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Study on Effect Elements of Exopolysaccharide Production of *Lactobacillus Kimchi* SR8 and DPPH Radical Scavenging Activity

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Abstract The correlation between exopolysaccharide production of *Lactobacillus kimchi* SR8 under different culture conditions and the DPPH radical scavenging activity of exopolysaccharide was studied. *Lactobacillus kimchi* SR8 produced 228.24 ± 2.23 mg/L exopolysaccharides with sucrose as the carbon source, Beef Extract-Peptone (2:1) as the nitrogen source and an initial pH of 6.50, whilst the DPPH radical scavenging activity of exopolysaccharides was only 6.85% ± 0.77% at a concentration of 0.20 mg/mL. However, the strain produced 206.79 ± 2.23 mg/L exopolysaccharides with glucose as the carbon source, Peptone-Tryptone (1:1) as the nitrogen source and an initial pH of 7.00, and the DPPH radical scavenging activity of exopolysaccharides increased to 28.34% ± 0.32% at the same concentration. The results showed no correlation between the exopolysaccharide production of lactic acid bacteria and the DPPH radical scavenging activity of exopolysaccharide. Therefore, the antioxidant activity should be taken into consideration when measures are taken to increase exopolysaccharide production if greater antioxidant activity of exopolysaccharides is preferred.

Keywords: *characterization, exopolysaccharide, free radical, scavenging activity, relationship*

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1. Introduction

Lactic acid bacteria (LAB) exopolysaccharides (EPS) are high–molecular -weight polymers that are produced during growth and reproduction to adapt to the environment [1,2]. They are composed of repeating and branched units of sugars or their derivatives, such as glucose, fructose, mannose, lactose, rhamnose and galactose [3,4]. In recent years, LAB EPSs have received increasing attention because LAB strains are generally regarded as safe and of food-grade status [5,6,7]. Thus far, the main LAB strains that have been found to produce EPS are *Lactobacillus rhamnosus* [8], *Lactobacillus plantarum* [9], *Lactobacillus helveticus* [10], *Lactobacillus delbrueckii* subsp. *Bulgaricus* [11], *Lactococcus lactis* subsp. *Lactis* [12], *Bifidobacterium bifidum* [13], *Pediococcus pentosaceus* [14] and *Streptococcus thermophiles* [15]. The production of EPS is influenced by parameters such as the starter culture, medium, incubation time and extraction methods. A report has shown that a probiotic *Lactobacillus plantarum* MTCC 9510 produced 0.14 g/L, but its production was increased up to 1.08 g/L when ammonium sulphate was used as an inorganic nitrogen source [1].

Studies have demonstrated that the antioxidant effects of LAB EPS may involve non-enzymatic antioxidant activities, up-regulation of enzymatic antioxidant activities, inhibition of lipid peroxidation, and scavenging of reactive oxygen species [9,16,17]. They also scavenge free radicals, including hydroxyl radical, superoxide anion radical and 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH radical). Catalase, superoxide dismutase and glutathione peroxidase activity were increased in the serum and livers of mice treated with EPS and phosphorylated exopolysaccharide (P-EPS) [18]. In addition, EPSs show immunity stimulation, cholesterol reduction, anti-allergy and antitumor activity [19,20,21,22].

Due to the different functions of LAB EPS, such as antioxidant activity, anticancer activity and dairy stabilization, measures have been taken to increase EPS production. After observing the anticancer activity of LAB EPSs, Deepak et al. [23] used response surface methods to increase cancer EPS production from *Lactobacillus acidophilus*, and the level of EPS increased to 597 mg/L. The optimization models and methods were developed only to enhance EPS production. Desai et al. [24] applied artificial intelligence–based techniques in the optimization of fermentation media to enhance the EPS production from *Lactobacillus plantarum* and increased production to 7.14 g/L. Further studies include the optimization of EPS production and the functions of EPS from the optimized culture conditions. Hsieh et al. [25] optimized *Lactobacillus acidophilus* cultivation using taro waste and evaluated the anti-tumour and immunomodulatory activities of heat-killed cells, cytoplasmic fraction and EPSs extracted from culture in the optimized conditions. However, most studies have focused only on EPS production or functions and

neglected EPS activities in the same conditions and few reports have combined EPS production with antioxidant activity.

In our previous studies, a LAB strain of *Lactobacillus kimchi* SR8 was screened from 36 LAB strains isolated from fermented meat products. This strain has high EPS production ability and the EPS also has antioxidant activity. The aim of this study was to use *Lactobacillus kimchi* SR8 as the research strain to explore the relationship between EPS production extracted from different LAB culture conditions and the DPPH radical scavenging activities of EPS. The DPPH radical scavenging activity was used as an indicator of antioxidant activity of EPS. Much attention has been paid if greater antioxidant activity of EPS is preferred when measures are taken to increase EPS production of LAB.

2. Materials and Methods

2.1. LAB Strain

Lactobacillus kimchi SR8 was isolated and identified from traditionally fermented sour meat from China and was screened from 36 strains of LAB and identified as a strain with high production of EPS [26]. The strain was maintained at 4°C and renewed for 4 weeks for short-term preservation. In the study, it was inoculated in MRS broth (Beijing Land Bridge Co., Ltd) two or three times to measure the production and DPPH radical scavenging activity of its EPS.

2.2. Growth Characteristics

Lactobacillus kimchi SR8 was grown in fresh MRS broth with an inoculum size of 3% (v/v). All samples were cultivated at 37°C from 0 h to 24 h. The absorbance at 600 nm (A_{600}), soluble solid proportion (SSP) and pH values were measured respectively using T6 New Century UV-Vis spectro-photometer (Beijing Persee General Instrument Co., Ltd), WZS-20 Portable Refractometer (Shanghai physical optics instrument Co., Ltd) and Starter 3100 pH meter (Ohaus Instrument (Shanghai) Co., Ltd).

2.3. Production Properties of Exopolysaccharide

Lactobacillus kimchi SR8 was grown in fresh MRS broth with an inoculum size of 3% (v/v). A third of all samples were cultivated at 37°C from 26 h to 34 h. One third of samples were cultivated respectively at 33, 35, 37, 39 and 41°C for 24 h. The pH values of the last one third of samples were respectively regulated to 5.50, 6.00, 6.50, 7.00 and 7.50 and those were cultivated at 37°C for 24 h. The EPS-producing capacity on conditions of culture time, temperature and pH were measured.

2.4. Exopolysaccharide Determination

Fermented culture with *Lactobacillus kimchi* SR8 was centrifuged at 10 000 rpm at 4°C for 20 min. The protein was then precipitated from the supernatant overnight by the addition of 80% trichloroacetic acid solution at a final concentration of 10% and centrifuged at 10 000 rpm at 4°C for 20 min for removal. Furthermore, small molecular carbohydrates were dialysed out of the dialysis bag MD34 (Molecular cut off: 8 000-14 000, Solarbio, Beijing) by overnight dialysis. The dialyzed solution was added to triple volumes of absolute ethanol (Tianjin Fuyu, Tianjin) to precipitate EPS and then centrifuged at 10 000 rpm at 4°C for 20 min. The precipitated EPS was dissolved for measurement of the contents. The absorbance of the EPS sample solution was measured at 490 nm with a phenyl hydroxide (Sinopharm chemical reagent Co. Ltd, Shanghai)-sulfuric acid (Chuandong Chemical, Chongqing) procedure [20] and calibrated using glucose as a standard.

2.5. Free Radical Scavenging Activity

The DPPH radical scavenging activities of EPSs were measured with Choi's method [21]. The concentration of EPSs was respectively regulated to 0.08, 0.16, 0.24, 0.32 and 0.40 mg/mL and then all samples were used to determine the radical scavenging activity. The DPPH radical scavenging activity of EPS was calculated with the following equation:

$$\text{Scavenging activity (\%)} = \left(1-\frac{A_{517}\ \text{of sample}}{A_{517}\ \text{of control}}\right)\times 100. \quad (1)$$

2.6. Relationship between EPS Production and Radical Scavenging Activity

2.6.1. Effect of Carbon Source on EPS Production and the DPPH Radical Scavenging Activity

Lactobacillus kimchi SR8 was inoculated in fresh modified MRS culture including lactose, glucose, maltose or sucrose as carbon source with an inoculum size of 1%. All samples were inoculated at 37°C. After 24 h of incubation at 37°C, the culture was centrifuged at 10000 rpm at 4°C for 20 min to remove the LAB cells. EPSs were then obtained by addition of 80% trichloroacetic acid solution for precipitating proteins and by dialysis to remove small molecular carbohydrates. The precipitated EPS was used to measure the DPPH radical scavenging activity at the EPS concentration of 0.16 mg/mL.

2.6.2. Effect of Nitrogen Source on EPS Production and the DPPH Radical Scavenging Activity

Lactobacillus kimchi SR8 was grown in fresh modified MRS culture including beef extract-peptone (1:2, 1:1 and 2:1), beef extract-tryptone (1:1) or peptone-tryptone (1:1) as nitrogen source with an inoculum size of 1%. After 24 h of incubation at 37°C, the EPS was precipitated by centrifugation to remove cells, precipitation of proteins by the addition of 80% trichloroacetic acid and dialysis for the removal of small molecular carbohydrates. The DPPH radical scavenging activity of precipitated EPS was measured at 517 nm at the EPS concentration of 0.16 mg/mL.

2.6.3. Effect of pH on EPS Production and the DPPH Radical Scavenging Activity

Lactobacillus kimchi SR8 was inoculated in fresh culture adjusted to pH values of 5.50, 6.00, 6.50, 7.00 and

7.50 with an inoculum size of 1%. After 24 h of incubation at 37°C, the EPS was extracted from the cultures with different pH values to examine DPPH radical scavenging activity at the EPS concentration of 0.16 mg/mL.

2.7. Statistical Analysis

All of determinations were carried out in triplicate, and all mean values were used for statistical analysis. Analysis of variance was performed with SPSS software (version 16.0, IBM, USA).

3. Results

3.1. Growth Characteristics of *Lactobacillus kimchi* SR8

Adequate nutrients improve growth and increase the production of active substances. The fermentation characteristics of *Lactobacillus kimchi* SR8 were measured with MRS broth as a substrate (Figure 1). With extension of the culture time, the absorbance at 600 nm showed an ascendant trend and reached its absorbance peak value (2.359 ± 0.007) when the culture time reached the tenth hour. It illustrated an adaptive phase in the range of 0 to 4 h when the activated culture was homogenized with fresh culture, a logarithmic phase in the range of 4 to 8 h because of adequate nutritious substrates and a stationary phase in the range of 8 to 24 h due to the consumption of substrates (Figure 1a). With continuing fermentation, LAB strains consumed substrates for growth and reproduction so that the soluble composition content decreased. The soluble solid proportion of zymotic fluid declined until the culture time reached the eighth hour and then reached a plateau as the biomass changed. The initial pH value of the zymotic fluid was 6.47 ± 0.05. Along with the reproduction and fermentation from the isolate, the pH gradually decreased and then reached a plateau when the culture time reached the eighth hour. At that moment, the reproduction and growth of the isolate also remained steady. Therefore, acid production mainly appeared during the reproduction and growing phases. The pH of the zymotic fluid changed from an initial value of 6.47 ± 0.05 to a steady-state value of 4.86 ± 0.01 (Figure 1b).

3.2. Effect of Incubation Time, Temperature and pH on Production

The glucose standard curve for EPS production (y = 0.0112x + 0.0271; R2 = 0.99997) illustrates fitness for the calculation of EPS production (Figure 2).

As illustrated in Figure 3a, the EPS production from *Lactobacillus kimchi* SR8 increased slowly from the 26th to the 34th hour and reached a peak value of 210.37 ± 3.57 mg/L at the 32nd hour. Afterward, the production of EPS showed a declining trend. The growing environment has a significant effect on secretion of EPS. A harsh environment can enhance the secretion of some functional compounds of microorganisms. The EPS production changed as fermentation temperature rose (Figure 3b). When fermentation temperature increased, the production of EPS produced by LAB gradually increased. The LAB produced EPS that was used for their survivals to resist the high-temperature environment. The EPS production reached 236.28 ± 1.79 mg/L when fermentation temperature was up to 41°C. The pH also showed effects on the secretion of EPS of the isolate *Lactobacillus kimchi* SR8 (Figure 3c). The alkaline conditions inhibited the secretion of EPS of *Lactobacillus kimchi* SR8. It was drawn that the secretion of EPS raised just on suitable conditions.

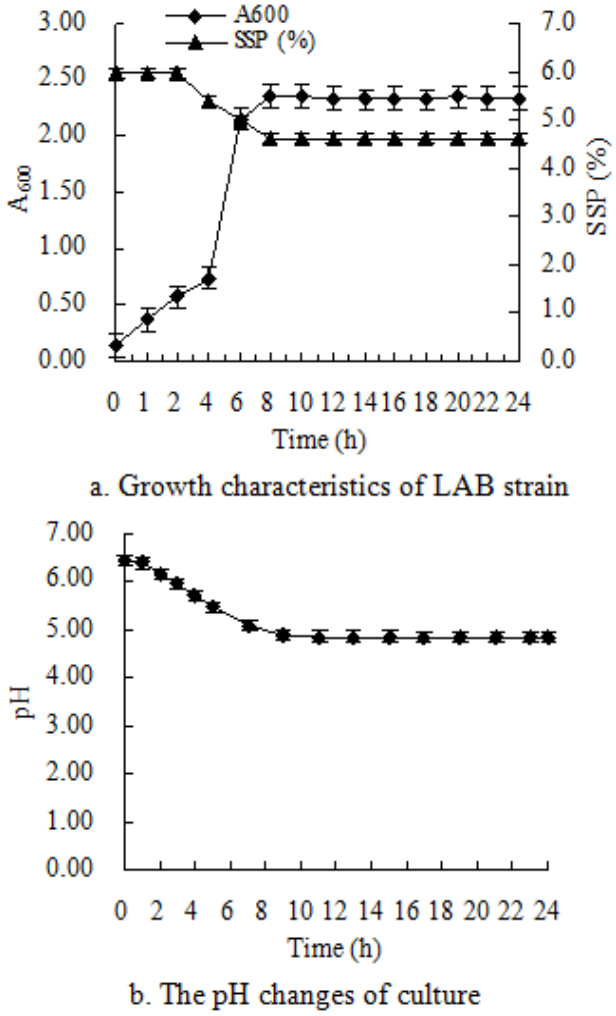


Figure 1. Growth and acid-producing properties of *Lactobacillus kimchi* SR8

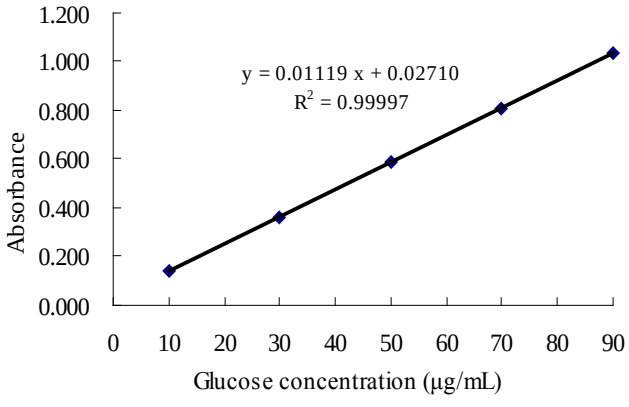
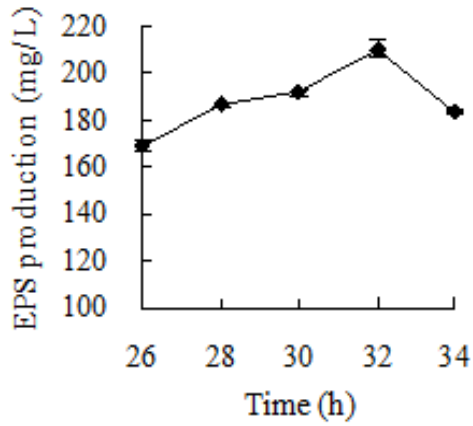
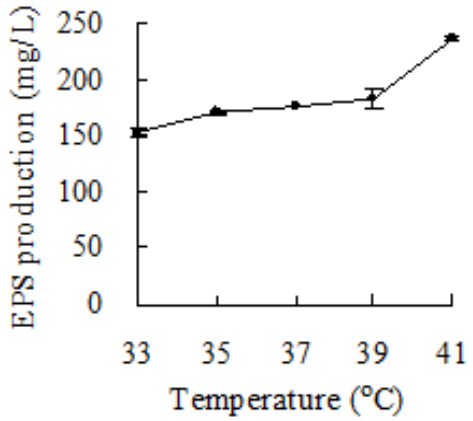


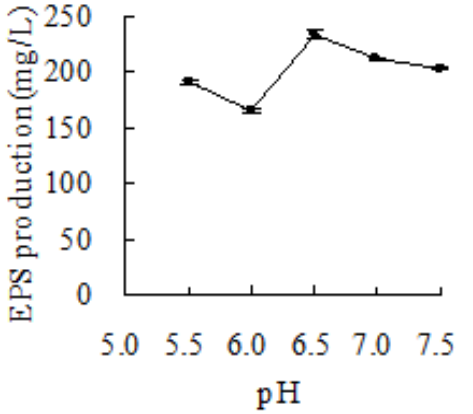
Figure 2. The glucose standard curve



a. Effect of time on EPS production



b. Effect of temperature on EPS production



c. Effect of pH on EPS production

Figure 3. Effect of incubation time and temperature on EPS production

3.3. Free Radical Scavenging Activity

DPPH radical scavenging activity was chosen as a free radical scavenging activity index for antioxidant activity of EPS. The DPPH radical scavenging activity of EPS at different concentrations was shown in Figure 4. With EPS concentration rising, the DPPH radical scavenging activity of EPS rose steadily. The scavenging activity suddenly rose rapidly when EPS concentration exceeded 0.32 mg/mL.

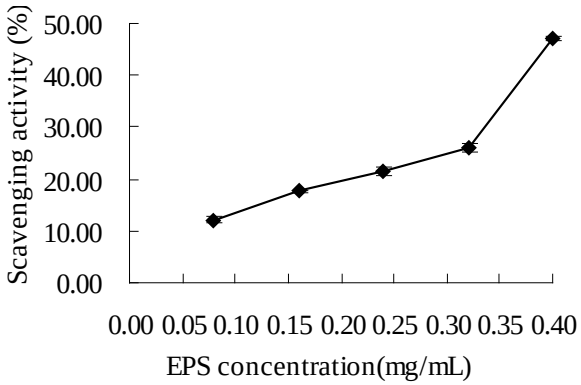


Figure 4. Effect of EPS concentration on DPPH radical scavenging activity

3.4. Relationship between Production and Free Radical Scavenging Activity

3.4.1. Effect of Carbon Source on Production and DPPH Radical Scavenging Activity

To select the optimal carbon source for EPS production and DPPH radical scavenging activity, the glucose in the MRS broth was replaced with lactose, maltose or sucrose (a final concentration, 20.00 g/L). The different kinds of sugar showed different effects on EPS production and DPPH radical scavenging activity. Sucrose showed the most effective carbon source for the production of LAB EPS (263.84 ± 2.98 mg/L). However, although EPS production was the greatest, the DPPH radical scavenging activity was the slowest than those of other carbon sources. According to Figure 5, EPS from fermented supernatants with glucose as the carbon source showed a higher DPPH radical scavenging activity (17.95 ± 0.36%). The EPS from the three kinds of reducing monosaccharides showed a good DPPH radical scavenging activity, while EPS from a non-reducing disaccharide (sucrose) illustrated a low DPPH radical scavenging activity (7.84 ± 2.35%).

3.4.2. Effect of nitrogen source on production and DPPH radical scavenging activity

The nitrogen source, a vital growth factor to LAB, showed an insignificant influence on their physiological-biochemical characteristics. This study showed the production and DPPH radical scavenging activity of EPS with different nitrogen sources and their ratios (Figure 6). The production of EPS was highly enhanced (284.99 ± 3.18 mg/L) when the nitrogen source was BP2:1, followed by those with BP1:2, BT1:1 and PT1:1 as nitrogen sources. Compared to former production of EPS, the production of the former EPS production was more than 19%. However, EPS production from different nitrogen sources was shown to be irrelevant to its DPPH radical scavenging activity at the same final EPS concentration. EPS from BP2:1 used as a nitrogen source illustrated higher production and lower DPPH radical scavenging activity than those from other nitrogen source.

In addition, EPS from PT1:1 culture showed a great DPPH radical scavenging activity (31.03 ± 0.90%), followed by those of EPSs from those with BP1:2, BP1:1 and BT1:1 as nitrogen sources, with an EPS production of 245.67 ± 2.91 mg/L. The nitrogen sources and their ratios

had a significant effect on the EPS's DPPH radical scavenging activity.

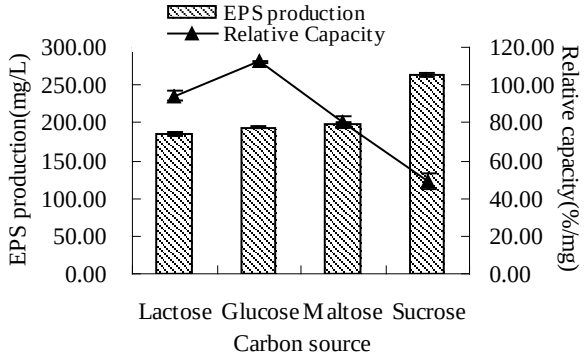


Figure 5. Influence of carbon source on the production of EPS and DPPH radical scavenging activity

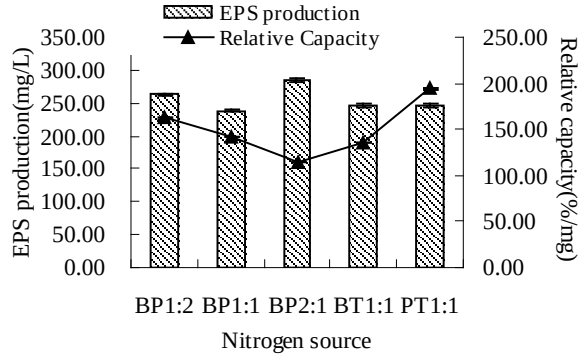


Figure 6. Influence of nitrogen source on the production of EPS and DPPH radical scavenging activity

Note: BP1:2, BP1:1, BP2:1, BT1:1 and PT1:1 are respectively represented as Beef Extract-Peptone (1:2), Beef Extract-Peptone (1:1), Beef Extract-Peptone (2:1), Beef Extract-Tryptone (1:1) and Peptone-Tryptone (1:1).

3.4.3. Effect of pH on Production and DPPH Radical Scavenging Activity

About effect of pH on microbial EPS, pH may stimulate the activities of enzymes and intermediates. LAB EPS production showed differences due to the initial pH values of the cultures (Figure 7). The EPS production showed a trend from rising to declining. EPS production reached a peak at a pH of 6.5 (234.05 ± 3.57 mg/L). With the pH rising continuously, the EPS production showed downtrend.

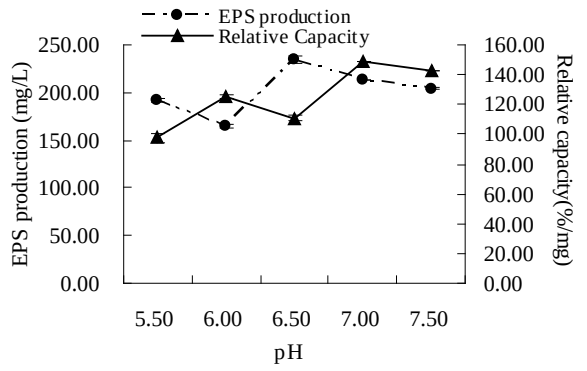


Figure 7. Influence of pH value on the production of EPS and DPPH radical scavenging activity

In addition, the DPPH radical scavenging activity of EPS showed differences in the different initial pH values of the cultures. As the pH increased, the scavenging activity showed an increasing trend (Fig.7). The EPS extracted from the medium with an initial pH of 7.00 showed the highest DPPH radical scavenging activity (23.85 ± 0.00%). When the pH value rose continuously, the scavenging activity weakened. It illustrated that the initial pH value had an effect on the DPPH radical scavenging activity of EPS.

3.5. Production and Radical Scavenging Activity of Exopolysaccharides on Combined Conditions

With reference to the effects of the carbon source, nitrogen source and initial pH value on DPPH radical scavenging activities, the production and DPPH radical scavenging activity of EPS from the combination of the following conditions were measured (Table 1). *Lactobacillus kimchi* SR8 produced 228.24 ± 2.23 mg/L EPS with sucrose as the carbon source, BP2:1 as the nitrogen source and an initial pH 6.50, and the EPS's DPPH radical scavenging activity was just 6.85% ± 0.77% with a 0.20 mg/mL EPS concentration. However, *Lactobacillus kimchi* SR8 produced 206.79 ± 2.23 mg/L EPS with glucose as the carbon source, PT1:1 as the nitrogen source and an initial pH of 7.00, and the DPPH radical scavenging activity reached 28.34% ± 0.32% in the same conditions. These combined test results verify the previous finding that the effect elements of the carbon source, the nitrogen source and the initial pH value have different influences on the EPS production of *Lactobacillus kimchi* SR8 and the DPPH radical scavenging activity.

Table 1. Production and DPPH radical scavenging activity of EPS from different culture conditions

Carbon	Nitrogen	pH	Results	
			EPS Production (mg/L)	DPPH radical scavenging activity (%)
Sucrose	BP2:1	6.50	228.24±2.23	6.85±0.77
Glucose	PT1:1	7.00	206.79±2.23	28.34±0.32

Note: BP2:1 and PT1:1 are represented as Beef Extract-Peptone (2:1) and Peptone-Tryptone (1:1), respectively.

4. Discussion

Many LAB isolates, such as *Lactobacillus sp.* Ca6, *Lactobacillus kefiranofaciens*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus*, can produce EPS [4,5,6,8,9]. The EPS produced by *lactobacilli* was an important functional compound for their survivals in hypertonic environment [29]. Reported lactobacilli with producing EPS were approximately 30 species [6]. However, EPS production of lactic acid bacteria was influenced by many factors, such as medium components, culture conditions, initial pH value and so on except for species of LAB. Wang's study showed a great influence of fermentation temperature, initial pH and nitrogen source on EPS production [30]. In our study, EPS production was also influenced by fermentation temperature and time, initial pH and medium. Imran et al. reported that the EPS

production of *Lactobacillus plantarum* reached a maximum in stationary phase of growth (32 h), and then decreased in declining phase [31], which was consistent with our results. This decrease in EPS production after prolonged culture was reported earlier [32]. For the effect of pH value, pH may stimulate the activities of enzymes and intermediates. It has been reported that lipid intermediate has an important effect on the biosynthesis of microbial polysaccharides. With the pH changing, EPS production changed and reached the maximum 234.05 ± 3.57 mg/L at pH 6.50, which was same to initial pH (pH 6.5) for the maximum production of EPS from *Lactococcus lactis subsp. Lactis* reported in Tao's study [33], which was consistent with the results of *Lactobacillus kimchi* SR8 in this study. Ibarburu et al. [34] studied production and partial characterization of EPS from *Lactobacillus suebicus* isolates, and found that EPS production was influenced by fermentation time, initial pH and LAB strains. As potential functional composition, antioxidant activity of EPS from LAB received much attention. The scavenging activity of DPPH radical was chosen to evaluate antioxidant activity of EPS in this study due to that DPPH radical, a synthetic free radical, has a proton free radical with a characteristic absorption, which significantly decreased when it was exposed to proton radical scavengers when it was compared to other free radicals [35]. It was found that the scavenging activity of DPPH radical of exopolysaccharides from *Lactobacillus kimchi* SR8 was up to 47.20% at 0.40 mg/mL. Zhang et al. [9] reported antioxidant activity of EPS from *Lactobacillus plantarum* C88, and its scavenging activity of DPPH radical was influenced by EPS concentration and the activity was about 43% at 1 mg/mL, which was lower than EPS's activity in our study. Based on antioxidant activity of EPS, the production of EPS has been optimized with an orthogonal experimental design, response surface method and artificial intelligence-based techniques [23,24,30], but optimization of antioxidant activity of EPS is still not seen. No research shows relationship between optimization of EPS production and its antioxidant activity. In our study, no significant relationship between production and antioxidant activity was found, and EPS production on combined conditions just for production was higher than that on combined conditions only for antioxidant activity of EPS while antioxidant activity of EPS from the former fermentation conditions was far lower than that from the later conditions. Antioxidant activity of EPS should be taken into consideration at the same time when optimization of EPS production is carried out. For that, the aim of high production and high antioxidant activity of EPS will be reached to ensure further study and development of antioxidant EPS products.

5. Conclusions

The parameters, including the fermentation time, temperature, initial pH, carbon source and nitrogen source, played important roles in the EPS production of LAB. *Lactobacillus kimchi* SR8-producing acid mainly appeared during the reproduction and growing phases, and the EPS production reached a peak value (210.37 ± 3.57 mg/L) at the 32nd hour and increased further to 236.28 ± 1.79 mg/L when cultured at 41°C. *Lactobacillus kimchi* SR8 was then studied to improve EPS production under different conditions, including carbon source, nitrogen source and the initial pH value of the medium. The antioxidant activity of EPS was taken into consideration when optimization was carried out to enhance the quantity of EPS. The results from single-factor experiments and combined test showed no positive correlation between the EPS production of LAB and its DPPH radical scavenging activity. So far, few reports have combined EPS production with antioxidant activity.

Acknowledgements

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Abbreviations

LAB	lactic acid bacteria
EPS	exopolysaccharide
DPPH	1,1-diphenyl-2-picrylhydrazyl
SSP	soluble solid proportion

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