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# Research Article

# Optimization of Extracellular Polysaccharide Substances from Lactic Acid Bacteria Isolated from Fermented Dairy Products

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## Abstract

**Background and Objective:** The extracellular polysaccharide secreted by Lactic Acid Bacteria (LAB) has been used in the food industry due to their viscosity, bio-thickening properties as well as immunomodulatory potentials. Due to the increase in the demand for EPS, there is a need to study the production of extracellular polysaccharides produced by Lactic Acid Bacteria (LAB) under different production conditions for optimum EPS production. **Materials and Methods:** Five EPS producing LAB isolated from fermented dairy products were collected from the culture collection Centre, maintained in De Ma, Rogosa and Sharpe (MRS) broth and the EPS producing LAB were molecularly characterized. Modified Exopolysaccharide Selection Medium (mESM) was used to produce the EPS. **Results:** The EPS producers were molecularly characterized as *Lactobacillus tucetti* FASHADFF2, *Lactobacillus delbrueckii* FASHADYG2, *Weissella* sp. FASHADFF1, *Weissella* sp. FASHADWR2 and *Leuconostoc mesenteroides* FASHADWR1. The EPS produced by the five LAB strains ranged from 109.11-185.02 mg $L^{-1}$. About 35°C and pH 5 supported the highest EPS production by the isolates. Glucose, sucrose, yeast extract, alanine and folic acid supported the highest EPS production. **Conclusion:** This study demonstrated that all the five LAB strains were EPS producers and they were 99-100% molecularly related to the identified species of LAB. Optimizing the production of EPS by the LAB strains yields a higher amount of EPS.

Key words: Exopolysaccharide, lactic acid bacteria, production, optimization condition, molecular characterization, glucose, isolates

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

An increase in the demand for Bacteria Exopolysaccharide (EPS) produced by Lactic Acid Bacteria (LAB) has been greatly researched[1,2]. Exopolysaccharides (EPS) are long-chain polysaccharides with high molecular weight secreted by microbial cells consisting of non-carbohydrate substitutes such as acetate, pyruvates and phosphate[3,4]. Microbial EPS is mainly in two distinct forms depending on their locations. They could be capsular EPS which are cell-bound EPSs that closely adhere to the bacterial surface (cEPSs) or Free EPS which are EPSs that are released into the surrounding medium (fEPSs). However, the production of EPSs by LAB is often distinguished as ropy or non-ropy EPS. The ropy EPS is secreted into the surrounding medium while the non-ropy EPS remains or adhered to the cell surface creating a discrete covering[5]. Due to the costs, fluctuating prices of plant and algal gums as well as the increased demand for polymers of natural origin used in different industries, it has encouraged manufacturers to look industrially into the making of gums which are polysaccharides derived from microbial origin[6]. Microbial polysaccharides may be natural or semisynthetic polymers with different structures and they are manufactured via sugar fermentation by some microorganisms like lactic acid bacteria. EPS is a water-soluble polymer, thereby majorly used as a thickening, gelling and suspending agent due to their ability to dissolve, disperse or swell in water are mainly[7,8]. LAB involved in food fermentation is considered as "generally recognized as safe" (GRAS) due to their widespread occurrence in food, ability to produce EPS in addition to their long-lived applications[9,10]. LAB enhanced shelf life, improved digestibility, pleasant sensory profile, improve the texture and flavor of food products. These properties are ensured through the production of certain useful metabolites which include organic acids, ethanol, aroma compounds bacteriocins, enzymes and exopolysaccharides[11]. *Streptococcus, Lactococcus, Pediococcus, Lactobacillus, Leuconostoc* and *Weissella* species are LAB strains that frequently produced EPSs[12,13]. Microbial EPS is known for its antioxidant potential with varied activity. The EPS produced by *Weissella confusa* also have immunomodulatory potential[2]. Optimization conditions such as the composition of the culture medium (carbon, nitrogen and cation sources), incubation time, pH, temperature, oxygen concentration, agitation made EPS released by LAB have a great effect in microbial polysaccharide production[14,15] thereby enhancing taste perception[16,17]. Optimization of the growth environment is a critical point for the largest EPS yield[18]. The amount as well as the EPS composition produced by LAB are strongly influenced by culture medium and fermentation conditions[19,20]. An additional physiological benefit of EPS is that it enhances the colonization of probiotic bacteria because it can be retained in the gastrointestinal tract for a long period. The EPS released by LAB is being used in the food industries because of its emulsifying and bio-thickening potential. In recent times, it was discovered that there is a need to develop healthy foods and this development has posed lots of challenges in the food industry which resulted in a lot of research into the production of EPSs by LAB. The research work is therefore aimed at the production of EPS from LAB, molecularly characterize and determine the effect of physicochemical parameters on EPS produced by LAB strains.

## MATERIALS AND METHODS

**Study area:** This study was carried out at the Department of Microbiology, Physiology Laboratory and the Research Central Laboratory, University of Ibadan, Oyo State from June, 2017-January, 2018.

**Collection of cultures:** Five EPS producing LAB isolated from fermented milk and yogurt were collected from the culture collection Centre of our previous work in the Microbial Physiology Unit, Department of the Microbiology University of Ibadan, Ibadan, Nigeria[21]. The LAB was maintained in De Man, Rogosa and Sharpe (MRS) broth (Difco)[22] and stored in a refrigerator at 28°C for three days.

### Molecular characterization of EPS producing LAB strains

**Genomic DNA extraction:** In this study, an amplified segment of the 16S rDNA gene of each LAB isolate was sequenced and the sequences were compared to the strains in the National Centre for Biotechnology Information (NCBI) BLAST library. The five LAB isolates were subcultures on MRS agar and incubated at 30°C for 18 hrs. The DNA was extracted from the LAB isolates and purified using the DNA Extraction kit (Invisorb Spin) as instructed by the manufacturer[23]. The 18 hrs old LAB culture of each isolate were centrifuged at 10,000 $\times$g for 10 min at 4°C. The twice with Dnase-free deionized water and re-suspended in 100 µL Chelex. The suspension was incubated for 20 min at 56°C, vortexed, boiled and freeze cycle for 5 min. Centrifugation was done at 14,000$\times$g for 5 min at 4°C. Total 20 µL Proteinase K (20 mg mL$^{-1}$) (Thermo Scientific, USA) was inoculated into 80 µL of the supernatant and was incubated at 65°C for 60 min. The Cell debris was collected by centrifugation at 14,000$\times$g for 5 min at 4°C and the

supernatant was stored at -20°C. A spectrophotometer (Shimadzu UV-1800, Japan) was used to measure the concentrated and pure extracted DNA[24].

**Molecular identification of LAB by sequencing of 16S rRNA gene:** Fragments of the 16S rRNA gene of LAB isolates were amplified distinctly using PCR and was performed in a thermal cycler (Bio-Rad Laboratories Inc., Hercules, USA). Each PCR contained 10 μM of each 27 F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492 R (5' GGTTACCTTGTTACGACTT 3') universal primer (Lane, 1991), 1 μL of the extracted DNA, 2.5 mM of each dNTP, 1 μL taq™ DNA polymerase (Fermentas, St. Leon-Rot, Germany) with 5 μL PCR buffer. Cycling conditions were set at an initial denaturation at 95°C for 60 sec followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 50°C for 30 sec and primer elongation at 72°C for 60 sec and final elongation at 72°C for 5 min. Gel electrophoresis (3% Agarose gel, 1 μL loading dye with 5 μL PCR products and stained with ethidium bromide for gel documentation) was used to separate the amplicons of LABs. PCR yields were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced with universal primer 27 F. The nucleotide sequences were analyzed by BLAST algorithm packages (Biological sequence alignment editor for Win95/98/NT/XP/7).

**Production of EPS:** Modified Exopolysaccharide Selection Medium (mESM) was used to produce the EPS from the LAB[22]. Seed culture of the EPS producing LAB was prepared by transferring 0.5 mL of the stock frozen culture to 10 mL of MRS broth and incubated for 16 h at 30°C. The resulting culture was transferred (2% v/v) into Exopolysaccharide Selection Medium (mESM). The medium contains 5% skimmed milk (Oxoid), 0.35% yeast extract (Oxoid), 0.35% peptone (Difco) and 5% glucose (BDH) and incubated at 30°C for 16 hrs[25,26]. The fermentation medium was heated in a boiling water bath for 15 min. to inactivate enzymes and then cooled to room temperature. 250 μg of 80% (w/v) trichloroacetic acid was added to the medium and stored at 4°C to precipitate the protein. The cleared supernatant was obtained by centrifugation (Sigma 3-18 K centrifuge, Germany) at 15,000 g for 20 min at 4°C to remove cells and coagulated proteins. The EPS was precipitated from the supernatant with three volumes of old ethanol (4°C, 100%) followed by overnight incubation at -20°C. The resulting precipitate (EPS) was collected after centrifugation.

**Extraction and quantification of EPS:** The total sugar concentration was determined by using the phenol-sulfuric acid method. 0.1 mL of EPS samples were diluted in 2.0 mL of distilled water. 1.0 mL of 6% phenol and 5.0 mL of 95% (v/v) sulfuric acid was added to the solution. Absorbance was read at 490 nm. The concentration of EPS was determined in triplicate and 2.0 mL distilled water was used as blank. The EPS content of each sample was calculated by the standard curve. The glucose standard curve was prepared for the quantitative determination with some modifications[27]. The fermentation medium was centrifuged at 15,000 × g for 15 mins at 4°C. The EPS was precipitated at 4°C by the addition of 2 volumes of ethanol (100%). The resulting precipitate was collected after centrifugation (15,000 xg for 15 mins at 4°C). Total sugar was determined using the phenol-sulphuric acid method[27].

**Effect of physicochemical parameters on EPS production:** To increase the yield of EPS, the following media components and cultivation conditions were investigated[28]. The conditions include: cultivation temperature (15, 20, 25, 30, 35, 40 and 45°C), pH (3, 4, 5 and 6), carbon sources in the fermenting medium was replaced with glucose, sucrose, mannitol, lactose, maltose and fructose, nitrogen source was replaced with peptone, yeast extract, urea and sodium acetate, amino acid was substituted with alanine, lysine, tryptophan and valine while vitamins were replaced with riboflavin and folic acid. The supplemented medium was sterilized, inoculated and incubated for 24 hrs.

**Statistical analysis:** Results from the experimental design were statistically subjected to analysis of variance (ANOVA) using SPSS (version 11.0, Chicago, IL). Probability values ($p \leq 0.05$) was considered significant to indicate the difference.

## RESULTS AND DISCUSSION

**Molecular characterization by gene sequencing:** The results of the sequence similarity (%) by BLASTN in the GenBank of the NCBI are shown in Table 1. The 16S rRNA gene sequences obtained in this study exhibited 99-100% of sequences in NCBI. Genotypic identification of the LAB strains includes 2 strains of *Lactobacillus*, 2 strains of *Weissella* and one strain of *Leuconostoc. Lactobacillus tucetti* FASHADFF2 had 100% relatedness to *Lactobacillus tucceti*, *Lactobacillus delbrueckii* FASHADYG2, *Weissella* sp. FASHADFF1, *Weissella* sp. FASHADWR2 and *Leuconostoc mesenteroides* FASHADWR1 had 99 and 100% relatedness to *Lactobacillus delbrueckii, Weissella confusa*, *Weissella koreensis* and *Leuconotoc mesenteroides.* The molecular methods are important for bacterial identification. The molecular method is more reliable in identifying LAB by giving the precise and accurate strains of

Table 1: Comparative taxonomy identification of LAB isolated in the study by sequencing of 16S rRNA coding gene according to the database

| Isolates code | Phenotypic Identification | Genotypic identification | Similarities (%) | Accession number |
|---|---|---|---|---|
| WCFF1 | *Weissella confuse* | *Weissella confusa* JCM 1093 | 99 | *Weissella confusa* FASHADFF1 | MH790313 |
| LTFF2 | *Lactobacillus tucceti* | *Lactobacillus tucceti* strain CECT 5920 | 100 | *Lactobacillus tucceti* FASHADFF2 | MH790316 |
| LMWR1 | *Leuconostoc mesenteroides* | *Leuconostoc mesenteroides* srain ATCC8293 | 100 | *Leuconostoc mesenteroides* FASHADWR1 | MH790314 |
| WKWR2 | *Weissella koreensis* | *Weissella koreensis* strain S-5623 | 99 | *Weissella koreensis* FASHADWR2 | MH790312 |
| LDYG2 | *Lactobacillus delbrueckii* | *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 | 99 | *Lactobacillus delbrueckii* FASHADYG2 | MH790315 |

microorganisms. Molecular methods have gained acceptance for accuracy and reliability for microbial identification to validate phenotypically determined taxa, it was however observed that they do not always correlate with phenotypic characterization[29,30]. Polymerase Chain Reaction (PCR) based methods is essential for specific classification and detection of lactic acid bacteria strains[31]. Blast search analysis using the whole 16S rRNA sequence resulted in identification above 90% level, which is close to the considered cut-off value indicating species identity[32]. Gene sequencing, BLAST searching at NCBI and submission to GenBank identified the five EPS producing LAB isolates as *Weissella confusa* FASHADFF1, *Weissella koreensis* FASHADFF2, *Leuconostoc mesenteroides* FASHADWR1, *Lactobacillus tucceti* FASHADFF2 and *Lactobacillus delbrueckii* FASHADYG2.

**Phylogenetic trees:** The phylogenetic trees of the isolated LAB strains based on 16S rDNA sequences (Neighbor-Joining Analysis of CLC bio software) are shown in Fig. 1(a-e).

**Quantification of EPS produced by the LAB strains:** The EPS production by the LAB strain is shown in Fig. 2. There was a significant difference ($p \leq 0.05$) in EPS produced by all the LAB isolates. The EPS production of five (5) LAB strains (*L. tucceti* FASHADFF2, *L. delbrueckii* FASHADYG2, *Weissella koreensis* FASHADWR2, *Weissella confusa* FASHADFF1 and *L. mesenteroides* FASHADWR1) ranged from 109.11-185.02 mg $L^{-1}$. *L. delbrueckii* FASHADYG2 had the highest amount of EPS (185.02 mg $L^{-1}$) production while the least EPS production was observed in *Leuconostoc mesenteroides* (109.11 mg $L^{-1}$). This result is in support of the work of Mostefaoui *et al.*[33], who observed that *Lactobacillus* strain had the highest EPS yield ranging from 60-740 mg $L^{-1}$. Laws and Marshall[3] obtained an EPS yield of 175 mg $L^{-1}$ produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* LY03. However, Mozzi *et al.*[12] reported that *Lactobacillus sakei* 0-1 produced an EPS yield of 1375 mg $L^{-1}$.

**Physicochemical parameters:** The effect of temperature on EPS production by the selected LAB isolates is shown in Fig. 3. There was a significant increase in EPS production as the temperature increases to 35°C and then gradual reduction began to occur in the EPS yield. *L. delbrueckki* FASHADYG1 and *W. confusa* FASHADFF1 produced the highest quantity of EPS (1841 and 1816 mg $L^{-1}$) at 35°C. *W. confusa* FASHADFF1, *Leuconostoc mesenteroides* FASHADWR1 and *W. koreensis* FASHADWR2 produced the least EPS (908, 807 and 741 mg $L^{-1}$) at 35°C. EPS yield reduced generally at 40°C. Temperature is a critical factor in the synthesis of

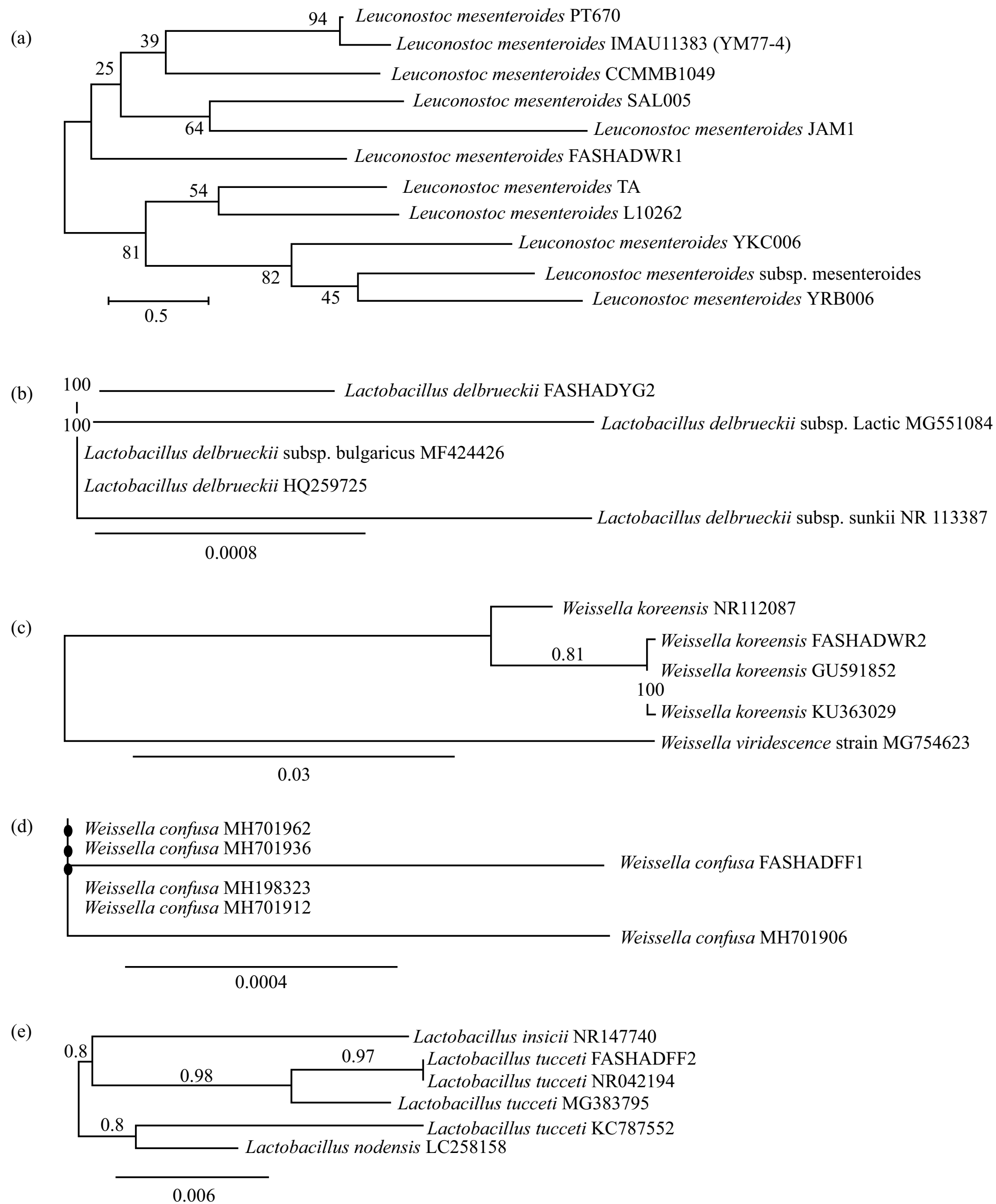


Fig. 1(a-e): Phylogenetic tree of different isolated LAB strains

(a) *Leuconostoc mesenteroides* FASHADWR2 (LMWR1), (b) *Lactobacillus delbrueckii* FASHADYG2 (LDYG2), (c) *Weissella koreensis* FASHADWR2 (WKWR2), (d) *Weissella confusa* FASHADFF1 (WCFF1) and (e) *Lactobacillus tucceti* FASHADFF2 (LTFF2)

polysaccharides. The highest EPS production was recorded at 35°C by *Lactobacillus delbrueckii* FASHADYG2. This could be because the LAB isolates in this research work are mesophiles. Joshi and Koiji[34] reported that the maximum temperature for EPS production was at 37°C. This correlates with the research of Gamar *et al.*[35] where the optimal temperature for its growth was in the range of 30-37°C. Prasanna *et al.*[36] also observed that the highest production of polysaccharides occurred between 25-35°C. However, studies have shown that species of thermophilic LAB such as *Streptococcus thermophilus* LY03 and *Lactobacillus delbrueckii* have a better EPS production at 42°C and above 45°C, respectively[37,3].

The effect of pH on EPS production by the LAB strains is shown in Fig. 4. *W. confusa* FASHADFF1, *W. koreensis* FASHADWR2 and *Lactobacillus delbrueckii* FASHADYG2 supported the highest EPS yield at pH 5. pH 6 also supported the best for EPS production (1369 mg $L^{-1}$) by *W. confusa* FASHADFF1. However, *Leuconostoc mesenteroides* FASHADWR1 produces the least EPS at pH 5 and pH 6. Generally, Acidic pH leads to high EPS production until pH 5

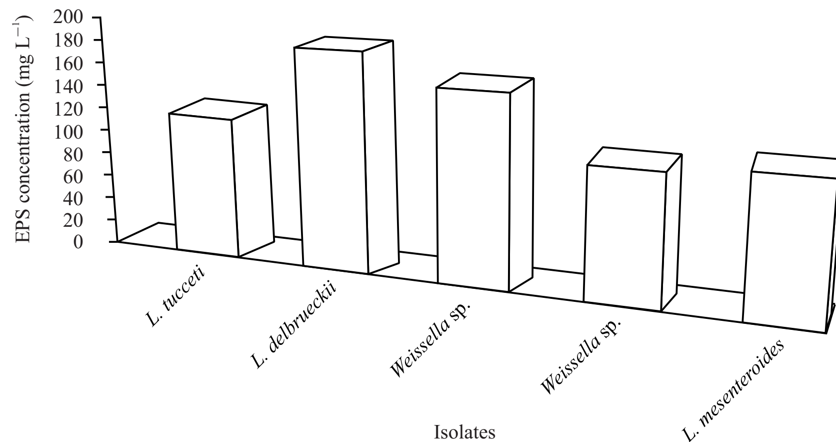


Fig. 2: Production of EPS by the LAB strains

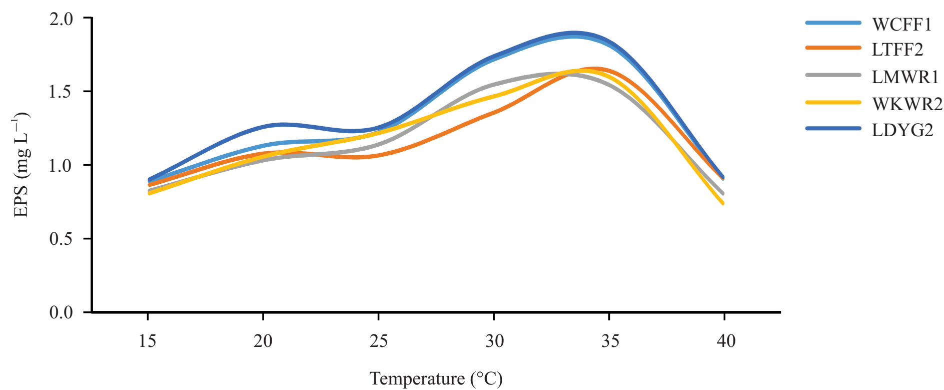


Fig. 3: Effect of temperature on EPS production by the selected LAB strains

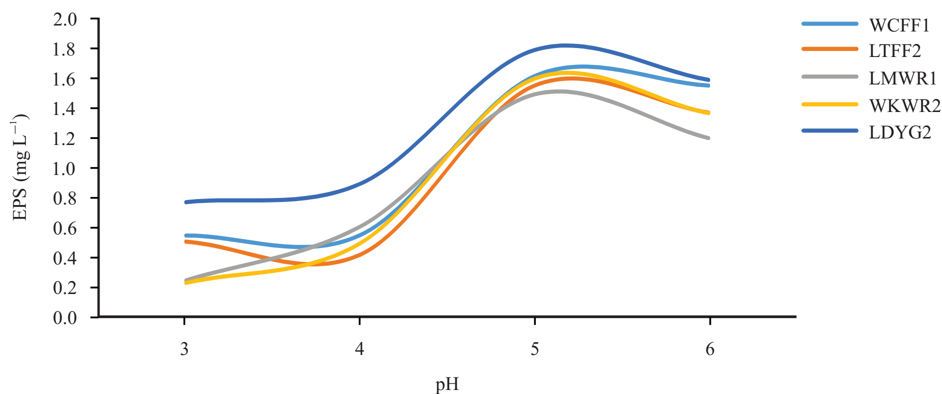


Fig. 4: Effect of pH on EPS production by the selected LAB strains

where there was a reduction. Reduction in pH during fermentation occurs due to the fermentative transformation of carbohydrates to lactic acid and acetic acid by the isolates. The ability of LAB to lower the pH of fermented food leads to a hindrance of food spoilage which brings about an increase in its shelf life. By lowering the pH and acid production (acetic, lactic and carbonic), LAB adds to the preservation of food by releasing a vast array of antimicrobial compounds and proteins[38]. Production of lactic acid by lactic bacteria strains reduces the pH and increases its acidity[39].

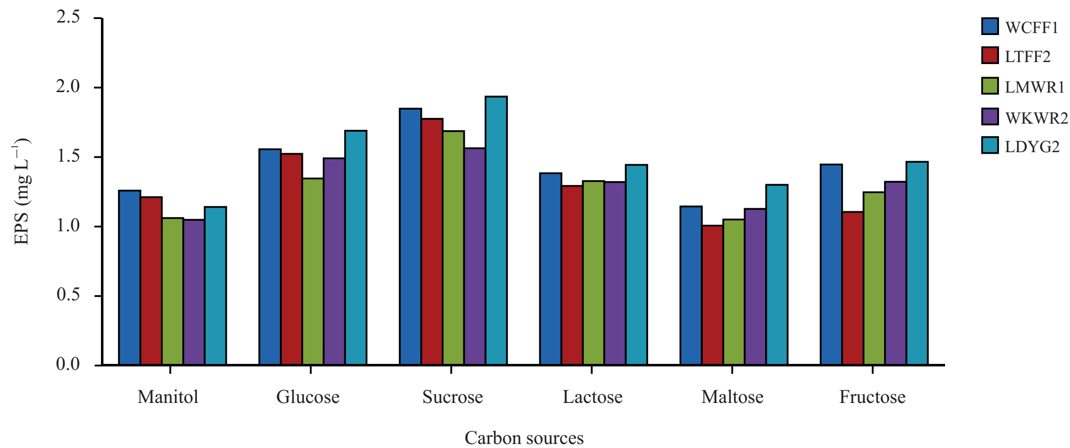


Fig. 5: Effect of Carbon sources on EPS production by the selected LAB strains

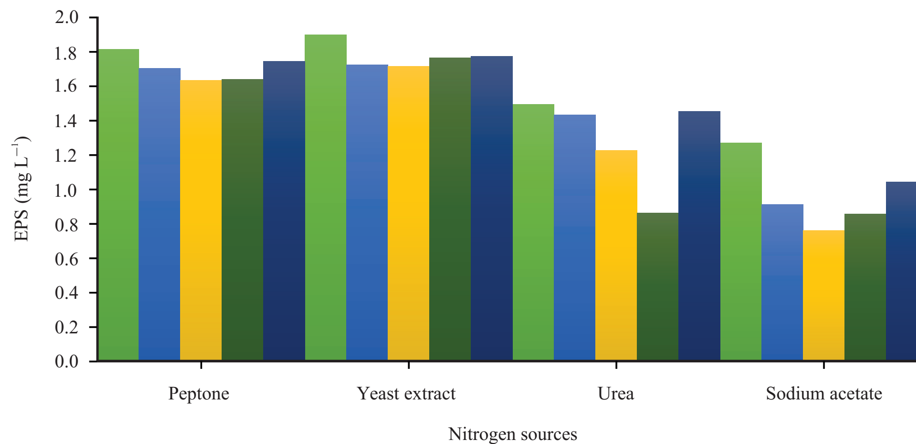


Fig. 6: Effect of Nitrogen sources on EPS production by the selected LAB strains

The effect of different carbon sources on EPS production by the selected LAB strains is shown in Fig. 5. Generally, *W. confusa* FASHADFF1 and *L. delbrueckii* FASHADYG2 utilizes glucose and sucrose (1684 and 1774 mg $L^{-1}$) followed by lactose and fructose for high EPS yield. The least EPS production was observed when mannitol and maltose were used as carbon sources. Generally, *Leu. mesenteroides* FASHADWR1 (1004 mg $L^{-1}$) and *Weissella koreensis* FASHADFF2 (1047 mg $L^{-1}$) produced the least quantity of EPS. The highest EPS yield utilizing sucrose as a carbon source could be because sucrose is a disaccharide and it is broken down to glucose and fructose. LAB is also able to efficiently utilize sucrose and glucose than other sugars as an energy source or precursor for the EPS synthesis of this strain[40]. Carbon is required for growth and EPS production by bacteria. In this study, it was found that all the isolates metabolized all tested sugars. Aslim[41] and Lane[42] reported that LAB strains can utilize carbohydrate sugars, such as glucose, lactose, sucrose and mannose as the carbon source for EPS production. Zotta *et al.*[43] reported that sucrose has to be one of the most suitable carbon sources for EPS production by LAB including *Weissella* sp.

The effect of nitrogen sources on EPS production by the LAB is shown in Fig. 6. *W. confusa* FASHADFF1, *L. tucetti* FASHADFF2, *Leu. mesenteroides* FASHADWR1, *W. koreensis* FASHADWR2 and *L. delbrueckii* FASHADYG2 had the highest EPS production (1897, 1725, 1765, 1717 and 1774 mg $L^{-1}$) when yeast extract was used as a nitrogen source. The least EPS (856, 912, 758, 127 and 1043 mg $L^{-1}$) was produced when Sodium acetate was used. Generally, *L. delbrueckii* FASHADYG2 and *W. confusa* FASHADFF1 produced the highest amount of EPS by utilizing all the nitrogen sources used for this research work. Yeast extract, however, supported the highest production of EPS which is in line with the work of Razack *et al.*[44] in which yeast extract gave the highest EPS production by *Bacillus*. Himanshu *et al.*[45] reported

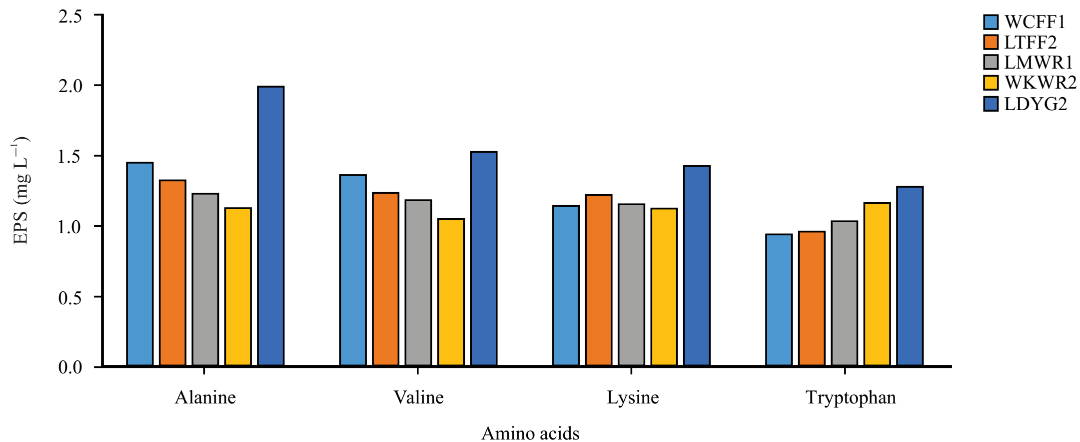


Fig. 7: Effect of amino acid on EPS production by the selected LAB strains

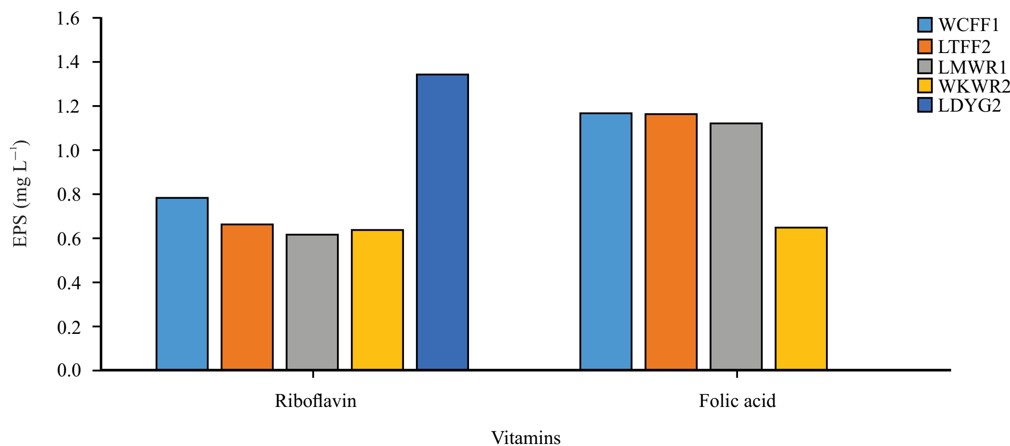


Fig. 8: Effect of vitamins on EPS production by the selected LAB strains

that ratios of carbon and nitrogen sources play the most important role in cellular growth and exo-biopolymer production.

The effect of different amino acids on EPS production by the LABs is shown in Fig. 7. *W. confusa* FASHADFF1, *L. tucetti* FASHADFF2 and *Leu. mesenteroides* FASHADWR1 had the highest EPS production (1416, 1292 and 1199 mg L$^{-1}$) when alanine was used as a source of amino acid with *L. delbrueckii* FASHADYG2 having the highest EPS production (1946 mg L$^{-1}$). *W. koreensis* FASHADWR2 had the highest EPS production (1133 mg L$^{-1}$) when tryptophan was used and the least EPS production (1023 mg L$^{-1}$) was observed when valine was utilized. The least production (1248 mg L$^{-1}$) was recorded when tryptophan was utilized. However, alanine supported the highest amount of EPS production while lysine had the least EPS production. This could be a result of alanine being an essential nutrient for the growth and production of EPS. Othman *et al.*[46] and Zhongchun *et al.*[47] reported that *Lactobacillus* sp. consumes more of alanine, then lysine, valine, tryptophan, aspartic acid, etc. Studies have shown that concentrations of most of the amino acids slightly increase due to fermentation. There is evidence that these amino acids have unique roles in human metabolism. In addition to providing substrates for protein synthesis, suppressing protein catabolism and serving as substrates for gluconeogenesis, they also trigger muscle protein synthesis and promote protein synthesis[48].

The effect of vitamins on EPS production by the LAB strains is shown in Fig. 8. Folic acid supported the highest EPS production by three LAB strains (*W. confusa* FASHADFF1, *L. tucetti* FASHADFF2, *Leu. mesenteroides* FASHADWR1). Riboflavin supported EPS production by two (*W. koreensis* FASHADWR2 and *L. delbrueckii* FASHADYG2). The ability of folic acid to support EPS production by *L. delbrueckii* FASHADYG2 (LDYG2) maybe because *Lactobacillus* spp can utilize folate. As suggested by Wouters *et al.*[49], the amount of

folate may be increased depending on the starter cultures. Among LAB, many *Lactobacillus* spp. and *Lactococci* spp. including *L. plantarum, L. bulgaricus, Lc. lactis, Streptococcus thermophilus* and *Enterococcus* spp. can utilize folate. On the other hand, some Lactobacilli (*L. gasseri, L. salivarius, L. acidophilus* and *L. johnsonii*) used as both starter cultures and probiotics, cannot utilize folate because they lack few specific genes involved in folate biosynthesis[50]. The ability of Riboflavin to support the production of EPS may because riboflavin is a component of flavin coenzymes which appears to be essential for the growth of lactic acid bacteria[51]. Although diverse LAB EPS have been studied, little information is available regarding their physicochemical parameters. Factors such as medium composition and fermentation conditions (pH and temperature) can affect EPS production. This study describes some molecularly characterized microbial EPS producers and fermentation parameters necessary to enhance EPS yield in the chemical, food and pharmaceutical industries. Exopolysaccharides produced by lactic acid bacteria are natural and nontoxic thereby highly recommended for use as a bio-thickener. However, the production of EPS from Modified Exopolysaccharide Selection Medium (mESM) is tedious, expensive and time-consuming. Although milk is relevant in most food industries, other mediums that are cheap, readily available with the ability to support the growth and EPS production in industries should be studied.

## CONCLUSION

The LAB isolated from fermented foods were molecularly identified as *Leuconostoc mesenteroides* FASHADWR1, *Weissella confusa* FASHADFF1, *Lactobacillus tucceti* FASHADFF2, *Weissella koreensis* FASHADWR2 *and Lactobacillus delbrueckii* FASHADYG2. This study revealed that the Physico-chemical parameters and their optimization greatly influence the production of EPS when compared with the number of EPS produced before optimization. From the findings, it may be suggested that sucrose, yeast extract, alanine and riboflavin are the best carbon, nitrogen, amino acid and vitamin sources for EPS production. The best pH was 5 while 35°C was the best temperature for maximum EPS production.

## SIGNIFICANCE STATEMENT

This study discovers the optimization of exopolysaccharides produced by some lactic acid bacteria that can be beneficial as a bio-thickener and viscosifier in most food, pharmaceutical and chemical industries. This study will help the researcher to uncover the critical areas of the optimum temperature, pH and the best carbon, nitrogen, amino acid and vitamin sources for higher EPS yield that many researchers were not able to explore. Thus, a new theory on exopolysaccharide production obtained from lactic acid bacteria using other sources for optimization may be arrived at.

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