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The Use of Molasses in Producing Bioethanol Catalyzed by *Candida tropicalis* (Isolated from *Cocos nucifera*. L) Immobilized $MnFe_2O_4$ Coated-Chitosan

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ABSTRACT. Bioethanol is a sustainable fuel product to be an alternative energy source. Therefore, the study aims to observe and analyze the effect of $MnFe_2O_4$ coated-chitosan in increasing bioethanol production. This bioethanol was produced from molasses with a high total sugar content of up to 50% with *Candida tropicalis* as the microorganism. Fermentation is divided into two parts, namely using free *C. tropicalis* and *C. tropicalis* immobilized $MnFe_2O_4$ coated-chitosan. There was electrostatic interaction between $MnFe_2O_4$ and chitosan in 578 cm^{-1} and 659 cm^{-1} of FTIR, cubic spinel pattern in XRD, and SEM surface image of *C. tropicalis* immobilized $MnFe_2O_4$ coated-chitosan interaction. These characterization results show very good properties as a biocatalyst. The highest concentration was produced by fermentation using *C. tropicalis* immobilized $MnFe_2O_4$ coated-chitosan about 4.35% with residual sugar of 8.46 g/L. In summary *C. tropicalis* immobilized $MnFe_2O_4$ coated-chitosan have the potential to improve bioethanol products.

Keywords: *Candida tropicalis,* fermentation, $MnFe_2O_4$ coated-chitosan, molasses

INTRODUCTION

Bioethanol is an ethanol that is produced from biomass through the fermentation process which is contributed as an alternative energy source. Bioethanol production continues to increase from 2007 to 2022 about 15,400 million gallons based on Renewable Fuel Association data analysis. The growth numbers explain how bioethanol is highlighted as an interesting research topic. Bioethanol can be categorized as green and sustainable fuel whereas the raw material is renewable and it produces lower emissions (environmentally friendly) with three times ignition point higher compared to gasoline. Moreover, the purpose of increasing bioethanol production is carried out using many approaches that have been developed including genetically engineered microorganisms, substrate pretreatment, high gravity fermentation, the use of magnetic and nonmagnetic nanoparticles in the process, or the immobilization of yeast cells and enzymes into magnetic nanoparticles. The progress of the bioethanol production process has been carried out in various aspects with various challenges. Building upon it, the novelty of this study is observing the effect of adding nanoparticles to the molasses fermentation process.

The bioethanol process consists of three main points which are the preparation of raw materials, fermentation, and yield purification. The raw materials must contain sugar or starch that mostly found in biomass or agricultural industry residues. The fermentable sugars are glucose, xylose, mannose, arabinose, and galactose (Boateng, 2020). There is high possibility those sugars come from empty fruit bunches (Sukhang et al., 2019), sugarcane bagasse (Guilherme et al., 2019), napier grass (Ismail et al., 2022), eucalyptus sawdust (Guigou et al., 2018), banana pseudostem (Silva et al., 2020), corncobs (Zheng et al., 2019), rice straw (Nandal et al., 2020), potato waste (Izmilioglu & Demirci, 2017), and sugarcane molasses (Rasmey et al., 2018). Furthermore, the production of second-generation bioethanol from molasses is a good alternative for energy recovery. Sources of molasses for bioethanol production from the sugar industry waste in Indonesia are very abundant approximately 815,488 kL.

Sugarcane molasses is a high viscosity liquid which is a byproduct of the sugar crystallization process. The composition of molasses affects the results of bioethanol production which is greatly influenced by processing conditions, for example the number of centrifugations, the quality of raw materials (sugar

cane and juice), as well as environmental factors such as changes in weather and soil. Molasses consists of sugars, water, minerals, and ash. The total sugars are close to 62%. It is dominantly composed 48.8 % of sucrose and others: glucose, fructose, raffinose, and galactose (Palmonari et al., 2020). The sugar numbers in molasses validate it as a potential and low-cost carbon source for bioethanol production. In addition, a sufficient number of fermentable sugars in the fermentation process is an essential aspect.

Although, how the quality of the fermentation source is important, microorganisms are also the success key to bioethanol production. Microorganisms can convert sugar into ethanol like *Candida tropicalis*. *C. tropicalis* is the chosen fermenter agent in this study. *C. tropicalis* is a type of Candida with the ability to grow in media containing sucrose, glucose, galactose, xylose, cellobiose and ethanol. *C. tropicalis* has been isolated from honey (Kim et al., 2019), marine mud (Tan et al., 2019), pineapple (Kanakdande et al., 2019), and oil fields (Al-Dabhaan, 2021). While, the application of *C. tropicalis* in this research came from coconut water (*Cocos nucifera*. L) from the previous research of Kasmiarti et al (2022). It has Osmo, thermos, and high molasses concentration tolerance. Apart from that, *C. tropicalis* has the capability of producing enzymes (proteinase, phospholipase, and hemolysin), morphogenesis, and phenotypic transition (Alves et al., 2017). Kim et al (2019) as the setting example stated that *C. tropicalis* was growing well at a temperature of 45°C, 16% ethanol content, and 1 M NaCl. Moreover, *C. tropicalis* is resistant to some inhibitors such as acetic acid, furfural, and hydroxymethylfurfural and detoxification is not necessary for this yeast (Singh et al., 2023). These advantages suggested *C. tropicalis* a promising yeast in bioethanol production and worthy of research.

Nanoparticles are also used as catalysts in bioethanol production. Observing the interaction between nanoparticle with yeast in order to improve bioethanol production is considered as something new in the nanobiotechnology field. The addition of nanoparticles will be carried out during the substrate bioconversion process to increase bioethanol production. This process can shorten the process time which results in high productivity by simplifying product separation and purification, and the involvement of biocatalysts (Fardelone et al., 2020). $MnFe_2O_4$ coated-chitosan is the underline nanoparticle. $MnFe_2O_4$ coated-chitosan nanoparticles have good stabilization and capping capabilities, biocompatibility, biodegradability, environmentally friendly and non-toxic properties (Verma et al., 2021). It also can be synthesized easily through a one-time co-precipitation method. The findings of Caraballo et al (2021) were successful in shortening fermentation time with higher ethanol titers and productivity which was demonstrated due to the application of $MnFe_2O_4$ nanoparticles with an immobilized system. It produced 56.15 g/L ethanol for 24 hours. Surprisingly, it did not cause an increase in the activity of amino acid groups in fermentation. The use of $MnFe_2O_4$ coated-chitosan nanoparticles is expected to increase the yield of bioethanol production made from sugarcane molasses.

Optimization of the fermentation process needs to be done to improve the yield and quality of bioethanol. This improvement can focus on the basic ingredients, fermentation process, and/or extraction and purification of the results. Molasses, *C. tropicalis*, and $MnFe_2O_4$ coated-chitosan are three important aspects of this research. In conclusion, the research aims to increase the yield of molasses-based bioethanol using *C. tropicalis* with the addition of $MnFe_2O_4$ coated-chitosan.

EXPERIMENTAL SECTION

Materials

Sugarcane molasses is obtained from one of the sugar industries in Ogan Ilir Regency, South Sumatra, Indonesia. *C. tropicalis* strain MYA-3404 is the result of isolation and identification from Kasmiarti et al (2022). Chitosan (Himedia TC242), $MnFe_2O_4$ and $MnFe_2O_4$ coated-chitosan was obtained through laboratory synthesis.

Molasses Characterization and Preparation

°Brix molasses was analyzed using a pycnometer. The water content of the pycnometer is measured by putting water into the pycnometer and then weighing it. 25 mL of 2.7 g molasses solution was put into a dry pycnometer and weighed. Molasses Brix analysis uses the Mahling (1965) method described by Jazaeri et al (2018). Ash content was determined by heating 5 g of molasses added with H_2SO_4 (merck 1007310510) at 600°C for 1.5 hours (Azonwade et al., 2018). The mixed ash was weighed and its content was determined. Molasses is diluted to 5, 15, 25, and 35% to reduce its viscosity. The pH of the diluted molasses was adjusted to 3 by adding 2M H_2SO_4 and incubated at room temperature for 24 h. After incubation, the mixture was centrifuged (6000 rpm), then the precipitate formed was discarded and the diluted molasses was adjusted to pH 4.5. The molasses was then sterilized by autoclaving at 121°C and 1.2 bar for 15 min.

C. tropicalis Inoculum Preparation

The inoculum was made using 50 mL of yeast extract peptone dextrose broth media. It was added with 2 rounds of yeast culture and incubated using an incubation shaker for 24 h. Yeast growth was observed through Optical Density (OD) using a UV-VIS spectrophotometer (Orion AquaMate 8000 UV-VIS) at 600 nm.

$MnFe_2O_4$ Coated-Chitosan Synthesis

$MnFe_2O_4$ coated-chitosan preparation is a modification of the procedure from Caraballo et al

(2021). $MnSO_4.H_2O$ (merck 102786), $FeCl_3.6H_2O$ (merck 103943), NaOH (merck 109139), and chitosan (low molecular weight and deacetylation degree 75%) are the ingredients used to make nano-sized $MnFe_2O_4$ powder which is made using the co-precipitation method. $FeCl_3.6H_2O$ (0.3 M) and $MnSO_4.H_2O$ (0.15 M) were dissolved together in 50 mL of water each with a Fe:Mn cation ratio of 2:1 at a temperature of 70°C. Chitosan was added at 0.125% (w/v) and stirred until dissolved. 20 mL of NaOH solution (2M) was added slowly to 100 mL of the mixture until the pH was 10-11. A dark brown precipitate will form after the reaction and then be separated with a magnet. The solid was washed with distilled water to remove impurities until the solution reached pH 7. The sample was dried in an oven at 40°C for 3 h. The solid obtained is ground with a mortar. $MnFe_2O_4$ is made in the same way. $MnFe_2O_4$ coated-chitosan was stored in a desiccator at room temperature before being used for fermentation.

$MnFe_2O_4$ Coated-Chitosan Characterization

Changes in the structure of $MnFe_2O_4$ and $MnFe_2O_4$ coated-chitosan can be seen from X-ray patterns recorded using a Siemens D-5000 diffractometer operating with Cu-Kα radiation (λ = 1.54056 Å) at 35 kV and 25 mA in the range of 2 to 80°C with scanning speed 0.02°C/s. The functional groups of $MnFe_2O_4$ coated-chitosan particles and $MnFe_2O_4$ coated-chitosan immobilized yeast were characterized using an FTIR (Fourier transformed infrared) spectrometer (Thermo Scientific Nicolet iS10 FT-IR Spectrometer). KBr pellets containing samples of 1.5 mg and 15 mg KBr were run at wave numbers of 400-4000 cm-1. The morphology of $MnFe_2O_4$, $MnFe_2O_4$ coated-chitosan, and yeast immobilized $MnFe_2O_4$ coated-chitosan was analyzed using the Scanning Electron Microscope-Energy Dispersive X-Ray Spectroscopy method (JEOL-JSM-6510 LA).

Fermentation

Fermentation was carried out using Arshad et al (2017) method modification whereas an anaerobic fermentation was carried out in a 500 mL Erlenmeyer using 150 mL of molasses media with variations in % (5, 15, 25, 35) using a solution of 1.0 g/L KH_2PO_4 (Merck 529568), 1.59 g/L $(NH_4)_2SO_4$ (Merck 101217), and 0.5 g/L $MgSO_4.7H_2O$ (Merck 172572), then this solution was sterilized by autoclaving. The inoculum of each yeast was added as much as 3 mL (30 mL of stock solution). $MnFe_2O_4$ coated-chitosan was added to each yeast inoculum 30 minutes before fermentation around 460 mg/mL. Fermentation was carried out for 48 h at a speed of 150 rpm and 30 °C where measurements of ethanol content were carried out every 12 h using Gas Chromatography (Shimadzu Gc-2010 plus). Analysis of glucose reduction levels was carried out using the DNS (Dinitrosalicylic Acid) method by measuring the absorbance of the sample at a wavelength of 540 nm with a UV-Vis (Spectrophotometer Orion AquaMate 8000 UV-VIS). The absorbance value is entered into the linear equation of the glucose standard curve.

RESULTS AND DISCUSSION

Molasses Characterization and Preparation

Sugarcane molasses is a thick liquid by-product of the sucrose crystal in the centrifugation process during sugar production. Blackstrap molasses is a type of molasses used in bioethanol production with a content of 36-55% total sugar with 80-90°brix (Raby et al., 2023). The blackstrap molasses sample in the study had 87°brix with 8.6% ash content. In general, samples with this value contain ≥40% sucrose, 14% glucose, 25% water and organic salts to amino acids. These results indicated that molasses sample is a suitable media for fermentation.

FTIR (Fourier Transformed Infrared)

FTIR analysis was carried out in the absorption range of 400-4000 cm^{-1} which is the basic region for detecting the presence of chemical bonds and functional groups. **Figure 1a** is $MnFe_2O_4$ coated-chitosan and **Figure 1b** is bare $MnFe_2O_4$. In general, spinel ferrite is detected in the 400-600 cm^{-1} band. In **Figure 1b**, the band at 578 cm^{-1} is Fe-O and 421 cm^{-1} is Mn-O. The spectrum at 3423 cm^{-1} and 3416 cm^{-1} (**Figure 1a**) is possible as the amino group of chitosan ovulates with O-H from the stretching vibration of $MnFe_2O_4$. The electrostatic interaction of Fe ($MnFe_2O_4$) with NH_2 (chitosan) is proven by the presence of absorption at 578 cm^{-1} and 659 cm^{-1} (Haghiri & Izanloo, 2018). $MnFe_2O_4$ is a group of ferrite compounds that have a large absorption capacity with a high volume to surface area. This property makes $MnFe_2O_4$ can enhance and surround the yeast cells. Chitosan functions as a host and stabilizer for $MnFe_2O_4$ because of its chelating ability. Liew et al (2019) was described the coating process acts as a protector to prevent degradation of $MnFe_2O_4$ during the catalytic reaction process. This activity run under the cross-linked bonding between chitosan and $MnFe_2O_4$.

XRD analysis was done to obtain the diffraction patterns of $MnFe_2O_4$ and $MnFe_2O_4$ coated-chitosan compounds. Based on research results published by Tang et al (2020), the structure of cubic spinel $MnFe_2O_4$ (JCPS No. 29-0713) is at 29.65°, 34.92°, 42.43°, 56.08° and 61.56° with (220), (400), (511), and (440) sequentially at the 2θ. In this study, 2θ peaks were found at 29.7°, 34.9°, 42.5°, 56.2°, and 61.6° which were followed by intensities in sequence (220), (400), (511), (422) and (440). According to Scherrer equation, the average crystallite size was 14.662 nm. In addition, at 18° a slight peak appears indicating chitosan. The obtained pattern and nanoparticle size of XRD data are strongly describe the $MnFe_2O_4$ nanoparticle.

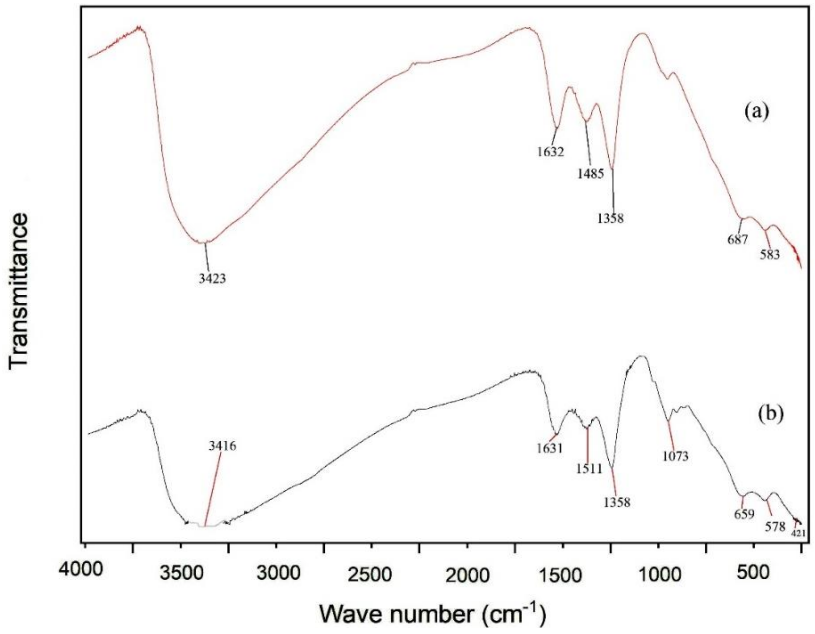

Figure 1. Fourier transform infrared (FTIR) spectrum of (a) $MnFe_2O_4$ coated-chitosan (b) $MnFe_2O_4$.

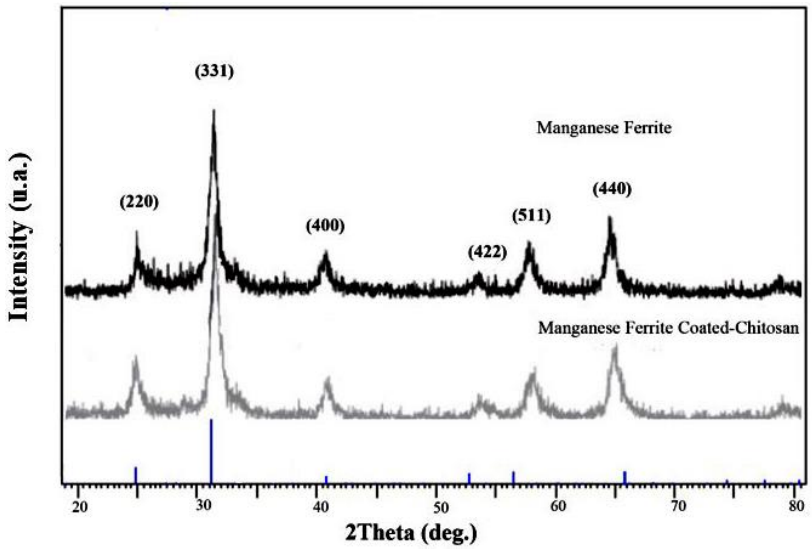

Figure 2. XRD pattern of $MnFe_2O_4$ and chitosan-coated $MnFe_2O_4$.

Scanning Electron Microscopy

SEM (Scanning Electron Microscopy) is an instrument that is used to analyze the surface structure of yeast, $MnFe_2O_4$ coated-chitosan, yeast immobilized $MnFe_2O_4$ coated-chitosan. **Figure 3a** shows the surface of *C. tropicalis* where the yeast colonies are cream-white in color, oval, and have a smooth texture. In the picture, it can be seen that the *C. tropicalis* cells are of the multipolar budding type. **Figure 3b** is the surface structure of $MnFe_2O_4$ coated-chitosan. These nano particles are made using a one-way precipitation method so that the process becomes more effective and efficient.

Figure 3c describes the results of immobilization of *C. tropicalis* with chitosan-coated $MnFe_2O_4$. In this image, there are $MnFe_2O_4$ coated-chitosan granules between the gap of *C. tropicalis*. Modification occurs through the process of attracting chitosan to the surface of $MnFe_2O_4$, then this compound interacts with free cells of *C. tropicalis*. This process takes place before molasses fermentation takes place where several nanoparticles are reacted into the broth containing *C. tropicalis*. Faramarzi et al (2019) and Ingale et al (2019) also proved that yeast does have the ability to interact with metal compounds such as selenium and magnetic beads.

Fermentation

Molasses fermentation using *C. tropicalis* was put through concentrations of 5, 15, 25, and 35% as a screening step in which the concentration of the yeast worked optimally. Fermentation was conducted for 48 hours and each sample was analyzed every 12 h in **Figure 4**. Based on data from the beginning to the end of fermentation, the media with a concentration of

25% was the best compared to the others. A total of 2.45% bioethanol was produced, which is also the highest value among others.

Moreover, there was a decreasing trend of bioethanol yield at 36-48 h at 35% concentration. This decline may have occurred due to the influence of media tolerance experienced by *C. tropicalis*. Even though the concentration of the carbon source is large but it exceeds the capacity of *C. tropicalis* to convert it into ethanol. Further observation shows that *C. tropicalis* was in a stagnant phase after 36 h and then decreased significantly.

Unusual changing is shown by the graph of molasses fermentation at 5% in **Figure 4**. This is the smallest concentration in fermentation. It drastically dropped at 48 h because the low content of fermentable sugars in the media. In addition, the best growth of *C. tropicalis* was after 18 h (Nordin & Razak., 2014). This is proven by the appearance of an exponential peak point at 36 hours as a sign of the maximum process in sugar to ethanol conversion. In conclusion, sugar was absent after this accomplishment.

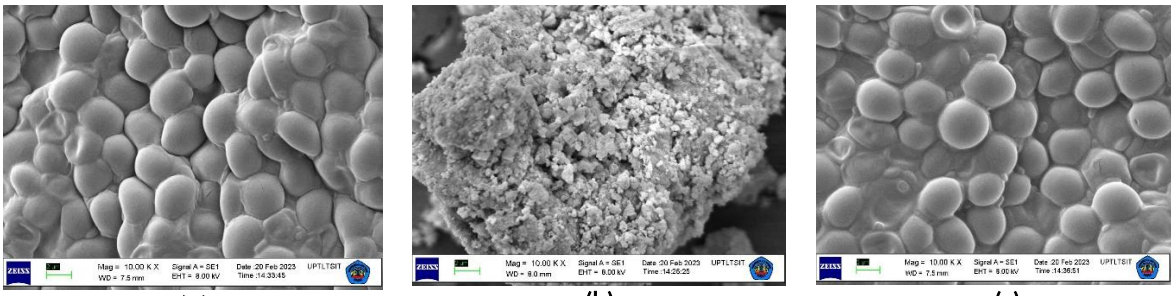

Figure 3. SEM images of (**a**) *C. tropicalis*, (**b**) $MnFe_2O_4$, and (**c**) *C. tropicalis* with $MnFe_2O_4$ coated-chitosan.

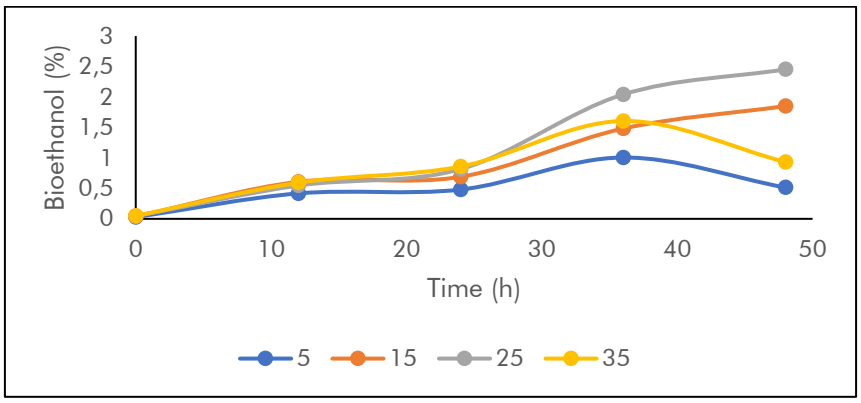

Figure 4. Bioethanol yield from molasses fermentation in 5, 15, 25, and 35% using *C. tropicalis*.

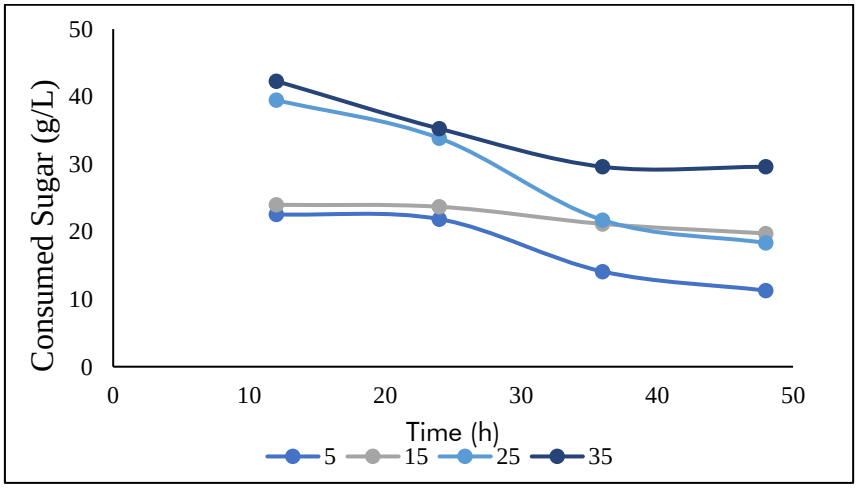

Figure 5. The concentration of sugar consumed from molasses fermentation in 5, 15, 25, and 35% using *C. tropicalis*.

Figure 5 is a consumed sugar graph during fermentation. The media with a concentration of 25% undergo the largest alleviation from 38 g/L to 18 g/L after 48 hours of fermentation whereas the bioethanol produce was 2.45%. The largest reduction of the sugar consumption was in line with the highest number of bioethanol produce. This result even exceeds the values of 15% and 35%. This difference supports the argument that 25% is the best composition of media for the current fermentation. Furthermore, *C. tropicalis* able to ferment half or even more sugar than the initial concentration due to its good bioconversion metabolic capabilities (Madian et al., 2022).

A comparison of bioethanol results from fermentation by free *C. tropicalis* and *C. tropicalis* immobilized chitosan is presented in **Figure 6**. Fermentation using immobilized *C. tropicalis* produces more bioethanol, namely 4.35%. This result is twice compared to fermentation with free *C. tropicalis*. Previously, Sanusi et al (2021) were also able to increase bioethanol production by 1.6 times through the application of yeast inclusions with nanoparticle. This is related to the role of $MnFe_2O_4$ coated-chitosan because of its catalytic properties which play an important role in electron transfer when reacting. The surface of *C. tropicalis* cells is attached to nanoparticles through electrostatic and hydrophobic bonds. The presence of chitosan in the $MnFe_2O_4$ surrounding the cells also protects *C. tropicalis* from toxicity that causes cell death (Muhammad & Badshah, 2019). This chemical interaction is one of the factors in the large yield obtained because there is more *C. tropicalis* in fermentation over a long period.

Figure 7 shows a comparison of sugar consumption values during fermentation that lasted 48 hours. Initially, both media had relatively the same total amount of sugars 43.71 g/L. During fermentation time, bioconversion of *C. tropicalis* with $MnFe_2O_4$ coated-chitosan consumes more sugar due to the high yield of bioethanol. Sugar consumption looks stable in **Figure 7 25b.** This consistency is related to the *C. tropicalis* being immobilized by $MnFe_2O_4$ coated-chitosan due to the high cell density against the inhibitors.

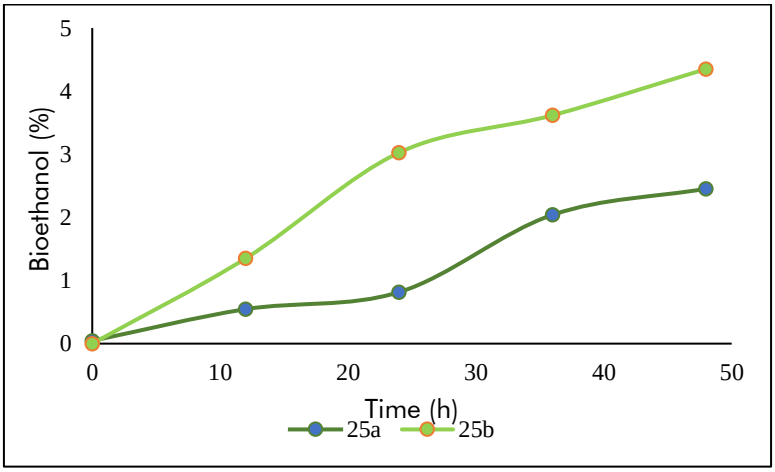

Figure 6. The comparison of bioethanol yield from molasses fermentation 25a (*C. tropicalis* with $MnFe_2O_4$ coated-chitosan) and 25b (*C. tropicalis*).

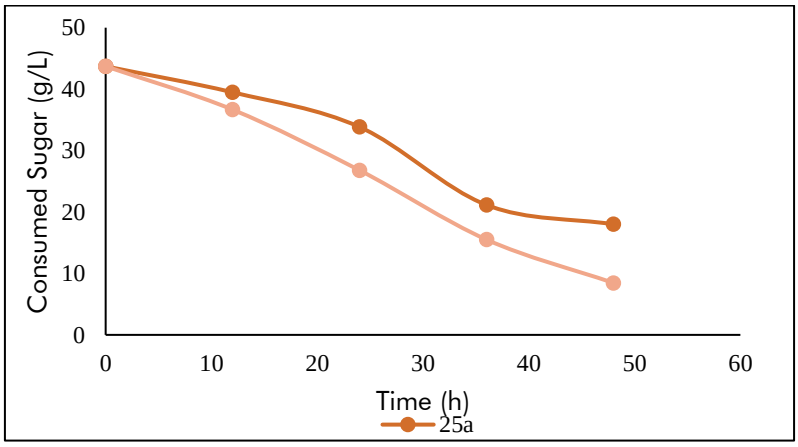

Figure 7. The comparison of consumed sugar from molasses fermentation 25a (*C. tropicalis*) and 25b (*C. tropicalis* with $MnFe_2O_4$ coated- chitosan).

Table 1. Fermentation results of free *C. tropicalis*

Media (%)	Final Bioethanol (%)	Final Consumed Sugar (g/L)	
		12 (h)	48 (h)
5	0.51	22.56	11.28
15	1.85	23.7	19.74
25	2.04	39.48	18.33
35	0.93	42.3	29.61

Table 2. Fermentation results of free *C. tropicalis* immobilized $MnFe_2O_4$ coated-chitosan

Media (%)	Final Bioethanol (%)	Final Consumed Sugar (g/L)	
		0 (h)	48 (h)
25a	2.45	43.71	18
25b	4.35	43.71	8.46

Table 1 shows that there was a decreasing in sugar concentration for each fermented molasses concentration. The difference in initial and final sugar concentrations using *C. tropicalis* was 11.28, 3.96, 21.15, and 12.69 g/L respectively from the concentration of 5, 15, 25, and 35%. The data comparison of fermentation results in molasses concentration of 25% using and not using $MnFe_2O_4$ coated-chitosan was in Table 2. The difference in sugar consumption concentration was 25.71 g/L and 35.25 g/L for *C. tropicalis* and *C. tropicalis* immobilized $MnFe_2O_4$ coated-chitosan respectively. Fermentation of molasses using *C. tropicalis* immobilized $MnFe_2O_4$ coated-chitosan was consumed more sugar with highest bioethanol concentration 4.35%.

It is necessary to conclude that $MnFe_2O_4$ coated-chitosan brings the desired impact on fermentation results. Whereas *C. tropicalis* immobilized $MnFe_2O_4$ coated- chitosan improve the final amount the bioethanol. It is because the immobilized activity can provide the ideal conditions for the fermentation. The media conditions are less intrusion of inhibitors, abundant cell density, effective and efficient utilizing time, and a relatively high source of carbons.

CONCLUSIONS

Molasses is a good fermentation medium for producing bioethanol because of its large sugar content (87°brix). The highest concentration was produced by fermentation using *C. tropicalis* immobilized $MnFe_2O_4$ coated-chitosan about 4.35% with residual sugar of 8.46 g/L. This result was two times higher compared to fermentation only with free *C. tropicalis.* Synthesizing, characterizing, and applying $MnFe_2O_4$ coated-chitosan to *C. tropicalis* used in molasses fermentation has been proven to increase bioethanol yield.

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