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LIPID PRODUCTION FROM BR. 2.2 OLEAGINOUS FUNGAL ISOLATE USING ACETATE, GLYCEROL, AND MOLASSES AS CARBON SOURCES

Produksi Lipid Kapang Oleaginous Isolat BR. 2.2. dengan Sumber Karbon Asetat, Gliserol, dan Molase

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ABSTRACT
Microorganisms that accumulate more than 20% of their dry cell weight as lipids are called oleaginous microorganisms. Oleaginous microorganisms can grow well on various carbon sources other than glucose. These non-glucose alternative carbon sources could potentially reduce high biofuel manufacturing costs. BR. 2.2 isolate is an oleaginous fungus that accumulates 0.62 g L-1 lipids using glucose as a carbon source. This study aims to determine the effect of acetate, glycerol, molasses, and C/N ratios on lipid accumulation of the BR.2.2 isolate. The highest lipid produced by the BR. 2.2 isolate using acetate is 0.196 g L-1 at a C/N ratio of 400, 0.229 g L-1 at a C/N ratio of 225 using glycerol, and 1.97 g L-1 at a C/N ratio of 25 using molasses in 144 hours of incubation. The results revealed that the accumulation of lipids increased with the rising acetate and glycerol C/ N ratios and incubation period. Meanwhile, the accumulation of lipids decreased with increasing molasses C/N ratio.

Keywords: *acetate, glycerol, lipid, molasses, oleaginous*

ABSTRAK
Mikroorganisme yang memiliki kandungan minyak lebih dari 20% dari berat keringnya disebut sebagai mikroorganisme oleaginous. Mikroorganisme oleaginous dapat tumbuh dengan baik pada berbagai sumber karbon selain glukosa. Penggunaan sumber karbon selain glukosa merupakan potensi yang menjanjikan karena dapat mereduksi biaya produksi biodiesel yang tinggi. Isolat BR. 2.2 tergolong kedalam mikroorganis me oleaginous yang dibuktikan mampu mengakumulasi lipid sebesar 0,62 g L-1 dari biomassa keringnya dengan sumber karbon glukosa. Penelitian ini dilakukan untuk mengetahui pengaruh gliserol, molase, dan asetat serta rasio C/N terhadap produksi lipid. Hasil yang diperoleh Isolat BR. 2.2 mampu mengakumulasi lipid tertinggi sebesar 0,196 g L-1 pada rasio C/N 400 dengan sumber karbon asetat, 0,229 g L-1 pada rasio C/N 225 dengan sumber karbon gliserol dan. Akumulasi lipid tertinggi didapatkan ketika menggunak an sumber karbon molase dengan rasio C/N 25 dengan jumlah lipid 1,97 g L-1 pada inkubasi 144 jam. Berdasarkan penelitian dapat disimpulkan bahwa jumlah lipid yang dihasilkan mengalami peningkatan selama peningkatan waktu inkubasi dan rasio C/N dengan menggunakan sumber karbon gliserol dan asetat sementara pada molase mengalami penurunan jumlah lipid.

Kata Kunci: asetat, gliserol, lipid, molase, oleaginous

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INTRODUCTION

Lipids are one of the largest macronutrient groups that include oils and fats. Lipids have various roles in metabolism, including as an energy reserve in the bodies of animals and plants, the main constituent component of cell membranes, and as a precursor to the biosynthesis of several hormones (Akpinar-Bayizit 2014). Lipids are also used as biodiesel manufacturing materials. Biodiesel consists of esters of fatty acids and short-chain alcohols that are used in diesel engines as an alternative fuel. Biodiesel is produced on a large industrial scale by esterifying and trans-esterifying vegetable or animal oils (Chew et al. 2018). As biodiesel manufacturing has increased, agricultural products have become a more common source of lipids. Using agricultural products as the source of lipids reduces agricultural land availability for food supply (Tudge et al. 2021). Thus, finding other alternative materials to replace agricultural products as a source of lipids is crucial.

Microorganisms that accumulate more than 20% of their dry cell biomass as lipids are called oleaginous microorganisms (Chebbi et al. 2019). Oleaginous microorganisms have recently been recognized as potential feedstock in fuel production, called third-generation biofuels (Leong et al. 2018). Oleaginous fungi are one of the potential microorganisms to replace crops as a source of lipids in biodiesel production. These fungi can synthesize and accumulate lipids (Bagy et al. 2014). The composition and energy value of lipids produced by oleaginous fungi are identical to those produced by vegetable or animal oils (Kumar et al. 2019). Lipid accumulation occurs when excess carbon and limited nutrients are in the medium (e.g., nitrogen, sulfur, or phosphorus) (Karamerou and Webb 2019). Nitrogen deficiency stimulates lipogenesis and activates AMP-deaminase, which linearly decreases AMP (adenosine monophosphate) to supply ammonium to nitrogen-deficient cells (Subhash and Mohan 2015).

Furthermore, the activity of the isocitrate dehydrogenase enzyme will be inhibited, resulting in citrate production in the mitochondria. Citrate is carried to the cytosol by the ACL (ATP citrate lyase) enzyme, which is broken down and converted into acetyl CoA for fatty acid synthesis (Akpinar-Bayizit 2014). When nitrogen is no longer available or has been depleted, the carbon substrate will begin to be assimilated and then converted into lipids (Garay et al. 2014).

Using oleaginous fungi as a source of lipids in biodiesel production provides advantages over vegetable or animal oils, such as a shorter life cycle, rapid growth rate, and lipid production unaffected by the seasons or climate. The source of carbon used for the microbial oil production process is one of the critical factors in the biodiesel industry. Microorganisms that produce oil can use various carbon sources as substrates, such as carbon dioxide, glucose, molasses, xylose, glycerol, acetic acid, and ethanol, to accumulate oils. This carbon substrate is used in various metabolic pathways before finally entering the triacylglycerol synthesis pathway (TAG) (Nouri et al. 2019). Although most oleaginous microorganisms prefer simple sugars to grow, using glucose or other pure sugars to obtain microbial biomass for biodiesel production is not economically viable. Several studies have reported lipid accumulation by oleaginous microorganisms on cheaper substrates, such as glycerol (Nouri et al. 2019), molasses (Vieira et al. 2014), and citrate, which could reduce high biodiesel manufacturing costs.

BR. 2.2 isolate is an oleaginous fungus that belongs to soil fungi. Research conducted by Rizki and Ilmi (2021) revealed that BR. 2.2 isolate can accumulate lipids using glucose, resulting in a total lipid of 0.62 g L-1 with a lipid content of 28.44% at a 144-hour incubation time with a 200-rpm agitation rate. However, the ability of BR. 2.2 isolate to produce lipids in media other than glucose has yet to be explored. This study aims to determine the ability of BR. 2.2 isolate to produce lipids in a medium containing acetate, glycerol, and molasses as carbon sources.

MATERIALS AND METHODS

Materials

The materials used in this study were as follows: BR. 2.2 isolate from Faculty of Biology University of Gadjah Mada (UGM); glycerol (PT. BRATACO); acetic acid (MERCK); sugar cane molasses (PG-PS

Madukismo); Potato Dextrose Agar (PDA) (Merck); chloroform (Merck); methanol (Merck); KH2PO4 (Merck); ZnSO4·7H2O (Merck); CuSO4·5H2O (Merck); MnSO4 (Merck); MgSO4·7H2O (Merck); FeSO4·7H2O (Merck); CaCl2 (Merck); yeast extract (Himedia); KNO3 (Merck); HCl 4M (Merck); triton 0.01%; NaOH (Merck); 4M HCl (Merck); acid sand; and agar (Merck).

Subculturing of BR.2.2 isolate culture

The BR. 2.2 isolate was subcultured on PDA medium using the streak method and incubated for 7-14 days until spores were produced. The incubation temperature was set to 28 ºC. Some cultures were frozen for stock, while others were used to create a spore suspension.

Inoculum preparation

The spore suspension was prepared by growing the fungal isolate on a PDA agar slant in a test tube for 14 days until sporulation occurred. After sporulation, 0.01% triton-X was obtained by diluting 100% triton-X with distilled water. The sterilized 0.01% triton-X (7-10 mL) was poured onto the PDA agar slant until the entire surface was submerged. The surface of the slant was then scraped using a loop inoculation needle to collect the spore suspension. The spore suspension was transferred to a bottle and stored in the refrigerator.

The number of spores was determined by multilevel dilution of the suspension stock. Then, 1 mL of each dilution was taken, and the concentration was estimated using a spectrophotometer based on the absorbance reading at λ 540 nm. Subsequently, 0.1 mL of each dilution was inoculated onto PDA media using the spread plate method and incubated at 28 ºC for 14 days. The entire mold colony was then counted (Silva et al. 2013).

Lipid production with C/N variation in the medium

Lipid production of the BR. 2.2 isolate was conducted using a medium containing acetic acid, glycerol, and molasses as carbon sources. The medium composition included KH_2PO_4 (2.5 g L^{-1}), $ZnSO_4·7H_2O$ 0.01 g L^{-1}, $CuSO_4·5H_2O$ 0.001 g L^{-1}, $MnSO_4$ 0.01 g L^{-1}, $MgSO_4·7H_2O$ 0.5 g L^{-1}, $FeSO_4·7H_2O$ (0.02 g L^{-1}), $CaCl_2$ (0.1 g L^{-1}), yeast extract (1.0 g L^{-1}), KNO_3 (1.0 g L^{-1}) (Somashekar et al. 2003) and the respective carbon sources. The pH of the medium was adjusted to 5.5 using HCl or NaOH. Sterilization was carried out using an autoclave at 121 ºC and 1 atm for 15 minutes (Rizki and Ilmi 2021). To assess the effect of the C/N ratio on lipid accumulation in the BR. 2.2 isolate, the C/N ratio was varied by adding different amounts of carbon sources (Table 1).

Determination of total biomass

The fungal mycelia were cultivated in a fermentation medium and harvested at the 48th, 96th, and 144th hours. The harvested mycelial biomass was processed by f iltration using a f ilter paper (8 × 8 cm). The biomass on the f ilter paper was washed with distilled water, dried at 50 º C in an oven until a constant weight was achieved, and then weighed using an analytical balance to obtain the total biomass.

Table 1. C/N ratio and concentration variation of acetic acid, glycerol, and molasses used in the production medium

Acetic acid		Glycerol		Molasses	
C/N Ratio	Acetic Acid (g L^{-1})	C/N Ratio	Glycerol (g L^{-1})	C/N Ratio	Molasses (gr L^{-1})
50	29.08	60	36.58	25	36.60
120	71.08	90	55.35	40	81.80
200	119.08	120	74.13	55	189.8
300	179.08	150	92.90	70	733.6
400	239.08	225	139.84	-	-

Lipid extraction and lipid content calculation

The dried fungal biomass was macerated and homogenized with acid sand at a ratio of 1:2 (Somashekar et al. 2003). The fungal biomass and acid sand mixture were ground into a powder with a mortar and pestle, and then transferred into a conical flask for extraction with organic solvents (Kamoun et al. 2018). Next, the lipids were extracted by adding chloroform and methanol in a 2:1 ratio and centrifuged at 4000 rpm for 10 minutes to separate the layers. The top layer contained chloroform and lipids, while the bottom layer contained non-lipid biomass and sand. The top layer was collected in a bottle, and the solvent was evaporated. Subsequently, the lipid bottle was weighed (Rizki and Ilmi 2021).

RESULTS AND DISCUSSION

In this study, the BR. 2.2 isolate was cultivated using various carbon sources: acetic acid, glycerol, and molasses to accumulate lipids. The process of lipid accumulation in oleaginous microorganisms begins when there is a limited source of nutrients and an excess amount of carbon in the fermentation medium. This process induces lipogenesis, and metabolism is directed towards lipid synthesis.

As a result, lipid accumulation gradually increases as the concentration of acetates increases in the fermentation medium. This research investigated the influence of acetate concentration between 29.08 g L-1 and 239.08 g L-1. The highest lipid accumulation was reached with 239.08 g L-1 acetate, occurring at 144 hours of incubation, resulting in 0.196 g L-1 of lipids. Xu et al. (2017) also found a similar result, where a high amount of acetate use caused significant carbon loss due to cell consumption and resulted in better lipid production compared to low amounts of acetate. Additionally, a C/N ratio of 300 showed a high lipid content, resulting in 0.101 g L-1 with a total acetate concentration of 179.08 g L-1. In contrast, C/N ratios of 50, 120, and 200 resulted in lower lipid accumulation, with values of 0.014 g L-1, 0.017 g L-1, and 0.058 g L-1, respectively.

As illustrated in Figure 1, lipid accumulation of BR. 2.2 in a medium containing acetate as a carbon source showed a significant increase over time. The graph (Figure 1) reveals that higher lipid accumulation occurred at 144 hours of incubation. Qian et al. (2020) published similar results, stating that single acetate as a carbon source in batch culture accumulates lipids after 72 hours. This lag phase represents the time when the inoculum conditions are adapting to the fermentation medium. Additionally, it can be observed that

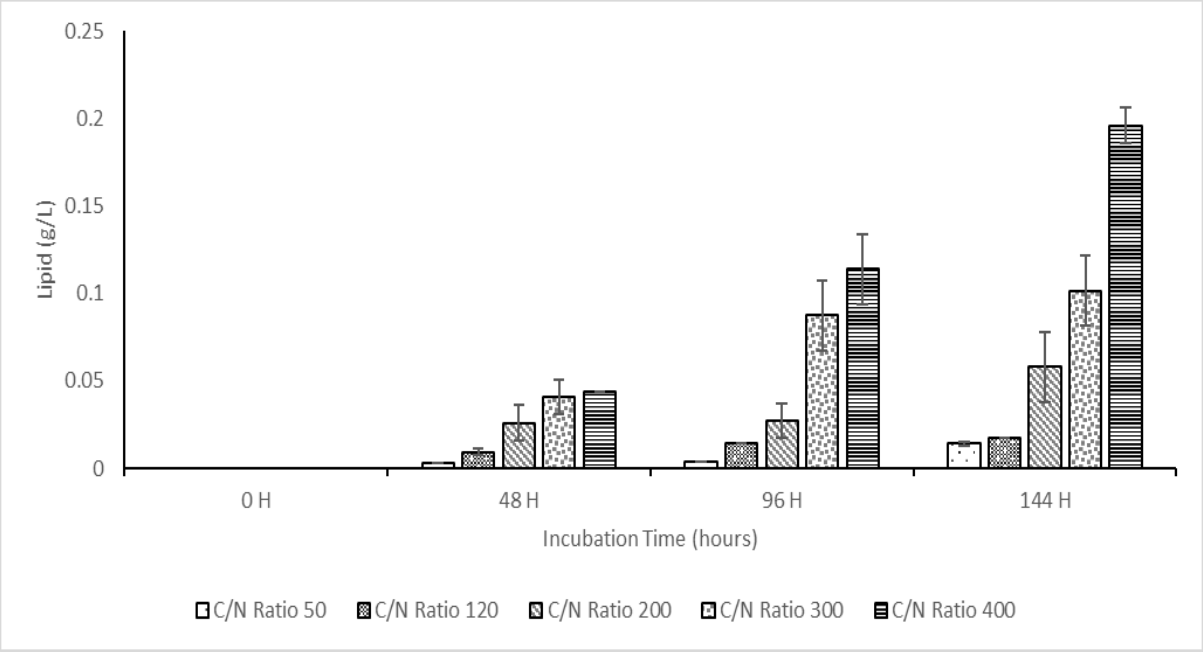

Figure 1. Comparison of lipid accumulation by BR. 2.2 isolate with varying C/N ratios of acetate and incubation time

the accumulation of lipids at 144 hours demonstrated more significant results compared to 96 hours. This is because at 96 hours of incubation, there is a build-up of citrate that inhibits lipogenesis. Therefore, maintaining an appropriate pH is crucial when using acetate as a single carbon source for microbial growth (Qian et al. 2020). According to Rossi et al. (2011), this phenomenon is likely to occur in batch culture with high carbon availability from the beginning of fermentation, which increases carbon consumption by cells. In fed-batch culture, nutrients are controlled to maintain cels in optimal metabolism. Apart from carbon availability, the pH of the culture also plays an important role in carbon consumption. Acetate has an acid dissociation value (pKa) of 4.75, which means that if the pH used is higher than its pKa, acetate can only enter the cellular membrane with active transport (Gong et al. 2016). Although the pH used in this culture is higher than the pKa of acetate, Béligon et al. (2015) showed that using a pH of 5.5 resulted in only 85% of acetate being dissociated, while at a neutral pH, 99% of acetate will be dissociated, reducing the toxic effects of acetate optimally. Based on these results, the increasing lipid accumulation of BR. 2.2 with acetate occurred along with the increasing C/N ratio. However, further research is needed to investigate lipid accumulation in fed-batch culture or continuous culture with a pH above 5.5.

As shown in Figure 2, the lipid accumulation in media containing glycerol as a carbon source exhibited a significant increase over the incubation time. The data above reveals that lipid accumulation was highest at 144 hours of incubation. Signori et al. (2016) reported similar results, stating that the lipid accumulation process consists of several phases. The initial phase, which usually lasts from 0 to 28 hours, is when the cells adapt to the conditions of the fermentation medium. At 48 hours of incubation, lipid accumulation is stil very low compared to other incubation times. The next phase is the "feeding" stage, which occurs from 28 to 48 hours of incubation, followed by the accumulation phase. In this phase, which occurs after 48 hours, glycerol as a carbon source is used as a precursor in lipid accumulation until the glycerol concentration in the fermentation medium is depleted. This phenomenon does not occur with other carbon sources. In this study, lipid production using molasses indicated lower results as the concentration of molasses in the fermentation medium increased.

Lipid accumulation showed a considerable increase with an increasing C/N ratio of glycerol. This experiment investigated the impact of glycerol

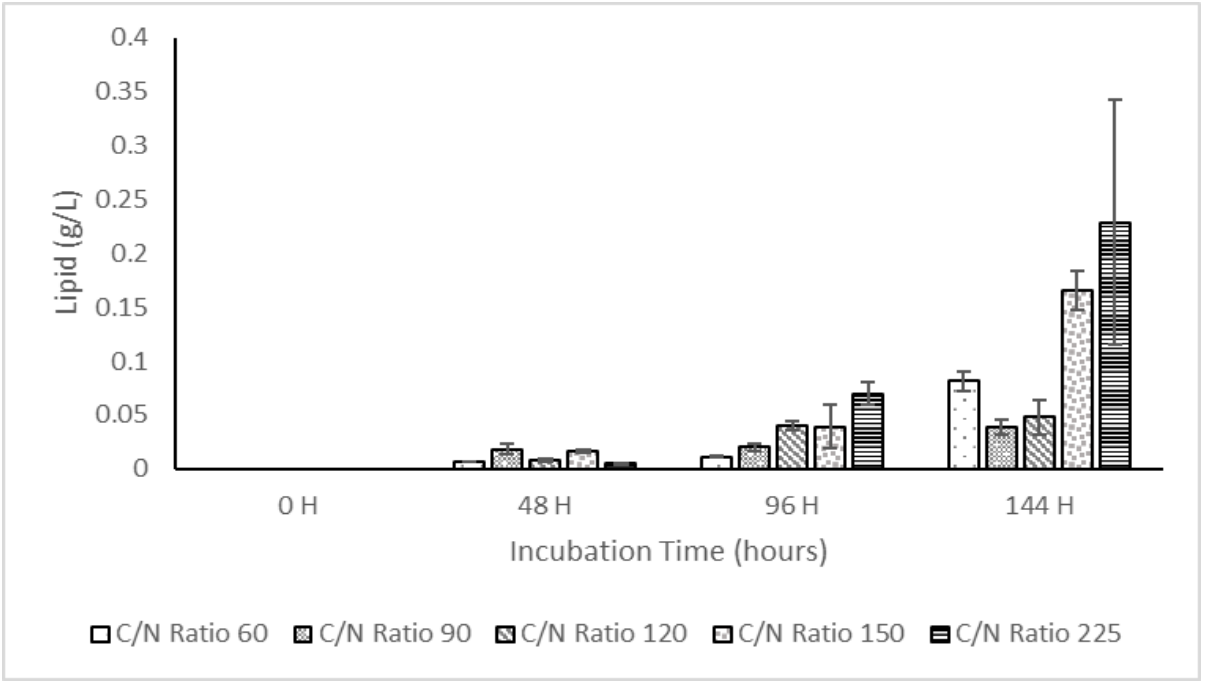

Figure 2. Comparison of lipid accumulation by BR. 2.2 isolate with varying C/N ratios of glycerol and incubation time

concentration ranging from 36.58 g L-1 to 139.84 g L-1 on lipid accumulation. The highest lipid accumulation was achieved with a C/N ratio of 225, occurring at 144 hours of incubation, resulting in a total lipid content of 0.229 g L-1 with a total glycerol supplementation of 139.84 g L-1. A C/N ratio of 150 also showed a high lipid content, resulting in a total lipid of 0.166 g L-1 with a total glycerol supplementation of 92.90 g L-1. In contrast, C/N ratios of 60, 90, and 120 indicated lower lipid accumulation, with values of 0.081 g L-1, 0.039 g L-1, and 0.048 g L-1, respectively, obtained with glycerol supplements of 36.58 g L-1, 55.35 g L-1, and 74.13 g L-1, respectively.

Based on the amount of lipid produced, it is clear that BR. 2.2 isolates are capable of producing high and optimal lipid content when the concentration of glycerol in the medium exceeds 100 at a C/N ratio. The increase in lipid accumulation is due to a limited concentration of nitrogen, while the glycerol concentration increases with the C/N ratio. Carbon is converted into triacylglycerol (TAG) and stored in lipid bodies, while nitrogen is necessary for proliferation and the growth of BR. 2.2 isolates. When the nitrogen concentration decreases, oleaginous cells show a fivefold increase in the activity of AMP deaminase before nitrogen restriction (Athenaki et al. 2018), leading to lipid accumulation. This study has shown that a C/N ratio of 225 is the optimal ratio for obtaining a high amount of lipid in BR. 2.2 isolate.

The lipid accumulation process using molasses as a carbon source showed that the highest lipid production occurred with a C/N ratio of 25 at 144 hours of incubation time, resulting in 1.97 g L-1 of lipids. This was the highest lipid yield compared to the other ratio treatments. Similarly, lipid production increased proportionally with the increasing incubation time in the C/N ratio. The highest lipid produced with a C/N ratio of 40 was 0.87 g L-1 at 144 hours of incubation time. In contrast, the treatments with C/N ratios of 50 and 70 resulted in lower lipid amounts, which likely decreased with increasing incubation time. In the case of the C/N ratio of 55, the highest lipid yield was 0.25 g L-1 at 48 hours of incubation time, while the lowest lipid yield was 0.09 g L-1 at 144 hours of incubation time. For the C/N ratio of 70, the highest lipid yield was 0.34 g L-1 at 48 hours of incubation time, while the lowest lipid yield was 0.17 g L-1 at 144 hours of incubation time.

The results presented in Figure 3 show that a higher C/N ratio of molasses leads to lower lipid accumulation. The low lipid accumulation with C/N ratios of 55 and 70 occurred due to stress from osmotic pressure on BR. 2.2 cells. High concentrations of molasses in the medium reduce free H2O molecules as they bind to sugar. This

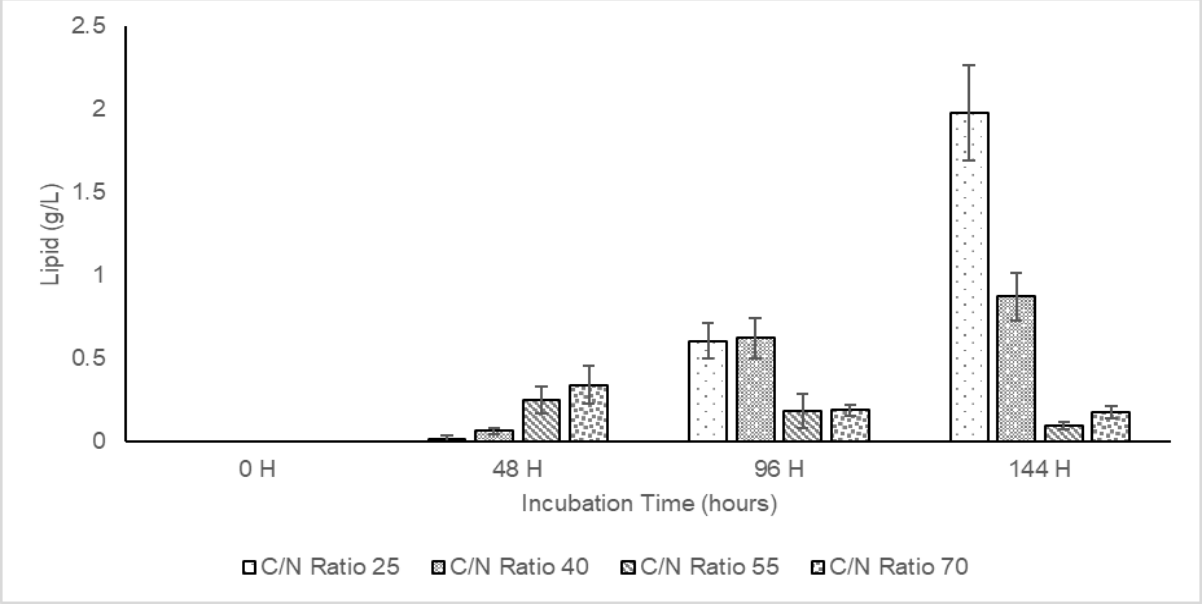

Figure 3. Comparison of lipid accumulation by BR. 2.2 isolate with varying C/N ratios of molasses and incubation time

condition causes H2O molecules to move out of the cells, resulting in cytoplasmic dehydration and decreased turgor pressure. Consequently, the cells lose their ability to perform biosynthesis, including lipid accumulation (Bremer and Kramer 2019).

According to Acosta-Piantini et al. (2023), the use of high concentrations of molasses may cause several issues during the fermentation process, such as inhibiting cell growth and inactivating enzymes involved in product biosynthesis. Molasses, as a by-product from the sugar processing industry, potentially contains organic and inorganic inhibitors for some microorganisms produced during the processing and heating of sugarcane in the plant (Sun et al. 2019). Some harmful substances, such as 5-hydroxymethylfurfural and metal ions produced during sugar production, are highly toxic to cell growth, resulting in low cell conversion and productivity (Sun et al. 2019).

CONCLUSION

BR. 2.2 isolate accumulated lipid on several alternative carbon sources such as acetate, glycerol, and molasses at various variations of C/N ratios. The increase in C/N ratio significantly increased lipid accumulation of the BR. 2.2 isolate when using acetate and glycerol as carbon sources. Specifically, acetate at a C/N ratio of 400 resulted in an amount of lipid of 0.196 g L-1, while glycerol at a C/N ratio of 225 resulted in 0.229 g L-1. Both measurements were taken at 144th incubation hours. The highest lipid accumulation occurred when molasses was used as the carbon source, with a C/N ratio of 25, resulting in an amount of lipid of 1.97 g L-1 at 144th incubation hours.

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