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Production and profiling bioflavor compound from fermentation OPEFB hydrolysate and CPO by *Lactobacillus* sp.

Produksi dan identifikasi profil senyawa bioflavor dari fermentasi hidrolisat TKKS dan CPO menggunakan Lactobacillus *sp.*

Firda DIMAWARNITA[1)*)], Azzakiyya Salsabila Syifa KUSUMA[2)], Urip PERWITASARI[3)], Elok ZUBAIDAH[2)], Yora FARAMITHA[1)], Pijar RELIGIA[4)] & Ario Betha JUANSSILFERO[3)]

[1)]Indonesian Oil Palm Research Institute-Bogor Unit. Jalan Taman Kencana no 1, Bogor 16128, West Java, Indonesia
[2)]Department of Food Science and Biotechnology, Faculty of Agricultural Technology, Universitas Brawijaya. Jalan Veteran no 10-11. Malang 65145, East Java, Indonesia
[3)]National Research and Innovation Agency. Jalan Raya Jakarta-Bogor no 32, Bogor 16915, West Java, Indonesia
[4)]International Center for Biotechnology, Osaka University. 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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Abstrak

Bioflavor merupakan rasa alami yang diperoleh dari metabolit mikroba selama proses fermentasi, dan sebagian besar bakteri yang berperan dalam fermentasi makanan adalah bakteri asam laktat, termasuk Lactobacillus *sp. Media optimal untuk pertumbuhan* Lactobacillus *sp. adalah* de Man Rogosa *dan* Sharpe *(MRS), dinilai kurang ekonomis sehingga diperlukan alternatif sumber karbon dan nitrogen. Tujuan penelitian ini untuk mengetahui bioflavor yang dihasilkan pada media* de Man Rogosa *dan* Sharpe Broth *(MRSB) yang disubstitusi dengan hidrolisat Tandan Kosong Kelapa Sawit (TKKS) dan* Crude Palm Oil *(CPO) masing-masing dengan konsentrasi 5, 15, dan 30% menggunakan kromatografi gas-spektrometri massa. Berdasarkan hasil penelitian, substitusi MRSB dengan CPO 15% menunjukkan hasil terbaik terhadap pertumbuhan* Lactobacillus *sp. Namun setiap media menghasilkan senyawa bioflavor yang berbeda-beda. Pada standar media kontrol (MRSB), senyawa bioflavor tertinggi adalah 2,3-dihidro-3,5-dihidroksi-6-metil-4H-Pyran-4-one (*rose tea*). Pada media tersubstitusi hidrolisat TKKS senyawa bioflavor tertinggi adalah benzenasetaldehida (manis, roti, mawar), pada media tersubstitusi CPO senyawa bioflavor tertinggi adalah furaneol (nanas dan stroberi) serta pirazin (kacang-kacangan, kopi panggang).*

*[Kata kunci: sumber karbon nitrogen, aroma, GC-*MS, *mikroorganisme]*

Abstract

Bioflavor is a type of natural flavor that is obtained from microbial metabolites during the process of fermentation. Most of the bacteria involved in food fermentation are lactic acid bacteria, including *Lactobacillus* sp. The optimal medium for *Lactobacillus* sp. growth is de Man Rogosa and Sharpe (MRS), but it is considered to be less economical. Therefore, alternative carbon and nitrogen sources are needed. This study aimed to determine the bioflavor produced in *de Man Rogosa dan Sharpe Broth* (MRSB) media that was substituted with Oil Palm Empty Fruit Bunch (OPEFB) hydrolysate and Crude Palm Oil (CPO) at concentrations of 5, 15, and 30%, respectively by using gas chromatography-mass spectrometry. The results showed that substituting MRSB with 15% CPO produced the best results for the growth of *Lactobacillus* sp. However, each medium produced different bioflavor compounds. In the control media (MRSB), the highest amount of bioflavor compound was 2,3-dihydro-3,5-dihydroxy-6-methyl -4H-Pyran-4-one (rose tea). In the OPEFB hydrolysate-substituted medium, it was benzene-acetaldehyde (sweet, bread, rose), in the CPO-substituted medium, it was furaneol (pineapple and strawberry) and pyrazine (nutty, roasted coffee).

[Keywords: carbon nitrogen sources, flavor, GC-MS, microorganism]

*)] Corresponding author: firda.dimawarnita@gmail.com

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Introduction

The flavoring agent is the food additive used to enhance and emphasize the flavor of a food product. During food processing, the ingredients' natural flavor intensity may be decreased, resulting in undesirable flavors for the consumer (van Zwanenberg & Millstone, 2015). In such cases, adding flavoring agent can help to restore the lost taste and aroma to meet consumer expectations. Flavoring agents are generally classified into two categories, i.e. a natural flavor, which is made by extracting specific plants, and chemical materials which make synthetic flavors (Aljaff et al., 2013). Nowadays, consumers are increasingly aware of their health and prefer natural ingredients over synthetic ones that can have negative health impacts, such as nausea, chest pain, headache, allergy, kidney failure, and cancer (Román et al., 2017).

Bioflavor is a natural flavor produced from microorganism metabolism biotechnologically (Vandamme & Soetart, 2002). Bacteria and yeast are microorganisms that can generally play a role in this process. Previous studies have shown that these microorganisms produced flavor compounds during growth. For example, *Kluyveromyces marxianus* produces benzeneactealdehyde (rose, flower) and isobutyl acetate (bubble gum) (Güneşer et al., 2015), lactic acid bacteria in yogurt products produce acetaldehyde (fruity), 2-hexanone (grape) and 2-undecanone (orange) (Tian et al., 2019), *Nocardia iowensis* produces vanillin (vanilla) (Carroll et al., 2016), *Saccharomyces cerevisiae* produces butyl butyrate (pineapple) (Aggelopoulos et al., 2014), and *Rhizopus oryzae* produces limonene (citrus) (Guneser et al., 2017).

In food fermentation, *Lactobacillus* sp. is a type of bacteria that can produce lactic acid and is known as lactic acid bacteria (LAB). *Lactobacillus* sp. is commonly found in fermented food such as yogurt, cheese, *dadih* (fermented buffalo milk product), and others that give a buttery flavor. These bacteria require optimal sources of carbon (C) and nitrogen (N) to grow, and the standard medium for their growth is de Man Rogosa and Sharpe (MRS). However, MRS is relatively expensive, so an alternative medium growth is needed (Ayad et al., 2020; Dewi et al., 2020).

Waste from the palm oil extraction from fresh fruit bunches is called oil palm empty fruit bunches (OPEFB). One ton of fresh fruit bunches of palm oil can yield up to 23% OPEFB (Susanto et al., 2017). OPEFB is an environmentally friendly ligno-cellulose waste. The high carbon content can make OPEFB hydrolysate as a medium-growth of microorganisms material (Destyorini & Indayaningsih, 2018; Salmina, 2017). In Kresnowati et al. (2016) research, OPEFB hydrolysate is utilized as medium growth for *D. hansenii* in the production of xylitol. The results showed the medium of OPEFB hydrolysate produced higher xylitol yields. Therefore, the hydrolysate empty palm fruit bunches may also be used as a carbon and nitrogen source in the *Lactobacillus* sp. growth medium. In addition, empty palm fruit bunches can also be used as a carbon and nitrogen source for *Lactobacillus* sp. (Ye et al., 2014). The use of hydrolysate in this research may increase the value of OPEFB because of its abundant availability and lower production costs compared to using MRS. Moreover, crude palm oil (CPO) is another potential growth medium for microorganisms, as it is rich in nutrients and fatty acids. Based on Suryanti et al. (2017), the addition of CPO to the *P. putida* resulted in more optimal results in biosurfactant production. Therefore, this research aims to investigate the flavor compounds profile produced by *Lactobacillus* sp. in substituted media using OPEFB hydrolysate and CPO, which could lead to better cost-effectiveness. CPO is also used in the treatment.

Material and Methods

Materials

The research uses *Lactobacillus* culture (collection of Indonesian Oil Palm Research Institute, Bogor), MRSB (HiMedia), MRSA (HiMedia), OPEFB from Oil Palm Research Institute, Bogor, CPO from PTPN IV Medan, aluminum foil, H_2SO_4 97% (Merck), monel fabric, 3,5 Dinitrosalicylic (Sigma Aldrich), Ninhydrin (Merck), $Na_2HPO_4.2H_2O$ (Merck), fructose (brand), KH_2PO_4 (Merck), KIO_3 (Merck), ethanol 99% (Merck), glycine (Merck), phenolphthalein indicator, NaOH (Merck), chloroform (Merck) and sodium sulfate (Merck).

Preparation for Lactobacillus *sp*.

Lactobacillus sp. stocks that are cultivated on MRSA media undergo a secondary growth phase on fresh MRSA media in separate Petri dishes. This step is designed to revitalize bacterial metabolism and serve as a means of reactivation after prolonged storage (Wijayanti et al., 2015). Incubation lasts for 72 h, after which the cultured bacteria are stored in a refrigerator at 0-10 ºC.

Preparation for hydrolysate oil palm empty fruit bunches

Oil palm empty fruit bunches are hydrolyzed using a two-stage hydrolysis process with H_2SO_4 (Lalou et al., 2013). The acid hydrolysis process breaks down the polysaccharides in OPEFB into monosaccharides. First, 36 g of OPEFB is hydrolyzed by 300 mL H_2SO_4 0.5% (v/v) at 121 °C for 15 min. Next, the filtrate and the solid part are separated by monel fabric. The solid part is

hydrolyzed in the same condition for the next stage for 30 min. The results of both stages are filtered and stirred until evenly mixed.

Production of bioflavor in substituted medium

Lactobacillus sp. was inoculated on media substituted with sterilized OPEFB hydrolysate and CPO, respectively, in jars with 5%, 15%, and 30% (v/v) substitution percentages in the different Erlenmeyer. Each substitution medium also contains MRSB with 95%, 85%, and 70% (v/v) as the standard medium for *Lactobacillus* sp., with the alternative carbon and nitrogen sources added in specified percentages. The incubation was carried out for 72 h on a shaker at room temperature. Optical density (OD) values were analyzed every 24 h using a 600nm wavelength spectrophotometer (Nguyen-Sy et al., 2020). The OD value was used to determine cell density to observe the growth of *Lactobacillus* sp. in the substituted media.

pH measurement and titrable acidity (% lactic acid)

pH and titrable acidity measurements were carried out on media that had been inoculated with *Lactobacillus* sp.. During the fermentation process, the pH of the media decreased due to the production of an organic acid compound. This acid compound is lactic acid produced by bacteria that convert carbohydrates into lactic acid (Bintsis, 2018). The pH of the media was measured using a pH meter (Thermo Scientific) calibrated with standard buffer solution of pH 4, 7, and 10. Titratable acidity analysis was used to determine the amount of lactic acid produced in the media. According to Suhaeni (2018), a 10 mL sample was dissolved with distilled water in Erlenmeyer flask, and three drops of 1% phenolphthalein indicator were added. The solution was titrated with 0.1 N NaOH until the color changed to pink and the volume of NaOH was inserted into Equation (1) to determine the percentage of lactic acid content.

$$\text{Total acid (\%)} = \frac{V\ NaOH \times N\ NaOH \times MW\ lactic\ acid}{V\ sample \times 1000}\ 100 \quad (1)$$

Note:
V NaOH= volume of NaOH (mL)
N NaOH= normality of NaOH
MW lactic acid= molecular weight of lactic acid
V sample= volume sample (mL)

Total carbon and nitrogen content

Carbon sources for *Lactobacillus* sp. growth is carbohydrate in the form of glucose. During the fermentation process, *Lactobacillus* sp. consumes glucose to produce organic acid, resulting in a decrease in glucose levels in the media (Wang et al., 2021). Total carbon content analysis was performed using a DNS reagent to determine the amount of carbon consumed. The reaction in the DNS analysis is redox, which DNS reagent is reduced to 3-amino-5-nitrosalicic, which results in a reddish-orange color. Meanwhile, the aldehyde group from reducing sugar is oxidized to carboxyl. The more aldehyde groups reduced the DNS reagent, the more intense the reddish-orange color produced. The reddish-orange color is measured with a UV-Vis spectrophotometer at 575 nm. Analysis was performed using modified Arifin et al. (2006) and Habibah et al. (2018) methods by adding 1 mL of distilled water to 1 mL of dissolved sample. Then 3 mL of DNS reagent was added and homogenized using a vortex. The solution was boiled in water for 15 min and cooled for 20 min before measuring the absorbance with a spectrometer.

Apart from carbon sources, microorganisms also require nitrogen sources for growth. A modified Lie (1973) and Zaini et al. (2019) method were employed to determine the amount of nitrogen consumed. In this method, a 2 mL diluted sample in a test tube was mixed with 1 mL of a color reagent composed of 4.971 g of $Na_2HPO_4.2H_2O$, 0.5 g of ninhydrin, 0.3 g of fructose, and 4 g of KH_2PO_4 dissolved in 100 mL of distilled water and then, sealed and heated to 100 °C for 16 min. Afterward, it was allowed to cool for 20 min, and 5 mL of a dilution solution (consisting of 2 g of potassium iodate dissolved in 616 mL of distilled water and 348 mL of 96% ethanol) was added. The absorbance of the solution is measured using a spectrophotometer at a wavelength of 570 nm at 0 and 72 h. During this process, ninhydrin acts as an oxidizing agent, leading to the oxidative decarboxylation of α-amino acids. This reaction results in the production of CO_2, NH_3, and aldehydes. Fructose, on the other hand, acts as a reducing agent for ninhydrin. The reduced ninhydrin then reacts with unreacted ninhydrin and ammonia to form a purplish-blue complex.

Analysis of bioflavor compounds

Flavor compounds from the fermentation process are investigated by Gas Chromatography-Mass and Spectrum (GC-MS) analysis with specification GCMS-QP2010 Ultra (Shimadzu), column RTx-5MS (25 m x 0.2mm ID, thickness 0.33 μm). The identified flavor compounds are compared with mass spectra from the National Institute of Standard and Technology data and the Wiley Registry of Mass Spectral Data. GC-MS results also show the area compound, indicating the amount of a flavor compound in the sample.

Statistical analysis

The research examined two factors: the media type and the percentage of media substitution. The study used MRSB media to substitute OPEFB hydrolysate and CPO. The media substitution percentage comprised three levels: 5%, 15%, and 30% (v/v) of the total growth media. Each combination of media type and percentage of substitution was replicated three times. All data underwent statistical analysis using ANOVA with a 95% confidence interval. The Tukey test assessed the significance of variations between the different treatments. All the statistical analyses were conducted utilizing Minitab version 19 software.

Results and Discussion

Growth of Lactobacillus *sp. in substituted medium*

The number of cells can be determined by the density of the bacteria cell population, and enhancements of the number of cells shows the bacteria's growth and metabolism. The number of cells in liquid media can be measured by optical density (OD) (Ram et al., 2019). Besides, the environment influences the growth of bacteria by looking at changes in the number of cells (Sharah et al., 2015). The growth medium will affect bacterial growth, and the growth also differs based on the amount and the sources of nutrition in the environment (Ukalska & Jastrzębowski, 2019). In this study, MRSB media was substituted with two types of materials, i.e. OPEFB hydrolysate and CPO, with the three levels of substitution: 5%, 15%, and 30% for media of growth for *Lactobacillus* sp.

The results of measuring the OD value for 72 h are shown in Figure 1. In the first 24 h, *Lactobacillus* sp. adapts to the new environment and experiences exponential growth, resulting in a quite large increase. This section can be used to determine a suitable medium for *Lactobacillus* sp.(Putri et al., 2021; Rolfe et al., 2012). After 72 h of incubation, the highest growth in media substituted with OPEFB hydrolysate was obtained on 5% of substitution, with an increase of 0.7. The highest increase in CPO-substituted media was obtained with a 15% substitution with an increase of 1.3 OD value after incubation for 24 h.

Figure 1 shows that increasing the OD value in the control media (OPEFB 0%) is better than the substituted media. The control media, de Man Rogosa and Sharpe (MRS) is a standard medium for *Lactobacillus* sp. growth. The nutrient content in the form of carbon and nitrogen sources has been adjusted to be suitable for bacterial growth. The carbon source contained in MRS media is glucose, and the nitrogen source is tryptic digest casein, beef extract, and yeast extract. Moreover, this is also caused by hydrolysate OPEFB and CPO, which have lower N sources than standard media sources (MRSB) (Ye et al., 2014).

Additionally, the pH of MRSB media is the optimal pH of *Lactobacillus* sp. growth, which is 6.5. The pH of OPEFB hydrolysate is low, which is 1.25 because the solvent used for the hydrolysis process was sulfuric acid. The higher the substitution percentage, the increase in the number of bacterial cells is also lower. The substitution decreased the fermentation media's pH (Suhaeni, 2018). It made the growth of *Lactobacillus* sp. not optimal because *Lactobacillus* sp. can grow at pH 4.0 - 7.0 and optimally at pH 6.5 (Hamilah et al., 2018). Moreover, hydrolysis will degrade glucose into 5-(hydroxymethyl)-furfural (HMF). The presence of HMF in the media can affect bacterial growth because HMF has the properties of a fermentation inhibitor (Koruda et al., 2016).

In contrast to OPEFB hydrolysate, CPO substitution gave a good increase compared to the control media. The best increase in cell number after incubation for 24 h was in media substituted with 15% CPO (Figure 1B). The source C from OPEFB hydrolysate is less than CPO. CPO consists of a lot of carbon because it contains fatty acids. However, the OPEFB hydrolyzate contains impurities from solvents that are still present. CPO contains a lot of fatty acids, and fatty acids are helpful in the formation of cell walls and cell membranes in *Lactobacillus* sp.. Fatty acids become an essential component of cell membranes because they become constituents of phospholipid bilayers. While in the cell wall, fatty acids become constituents of lipoteichoic acid components (Mezo et al., 2022; Szentirmai et al., 2021). In the research of Pato et al. (2021), there was a decrease in the amount of lauric acid, palmitic acid, stearic acid, oleic acid, and linoleic acid in the fermentation process of lactic acid bacteria. Therefore, the content of lauric acid, palmitic acid, stearic acid, oleic acid, and linoleic acid in CPO positively impacts the growth of *Lactobacillus* sp. In addition, linoleic acid also plays a role in cell gene activity (Orsavova et al., 2015).

Acidity media

The pH of the media decreases more significantly during incubation when substituted with 5% OPEFB hydrolysate compared to other media with 15% and 30% OPEFB hydrolysate substitution. Control MRSB also experienced pH reduction (from 6.04 to 4.38) after 72 h of incubation. OPEFB hydrolysate has a low pH because using sulfuric acid solvent. Increasing the proportion of OPEFB hydrolysate in the media further lowers the initial pH level, adversely affecting Lactobacillus sp.'s growth (Table 1). During fermentation, the acidity level plays a crucial role because each microorganism has an optimal pH

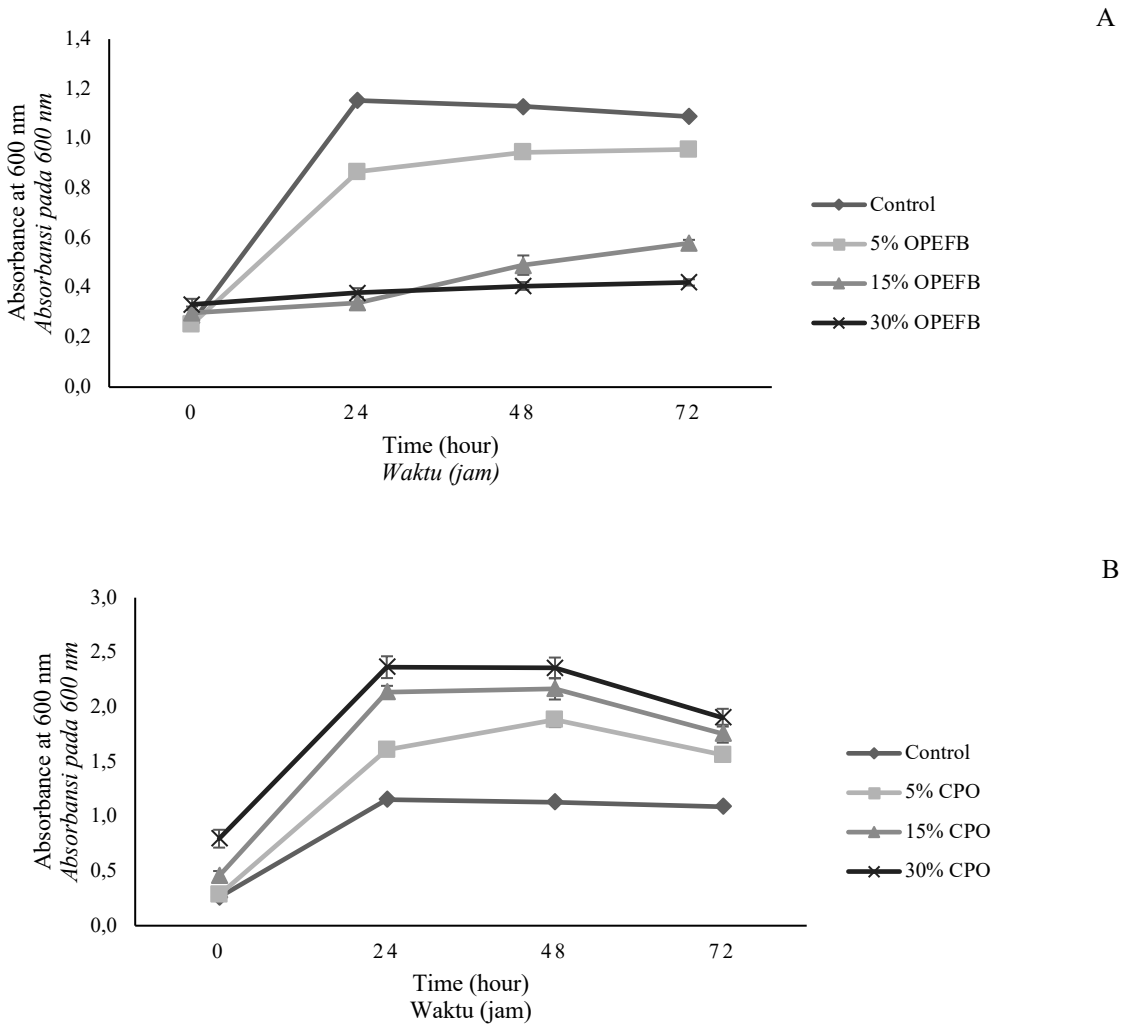

Figure. 1. A. OD value of *Lactobacillus* sp. growth in MRSB media substituted with OPEFB hydrolysate. B. OD value of *Lactobacillus* sp. growth in MRSB media substituted with CPO

Gambar 1. A. Nilai OD dari pertumbuhan Lactobacillus *sp. pada media MRSB tersubstitusi hidrolisat TKKS. B. Nilai OD dari pertumbuhan* Lactobacillus *sp. pada media MRSB tersubstitusi CPO*

for growth. *Lactobacillus* sp. can grow in media with a pH of 4 – 7 and is optimal at a pH of 6.5. As the fermentation process continues, the pH of the media will continue to decrease due to the metabolism of *Lactobacillus sp*. in the form of organic acids. The pH will continue to decline during the fermentation process until it reaches a certain point where the pH of the media is too acidic and inhibits further growth (Fadilah et al., 2018).

The presence of lactic acid that continues increasing during incubation causes the pH of the media to continue decreasing along with the length of the fermentation process (Aliya et al., 2015). The ANOVA test results indicate that the type and percentage of media substitution and their interactions significantly affect the fermentation process (P-value<0.05). Based on the Tukey test, media substituted with 15% CPO was the most favorable treatment, with an average pH decrease of 1.75 at 72 h. However, this treatment is similar to treatments involving 5% and 30% CPO substitution, and 5% OPEFB hydrolysate. The initial pH of the media is influenced by the type of substitute substance used and the substitute is still at the optimum pH for the growth of *Lactobacillus* sp.. The pH value of the media is also influenced by total titratable acid, which is obtained from the percentage of lactic acid present in the solution. Total acid measures the acid naturally possessed by the material, the acid added, and the acid produced from a microorganism's metabolic process, as explained by Santoso (2020). Figure 2 shows that each substituted media undergoes an increase in total acid content after a 72-h fermentation process.

Table 1. pH changes in substituted media before and after incubation
Tabel 1. Perubahan pH pada media tersubstitusi sebelum dan setelah inkubasi

Media substitution *Substitusi media*	pH of media *pH media*		
	0 h *0 jam*	72 h *72 jam*	Δ[1]
Control/ *Kontrol*	6.04 ± 0.020	4.38 ± 0.080	1.66[a]
MRSB + 5% OPEFB	6.02 ± 0.000	4.48 ± 0.232	1.54[a]
MRSB + 15% OPEFB	4.80 ± 0.006	4.41 ± 0.000	0.39[b]
MRSB + 30% OPEFB	3.95 ± 0.036	3.94 ± 0.010	0.01[b]
MRSB + 5% CPO	6.16 ± 0.020	4.55 ± 0.000	1.61[a]
MRSB + 15% CPO	6.14 ± 0.006	4.40 ± 0.055	1.75[a]
MRSB + 30% CPO	5.96 ± 0.234	4.44 ± 0.075	1.52[a]

[1] lowercase letters indicate significant differences between the factor interactions ($p < 0.05$)
[1] huruf kecil menunjukkan perbedaan nyata antar interaksi faktor ($p < 0.05$)

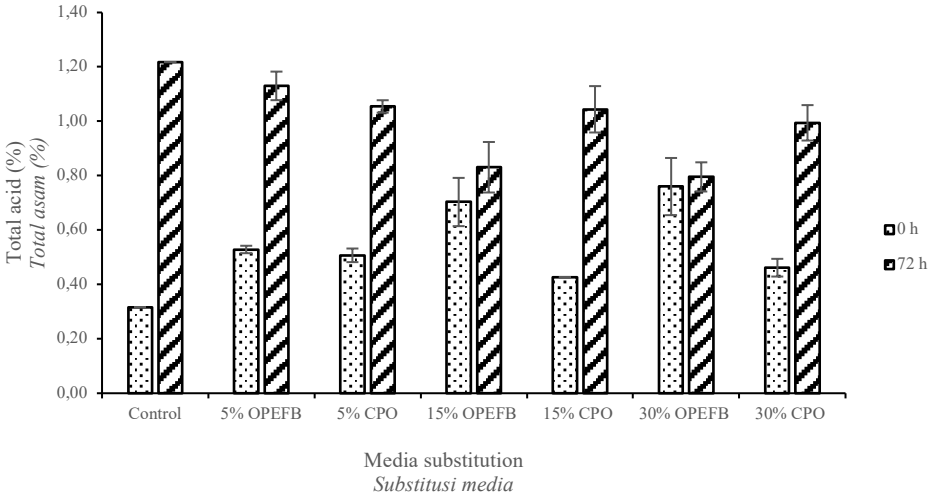

Figure 2. Total acid in substituted media before and after 72 h of incubation
Gambar 2. Total asam dalam media tersubstitusi sebelum dan sesudah inkubasi 72 jam

The levels of lactic acid contained in each experiment. and may be related to the data in Figure 1B, between total acid and the OD value of each treatment. The results showed that the rise in total acid content is related to the growth of *Lactobacillus* sp. on the substituted media and its ability to produce lactic acid (Adisa et al., 2019). Melia et al. (2021) noted that prolonged fermentation leads to higher lactic acid production. The ANOVA test results indicate that the type and percentage of media substitution significantly affect the total acid level (P-value<0.05). The Tukey test results also reveal noteworthy distinctions between treatment factors. The most effective treatment, as determined by the highest average increase of total acids is 0.618%, is achieved with a 15% CPO substitution. A higher total acid content in the media can lower the media's pH, which can adversely affect the growth of *Lactobacillus* sp., potentially leading to poor growth or even cell death (Arfianty et al., 2017). The reduced pH can also result in protein denaturation within living cells, disrupting their growth activities and potentially causing bacterial death (Iswari et al., 2022).

This parameter determines the carbon content. The presence of a carbon source will help optimize the fermentation process. Glucose as a carbon source will be utilized to produce organic acids. The outcomes, indicating the reduction in total sugar levels after a 72-h incubation period, are presented in Table 2. The ANOVA test results at a 95% confidence level indicated that the type of media substitution and the percentage of media substitution significantly affect the total sugar levels. The Tukey test outcomes indicated that the most effective treatment is associated with a 15% CPO substitution, which results in an average reduction in total sugar content of 21.9%. Interestingly, this treatment factor does not exhibit a statistically significant difference compared to the results obtained with a 30% CPO

substitution. Compared to the control medium (MRSB), this treatments factors showed higher carbon consumption, while no significant difference was observed in media containing OPEFB hydrolysate at 5% and 15% substitution levels. According to the Tukey test results, the medium substituted with 30% OPEFB showed the lowest carbon consumption.

The reduction in total sugar content indicates that the carbon source is being consumed as an energy source for the production of lactic acid, as observed in the study by Hassan et al., (2014). The substitution with CPO as one of the media's carbon sources is effectively utilized by *Lactobacillus* sp., which is also supported by the increase in the OD value shown in Figure 1B, which has the best increase in cell density. Bacteria are capable of using different types of carbon sources for their metabolic processes (Cheng et al., 2019), including fatty acids, which are abundant in CPO. A greater variety of carbon sources can promote increased bacterial cell growth, as highlighted by Subagiyo et al. (2015). However, this phenomenon was not observed when 30% of the CPO was substituted with OPEFB hydrolysate, which has a higher glucose content. This is due to the extremely low pH of OPEFB hydrolysate, which inhibits bacterial growth, as shown in Figure 1A. Furthermore, CPO as a carbon source for fatty acids, OPEFB as a carbon source for the hydrolyzate, and it is related to the pH and total acid in each treatment.

Nitrogen consumption

Nitrogen is one of the sources of nutrients in microorganism fermentation media. Amino acids in the growth media are consumed by bacteria to facilitate their metabolic processes. The process decreased amino acids in the growth media (Khan et al., 2018). A ninhydrin test was conducted to assess the amount of free amino acids both initially available and remaining in the media. The results reflecting nitrogen consumption are presented in Table 3.

Table 2. Carbon consumption in substituted media before and after 72 hours incubation
Tabel 2. Konsumsi karbon pada media tersubstitusi sebelum dan sesudah inkubasi 72 jam

Media substitution *Substitusi media*	Total sugar (%) *Gula total (%)*		
	0 h 0 *jam*	72 h 72 *jam*	Δ[1]
Control/ *Kontrol*	15.5 ± 0.653	11.7 ± 0.653	3.81[c]
MRSB + 5% OPEFB	15.9 ± 0.593	12.2 ± 0.495	3.70[c]
MRSB + 15% OPEFB	25.3 ± 1.582	19.9 ± 0.540	5.45[c]
MRSB + 30% OPEFB	22.7 ± 0.443	22.3 ± 0.677	0.40[d]
MRSB + 5% CPO	23.6 ± 0.822	14.4 ± 0.047	9.19[b]
MRSB + 15% CPO	39.0 ± 0.859	17.1 ± 1.552	21.95[a]
MRSB + 30% CPO	36.8 ± 0.836	17.8 ± 0.593	18.99[a]

[1] lowercase letters indicate significant differences between the factor interactions ($p < 0.05$)
[1] huruf kecil menunjukkan perbedaan nyata antar interaksi faktor ($p < 0.05$)

Table 3. Nitrogen consumption in substituted media before and after 72 hours incubation
Tabel 3. Konsumsi nitrogen pada media tersubstitusi sebelum dan sesudah inkubasi 72 jam

Media substitution *Substitusi media*	Free amino acid (g L^{-1}) *Asam amino bebas (g L^{-1})*		
	0 h 0 *jam*	72 h 72 *jam*	Δ[1]
Control/ *Kontrol*	1.38 ± 0.043	1.34 ± 0,043	0.05[a]
MRSB + 5% OPEFB	1.30 ± 0.062	1.21 ± 0.084	0.09[a]
MRSB + 15% OPEFB	1.19 ± 0.071	1.05 ± 0.092	0.15[a]
MRSB + 30% OPEFB	0.88 ± 0.081	0.82 ± 0.077	0.06[a]
MRSB + 5% CPO	1.77 ± 0.090	1.45 ± 0.177	0.31[a]
MRSB + 15% CPO	1.91 ± 0.039	1.61 ± 0.093	0.30[a]
MRSB + 30% CPO	2.07 ± 0.088	2.03 ± 0.097	0.04[a]

[1] lowercase letters indicate significant differences between the factor interactions ($p < 0.05$)
[1] huruf kecil menunjukkan perbedaan nyata antar interaksi faktor ($p < 0.05$)

The Tukey test showed no significant difference between the control medium (MRSB) and the other treatments. However, an increase in the amount of free amino acids was observed after adding CPO (0 h). This was likely due to the carryover of MRS media from the pre-culture isolate used. Nonetheless, nitrogen consumption in the media is important for the growth of microorganisms, especially in forming bacterial cell walls composed of peptidoglycan. Lactic acid bacteria also have limitations in synthesizing amino acids independently, so nitrogen sources are crucial for their growth in the media, as Cheng et al. (2019) outlined.

Although medium substitution did not result in a significant difference for nitrogen consumption in this study, the carbon source and environmental pH significantly impacted *Lactobacillus* growth. Increasing the amount of OPEFB hydrolysate substitution lowered the pH (Table 1), resulting in low *Lactobacillus* cell growth (Figure 1A), and carbon consumption did not occur properly (Table 2). On the other hand, CPO substitution provided nutrients and created favorable environmental conditions to support *Lactobacillus* growth.

Profiling bioflavor compound

Table 4 displays the bioflavor compounds found in *Lactobacillus* sp. growth media under different conditions, with and without substitution of carbon and nitrogen sources, after 72 h of incubation. In total, we identified 26 flavor compounds and the number of compounds produced in various media types were as follows: 12 compounds in the control media, 16 compounds in the OPEFB hydrolysate substituted media, and 13 compounds in the CPO substituted media. These compounds include a range of aromatic substances such as furan, pyrazine, pyridine, isothiocyanate, pyrrole, and more. The following are the compounds detected with the highest concentrations in the control medium: 2,3-dihydro-3,5– dihydroxy-6-methyl-4H–Pyran-4-one and 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one. The OPEFB hydrolysate substituted media compounds were 2,5-dimethyl-3-propyl-pyrazine, benzenacetaldehyde, and isopropyl isothiocyanate. In CPO-substituted media, the compounds were Pyrrolo[1,2-apyrazine-1,4-dione], hexahydro-3-(2-methylpropyl)-, 2-methyl-5-propyl-Pyrazine and Furaneol.

Furan can be formed from the degradation process of sugars such as glucose and fructose that react with amino acids and the degradation process of amino acids into acetaldehyde and glycolaldehyde, forming furan compounds. These furan compounds contribute to fruity, sweet, and caramel flavors (De Vivo et al., 2022). One of the furan compounds detected was 5-methylfurfural in the control media, with the largest area of 7.28%. 5-Methylfurfural imparts flavors reminiscent of caramel, coffee, almond, spiciness, and burnt sugar (De Vivo et al., 2022). Furaneol, another furan compound, is associated with flavors resembling strawberry and pineapple and is sometimes referred to as strawberry furanone and pineapple furanone (Weerawatanakorn et al., 2015). The highest concentration of furaneol was detected in the fermentation results of *Lactobacillus* sp. on CPO-substituted media. Furoic acid compounds are also produced during the *Lactobacillus* sp. fermentation process, and this study was obtained in MRSB media with an area of 38.86%. Furoic acid contributes to a sweet and oily flavor (Escobar et al., 2015). The formation of furan compounds can occur due to the hydrolysis and degradation of sugar precursors, with or without the presence of amino acids. Amino acids, particularly serine and cysteine derivatives, can facilitate furan formation. Additionally, the decomposition of unsaturated fatty acids, specifically 4-hydroxy-2-alkenals, can serve as precursors for furan compound formation (Guo et al., 2019).

Pyrazine is a type of nitrogen-containing heterocyclic compound. It is formed when amino acids and sugars react with each other. Pyruvate, which results from glucose metabolism, will be converted into acetoin and react with ammonium to produce pyrazine compounds. Apart from this, pyrazine compounds can also be synthesized using amino acids such as valine, leucine, isoleucine, and L-threonine. Pyrazine compounds are not solely a byproduct of heating processes in food preparation but can also be generated during fermentation (Mortzfeld et al., 2020; Verma et al., 2022; Zhang et al., 2019).

One of the Pyrazine compounds detected in the study was 2-methyl-5-propyl-pyrazine. It was found in the fermentation process of *Lactobacillus* sp. in CPO-substituted MRSB media with an area of 4.59%. Pyrazine is known for its nutty, hazelnut, corn, and potato flavors which depend on the type of compound that binds to the pyrazine ring (Dippong et al., 2022). Pyrazine also has a distinctive roasted coffee-like flavor, which it shares with pyridine and pyrrole compounds. However, pyridine compounds can also cause sour and fishy flavors, while pyrrole compounds can also cause nutty flavors (De Vivo et al., 2022). Table 5 shows other compounds that have different flavors.

Table 4. Bioflavor compound produced by *Lactobacillus* sp in various media
Tabel 4. Senyawa bioflavor yang diproduksi oleh Lactobacillus *sp. pada berbagai media*

Bioflavor compound *Komponen bioflavor*	Control *Kontrol*		Hydrolysate of OPEFB *Hidrolisat TKKS*		CPO *CPO*	
	RT	Area (%)	RT	Area (%)	RT	Area (%)
1H-pyrrole, 2,5-dimethyl-1-phenyl-	ND		13.4	4.4	13.4	2.2
2(5H)-furanone	ND		4.9	1.8	ND	
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	5.8	48.8	5.8	25.4	5.8	17.3
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	4.2	1.9	4.3	1.2	4.1	0.4
2,5-dimethyl-3-propyl-pyrazine	ND		9.1	2.4	9.1	3.4
2,5-Dimethylpyridine N-oxide	7.9	0.6	ND		ND	
2-Amino-3-hydroxypyridine	ND		ND		ND	
2-methyl-3-propylpyrazine	ND		8.4	0.9	ND	
2-methyl-5-propyl-pyrazine	ND		8.4	4.0	8.4	4.6
2-Propenal, 3-(2-furanyl)-	ND		5.5	2.1	ND	
2-tetrahydrofurfuryl isothiocyanate	ND		ND		5.1	2.8
3-methyl-1-oxide-pyridine	11.5	1.7	ND		ND	
4-ethyl-1-oxide-pyridine	9.9	2.4	ND		ND	
4-oxid-methoxy-pyrazine	12.32	3.57	ND		ND	
5-hydroxymaltol	6.6	0.8	8.8	1.0	ND	
5-methylfurfural	3.8	7.3	ND		ND	
Benzenacetaldehyde	ND		6.2	10.1	6.2	7.4
Cyclo(L-prolyl-L-valine)	ND		13.2	3.4	13.2	2.5
Cyclododecyl isothiocyanate	ND		ND		11.2	1.8
D5-pyridine-	11.2	2.8	ND		ND	
Furaneol	5.0	4.8	6.5	4.3	5.8	9.0
Furoic acid	5.85	38.86	ND		ND	
Isopropyl isothiocyanate	10.9	0.4	6.4	5.0	6.1	1.1
Methoxymethyl isothiocyanate	ND		11.0	0.5	ND	
Neopentyl isothiocyanate	ND		8.9	0.9	ND	
Propionic acid	6.3	2.2	14.1	4.2	12.6	1.1
Pyrazine, 1,4-dioxide	5.5	1.4	ND		ND	
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	ND		12.8	1.4	14.0	2.3

RT = retention time ; ND = not detected ; CPO = crude palm oil

Lactic bacteria have been reported to produce similar flavor compounds such as benzeneacetaldehyde, furaneol, and 2(5H)-furanone. Li et al. (2022), has conducted a study to investigate the presence of volatile compounds, specifically benzeneacetaldehyde, generated through the metabolic activities of *Lactobacillus plantarum* RLL68 during the fermentation process using by-products from black tea production. Additionally, Zhu et al. (2019) discovered abundant furaneol compounds during the fermentation process of producing Msalais Wines, with concentrations ranging from 27.59 ± 0.493 mg L^{-1} to 117.6 ± 0.235 mg L^{-1}. Meanwhile, under various experimental conditions, Ndagijimana et al. (2006) identified volatile compounds, specifically 2(5H)-furanone, in *Lactobacillus helveticus*.

Table 5. The representative flavor from bioflavor compounds produced by *Lactobacillus* sp.
Tabel 5. Senyawa bioflavor yang diproduksi oleh Lactobacillus *sp.*

Bioflavor compound *Senyawa bioflavor*		Flavor *Aroma*
Furan	• 5-methylfurfural • furaneol • 2,4-dihidroxy-2,5-dimethyl-3(2H)-furan-3-one • Furoic acid • 2(5H)-furnanone	Fruity, sweet, caramel, pineapple, strawberry, coffee, almond, spicy, burned sugar (De Vivo et al., 2022)
Pyrazine	• 1,4-dioxide-pyrazine • 4-oxide-methoxy-pyrazine • 2-methyl-5-propyl-pyrazine • 2,5-dimethyl-3-propyl-pyrazine • 2-methyl-3-propylpyrazine	Roasted coffee, nutty, roasted flavor (De Vivo et al., 2022)
Pyridine	• 3-methyl-1-oxide-pyridine • 4-ethyl-1oxide-pryridine • D5-Pyridine • 2,5-dimethylpyridine N-oxide • 2-methyl-1-oxide-pyridine • 2-Amino-3-hydroxypyridine	Sour, fishy, burnt (De Vivo et al., 2022)
Isothiocyanate	• Isopropyl isothiocyanate • Neopentyl isothiocyanate • Methoxymethyl isothiocyanate • 2-tetrahydrofurfuryl isothiocyanate • Cyclododecyl isothiocyanate	Bitterness (Bell et al., 2018)
Pyyrole	• 1H-pyrrole, 2,5-dimethyl-1-phenyl- • Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Roasted coffee, sweet, nutty (De Vivo et al., 2022)
Cyclo(L-propyl-L-valine)		Bitter taste cocoa (Andruszkiewicz et al., 2019)
5-Hydroxymaltol		Caramel-like (ZhiLei et al., 2014)
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one		Rose tea(Chen et al., 2021)
Propionic acid		Sweet, nutty aroma(Bücher et al., 2021)
Benzenacetaldehyde		Sweet, bread, rose (Fan et al., 2020)
2-propenal, 3-(2-furanyl)		Fruity, spicy, woody aromas (Ma et al., 2022)

Conclusion

Substituting MRSB media with CPO for *Lactobacillus* sp. growth produced better results than OPEFB hydrolysate, especially at a 15% substitution percentage. Although there were insignificant differences in several parameters, the positive effects of the 15% substitution of CPO in the MRSB control media indicate that some of the nutrients such as carbon and nitrogen and their content can be replaced with those contained in CPO for *Lactobacillus* sp. growth. *Lactobacillus* sp.'s growth resulted in bioflavor compounds being produced in all three media types: CPO-substituted media, OPEFB hydrolysate-substituted medis, and the control media (MRSB). The highest number of flavor compounds in the control media (MRSB) was 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one (Rose tea), in the OPEFB hydrolysate substituted media, it was benzenacetaldehyde (sweet, bread, rose). In the CPO substituted media, the highest number of flavor compound was furaneol (pineapple and strawberry) and pyrazine (nutty, roasted coffee).

To summarize, this study suggests that both CPO and OPEFB hydrolysate can substitute the carbon and nitrogen sources in the growth medium to

produce bioflavor compounds in *Lactobacillus* sp. As both CPO and OPEFB hydrolysate are abundant and OPEFB is a waste product, this method could potentially reduce production costs. However, further study such as a techno-economic analysis, is necessary to determine the extent of cost production.

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