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Exopolysaccharides production by *Lactobacillus fermentum* under different growth conditions in coconut water medium

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Abstract

Background: Exopolysaccharide (EPS) production has gained a lot of attention over recent decades because EPS can provide beneficial effects not only on industrial applications but also on the health sector. An understanding of the optimal condition for EPS production will increase the productivity of EPS and can develop EPS with desirable properties. The factors that affect EPS production are additional sugar concentration, temperature, fermentation time, and others. The current work aimed to optimize the utilization of a byproduct leftover from coconut water in EPS production from *Lactobacillus fermentum*. **Methods:** The EPS synthesis was analyzed under various growth conditions in coconut water, such as additional sucrose concentration and incubation times. EPS production of *Lactobacillus fermentum* was performed by adding 1%, 2%, and 3% of sucrose and 12, 24, 36, and 48 h incubation periods. The obtained data were analyzed statistically using a two-way ANOVA test. **Results:** The EPS production increased as the sucrose concentration and incubation time were increased. The optimal output was in the media supplemented with 3% sucrose and 48 h of incubation, giving 12.613 g/L of EPS production. **Conclusions:** The press of coconut water is suitable for EPS production by *Lactobacillus fermentum*. It produced more EPS than other conditions under 3% additional sucrose concentration and 48 h incubation time.

Keywords: Byproduct left over, coconut water, Exopolysaccharides, *Lactobacillus fermentum*, Optimization.

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Introduction

Exopolysaccharides (EPS) are polysaccharides produced extracellularly and present on the cell's surface (Nwodo et al., 2012). It is a kind of biopolymer that has various advantages. EPSs are beneficial in food industries, such as stabilizers, emulsifiers, thickeners, and plasma substitutes (Mekhici et al., 2017; Nwodo et al., 2012). EPSs have attracted much attention due to their various bioactivities and pharmacological activities (Abdalla et al., 2021; Yang et al., 2023). In regards to their primary structure, EPS varies in composition and sequences. However, their structure determines their bioactivity and physicochemical properties (Hu et al., 2022; Wei et al., 2023).

Microbial is a source to obtain EPSs. Microbial exopolysaccharide is an extracellular polysaccharide (EPS) produced by microorganisms, which usually function in cell adhesion and protection (Kumar & Dey, 2007). Among other organisms, EPS of Lactic Acid

Bacteria (LAB) gained much attention and has been widely explored for their potential effects on industrial properties (Pham et al., 2000). LABs are food-grade organisms, so they have been used extensively in the food industry (Pham et al., 2000). In addition to their function in the industrial sector, they also have pharmacological activities as antitumor, antimutagenicity, antibacterial, and immunomodulator (Abdalla et al., 2021; Doleyres et al., 2005; Kawanabe-Matsuda et al., 2022; Zhang et al., 2019). Because of their broad advantages, EPS by LAB is usually synthesized in large numbers.

The study to improve the synthesis of potential EPS to avoid low production yields has been widely conducted during the last decades. A study reported that EPS quantity is affected when it grows on the media supplemented with glucose or lactose (Pham et al., 2000). Effects of the incubation period, pH, and temperature variations influenced EPS production (Chug et al., 2021; Hereher et al., 2018; R. Zhang et al., 2023). The variations of media production are analyzed to examine EPS production, and it was reported that the highest purified EPS production was obtained when microorganisms were grown in MRSB and added to glucose (El-Waseif et al., 2013). Adding essential oils into the media increased the EPSs (Mekhici et al., 2017). Several reports stated that a byproduct of the food industry, coconut water, improved the production of microbial EPS of *Agrobacterium* sp. CFR-24 and *Bacillus velezensis* FCW2 MCC4686 significantly (Dhanya Raj et al., 2023; J. Zhang et al., 2019). Despite the previous studies, the exploration of EPS by LAB using coconut water is yet limited. In this present study, the optimization of EPS production by lactic acid bacteria, Lactobacillus fermentum, was conducted using a byproduct left over from the coconut industry. Various growth conditions for Lactobacillus fermentum to produce EPS were performed to reach the maximum yield of EPS, which were additional sucrose and variation of incubation times.

Methods

Material and Instruments

The materials used in this study were *Lactobacillus fermentum* culture, coconut water, NaOH, *de Mann Rogose Sharpe Agar* (MRSA), violet crystal, *de Man Rogosa and Sharpe Broth* (MRSB), ethanol 96%, and starter *Lactobacillus fermentum*. The instruments used in this study were Erlenmeyer, beaker glass, petri dish, autoclave, and Laminar Air Flow (LAF).

Coconut water medium

Coconut water with a volume of 150 mL was obtained from old coconuts and then filtered to separate the remaining residues. The pH was measured, and the ideal pH was around 6.5. If the pH was less than 6.5, a few drops of 0.1 N NaOH were added. If the pH exceeded 6.5, a few hydrochloric acid (HCl) drops were added. The coconut water was sterilized using an oven for 10 minutes at 90°C.

MRSA and MRSB medium

The weight of MRSA (7 g) and MRSB (6 g) were prepared to make media. Each MRSA and MRSB was then dissolved in 100 mL of distilled water. Then, all the presses were sterilized in an autoclave. The solutions were then cooled and solidified. This MRSA and MRSB medium were used to generate *L. fermentum* and create a starter culture, respectively.

Lactic acid strains

Lactobacillus fermentum was obtained from the Center for Good and Nutrition, University of Gadjah Mada (Yogyakarta, Indonesia). Stock cultures were stored at cold temperatures. *Lactobacillus fermentum* was prepared by putting the solution into the solid of MRSA. It was then incubated for about 24 h at 37°C.

Starter culture of Lactobacillus fermentum

A starter culture of *Lactobacillus fermentum* was prepared in the MRSB media with 30 mL. The starter culture was incubated for about 24 hours at 37°C.

Optimization of the production of exopolysaccharides

The fermentation method was used to synthesize EPS. Initially, 30 mL of coconut water was put into a 100 mL bottle and added with sucrose in 1%, 2%, and 3% until completely dissolved. Then, the medium was inoculated with 3 mL of *L. fermentum* starter (10% v/v) and incubated according to the independent variable incubation time (12, 24, 36, and 48 hours). Each of the above treatments was carried out three times.

Density calculation

The suspension of *L. fermentum* culture was measured in the optical density (OD) at 600 nm to identify the density of the microbial growth. The Colony Forming Unit per milliliter representing the cell numbers was analyzed. It was conducted by growing the culture on the solid MRSA. The colonies were then calculated using a Haemocytometer.

Isolation, purification, and quantification of exopolysaccharides

The yields of EPS were quantified as described in the previous study (Cerning et al., 1992). The culture was treated at 100°C for about 15 min to inactivate the enzyme. Centrifugation was performed at 15.000 g for 30 min to isolate and purify the EPS. The supernatant containing EPS was precipitated by adding chilled ethanol and centrifuged. The supernatant was then dissolved in the deionized water. It was then dialyzed to deionized water for 24 h and lyophilized. They were then dissolved with 10% trichloroacetic acid to remove the proteins. These preparations resulted in the purified EPS.

Statistical analysis

The works were repeated in triplicate. All the obtained data were analyzed using a two-way ANOVA test. The Least Significant Difference (LSD) test was also performed to investigate significant differences.

Result

To find the optimal output in the coconut water medium, the effect of additional sucrose and incubation times on EPS production by Lactobacillus fermentum was studied.

Growth of Lactobacillus fermentum

Lactobacillus fermentum was grown on the coconut water media supplemented by sucrose in various concentrations and incubation periods. During the incubation, pH was measured, as shown in Figure 1. The pH measurement can provide information about the environmental conditions of the microorganisms when they produce EPS. The physicochemical characteristics of EPS can be influenced by the pH of the environment (Nguyen et al., 2020). At 48 h, all variations of sucrose concentration had the same average pH of 3.7. The results showed that the longer the incubation time, thus the lower the pH.

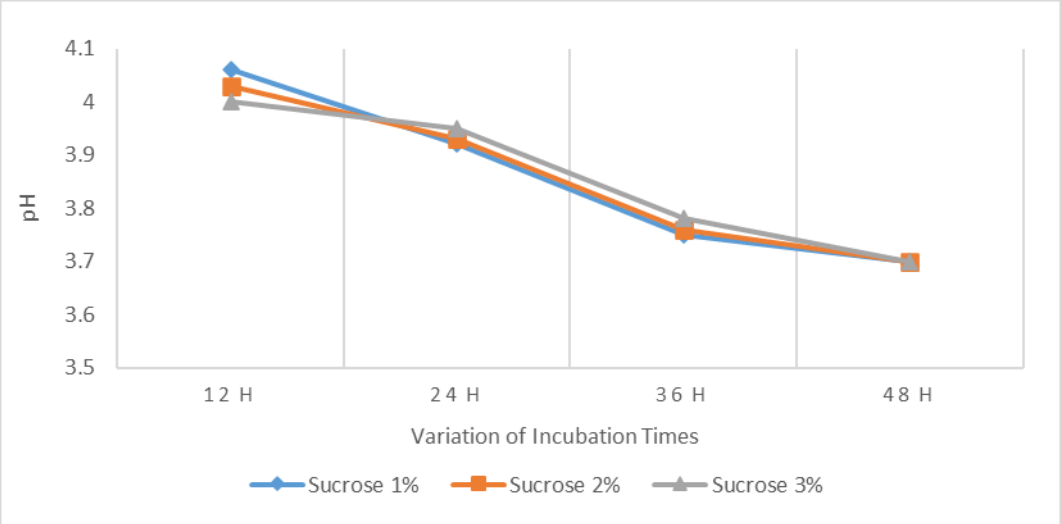

Figure 1. pH measurement of EPS production by *Lactobacillus fermentum*

The results showed a significant difference between the results of cell numbers calculation in the sucrose concentration and incubation time. In this study, 2% sucrose concentration and 36 h incubation gave the highest cell number, which was 67 $\times 10^5$ cells. In contrast, 3% sucrose gave lower cell numbers compared to 2% sucrose concentration, which was 65.17 $\times 10^5$ in 12 h of incubation. Meanwhile, the lowest cell density was found at a 1% sucrose concentration and 24 h of incubation times with an average of 39.5 $\times 10^5$ cells. In general, the longer the fermentation takes, the sugar concentration decreases. The sugar level decreases because bacteria need a substrate for growth, and sugar produces ethanol as a primary metabolite. Total bacterial cell density can vary due to high sucrose concentrations, which can cause a decrease in osmotic stress and ultimately affect cell density and biomass production. According to the previous finding, a high level of sucrose also causes an inhibitory effect on cell numbers (Manochai et al., 2014).

Sucrose concentration and incubation times to the EPS production

The suspension incubated for 12, 24, 36, and 48 hours was subjected to centrifugation to separate the supernatant and pellet. The supernatant was precipitated by adding 96% ethanol. The precipitation process was carried out for one night in the refrigerator. After forming a precipitate containing EPS, a drying process was carried out using an oven at 90°C to determine the dry weight.

Table 1. EPS production by *Lactobacillus fermentum* (g/L) under various conditions (addition of sucrose concentration and incubation times)

Sucrose concentration (b/v)	Incubation times			
	12 h	24 h	36 h	48 h
1 %	3,787	3,690	8,380	7,553
2 %	8,403	8,980	6,460	9,327
3 %	9,977	11,467	7,267	12,613

Our result showed that the highest yield of EPS was achieved in the 3% of sucrose and 48 h incubation. This was followed by 3% sucrose and 24h incubation. The lower yield of EPS was obtained in the 1% sucrose and 12h incubation, as shown in Table 1.

Discussion

In this study, *Lactobacillus fermentum* was used as one of the microorganisms from the Lactic Acid Bacteria (LAB) groups. The other LAB strains, such as *Leuconostoc, Streptococcus*, *Weissellale*, *Pediococcus*, and *Lactococcus,* are known to produce ESPs (Nguyen et al., 2020). This study chose coconut water as a medium because it is suitable for microorganisms' growth, such as LAB. Coconut water is a rich source of nutrients, electrolytes, and antioxidants (Widhorini et al., 2021). Despite its presence as a waste or byproduct left over in the food industry, it contains a certain number of calories, sugars, carbohydrates, and proteins needed for the growth of microorganisms (Shivakumar & Vijayendra, 2006; Widhorini et al., 2021). More details: It contains dextrose, fructose, sucrose, vitamin B complex, and phytohormone, all of which are beneficial for the growth of microbes (Andriani, 2020). It has been reported to be used for EPS production in *Agrobacterium sp*. CFR-24 and *Bacillus velezensis* FCW2 MCC4686 (Dhanya Raj et al., 2023; Shivakumar & Vijayendra, 2006). It can also be used as a substitute for *Dextrose Agar* (SDA) medium to grow *Aspergillus flavus* (Widhorini et al., 2021).

It is known that under environmental stresses, LABs react with different adaptation behaviors, which can influence their metabolite production, including EPS production. The ecological stress used in our study was the addition of sucrose and variation of incubation times, which were considered nutrient and physiological factors, respectively. The consumption of nutrients is one of the factors that influences the growth and metabolism of cells (Phillips et al., 2017). Previous studies reported that limiting or excessive nutrients changed EPS levels (Cirrincione et al., 2018; Mbye et al., 2020; Ninomiya et al., 2009).

Sucrose is one of the disaccharide sugar groups composed of glucose and fructose subunits. In this study, an additional 3% sucrose concentration increased EPS production compared to the lower concentrations of sucrose (1% and 2%). This result is consistent with a previous study that stated that an oversupply of sugar in the culture medium elevated EPS produced by LAB (Nguyen et al., 2020). The reasons for the higher EPS production under stress of high sugar concentration are osmosis, unlimited supply of sugar building blocks, and high energy availability (Cirrincione et al., 2018). A similar result has been reported for the higher EPS production in *Lactobacillus confusus* caused by high sucrose concentration in the media (Seesuriyachan et al., 2012). It has also been showed that the highest yield in EPSs from *Lactobacillus* strains (*L. delbrueckii bulgaricus, L. helveticus, and L. casei*) were in the medium containing 20% sucrose as a carbon source (Helal et al., 2015). The EPS synthesis in *Fructilactobacillus sanfranciscensis* LTH2590 was highly increased by about 40 g/L at a sucrose concentration of 160 g/L (Korakli et al., 2003). Like sucrose, media supplemented with higher glucose levels can also increase EPS yields in the *Streptococcus thermophilus* (W22) and *L. delbrueckii* subsp. *Bulgaricus* (Yuksekdag & Aslim, 2008). Other studies also showed that excess sugar in the medium improved EPS production in *L. casei* and *Lacticaseibacillus rhamnosus* (Cerning et al., 1994; Gamar et al., 1997).

Incubation times are considered one of the physical stress factors in the growth of microbes to produce EPS. Other physical factors that may affect microbial EPS production are variations in pH, osmotic stress, and temperature (Ananta et al., 2005; H.-T. Nguyen et al., 2016; Sanhueza et al., 2015; Seesuriyachan et al., 2012). However, in our study, there was a direct relationship between incubation times and EPS, in which EPS production increases when the incubation times increase—the 48 h incubation times produced a higher yield of EPS than the duration of 12 and 24 h. The result is consistent with the finding that the incubation time of 48 h was reported to be an optimal incubation period to produce a high number of EPS in indigenous lactic acid bacteria (Mıdık et al., 2020). Some studies revealed that the EPS production increased linearly to reach a maximum after 18 h to 96 h, then decreased after 96 h (Hereher et al., 2018; Mekhici et al., 2017).

We performed a two-way ANOVA test to compare the data on EPS production at various levels of sucrose and incubation. Since the results of the previous normality and homogeneity tests have met these requirements, thus the ANOVA test can be carried out. The two-way ANOVA test showed the significance value of sucrose concentration and incubation times were 0.000 and 0.000, respectively (p-value < 0.05), suggesting that sucrose concentration and incubation times affect EPS production. This study's interaction between sucrose concentration and incubation times affected EPS production. It was shown by the significance value of 0.002 (p < 0.05). However, the Least Significance Difference (LSD) test showed that all of the obtained data for sucrose concentration and incubation times gave a significance value of less than 0.05, suggesting that all received data were statistically different.

Generally, LAB responds to environmental stress by producing EPS, forming a protective barrier around the cells (Nguyen et al., 2020). The genes express the synthesis of EPS in the *EPS* cluster (Nguyen et al., 2020). The external stress can alter the expression of these genes in a way that increases EPS production. In other words, external conditions may control the biosynthesis of EPS in LAB. Since EPS produced by LAB can be promising for the industrial sector, understanding their basic need for growth is necessary. The optimization in their production can, of course, reduce costs; thus, it is beneficial to the industry. Regarding future work, exploring other pharmacological activities of EPS produced by *L. fermentum*, such as anti-inflammatory, antioxidant, anticancer, antitumor, etc, is fascinating. Understanding their role in their sequences and crystal structure of EPS produced by *L. fermentum* is also necessary to elucidate their structure-activity relationship.

Conclusions

Our findings suggest that increasing sucrose levels and incubation times could increase the EPS produced by *Lactobacillus fermentum*. In this study, the optimal conditions for L. fermentum to produce higher yields of EPS were the addition of 3% sucrose concentration and 48 hours of incubation times, which resulted in 12,613 g/L of EPS.

Declaration statement

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