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Technical Optimization of Culture Conditions for the Production of Exopolysaccharide (EPS) by *Lactobacillus rhamnosus* ATCC 9595

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Abstract Microbial exopolysaccharide (EPS) is a biothickener that can be added to a wide variety of food products, where it serves as a viscosifying, stabilizing, emulsifying, and gelling agent. The objective of this study was to investigate the optimum conditions of pH, incubation temperature, and whey protein concentration (WPC) for EPS production by *Lactobacillus rhamnosus* ATCC 9595. We found that maximal EPS production was achieved at a pH of 5.5 and temperature of 37°C. At the same fermentation conditions, EPS production was affected by the addition of *L. rhamnosus* GG (a weak-EPS producer). After growth for 24 hr, total EPS production was 583±15.4 mg/L in the single culture system, and 865±22.6 mg/L in the co-culture system with *L. rhamnosus* GG. Based on the presence of WPC, EPS production dramatically increased from 583±15.4 (under no WPC supplementation) to 1,011±14.7 mg/L (under supplementation with 1.0% WPC). These results suggest that WPC supplementation and the co-culture systems coupled with small portions of weak-EPS producing strain can play an important role in the enhancement of EPS production.

Keywords: *Lactobacillus rhamnosus*, exopolysaccharide, optimal condition, whey protein concentrate

Introduction

Most of the biothickeners in current use by the food industry are polysaccharides from plants (e.g., starch, pectin, locust bean gum, and guar gum) or seaweeds (carrageenan and alginate). Animal protenaceous hydrocolloids such as gelatin and casein are also used. However, these polysaccharides may not always be readily available in the quality needed or have the correct rheological properties (1). Most utilized plant carbohydrates are chemically modified to improve their structure and rheological properties (2). Hence, their use is strongly restricted. In the dairy industry, notably in the European yoghurt industry, additions of stabilizers are prohibited, and food products need to be labeled with an E-number. Therefore, microbial exocellular polysaccharides may be regarded as an alternative class of biothickeners.

Exopolysaccharide (EPS), one of the primary metabolic products of lactic acid bacteria (LAB), have received increasing amounts of attention in recent years (3). EPS has thickening properties, and also appears to improve both the texture and mouthfeel of several dairy products. Moreover, there are a number of reports referring to the health benefits of EPS from LAB. Kitazawa *et al.* (4) claimed that EPS has various beneficial physiological effects on humans. Based on these characteristics, EPS from some LAB is actually used as a natural additive in food products and has become an alternative to chemical, plant, or animal additives as a stabilizing, thickening, gelling, and waterbinding agent (5,6).

Unfortunately, the EPS amounts produced by LAB cultures are generally very low. Currently, a lot of effort is put into the selection of LAB strains and the optimization of culture conditions to achieve a higher yield of EPS that is already commercially successful. Moreover, there is considerable interest in finding a new EPS suitable for special applications, or that has industrial relevance, either by applying different culture conditions or by using novel bacterial strains. Manipulation of the growth medium may consist of altering the carbon source, medium composition, temperature, or pH (7). Among these factors, the presence of casein hydrolysate, co-culture systems, and the amount of glucose (8) have been considered.

Lactobacillus strains including *L. rhamnosus*, members of the *L. casei* group, are recently become important as adjunct cultures for the production of fermented foods (9-11). However, few studies have been performed on EPS production by these strains, including their comparison to other LAB. Recently, we reported on the characterization and purification of EPS produced by *L. rhamnosus* ATCC 9595 as one of the LAB which produces high levels of EPS (6).

The purpose of the present study was to investigate the optimial technical conditions for EPS production by *L. rhamnosus* ATCC 9595, according to pH, fermentation temperature, co-culture systems, and whey protein concentration (WPC).

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Materials and Methods

Bacteria and medium *L. rhamnosus* ATCC 9595, the EPS-producing strain, and the *L. rhamnosus* GG strain (ATCC 53103), were acquired from a frozen stock collection obtained from the Dairy Food Microbiology laboratory at Korea University (Seoul, Korea), and were sub-cultured 3 times in MRS broth (Difco Laboratories, Detroit, MI, USA) at 37°C for 18 hr. The stock cultures were stored at −80°C in 10% reconstituted skim milk (RSM) containing 20%(v/v) glycerol. Prior to use, the bacteria were sub-cultured twice in RSM at 37°C for 18 hr.

Fermentation for EPS production For high production of EPS, batch fermentation was carried out for 48 hr in a laboratory fermentor (Biostat C; B. Braun Biotech International, Melsungen, Germany) controlled with a pH controller (the pH was maintained at 5.5 by the addition of 5 N NaOH; Whatman Lab Sales, Clifton, NJ, USA), as previous described by Kim *et al.* (6). The EPS was quantified using the phenol-sulfuric method (12) and was expressed as a glucose standard. In addition, cell growth was evaluated by the number of viable cells after anaerobic incubation (BBL Gas-Pak System, Sparks, MD, USA) at 37°C for 72 hr in MRS agar (Difco).

Effects of pH and temperature on cell growth and EPS production The effects of pH on EPS production were examined in separate fermentations at pH 4.5, 5.5, and 6.5 at 37°C, respectively. Inoculation was carried out as previously mentioned. The desired pH was maintained automatically over 24 hr using 5 N NaOH. Samples were aseptically removed at 0 hr and every 6 hr thereafter. Additionally, the effect of pH on EPS production was examined by removing pH automation, and allowing the pH to drop freely during fermentation for 24 hr at 37°C. In addition, fermentations were conducted separately at 30, 37, 40, and 42°C at pH 5.5 (optimum pH was found to be pH 5.5 in this study). Temperature and pH were maintained automatically, as described earlier, over 24 hr, and the samples were aseptically collected every 6 hr.

Effects of co-culturing with the weak-EPS producing *L. rhamnosus* culture on cell growth and EPS production The effects of co-culturing with the weak-EPS producing *L. rhamnosus* strain GG as an adjunct culture on EPS production were studied. Three combinations of *L. rhamnosus* ATCC 9595 and *L. rhamnosus* GG (75:25, 50:50, and 25:75, v/v) were examined based on the initial medium volume. Bacterial cultures were grown separately at the conditions described earlier and an appropriate quantity of each strain was transferred into the fermentation vessel. The fermentations were conducted for 24 hr at pH 5.5 and 37°C.

Effects of supplementation with WPC on cell growth and EPS production WPC containing 80% protein (Davisco, Eden Prairie, MN, USA) was used as an additional nitrogen source. The WPC was supplemented in RSM at 0.5, 1, and 1.5%(w/v) prior to sterilization. The fermentations were conducted at pH 5.5 and 37°C. Furthermore, the effects of pH and supplementation with WPC on EPS production were examined by removing the automatic pH controller and allowing the pH to drop freely during fermentation for 24 hr at 37°C.

Statistical analysis All of the experiments conducted in this study were repeated 3 times. Statistical analyses were performed by SAS using the general linear model procedures. The level of significance was defined at $p<0.05$ using Tukey's test.

Results and Discussion

Effects of the growth medium pH and temperature on bacterial growth and EPS production There are significant relationships among pH, EPS production, and bacterial growth. Most researchers have shown the optimum pH for EPS synthesis to be around pH 6.0. Many have reported a pH of 6.2 as the optimum pH for *Streptococcus thermophilus* (13).

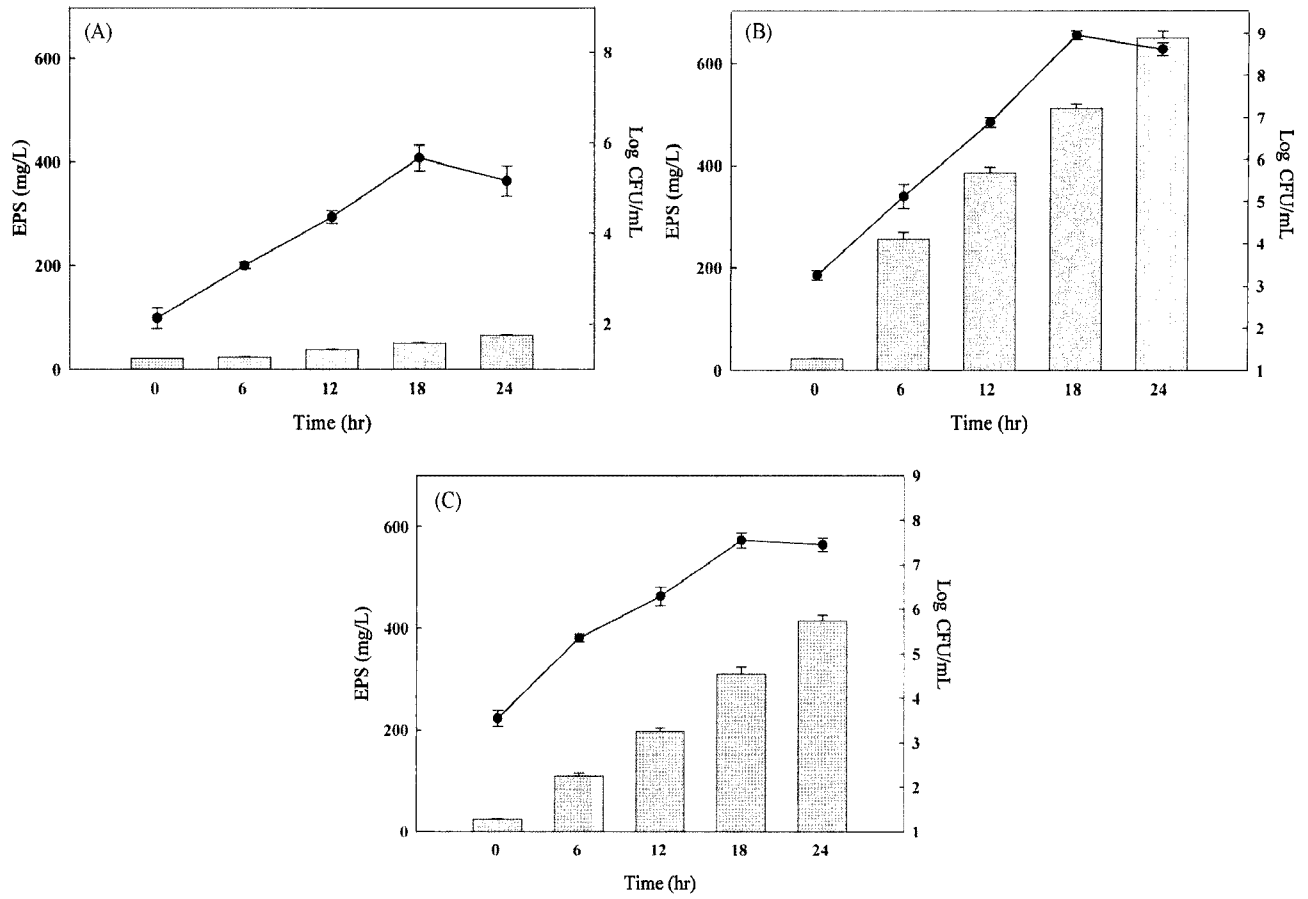
Figure 1 presents the effects of pH on EPS production and bacterial growth for *L. rhamnosus* ATCC 9595 fermented in 10%(w/v) RSM at 37°C over 24 hr, at constant pH levels of 4.5, 5.5, and 6.5, respectively. As expected, there were significant differences in EPS production at pH 4.5, 5.5, and 6.5 after 24 hr of fermentation ($p<0.05$). EPS production dramatically decreased at pH 4.5, and this pH also affected the bacterial population as compared to the other pH conditions. Similar to previous data described by Zisu and Shah (14), at pH 5.5, profound EPS production was observed in *L. rhamnosus* ATCC 9595, yielding 650± 14.3 mg/L in 24 hr of fermentation.

In addition, to understand the effects of different temperatures on EPS production yield, various temperatures were investigated during separate fermentations at 32, 37, and 42°C, respectively. The pH was maintained at 5.5, which we found to be adequate for EPS production, as stated above. As shown in Fig. 2, the optimum temperature was demonstrated as 37°C. At this temperature, the total yield of EPS was 650±14.3 mg/L, while the EPS levels at 32, 40, and 42°C were 303±11.5, 456±18.6, and 272±13.3 mg/L, respectively. In the case of the cell population, similar trends were observed during 24 hr of fermentation.

Different optimal temperatures have been reported according to strains. For *S. thermophlus*, De Vyust *et al.* (13) demonstrated 42°C as the optimal temperature. In other reports, mesophilic microorganisms showed greater EPS production at temperature below 37°C (8,15). These reports indicate that the optimal temperature is dependant on the LAB strain.

Based on our results, pH 5.5 and 37°C were selected as the basal fermentation conditions for performing the remaining experiments of this study.

Effects of co-culturing systems with weak-EPS producing *L. rhamnosus* culture on cell growth and EPS production The effects of the co-culturing system and the weak-EPS producing *L. rhamnosus* strain on cell growth and EPS production are shown Fig. 3. Three combinations were carried out as follows: a 50% mixture of each strain, a mixture of 75% *L. rhamnosus* ATCC 9595 and 25% *L. rhamnosus* GG, and another mixture of 25% *L. rhamnosus* ATCC 9595 and 75% *L. rhamnosus* GG,



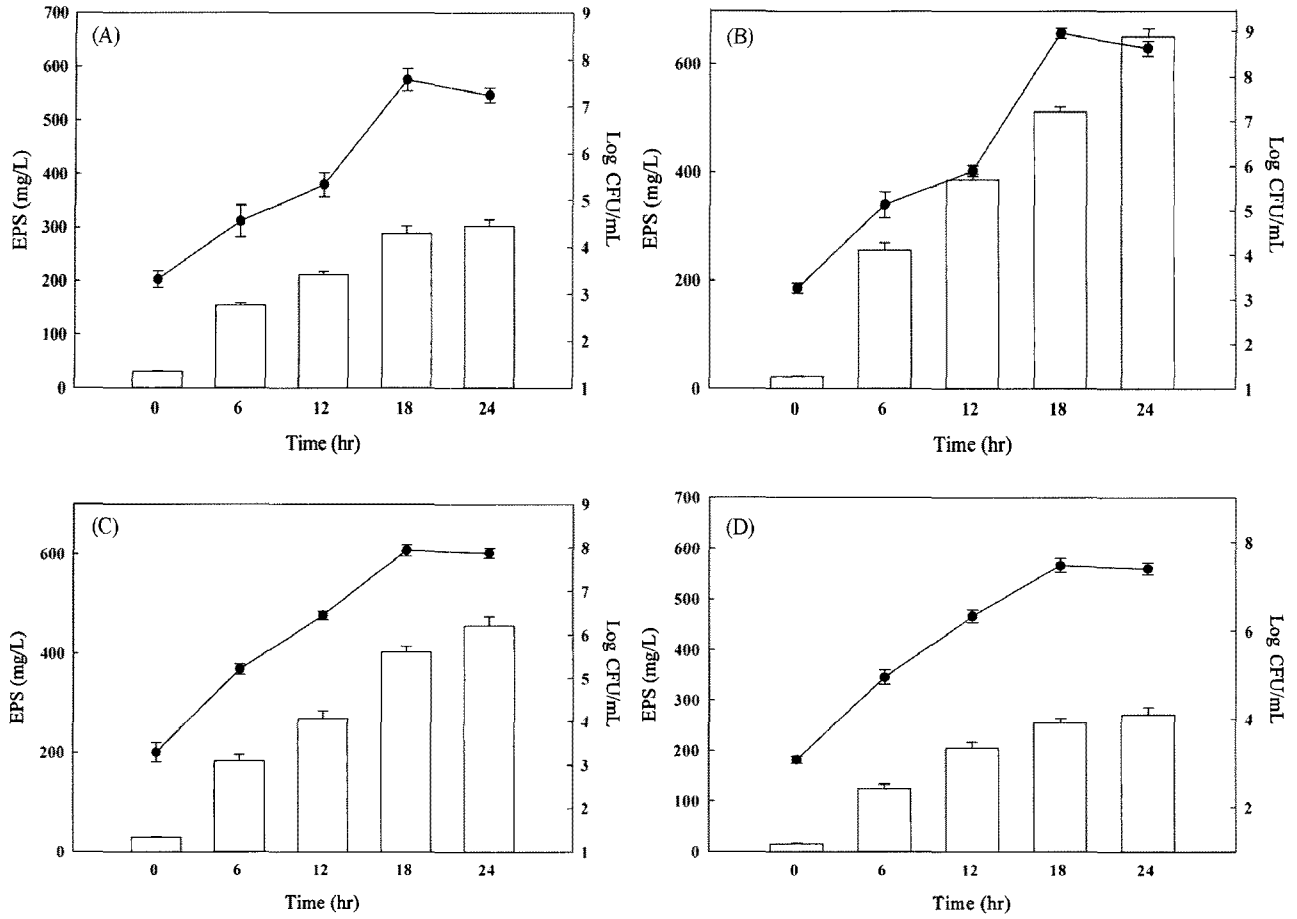
pH	Fermentation time (hr)	EPS (mg/L)	Bacterial count (log CFU/mL)
4.5	24	65±1.3^{c}	5.15±0.34^{c}
5.5	24	650±14.3^{a}	8.63±0.16^{a}
6.5	24	415±10.9^{b}	7.45±0.15^{b}

Fig. 1. Bacterial counts and quantity of exopolysaccharide (EPS) produced during fermentation in 10% RSM by *L. rhamnosus* ATCC 9595 at 0, 6, 12, 18, and 24 hr, at various pH levels and 37°C. A represents fermentation at pH 4.5; B at pH 5.5; and C at pH 6.5, respectively. The line plot indicates bacterial counts and the vertical bar indicates the quantity of EPS, respectively. Different letter superscripts within the same column indicate differences at $p<0.05$.

respectively.

Interestingly, the co-culturing system showed a significant induction of EPS production during 24 hr of fermentation. The 75% *L. rhamnosus* ATCC 9595 and 25% *L. rhamnosus* GG mixture showed the highest quantity of EPS production at 865±22.6 mg/L for 24 hr of fermentation. Moreover, an EPS production of 726±14.9 mg/L in each 50% mixture was higher than that of 650±14.3 mg/L in the 100% *L. rhamnosus* ATCC 9595. As expected, the quantity of EPS produced in the 25% *L. rhamnosus* ATCC 9595 and 75% *L. rhamnosus* GG mixture was lowest. However, cell counts were slightly lower when both strains were co-cultured than single cultured. Unfortunately, in this study, it is impossible to enumerate the portions of *L. rhamnosus* ATCC 9595 and *L. rhamnosus* GG, separately.

Overall, one may suggest that a co-culturing system with a weak-EPS starter culture has the potential to increase EPS production, and may be a convenient alternative for the elevation of EPS production. Limited research with regard to EPS production influenced by co-culturing systems with no, or with weak-EPS producing LAB has been published. EPS produced by *S. thermophilus* increases when it is grown in the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* (16,17). In the present study, the increase in EPS synthesis by the addition of *L. rhamnosus* GG may indicate a complementary relationship that existed between the 2 strains of *L. rhamnosus*. The co-culturing combinations showed sustained increases in EPS production for 24 hr of fermentation, whereas, in the case of pure strain, the rate of EPS synthesis had slowed after 12 hr fermentation (Fig. 3). The components of the medium are crucial factors to the production of EPS. Especially, the essential nutrients such as vitamins and minerals resulted in an enhanced EPS production (13,14). Therefore, it is likely that induced EPS production in co-culture systems were caused, in part at least, by a result of supplementation of specific EPS-inducing components produced by *L. rhamnosus* GG fermentation from RSM medium.

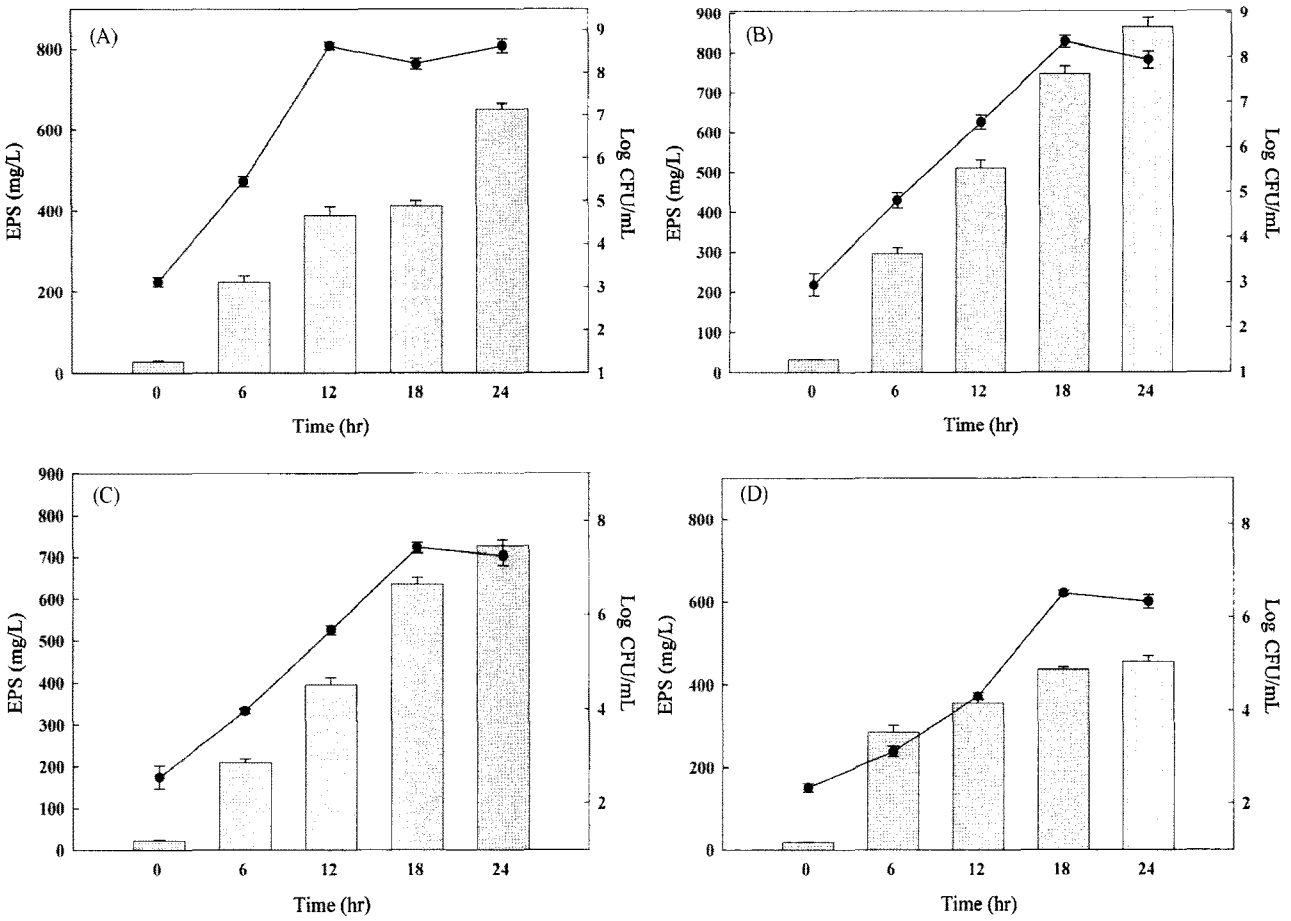


Temperature (°C)	Fermentation time (hr)	EPS (mg/L)	Bacterial count (log CFU/mL)
32	24	303±11.5^{c}	7.25±0.16^{b}
37	24	650±14.3^{a}	8.63±0.16^{a}
40	24	456±18.6^{b}	7.88±0.11^{b}
42	24	272±13.3^{c}	7.41±0.14^{b}

Fig. 2. Bacterial counts and quantity of exopolysaccharide (EPS) produced during fermentation in 10% RSM by *L. rhamnosus* ATCC 9595 at 0, 6, 12, 18, and 24 hr, at various temperatures and pH 5.5. A represents fermentation at 32°C; B at 37°C; C at 40°C; and D at 42°C, respectively. The line plot indicates bacterial counts and the vertical bar indicates the quantity of EPS, respectively. Different letter superscripts within the same column indicate differences at $p<0.05$.

In addition, this suggests there was a gradual release of peptides and amino acids that were readily available to the bacterial cells; similar to what was observed in the symbiotic relationship that exists between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in yogurt, which was found to improve the growth of the bacterial population (18). Moreover, our results indicate that *L. rhamnosus* GG, weak-EPS producer, provided essential components to induce EPS synthesis of *L. rhamnosus* ATCC 9595 as well as not affected to cell growth. Our studies are currently underway to evaluate the purification and characterization of EPS-inducing components in the *L. rhamnosus* GG, specifically with regard to fermentation strategies for optimal EPS production in dairy food industry.

Recently, many bacteria, including pathogens and commensals, communicate via diffusible signal molecules to coordinate multi-cellular behavior in a process referred to as 'quorum sensing' (19). Regarding the high density and diversity of the gastrointestinal microbiota, it is postulated that bacterial communication fulfills an important role in coordinating various processes in the gut. Quorum sensing, however, is not limited to pathogenic bacteria. In fact, many commensal and potentially probiotic bacteria such as *Bifidobacterium* and *Lactobacillus* strains possess a *luxS* homologue and can produce autoinducer (AI)-2, a universal quorum sensing signal, but a role for the AI-2/LuxS system has not yet been assigned (20). Interestingly, a co-culture system can act as an environmental signal that is able to switch on bacteriocin production in *L. plantarum* NC8 via a quorum-sensing mechanism (21). At present, we are investigating the specific relationships of co-culturing systems and quorum sensing signals in EPS production.



The ratio of strain (9595:GG)	Fermentation time (hr)	EPS (mg/L)	Bacterial count (log CFU/mL)
100:0	24	650±14.3[c]	8.63±0.16[a]
75:25	24	865±22.6[a]	7.95±0.19[b]
50:50	24	726±14.9[b]	7.25±0.21[b]
25:75	24	456±13.7[d]	6.34±0.15[c]

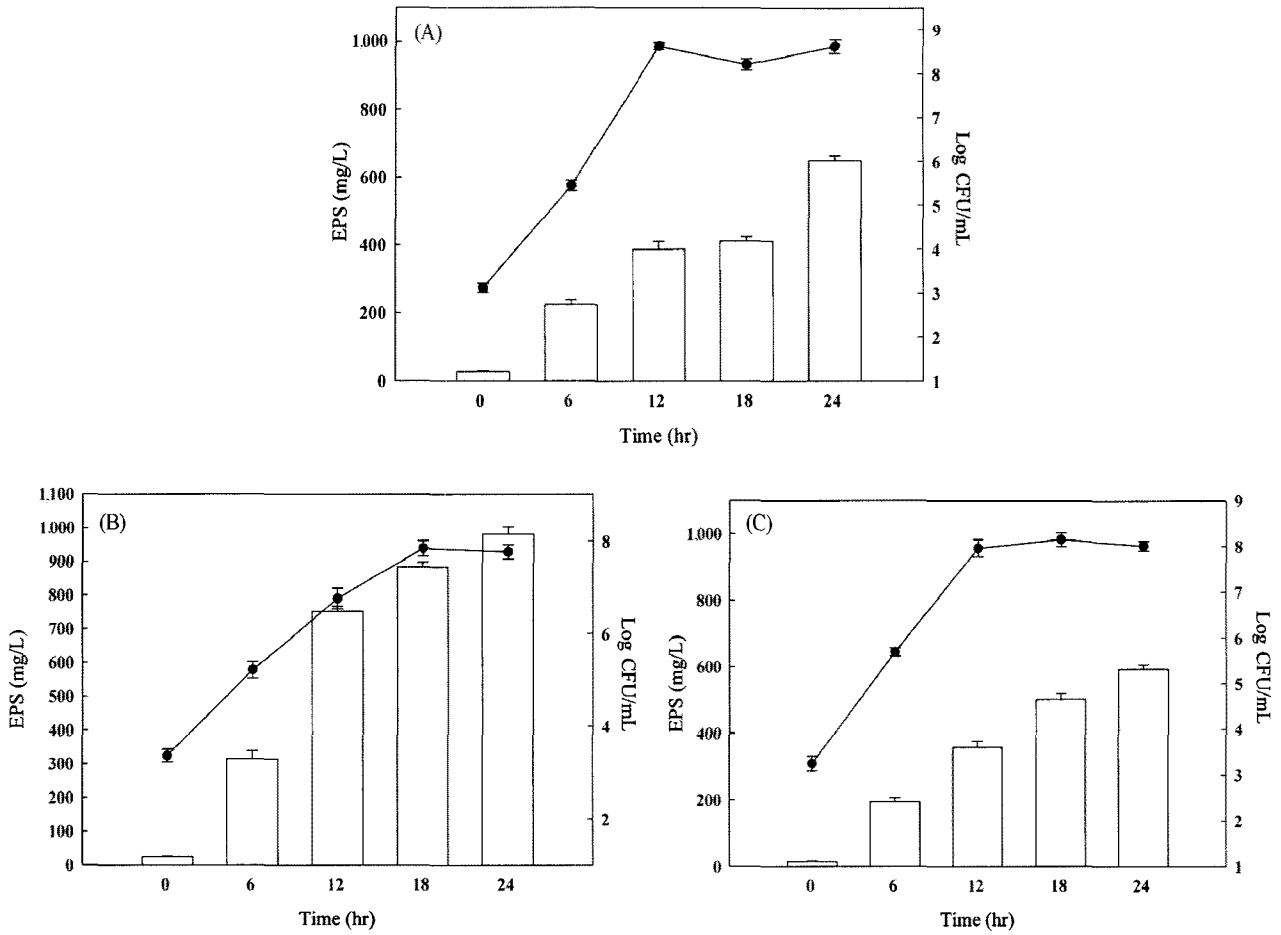
Fig. 3. Bacterial counts and quantity of exopolysaccharide (EPS) produced during fermentation in 10% RSM by *L. rhamnosus* ATCC 9595 and *L. rhamnosus* GG at 0, 6, 12, 18, and 24 hr, at pH 5.5 and 37°C. A represents fermentation when the ratio of 9595:GG=100:0; B=75:25; C=50:50; D=25:75, respectively. The line plot indicates bacterial counts and the vertical bar indicates the quantity of EPS, respectively. Different letter superscripts within the same column indicate differences at $p<0.05$.

Effects of WPC supplementation on cell growth and EPS production Table 1 shows the effects of different concentration of WPC on cell growth and EPS production during 24 hr of fermentation. After 24 hr of fermentation, the quantity of EPS produced in the media supplemented with WPC dramatically increased from 650±14.3 (under no WPC supplementation) to 1,011±14.7 mg/L (under supplementation of 1.0% WPC), whereas the bacterial counts grown on media supplemented with WPC were lower than those grown without WPC. This suggests that WPC was mainly utilized for EPS production rather than bacteria growth. Previously, increases in EPS quantity were observed by additions of nitrogen sources such as yeast extract or specific amino acids (13,22). Furthermore, there were no significant difference in EPS production between the concentrations of 0.5 and 1.0% WPC. From these results, it is suggested that a 0.5% addition of WPC would be appropriate for cost reduction. However, preliminary studies have reported that WPC supplementation over 0.5% would not be effective in food applications (5,14).

Table 1. Bacterial counts and quantity of exopolysaccharide (EPS) produced by *L. rhamnosus* ATCC 9595 for 24 hr of fermentation in 10% RSM supplemented with various concentrations of WPC at pH 5.5 and 37°C[1)]

Concentration of WPC (%)	EPS (mg/L)	Bacterial count (log CFU/mL)
0	650±14.3[b]	8.63±0.16[a]
0.5	983±21.3[a]	7.76±0.16[b]
1.0	1,011±14.7[a]	7.84±0.13[b]
1.5	979±23.5[a]	7.66±0.29[b]

[1)]Different letter superscripts within the same column indicate differences at $p<0.05$.



	Fermentation time (hr)	EPS (mg/L)	Bacterial count (log CFU/mL)
Control (No WPC)	24	650±14.3[b]	8.63±0.16[a]
0.5% WPC (pH controlled)	24	983±21.3[a]	7.76±0.16[c]
0.5% WPC (pH uncontrolled)	24	594±13.8[c]	8.01±0.11[b]

Fig. 4. Bacterial counts and quantity of exopolysaccharide (EPS) produced by *L. rhamnosus* ATCC 9595 during 24 hr fermentation in 10% RSM supplemented with a 0.5% concentration of WPC at 37°C. A represents fermentation on RSM; B is supplementation with 0.5% WPC (pH controlled); and C is supplementation with 0.5% WPC (pH uncontrolled), respectively. The line plot indicates bacterial counts and the vertical bar indicates the quantity of EPS, respectively. Different letter superscripts within the same column indicate differences at $p<0.05$.

The effects of controlled pH and WPC supplementation on EPS production, during fermentation for 24 hr at 37°C are shown in Fig. 4. The concentration of WPC was fixed at 0.5% as stated above. Interestingly, under uncontrolled pH, a robust reduction in EPS production was observed compared to controlled pH conditions at 5.5 in the presence of WPC. Importantly, EPS production of LAB is influenced by pH conditions (14). Especially, as the bacteria population entered the stationary phase with relative low pH condition (pH 4.2-4.5), there was significant reduction on the production of EPS (23). These results indicated that, in the RSM medium including WPC, pH conditions during fermentation were one of crucial factors on the EPS production of LAB. On the other hand, it has been shown that enhanced EPS production was achieved in the uncontrolled pH condition supplemented with 0.5% WPC (594±13.8 mg/L) compared to that of uncontrolled pH condition without WPC (483±10.3 mg/L; data not shown). As described in previous reports (14,23), inducing EPS production in the presence of WPC may partially be due to the buffering effects of WPC. In particular, Kailasapathy *et al.* (23) demonstrated that protein and phosphates from the addition of WPC improved buffering capacity.

Furthermore, it seems that WPC is apparently useful for the metabolism of EPS synthesis. Simple peptides or amino acids are responsible for increased EPS production, and providing a nitrogen source such as WPC plays an important role in enhancing EPS production yields, as described by De Vuyst and Degeest (5).

In conclusion, our data clearly show that fermentation factors such as pH and temperature influenced bacterial growth and EPS production. In addition, EPS production significantly increased in mixed culture systems with *L. rhamnosus* GG, a weak-EPS producer. Furthermore, in the

presence of WPC, EPS production dramatically increased as compared to the control, whereas bacterial counts on media supplemented with WPC were lower than those of bacteria grown without WPC. These results suggest that specific supplementation and culture conditions, such as WPC and co-culture systems, can play important roles in the enhancement of EPS production for applications in the food industry.

Acknowledgments

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