Enhancement of the ligninolytic activity of *Lysinibacillus sphaericus* by the addition of MnSO₄ and its impact on subsequent methane production from Oil Palm Empty Fruit Bunches (OPEFB)

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**A R T I C L E  I N F O**

Keywords:  
Inducer  
Lignocellulose  
*Lysinibacillus sphaericus*  
Methane  
OPEFB

**A B S T R A C T**

Palm empty fruit bunches (OPEFB) is a recalcitrant agricultural waste not readily digested which is produced in abundance. The recently isolated bacteria, *Lysinibacillus sphaericus* is a facultative anaerobe which can digest lignin. The objective of this study was to determine if the addition of *L. sphaericus* and MnSO₄ could aid the bioconversion of OPEFB, and if this could enhance methane production during subsequent anaerobic digestion (AD). The untreated OPEFB had a specific methane potential of 0.099 L/g VS which was doubled following pretreatment with *L. sphaericus*. In the presence of 0.25 mM MnSO₄ a further 1.5-fold increase in methane production was observed. This illustrates for the first time how the addition of inducers (MnSO₄) can significantly enhance this bacteria's ability to degrade lignocellulose through enhancement of its lignin degrading enzymes. Thus, enabling this agricultural waste to be utilised more effectively for biogas production.

1. Introduction

Oil palm empty fruit bunches (OPEFB) is a solid agricultural residue produced from the oil palm industry that has potential as a feedstock for the development of biomass-based biorefineries. Indonesia is one of the largest crude palm oil (CPO) producers in the world with a total oil palm production 45.12 million tons in 2021 (BPS, 2022). 21.8 % of fresh fruit bunches (FFB) is CPO while 22.5 % of the residues are labelled oil palm empty fruit bunches (OPEFB). This represents 14.3 % fiber, 6.7 % shell and 5.4 % kernel (Rohma et al., 2021). Generally, the OPEFB is used as raw material for incinerations in palm oil mills, that has a negative impact on the environment. OPEFB burning process is now regarded as something to be avoided due to the significant greenhouse gas emission associated with it (Kahar et al., 2022). As a result, large amounts of OPEFB are building up as it is recalcitrant to many types of processing. According to Suksong et al. (2016), the OPEFB contains cellulose (35–45 %), hemicellulose (25–40 %) and lignin (15–25 %) OPEFB has a relatively high cellulose and hemicellulose content compared to many types of biomass that can provide abundant sugars following adequate pretreatment. Due to its abundance and environmentally friendly an economic means of utilizing this biomass is needed. The aim of pretreatment is to break down the lignocellulosic structures and depolymerize associated polymers such as lignin and hemicellulose (Jönnsson and Martin, 2016; Mood et al., 2013; Taufikurahman and Delimanto, 2020) to release the sugar content. However, lignin is a recalcitrant compound which is associated with hemicellulose forming covalent and non-covalent bonds in the cell wall which makes its separation from the polysaccharides difficult (Nishimura et al., 2018; Terrett and Dupertre, 2019).

Certain bacteria such as *Lysinibacillus sphaericus* have been isolated from municipal solid waste that are capable of degrading lignin (Rashid et al., 2017). Previous research revealed that *L. sphaericus* was able to reduce lignin by 24 % and enhance gas production by 2.6-times under anaerobic conditions, compared to untreated samples of pine wood chippings (Rashid et al., 2017). A group of four facultative anaerobe lignin-degrading bacteria, *Lysinibacillus sphaericus*, *Comamonas testosteroni*, *Agrobacterium* sp., and *Paenibacillus* sp., have been shown to enhance methane production during anaerobic digestion (AD) (Nurika et al., 2022). The lignin content of lignocellulose can inhibit the hydrolysis process in AD and affect the volume of biogas production.

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https://doi.org/10.1016/j.biteb.2023.101394

Received 7 January 2023; Received in revised form 7 March 2023; Accepted 9 March 2023

Available online 16 March 2023

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(Hamado et al., 2020). Other lignocellulose pretreatments for OPEFB involve either phosphoric acid pretreatment (Nieves et al., 2011), or pretreatment using Bacillus sp. (Shah et al., 2019). The advantage of using a facultative anaerobe for microbial pretreatment is that the initial pretreatment can be carried out under aerobic conditions, but the microbe can then continue to break down lignocellulose under the anaerobic conditions of AD.

The ability of *L. sphaericus* to produce ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase plays a pivotal role in its lignin degradation capacity (Ayeronte et al., 2019; Bilal et al., 2023). Some metals such as manganese, copper, iron, zinc and nickel are necessary for the growth of microbes including bacteria (Vršanska et al., 2015). In this study OPEFB was inoculated by *L. sphaericus* in combination with MnSO₄ as it has been reported that laccase activity of white-rot fungi can be increased by the addition of inducers such as copper (II) sulfate (Usha et al., 2014; Vršanska et al., 2015). The addition of 1 mM MnSO₄ to *S. commune* incubated for 5 days was reported to increase the production of manganese peroxidase (MnP) up to 400 IU/ml, LiP 2400 IU/ml, and laccase 300 IU/ml (Irshad and Asgher, 2011).

Nakhate et al. (2022) found that the biogas as measured by the biochemical methane potential (BMP) test from agricultural waste is inversely proportional to the lignin content. This indicates that the lignin concentration in the biomass needs to be reduced to increase the BMP rate. In this study, the effect of similar concentrations of MnSO₄ on the expression of ligninolytic enzymes as well as their effect on lignocellulose breakdown was investigated. It is expected that the addition of MnSO₄ at a certain concentration can increase enzyme activity and the bacteria’s ability to degrade lignocellulosic OPEFB, and therefore increase methane production through increasing the availability of sugars and reducing the lignin content.

2. Materials and methods

2.1. Microorganism and culture preparation

The bacteria *L. sphaericus* was obtained from the Department of Chemistry, University of Warwick, UK. This was cultured and maintained on Luria Bertani (LB) agar plates then grown on in LB broth under sterile conditions using a shaking incubator (Heidolph); 150 rpm at temperature of 30 °C. The bacteria were then precipitated by centrifugation for 10 min (5000 rpm) and suspended with 2 ml of M9 minimal media.

2.2. Feedstock and pretreatment

The OPEFB was obtained from PT Sawit Arum Mardani, Indonesia. OPEFB was chopped into ±1 cm fragments and dried for 48 h to reduce the moisture content. The dried OPEFB was subjected to proximate analysis including moisture content, ash content, total solid (TS), volatile solid (VS), calorific value (CV), C, H, O, and N quantification. OPEFB samples were double sterilized at a temperature of 121 °C and a pressure of 1 atm for 15 min. The OPEFB was cooled down to 40–50 °C, then 30 g OPEFB was placed into a 500 ml honey jar and 300 ml of M9 minimum media added. 0.1 mM, and 0.25 mM MnSO₄ were prepared and 15 ml added along with 3 ml of suspended *L. sphaericus*. The cultures were then incubated in a shaking incubator for 5 days at a temperature of 30 °C and 150 rpm (Heidolph, model Incubator 1000). The samples were subsequently filtrated according to the method of Rashid et al. (2017) and used for analysis of moisture content, ash content, TS, VS, CHON, CV, pH, weight loss, total reducing sugar (TRS), total soluble phenols (TSP), enzyme activity, and lignocellulose concentration before and after pretreatment.

2.3. Preparation of AD inoculum

The inoculum for the BMP test was prepared from digestate taken from a full-scale mesophilic digester treating cattle slurry at Balai Besar Pelatihan Peternakan (BBPP), Batu City. The organic residue was sieved through a 1 mm screen to remove larger particles, then put into a bottle and degassed for 48 h at 37 °C. The inoculum was analyzed for temperature (°C), moisture content, ash, pH, TS, and VS.

2.4. Total soluble phenol (TSP), total reducing sugar (TRS), pH, and weight loss analysis

Total soluble phenols were measured colorimetrically using the Folin-Gioacalteau method with gallic acid as the standard and using a UV–Vis spectrophotometer at a wavelength of 765 nm (Singleton and Rossi, 1965). Total reducing sugar was measured colorimetrically by the dinitrosalicylic acid (DNS) method using glucose as the standard and using a UV–Vis spectrophotometer at a wavelength of 540 nm (Miller, 1959). Measurement of pH is carried out using a calibrated pH meter (Kulasekaran et al., 2015; Lopez et al., 2002). Weight loss calculations were carried out by drying the sample in an oven at 80 °C for 16–24 h then the weight taken (Pitt and Hocking, 2009).

2.5. Enzyme activity

1 g of sample was placed into a 250 ml Erlenmeyer and 50 mM sodium phosphate buffer pH 6.5 (10:1 v/w) added. To measure the activity of the ligninolytic enzymes extracts were taken at 0 and 5 days as follows. A total of 1 g of sample was put into a 250 ml Erlenmeyer and added 50 mM sodium phosphate buffer pH 6.5 (10:1 v/w). The extract obtained was then centrifuged for 20 min at 4000 rpm (Fisher Scientific) at 4 °C to separate the mycelium from the supernatant. The supernatant was then used for testing the activity of LiP, laccase, and MnP enzymes using spectrophotometry. The laccase enzyme activity test was determined at a wavelength of 420 nm. A total of 0.1 ml of 1 mM ABTS, 0.5 ml of 0.5 M pH 5 acetate buffer, and 0.4 ml of enzyme filtrate were mixed in a cuvette and homogenized. Oxidation was carried out with a wavelength of 420 nm (ε = 3.6 × 104 cm⁻¹ M⁻¹) with time intervals of 0 and 30 min. LiP enzyme activity test was measured based on the reaction with veratryl alcohol. A total of 0.2 ml of enzyme filtrate; 0.05 ml H₂O₂ 5 mM; 0.1 ml veratryl alcohol 8 mM; 0.2 ml 0.05 M acetate buffer pH 3; and 0.45 ml of distilled water was homogenized then the solution added to a cuvette. Oxidation was measured using a spectrophotometer with a wavelength of 310 nm at time intervals of 0 and 30 min (del Pilar Castillo et al., 1997).

The MnP enzyme activity assay was determined at a wavelength of 465 nm. The principle of this enzyme activity test is to oxidize Mn²⁺ to Mn⁴⁺. A total of 0.25 ml citrate-phosphate buffer pH 5.5 was mixed with 0.25 ml MnSO₄ 1 mM, 0.25 ml enzyme filtrate, 0.25 ml H₂O₂ 50 mM, 0.5 ml guaiacol 4 mM, and 0.375 ml of distilled water. The solution was then mixed with a vortex and then put into a cuvette. The MnP activity was measured using spectrophotometry at a wavelength of 465 nm with time intervals of 0 and 30 min. Measurement of MnP activity was obtained by carrying out a reaction with the same reagent composition as mixture A, only without the addition of MnSO₄, so that 0.625 ml of distilled water was added (mixture B). One unit of MnP activity indicates a change of 1 M Mn⁴⁺ per minute (Astuti et al., 2021; Pazla et al., 2020).

2.6. Lignocellulose content analysis

The test for lignocellulose content in the form of cellulose, hemicellulose, and lignin was carried out according to the Van Soest method (Van Soest, 1977).
2.7. BMP test

The BMP test was carried out based on Suhartini et al. (2019). Substrate and inoculum were added with a ratio of inoculum to substrate of 6:1 with a substrate ratio of 100 % OPEFB. The weight of inoculum and substrate added is based on the calculation of VS inoculum, VS substrate, and the ratio of inoculum to the substrate. 150 ml of substrate and inoculum were added to a bottle with working volume of 400 ml. The cover used is connected to the triple headcover for gas observation. Three sets of blank samples and positive controls (alpha cellulose) are included in the test. The bottle was placed in a waterbath at 37 °C. The pressure was measured using a manometer for 28 days of observation. The organic residue from the BMP test was calculated for moisture content, TS, ash content, and VS.

2.8. Kinetic analysis of BMP test

In this study, a comparison was also made between three different kinetic models, referring to Panigrahi et al. (2022), to estimate the kinetic parameters and the process performance in anaerobic digestion of the various observed substrates. The equations of the three kinetic models are shown as follows (Fernández-Rodríguez et al., 2022; Panigrahi et al., 2022; Parra-Orobio et al., 2022; Veluchamy and Kalamdhad, 2017):

Transference function model:

$$M_t = M_0 \times \left(1 - \exp \left(- \frac{M_R \cdot (t - L)}{M_0}\right)\right)$$  \hspace{1cm} (1)

Modified Gompertz model:

$$M_t = M_0 \times \exp \left(- \exp \left(\frac{M_R \cdot e^{M_0 \cdot (L - t)} + 1}{M_0}\right)\right)$$  \hspace{1cm} (2)

Logistic function model:

$$M_t = \frac{M_0}{1 + \exp \left(- \frac{e^{M_0 \cdot (L - t)} + 2}{M_R}\right)}$$  \hspace{1cm} (3)

where: $M_t =$ specific methane yield at t destruction time (L/g VS); $M_0 =$ maximum methane potential (L/g VS); $M_R =$ maximum methane production rate (L/g VS); $L =$ lag phase time (days); and $e =$ Euler’s constant (2.72). The applicability of the three models is evaluated by statistical
3. Results and discussion

3.1. Feedstock characteristics

The OPEFB characteristic including water content, ash content, TS, VS, CV, C/N ratio, C, H, O, and N were measured (Table 1). The results showed the TS and VS values of the non-treated OPEFB are 85.90 % and 80.97 % respectively. This indicated that the OPEFB is suitable to be used as a raw material in the AD process.

The C/N ratio can affect the activity of microorganisms in producing methane. Nathia-Neves et al. (2018) stated that the growth of microorganisms requires nitrogen as a nutrient for protein synthesis. In this study the C/N ratio of the OPEFB was 25.6, which could be seen as ideal. Others have reported slightly higher figures for example Hayawin et al. (2014), Tepsour et al. (2019), and Suksong et al. (2020), i.e. 53.5, 88.76, and 70.1, respectively. If the C/N ratio is too high, it will cause the degradation process to be slow. This is due to the high carbon content which causes microorganisms to spend nitrogen more quickly, resulting in a buildup of carbon (Nathia-Neves et al., 2018).

The OPEFB was found to have a calorific value of 17.394 MJ/kg, higher than the theoretical calorific value of 17.201 MJ/kg. The calorific value of biomass is a useful indicator of the potential of OPEFB to be used as a substrate for biogas production. According to Sutanto and Darmo (2022), a high calorific value indicates high methane (CH₄) potential, while a low calorific value indicates a propensity for higher carbon dioxide (CO₂) production compared to methane.

3.2. Total reducing sugar, total soluble phenols, pH and weight loss

The change of total reducing sugar before and after pretreatment can indicate the degree of depolymerization of lignocellulose. Results showed the amount of TRS from pretreated OPEFB was higher compared to non-treated samples (Fig. 1a). The highest TRS (6.504 mg g⁻¹) was obtained from pretreated OPEFB with the addition of 0.25 mM MnSO₄ incubated for 5 days. Under these conditions it increased by 62.38 % compared to the non-treated samples. The lowest TRS (5.035 mg g⁻¹) was obtained from non-treated sample. The TRS level of this study is similar to those previously observed for this feedstock (Nurika et al., 2022). The increase in TRS indicates that the cellulose and hemicellulose content of OPEFB was depolymerized by the activity of lignin-degrading bacteria which produces a certain number of sugars (Nurika et al., 2017). The degradation of lignocellulose can increase the accessibility of cellulose enzymes to hydrolyze polysaccharides into monosaccharides. According to Mahalik et al. (2018), L. sphaericus can produce enzymes such as xylanase and pectinase which can potentially degrade lignocellulosic biomass. Xylanase and cellulase enzymes in the lignocellulosic extracellular complex will interact in degrading lignocellulose (Chang et al., 2010), especially in delignifying cellulose and hemicellulose in OPEFB. The release of the sugars indicates the degree of depolymerization of the lignocellulosic components thus indicating that the bacteria are modifying the biomass. The greater the modification the more digestible components are made available for subsequent conversion during the AD phase.

Total soluble phenol indicates the amount of phenol released through the degradation of lignin by L. sphaericus. The pretreated OPEFB with MnSO₄ increased the yield of TSP compared to non-treated OPEFB sample (Fig. 1b). The highest value of TSP (0.132 mg g⁻¹) was obtained from pretreated OPEFB to which was added with 0.25 mM MnSO₄ and which was incubated for 5 days. The TSP was 57.43 % higher compared to non-treated OPEFB. The increased TSP value indicates that the lignin fraction has been degraded most likely by the ligninolytic enzymes produced by L. sphaericus. This is higher than that found with the delignification of pine lignocellulosic biomass by L. sphaericus, increased in which the phenol levels increased by up to 19.1 % of 4 days incubation and 10.0 % of 7 days incubation (Rashid et al., 2017).

In this study the pretreated OPEFB with L. sphaericus and 0.25 mM MnSO₄ resulted in a lower pH compared to other treatments. The pH value in each sample decreased following 5 days of incubation and ranged from 6.0 to 7.0 (Fig. 1c). The decrease of pH indicates the presence of organic acids produced by the bacteria during pretreatment.
The bacterium *L. sphaericus* is known to be capable of producing organic acids (Naureen et al., 2017). The longer the incubation time, the greater the amount of the organic acids produced by bacteria, which causes a decrease in pH. The optimum pH of the amount of the organic acids produced by bacteria, which causes a decrease in pH. The optimum pH of the organic acids produced by bacteria, which causes a decrease in pH.

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enzyme</th>
<th>Laccase (U/ml)</th>
<th>MnSO₄ oxidation (U/ml)</th>
<th>Peroxidase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPEFB + LS + 0 mM MnSO₄</td>
<td></td>
<td>39.2</td>
<td>330</td>
<td>291</td>
</tr>
<tr>
<td>OPEFB + LS + 0.1 mM MnSO₄</td>
<td></td>
<td>42.5</td>
<td>423</td>
<td>380</td>
</tr>
<tr>
<td>OPEFB + LS + 0.25 mM MnSO₄</td>
<td></td>
<td>56.7</td>
<td>521</td>
<td>450</td>
</tr>
</tbody>
</table>

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The percentage of weight loss from pretreated OPEFB with addition of MnSO₄ increased compared to the non-treated sample (Fig. 1d), indicating the lignocellulosic biomass is degraded by the bacteria. The highest percentage weight loss (71.4 %) was obtained from pretreated OPEFB incubated by the bacteria for 5 days with 0.25 mM MnSO₄. This could be due to the oxidation of Mn²⁺ to Mn³⁺, which can in turn oxidize phenolic lignin units through the formation of phenoxy radicals, which then causes a decrease in lignin levels in the OPEFB substrate (Datta et al., 2017). The weight loss observed indicates a highly effective breakdown of the lignocellulose. When considering how best to optimize the BMP in AD the consideration of how far this breakdown should be allowed to occur to allow the biomass breakup while maximizing the amount of methane that can subsequently be collected would need to be optimized. Currently the use of organic solvents in the recovery of sugars prior to submitting the residues for AD could inhibit AD activity. The use of bacteria would remove this risk as water can be used instead.

3.3. Lignocellulosic fractionation

The lignocellulose content of non-treated OPEFB and pretreated OPEFB with *L. sphaericus* was analyzed (Fig. 2). After 5 days treatment with *L. sphaericus*, the cellulose and hemicellulose content were unchanged or slightly increased, but the lignin content had reduced from 19.0 % to 16.8 % (Fig. 2), a reduction of 12 %. This indicates that *L. sphaericus* can degrade the lignin structure of OPEFB through pretreatment without the addition of inducers. Rashid et al. (2017), similarly showed 7 days treatment of pine lignocellulose with *L. sphaericus* is able to reduce the lignin levels by 24 %.

With the addition of the MnSO₄, the percentage of lignin removal increased compared to the sample without (see Fig. 2). The OPEFB samples with the addition of 0.25 mM MnSO₄ showed the highest lignin removal of 38.5 %, followed by samples with the addition of 0.1 mM MnSO₄ with a lignin removal of 30.1 %. This indicates that the addition of MnSO₄ can increase the lignin degrading activity of the bacteria.

3.4. Enzyme activity

*L. sphaericus* is a type of lignin-degrading bacteria that produces extracellular ligninolytic enzymes in the form of laccase and peroxidase enzymes (Ayorene et al., 2019). Enzyme assays were carried out on the bacterial supernatant for laccase activity, peroxidase activity, and manganese oxidation activity, as shown in Table 2. The results show that the addition of MnSO₄ can increase laccase, peroxidase, and manganese oxidation activity of *L. sphaericus*. The OPEFB pretreated with *L. sphaericus* in the presence of 0.25 mM MnSO₄ showed the highest manganese oxidation activity of 521 U/ml, compared to *L. sphaericus* in the presence of 0.1 mM MnSO₄ (423 U/ml) or without the addition of MnSO₄ (330 U/ml) (Table 2). MnSO₄ in the pretreated lignocellulosic biomass acts as a source of micronutrients that can increase the production of lignolytic enzymes (Acevedo et al., 2011; Asgher et al., 2011).

The addition of 0.25 mM MnSO₄ to the fungus *A. discolor* Sp4 can increase the MnP enzyme up to 1.354 U/L after 13 days incubation.

The addition of MnSO₄ was found to enhance the activity of laccase, peroxidase and manganese oxidation enzymes. Highest activity was observed in the presence of 0.25 mM MnSO₄, which led to increases of 45% in laccase activity, 58% in manganese oxidation activity, and 55% in peroxidase activity. According to Irshad and Asgher (2011), the addition of 1 mM MnSO₄ to *S. commune* was able to increase the activity of laccase up to 345 IU/ml for 8 days incubation.

3.5. Scanning electron microscopy (SEM)

The SEM analysis showed a significant change in the structure and morphology of the pretreated OPEFB by *L. sphaericus* (Fig. S1). The non-treated OPEFB samples showed a rough, stiff, dense, firm surface and irregular fiber arrangement. The rigid and dense structure of OPEFB can be due to the presence of lignin and silica bodies (Roshi et al., 2017), that act as barrier in the enzymatic digestibility (Akhtar et al., 2015). Besides containing high content of lignin, cellulose, and hemicellulose, OPEFB is also rich in inorganic elements such as silica and metal ions.

The rupture of the fiber wall surface is caused by the breakdown of lignocellulose (Brännvall et al., 2021). According to Sari et al. (2021), pretreatment can damage the fiber structure and increase the surface area of OPEFB fibers, which indicates the presence of lignin removal. In addition, there are some silica bodies that are damaged or cracked and cause open pores, which are indicated by red circles (Fig. S1). The increase in the number and size of pores also indicates that lignocellulosic delignification of the OPEFB structure has occurred (Rezende et al., 2011). Pretreatment of OPEFB for 2–12 weeks with the fungus *Ganoderma lucidum* also caused damage to the fiber structure, removing silica bodies, and opening of the pores (Nur-Nazarul et al., 2021). Singh et al. (2021) stated that the structure of lignocellulosic biomass was modified through pretreatment which introduced pores and cracks caused by the weakening of the cell wall during delignification. This led to increased efficiency of the dissolution of cellulose and increased yield of reducing sugars. However, despite changes in structure and increase in porosity, in this study the silica bodies did not disappear completely. This is probably because it was cultivated for only 5 days, which is a relatively short period for the lignocellulosic degradation to occur.

3.6. FTIR spectroscopic analysis

FTIR analysis was carried out to determine the change of molecular structure through the functional groups of OPEFB. The FTIR spectrum analysis of non-treated OPEFB and pretreated OPEFB followed by the addition of MnSO₄ is presented in Fig. S2. The results showed that the pattern of non-treated and pretreated OPEFB is almost the same, but the transmittance of pretreated OPEFB is stronger than that of non-treated OPEFB.

The pretreatment did not show any new formation of functional groups but changed the intensity of the absorption band resulting in a shift in the band. The non-treated and pretreated OPEFB showed peak clusters at a wavelength of 3200–3600 cm⁻¹ which indicates the presence of a hydroxyl group (OH) and at wavelength of 2850–2970 cm⁻¹ which indicates the presence of the CH functional group which indicates the absorption of the cellulose functional group (Sepevani et al., 2017). Rosli et al. (2017) showed that the FTIR spectrum of non-treated OPEFB, shows a strong absorption at a wavelength 3355 cm⁻¹ which is related to the stretching of the H-bonds on the hydroxyl groups of cellulose. In addition, research by Hermawan et al. (2019) and Chambre and Dochia (2021) showed an absorption area at a wavelength of 2900 cm⁻¹ which corresponds to the CH stretching of the CH₂=OH bond of cellulose.

At a wavelength of 1340–1470 cm⁻¹ there is also a peak functional group of CH indicative of the presence of cellulose and hemicellulose (Raharjo et al., 2019). In OPEFB the presence of a peak at wavelength of 1500–1600 cm⁻¹ indicates the presence of a functional group C=O.
content in the substrate can affect the performance in anaerobic digestion according to Orhorhoro et al. (2017), an increase in the percentage of volatile solids can result in a higher cumulative biogas quantity. This is in accordance with Mahat et al. (2020), who found in 100 % inoculum samples without the addition of substrate, there is no organic material that can be degraded by bacteria therefore the biogas production is low. The pretreated OPEFB with L. sphaericus resulted in a 33 % increase in cumulative biogas production. Achinas and Euvrlink (2019), stated the biomass containing a high percentage of lignin will be a challenge for the production of high yields of biogas. Therefore, pretreatment with lignin-degrading bacteria such as L. sphaericus is imperative to breakdown the lignin within the biomass. This facilitates the hydrolysis process and increases biogas production (Jos et al., 2018). In this experiment’s treatment of OPEFB with L. sphaericus in the presence of 0.1 mM MnSO$_4$ or 0.25 mM MnSO$_4$ resulted in 44 % and 52 % increases in cumulative biogas production, respectively. Pre-treatment of the sample with 0.25 mM MnSO$_4$ produced the highest cumulative net biogas volume when compared to other treatments. These results indicate that the addition of inducers in the pretreatment can increase the activity of ligninolytic enzymes, and the percentage of lignin removal (Acevedo et al., 2011). According to Sari and Budiyono (2014), a higher lignin removal content causes an increase in biogas production, so based on the results of the study, the addition of 0.25 mM MnSO$_4$ able to increase the decrease in lignin levels which was higher in OPEFB biomass compared to the addition of 0.1 mM MnSO$_4$ or without the addition of inducer.

3.7. BMP test results

3.7.1. Characteristics of samples

Proximate analysis (Table 3) in the form of measurement of water content, ash content, TS, and VS on OPEFB substrate and inoculum was carried out before the BMP test to determine sample weight and inoculum used based on the L/S ratio. The addition of 0.1 mM MnSO$_4$ to the OPEFB substrate was found to result in the highest water content of 69.43 % WW, while the non-treated OPEFB showed the lowest water content of 14.10 % WW. The highest ash content was found in non-treated OPEFB, (4.93 % WW), while the lowest ash content was found in OPEFB with the addition of 0.25 mM MnSO$_4$ (0.86 % WW). The highest VS value was found in the pretreated OPEFB sample with the addition of 0.25 mM MnSO$_4$ (97.69 % TS) and the lowest VS value was found in non-treated OPEFB (94.26 %TS).

The VS value indicates the content of organic matter in the substrate (Haryanto et al., 2019). The results showed that OPEFB pretreated with L. sphaericus contains 2.3-fold higher organic content, compared with non-treated OPEFB, and the addition of 0.1 mM or 0.25 mM MnSO$_4$ gives 2.6-fold and 2.8-fold higher organic content respectively. The VS content in the substrate can affect the performance in anaerobic digestion and thus affect the biogas production (Saraghi et al., 2019). According to Orhorhoro et al. (2017), an increase in the percentage of volatile solids can result in a higher cumulative biogas quantity. This was supported by Bacab et al. (2020) and Darwin et al. (2021), as the high volatile content indicates that the substrate or biomass contains a higher proportion of organic matter, that can be converted into biogas.

3.7.2. Specific biogas production

The average cumulative biogas production in the control sample (blank) is 48.56 ml (Fig. 3a). According to Halim et al. (2017) and Ambarsari et al. (2018), cow dung inoculum contains methanogen bacteria and the main nutrients that potentially can be used as a medium for biogas production, and therefore the increase in biogas production in the blank sample indicates that the bacteria was able to degrade the organic content (Elarsi and Afila, 2016). In this study, the blank sample exhibited lower biogas production than the alpha cellulose. This is in accordance with Mahat et al. (2020), who found in 100 % inoculum samples without the addition of substrate, there is no organic material

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water content (%) WW</th>
<th>TS (%)</th>
<th>Ash content (%) WW</th>
<th>VS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank control</td>
<td>96.94</td>
<td>3.06</td>
<td>0.58</td>
<td>80.96</td>
</tr>
<tr>
<td>Alpha cellulose</td>
<td>52.53</td>
<td>47.65</td>
<td>0.05</td>
<td>99.90</td>
</tr>
<tr>
<td>OPEFB (non-treated)</td>
<td>14.10</td>
<td>85.90</td>
<td>4.93</td>
<td>94.26</td>
</tr>
<tr>
<td>OPEFB + LS + 0 mM MnSO$_4$</td>
<td>68.27</td>
<td>31.73</td>
<td>1.33</td>
<td>95.80</td>
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<tr>
<td>OPEFB + LS + 0.1 mM MnSO$_4$</td>
<td>69.43</td>
<td>30.57</td>
<td>1.08</td>
<td>96.45</td>
</tr>
<tr>
<td>OPEFB + LS + 0.25 mM MnSO$_4$</td>
<td>62.68</td>
<td>37.2</td>
<td>0.86</td>
<td>97.9</td>
</tr>
</tbody>
</table>

The pretreated OPEFB with L. sphaericus resulted in a 33 % increase in cumulative biogas production. Achinas and Euvrlink (2019), stated the biomass containing a high percentage of lignin will be a challenge for the production of high yields of biogas. Therefore, pretreatment with lignin-degrading bacteria such as L. sphaericus is imperative to breakdown the lignin within the biomass. This facilitates the hydrolysis process and increases biogas production (Jos et al., 2018). In this experiment’s treatment of OPEFB with L. sphaericus in the presence of 0.1 mM MnSO$_4$ or 0.25 mM MnSO$_4$ resulted in 44 % and 52 % increases in cumulative biogas production, respectively. Pre-treatment of the sample with 0.25 mM MnSO$_4$ produced the highest cumulative net biogas volume when compared to other treatments. These results indicate that the addition of inducers in the pretreatment can increase the activity of ligninolytic enzymes, and the percentage of lignin removal (Acevedo et al., 2011). According to Sari and Budiyono (2014), a higher lignin removal content causes an increase in biogas production, so based on the results of the study, the addition of 0.25 mM MnSO$_4$ able to increase the decrease in lignin levels which was higher in OPEFB biomass compared to the addition of 0.1 mM MnSO$_4$ or without the addition of inducer.

3.7.3. Specific methane potential (SMP)

The specific methane potential (SMP) was obtained by comparing the methane produced with the VS content of the OPEFB substrate. The graph of the specific methane potential (Fig. 3b) shows that pretreated OPEFB was able to increase the yield of methane compared to the non-treated OPEFB. The highest SMP value on the control sample blank and alpha cellulose were 0.038 L/g VS and 0.192 L/g VS, respectively. The control sample blank showed that there was an activity of a consortium of microorganisms in the inoculum to produce methane, while the higher SMP value of alpha cellulose indicated that the consortium of microorganisms was able to work well in breaking down organic matter. In addition, the highest SMP value in non-treated OPEFB was 0.125 L/g VS (incubated for 28 days) and increased after going through the pretreatment process. The highest SMP value produced by pretreated sample without the addition of an inducer (0 mM MnSO$_4$) is 0.301 L/g VS, while the sample with the addition of 0.1 mM MnSO$_4$ is 0.340 L/g VS, and the sample with the addition of 0.25 mM MnSO$_4$ was 0.368 L/g VS. The low SMP value of non-treated OPEFB may be due to the high content of lignin in OPEFB which can inhibit the production of methane. Hidayat et al. (2020), also stated that pre-treated OPEFB was able to produce higher methane yields than non-treated samples, due to a decrease in lignin content and an increase in organic matter. Lower organic content can reduce the activity of microorganisms due to insufficient nutrients, so the gas production produced will be lower (Orhorhoro et al., 2017).

The higher volume of methane in the pretreated compared to non-treated samples support the reasoning that the pretreatment of the lignocellulosic biomass was able to increase the production of methane. The addition of MnSO$_4$ produced a higher volume of methane compared to the sample without MnSO$_4$. These results indicate that the addition of MnSO$_4$ can enhance lignin removal thus increasing methane production. It is known that metallic nutrients can increase AD performance. This is reflected in the enhancement of the control through the addition of the MnSO$_4$ alone as shown in Fig. 3b. As the figure indicates that the further enhancement cannot be due to the addition of MnSO$_4$ alone but is also attributable to the activity of the bacteria. Pretreatment breaks down the complex lignocellulose structure so that the cellulose and hemicellulose can be accessed by the hydrolytic enzymes and thus facilitate the activity of microorganisms in breaking down the organic matter providing nutrients for growth thus increasing methane production.

The average value of SMP in each sample is shown in Fig. 4. The average value of SMP in the blank sample is 0.029 L/g VS and the alpha cellulose sample is 0.152 L/g VS. The low value of SMP in the blank sample could be caused by inoculum source, poor inoculum storage or...
handling, lack of nutrients in the inoculum, and the ratio of inoculum to the substrate (Chen et al., 2019; Suhartini et al., 2021). Angelidaki et al. (2009) suggested that additional nutrients are needed to increase the metabolism of the anaerobic microorganism consortium. The aim is to increase the ability of microbes in the inoculum to degrade organic matter from biomass.

Despite the limitations of the inoculum used in this study, the SMP values in each sample still showed a significant increase in methane production (Fig. 4). The average value of SMP in the treated sample showed higher results than the non-treated OPEFB. The average SMP value in non-treated OPEFB sample was 0.099 L/g VS. The lower value of SMP is due to the higher lignin content in OPEFB, which can inhibit the production of methane. The results of this study indicate that pretreatment on OPEFB can increase the value of SMP. In addition, the pretreated sample with the addition of 0.25 mM MnSO$_4$ resulted in the highest SMP average value compared to other treatments (0.296 L/g VS). These results indicate that pretreated samples resulted in higher methane production compared to non-treated samples.

### 3.7.4. Kinetic assessments

A kinetic assessment of methane yield was applied to better understand the process of anaerobic digestion. The transference function

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Fig. 3. Graph of cumulative biogas production (ml) (a) and specific methane potential (SMP) (L/g VS) (b) from pretreated and non-treated OPEFB against blank control (inoculum) and alpha cellulose during the 28 days of observation.
model was chosen because this model follows a first order curve to relate methane production to microbial activity (Veluchamy and Kalamdhad, 2017). Meanwhile, the logistic function and modified Gompertz models were chosen because these models follow a sigmoidal function which correlates the methanogenic bacteria growth with methane production in anaerobic reactors (Li et al., 2012; Veluchamy and Kalamdhad, 2017). Based on Table 4, there are differences between non-treated OPEFB and pretreated OPEFB that has been observed. The results showed the modified Gompertz model as the best fit for all treatments, both non-treated OPEFB and pretreated OPEFB, with the lowest RMSE value and highest R-square compared to the other models. Similarly, in research by Veluchamy and Kalamdhad (2017) on methane production from thermally pretreated lignocellulosic waste, the modified Gompertz model provided the best fit for all treatments compared to either the logistic function model or the transference function model. Rosada and Budiyono (2018) also showed that the modified Gompertz model provides better predictive results compared to a first-order kinetic model, for the co-digestion of cow dung and OPEFB.

The maximum methane yields for non-treated OPEFB, pretreated OPEFB, pretreated OPEFB with 0.1 mM MnSO₄ addition, and pretreated OPEFB with 0.25 mM MnSO₄ addition were 0.125 L/g VS, 0.301 L/g VS, 0.340 L/g VS and 0.368 L/g VS, respectively. Methane production rates for non-treated OPEFB, pretreated OPEFB, pretreated OPEFB with 0.1 mM MnSO₄ addition, and pretreated OPEFB with 0.25 mM MnSO₄ addition were 0.080 L/g VS, 0.235 L/g VS, 0.266 L/g VS and 0.288 L/g VS, respectively. It is known that pretreated OPEFB (all treatments) produces higher methane than non-treated OPEFB. This is presumably because the lag phase in pretreated OPEFB (3 and 4 days) is lower than for the non-treated OPEFB (8 days). The lag phase is an important parameter to determine the initial time of biogas formation (Rosada and Budiyono, 2018). A lower lag phase is expected as a result of the delignification within the pretreated OPEFB by L. sphaericus.

![Graph showing Specific Methane Potential (SMP) of treated and untreated OPEFB against the control (blank inoculum) and alpha cellulose over 28 days of observation. The error bar shows the standard deviation for each treatment.](image)

**Table 4**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Models</th>
<th>M₀ (L/g VS)</th>
<th>Mᵣ (L/g VS)</th>
<th>L (days)</th>
<th>RMSE</th>
<th>R²</th>
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<tr>
<td>OPEFB (non-</td>
<td>Transference</td>
<td>0.125</td>
<td>0.055</td>
<td>9</td>
<td>0.328</td>
<td>0.246</td>
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<tr>
<td>treated)</td>
<td>function</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Modified</td>
<td>0.125</td>
<td>0.080</td>
<td>8</td>
<td>0.059</td>
<td>0.506</td>
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<td>0.081</td>
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<td>0.061</td>
<td>0.492</td>
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<td>Transference</td>
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<td>0.168</td>
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<td>0.232</td>
<td>0.248</td>
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<tr>
<td>0 mM MnSO₄</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Modified</td>
<td>0.301</td>
<td>0.235</td>
<td>3</td>
<td>0.178</td>
<td>0.299</td>
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<tr>
<td></td>
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<td>0.238</td>
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<td>0.186</td>
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<td>0.257</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Modified</td>
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<td>0.266</td>
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<td>0.197</td>
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<tr>
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<td>0.268</td>
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<td>0.199</td>
<td>0.305</td>
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<tr>
<td>OPEFB + LS +</td>
<td>Transference</td>
<td>0.368</td>
<td>0.200</td>
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<td>0.298</td>
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<tr>
<td>0.25 mM MnSO₄</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Modified</td>
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<td>0.288</td>
<td>4</td>
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<tr>
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<td>Gompertz</td>
<td>0.368</td>
<td>0.291</td>
<td>2</td>
<td>0.212</td>
<td>0.321</td>
</tr>
</tbody>
</table>

**Table 5**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Annotated function</th>
<th>Highest sequence similarity match</th>
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</thead>
<tbody>
<tr>
<td>LYSIN_0256</td>
<td>Flavin-dependent phenol mono-oxygenase</td>
<td>NPHA1_RHOSO 4-nitrophenol 2-mono-oxygenase, 57.7 % ID</td>
</tr>
<tr>
<td>LYSIN_0257</td>
<td>Manganese-dependent catechol 2,3- dioxygenase</td>
<td>PHEB_GEOSE catechol 2,3-dioxygenase, 59.0 % ID</td>
</tr>
<tr>
<td>LYSIN_0258</td>
<td>NADH-dependent flavin reductase</td>
<td>NPHA2_RHOSO NADH-dependent flavin reductase, 43.6 % ID</td>
</tr>
<tr>
<td>LYSIN_0259</td>
<td>2-keto-4-pentenoate hydratase</td>
<td>MIP0_BURXL 2-keto-4-pentenoate hydratase, 44.2 % ID</td>
</tr>
<tr>
<td>LYSIN_0260</td>
<td>Acetaldehyde dehydrogenase</td>
<td>ACDH_GEOSE Acetaldehyde dehydrogenase 4, 65.1 % ID</td>
</tr>
<tr>
<td>LYSIN_0261</td>
<td>4-hydroxy-2-oxovalerate aldolase</td>
<td>HOA_BACAH 4-hydroxy-2-oxovalerate aldolase, 61.8 % ID</td>
</tr>
<tr>
<td>LYSIN_0262</td>
<td>Oxalocrotonate decarboxylase</td>
<td>DMPH_PSEUF oxalocrotonate decarboxylase, 41.1 % ID</td>
</tr>
<tr>
<td>LYSIN_0263</td>
<td>2-hydroxymuconate tautomerase</td>
<td>Y4393_RAL50 2-hydroxymuconate tautomerase, 52.7 % ID</td>
</tr>
<tr>
<td>LYSIN_0264</td>
<td>2-hydroxymuconate semi-aldehyde dehydrogenase</td>
<td>DMPC_PSEUF 2-hydroxymuconate semi-aldehyde dehydrogenase 51.3 % ID</td>
</tr>
</tbody>
</table>
3. Bioinformatic analysis of L. sphaericus genome

Analysis of the genome of L. sphaericus (Table 5) revealed the presence of a putative laccase domain protein YlmD (LYSIN_01914, NCBI accession POZ57131), with sequence similarity to bacterial polyphenol oxidase enzymes. A YlmD polyphenol oxidase from Ochrobactrum sp. S10 has been characterized biochemically and found to show activity for oxidation of a lignin b-aryl ether model compound, and activity for lignocellulose oxidation (Yang et al., 2021). A YlmD homologue has also been identified and expressed in a lignin-degrading Paenibacillus strain (Granja-Travez et al., 2018). This enzyme could therefore be responsible for the observed laccase activity. There is one putative deferrochelatase/peroxidase EfeH enzyme in the genome (LYSIN_01546, NCBI accession POZ56763), which bears sequence similarity to the DypA class of dye-decolorizing peroxidases, but DypA enzymes typically lack Mn$^{2+}$ oxidation activity and activity for degradation of polymeric lignin (Ahmad et al., 2011). Microbial laccase enzymes have been reported to have activity for Mn$^{2+}$ oxidation (Höfer and Schlosser, 1999; Ridge et al., 2007), therefore, the YlmD polyphenol oxidase present in L. sphaericus could be responsible for the observed manganese oxidation activity.

Although there is no gene cluster encoding the b-ketoadipate pathway that is most commonly used by bacterial lignin degraders for conversion of low molecular weight aromatic compounds to central metabolism (Granja-Travez et al., 2020), there is a gene cluster for metabolism of catechol via LYSIN_03257 to LYSIN_03264 (Fig. 5), which bears sequence similarity to the DypA class of dye-decolorizing peroxidases, but DypA enzymes typically lack Mn$^{2+}$ oxidation activity and activity for degradation of polymeric lignin (Ahmad et al., 2011). Microbial laccase enzymes have been reported to have activity for Mn$^{2+}$ oxidation (Höfer and Schlosser, 1999; Ridge et al., 2007), therefore, the YlmD polyphenol oxidase present in L. sphaericus could be responsible for the observed manganese oxidation activity.

4. Conclusion

The lignin-degrading bacteria L. sphaericus together with the addition of MnSO$_4$ were proven to increase the OPEFB degradation and increase the ability to produce methane from the residue. The addition of 0.25 mM MnSO$_4$ resulted in the greatest observed increase (44–57 %) in enzyme activity, and also resulted in the highest lignin removal (38.6 %) and increase in methane production. This indicates that this concentration of MnSO$_4$ can increase the activity of the enzymes in degrading lignocellulose. Thus, biological pre-treatment of lignocellulose could be a valuable method for enhancement of gas production from OPEFB biomass via anaerobic digestion and could also help to reduce the amount of this lignin-rich agricultural waste thus reducing the environmental pollution which is currently becoming of great concern.

CRediT authorship contribution statement

Irina Nurika: writing original draft manuscript, conceptualization, methodology, supervision; project administration, writing review, editing, funding acquisition; Yuvira Ivana Aristya: investigation, data analysis; Nurul Azizah: data curation, visualisation; Nimas Mayang Sabrina Sunyoto: review and editing; Sri Suhartini: formal analysis, supervision; T.D.H. Bugg: writing-review and editing, supervision; G.C. Barker: writing-review and editing, supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work supported by the grants “Hibah Penelitian Unggulan” Universitas Brawijaya, Indonesia (Grant No.537.26.2/UN10.C.10/PN/


