

Optimization on production and technological properties of exopolysaccharide from *Pediococcus pentosaceus* SL4 isolated from sourdough Tomi-tomi (*Flacourtia Inermis* Roxb)

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Abstract

Sourdough is a fermented food made by a complex and diverse consortium of microorganisms, predominantly wild yeasts and lactic acid bacteria (LAB), positioning it as a valuable source for the isolation of specific LAB strains. LAB are capable of synthesising metabolites known as exopolysaccharides (EPS) during fermentation which enhanced the quality of food product. The characteristics of exopolysaccharides which are produced by LAB vary greatly depending on structure, molecular size, and physicochemical properties. This study aims to determine the optimal conditions for EPS production and characterize the exopolysaccharide from *Pediococcus pentosaceus* SL4 isolated from *Flacourtia inermis* sourdough. The investigation utilized a modified de Man Sharpe Rogosa medium and response surface methodology (RSM). The screened factors included monosaccharide types and concentrations, inoculum size, agitation speeds, and fermentation time. Using the Box Behnken design, the optimized conditions for EPS production were sucrose concentration of 5%, inoculum size of 6%, and agitation at 100 rpm, resulting in a maximum yield of 426.2 mg/L after 24 h incubation. The partially purified EPS had a molecular weight of 7.35×10^5 kDa with a degradation temperature of 91.8°C. The irregular, porous, and spongy morphology of EPS was observed by scanning electron microscopy. The EPS exhibited physiological properties such as water solubility index, holding capacity, and emulsifying activity. Overall, EPS exhibits technological properties as a stabilizer in the food industry.

1. Introduction

The demand for biomolecules that offer technological and health benefits has been increasing and must be addressed by exploring alternative biomolecule sources with specific properties. Exopolysaccharides (EPS) are a type of extracellular polysaccharide produced by bacteria that have wide applications in the food, cosmetic and pharmaceutical industries, functioning as a stabilizer, texturizer, emulsifier, and gelling agent (Wang et al. 2020; Balyan et al. 2024a). Some groups of lactic acid bacteria (LAB), such as *Lactobacillus*, *Enterococcus*, and *Pediococcus*, produce EPS through sucrose supplementation in the fermentation medium (Jayamanohar et al. 2018; Wang et al. 2019; Jiang et al. 2022) to protect themselves from environmental stresses such as insufficient nutrients, pH, salinity, temperature, or co-cultivation (Wang et al. 2017; Vinothini et al. 2019; Oleksy-Sobczak and Klewicka 2020; Jiang et al. 2020). Depending on the sugar composition, EPS can be either homopolysaccharides or heteropolysaccharides. The properties of EPS are associated with their characteristics, such as molecular weight, thermal properties, and sugar composition. EPS produced by LAB has the potential to play an important role because it has been classified as Generally Recognized as Safe (GRAS). The properties of EPS vary depending on the strain and fermentation conditions. Consequently, screening for LAB shows potential EPS production is necessary. Fermented foodstuffs like sourdough could be an ideal source for EPS-producing LAB. LABs exist spontaneously in sourdough due to the materials utilized or the environment where the sourdough is produced (Ripari et al. 2016; Yu et al. 2018). Some LABs, including *P. pentosaceus*, *L. plantarum*, and *L. brevis*, have been isolated from sourdough and produce EPS (Ripari et al., 2016; Fu et al., 2022). The presence of exopolysaccharides produced by LAB has been

shown to improve the rheological properties of sourdough (Zhang et al. 2023a) and enhance health benefits such as antioxidants (Wang et al. 2020), maintain blood sugar stability, and reduce cholesterol (Dilna et al. 2015). Other studies reported that the utilization of lactic acid bacteria, such as *Lactobacillus spp.*, *Pediococcus spp.*, and *Leuconostoc spp.*, in the production of EPS has been demonstrated to enhance the stability and quality of food products through its application as a stabilizer and a coating agent (Wang et al, 2019; Jiang et al. 2022; Balyan et al. 2024).

We developed the sourdough using some tropical fruits as a starter (Kemhay et al. 2024) and we also isolated *Pediococcus pentosaceus* SL4 were among the LAB species and had been screened for its potential EPS production (unpublished data). *P. pentosaceus* cannot produce large amounts of exopolysaccharides without optimal conditions, while under optimal conditions, LAB produces EPS that can be 2 to 5 times higher depending on the bacterial strain (Xing et al. 2018; Wang et al. 2019; Jiang et al. 2020). The optimization of carbon sources and fermentation process conditions has been identified as a strategy to enhance EPS production (Oleksy-Sobczak et al. 2020; Zanzan et al. 2023). However, it is crucial to note that each strain exhibits distinct optimal conditions for EPS production. The objectives of this study are to optimize EPS production of *Pediococcus pentosaceus* SL4 by adjusting carbon source, agitation speed, inoculum size, and fermentation time and subsequently derive a quadratic model to biosynthesize EPS and to characterize the EPS using various instrumental analyses and estimate its technological properties as a stabilizer in food processing.

2. Materials and Methods

Materials

The high-yield EPS-producing strain *P. pentosaceus* SL4 was isolated from *tomi-tomi* sourdough and was stored at -80°C in the Biotechnology Laboratory Faculty of Agricultural Technology Gadjah Mada University. de Man Rogosa Sharpe (MRS) broth (Himedia, India) supplemented with saccharides was used to cultivate the SL4 for EPS production. Glucose, fructose, galactose, and lactose as supplemented sugar were from Merck (Darmstadt, Germany) and sucrose from Phytotech (Kansas, USA). All chemicals used for analysis were analytic grade from Sigma Aldrich (Missouri, USA), and virgin red palm oil was from the local Indonesian market.

Preliminary screening of fermentation variables and optimization

Screening design is a methodical approach that facilitates the comprehension of the factors potentially implicated in the system. The bacteria were inoculated in MRS broth for 18 hours at 37°C for pre-culture. The first stage in screening was determining the right carbon source. Five types of carbon sources at 20 g/L were supplemented to MRS broth, culture at concentration 2% (v/v) was inoculated into sterile broth, and fermentation was carried out at 37°C with 80 rpm agitation for 48 hours. The best carbon source screening results were used for the next screening stage. In the second stage, the variables utilized were sucrose (25–125 g/L), inoculum size (2–10%), agitation speeds (0–200 rpm), and cultivation time (8–56 h) used to evaluate the maximum EPS production. The starter was inoculated at specific conditions

based on one factor at a time (OFAT) with four numerical factors. After identifying the factors affecting EPS production by OFAT design, a Box-Behnken design (BBD) was employed to assess the impact of three additional factors. The 2% culture (v/v) was inoculated to MRS broth (5% glucose) and incubated at 80 rpm at 37°C for 48 h, which was used as a non-optimized fermentation condition.

Extraction and purification of EPS

The extraction of exopolysaccharide (EPS) from *P. pentosaceus* was performed according to the method of Zanzan et al. (2023) with modifications. After incubation, the cultures were heated at 100°C for 10 minutes to inactivate EPS degrading enzymes, followed by centrifugation at 5000×g for 30 minutes. The pre-chilled 96% (v/v) ethanol was added to the resultant precipitate twice the initial volume. The mixture was then stored at 4°C for 24 hours, followed by precipitation using centrifugation at 5000×g for 30 minutes at a temperature of 4°C. Subsequently, 8% (w/v) trichloroacetic acid (TCA) was added to the precipitates and maintained at 4°C for 30 minutes to yield a protein precipitate. The protein precipitates were then removed by centrifugation at 5000 × g for 30 minutes. Subsequently, two volumes of pre-chilled 96% (v/v) ethanol were added to the filtrate and stored at 4°C for 24 hours, and the precipitate was subjected to centrifugation at 5000×g for 30 minutes at 4°C. The polysaccharide pellet was dissolved in 5 mL of deionized water and dialyzed using a 12–14 kDa cut-off dialysis membrane. The resulting EPS was then lyophilized, resulting in a powder form of the EPS suitable for further analysis.

Characterization of EPS

Molecular weight analysis

Size exclusion chromatography was used to determine the molecular weight distribution of EPS. Ten milligrams of EPS sample were dissolved in 1 mL of ddH₂O and filtered through a 0.22 µm filter. Then, the EPS solution (10 mg/mL, 20µL) was loaded into the gel permeation chromatography system, TOSOH HLC-8320GPC Japan, with TSKGel Super AW5000 as column and coupled to the Refractive Index detector. Isocratic elution was carried out using deionized water at a constant rate of 0.3 mL/min, and the detector temperature was set to 40°C.

Fourier transform infrared (FT-IR) analysis

Ten milligrams (10 mg) of the lyophilized EPS sample were ground with KBr powder. The spectrum was recorded using a Bruker vertex 70 DTGS FTIR spectrometer (Bruker, Germany) with a resolution of 4 cm⁻¹ in the 400–4000 cm⁻¹ range.

Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray analysis of EPS

The morphology of lyophilized EPS was observed under SEM JEOL JSM-IT200 (Tokyo, Japan). In brief, 5 mg of lyophilized EPS was placed on SEM stubs, tapped with double-sided, and coated with a carbon layer of approximately 10 nm thickness. The image has been taken at 10 kV at a resolution of 250×,

2000×, and 15000× magnification. The same samples for SEM were used for EDX analysis but with an acceleration voltage of 20 kV and at 250x magnification.

Thermal properties

Differential scanning calorimetry (NETZSCH DSC 300 Caliris Select, Germany) was used to investigate the thermal properties of EPS. The EPS sample (3–5 mg) was put in an alumina pan and sealed. The system was heated from 25°C to 400°C at a linear heating rate of 10°C/min in a nitrogen atmosphere (50 mL/min). The empty aluminium crucible was treated as a reference.

Water Holding Capacity

Water holding capacity (WHC) was determined by dissolving 10 mg of EPS in 1 mL of double distilled water and mixing in the vortex for 5 min. The sample was then centrifuged at 16000 rpm for 25 min. The pellet was collected and weighed (Marimuthu and Rajendran 2023). The WHC was determined using Eq. (1)

$$\% \text{ WHC} = \frac{\text{the dry pellet}}{\text{actual weight of EPS taken for the experiment}} \quad (1)$$

Solubility Index

The EPS sample (10 mg) was dissolved in 1 mL double distilled water under constant agitation for 24 h, then centrifuged at 6000 rpm for 20 min. A triple volume of chilled ethanol 96% was added to the supernatant to precipitate EPS. It was then centrifuged at 12000 rpm for 20 min. The pellet was dried at 55°C and weighed (Marimuthu and Rajendran 2023). The solubility was calculated using Eq. (2)

$$\% \text{ solubility} = \frac{\text{the weight of dry pellet obtained from the supernatant}}{\text{actual weight of EPS taken for the experiment}} \times 100 \quad (2)$$

Emulsification properties of EPS

The emulsion index was evaluated by suspended EPS at various concentrations (0.25–1.25 mg/mL) in double distilled water as described previously (Marimuthu and Rajendran 2023) with slight modification. A 2 mL of EPS solution was added to an equal volume of virgin red palm oil, and the mixture was vortexed vigorously for 5 min. The emulsion activity (%EA) was defined as the percent retention of the emulsion after incubation for one hour at room temperature, whereas emulsion stability (%ES) was determined after 24, 48, and 72 h separately. The %EA and %ES calculated according to Eq. (3)

$$\% \text{EA or \%ES} = \frac{\text{height of emulsion layer}}{\text{total height of mixture}} \times 100 \quad (3)$$

3. Results

Effect of media composition and culture conditions on EPS production

A comprehensive experimental approach was employed to ascertain the optimal carbon source for enhancing exopolysaccharide production. This involved utilizing five distinct carbon sources, each at a concentration of 20 g/L, within a customized MRS broth medium (Table 1). The experimental design comprised a one-factor-at-a-time (OFAT) experimental framework meticulously designed to investigate the impact of medium composition and prevailing culture conditions on the production of exopolysaccharides. No statistically significant differences ($p > 0.05$) in EPS yield or cell growth were observed when five carbon sources were prepared as supplements in fermentation media. However, sucrose exhibited the highest EPS production at 85 mg/L, 1.5-fold higher than MRS without saccharide supplementation. After determining sucrose as the optimal carbon source, different sucrose concentrations (25–125 g/L) were examined to ascertain the appropriate sucrose concentration for EPS production. The preliminary screening revealed optimal EPS production was achieved at 150.1 mg/L with 50 g/L sucrose supplementation.

[Table 1]

A culture was also cultivated on a water bath shaker to ascertain the impact of agitation on EPS production. The agitation speed of the water bath shaker was adjusted within the range of 0 to 150 rpm and the quantity of produced EPS was examined after 48 hours. The application of an agitation speed of 100 rpm was found to enhance EPS production by up to 2-fold compared to fermentation conducted without agitation. A statistically significant difference ($p < 0.05$) was observed between cultures cultivated with and without agitation (Figure 1). Agitation speeds exceeding 100 rpm during fermentation did not lead to a significant enhancement in EPS production.

A preliminary study was conducted to evaluate the effect of inoculum size on EPS production using inoculum concentrations of 2%, 4%, 6%, 8%, and 10%. Fermentation was carried out at an agitation speed of 100 rpm, and EPS was harvested after 48 hours. The results indicated that the highest EPS yield was obtained with an inoculum size of 6%, reaching 204.9 mg/L—approximately 1.2 times higher than that obtained with lower concentrations. Increasing the inoculum size beyond 6% did not result in a significant enhancement of EPS production.

[Figure 1]

Fermentation time is critical in producing exopolysaccharides (EPS) due to different microorganisms varying growth phase rates. In this screening stage, the determination of the optimal fermentation time was carried out by culturing with a 6% starter concentration, 50 g/L sucrose supplementation, and an agitation speed of 100 rpm for 8, 16, 24, 32, 40, 48, and 56 hours. The results showed that the highest EPS production was achieved at 24 hours of fermentation. Extending the fermentation time did not result in a further increase in the number of EPS produced.

Optimization and validation of EPS yield

Following the implementation of a screening process employing the OFAT method, sucrose (X_1), inoculum size (X_2), and agitation speed (X_3) were identified as the variables to be investigated for their capacity to enhance the production of EPS from *P. pentosaceus* SL4. The optimal levels of these variables were determined by utilizing a Box-Behnken design, a statistical design methodology within the response surface methodology (RSM) framework. This experimental design involved the execution of three replicates, encompassing twelve factorial points and five central points (Table 2). The measurement of the resultant EPS yield served as the response variable in this study. Therefore, a quadratic model was developed for EPS yield as represented below in terms of core values:

$$EPS (mg L^{-1}) = 418.2 - 17.94X_1 - 21.96X_2 - 8.32X_3 - 18.9X_1X_2 - 17.27X_1X_3 - 12.62X_2X_3 - 11.96X_1^2 - 28.46X_2^2 - 56.08X_3^2 \quad (4)$$

X_1 is sucrose concentration, X_2 is agitation speed, and X_3 is inoculum size.

[Table 2]

[Table 3]

The triplicate responses from each run were analysed using one-way ANOVA (Table 3). The quadratic polynomial model derived from Response Surface Methodology (RSM) effectively characterized the relationship between sucrose concentration (X_1), agitation speed (X_2), and inoculum size (X_3) on exopolysaccharide (EPS) production (Figure 2). ANOVA results confirmed the statistical significance of the model (F-value = 14.56, $p = 0.001$), validating the inclusion of linear, interaction, and quadratic terms. Regression coefficients revealed significant negative linear effects of X_1 , X_2 , and X_3 , as well as notable antagonistic interactions between the factors (X_1X_2 , X_1X_3 , and X_2X_3), suggesting synergistic modulation of EPS synthesis. The quadratic terms were also significant, with inoculum size (X_3^2) exhibiting the greatest curvature effect, thereby delineating an optimal response surface. Optimization analyses, supported by three-dimensional response surface plots, identified the optimal fermentation conditions as approximately 45–50 g/L sucrose, 90–100 rpm agitation, and 6% inoculum size, corresponding to maximal EPS yield. These findings highlight the critical influence of both individual and combined fermentation parameters and emphasize the necessity of multifactorial optimization in maximizing EPS biosynthesis efficiency.

The optimal value, predicted using the BBD experimental model, was selected for the verification test. The verification test involved four repeated experiments by setting sucrose, agitation speed, and inoculum size at predicted levels: 50 g/L, 100 rpm, and 6%, respectively. The results showed no

significant difference ($p>0.05$) between the experimental and predicted values, suggesting that the model can predict the EPS yield.

[Figure 2]

The molecular weight of EPS

The molecular weight of EPS was estimated by the polyethylene glycol (PEG) as standard, which has a known molecular weight. Gel permeation chromatography was used to fractionate the crude EPS into two components eluted by deionized water. The polysaccharide content was detected at 94.81% (EPS-1) and 5.19% (EPS-2), indicating that the production of EPS from *P. pentosaceus* SL4 after purification was approximately 405.79 mg/L (peak 1) and 22.21 mg/L (peak 2). The presence of sharp, single, and symmetrical peaks in gel permeation chromatography suggests that EPS is a homogeneous fraction. The molecular weight of the exopolysaccharide (EPS) was estimated to be 7.35×10^5 KDa for EPS-1 and 2,7 KDa for EPS-2.

Functional group analysis of EPS

FT-IR spectroscopy has been used to investigate the presence of functional groups qualitatively and the linkage among the monosaccharides of the EPS. The absorbance was recorded using FT-IR at a wavenumber from 4000 to 500 cm^{-1} . The IR spectrum of the EPS from *P. pentosaceus* SL4 revealed absorption characteristics for polysaccharides, which had many peaks from 3406 to 581.19 cm^{-1} . A broad peak around 3406 cm^{-1} corresponds to O–H stretching vibrations, which are typical of polysaccharides due to the abundance of hydroxyl groups in sugar monomers. The peaks at approximately 2920 and 2850 cm^{-1} are attributed to C–H stretching vibrations of aliphatic $-\text{CH}_2$ and $-\text{CH}_3$ groups, indicating the presence of alkyl chains. A distinct peak at 1653 cm^{-1} is attributed to the C=O stretch bond vibration. In addition, the weak absorption peak observed at 1542 cm^{-1} is related to the N–H. A weak absorption at 1417 cm^{-1} is related to the presence of C–H, while intense absorption peaks at around 1219 and 1053 cm^{-1} are assigned to carboxylic acid and ester groups C–O stretching vibrations. Several minor peaks associated with α - or β -glycosidic configurations and ring vibrations of polysaccharides were also observed in the absorbance range of 914–581 cm^{-1} . Overall, the spectral features confirm the presence of hydroxyl, carbonyl, and glycosidic functional groups, supporting the identification of the sample as a complex exopolysaccharide.

[Figure 3]

Microscopic manifestation of EPS

Scanning electron microscopy (SEM) is a technique for illustrating common physical properties, including microstructure, surface morphology, and texture of EPS. As illustrated in Figure 4, the SEM of

EPS produced by *P. pentosaceus* under optimal conditions (sucrose concentration of 50 g/L, agitation speed of 100 rpm, and inoculum size of 6%) with incubation at 37°C for 24 h displays a compact and porous structure with a flake-like morphology, as well as irregular, overlapping aggregates of varying size.

The Energy Dispersive X-ray (EDX) has become a staple quantitative elemental sample analysis method. The EDX analysis revealed that carbon and oxygen were the predominant elements, with a mass ratio of 48.33% and 42.72%, respectively, suggesting EPS is comprised mainly of carbohydrates. Heteroatoms such as phosphorus (P) and nitrogen (N) were detected at approximately 0.29% and 7.91%, respectively. In addition, the analysis revealed traces of sulphur (0.21%) in the EPS.

[Figure 4]

Thermal properties of EPS

The thermal properties of the EPS were found to be critically important for the intended application. Differential Scanning Calorimetry (DSC) was employed to analyse the thermal behaviour of the sample, including melting temperature, glass transition temperature, and crystallization characteristics. The thermal characterization of the EPS revealed the presence of both endothermic and exothermic transitions, observed within the temperature range of 30–400 °C for the EPS derived from *Pediococcus pentosaceus* SL-4. The DSC thermogram of the EPS exhibited a broad endothermic peak, beginning at 46.8 °C and ending at 127.8 °C, with a melting point peak observed at 91.7 °C. The enthalpy of fusion (ΔH) was calculated to be 232.2 J/g. A second endothermic peak was detected at 226.1 °C, with an associated enthalpy change of 29.21 J/g, corresponding to partial melting or the onset of thermal degradation. No detectable glass transition temperature was observed in the DSC thermogram of the EPS sample. Finally, a mild exothermic drift beyond 250 °C was observed, suggesting the initiation of degradation of the polymer backbone.

[Figure 5]

Technological Properties

Microbial polysaccharides have been identified as a promising renewable source for hydrocolloids in the food industry, owing to their distinctive functional characteristics, including high solubility and substantial water-retention capabilities, and act as stabilizer. In this study, the findings indicate that the water solubility index and water holding capacity of EPS were $21.53 \pm 0.58\%$ and $130.52 \pm 1.44\%$, respectively. Thereby to preventing disintegration into their native phase and thus enhancing the stability of food products, in this study the probable effect of EPS concentration on suspension stability against virgin red palm oil was investigated. Emulsification activity (EA) values were tested by adjusting EPS at several concentrations (0.2-1.0%). A statistically significant difference ($p < 0.05$) in the EA values was observed when the emulsion was prepared with concentrations ranging from 0.2 to 1% (Figure 6). Therefore, EPS at 0.6% is the optimal concentration for EPS when used as a stabilizer. No significant

difference in its emulsifying capacity is observed at higher concentrations. The stability of the emulsion was determined by allowing it to sit for a more extended period, up to 72 hours, after which the emulsion gradually decreased after 24 hours. A common challenge in applying emulsifiers is maintaining the emulsion formed under extreme conditions, such as high and low temperatures and varying pH levels. This study evaluated the emulsion-forming and stabilizing properties at varying temperatures (0, 30, 60, and 90°C) and pH levels (3-7). The emulsion of EPS with virgin red palm oil exhibited stability within the pH range of 4-7 and emulsification stabilizer declined at storage temperature above 30°C.

[Figure 6]

4. Discussion

Sourdough prepared using tomi-tomi fruit (*Flacourtia inermis*) starter is a source of various lactic acid bacteria species (Kemhay et al. 2024). *Pediococcus pentosaceus* SL4, one of the lactic acid bacteria successfully isolated, has the potential to produce a considerable number of exopolysaccharides. In the screening process using MRS Agar medium supplemented with 0.1% Congo Red indicator, *P. pentosaceus* SL4 demonstrated the ability to produce exopolysaccharides, as indicated by a color change of the MRS Agar medium to dark brown. Several studies have also reported similar findings, where *P. pentosaceus* was successfully isolated from sourdough made with grape, apple, and pomegranate starters (Gordún et al. 2015; Ripari et al. 2016). The exopolysaccharides produced by certain lactic acid bacteria during dough fermentation have also been reported to enhance the sensory characteristics of sourdough (Zhang et al. 2023b). Other research suggests that exopolysaccharides hold potential as functional food ingredients or additives to improve the physical and chemical properties of food products (Loeffler et al. 2020). Those potentials can be further explored by enhancing *P. pentosaceus* SL4 to produce EPS then characterize them.

Many factors contribute to the enhanced production of exopolysaccharides (EPS) by lactic acid bacteria. The presence of an excess carbon source in the form of saccharides in the fermentation medium can enhance the ability of lactic acid bacteria to produce exopolysaccharides. This phenomenon is associated with an increase in osmotic pressure due to the high sugar content. Several studies have shown that the maximum EPS yield (53.79 g/L) was attained with *Leuconostoc mesenteroides* DRP105, utilizing sucrose at 86.83 g/L as a carbon source (Xing et al. 2018). A parallel finding was reported for *Leuconostoc citreum* B-2, which yielded 59.33 g/L with sucrose supplementation at 99.8 g/L (Wang et al. 2020). *P. acidilactici* MT41-11 was reported to be able to produce exopolysaccharides of 500 mg/L when fermented MRS media by adding 5% sucrose (Bai et al. 2021). However, other studies have acknowledged that alternative carbon sources, such as maltose and lactose, can stimulate EPS production by *Enterococcus* and *Lactobacillus* (Imran et al. 2016; Zanzan et al. 2023). The variation in EPS production can be attributed to differences in bacterial strains and the type of carbon source utilized (Imran et al. 2016). Each strain has a different metabolic pathway, so it uses different carbon sources. High sucrose concentrations in media induced the β -fructofuranosidase enzyme production from

microorganisms to catalyse the conversion of sucrose into glucose and fructose, which can later be used as substrates (Sheng et al., 2016).

In addition to the critical role of carbon source selection and concentration, agitation as a parameter of the fermentation process has been identified as a factor that also significantly affects EPS production. Agitation also plays a role in maintaining the homogeneity of the medium throughout the fermentation process. The inadequate mixing of high-viscosity media gives rise to non-homogeneity. Nevertheless, elevated mixing speed engenders elevated shear stress in the culture medium, diminishing the EPS yield (Prasad and Purohit 2023). Fermentation of *L. rhamnosus* in a mixed medium of fructose, glucose, and saccharose at 20 g/L with an agitation speed of 80 rpm, which exhibited augmented EPS production in comparison to fermentation conducted without agitation (Oleksy-Sobczak et al., 2020). Furthermore, previous studies have also investigated the optimization of exopolysaccharide production through the regulation of inoculum concentration; however, inoculum concentrations exceeding 10% did not result in a significant improvement in EPS yield from an economic standpoint (Oleksy-Sobczak et al., 2020).

Beyond agitation and inoculum concentration, fermentation time emerges as a critical parameter affecting EPS yield. Precise determination of the optimal fermentation duration for each microorganism is vital, as mismatches between the growth and EPS production phases can compromise both efficiency and yield. Extended fermentation periods often result in decreased EPS production, largely due to enzymatic degradation by glycosyl hydrolases (Haj-Mustafa et al. 2015; Amiri et al. 2019), including rhamnosidase produced by species such as *Lactobacillus plantarum* (Ferreira-Lazarte et al. 2021) and *Lactobacillus acidophilus* (Mueller et al. 2018). This pattern aligns with observations from Haj-Mustafa et al. (2015), who noted peak EPS synthesis by *L. rhamnosus* 519 and *L. acidophilus* LA5 within the first 49 and 24 hours, respectively, followed by gradual declines. Similarly, *Enterococcus mundtii* A2 demonstrated maximum EPS yield at 16 hours, with reduced production at longer incubation times (Zanzan et al. 2023). In the RSM optimization, by adjusting sucrose concentration, agitation speed, and inoculum concentration to optimal conditions, the EPS yield reached 428.0 mg/L, which is 7.8-fold higher than the original yield (55 mg/L in MRS medium, Table 1). Moreover, this yield was also higher than those reported for *Enterococcus mundtii* A2 (382.27 mg/L) (Zanzan et al. 2023), *Lactobacillus plantarum* NTMI05 (197 mg/L), and *Lactobacillus plantarum* NTMI20 (187 mg/L) (Imran et al. 2016). However, some other species such as *Leuconostoc lactis* L2 (58.1 g/L) (Jiang et al. 2020), *Lactobacillus plantarum* KX041 (599.52 mg/L) (Wang et al. 2017), and *Enterococcus faecium* MC-5 (16.48 g/L) (Tilwani et al. 2021) have been reported to produce even higher amounts of EPS. In addition to strain differences, variations in fermentation optimization parameters can also influence the EPS yield obtained.

The molecular weight of the first fraction of EPS is higher than that of other EPS produced by coccus genera of lactic acid bacteria, which range from 2.5×10^1 KDa to 2×10^3 KDa (Jia et al. 2019; Bai et al. 2021; Zhou et al. 2021; Jiang et al. 2022). However, it is nearly equivalent to the molecular weight of dextran, which is produced by fructophilic lactic acid bacteria, *Apilactobacillus waqarii* strain HBW1 (Ahmad et al. 2022). These discrepancies in molecular weight may be attributable to inter-species and

inter-strain variations. The elevated molecular weight of EPS produced by lactic acid bacteria renders them a viable alternative to stabilizers in the food industry, biomedicine, and pharmaceuticals.

The FTIR spectrum of the EPS in this study aligns with previous findings. A broad band at 3590–3389 cm^{-1} corresponds to O–H stretching, indicating abundant hydroxyl groups typical of polysaccharides (Amiri et al. 2019; Zanzan et al. 2023). This region is also associated with water solubility (Vinothini et al. 2019). The peak at around 2932 cm^{-1} , attributed to C–H and C–O stretching, matches that reported in EPS from *L. plantarum* KX041 (Wang et al. 2017). A peak at 1633 cm^{-1} corresponds to C = O stretching, as also found in EPS from *L. acidophilus* LA5 (Imran et al. 2016). A weak absorption peak at 1542 cm^{-1} is related to N–H bending of amide II from proteins (Wang et al. 2017). Notably, absorption in this region was not observed in EPS from other *P. pentosaceus* strains (Jiang et al. 2022). The fingerprint region (800–1200 cm^{-1}) shows typical polysaccharide features, with 1200–1000 cm^{-1} indicating C–O and C–O–C glycosidic linkages (Amiri et al. 2019). A band at 813.44 cm^{-1} suggests α -glycosidic linkages (Zanzan et al. 2023). These features confirm that the EPS contains characteristic functional groups commonly found in microbial exopolysaccharides.

Microscopic analysis revealed that the morphology of exopolysaccharides (EPS) produced by different bacterial strains can vary significantly, even among those belonging to the cocci group. For example, levan produced by *Enterococcus faecium* MC-5 exhibits a uniform spherical morphology (Tilwani et al. 2021), whereas glucan synthesized by *Enterococcus hirae* KX577639 presents an irregular and interconnected structure (Jayamanohar et al. 2018). Such variations in EPS microstructure are primarily attributed to differences in the composition of monosaccharide units, chain length, and the types of glycosidic linkages forming the polymer backbone. Furthermore, EPS characterized by porous and spongy textures, as observed in this study, possess enhanced water-binding capabilities, which may contribute to their functional properties. This property that lends itself to applications in the food industry as a gelling, thickening, and stabilizing agent. This characteristic renders it a promising candidate for producing edible films and coatings for food products (Jiang et al. 2022). The heteroatom P and N in the partially purified EPS *P. pentosaceus* SL4 proposed a trace of phospholipids and protein, respectively (Zaghloul and Ibrahim 2022; Zanzan et al. 2023). Also, the analysis revealed traces of another element, including sulphur, which were close to the EPS produced by *Leu. mesenteroides* DRP105 (Xing et al. 2018) and *sakei* L3 (Wang et al. 2019). The elemental composition of the SL4-EPS was similar to the EPS produced by *Enterococcus* sp.BE11 (Zaghloul et al. 2023) and *Enterococcus mundtii* A2 (Zanzan et al. 2023).

The DSC analysis of the exopolysaccharide (EPS) sample demonstrated thermal behaviour consistent with previous studies on similar biopolymers. This analysis was employed to ascertain the correlation between physicochemical properties and temperature during application (Vinothini et al. 2019). The prominent endothermic peak, attributed to the loss of bound water, which is facilitated by the abundant carboxyl groups in EPS that bind with water molecules. As the temperature rises, the hydrogen bonds that bind the water molecules weaken, releasing them (Tilwani et al. 2021). This is in line with the study that has been conducted by Jiang et al. (2022), who observed dehydration peaks in EPS from *P.*

pentosaceus E-8 occurring at 82.6°C due to the hygroscopic nature of polysaccharides. The relatively high enthalpy value in first endothermic peak in this study suggests a significant water-binding capacity, which is critical for the material's physical properties and stability. The second endothermic phase occurred between 226 and 233.8°C, corresponding to the higher protein content in the EPS. Higher temperatures associated with higher energy requirements are necessary to remove proteins covalently bound to polysaccharides (Wang et al. 2017). Studies on microbial EPS have demonstrated thermal stability up to approximately 220–260°C before degradation initiates, consistent with the mild exothermic transition observed here beyond 250°C. For instance, EPSF2 from *Enterococcus* sp. F2 maintained structural integrity up to 266.6°C (Jiang et al. 2021) and EPSE8 from *P. pentosaceus* exhibited stability up to 257°C in TGA analysis (Jiang et al. 2022). The slight variation in peak temperature and enthalpy values may be attributed to differences in molecular weight distribution, degree of branching, and molecule structure (Lakra et al. 2020; Jiang et al. 2021).

Solubility, a pivotal metric in assessing the saturation concentration of EPS in an aqueous environment at a designated temperature, is a critical factor in determining the textural characteristics of food products (Jiang et al. 2022). Water-holding capacity is the ability to hold an immense amount of water (Vinothini et al. 2019). The water solubility of EPS in this study was nearly higher than that of EPS extracted from *Leu. lactis* KC117496 (14.2%), yet the water-holding capacity value obtained was lower (Saravanan and Shetty 2016). In contrast, D-glucan produced by *Enterococcus hirae* KX577639 exhibited a water solubility index of 46.5% and a water holding capacity of 202.04%, which is higher than the results obtained in this study (Jayamanohar et al. 2018). The molecular weight and percentage of branching appear to influence their water solubility (Jayamanohar et al. 2018). Furthermore, the high water-holding capacity of EPS can be attributed to its porous polymer structure, which enables water retention through hydrogen bonds (Saravanan and Shetty 2016). The elevated WHC of EPS indicates its capacity to retain substantial amounts of water, thereby mitigating the dehydration levels of the product. A previous study has demonstrated that incorporating 4% EPS into minced meat can stabilize the meat during cooking, thereby reducing losses (Wu et al. 2024). EPS's high solubility and water-retention capacity render it a suitable stabilizer and bio-thickener. Xanthan gum polymer has been reported to have a solubility index of 64.86% and a water holding capacity of 1000.33% (Sarkar et al. 2018), respectively, and has been recommended by the US as a stabilizer, thickener, binder, and texturizer. EPS from *P. pentosaceus* SL4 has been identified as a potential alternative renewable hydrocolloid despite xanthan gum's higher solubility and water binding.

This finding is consistent with the observations reported for EPS derived from *L. plantarum* ATCC 8014, which exhibited emulsification activity of approximately 60% at 72 hours (Balyan *et al.*, 2024). However, a separate study demonstrated that the emulsifying properties of a mixture of lactic acid bacteria found in yogurt could stabilize limonene emulsions by over 70% after 72 hours (Balyan et al. 2024b). Notably, the optimal emulsifying capacity of EPS produced by *P. pentosaceus* SL4 is about 74.75% when employed in products with a temperature of 4°C and a pH range of 4–7. Furthermore, a comprehensive study established that a reliable emulsifier must retain at least 50% of the original emulsion volume after 24 hours of its formation (Vinothini et al. 2019).

Declarations

Conflict of Interest

The authors declare no conflicts of interest related to the results of this study with other parties

Author Contribution

DAP: study design, data acquisition, data analysis and interpretation, drafting the manuscript; RY: material support, critical revision of the manuscript; CH: study design, study supervision, critical revision of the manuscript; DAS: study concept and design, study supervision, data interpretation, critical revision of the manuscript.

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Tables

Table 1 Effect of carbon source on cell growth and EPS produced by *P. pentosaceus* SL4

Carbon Source	Saccharide Cons (g/L)	Growth (OD 600 nm)	EPS yield (mg L ⁻¹)
MRS+Glucose	20	0.138 ^a	75.0±0.00 ^a
MRS+Sucrose	20	0.154 ^a	85.0±10.61 ^a
MRS+ Lactose	20	0.141 ^a	60.0±7.07 ^a
MRS+ Fructose	20	0.133 ^a	60.0±0.00 ^a
MRS+ Galactose	20	0.149 ^a	76.0±17.67 ^a
MRS	-	0.139 ^a	55.0±5.67 ^a

^a Values in the same column not followed by a common superscript letter are significantly different (p < 0.05)

Table 2 BBD matrix along with actual and predicted EPS yield (n=3)

Variables				Response	
				EPS (mg/L)	
Run	Sucrose (g/L)	Agitation (rpm)	Inoculum (%)	Actual	Predicted
1	50	150	4	344,9	332,6
2	50	50	4	346,2	351,2
3	50	50	8	347,5	359,8
4	75	100	8	315,1	306,5
5	25	150	6	388,8	392,6
6	75	150	6	305.3	318,9
7	50	100	6	463.3	440,2
8	75	100	4	359	357,7
9	25	50	6	412,3	398,7
10	50	100	6	396,5	418,1
11	25	100	4	350,3	359,1
12	50	150	8	295,7	290,7
13	25	100	8	375,7	377,0
14	75	50	6	404,4	400,6
15	50	100	6	426,2	418,1
16	50	100	6	428	418,1
17	50	100	6	419,8	418,1

Table 3. Analysis of variance for quadratic model of exopolysaccharide production

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	28832.90	9	3203.66	14.56	0.0010	significant
A-Sucrose_Conc	2574.03	1	2574.03	11.70	0.0111	
B-Agitation	3858.81	1	3858.81	17.53	0.0041	
C-Inoculum_Conc	554.44	1	554.44	2.52	0.1565	
AB	1428.84	1	1428.84	6.49	0.0382	
AC	1193.70	1	1193.70	5.42	0.0527	
BC	637.56	1	637.56	2.90	0.1325	
A ²	602.28	1	602.28	2.74	0.1420	
B ²	3410.41	1	3410.41	15.50	0.0056	
C ²	13244.33	1	13244.33	60.18	0.0001	
Residual	1540.49	7	220.07			
Lack of Fit	903.42	3	301.14	1.89	0.2723	not significant
Pure Error	637.07	4	159.27			
Cor Total	30373.39	16				

Fit Statistics			
Std. Dev.	14.83	R²	0.9493
Mean	372.71	Adjusted R²	0.8841
C.V. %	3.98	Predicted R²	0.4913
Adeq Precision	11.2024		

Figures

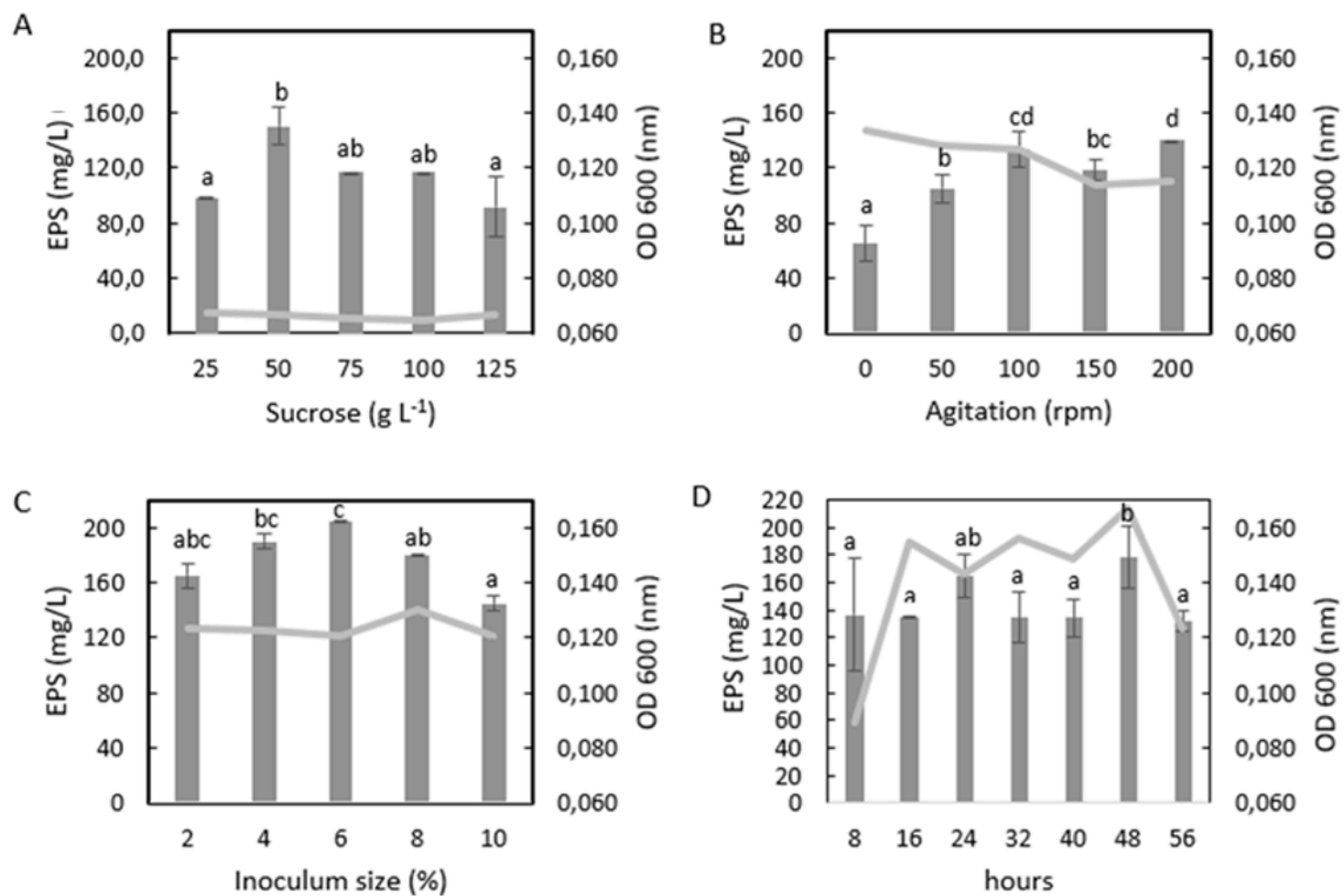


Figure 1

Effect of (A) sucrose, (B) agitation, (C) inoculum size, and (D) fermentation time on *P. pentosaceus* cell growth (OD 600 nm; line) and yield of EPS (bar)

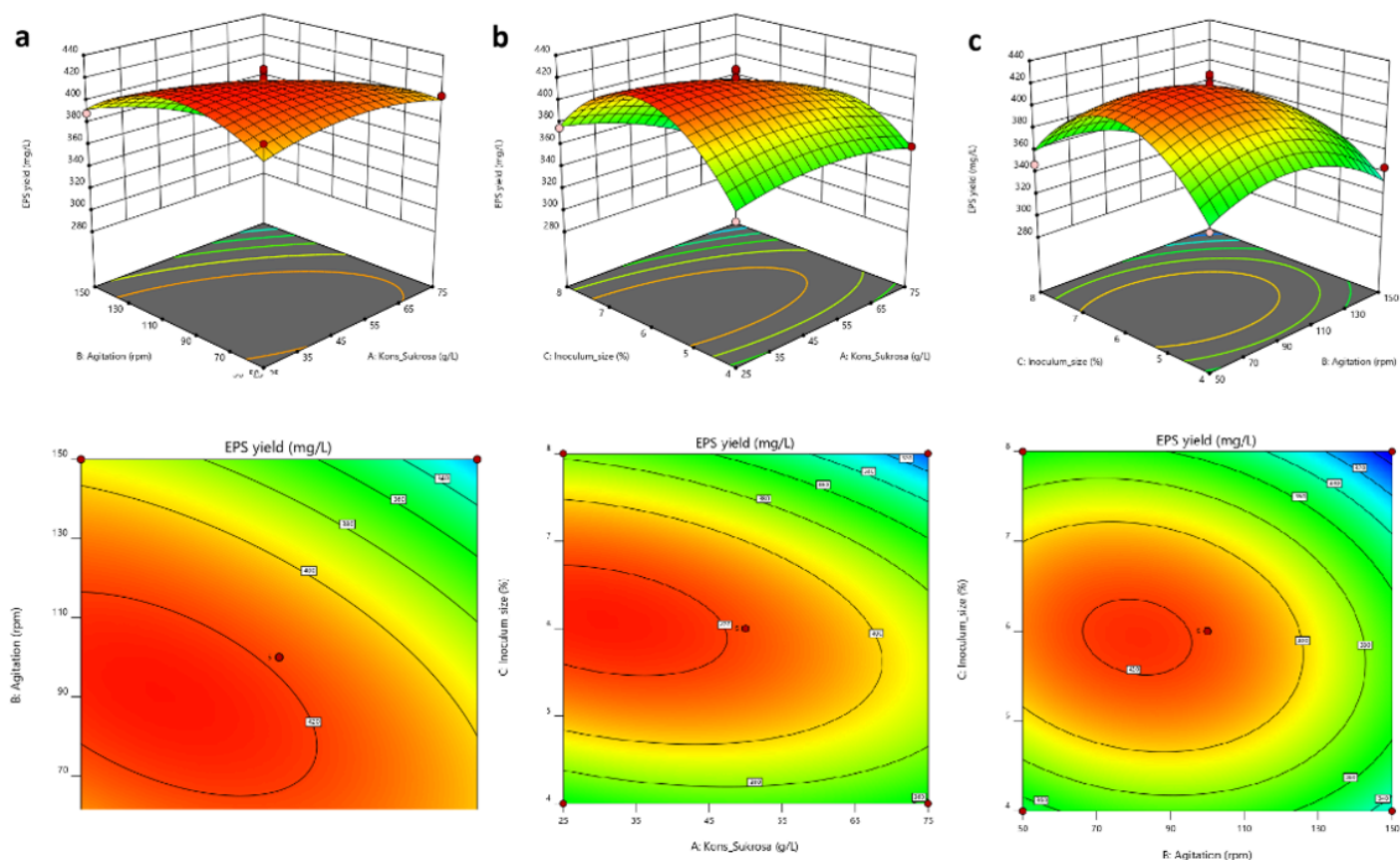


Figure 2

The surface plots of EPS production by *P. pentosaceus* vs. (a) agitation, sucrose (inoculum size and fermentation time: 6% and 24 h, respectively), (b) inoculum, sucrose (agitation and fermentation time: 100 and 24 h, respectively), (c) inoculum, agitation (sucrose concentration and fermentation time: 50 g/L and 24 h, respectively)

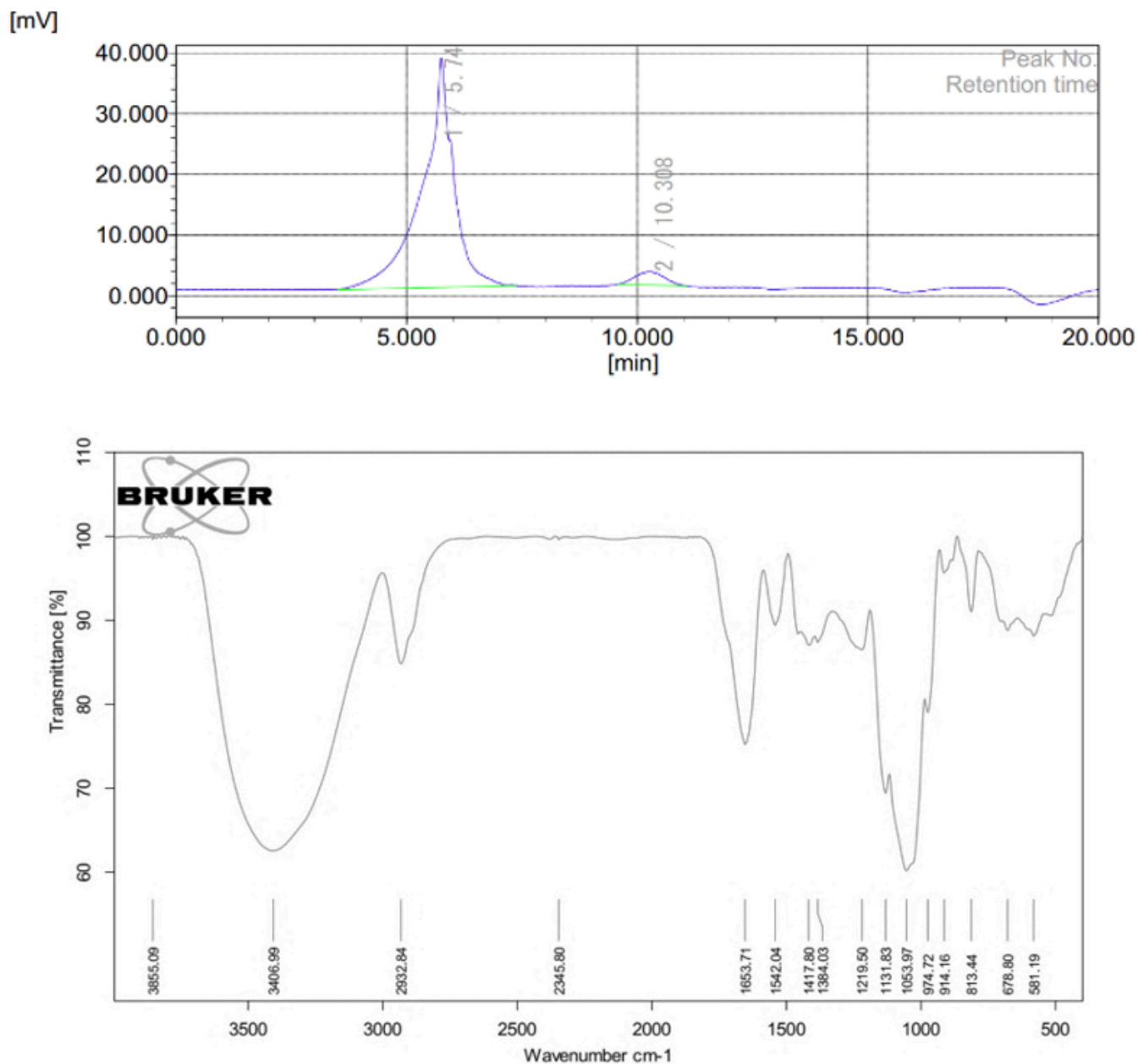


Figure 3

Elution profile of crude EPS on gel chromatography column at a flow rate of 0.3 mL/min and FT-IR spectrum of EPS produced by *P. pentosaceus* SL4

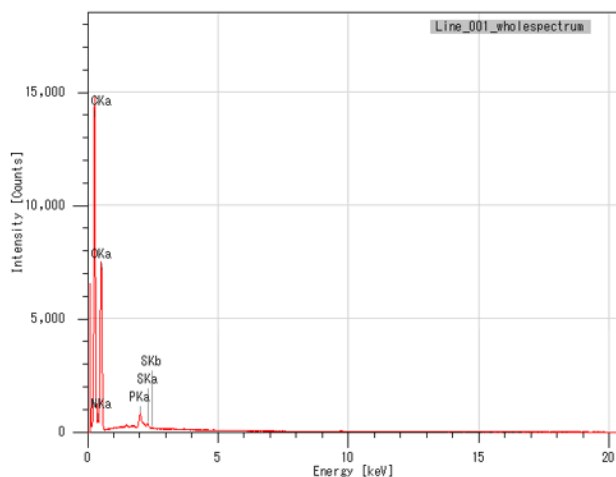
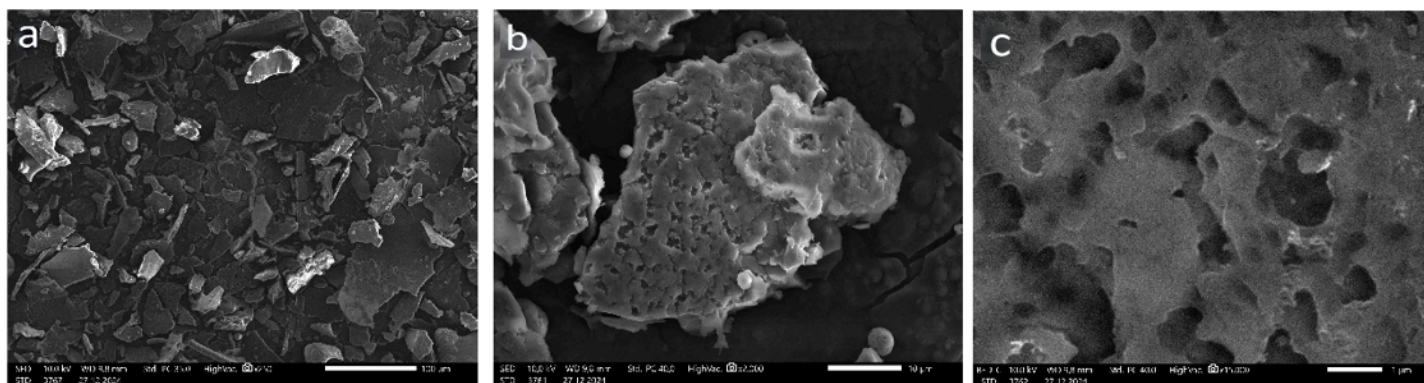


Figure 4

Scanning electron micrograph of EPS at different magnifications: a, 250 ×; b, 2000 ×; c, 15,000 × and the elemental composition of EPS produced by *P. pentosaceus* SL4

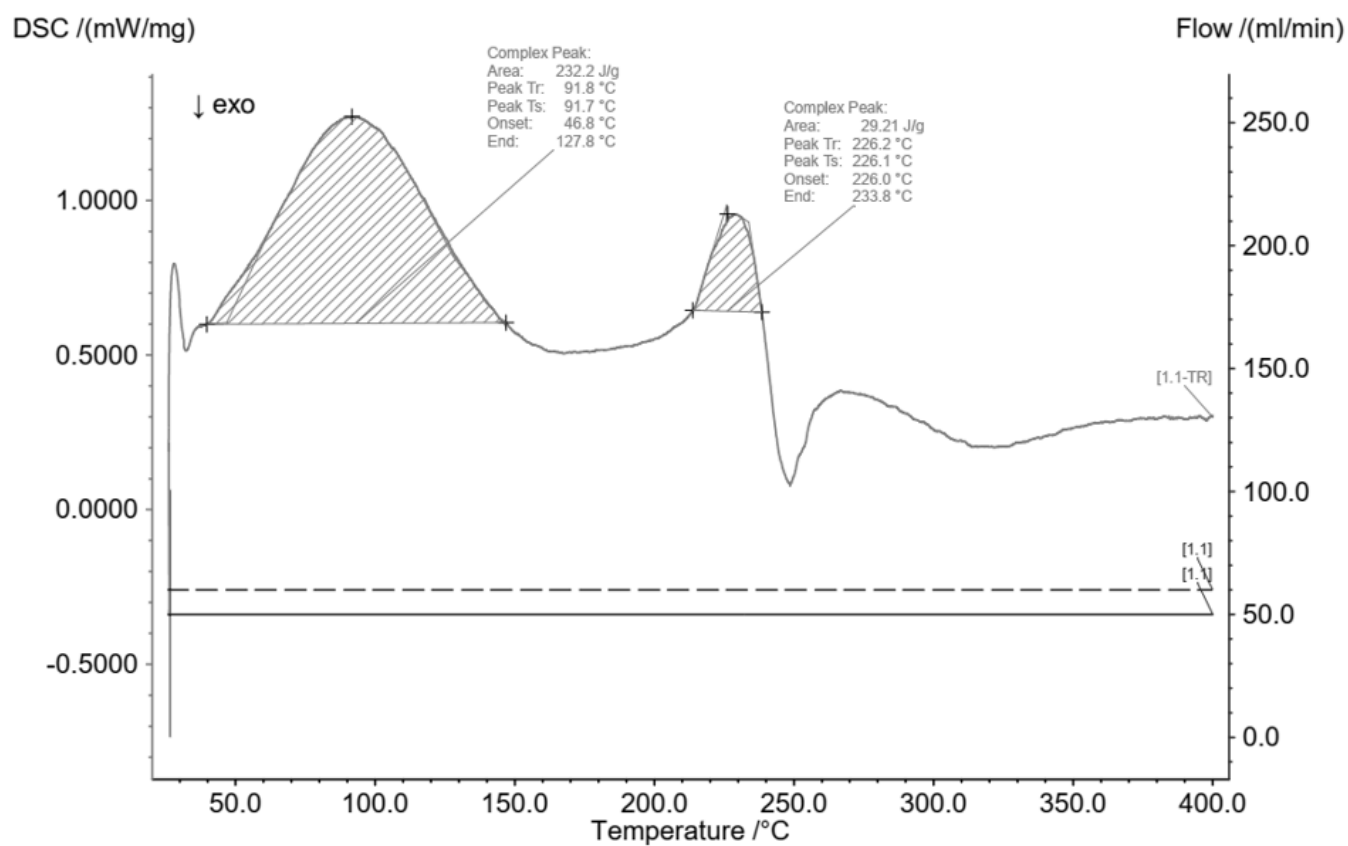


Figure 5

DSC thermogram EPS from *P. pentosaceus* SL4

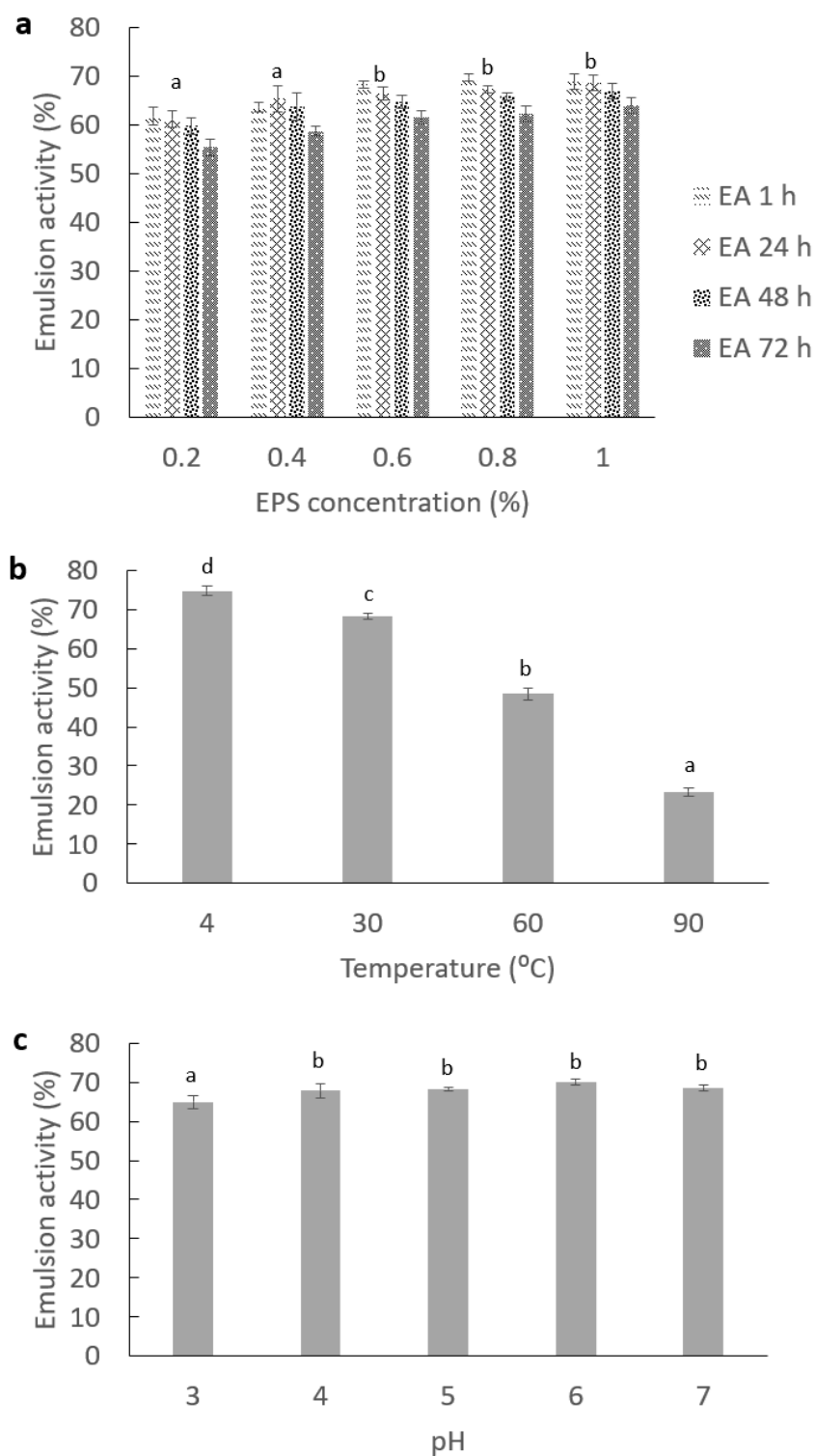


Figure 6

Emulsification activity of emulsion prepared with virgin red palm oil at a) different concentrations, influenced by b) temperature and c) pH. The statistic differences were indicated with different lowercase letters ($p < 0.05$)

Supplementary Files

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