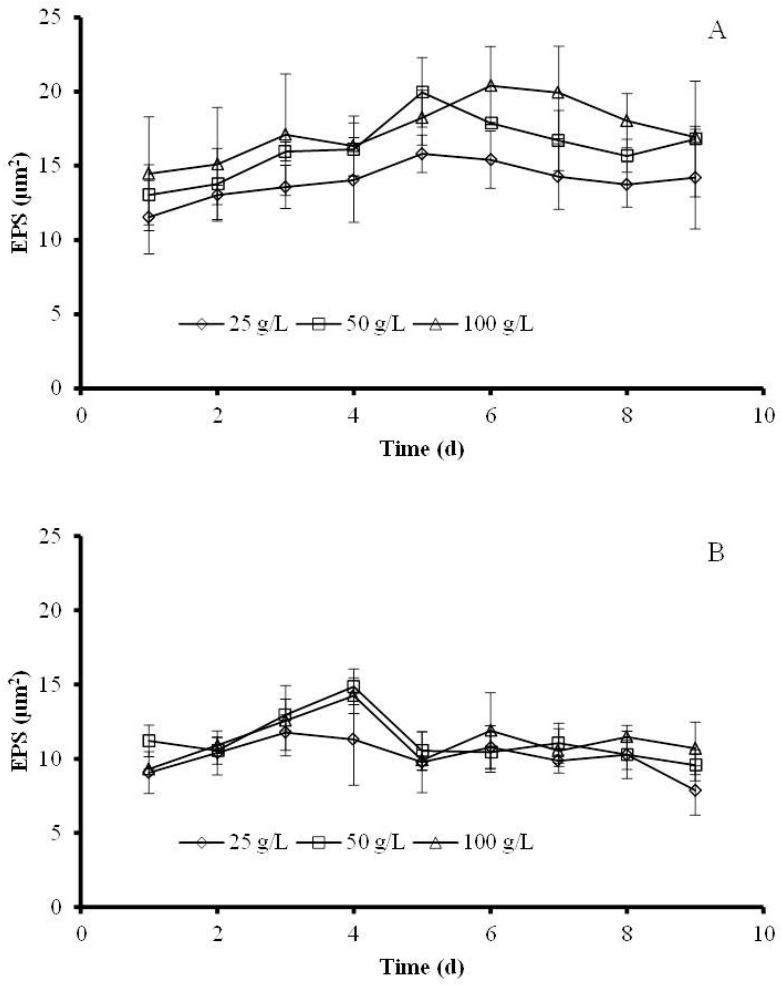

Figure 2. Effect of the glucose concentration on EPS production by *L. buchneri* GM3701 (A) and *L. plantarum* RB-3 (B). Bars represent the standard deviation from the mean (n=3). ◊: 25 g/L, □: 50 g/L, Δ: 100 g/L.

Table 2. EPS yields and the carbohydrate and protein concentrations in the lyophilized EPSs.

Strain	EPS yield (mg/L)	Carbohydrate (%)	Protein (%)	Composition of the EPS (%)			
				Glucose	Galactose	Mannose	Rhamnose
GM3701	340	76.0	9.6	73.2	-	23.7	trace*
RB-3	146	83.2	3.5	27.2	-	58.6	14.1
TYG broth	50.8	83.0	2.8	12.0	-	82.8	-

*: trace means less than 10%.

The correct yields and monosaccharide components of the EPSs produced by the LAB strains were estimated by subtracting the PS values, while taking the carbohydrate concentration into consideration (Table 3). The calculated EPS yields for the strains GM3701 and RB-3 were 216 and 79.0 mg/L, respectively. Glucose was found to be a major component of the EPS produced by strain GM3701, and glucose, mannose, and rhamnose

were found to be the predominant sugar residues in the EPS produced by the strain RB-3 (Table 3). Glucose and rhamnose are typical components of many EPSs produced by LAB. The quantities of the hetero-EPSs produced by LAB vary greatly. The production of EPS is 50-350 mg/L for *Str. thermophilus*, 80-600 mg/L for *Lc. lactis* subsp. *cremoris*, 60-150 mg/L for *Lb. delbrueckii* subsp. *bulgaricus*, 50-60 mg/L for *Lb. casei* (CERNING, 1995), and approximately 140 mg/L for *Lb. plantarum* (STAAF *et al*., 2000; TSUDA and MIYAMOTO, 2010). The highest recorded yields for hetero-EPSs are 2775 mg/L for *Lb. rhamnosus* RW-9595M (MACEDO *et al*., 2002) and 2500 mg/L for *Lb. kefiranofaciens* WT-2B (MAEDA *et al*., 2004). The EPS yields from *Lb. buchneri* GM3701 and *Lb. plantarum* RB-3 were 216 and 79.0 mg/L, respectively, and these values were thought to be a normal value for *Lactobacillus* EPSs.

Table 3. EPS yields and monosaccharide components of lyophilized EPSs, obtained by subtracting the values for the EPSs from the TYG broth.

Strain	EPS yield	Composition of the EPS (%)		
	(mg/L)	Glucose	Mannose	Rhamnose
GM3701	216	70.9	trace*	trace
RB-3	79.0	41.7	29.0	28.3

*: trace means less than 10%.

The PS from the TYG broth was thought to be mannan. It is well known that purified EPSs are contaminated with the mannan from the yeast cells in yeast extract. Glucose and rhamnose are the usual components of many EPSs produced by LAB (CAGGIANIELLO *et al*., 2016; DONOT *et al*., 2012; SANCHEZ *et al*., 2006), and the mechanisms of glucose incorporation into the polysaccharide chain are well known (DE VUYST *et al*., 2001). There are some reports about EPSs composed of glucose and mannose that are produced by *Lactobacillus* (HASHIGUCHI *et al*., 2011; SANCHEZ *et al*., 2006).

3.3. Growth enhancement by the EPSs

The growth rates of the EPSs against glucose for the 22 strains are shown in Fig. 3. The highest growth rate was observed with *Lb. farciminis* HM2001 ($P<0.05$). All of the 22 tested strains showed an OD_{660} of more than 0.3 when they were incubated in TYG broth for 24 h. The growth rates of the EPSs against glucose were calculated, and all of the tested strains showed a growth rate of less than 0.1, except for strain HM2001. The EPSs produced by strains GM3701 and RB-3 showed growth rates of 0.146 and 0.113 with strain HM2001, respectively.
Although the monosaccharide composition of the EPSs was different between the two EPSs (Table 3), the growth of *Lb. farciminis* HM2001 was enhanced by supplementation with either of the EPS produced by the LAB. The growth enhancement of this strain did not occur following supplementation with the PS from the TYG broth (data not shown). This suggested that the EPSs produced by *Lb. buchneri* GM3701 and *Lb. plantarum* RB-3 enhanced the growth of strain HM2001. On the other hand, the EPSs produced by *Lb. plantarum* RB-3 did not enhance the growth of the three tested *Lb. plantarum* strains.

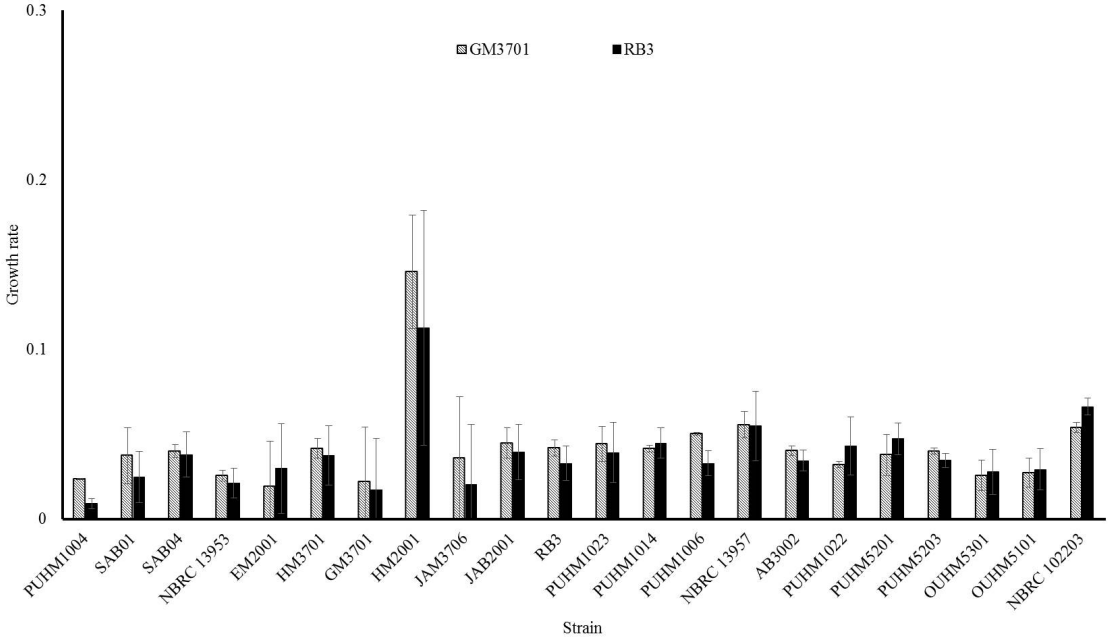

Figure 3. Growth rates of the EPSs against glucose for the 22 strains.

Therefore, no species specificity was shown for growth enhancement by EPSs in this study. We think that strain HM2001 may have the enzymes that degrade the EPSs produced by LAB, and the degraded sugars may be utilized by strain HM2001; the EPS or degraded carbohydrate chains may stimulate the growth by working like an extracellular signalling molecule. Another factor, such as the charge and linkage types of the EPS and the combinations of the enzymes that degrade EPSs, may be involved in the utilization of EPSs. RUSSO (2012) reported that β-D-glucan in EPSs enhanced the growth of *Lb. plantarum* and *Lb. acidophilus*. Therefore, a lot of β-D-glucan may exist in the EPSs used in this study. Further work is needed for a better understanding of the physiological importance of EPSs.

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