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PRODUCTION AND CHARACTERIZATION OF LACTIC ACID BACTERIA BIOFILMS SYNTHESIZED USING TOFU WASTEWATER

PRODUKSI DAN KARAKTERISASI SINTESIS BIOFILM BAKTERI ASAM LAKTAT MENGGUNAKAN LIMBAH CAIR TAHU

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ABSTRAK

Biofilm berfungi untuk melindungi mikroba dari kondisi lingkungan. Biofilm yang dihasilkan bakteri asam laktat (LAB) juga mampu menghambat pertumbuhan patogen. Medium de Man Ragosa Sharpe (MRS) adalah medium spesifik untuk pertumbuhan LAB dan sekaligus pembentukan biofilm, namun pada skala industri tidak efektif dikarenakan harga yang mahal. Medium alternatif yang dapat digunakan adalah limbah cair tahu, kandungan nutrisi yang lengkap sehingga dapat digunakan sebagai medium pembentukan biofilm LAB. Penelitian ini bertujuan mengetahui formulasi C dan N pada limbah cair tahu terhadap produksi dan karakter biofilm 4 strain LAB, meliputi Enterococcus casseliflavus F4IS5, E. casseliflavus F14IS5, E. casseliflavus F14IS5 dan E. casseliflavus F14IS6. Glukosa dan amonium sulfat, masing masing ditambahkan dalam limbah cair tahu sebagai sumber karbon dan sumber nitrogen. Kemampuan pembentukan biofilm pada LAB diuji dengan metode biofilm assay. Karakter biofilm LAB diuji berdasar kemampuan daya lekat, konsentrasi eksopolisakarida sebagai penyusun biofilm yang dianalisis dengan metode berat kering. Karakter aktivitas penghambatan biofilm LAB terhadap pertumbuhan bakteri patogen Escherichia coli dan Staphylococcus aureus diuji dengan microplate method. Produksi biofilm LAB terbaik diperoleh pada isolat E. casseliflavus F6IS4 dalam medium limbah cair tahu dengan penambahan glukosa 2% dan amonium sulfat 1% dengan waktu inkubasi 48 jam. Biofilm yang dihasilkan termasuk dalam kategori kuat dan mempunyai daya lekat dengan kategori kuat, sel lepas yang teramati hanya sebesar 19.25 %. Selain itu produksi EPS yang dihasilkan strain tersebut sebesar 63.4 %. Biofilm E. casseliflavus F6IS4 dalam limbah cair tahu dengan penambahan 2 % glukosa dan 1 % amonium sulfat tersebut juga mempunyai aktivitas penghambatan hambat bakteri E. coli maupun S. aurues paling tinggi yaitu masing-masing sebesar 2.7 % dan 2.1 %.

Kata kunci: *Aktivitas pengambatan; daya lekat; E. casseliflavus; E. coli; S. aureus*

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ABSTRACT

Biofilms serve to protect microbes from environmental conditions. Biofilms produced by lactic acid bacteria (LAB) can even inhibit the growth of pathogens. Medium de Man Ragosa Sharpe (MRS) is a specific medium for LAB growth and biofilm formation; however, it is not effective on an industrial scale due to its high cost. Tofu wastewater serves as an alternative medium because it contains complete nutrients that support the formation of LAB biofilms. This study aimed to determine the effect of C and N formulation in tofu wastewater on the production and characterisation of biofilms produced by four Lactobacillus (LAB) strains, including Enterococcus casseliflavus F4IS5, E. casseliflavus F14IS5, and E. casseliflavus F14IS6. Glucose and ammonium sulfate were added to the tofu wastewater as carbon and nitrogen sources, respectively. The biofilm-forming ability of LAB was tested by the biofilm assay method. The LAB biofilm characteristics were tested based on adhesion, while the exopolysaccharide concentration, a component of the biofilm, was analysed using the dry weight method. The inhibitory activity of LAB biofilms against the growth of pathogenic bacteria, specifically Escherichia coli and Staphylococcus aureus, was tested using the microplate method. The highest LAB biofilm production was obtained from the E. casseliflavus F6IS4 isolate in a tofu wastewater medium supplemented with 2% glucose and 1% ammonium sulfate, with an incubation time of 48 hours. The biofilm produced was categorised as a strong biofilm, which also exhibited strong adhesion; the separate cells accounted for only 19.25%. Besides, the EPS production by the strain was 63.4%. The biofilm of E. casseliflavus F6IS4 in tofu wastewater, supplemented with 2% glucose and 1% ammonium sulfate, also exhibited the highest inhibitory activity against E. coli and S. aureus, at 2.7% and 2.1%, respectively.

Keywords: *Inhibitory activity; adhesion; E. casseliflavus; E. coli; exopolysaccharide; S. aureus*

INTRODUCTION

Biofilms function as a barrier that protects bacterial communities from environmental conditions. They consist of bacterial cells and Extracellular Polymeric Substances (EPS). EPS also can inhibit the formation of pathogenic bacteria (Galie et al., 2018). Lactic Acid Bacteria (LAB) have been known to have the potential to form biofilms. Several LAB genera, including *Lactobacillus, Lactococcus, Pediococcus, Enterococcus,* and *Streptococcus,* have been shown to produce biofilms. The biofilms produced by the five LAB genera can inhibit the growth of pathogenic bacteria by producing various antimicrobial compounds, including organic acids, bacteriocins, fatty acids, hydrogen peroxide, and diacetyl (Faten et al., 2016). Biofilm-forming lactic acid bacteria are a source of edible biofilms (Bastard et al., 2016; Liu et al., 2021).

Four strains of *E. casseliflavus* isolated from specifically *ubi karet busuk* showed β-considers activities ranging from 4.15 to 4.29 U/mL. The four LAB strains have the potential to be used in the fermentation of plant-based foods that contain cyanogen. This antinutritional compound can be removed by the strains during the fermentation process (Hutajulu et al., 2021).

Medium de Mann Rogose and Sharpe (MRS) is a specific medium for LAB growth; however, it is ineffective on an industrial scale due to its high cost. Tofu wastewater can serve as an alternative medium in this case. Tofu wastewater contains a complete nutritional profile, particularly with a protein content of 40-60%, making it an ideal source of nitrogen and minerals for the growth and development of LAB biofilms. Nevertheless, to serve as an alternative medium for LAB growth, tofu wastewater has a weakness, namely, its carbon content is <1%. To address such weakness, carbon can be added after considering the available nitrogen levels (Safitri et al., 2016). Based on the above-mentioned description, research on the utilization of tofu wastewater as an alternative medium for LAB biofilm production is needed

Method

Materials

This study used four LAB strains, namely *E. casseliflavus* F4IS5, *E. casseliflavus* F14IS5, *E. casseliflavus* F14IS5, and *E. casseliflavus* F14IS6. The four LAB strains were isolated from *ubi karet busuk* in Sumba, NTT (Hutajulu et al., 2021). The two pathogenic bacteria used in this study were *Escherichia coli* and *Staphylococcus aureus*. The tofu wastewater

was obtained from a tofu factory located in Janten, Kasihan, Bantul, Yogyakarta.

Formulation of Tofu Wastewater Medium

There were six variations of medium formulation used to treat the four *E. casseliflavus* strains, as shown in Table 1. The medium was formulated by adding glucose as a carbon source and ammonium sulfate as a nitrogen source (Safitri et al., 2016; Yeni et al., 2016).

Table 1.
Formulation of liquid tofu waste medium with the addition of carbon and nitrogen sources

Formulation type	Composition
A	Tofu wastewater, 1% glucose, 1% ammonium sulfate
B	Tofu wastewater, 2 % glucose, 1% ammonium sulfate
C	Tofu wastewater, 5 % glucose, 1% ammonium sulfate
D	Tofu wastewater, 10 % glucose, 1% ammonium sulfate
E	Tofu wastewater
F	de Man Ragosa Sharpe broth (MRS) medium

Source: Research documentation (2024)

Biofilm Production

Four *E. casseliflavus* strains were prepared, each of which was cultured into six variations of medium formulation. The culture was incubated for 24 hours at 37˚C until the number of bacterial cells reached 10^8 CFU/mL (Gomez et al., 2016). A total of 3 mL of each bacterial suspension was inoculated into six variations of medium formulation, using 30 mL of tofu wastewater and 30 g of sterilised zeolites. The zeolites were used as a material to which the bacterial cell biofilm was attached. The culture was incubated at 37˚C for 72 hours. Biofilm production was observed at hours 0, 24, 48, and 72 of incubation, based on Optical Density (OD) values at a wavelength of 600 nm.

The biofilm production by the strains was categorized according to the reference by Borges et al. (2012). The cultures were not categorized as biofilm-forming if the OD value ≤ ODC (optical density cut-off). The ODC value was determined from the OD value of the negative control (MRS broth medium without LAB isolates). The cultures were categorized as a poor biofilm-forming medium if the OD value was >ODC and the OD value was ≤ 2 x ODC. The cultures were categorized as moderate biofilm-producing medium if the OD value > 2 x ODC and OD ≤ 4 x ODC. The cultures were categorized as strong biofilm-forming medium if the OD value was greater than 4 times the ODC.

Analysis of Biofilm Adhesion

An analysis of biofilm adhesion of *the E. casseliflavus* strain was carried out following the method of Siradje et al. (2017). The biofilm products from 48-hour incubation were put into test tubes. Each of the test tubes was then vortexed at three different speeds: 600 rpm, 1200 rpm, and 1800 rpm, for 20 seconds. After vortexing, the turbidity was observed and measured using a spectrophotometer with a 600 nm wavelength. If the bacteria remain attached to form a biofilm and the absorbance decreases, the bacteria are categorized as having strong adhesion. On the other hand, if the bacteria are detached, as evident from the turbidity in the test tube and the increased absorbance value, they are classified as having poor adhesion.

Test of Exopolysaccharide Concentrations

The concentration of exopolysaccharide (EPS) was tested based on the biofilm product formed after 48-hour incubation. A total of 10 mL of the LAB culture biofilm from the six medium formulations were put into a 50 mL centrifuge tube. The biofilm was then centrifuged at 4˚C and 4000 rpm for 40 minutes. The supernatant was collected, and cold ethanol (96%) was added in a volume twice that of the supernatant. The supernatant was stored overnight, then centrifuged at 4˚C and

4000 rpm for 40 minutes. The pellet was dried at 50˚C for 24 hours. Finally, the dry weight, as well as the EPS concentration, was determined (Giyatno & Retnaningrum, 2020).

Test of Inhibitory Activity of LAB Biofilm against Pathogenic Bacteria

The inhibitory activity of the LAB biofilm against pathogenic bacteria was tested using a 96-well plate. This test was conducted using the method described by Gomez et al. (2016). A total of 50 µL was inoculated into a 96-well plate containing 50 µL of MRS broth medium. The culture was then incubated for 48 hours at 37˚C. Once the cell count had reached 10^7 CFU/mL (equivalent to McFarland 0.2) and the biofilm had formed, the plate was washed with NaCl to remove any LAB planktonic cells.

The pathogenic bacteria used for this test were *Escherichia coli* and *Staphylococcus aureus,* each of which was 50 µL with a cell count of 10^7CFU/mL (equivalent to Mc Farland 0.2). These bacteria were inserted into the plate and incubated for 72 hours at 37˚C. Every 24 hours, half of the medium in the plate was replaced with a new medium, and the growth of the test pathogenic bacteria was analyzed based on the OD value, which was measured using an ELISA reader at λ600 nm. A decrease in the growth of the test pathogenic bacteria during the 72-hour incubation indicated the inhibitory activity of the LAB biofilm. As the control, non-biofilm plates were prepared and inoculated with the test pathogenic bacteria. Meanwhile, the number of viable pathogenic bacteria cells was determined using the TPC method.

The results of the biofilm-forming ability, exopolysaccharide concentration, and inhibitory activity of LAB biofilm against the two test pathogenic bacteria were statistically analyzed using analysis of variance (ANOVA) at a 95% confidence level. If an effect is observed, the Duncan test is then conducted to determine the differences between each treatment (Kumar et al., 2017).

RESULTS AND DISCUSSION

Production of LAB Biofilm

The four *E. casseliflavus* strains exhibited the ability to produce biofilms, as shown in Table 2. The biofilm-forming ability was determined by referring to Borges et al. (2012). All the OD values of the cultures of the four strains were higher than the ODC (OD value of 0.15), indicating that the four strains possessed biofilm-forming ability. The biofilm-forming ability of the four *E. casseliflavus* strains on various medium formulations showed significant differences ($p < 0.05$).

The biofilm produced by the four strains of *E. casseliflavus* on tofu wastewater supplemented with glucose and ammonium sulfate, as well as in MRS, was categorized as strong. Meanwhile, the biofilm produced on the tofu wastewater medium, without the addition of glucose and ammonium sulfate as carbon and nitrogen sources, was in the moderate category. Variations likely influenced such differences in both LAB strains' ability to form biofilms and the composition of nutrients contained in the medium (Garcia-Gonzalo & Rafael, 2015; Olszewska et al., 2019; Pannella et al., 2020).

Table 2.
Biofilm production by four strains of *E. casseliflavus* during incubation time of 0, 24, 48 and 72 hours in various medium formulations.

Medium formulation	*E. casseliflavus* strain	Biofilm Production (λ600 nm)			
		Incubation time (hour)			
		0	24	48	72
A	F4IS5	0.25	0.34	0.84	0.52
	F6IS4	0.25	0.41	1.02	0.98
	F14IS5	0.25	0.38	0.73	0.47
	F14IS6	0.25	0.44	0.70	0.45
B	F4IS5	0.25	1.10	1.21	0.92
	F6IS4	0.25	0.80	1.26	1.09
	F14IS5	0.25	0.58	0.74	0.68
	F14IS6	0.25	0.34	1.02	0.91
C	F4IS5	0.25	0.45	0.46	0.34
	F6IS4	0.25	0.74	1.11	0.87
	F14IS5	0.25	0.33	0.95	0.36
	F14IS6	0.25	0.38	0.77	0.67
D	F4IS5	0.25	0.22	0.75	0.19
	F6IS4	0.25	0.38	0.82	0.57
	F14IS5	0.25	0.21	0.43	0.37
	F14IS6	0.25	0.39	0.45	0.22
E	F4IS5	0.25	0.30	0.42	0.36
	F6IS4	0.25	0.34	0.45	0.31
	F14IS5	0.25	0.38	0.52	0.48
	F14IS6	0.25	0.39	0.41	0.21
F	F4IS5	0.25	0.97	1.08	0.84
	F6IS4	0.25	0.52	0.65	0.61
	F14IS5	0.25	0.88	0.91	0.48
	F14IS6	0.25	1.06	1.04	0.75

A.) tofu liquid waste with the addition of 1% glucose and 1% ammonium sulfate; B.) tofu liquid waste with the addition of 2% glucose and 1% ammonium sulfate; C.) tofu liquid waste with the addition of 5% glucose and 1% ammonium sulfate, D.) tofu liquid waste with the addition of 10% glucose and 1% ammonium sulfate, E.) tofu liquid waste, F.) MRS.
Source: Author's analysis (2024)

The addition of glucose and ammonium sulfate to the tofu wastewater increased biofilm production in all four *E. casseliflavus* strains. The biofilm production of the four strains also increased with increasing incubation time. At 48 hours of incubation, biofilm production reached an optimum value, as the lactic acid bacteria had entered the logarithmic phase at that time. At that phase, LAB biofilm synthesis reached an optimum value. The highest biofilm production was observed in *E. casseliflavus* F6IS4 grown in the tofu wastewater medium supplemented with 2% glucose and 1% ammonium sulfate, as measured by OD values of 1.26 and 1.02, respectively.

These results are consistent with previous studies, which reported that an optimum

concentration of carbon sources in the range of 2%-5% is necessary for growth and biofilm production (Kubota et al., 2008). However, excess addition of carbon can inhibit growth and biofilm production due to carbon catabolite repression (Pattnaik et al., 2005). The biofilms produced by the four *E. casseliflavus* strains served to protect the cells, allowing them to survive in stressful environmental conditions and experience an increase in growth and metabolism.

LAB biofilms typically consist of exopolysaccharides (EPS), which are composed of long-chain sugars, lipids, proteins, nucleic acids (RNA and DNA), phospholipids, and other biomolecules (Hooshdar et al., 2020). The EPS that makes up the biofilm also serves as an adhesive, allowing cells to adhere to the surface of the material (Nguyen et al., 2020). EPS compounds are grouped into two, namely homopolysaccharides, which contain only one type of sugar monomer, and heteropolysaccharides, which contain several types of sugar monomers (Xu et al., 2019).

Biofilm Adhesion

The biofilm adhesion of the four *E. casseliflavus* strains was tested at 48 hours of incubation, when biofilm production was at its maximum, as presented in Figure 1. On the MRS medium, the biofilms produced by the four *E. casseliflavus* strains exhibited strong adhesion, with the strongest adhesion observed in the biofilm of *E. casseliflavus* F14IS5, where only 3.7% of the cells were detached. However, in the case of tofu wastewater without the addition of glucose and ammonium sulfate, the biofilms of the four strains exhibited poor adhesion. Using a medium supplemented with 1% glucose and 1% ammonium sulfate, only the biofilm of *E. casseliflavus* F14IS6 exhibited strong adhesion, with 57% of the cells remaining detached.

On the tofu wastewater medium supplemented with 2% glucose and 1% ammonium sulfate, the biofilms of *E. casseliflavus* F4IS5, *E. casseliflavus* F6IS4, and *E. casseliflavus* F14IS5 exhibited strong adhesion, with the strongest adhesion observed in the biofilm of *E. casseliflavus* F14IS5, which had 23% detached cells.

Regarding tofu wastewater, the addition of 5% glucose and 1% ammonium sulfate resulted in strong adhesion of the biofilm of *E. casseliflavus* F6IS4, with 83% of the cells remaining attached (Figure 1C). Similarly, when tofu wastewater was supplemented with 10% glucose and 1% ammonium sulfate, only the biofilm of *E. casseliflavus* F6IS4 exhibited strong adhesion, with 45.5% of the cells remaining detached (Figure 1D).

Differences in the level and strength of bacterial adhesion are influenced by the characteristics of cell surface structure, cell surface hydrophobicity, as well as the presence of fimbriae and flagella (Mahami & Adu-Gyamfi, 2011). The presence of fimbriae determines the hydrophobicity of the cell surface; it is important in adhesion as hydrophobic interactions are likely to increase with an increase in the non-polar characteristics of the surfaces involved.

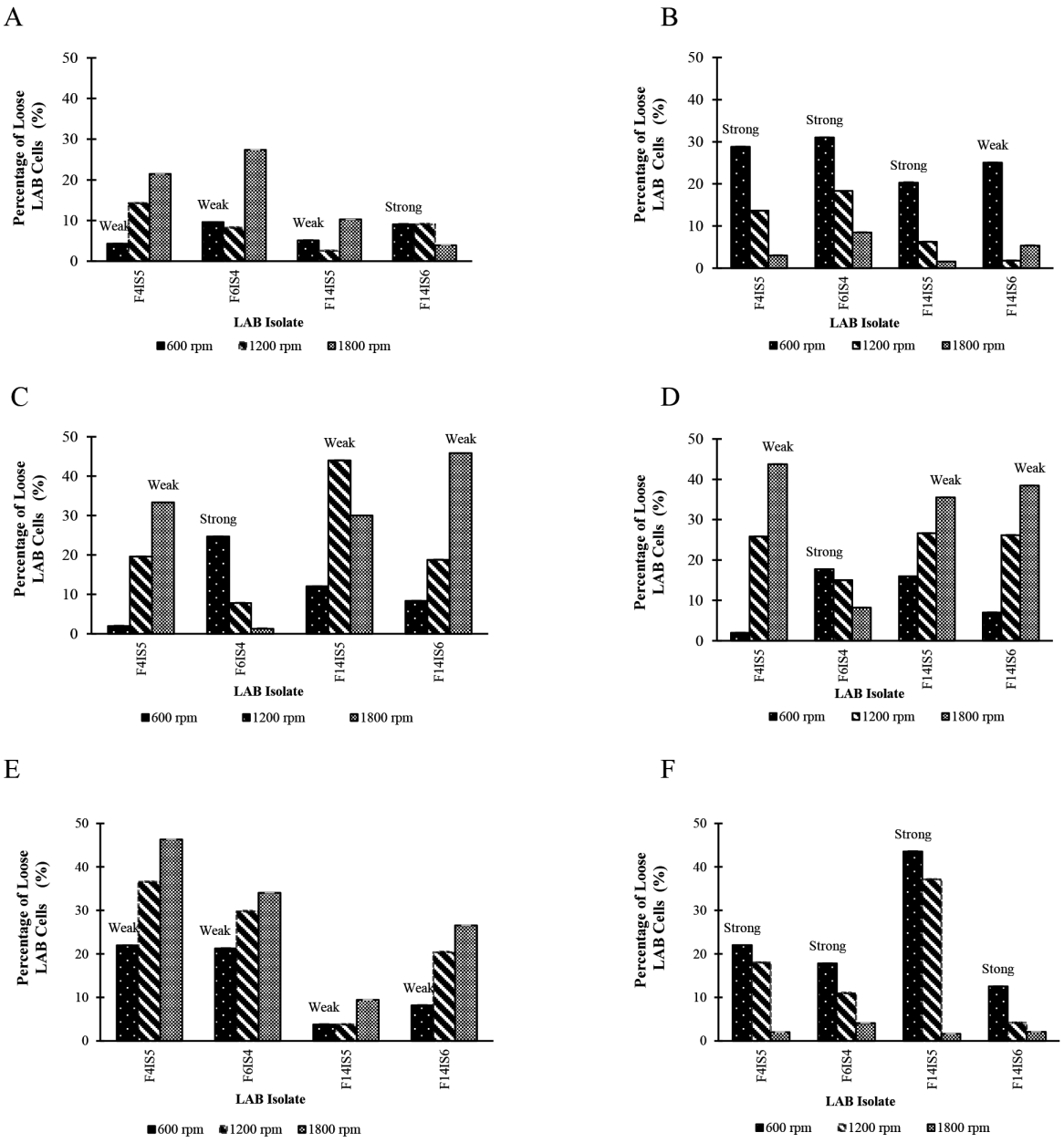

Figure 1.
Adhesiveness of four strains of *E. casseliflavus* during incubation times of 0, 24, 48 and 72 h in various medium formulations. A.) tofu liquid waste with the addition of 1% glucose and 1% ammonium sulfate; B.) tofu liquid waste with the addition of 2% glucose and 1% ammonium sulfate; C.) tofu liquid waste with the addition of 5% glucose and 1% ammonium sulfate, D.) tofu liquid waste with the addition of 10% glucose and 1% ammonium sulfate, E.) tofu liquid waste, F.) MRS
Source: Author's analysis (2024)

Exopolysaccharide Concentration

Exopolysaccharides (EPS) are metabolic products that accumulate on the surface of bacterial cells. EPS concentration is calculated by the dry weight of EPS divided by the volume of the medium. The EPS concentration in this study was tested at a 48-hour incubation time, and the results are presented in Figure 2.

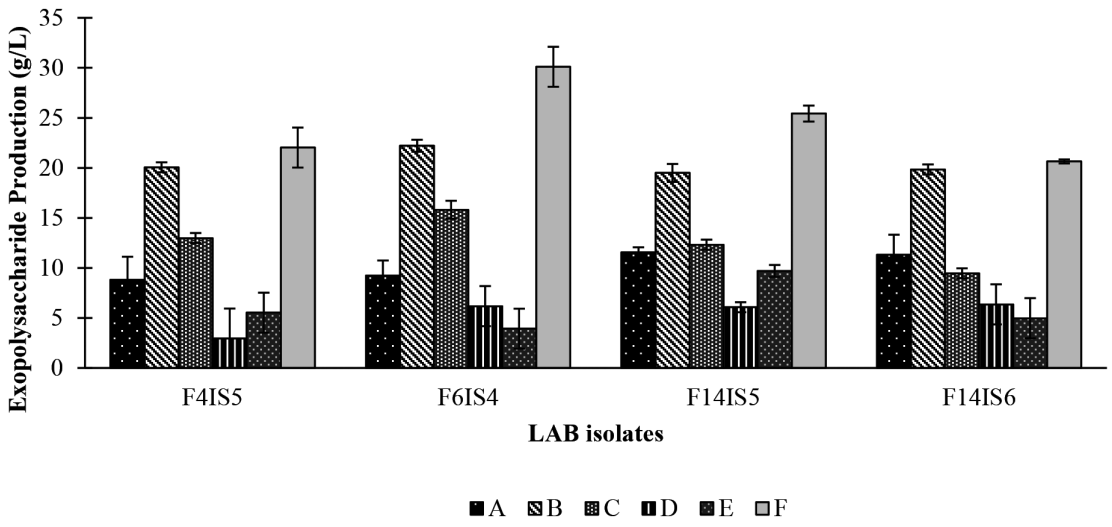

Figure 2.
Exopolysaccharide concentrations of four strains of *E. casseliflavus* during 48 h incubation time in various medium formulations. A.) tofu liquid waste with the addition of 1% glucose and 1% ammonium sulfate; B.) tofu liquid waste with the addition of 2% glucose and 1% ammonium sulfate; C.) tofu liquid waste with the addition of 5% glucose and 1% ammonium sulfate, D.) tofu liquid waste with the addition of 10% glucose and 1% ammonium sulfate, E.) tofu liquid waste, F.) MRS
Source: Author's analysis (2024)

The highest EPS concentration, namely 30.1 g/L, was observed in *E. casseliflavus* F6IS4 on MRS medium. In contrast, the lowest EPS concentration, namely 2.9 g/L, was produced by *E. casseliflavus* F4IS5 on a tofu wastewater medium supplemented with 10% glucose and 1% ammonium sulfate. The biofilm production of *E. casseliflavus* F6IS4 on tofu wastewater, supplemented with 2% glucose and 1% ammonium sulfate, was also relatively high, at 22.2 g/L. The results are relevant to research by Corning (1995), reporting that milk-based medium and MRS are the most recommended media for EPS production in LAB.

Tofu waste substrate with 10% glucose addition results in the lowest EPS production compared to other substrates. This is likely because of the unbalanced C/N ratio in the substrate. A pretty low amount of nitrogen can also affect EPS production by LAB. According to Czaczyk and Myszka (2007), the synthesis of extracellular biopolymers by microbial cells depends on the ratio of carbon to nitrogen in the culture medium.

Inhibitory Activity of Lactic Acid Bacteria Biofilm against Pathogenic Bacteria

Lactic acid bacteria play a crucial role in inhibiting the growth of pathogenic and spoilage-causing bacteria. Lactic acid bacteria have the potential to extend the shelf life of food with their metabolic products, such as bacteriocins (Retnaningrum et al., 2020; Speranza et al., 2020).

In this study, the inhibitory activity of the biofilms was tested using *E. casseliflavus* F6IS4, the strain with the highest biofilm-forming ability. The biofilms produced by *E. casseliflavus* F6IS4 in tofu wastewater, supplemented with 2% glucose and 1% ammonium sulfate, at an incubation time of 72 hours, showed the highest inhibitory ability against *E. coli* and *S. aureus* by 2.7% and 2.1%, respectively (Figure 3).

Such differences are likely due to variations in the constituents of cell walls. *E. coli* is a gram-negative bacterium that has a high lipid content and a small amount of peptidoglycan. At the same time, *S. aureus* is a Gram-

positive bacterium, the cell wall of which contains more peptidoglycan but less teichoic acid and lipid than that of Gram-negative bacteria.

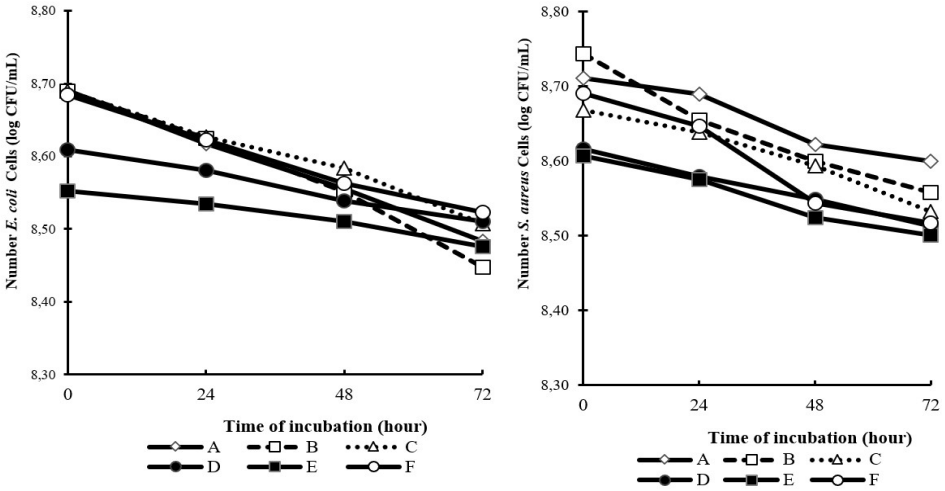

Figure 3.
Inhibitory activity of *E. casseliflavus* F6IS4 biofilm against (1) *Escherichia coli* and (2) *Staphylococcus aureus* during 72 hours incubation time in various medium formulations. A.) tofu liquid waste with the addition of 1% glucose and 1% ammonium sulfate; B.) tofu liquid waste with the addition of 2% glucose and 1% ammonium sulfate; C.) tofu liquid waste with the addition of 5% glucose and 1% ammonium sulfate, D.) tofu liquid waste with the addition of 10% glucose and 1% ammonium sulfate, E.) tofu liquid waste, F. MRS
Source: Author's analysis (2024)

CONCLUSION

E. casseliflavus F6IS4 demonstrated the highest ability to grow and form biofilms at a 48-hour incubation period using tofu wastewater supplemented with 2% glucose and 1% ammonium sulfate. The biofilm produced exhibited strong adhesion, with detached cells accounting for only 19.25%. Additionally, the strain was able to produce EPS at a concentration of 22.2 g/L. The biofilms of *E. casseliflavus* F6IS4 on tofu wastewater with an addition of 2% glucose and 1% ammonium sulfate also showed the highest inhibitory activity against *E. coli* and *S. aureus,* namely 2.7% and 2.1%, respectively.

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