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ORIGINAL ARTICLE

The isolation of exopolysaccharide-producing lactic acid bacteria from lontar *(Borassus flabellifer* L.) sap

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ABSTRACT

Background and Objectives: Lontar (*Borassus flabellifer* L.) is widely grown in Indonesia and one of its products is palm sap. Palm sap contains a high level of sugar, making it suitable as a medium to increase the lactic acid bacteria (LAB) production of exopolysaccharides (EPS). This study aimed to isolate the EPS-producing LAB from palm sap and evaluate its EPS production. LAB isolation was carried out on MRS agar containing 0.5% $CaCO_3$.
Materials and Methods: The screening and production of EPS were carried out on MRS media supplemented with 10% sucrose. The molecular identification of the selected EPS-producing LAB was based on 16S rDNA. A quantitative analysis of EPS polymer dry mass and total sugar was conducted using one-way ANOVA.
Results: In this study, five EPS-producing LABs were found: *Fructobacillus fructosus* N4, *Leuconostoc mesenteroides* N5, *Leuconostoc mesenteroides* N7, *Leuconostoc mesenteroides* N9, and *Fructobacillus fructosus* N10. The highest EPS yield in liquid media was 10.997 ± 1.591 g/L by *Leuconostoc mesenteroides* N7, whereas the lowest was 4.505 ± 0.459 g/L by *Fructobacillus fructosus* N10.
Conclusion: This study found *Fructobacillus fructosus* strains as EPS producers that have never been reported before.

Keywords: *Borassus flabellifer*; Exopolysaccharide; *Fructobacillus fructosus*; Lactic acid bacteria; *Leuconostoc mesenteroides*

INTRODUCTION

Exopolysaccharides (EPS) is a long-chain polysaccharide produced by bacteria. Some Gram-positive bacteria can produce EPS to protect the cell against osmotic stress, desiccation, antibiotics and phagocytosis (1, 2). The structure of EPS can be comprised of one type of sugar monomer (homopolysaccharide) or several types of sugar monomer (heteropolysaccharide) (2). Moreover, EPS also contains several non-carbohydrate substituents, such as acetate, pyruvate, succinate, and phosphate (3), and biomolecules, such as proteins, nucleic acids, lipids and humic substances (4). Lactic acid bacteria (LAB) can produce EPS that are attached to cell walls or released to the environment (5).

LAB is regarded as Generally Recognized as Safe and probiotics so the produced EPS are non-toxic (6). LAB can effectively perform the biosynthesis of functional EPS through sugar fermentation (7); therefore, the produced EPS by LAB has the advantages and potentials if used as pharmaceutical ingredients and food additives. It plays a role in the

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formation of texture, taste and viscosity in fermented milk products. Additionally, EPS are currently being developed play a beneficial role in health, e.g., antitumor, immunomodulatory activities, cholesterol reduction, prebiotic, antioxidant and antidiabetic (8-10).

Indonesia has a diverse palm family plants that are dispersed in every region, including Java island, one of which is lontar (*Borassus flabellifer* L.) (11). The main product of this plant is the sap which is obtained by tapping the male flowers. Palm sap contains about 10%-15% sugar, which is sucrose and reducing sugar. The high sugar contents and other nutrients in palm sap are suitable as a growth medium for a LAB that requires complex natural nutrients. It must be considered that nonpathogenic bacteria produce high amounts of EPS. Therefore, this study aimedto isolate the EPS-producing LAB from palm (*Borassus flabellifer* L.) sap and evaluate its EPS production.

MATERIALS AND METHODS

Study design. This study used anexperimental design utilizing *in vitro* methods. The first step was the isolation and identification of EPS-producing LAB from palm sap and then the evaluation of the EPS production of the selected LAB. The experiment for EPS production was conducted in triplicate.

Isolation and screening of EPS-producing LAB. Palm (*Borassus flabellifer* L.) sap was taken from the city of Lamongan, East Java, Indonesia. The palm sap was put into the laboratory bottle aseptically overnight and then stored in a cooling box to be brought to the laboratory. As much as 300 mL of palm sap was kept for 18 h for spontaneous fermentation (aerobic conditions), then 25 mL of those were transferred into 225 mL buffered peptone water (Merck, Germany) and diluted to 10^{-10}. As much as 1 mL of diluted sample was taken to be grown on De Man Rogosa and Sharpe (MRS) agar (Merck, Germany) containing 0.5% (w/v) $CaCO_3$ (HiMedia, India) then incubated at a 30°C incubator for 48 h. Bacterial colonies that produce clear zones were observed for morphology, transferred to an agar slant, and then purified by streaking on MRS agar plates. The screening of EPS-producing LAB was performed by growing on MRS agar supplemented with 10% (w/v) sucrose (Pronadisa, Spain). The LAB is considered as EPS producers if mucus production occurs in the medium.

EPS production and extraction. The inoculum was produced by transferring LAB into 25 ml of MRS broth (Pronadisa, Spain) and then incubated at 30°C for 18 h. The inoculum turbidity was adjusted to an optical density (OD) of 0.5 at 600 nm, which was equivalent to 10^9CFU mL^{-1}. The EPS was produced using 100 mL of MRS broth supplemented with 10% (w/v) sucrose, then added with 10% (v/v) of inoculum, and incubated at 30°C for 24 h (12). The fermentation medium was centrifuged at 6000 rpm at 4°C for 15 min. The crude EPS was precipitated by the addition of 2 × volume of cold ethanol (95%) (13) and kept at 4°C for 24 h. The crude EPS was *freeze-dried* for 18 h and the results were expressed as polymer dry mass.

Total sugar determination. The EPS total sugar was determined by the phenol sulfuric acid method using glucose as a standard (14). The EPS (0.01 g) was dissolved in 250 ml of distilled water and 2 ml of the solution was added with 1 ml of 5% phenol and 5 ml of 96% sulfuric acid (v/v). Subsequently, the solution was heated in a boiling water bath for 30 min. The absorbance was measured at 490 nm. The total sugar of EPS was calculated by the standard curve.

Infrared (IR) spectrum analysis. The IR spectrum of EPS was determined using Fourier transform infrared (FTIR) spectrophotometer (Shimadzu, Japan). The EPS was mixed with KBr and pressed on a mold. The spectrum was recorded in the region of 4000-400 cm^{-1} (15).

Selected LAB identification. The observed physiological characteristics of EPS-producing LAB including colony morphology, Gram staining, endospore staining and catalase activity. The biochemical characteristics were identified using API 50CHL (bioMerieux, France) according to the manufacturer's instructions to find out the fermentation patterns in carbohydrate substrates and their derivatives.

Selected LAB molecular identification. The selected LAB strains were grown on MRS agar and

incubated for 48 h. Three to five bacterial colonies were used for DNA isolation using commercial DNA kits (iNtRON Biotechnology, Inc.) according to the manufacturer's instructions. The DNA suspension was confirmed using agarose gel electrophoresis 0.7% (w/v). DNA templates were amplified by polymerase chain reaction (PCR) method with 16S rDNA using the following primers: 27F (5`-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5`GGT TAC CTT CTT ACG ACT T-3'). The PCR operating conditions were 94°C pre-denaturation for 30 s, 55°C denaturation for 30 s, 55°C annealing for 45 min, and 72°C extension for 1.5 min over 35 cycles. The obtained amplicon was confirmed with agarose gel electrophoresis 1.5% (w/v). The obtained nucleotide base sequences were analyzed using sequence scanners, BioEdit, and MEGA 6 software. The sequences were analyzed using BLASTN compared with the sequences in the NCBI.

Statistical analysis. The quantitative analysis of polymer dry mass and total sugar of EPS was conducted using one-way ANOVA with Tukey's post-hoc test utilizing SPSS Statistics 23.0 for Windows (IBM).

RESULTS

Isolation and screening of EPS-producing LAB. Palm (*Borassus flabellifer* L.) sap contains a high level of sugar, especially sucrose, making it suitable to isolate EPS-producing LAB. This study found 18 isolates of LAB, characterized by the clear zone formation around the colony on the MRS agar containing 0.5% $CaCO_3$. All strains were Gram-positive, non-spore-forming, and catalase-negative. The results of screening on MRS media supplemented with 10% sucrose revealed five strains were capable to produce EPS, namely, N4, N5, N7, N9 and N10. The EPS-producing LAB strains were rod cells for the N4 and N10 strains and ovoid cocci for the N5, N7 and N9 strains. The EPS production by the LAB is presented in Fig. 1.

The ability of EPS-producing LAB to ferment 49 types of carbohydrates and hydrolyze esculin was analyzed using API 50CHL (Table 1). All strains fermented D-glucose, D-fructose, N-acetylglucosamine, D-sucrose, D-trehalose, and potassium gluconate. Also, N7 strain fermented D-mannose and potassium 5-ketogluconate. N9 strain fermented potassium 2-ketogluconate and potassium 5-ketogluconate. N10 strain fermented D-mannose. These results indicated the biochemical differences of EPS-producing LAB from palm sap.

Molecular identification of selected LAB. EPS-producing LAB was identified based on its 16S rDNA by PCR with the amplicon size of 1500 bp to determine the genus and strain (Fig. 2). The results of the molecular identification (Fig. 3) revealed that the N4 strain was *Fructobacillus fructosus* with 99.9% similarity to *Fructobacillus fructosus* JCM1119 and *Fructobacillus fructosus* ATCC3516. N5 strain was *Leuconostoc mesenteroides* with 99.9% similarity to *Leuconostoc mesenteroides* F16, *Leuconostoc mesenteroides* SD7002, and *Leuconostoc mesenteroides* SD1S2L1. N7 strain was *Leuconostoc mesenteroides* with 100% similarity to *Leuconostoc mesenteroides* SD7002. N9 strain was *Leuconostoc mesenteroides* with 99.9% similarity to *Leuconostoc mesenteroides* SD7002, while the N10 strain was *Fructobacillus fructosus* with 99.9% similarity to *Fructobacillus fructosus* V5. This study found two species of LAB as EPS producers, *Leuconostoc mesenteroides* and *Fructobacillus fructosus*.

Production of EPS. Different bacterial strains exhibited significant effects ($P < 0.05$) on polymer dry mass and total sugar of EPS. The level of polymer dry mass production by selected LAB ranged from 4.505 to 10.997 g/L (Table 2). *Leuconostoc mesenteroides* N7 produced the highest EPS yield, whereas the lowest was *Fructobacillus fructosus* N10. The total sugar of produced EPS by *Leuconostoc mesenteroides* N5 and *Leuconostoc mesenteroides* N7 was 73% and 73.844%, respectively (Table 2). These strains are capable to produce highly pure EPS. The total sugar of EPS produced by *Leuconostoc mesenteroides* KIBGE-IB22 was 83% (16), whereas the total sugar of EPS produced by *L. plantarum* YML009 was 68.1% (17).

Infrared spectrum analysis. The spectrum results in this study were presented in Table 3. The FTIR spectrum analysis of EPS showed the identical spectrum were hydroxyl stretching vibrations, CH stretching, C=O stretching, (CH) (CH2) bending vibrations, covalent vibrations of C–O–C bonds, and glycosidic bonds (Fig. 4).

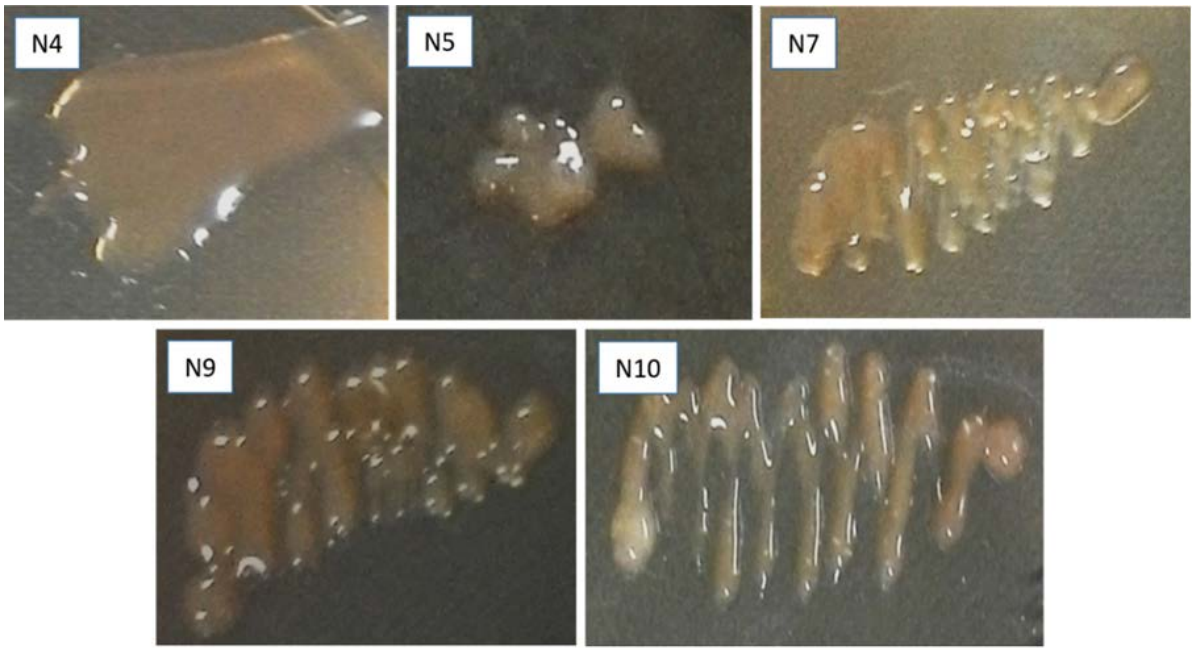

Fig. 1. EPS production by selected LAB on sucrose-containing medium

Table 1. Carbohydrate fermentation characteristics by selected LAB

Carbohydrates	Result					Carbohydrates	Result				
	N4	N5	N7	N9	N10		N4	N5	N7	N9	N10
Glycerol	−	−	−	−	−	Salicin	−	−	−	−	−
Erythritol	−	−	−	−	−	D-cellobiose	−	−	−	−	−
D-arabinose	−	−	−	−	−	D-maltose	−	−	−	−	−
L-arabinose	−	−	−	−	−	D-lactose	−	−	−	−	−
D-ribose	−	−	−	−	−	D-melibiose	−	−	−	−	−
D-xylose	−	−	−	−	−	D-sucrose	+	+	+	+	+
L-xylose	−	−	−	−	−	D-trehalose	+	+	+	+	+
D-adonitol	−	−	−	−	−	Inulin	−	−	−	−	−
Methyl-βD-xylopyranoside	−	−	−	−	−	D-melezitose	−	−	−	−	−
D-galactose	−	−	−	−	−	D-raffinose	−	−	−	−	−
D-glucose	+	+	+	+	+	Amidon (starch)	−	−	−	−	−
D-fructose	+	+	+	+	+	Glycogen	−	−	−	−	−
D-mannose	−	−	+	−	+	Xylitol	−	−	−	−	−
L-sorbose	−	−	−	−	−	Gentiobiose	−	−	−	−	−
L-rhamnose	−	−	−	−	−	D-turanose	−	−	−	−	−
Dulcitol	−	−	−	−	−	D-lyxose	−	−	−	−	−
Inositol	−	−	−	−	−	D-tagatose	−	−	−	−	−
D-mannitol	−	−	+		+	D- fucose	−	−	−	−	−
D-sorbitol	−	−	−	−	−	L-fucose	−	−	−	−	−
Methyl-αD- Mannopyranoside	−	−	−	−	−	D-arabitol	−	−	−	−	−
Methyl-αD-Glucopyranoside	−	−	−	−	−	L-arabitol	−	−	−	−	−
N-acetylglucosamine	+	+	+	+	+	Potassium gluconate	+	+	+	+	+
Amygdalin	−	−	−	−	−	Potassium 2-ketogluconate	−	−	−	+	−
Arbutin	−	−	−	−	−	Potassium 5-ketogluconate	−	−	+	+	−
Esculin	+	+	−	−	+		−	−	−	−	−

Abbreviations: (−) negative reaction; (+) positive reaction

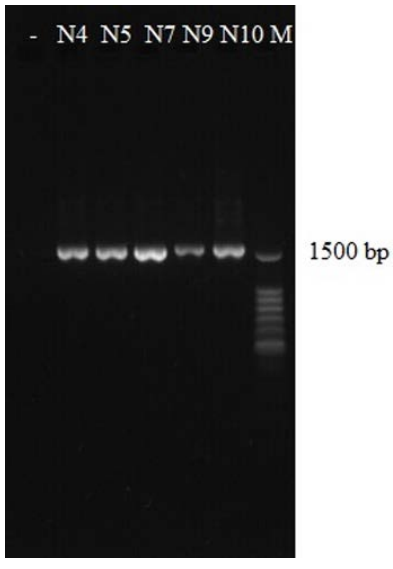

Fig. 2. PCR product of selected LAB based on 16s rRNA. Dash (–) means negative control (ddH_2O); N4-N10 = selected LAB; M = DNA marker.

DISCUSSION

Based on the phenotypic observations, N4, N5, N7, N9 and N10 strains were shown to produce visible EPS with the formation of shiny, mucoid, and ropy colonies on MRSA sucrose media. The *Leuconostoc* strains can produce EPS with shiny, mucoid, or viscous colonies on sucrose-containing media (12).

The different biochemical properties indicate different genotypes. The previous study reported that *Leuconostoc mesenteroides* G2d fermented D-galactose, α-methyl-D-glucoside, D-glucose, D-fructose, N-acetylglucosamine, D-lactose, D-maltose, D-mannose, D-sucrose, D-trehalose, and D-turanose but cannot hydrolyze esculin; whereas *Leuconostoc mesenteroides* G5b fermented D-glucose, D-fructose, N-acetylglucosamine, arbutin, salicin, D-lactose, D-maltose, D-mannose, D-sucrose, D-trehalose, D-turanose, and potassium gluconate, as well as hydrolyzed esculin (18).

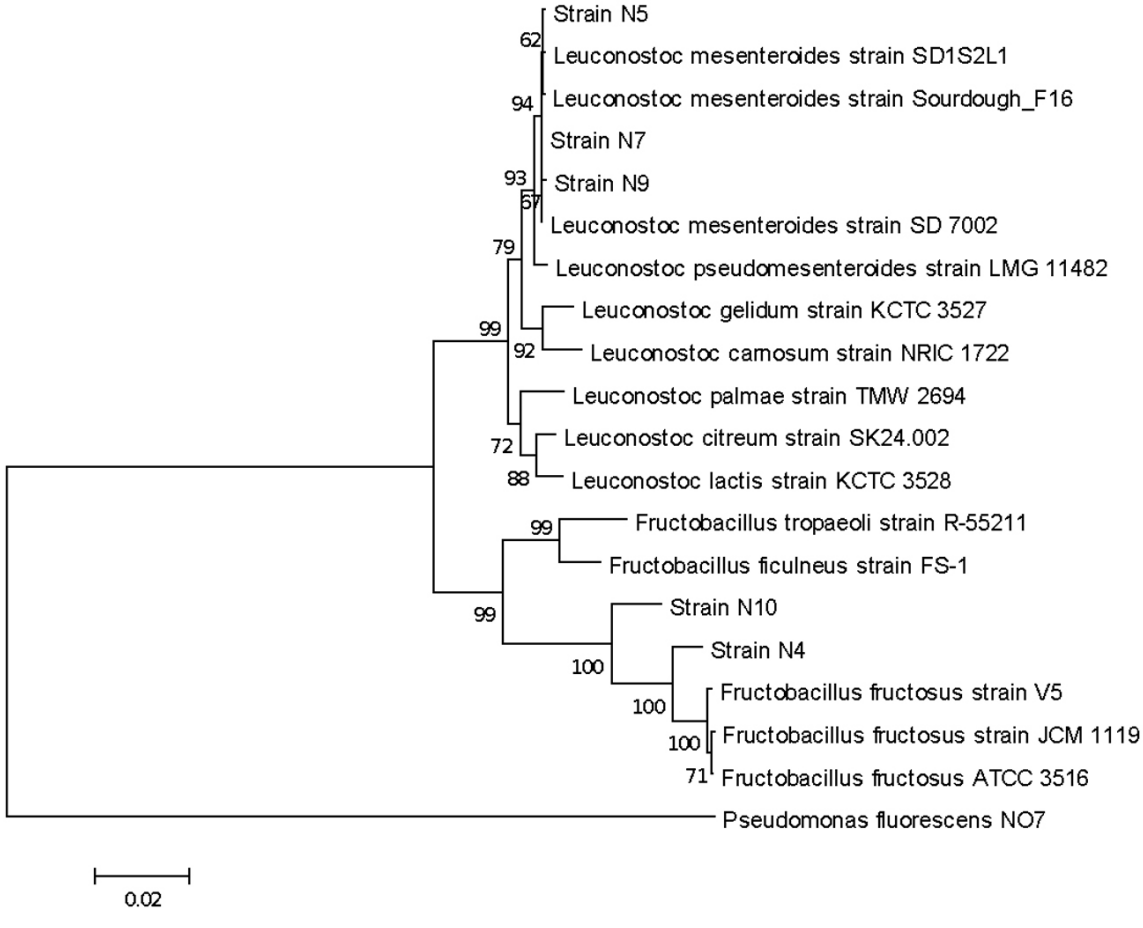

Fig. 3. Phylogenetic tree of EPS-producing LAB with reference bacteria based on the 16S rDNA sequences with a maximum-likelihood method

Table 2. EPS production by selected LAB strains

Strain	Polymer dry mass (g/L)	Total sugar (%)
F. fructosus N4	6.14 ± 2[a]	61.729 ± 1.006[ab]
Leuc. mesenteroides N5	9.849 ± 0.960[b]	73 ± 1.434[b]
Leuc. mesenteroides N7	10.997 ± 1.591[b]	73.844 ± 4.913[b]
Leuc. mesenteroides N9	9.713 ± 0.212[b]	47.244 ± 7.834[a]
F. fructosus N10	4.505 ± 0.459[a]	70.327 ± 5.460[b]

Table 3. FTIR spectra of produced EPS by selected LAB strains

Functional Group	Wavenumber (cm^{-1})				
	A	B	C	D	E
(OH) stretching	3416	3389	3426	3449	3381
(CH) stretching	2924	2926	2928	2930	2932
(C = O) stretching	1647	1653	1649	1651	1655
(CH) (CH2) bending vibration	1372	1381	1345	1346	1348
Covalent vibration COC bond	1144	1154	1152	1154	1154
α (1.6) glycosidic	1048	1015	1015	1015	1013
α-glycosidic	920	916	916	916	916

The results revealed that EPS-producing LAB isolated from palm (*Borassus flabellifer* L.) sap were *Leuconostoc mesenteroides* and *Fructobacillus fructosus*. *Fructobacillus* was distinguished from *Leuconostoc* based on its preference for fructose over glucose as a carbon source (19). This study found strains of *F. fructosus* as EPS producers that have never been reported before.

The previous study showed that adding sucrose to the fermentation media of *Leuconostoc mesenteroides* could produce higher EPS than without. EPS production requires a substrate with high sugar content. MRS broth media supplemented with 10% sucrose was utilized in the EPS production in this study. Dextran biosynthesis by *Leuconostoc* spp. showed that most fructose, derived from sucrose, will accumulate in the media, whereas glucose is used as a substrate for dextran synthesis by dextran sucrase enzyme (20).

In this study, the EPS levels were greater than previously reported. Dextran EPS types can be produced by *Leuconostoc mesenteroides* in high quantities (16). The EPS level produced by *Leuconostoc lactis* was 340.82 mg/L (21), whereas that produced by mutant *Weissella confusa* was 5580.72 mg/L (13).

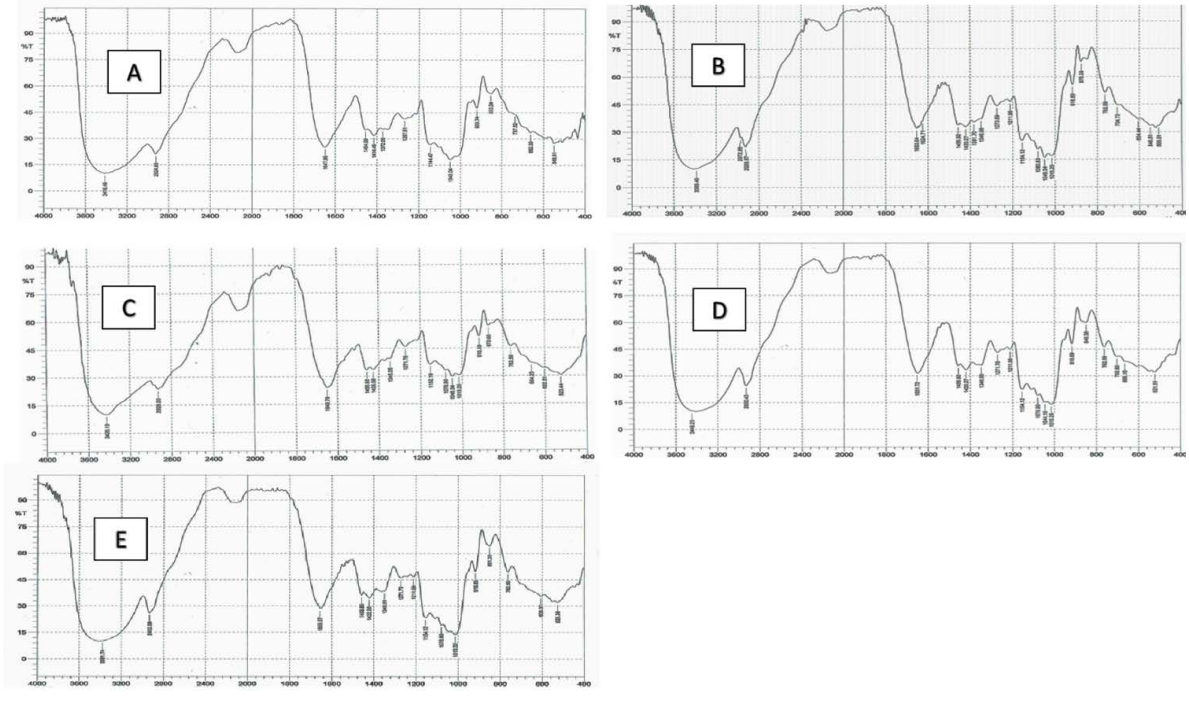

Fig. 4. FTIR spectra of EPS produced by *F. fructosus* N4 (A), *Leuc. mesenteroides* N5 (B), *Leuc. mesenteroides* N7 (C), *Leuc. mesenteroides* N9 (D), and *F. fructosus* N10 (E)

Furthermore, *Leuconostoc mesenteroides* NCDC744 and *Leuconostoc citreum* was reported to be able to produce higher EPS levels, which were 12.7 g/L and 28.3 g/L, respectively (12;22). The different EPS levels by each LAB in the media were influenced by the microbial strains used (17).

FTIR spectrophotometer was used to determine the functional groups and types of bonds in EPS. The characteristics of polysaccharides could be observed at wavenumbers of 1650, 1400 and 1250 cm^{-1} (23). Previous studies have shown that dextran produced by *Leuconostoc mesenteroides* FT045B indicated α-(1,6) bonds by the peaks at 1010, 916 and 1159 cm^{-1} due to the covalent vibrations from C–O–C bonds and glycosidic bridge (24). Dextran produced by *Leuconostoc mesenteroides* KIBGE-IB22 revealed the presence of stretching vibrations of hydroxyl at 3430 cm^{-1}, stretching vibrations from C–H at 2929 cm^{-1}, and a carboxyl group at 1635 cm^{-1} (16).

Further research is underway to optimize the EPS production by selected LAB from palm sap on various modified media and to purify EPS for its application as a bioactive compound.

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