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Powdered Inoculum: A New Approach for Improving Bioethanol Production During Simultaneous Saccharification and Fermentation (SSF) of Lignocellulosic Biomass

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This study presents a practical approach to enhancing microbial inoculum availability through the development of powdered inoculum for use in simultaneous saccharification and fermentation (SSF) of lignocellulosic materials for bioethanol production. The powdered inoculum is composed of a consortium of three microbes: *Trichoderma reesei*, *Saccharomyces cerevisiae*, and *Zymomonas mobilis*. Rice, corn, and soybean flour, supplemented with 5% (w/w) glucose, serve as carriers for the microbial consortium. The stability and viability of the powdered inoculum were evaluated using the Total Plate Count (TPC) method. The inoculum was applied to lignocellulosic material in the form of Dewaka banana pseudostem (DBP), which underwent pretreatment involving chemical-free pressure heating and drying. The powdered inoculum was applied to the lignocellulosic material for a 15-day fermentation period. Parameters monitored included optical density, reducing sugar levels, and bioethanol concentration. The results showed that microbial growth in the inoculum at the time of preparation was 1.26×10^7 CFU g^{-1}, and after four weeks of storage, it was 9.42×10^8 CFU g^{-1}. The bioethanol yield was 34.85 g g^{-1} substrate on the third day of inoculation, with a bioethanol concentration of 12.25% achieved by the 12th day.

Keywords: Powdered inoculum, simultaneous saccharification and fermentation (ssf), bioethanol production, lignocellulosic biomass, microbial inoculum.

INTRODUCTION

Currently, fossil fuels are the most widely used source of energy. However, despite increasing consumption rates in Indonesia and globally, production levels are decreasing. This widening gap between production and consumption highlights the need for alternative energy sources, such as biomass. Bioethanol is a type of fuel produced from biomass. The process of creating bioethanol from biomass involves three distinct stages: pretreatment, hydrolysis, and fermentation. During the pretreatment stage, cellulose is separated from hemicellulose and lignin. The hydrolysis stage follows, where the cellulose is converted into sugar (also known as saccharification), which is then fermented to produce bioethanol. Producing bioethanol from biomass is a lengthy and expensive process. Originally, it involved separate treatments for hydrolysis, fermentation, and pretreatment. However, for greater efficiency and better time management, bioethanol production now utilizes an integrated hydrolysis and fermentation process. Efficient simultaneous saccharification and fermentation (SSF) in high solids is one of the keys to the successful commercialization of lignocellulose-based bioethanol manufacturing processes. Based on techno-economic calculations, the SSF process for bioethanol production from lignocellulosic materials is designed with (1) the fermentation of C_5 and C_6 sugars by a consortium of microbes, and (2) a cellulose and hemicellulase enzyme mixture that works synergistically in the saccharification process (Khajeeram and Unrean, 2017). These processes have been developed by utilizing several types of microbes simultaneously, either in the form of microbial consortia or recombinant microbes (Gusakov,

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2011; Vats *et al.*, 2013; Kricka *et al.*, 2015; Bu *et al.*, 2019). Microbes, whether as microbial consortia or recombinant microbes, are employed by leveraging their differing characteristics. The use of promising microbial consortia faces challenges related to providing microbes that require specific conditions, such as those found in laboratory settings. One approach to address this challenge is to temporarily weaken the microbes in the form of inoculum, similar to the yeast used in the production of tape and tempeh. For generations, inoculum/yeast has been a consortium of microorganisms used in the fermentation process to produce *tape* and *tempeh*. Inoculum with a consortium of microorganisms, such as the yeast used for *tape* or *tempeh*, can serve as an alternative to laboratory-prepared inoculum or starter cultures. Previous research has explored the use of powdered inoculum for the decomposition of lignocellulosic materials into bioethanol. This powdered inoculum is made from a carrier material consisting of a mixture of corn flour, tapioca, rice flour, and wheat flour. The microbial consortium used includes *S. cerevisiae*, *Z. mobilis*, and *K. marxianus* for the production of bioethanol from used newspaper (Safitri *et al.*, 2017). The consortium of microorganisms is first grown in a medium containing the essential nutrients needed for the microorganisms to thrive. A powdered inoculum has been created from a consortium of *T. reesei*, *S. cerevisiae*, and *Z. mobilis* on a mixture of rice, corn, soybeans, and 5% (w/w) glucose, which can be used in bioethanol production. The powdered inoculum will be tested on lignocellulosic material, in this case, chopped Dewaka banana pseudostem (DBP), which has undergone pretreatment involving pressurized heating for 2 hours and soaking in distilled water for 24 hours. The pretreatment used is chemical-free. The powdered inoculum has an effectiveness of 1.26 x 10^7 CFU/g. This study aims to determine the effectiveness of this powdered inoculum in the process of producing bioethanol from lignocellulosic raw materials. The effectiveness of the inoculum will be evaluated based on optical density, reducing sugar content, and bioethanol levels.

MATERIALS AND METHODS

This research applies a trial and error method, where various approaches are tested directly to find the most effective solution. This process involves repeated experiments by modifying variables until the desired result is achieved. This method allows for empirically identifying the optimal approach through observation and evaluation of different experimental results. This research was carried out from August 2022 to May 2023.

***Preparation of carrier materials*:** The complete process for producing powder inoculum is illustrated in Figure 1. The procedure involves several stages, beginning with the preparation of the growth medium. This medium is made from coarsely ground rice, corn, and soybeans, which provide essential nutrients for microbial growth (Aljammas, 2021). The raw materials are carefully selected and ground to the desired consistency to create an optimal environment for the inoculum (Veerabadhran *et al.*, 2021). In Figure 1 the carrier materials rice, corn, and soybeans are first cleaned and soaked separately in clean water for 6 hours. After soaking, they are drained and dried until slightly moist, then coarsely ground and dried at 60°C for 8 hours. The processed materials are stored separately until use. Each step of the process is designed to ensure the quality and effectiveness of the final powdered inoculum (Luangthongkam *et al.*, 2021).

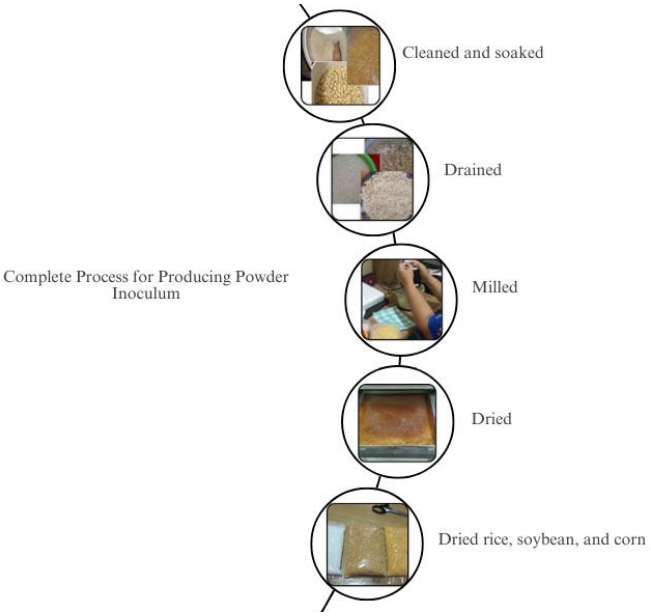

Figure 1. Process for producing carrier materials.

***Microbial Propagation*:** The next stage is microbial propagation (see Figure 2). Pure cultures of *T. reesei*, *S. cerevisiae*, and *Z. mobilis*, obtained from the Food and Nutrition Culture Collection (FNCC) at the Center for Food and Nutrition Studies, Gadjah Mada University, are used. Potato Dextrose Broth (PDB) and Nutrient Broth (NB), both Merck products prepared according to packaging instructions, serve as the growth media. After sterilization, these media are inoculated with the pure cultures at a 1:9 ratios. *S. cerevisiae* is incubated at 30°C for 2 days in PDB, *Z. mobilis* at 37°C for 2 days in NB, and *T. reesei* at 25°C for 5 days in PDB.

Powdered Inoculum Preparations: Subsequently, the complete process for making powder inoculum begins with the preparation of a growth medium from pre-processed rice, corn, and soybeans, mixed in a 1:1:1 ratio and supplemented with 5% (w/w) glucose. This mixture is thoroughly combined in an Erlenmeyer flask, sealed with cotton and aluminum foil, and sterilized using an autoclave at 121°C and 15 psi for 15 minutes before cooling. The medium was then aseptically inoculated with 3% (v/v) of *T. reesei*, *S. cerevisiae*, and *Z.*

mobilis starters and incubated at room temperature for 48 hours. Following incubation, the inoculum is dried at 40°C for six hours, ground, and sieved to produce the powder inoculum.

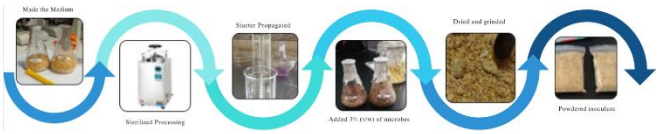

Figure 2. The stages of powdered inoculum manufacture.

***Pretreatment*:** The lignocellulosic material used in this research is Dewaka banana pseudostem (DBP) from Merauke. The pretreatment process for the material used for this test can be seen in Figure 3. First, the DBP is chopped into a uniform size. Next, pressure heating was carried out using an autoclave at a temperature of 121 °C and a pressure of 15 psi for 2 hours. The material remains stored in the autoclave for 12 hours in the cooling process. The material is then removed from the autoclave and soaked in distilled water in a ratio of 1:2 (material and distilled water) for 24 hours. Next, the material was dried at 40 °C for 12 hours and ground.

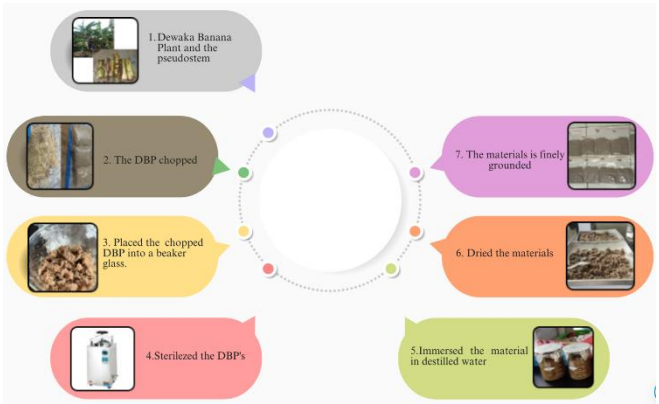

Figure 3. Pretreatment.

***Bioethanol Production Process*:** An overview of the sample inoculation process complete with starter can be seen in Figure 4. The process of making bioethanol begins by preparing the starter. The starter was made by mixing 0.5 gram powdered inoculum, 0.5-gram brown sugar, 3 grams per kilogram of ZA, and 9.0 milliliters of distilled water in a test tube, sealed with cotton wool and aluminum foil. The mixture was homogenized using a vortex. The starter is incubated at room temperature for 2 days and is ready to use. The substrate in the form of dry DBP is placed in a fermentation bottle. Water content was adjusted to 90% by adding distilled water. 2 ml of starter was added for every 30-gram weight of substrate. The process of making bioethanol was carried out for 15 days, with data collection carried out on days 3, 6, 9, 12, and 15.

Each time data was collected, 3 cm^3 of the sample was separated to observe optical density, and another sample was used to observe the levels of reducing sugar and bioethanol. Samples to observe the levels of reducing sugar and bioethanol were centrifuged at 400 rpm for 30 minutes. The supernatant solution was then filtered using Whatman filter paper No. 1. 0.75 ml filtrate is used to measure reducing sugar. Another filtrate will be distilled at 70 °C using a rotary evaporator to determine the ethanol content (Itelima *et al.*, 2013). The optical density value was measured using a SHIMADZU UV-VIS UV-1280 spectrophotometer at a wavelength of 690 nm. Reducing sugar levels were measured using a SHIMADZU UV-VIS UV-1280 spectrophotometer at a wavelength of 510 nm. Bioethanol content was measured using an alcohol meter using dilution theory (Tenkolu *et al.*, 2024).

Figure 4. Inoculation samples with starter.

***Analysis Procedure*:** During the preparation of the inoculum, a test is carried out on the total number of microbes to determine the ability of the microbes to survive in the inoculum. Testing was carried out using the TPC (Total Plate Count) method (Fardiaz, 1993; Safitri *et al.*, 2011). TPC testing was carried out every 8 hours for 2 days. During fermentation, microbial growth is measured using optical density values. The optical density value was measured with a SHIMADZU UV-VIS UV-1280 spectrophotometer at a wavelength of 690 nm. The measured sample is 3 cm3. Blank measurements are carried out by measuring the sample solution that is not inoculated with a starter (Itelima *et al.*, 2013). The testing process for reducing sugar levels begins with the preparation of DNS (dinitrosalicylic acid) reagent and the creation of a standard curve. The sample, consisting of 0.75 ml of filtered supernatant solution and 2.25 ml of DNS reagent, was homogenized. The sample solution was heated to 100 °C for 15 minutes, then allowed to cool to room temperature. A SHIMADZU UV-VIS UV-1280 spectrophotometer with a wavelength of 510 nm was used to measure the absorbance of the sample solution. The absorbance value is used to calculate the concentration of reducing sugar using a previously established standard curve equation. The actual concentration is determined based on the dilution factor (Miller, 1959; Julaeha *et al.*, 2016). Bioethanol levels were measured using an alcohol meter based on dilution theory. The bioethanol to be measured is obtained from distillation using a rotary evaporator at a temperature of 70 °C and a speed of 85 rpm. Calculation of bioethanol content is carried out using the dilution formula. All samples used in this study were repeated twice, and the average values

obtained were used for graphical and statistical analysis.

RESULTS

***Microbial Growth in Powdered Inoculum*:** Microbes perform important ecological functions in their life cycle, such as recycling organic materials trapped in cellulose and lignin (Faesal *et al.*, 2020). However, due to its short lifespan, special handling is required to maintain high microbial viability and effectiveness during storage so that it can be used at any time. Appropriate carrier materials are needed to carry microbes into the biomass to be decomposed. During storage, carrier materials suitable for microbial growth can maintain the high viability and effectiveness of microorganisms. The ideal carrier medium should be non-toxic to microbes, non-abrasive, easy to tamper with and clean, easily accessible, inexpensive, able to store water and contain sufficient nutrients for microbial growth (Djaenuddin *et al.*, 2018). The inoculum that has been made according to the inoculum production procedure in the methodology consists of three carrier ingredients: rice, corn, and soybeans, which are mixed in a ratio of 1:1:1, and the addition of 5% (w/w) glucose. Rice and corn function as sources of carbohydrates, while soybeans function as sources of protein. As is known, the largest component in the elemental composition of microorganisms is carbon. Carbon consists of 50-53% (dry weight) in bacteria, 45-50% (dry weight) in fungi, and 40-63% (dry weight) in molds. Nitrogen is the second most abundant element in microorganisms, constituting 12-15% (dry weight) in bacteria, 7.5-11% (dry weight) in fungi, and 7-10% (dry weight) in molds. Apart from these two elements, microorganisms also contain hydrogen, phosphorus, sulfur, potassium, sodium, calcium, magnesium, chloride, and iron. Other elements (such as Zn, Cu, Mn, Co, Mo, B, and W) are also needed in making growth media (Stanbury *et al.*, 2017a). The elements needed by microorganisms for growth are available in milled rice, yellow corn flour, and soybean flour as listed in the Indonesian Food Composition Table (Direktorat Jenderal Kesehatan Masyarakat, 2018). Carbon, nitrogen, and hydrogen are available in the form of carbohydrates and proteins. In rice, corn, and soybeans, carbon and hydrogen are found in the form of carbohydrates, with amounts of 80, 73.7, and 29.9 mg per 100 gram edible materials (EM), respectively. Nitrogen is available in the form of protein, with rice, corn, and soybeans containing 7.0 mg, 9.2 mg, and 35.9 mg of protein per 100-gram EM, respectively. Other elements such as calcium, phosphorus, potassium, sodium, iron, copper, and zinc are also available in these three materials. The optimal and appropriate inoculum condition is an inoculum with a minimum microbial number of 10^7 CFU gr^{-1}. Therefore, TPC analysis was carried out to assess the growth of the three microbes on the carrier material. Analysis during the inoculum production process was carried out every 8 hours for 2 days, while analysis during storage was carried out every week for a total of 4 weeks. The microbial growth curve on the powder inoculum can be seen in Figure 5. Figure 5 depicts microbial growth on the carrier material. When the three microbes were added to the carrier material, based on the results of the TPC analysis at hour 0, the number of microbes was $2.85x10^7$ CFU gr^{-1}. Furthermore, it was observed that the number of microbes experienced a slight decrease. The decrease occurred from 8 to 24 hours. The decrease in the number of microbes could occur due to the microbial adaptation process to the carrier material.

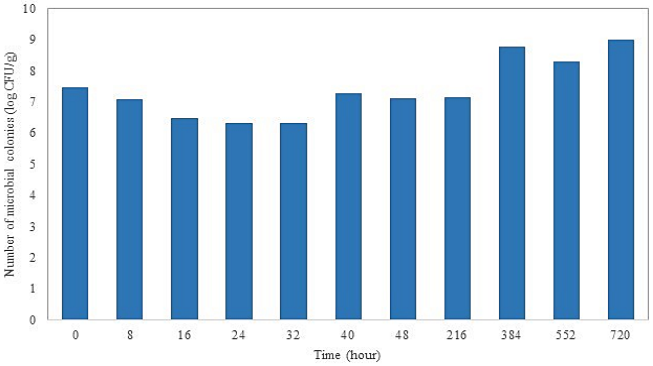

Figure 5. The microbial growth in powdered inoculum.

The number of microbes in the inoculum remained constant after 24 hours and continued to increase thereafter, reaching a microbial number of $1.26x10^7$ CFU gr^{-1}. This number represents the microbial population at the end of powder inoculum production or 48 hours. During storage conditions after 48 hours, an increase in the number of microbes was observed up to $9.42x10^8$ CFU gr^{-1} in the fourth week of storage. This condition can occur due to the general availability of elements needed for microbial growth in the three carrier materials. The main elements required by microbes include carbon, hydrogen, and nitrogen. Likewise, other minerals are needed (Stanbury *et al.*, 2017b; Direktorat Jenderal Kesehatan Masyarakat, 2018). Selection of the most suitable carrier material for the bioethanol production process involves several basic requirements to support the life of the microbes involved in the microbial life process. Microbes need water, an energy source, carbon, nitrogen, mineral elements, vitamins, and oxygen if fermentation occurs aerobically (Stanbury *et al.*, 2017a). The incorporation of microorganisms in carrier materials allows easy handling, long-term storage, and high effectiveness, as observed in the production of biofertilizers (Mukhtar *et al.*, 2017), as well as in the bioethanol production process (Safitri *et al.*, 2017). DBP which has gone through a pretreatment process is used as a medium in this research. Microbial growth in the medium was analyzed by measuring the absorbance of the sample at a wavelength of 690 nm. This measurement is expressed as an optical density (OD) value. Changes in the OD value of the sample can be seen in Figure 6.

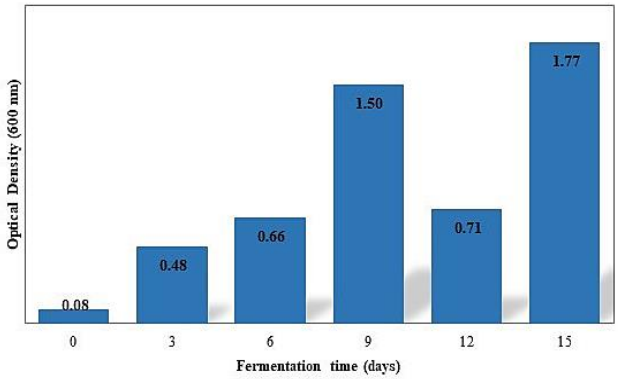

Figure 6. The optical density (600 nm) of the fermentation process.

Figure 6 shows the microbial growth pattern that can be associated with the availability of various types of sugar in the substrate, which causes diauxic occurrence. Diauxic growth occurs when a microbial population uses two carbon sources, resulting in two-phase exponential growth interrupted by a minimal growth lag phase (Chu and Barnes, 2016). Certain substrates can prevent the expression of genes encoding catabolic enzymes and/or transporter proteins. This is known as diauxic or "catabolic repression". However, these mechanisms vary among bacterial strains and require further research (Buendia-Kandia *et al.*, 2018).

***Bioethanol Production*:** Figure 5 presents the results of bioethanol production based on the yield and concentration of bioethanol. The bioethanol yield is calculated as the total amount of bioethanol produced per unit of raw material used. This involves measuring the total volume of bioethanol generated and relating it to the initial amount of substrate. On the other hand, the bioethanol concentration is determined by measuring the percentage of bioethanol in the final product using an alcohol meter, which provides an accurate quantification of ethanol content.

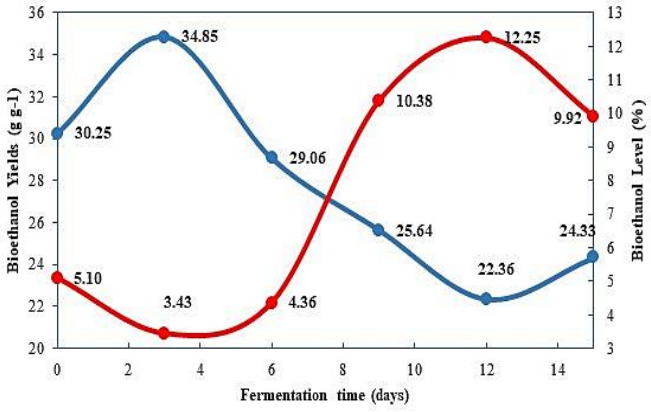

Figure 7. Bioethanol yields (g g^{-1}) and bioethanol level (%).

The graph indicates that the highest bioethanol yield, 34.85 g per gram of substrate, was achieved on the third day of fermentation, suggesting an optimal conversion rate of the raw materials into ethanol during this period. Meanwhile, the peak bioethanol concentration of 12.25% was recorded on the twelfth day of fermentation. This higher concentration over time implies that while the yield may stabilize after an initial surge, the ethanol concentration continues to increase as the fermentation progresses, likely due to the prolonged metabolic activity of the microorganisms breaking down the remaining substrates.

DISCUSSION

This research focuses on developing a powder inoculum consisting of a consortium of three microorganisms *T. reesei*, *S. cerevisiae*, and *Z. mobilis*. This consortium of microorganisms was chosen because each of them has a specific role in the hydrolysis and fermentation processes. *T. reesei* is known for its ability to produce cellulase enzymes which are effective in hydrolyzing cellulose into simple sugars, while *S. cerevisiae* and *Z. mobilis* play a role in fermenting sugar into ethanol. This inoculum uses a carrier material in the form of a mixture of rice, corn, and soybean flour in a ratio of 1:1:1, and 5% w/w of glucose is added. This mixture was chosen because it can provide sufficient nutrients and ensure the stability and viability of microorganisms during storage and application. This powder inoculum was tested in the Simultaneous Saccharification and Fermentation (SSF) process which uses lignocellulose as raw material. The lignocellulose used underwent pretreatment without chemicals, using pressure heating in an autoclave at a temperature of 121 °C and a pressure of 15 psi for 2 hours. After heating, the material is cooled in an autoclave for 12 hours, then soaked in distilled water with a ratio of 1:2 (material to distilled water) for 24 hours. This pretreatment aims to reduce the structural complexity of lignocellulose, making cellulose more accessible to cellulase enzymes without producing toxic compounds that can inhibit the fermentation process (Chukwuma *et al.*, 2020).

This chemical-free pretreatment approach has major advantages in terms of environmental sustainability and ease of operation (Abolore *et al.*, 2023). No additional washing steps are required to remove chemical residues, which are usually required in chemical pretreatment. This makes the process more efficient and environmentally friendly. This pretreatment process, similar to the Liquid Hot Water (LHW) method, has proven effective in changing the chemical composition of lignocellulosic raw materials, producing soluble oligosaccharides and phenolic compounds and acids important for subsequent fermentation. After pretreatment, the treated lignocellulosic material then undergoes the SSF process with the addition of powder inoculum. *T. reesei* produces cellulase enzymes consisting of 60-80%

cellobiohydrolases, 20-36% endoglucanases, and 1% β-glucosidases. These enzymes work synergistically to hydrolyze cellulose into glucose. *S. cerevisiae* and *Z. mobilis* then ferment glucose into ethanol. Fermentation conditions were carried out at room temperature (25-30 °C) for 15 days, which allowed the microorganisms to work effectively even though the optimal temperature for enzymatic hydrolysis (50 °C) was different from the optimal temperature for fermentation (30-35 °C). This study shows that the powdered inoculum formulation can extend the life span of microorganisms and ensure high catalytic activity during storage and fermentation processes. This innovation utilizes methods that have long been used in the fermented food industry, such as tape or *peyeum* production, which are applied in bioethanol production to increase efficiency and reduce complex preparations in the laboratory. The results of this research open new opportunities for a more practical and sustainable approach to the production of bioethanol from lignocellulosic biomass. The use of powder inoculum in this study showed significant advantages in storage stability compared to liquid inoculum (Xu *et al.*, 2020). Liquid inoculum usually requires strict storage conditions, such as refrigeration or certain temperature settings, to maintain the viability of the microorganisms. These conditions are often difficult to meet, especially on a large scale and in remote locations. In contrast, the powdered inoculum developed in this study can be stored under ordinary environmental conditions without significant loss of microorganism viability. This success is largely due to the dehydration process used to convert the microorganisms into powder form. Dehydration reduces the metabolic activity of microorganisms, which in turn slows down the degradation process of important components in microorganism cells (Hamill *et al.*, 2020). With reduced metabolic activity, microorganisms can survive longer in non-ideal storage conditions.

Powder inoculum formulation involves adding glucose and carrier ingredients such as rice, corn, and soybean flour in a 1:1:1 ratio. Glucose serves as an energy source that is easily accessible to microorganisms during storage and initiating the fermentation phase (Sharma *et al.*, 2020). This energy source is important for maintaining the viability of microorganisms over long storage periods. Without the presence of glucose, microorganisms may not have enough energy to survive dry conditions (Manzanera, 2021). Rice, corn, and soybean flour are used as carriers. These materials not only provide a physical matrix that supports the powder inoculum structure but also provide the nutrients required by the microorganisms. Rice, corn, and soybean flour are rich in nutrients such as protein, carbohydrates, and lipids, all of which are important for maintaining the viability of microorganisms. Proteins provide essential amino acids necessary for protein synthesis and cell repair, carbohydrates provide an energy source, and lipids help maintain the integrity of cell membranes (de Albuquerque *et al.*, 2023). Research shows that nutrient-rich carriers can significantly extend the shelf life of microorganisms by providing a stable, nutrient-rich environment. In this study, rice, corn, and soybean flour were proven effective in maintaining high catalytic activity of the inoculum during the storage and fermentation processes. For example, the enzymatic activity of *T. reesei*, which is essential for the hydrolysis of cellulose to glucose, remains high thanks to the stability provided by this carrier. Likewise, *S. cerevisiae* and *Z. mobilis*, which are responsible for the fermentation of glucose to ethanol, remained viable and active throughout the fermentation period. This powdered inoculum formulation offers a practical and effective solution to overcome the challenges of inoculum storage and stability in bioethanol production (Singhania *et al.*, 2021). By using easily accessible and nutrient-rich carrier materials and additional energy sources such as glucose, this research succeeded in developing a stable and efficient inoculum. This approach not only increases the efficiency of bioethanol production from lignocellulosic biomass but also ensures the sustainability of the production process by reducing dependence on stringent storage conditions and complex preparation in the laboratory. This study revealed that the SSF process using powder inoculum from a consortium of *T. reesei*, *S. cerevisiae*, and *Z. mobilis* produced encouraging results in the production of bioethanol from lignocellulosic biomass. The main role in the success of this process is the enzymatic activity of *T. reesei*, with a composition consisting of cellobiohydrolases (60-80%), endoglucanases (20-36%), and β-glucosidases (1%). The synergy between these three types of enzymes allows the effective decomposition of cellulose into glucose, providing the substrate needed for the fermentation process to ethanol. Although the optimal temperature for enzymatic hydrolysis by *T. reesei* is usually around 50°C and fermentation by *S. cerevisiae* and *Z. mobilis* around 30-35°C, fermentation in this study was carried out at room temperature (25-30 °C) for 15 days. Nevertheless, the powder inoculum was able to adapt well to less-than-ideal temperature conditions, showing high flexibility and efficiency in different environments. The results of fermentation at room temperature still produce quite high amounts of reducing sugar, indicating that enzymatic activity and fermentation take place effectively even at non-optimal temperature conditions (Liszkowska and Berlowska, 2021). Adaptation of powder inoculum to room temperature has significant practical implications (Luangthongkam *et al.*, 2021), especially in the context of field applications where temperature control may not always be optimally possible. Additionally, the stability of the inoculum in powder form provides additional advantages in terms of storage and transportation, reducing challenges related to storage infrastructure and maintaining the viability of microorganisms over longer periods. All research shows that powdered inoculum can be a practical and efficient solution

for large-scale bioethanol production. The chemical-free pretreatment approach also makes it more sustainable and environmentally friendly, which has potential for future commercial applications. Thus, this research makes a significant contribution to overcoming the challenges of bioethanol production from lignocellulosic biomass, as well as opening the door for further developments in this field.

The chemical-free pretreatment implemented in this research is a significant breakthrough in the production of bioethanol from lignocellulosic biomass (Rezania *et al.*, 2020). This method is based on pressure heating, similar to the Liquid Hot Water (LHW) technique, which has been proven effective in changing the chemical structure of biomass without the need for additional chemicals that have the potential to damage the environment or human health. One of the main advantages of this method is its ability to produce substrates that are easier to process enzymatically without leaving harmful residues. This chemical-free pretreatment provides several significant advantages. First, because it does not involve additional chemicals such as acids or bases, this method reduces the risk of environmental pollution and does not require complicated chemical waste management. This is in line with reducing the environmental impact of industrial processes. Second, because there is no washing step required to remove residual chemicals, the process is simpler, more efficient, and more cost-effective overall.

Pretreatment without chemicals has also proven effective in changing the chemical composition of lignocellulosic biomass (Bhatia *et al.*, 2020). The pressurized heating process at a certain temperature and pressure successfully destroys the structure of lignin and hemicellulose without damaging cellulose, facilitating the access of hydrolytic enzymes to the cellulose substrate. The result is a substrate that is more easily hydrolyzed into sugar by the cellulase enzyme, without producing toxic compounds that can inhibit fermentation. In comparison with more conventional alkaline pretreatment, this method shows competitive results in biomass conversion efficiency to sugars. Pretreatment without chemicals can produce sugar yields that are equivalent to or even higher than alkaline methods, without the need for additional chemicals and complicated washing stages. The use of this chemical-free pretreatment method brings great potential for more sustainable and efficient bioethanol production. However, further development is still required to optimize pretreatment parameters, understand the reaction mechanisms involved, and validate the economic and environmental feasibility of this approach on a larger industrial scale. The chemical-free pretreatment method applied in this study, as already mentioned, offers several significant advantages compared to conventional pretreatment methods that use chemicals (Diyanilla *et al.*, 2020). Chemical-free pretreatment, especially similar to the Liquid Hot Water (LHW) method, has advantages in terms of environmental sustainability and practicality in the bioethanol production process from lignocellulosic biomass. First of all, pretreatment without chemicals is a more environmentally friendly approach because it does not produce hazardous chemical waste that must be managed and disposed of carefully. In the context of environmental sustainability, the use of chemical-free pretreatment helps reduce the negative impacts on the environment and human health that are often associated with the use of toxic chemicals in industrial processes. Furthermore, pretreatment without chemicals is also more practical because it does not require additional washing steps to remove chemical residues from pretreated biomass. This reduces the complexity of the production process and speeds up the time required from pretreatment to the next fermentation process. Thus, the bioethanol production process becomes more efficient and economical. In addition, pretreatment without chemicals, especially the LHW method similar to that used in this study, can change the chemical composition of lignocellulosic biomass without producing toxic compounds that can inhibit the fermentation process (Alawad and Ibrahim, 2024). This is important to ensure that the final product, namely bioethanol, is not contaminated by harmful substances that can harm human health or inhibit the performance of fermentation microorganisms. The use of pretreatment without chemicals also shows quite competitive results in terms of biomass conversion efficiency into sugar compared to more conventional alkaline pretreatment methods. This shows that more environmentally friendly approaches such as pretreatment without chemicals have the potential to be a better alternative in the bioethanol production industry. The use of pretreatment methods without chemicals, especially those similar to LHW, in this research, makes a significant contribution to overcoming environmental challenges and technical in bioethanol production from lignocellulosic biomass. The advantages in terms of environmental sustainability, practicality, and process efficiency make this method attractive for further development on a larger industrial scale. Although the results of this study demonstrate significant progress in the development of powder inoculum and the application of chemical-free pretreatment in the production of bioethanol from lignocellulose, there are still several technical and environmental challenges that need to be overcome to make this technology more commercially acceptable. One of the main challenges is to achieve optimization of process conditions to increase the efficiency of simultaneous hydrolysis and fermentation. The difference in optimal temperatures for these two processes is a major obstacle in achieving the desired balance between enzyme and microorganism activity during the SSF process. Further research is needed to explore methods or strategies that can facilitate coordination between enzymatic hydrolysis and fermentation at optimal temperatures, perhaps using the latest approaches in process control or enzyme and microorganism engineering. In addition, waste management is a critical

aspect of maintaining the environmental sustainability of the bioethanol production process. The process of recycling wastewater and handling the resulting waste is a big challenge that needs to be taken seriously (Saravanan *et al.*, 2021). The development of efficient and environmentally friendly technology to process and recycle waste from production processes is very important to reduce negative impacts on the environment and ensure the sustainability of bioethanol production. Furthermore, further research needs to be focused on developing powder inoculum formulations that are more stable and can be applied on a larger industrial scale. This includes a better understanding of the interactions between microorganisms in consortia, optimal selection of carrier materials, and efficient inoculum production technologies. Testing and validation of powder inoculum on a larger industrial scale is also necessary to ensure overall production consistency and success. By overcoming these challenges through continued research and technological development, it is hoped that this powder inoculum and chemical-free pretreatment technology can become a more sustainable and efficient solution in the production of bioethanol from lignocellulose, and can make a significant contribution to reducing dependence on raw materials. fossil fuels and overcoming environmental problems related to greenhouse gas emissions.

***Conclusion*:** This research has developed a powder inoculum consisting of a consortium of *T. reesei*, *S. cerevisiae*, and *Z. mobilis* microorganisms with a mixture of rice, corn, and soybean flour as a carrier and the addition of 5% w/w glucose. This inoculum is designed for use in the Simultaneous Saccharification and Fermentation (SSF) process for the production of bioethanol from lignocellulosic biomass. The research results showed that this powder inoculum had high stability and viability during storage and was able to work effectively at room temperature. In addition, a chemical-free pretreatment approach using pressure heating, similar to the Liquid Hot Water (LHW) method, successfully breaks down the lignocellulose structure without producing toxic compounds, thereby allowing the hydrolysis and fermentation processes to run efficiently. The main advantage of this powdered inoculum is its flexibility in storage and fermentation conditions, which is important for field applications. The use of nutrient-rich carrier materials helps maintain the viability of microorganisms, while environmentally friendly chemical-free pretreatment methods increase the efficiency of the biomass conversion process to bioethanol. However, challenges such as optimization of SSF process conditions and waste management still need to be overcome for wider commercial applications. This research makes a significant contribution to the development of more practical, efficient, and sustainable bioethanol production technology, opening up opportunities to reduce dependence on fossil fuels and supporting climate change mitigation efforts. This research, although it has shown great potential in the development of powdered inoculum for bioethanol production, has several limitations that need to be overcome for wider commercial applications. One of the main limitations is the difference in optimal temperatures for the enzymatic hydrolysis and fermentation processes in SSF, which can reduce the overall efficiency because room temperature is not ideal for both processes. In addition, waste management from chemical-free production and pretreatment processes still needs to be further developed to ensure maximum environmental sustainability. Recommendations for further research include optimizing SSF process conditions to suit the optimal temperature of both processes, exploring more efficient and environmentally friendly pretreatment technologies, and developing more stable and effective inoculum formulations. In the future, contributions from this research may include increasing the efficiency of bioethanol production from lignocellulosic biomass, reducing the environmental impact of the production process, and developing sustainable solutions that can be implemented on an industrial scale, supporting the transition to renewable energy and reducing greenhouse gas emissions.

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