Identification of Superior Cellulose Microbes Producer for Bioethanol Production

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Abstract. The limited supply of fossil fuels in the world and increased in CO₂ emission problem causing the government has urged improving the supply of a source of energy, through the use of a new source of energy and renewable. Lignocellulose is one of the organic component in many was available an agricultural waste as a source of microbes to produce welfare fuel. In process of renewable energy, hydrolysis on source lignocellulose using a lignocellulose enzyme will be imported. Bioethanol produce there are have some problems, among other lack of strains superior enzyme lignocellulose microbes producer. Lignocellulose that degrades an enzyme is cellulose much used in various industries. An enzyme can be produced of a group of bacteria, like mold and yeasts. This article is determine to identify superior lignocellulose microbes producing an enzyme. Sample of the study obtained from the specimen land, litter, decayed wood, sand, water crater in various districts in Indonesia. Produce an enzyme lignocellulose identification microbes to bioetanol begins with exploration, isolation and selection, further testing and selection. From 15 bacteria producing cellulose isolates, and selection isolate superior obtained was that B93 with the activity of enzyme reached 25.3 U/ml. Identification of superior cellulose microbes producer is Enterobacteriaceae Sp. Based on this outcome was expected that B93 is a potential source of an cellulose enzyme producer. That can be used to producing welfare fuel (bioethanol). The next research can get reducing part cost of bioethanol production from lignocellulose is cost of cellulose and xillanase (commercial enzim), so it is very impact alternative to solve the global warming problem and ensure sustainable development of the economy leaf litter biomass from tree plantation sites can be collected and used as a promising feedstock for biofuel production to mitigate energy crisis.

1. Introduction
The supply of fossil fuels in the world is insecurity and carbondioxide emission problem causing the government has urged improving the supply of a renewable resource for energy. Many researches focused how to obtain new sources in the earth can be alternative for biofuel. The case is which resources can be substitute the renewable lignocellulosic and a low cost for enzyme hydrolysis. To solve the global warming problem and ensure sustainable development of the economy, it is necessary to increase the use of renewable biomass resources [1]. Lignocellulosic is one important source to produce a liquid fuel. That component can be collected through wood, hay, herbage, an agricultural crops, forest waste, the industrial wastes (wood, paper) and other fibrous material. The available biomass of the world is 220 billion oven dry tones (ODT) per year or 4500 EJ (10¹⁸ J) [2]. Therefore, leaf litter biomass from tree
plantation sites can be collected and used as a promising feedstock for biofuel production to mitigate energy crisis [3].

An enzyme this very active break ties cellulose that can dissolve. An enzyme lignocellulosic degradation is cellulose which is much used in various industries [4]. Generally an enzyme used still imports. An enzyme can be produced by group of bacteria, mildew and leavened. Biological and enzymatic pretreatments are moreover aimed at decomposition of lignin in milder conditions. The lack of strains microbes superior producer an enzyme lignocellulosic. There are some microbes derived from nature in indonesia like E. Cottonii from algae and Gracillaria verrucosa, fermentation by bacterium Clostridium acetobutylicum obtained at the fermentation conditions of pH 6.0 during 10 days period [5]. In addition, many microbes of a material substance natural no more expose for producing enzymes required. Ethanol produced through the catalytic hydration of ethylene or through the process of fermentation sugar using yeast Saccharomyces cerevisiae.

In addition to cellulosic feedstocks, municipal and industrial solid wastes are also a potential raw material for biofuel production [6]. Cellulose on biomass is natural resource which abundant, cheap and have potentially support for friendly fuel industry like etanol [7]. Biomass is materially left from plants or animals. Lignocellulosic material can generally be divided into three main components, such as cellulose (30-50%), hemicellulose (15-35%) and lignin (10-20%) [8]. Cellulose and hemicellulloses make up approximately 70% of the entire biomass and are tightly linked to the lignin component through covalent and hydrogenic bonds that make the structure highly robust and resistant to any treatment [9].

Research shows that corn cobs have yield in 14.22 %, which is 1 tons corn cobs can produces 142.2 liter ethanol [10]. Bioetanol of bunches palm oil can produce 25 % ethanol. But the budget to yield achieve 25 % ethanol was still high, so price liters of bioetanol is also high [11]. Technological can be isolating cellulolytic and xylolytic enzyme producing organisms and further extraction and application of these enzymes for various purposes [12]. Clostridium, Cellulomonas, Trichoderma, Penicillium, Fusarium, Aspergillus, and others also showed the presence of the ability of the activity of cellulolytic and hemicellulolytic high on the process of fermentation to produce sugar [13]. Bioetanol fuel having high value octane that can be used as a enhancing octane. It can replace ether and heavy metal compound as (Pb) as anti-knocking agent who have negative impact on environment. Although it has 68% lower energy content compared to gasoline, bioethanol’s high oxygen content makes the combustion cleaner and resulting lower emission of toxic substances [14]. Second generation production utilizes the non-edible lignocellulosic and starchy materials from agricultural and forestry biomasses and it becomes one fascinating solution in the popular deteriorating fuel demand and environmental complications [15]. Bioethanol production via this route is also expected to be economically preferable in the future for the observable reason of low feedstock cost.

The production of bioethanol from bunches empty palm oil, corn cobs, and sorghum stems having yield successive 14 %, 15 % and 13 %. To produce cellulose is facing a some problems, such as lack of strains microbe superior enzyme lignocellulose producer and knowledge of an enzyme technology production. Various groups of the microorganism of mildew, bacteria can produce cellulose. Abundant a source of lignocellulose and natural biodiversity needs to next studies on the utilization of that potential. This article is determined to identify superior lignocellulose microbes producing an enzyme. Sample of the study obtained from the specimen land, litter, decayed wood, sand, water crater in various districts in Indonesia. Produce an enzyme lignocellulose identification microbes to bioetanol begins with exploration, isolation and selection, further testing and selection.

2. Methodology
Produced an enzyme lignocellulose identification microbes to bioethanol begins with exploration the source, isolation from biomass, selection with good growth and identified for the last stages.

2.1 Exploration lignocellulose enzyme resources
Exploration has done in several areas, as soil samples from West Java, sample of a mine from former West Nusa Tenggara, water volcanic craters from Central Java, sea water and sand from Yogyakarta. In
the area where the set two or three tread exploration and inside each as there are 2-3 plotting. The road that explored varying in the range 1.3-2 km, depends on the condition of topography. Sample taken and put into plastic sample and given the label like sample code, the date of a collection, type and location of the sample, and saved in cooler-box.

2.2 Isolation cellulose microbes
Sample in form of soil and litter, isolation by suspension on one gram soil or litter (fine particles) into 10 ml sterile aquades. Then shaken and made series dilution $10^{-1}$ to $10^{-8}$ into test tube separate. Each sample concentration suspension, taken each one ml and is poured into a petri dish contains nine ml CMC media. CMC media composition consist of 1g CMC, 0.02 g MgSO$_4$7H$_2$O; 0.075 g KNO$_3$; 0.05g KH$_2$PO$_4$; 0.002g FeSO$_4$7H$_2$O; 0.004g CaCl$_2$.2H$_2$O; 0.2g khamir extract, 1.5g bacto jelly and 0.1g glucose [16]. Selection process based on comparisons between clear zone against the diameter of a colony. Next, incubation period is complete in each isolate selected and fourth combination treatment was kept for 48 hours. After the incubation period is complete done staining 0.1 % congo red to clarify formed the clear zone. After that, accounted for comparison between diameter of clear zone media against diameter of bacteria colonies.

2.3 Selection cellulose microbes
The next tested for producer isolates cellulose selected, they have a colony with diameter more than 3 mm. Moved by means of grow bacteria colonies of each isolates in a liquid media. This is means to know how far the ability to isolate bacteria in produce cellulose. In a statement to the same liquid media with medium to cellulose and isolation, as many as 50 ml in erlemeyer. After the harvest, was conducted a few observation, namely biomass level, dissolved of protein and cellulose activity. Parameters measured namely biomass with measuring optical density at wavelengths 660 nm using spektrofotometer. Proteins dissolved measured by Bradford method (1976). Incubated at a temperature 48°C for 30 minutes. To stop reaction added reagent DNS about 3 ml, simmer for 5 minutes. After chilled, sentrifugation at t speed of 3000xg for 15 minutes. Sugar reduction analysis done with reagent DNS (3.5 dinitro salicylic acid and at wavelengths 550 nm. As a standard used the standard solutions of glucose.

2.4 Identification cellulose producer superior microbes
Identification conducted based on sekuen 16S-ribosomal RNA. Reason to use utilize order this 16S-rRNA because the molecule rRNA containing order who is conservative in terms of evolution. Most conservative site can be used of sticking primary so it can be conducted amplification in invitro with PCR. In this way we can learn our range of genetic a neighborhood more detail because cannot be culture microbes have received had 16S-rRNA gen. The more diverse of molecules 16S-rRNA is well suited to distinguish an organism into lower stage as genus and species. The order 16S-rRNA is also to provide data which is statistically valid enough [17].

3. Result and Discussion

3.1 Isolation microbes cellulose producer
Isolation bacteria produced selulase started by dilution $10^{-1}$ to $10^{-9}$. On dilution $10^{-6}$ until $10^{-9}$ will be taken to used as observation. Colonies growing then observed celluloytic activity with determination of celluloytic index, they canned comparison between diameter of clear zone with a diameter of a colony. Isolates derived from soil samples in West Java consist of 15 isolates with clear zone. To clarify clear zone formed in done the staining with congo red 1 % and counted that comparisons. Less than 3 mm colonies, even though clear zone not used (Figure 1). Clear zone formed because of the hydrolysis activity of cellulose by an enzyme cellulose.
Isolates who tried out of 18, there are 15 isolates that produces the clear zone and there is 3 do not appear the clear zone. The ratio a zone pellucid that is produced have a range ratio 1.6 – 3.2 mm. Having the value of two isolates is > 3 mm namely B92, B94 and A92, with each value 3.2; 3.1 and 3. Three isolates growth has a good temperature 50°C as on a Table 1. Any cellulolytic bacterium produce different a complex cellulose of enzymes, hanging from a gene that owned and a source of carbon used [16]. Cellulose hydrolyzed on medium if at all submerged by congo red will produce clear zone, because of reaction between congo red and ties a β-1,4-glikosidik contained in a cellulose polymer. Cellulose can be hydrolyzed by own because the activity of an cellulolytic enzyme produced by the bacterium [18]. Flushing with a solution of NaCl 1M aims to swill congo red dye in the around area colony, so that clear zone more visible [19]. The utilization of waste that has lignocellulose using microorganisms can produce an enzyme extracellular capable of degrades material with lignocellulose into its constituent faction. An cellulose enzyme can be hydrolyzed cellulose into glucose [20].

### Table 1. The results of cellullolytic index decayed wood from (A) and with isolation derived (B)

| Sample | Colony diameter (cm) | Clear zone diameter (cm) | Cellulolytic index |
|--------|----------------------|--------------------------|--------------------|
| B62    | 1                    | 1.6                      | 1.600              |
| B63    | 1.15                 | 1.7                      | 2.125              |
| B64    | 0.8                  | 1.5                      | 1.765              |
| B65    | 0.8                  | 1.9                      | 2.375              |
| B66    | -                    | -                        | -                  |
| B61    | 1.1                  | -                        | -                  |
| B71    | 0.8                  | 1.5                      | 1.875              |
| B81    | 0.9                  | 1.6                      | 1.778              |
| B82    | 0.8                  | 1.7                      | 2.125              |
| B83    | 0.7                  | 1.7                      | 2.429              |
| A91    | 1.7                  | 2.4                      | 3.200              |
| A92    | 1                    | 2.2                      | 3.143              |
| B94    | 0.7                  | 3                        | 3.000              |

Remarks:
- A = Sample from decayed wood
- B = Sample from soil
- ** Behind the figures show a level dilution
### 3.2 Selection microbes cellulose producer

Colonies with clear zones will be analyzed by the Optical Density (OD), biomass and an enzyme cellulose activity. Cellulolytic activity in units per milliliter (U/ml) defined as the activity of bacteria isolates that produce glucose, as monomers cellulose, every minute [21]. Measurement of Optical Density (OD) at wavelengths 660 nm is the sum cell growth of each bacteria to support cell biomass observation (Table 2). The amount of OD and biomass which is cell growth not be correlate with high activity of an enzyme cellulose on certain isolates. Relations stage of dilution effect to OD and cellulose produce which is measured by the spectrophotometer, can be correlated in a linear with the number of cells in the culture (Figure 1).

**Table 2.** The result of the observation OD and isolate biomass bacteria cellulose producing

| Sampel | OD  | Biomassa (g/l) |
|--------|-----|----------------|
|        | 1   | 2  | 1  | 2  |
| B62    | 0.276 | 0.330 | 0.0320 | 0.082 |
| B64    | 0.323 | 0.335 | 0.023  | 0.042 |
| B65    | 0.288 | 0.396 | 0.0280 | 0.05  |
| B66    | 0.247 | 0.262 | 0.0300 | 0.04  |
| B81    | 0.620 | 0.602 | 0.0223 | 0.048 |
| B82    | 0.376 | 0.386 | 0.0283 | 0.0346|
| B84    | 0.442 | 0.423 | 0.0377 | 0.0806|
| B91    | 0.276 | 0.282 | 0.0351 | 0.0668|
| B93    | **0.427** | **0.465** | **0.0215** | **0.0328** |
| B94    | 0.345 | 0.355 | 0.0332 | 0.0632|
| B92    | 0.265 | 0.247 | 0.0246 | 0.0334|
| B62    | 0.276 | 0.330 | 0.0320 | 0.082 |

Remarks:
A = Sample from decayed wood
B = Sample from soil
**” Behind the figures show a level dilution

**Table 3.** OD observation to the standard

| Concentration | Absorbance |
|---------------|------------|
| 50            | 0.022      | 0.017 |
| 100           | 0.196      | 0.218 |
| 150           | 0.362      | 0.337 |
| 200           | 0.655      | 0.787 |
| 250           | 0.931      | 1.065 |

The results of the cultivation of protein content from isolates bacteria between 0.187 – 0.355 mg/ml. Several isolates it is protein content not similar. High proteins suspected show an enzyme higher too. However, formed enzyme has not been confirmed is cellulose. How far that protein are cellulose, so it is important to note the level of cellulose activity. The relationship between the activity of cellulose and proteins that are produced expressed with the specific activity. Relations between concentration of OD and absorbance liner.
Observation on the absorbansi parameter at the end of breeding (0.011 to 0.547) shows that there is a difference in the capability of every isolates to increase itself on tested condition. Indicate the bacteria is able to used one source of carbon in media growth, namely cellulose is isolates to grow up successful. Thus enzyme production would be better to used isolates can grow well on the substrate induced with cellulose (CMC). The activity of specific cellulose resulted, the highest attainable by isolates B93 namely 25.29 U/mg protein, sample A. The activity of an enzyme unusually high to followed by a high specific activity, so instead (Table 4). The measurement result cellulose activity in some isolates shows that the lowest activity 0.022 U/ml for three times test. When compared with other research the cellulolytic activity obtained from isolation were fairly higher. The activity of cellulolytic on *Cellvibrio japonicas* based on the research by Jarallah and Ali [22], 0.152 U/ml non-competitive purchase.

### 3.3 Identification Cellulose Producer Superior Microbes

From the selection and microbes on the highest produce activities cellulose are microbes B93. Those microbes identification based on genes 16sRNA (Figure 3). Zambare et al. [23] isolated cellulase and xylanase from thermophilic consortium of bacteria with maximum activities of 367 U/L and 489 U/L at 60 °C and 70 °C taking prairie cord grass and corn stover as substrates respectively. Identification of producing microbes superior selulase obtained the *Enterobacteria sp.*
Figure 3. Eletroforegam of microbes that are identified

**Figure 4.** Base sequence of isolate B93

4. Conclusion

From fifteen bacteria produced cellulose isolates, and selection isolate superior obtained that B93 with the activity of enzyme reached 25.3 U/ml. Identification of superior cellulose microbes producer is *Enterobacteria* Sp, based on identification of 16-S-RNA sequence. This outcome was expected that B93 is a potential source of cellulose enzyme producer. That can be used to producing welfare fuel (bioethanol). The next research can get reducing part cost of bioethanol production from lignocellulose is cost of cellulase and xillanase (commercial enzim), so it is very impact on the availability of domestic renewable energy.

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