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# BIOSYNTHESIS AND STRUCTURAL CHARACTERIZATION OF EXOPOLYSACCHARIDE FROM *Lactobacillus fermentum* MC3

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**Abstract.** Strain *Lactobacillus fermentum* MC3 isolated from fermented bamboo shoots was used in this study. This isolate had a high exopolysaccharide (EPS) production capability. It was inoculated in the medium with a supplement of various concentrations of carbohydrate source (2, 3, 4, 5, and 6% (w/v)). The mixtures were then cultured under different conditions of initial cell density, temperatures, pH, and incubation time to identify the optimum parameters for the EPS biosynthesis of this strain. The EPS yield was measured using the phenol-sulfuric acid method. The results showed that adding glucose, lactose, and sucrose to the culture medium significantly increased the EPS production with a maximum amount of 4% of sugars. The yield was the highest for glucose at 178.207 mg/L, and the obtained figures for lactose and sucrose were 148.614 mg/L and 152.272 mg/L, respectively. The results indicated that the EPS production by *L. fermentum* MC3 reached the maximum values at 200.728 mg/L in the medium supplemented with 4% glucose at 40 °C, pH 6.0, and initial cell density of $10^6$ CFU/mL for 48 h cultivation. By methylation and gas-chromatography mass spectrometry (GC-MS), it was found that the exopolysaccharide is composed of D-mannose, D-glucose, and D-galactose at the molar ratio of 1:0.74:0.09.

**Keywords:** glucose, exopolysaccharide, *Lactobacillus fermentum*

## 1 Introduction

Exopolysaccharides (EPS's) are long-chained polysaccharides that are mainly secreted by bacteria into their surroundings during growth. These bacterial EPS's are widely applied in the food industry as gelling and thickening agents. Among the wide variety of exopolysaccharide-producing microorganisms, lactic acid bacteria (LAB's) have received increasing attention because of their GRAS (generally recognized as safe) status and major importance for the food industry. Besides, most of these LAB's were isolated from dairy products, and the fermented food products are also served as a source for EPS-producing LAB strains [14]. Exopolysaccharides impart highly desirable rheological changes in the food matrix such as increased viscosity, improved texture, and reduced syneresis [5, 13]. Moreover, the EPS's from LAB's have beneficial effects to human

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health including anti-tumoral activity, antioxidant or prebiotic activities, and cholesterol-lowering ability [13].

Physiologically, the EPS synthesis by LAB's is influenced by many factors, e.g., temperature, pH, components of the growth medium, and fermentation time [7, 14]. The conditions of EPS production by LAB's vary considerably with the strains with respect to the EPS yield and their structural characterization [11, 12]. Therefore, this study was carried out to study the effect of different cultural conditions on the EPS production by *L. fermentum* MC3, which had the highest production capacity among tested strains. The aim of this study is to improve the EPS yield of this strain. In addition, the exopolysaccharide was isolated and studied for its structural properties.

## 2 Materials and methods

### 2.1 Microorganism

The bacterial strain *L. fermentum* MC3 was isolated from fermented bamboo shoots, one of Hue traditional fermented product, and was identified by Phenylalanyl-tRNA synthase (pheS) gene sequencing method. This strain was stored at –20 °C in MRS added glycerol.

### 2.2 Methods

**Effect of carbohydrate sources and their various concentrations**

To study the influence of various carbon sources and their concentrations on the EPS production by *L. fermentum* MC3, sucrose, lactose, and glucose (Scharlau, India) were added to MRS at concentrations of 1, 2, 3, 4, 5, 6%. These mixtures were incubated at 37 °C for 48 h in incubator Memmert IN30, Germany.

**Effect of culture conditions on EPS production**

The EPS production was investigated at different initial cell densities: $10^4$, $10^5$, $10^6$, $10^7$, and $10^8$ CFU/mL; different pHs: 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5; different temperatures: 30, 35, 40, and 45 °C; and different time: 12, 24, 36, 48, 60, and 72 h. The pH of the medium was adjusted with 0.1 M NaOH and 0.1 M HCl solutions.

**Isolation and quantification of EPS**

After incubation, the fermented broth was heated at 100 °C for 15 min to inactivate the enzymes potentially capable of polymer degradation, and the cells were removed by centrifugation (10 g, 10 min, 4 °C). The supernatant was precipitated with two volumes of chilled absolute ethanol. After standing overnight at 4 °C, the resultant precipitate was collected by centrifugation (12 g, 20 min, 4 °C), dissolved in distilled water, dialyzed against distilled water at 4 °C for 24 h, and lyophilized. The total of EPS (expressed as mg/L) was estimated in each sample with the phenol-

sulphuric method [3]. Briefly, a mixture containing 1 mL of EPS solution, 1 mL of phenol 5%, and 5 mL of concentrated sulfuric acid was vortexed and streamed for 2 minutes. Then, the mixture was kept at room temperature for 30 minutes. The absorbance of the characteristic yellow-orange color was measured at 490 nm. A blank was prepared by substituting distilled water for the EPS solution. The yield of EPS was then determined according to the reference standard curve of glucose.

**Monosaccharide composition and methylation analysis of EPS**

*Methylation analysis*: The polysaccharide samples were methylated using methyl disulfate and solid sodium hydroxide in dimethyl sulfoxide at 60 °C for 16 h. Two milligrams of purified EPS were hydrolyzed with 4 mL trifluoroacetic acid (TFA) 2 M at 120 °C for 2 h and followed by evaporation under a stream of $N_2$. The excess of TFA was removed by co-evaporation with MeOH under a stream of $N_2$.

*Converting monosaccharides into alditol acetates*: The resulting partially methylated monosaccharides were reduced with 4 mL $NaBH_4$ 0.25 M in $NH_3$ (30 minutes, room temperature). Then, the solution was neutralized with 5 mL acetic acid 10% in MeOH and lyophilized, and the boric acid was removed by co-evaporation with MeOH under a stream of $N_2$.

*Acetylation*: the alditols were acetylated with 2 mL acetic anhydride:pyridine mixture (1:1, v/v) at 100 °C for 20 minutes. The sample was dried under a stream of $N_2$. The resulting product was dissolved in ethyl acetate and analysed using gas chromatography-mass spectrometry (GC-MS) [9].

*GC-MS system* (*Shimadzu 2010*): the temperature programme is as follows: 65 °C for 1 minute, then 250 °C for 10 minutes, and 280 °C for 5 minutes. The total analysing time was 54 minutes.

**Statistical analysis**

The statistical analysis of variance (ANOVA) was performed using the SPSS software (version 22.0). Duncan's multiple range test was used to determine the significant difference ($p < 0.05$) among treatments. All experiments were repeated three times, and the results are the means of three replicates.

## 3 Results and discussion

### 3.1 Effect of carbon sources and their concentrations on EPS production

Table 1 shows that adding different sugars to the culture medium significantly increased the EPS production. The highest EPS yield was obtained at 178.207 mg/L, 148.614 mg/L and 152.272 mg/L

for 4% glucose, lactose, sucrose, respectively. The high sugar concentration might create an osmotic pressure that inhibits the growth of the LAB cells and results in a decreased EPS yield. Thus, for the *L. fermentum* MC3 strain, glucose was the most efficient carbon source in terms of EPS production. Glucose had the highest influence on *Lactobacillus delbrueckii Subsp. bulgaricus* (B3, G12) and *Streptococcus thermophilus* (W22) EPS production capacity among some carbohydrates added to the culture medium [15]. The EPS biosynthesis capacity of *Lactobacillus fermentum* F6 was the highest when glucose was supplemented to skim milk for the culture of this strain [17]. These results indicate that the influence of carbon sources on the yield of EPS produced of LAB strains also depends on the kind of sugars. Sucrose was found to be the most suitable carbon source for the EPS synthesis by *Streptococcus thermophilus* ST1. The EPS yield obtained in this medium was of 73.28mg/L [16]. Whereas, the EPS amounts produced by *Lactobacillus fermentum* TDS030603 in MRS in the presence of glucose, galactose, lactose, or sucrose were practically the same [4].

**Table 1.** Effect of carbon sources and their concentration on the production of EPS by *L. fermentum* MC3

| Supplement concentration of sugar (%) | EPS of the medium supplement various carbon sources (mg/L) | | |
|---|---|---|---|
| | Glucose | Sucrose | Lactose |
| 0 | 89.102$^{e}$ | 87.638$^{e}$ | 88.207$^{d}$ |
| 2 | 130.159$^{c}$ | 116.825$^{d}$ | 99.265$^{c}$ |
| 3 | 141.378$^{b}$ | 132.394$^{c}$ | 123.207$^{b}$ |
| 4 | **178.207$^{a}$** | **152.272$^{a}$** | **148.614$^{a}$** |
| 5 | 109.467$^{d}$ | 143.085$^{b}$ | 124.833$^{b}$ |
| 6 | 110.402$^{d}$ | 142.760$^{b}$ | 123.736$^{b}$ |

*The data in the same columns with different letters are significantly different at $p < 0.05$.*

### 3.2 Influence of culture condition on the EPS biosynthesis by strain *L. fermentum* MC3

**Effect of initial cell density**

In the medium with 4 % glucose added, the production of EPS increased at the initial cell density from $10^4$ CFU/mL to $10^6$ CFU/mL reaching a maximum of 184.427 mg/L at $10^6$ CFU/mL. At lower initial cell densities, the amounts of EPS were small (Fig. 1). The yields of EPS were 150.972 mg/L and 146.053 mg/L at the initial cell density of $10^7$CFU/mL and $10^8$ CFU/mL, respectively. The EPS yields were lower in the experiments at 4 and 5 lg CFU/mL of initial cell density may be due to the low concentration of the bacterial strain. In contrast, with a high initial density of cell (e.g., 7 and 8 lg CFU/mL), there is a nutritional competition resulting in a lower EPS yield than in case of 6 lg

CFU/mL. In summary, the optimal initial cell density for the EPS production by *L. fermentum* MC3 is $10^6$ CFU/mL.

**Effect of initial pH**

The EPS biosynthesis could take place at all test initial pHs (Fig. 2). The EPS production increased rapidly with pH from 5.5 to 6.0 and reached a maximal value of 189.996 mg/L at pH 6.0. The yields of EPS were 100.565 mg/L, 120.402 mg/L, and 139.752 mg/L at pH 4.0, 4.5, and 5.0, respectively. At pH 6.5, the yield decreased to 166.866 mg/L. The low EPS yields at pH 4.0, 4.5, 5.0 could be due to the high acidity of the medium that might cause acid stress to the cells or this acidity could protect the cells.

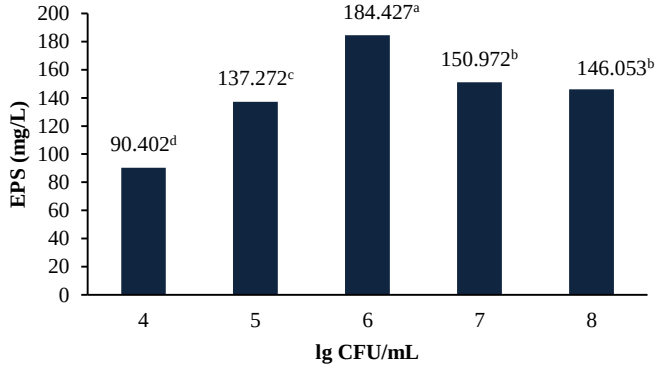


**Fig. 1.** Effect of initial cell density on EPS production (The data in the figure with different letters are significantly at $p < 0.05$).

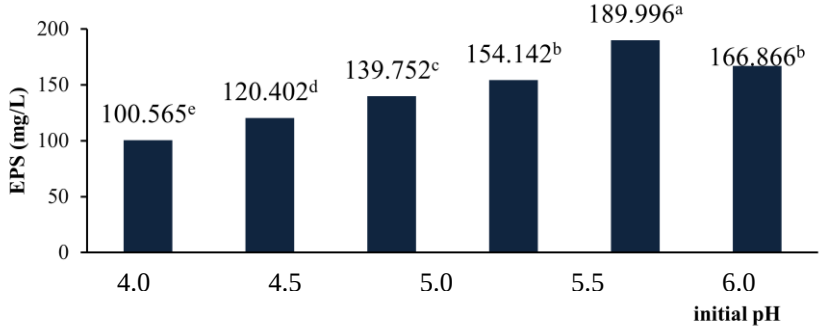


**Fig. 3.** Effect of initial pH on EPS production
(The data in the figure with different letters are significantly at $p < 0.05$).

When *L. sake* 0-1 was cultured in coconut water as an alternative carbon source, the best stimulation for EPS production (1375 mg/L) was at pH 5.8 [10]. At pH 5.5, the EPS production by *L. confusus* TISTR 1498 reached a maximal yield of 38.2 g/L using a mod-MRS-coconut water medium [8]. *L. plantarum* KF5 gave the highest yield of EPS with initial pH 6.3 [12]. Whereas, other

authors reported that 6.5 was the optimal pH for EPS synthesis by *L. fermentum* F6 [17], *L. fermentum* TDS030603 [4], and *S. thermophilus* ST1 [16].

Although the suitable pH for the EPS biosynthesis by different LAB strains is different, the strains usually grow best with better EPS production at pH nearly 6.0 [2]. In brief, the EPS production by *L. fermentum* MC3 was the highest at pH 6.0. This is nearly a neutral pH of the culture medium. This is an advantage to applying this strain in industry.

**Effect of temperature**

The EPS production by *L. fermentum* MC3 was studied at 30, 35, 40, and 45 °C in the MRS supplemented with glucose 4% and initial cell density of $10^6$ CFU/mL at pH 6.0 for 48 h.

The EPS production was observed at all tested temperatures (Fig. 3). The optimal temperature for EPS biosynthesis is 40 °C. At this temperature, the yield of EPS reached 191.378 mg/L. Therefore, in the temperature range from 37 to 40 °C, *L. fermentum* MC3 can grow and give high EPS yield.

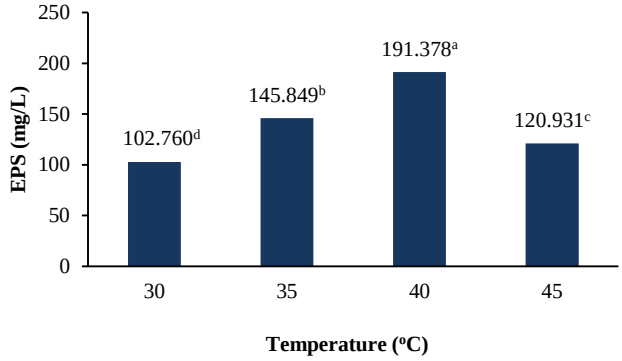


**Fig. 4.** Effect of incubation temperature on EPS production (The data in the figure with different letters are significantly at $p < 0.05$).

This report matches many previous studies investigating the culture conditions for EPS production by LAB strains. The growth and EPS production in skim milk by *L. fermentum* F6 isolated from traditional dairy products in Inner Mongolia of China were studied by Zhang et al. [17]. A maximum of 44.49 mg/L of EPS was produced by *L. fermentum* F6 in the skim milk medium supplemented with 2% (w/v) glucose and 0.5% (w/v) whey protein concentrate at 37 °C. Similarly, Yadav et al. reported that *L. fermentum* CFR 2195 produced the best EPS at 37 °C [14]. Similar results were shown in other reports [1, 11].

High temperature (45 °C) rapidly decreased the EPS production by *L. fermentum* MC3 due to the denaturalization of enzyme and the increased pressure on the cell membrane. Therefore, the

incubation temperature of 40 °C was chosen for further experiments for EPS production by *L. fermentum* MC3.

**Effect of culture time**

From 12 h to 48 h, there was a significant increase in the EPS production by *L. fermentum* MC3. At 12 h and 36 h, the yield of EPS reached 103.492 mg/L and 161.1947 mg/L, respectively. At 48 h, the EPS production was highest reaching 200.728 mg/L (Fig. 4). The EPS production started in the early growth phase and stopped when the culture reached the stationary phase. After 48 h, the EPS biosynthesis decreased substantially, first to 113.614 mg/L of EPS after 60 h and then to 67.963 mg/L after 72 h of growth. Like other LAB strains, *L. fermentum* MC3 produces the highest yield in the stationary phase and then the bacteria growth decreases [6]. The EPS biosynthesis by each bacterium can take place at different phases in their growth. Therefore, at the same physical parameters (pH, temperature), the obtained EPS yield is also relevant to the fermented time.

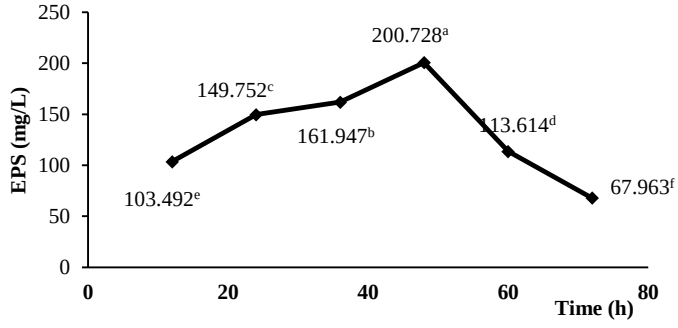


**Fig. 4.** Effect of time on EPS production
(The data in the figure with different letters are significantly at $p < 0.05$).

*L. fermentum* TDS08603 produced the highest yield after 72 h in the medium supplemented with 1% fructose. The EPS yield in purified form was 97.1 mg/L. The EPS production of this strain was maximum after a 24 h growth for MRS (130 mg/L) and galactose (40 mg/L), 48 h for the MRS supplemented with either glucose (70 mg/L) or lactose (50 mg/L) [4]. At 32 h, *L. fermentum* F6 produced a maximal yield of 44.49 mg/L of EPS when this strain was grown at 37 °C, pH 6.5 in 10% skim milk, 2% (w/v) glucose, and 0.5% whey protein concentrate [17].

### 3.3 Structural characterization of EPS

**Monosaccharide composition of EPS**

As can be seen in Table 2, the produced EPS is composed of mannose, glucose, and galactose. The EPS mainly consists of mannose with 54.70%, followed by glucose with 40.22% and D-galactose

with 5.08% corresponding to the molar ratio of 1:0.74:0.09. The monosaccharide composition of this EPS is the same as those of previous studies. Wang et al. noted that the EPS obtained by *L. plantarum* KF5 was composed of mannose, glucose, and galactose with a molar ratio of 4.99:6.90:1 [12].

**Glycosidic linkages of EPS**

The results of methylation analysis of EPS show the presence of five components (Table 3). Exopolysaccharide from *L. fermentum* M3 was mainly consisted of Glc-(1→. The presence of this terminal can exist in the branches and the first and final monomer of the polysaccharide.

D-mannose was the major monomer with two linkages: (2→6) and (1→3,6) mannopyranose glycoside. They were in a high molar ratio, therefore these linkages form the patterns of the backbone structure.

D-galactose with the lowest molar ratio has (1→6) galactopyranose glycoside and is also a part of the branch. Thus, EPS produced from *L. fermentum* MC3 is a heteropolysaccharide with a mannan-glucan structure backbone.

**Table 2.** The monosaccharide composition of EPS

| Monosaccharide composition | Molar ratio | Percent ratio (%) |
|---|---|---|
| D-mannose | 1.00 | 54.70 |
| D-glucose | 0.74 | 40.22 |
| D-galactose | 0.09 | 5.08 |

**Table 3.** GC-MS data for the alditol acetates derived from methylated EPS

| Methylated sugar (as alditol acetate) | Linkage pattern | Molar ratio |
|---|---|---|
| 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol | Glc (1→ | 1.00 |
| 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol | →6) Glc (1→ | 0.12 |
| 2,5,6-tri-O-acetyl-1,3,4-tri-O-methyl-D-mannitol | →6) Man (2→ | 0.87 |
| 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-D-mannitol | →3,6) Man (1→ | 0.65 |
| 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-galactitol | →6) Gal (1→ | 0.14 |

## 4 Conclusion

The EPS production by *L. fermentum* MC3 is affected by pH, temperature, and carbon sources. The EPS yield produced by *L. fermentum* MC3 reached the maximum value in the medium supplemented with 4% (w/v) glucose at 40 °C, pH 6.0, and initial cell density of $10^6$ CFU/mL for 48 h cultivation. These results indicate that the choice of the most suitable nutrition source and culture conditions

enables to produce EPS with high yield. Exopolysaccharide of *L. fermentum* MC3 isolated from fermented bamboo shoots is a heteropolysaccharide with D-mannose, D-glucose, and D-galactose residues in the molar ratio of 1.0:0.74:0.09. The obtained exopolysaccharide is a heteropolysaccharide with a mannan-glucan structure backbone.

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