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Exploration of Cellulolytic Microorganism as A Biocatalyst Candidate for Liquid Fertilizer Production

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Abstract. The raw materials of liquid fertilizer production are cellulose. Hence, cellulases are needed as biocatalisator of liquid fertilizer production. Cellulases are produced by cellulolytic microbes, bacteria and fungi. Soil is a habitat dominated by microorganisms such as bacteria, fungi, algae, and protozoa. Isolation of cellulolytic bacteria was done by using a screening medium containing 1% CMC. Cellulolytic bacteria was selected based on clear zone which form surrounding the colonies. Cellulase activity was measured by dinitrosalicylic acid (DNS) method. In this research, microorganisms that produce cellulases have been isolated from the soil of agricultural waste, and one with the highest activity has been studied for its benefits as a biocatalytic candidate in the production of liquid fertilizer. It was observed four isolates of microorganisms producing cellulases, the widest clear zone produced by NH1 isolate had diameter of 19.69 ± 0.3 mm. The optimum pH and temperature of crude extracts of cellulases were 8.0 and 60°C, respectively. The total nitrogen level in the production of liquid fertilizer with and without crude extracts of cellulases are 0.2073% and 0.1104%, respectively. So this crude extracts of cellulases increase 87,77% of total nitrogen level.

Keyword: *Liquid fertilizer, Cellulase, Soil, Microorganism*

1. Introduction

Enzymes are biotechnology products that attract attention because of their role in various fields. Enzymes are a biocatalyst in chemical processing. The use of enzymes as biocatalysts has several advantages over conventional methods. These advantages include faster reaction, more efficient and much more friendly to the environment.

There are several types of fertilizers, namely synthetic fertilizers and natural (organic) fertilizers. Synthetic fertilizers are mineral fertilizers made and distributed by the manufactory. Most Indonesian farmers use synthetic fertilizers. Excessive use of synthetic fertilizers causes damage to the soil and reduced plant nutrient needs. The use of organic fertilizer is the solution to this problems. Organic fertilizers are fertilizers made from natural materials such as plants or animals in solid or liquid forms.

A shortage of fertilizer inputs mainly nitrogen is faced by tropical regions [1]. Nitrogen is essential nutrient for maximum yield in most crops. Several studies related to these problem has been carried out by researcher to increase this need [2]. In addition to being friendly to the environment, application of liquid fertilizer is also able to reduce production costs. One of the problems in the process of making liquid fertilizer is the length of the manufacturing process which is about two weeks. To overcome these problem, a catalyst is needed to accelerate the production process, one of which is utilizing enzymes.

The basic ingredients of liquid organic fertilizer can be obtained from agricultural waste, such as straw and rice husks, peanut shells, bagasses, corn stalks, and other forage ingredients. Farmers usually use agricultural waste as compost. Composting is a process of degradation of biodegradable organic waste which is controlled by microorganisms [3,4]. Microorganism contained in the composting process are cellulolytic microorganisms [4]. The existence of these microbes is because many agricultural waste materials contain lignocellulose. Lignocelluloses consist of lignin, carbohydrates such as cellulose and hemicellulose, pectin, protein, ash, salt and minerals [5].

Celluloses are the main structural component of plants, which are the most abundant organic compound on earth [6]. Celluloses are chemical compounds that consist of long chains of glucose units bonded with the β -1,4-glycosidic bond [7]. Enzymes that can degrade celluloses are cellulases, that



consists of three main components namely endo- β -glucanase (EC.3.2.1.4), exo- β -glucanase (EC 3.2.1.91) and β -glucosidase (EC 3.2.1.21) [8].

Several cellulases have been successfully produced from various sources. Kshirsagar et al. has successfully obtained cellulase from *Amycolatopsis* sp. GDS isolated from agricultural waste biomass [9]. Imran, et al. reported that there are fungal and bacterial species isolated from agricultural waste capable of producing cellulose [10]. These species include *Trichoderma*, *Penicillium*, *Aspergillus*, *Pseudomonas*, *Bacilli*, *Actinomycetes*, *Streptomyces*, *Cellulomonas*, *Streptomyces* and *Actinomucor* [10][11].

The presence of cellulolytic microorganisms in the composting process of agricultural waste encourages the author to explore cellulase which will be used as a biocatalyst candidate in making liquid fertilizer. Lumbang sub-district, Probolinggo regency, East Java, Indonesia is one of the areas that have extensive agricultural land. The agricultural wastes produced are very abundant and have not been optimally utilized. In this study, we will explore cellulases produced by microorganisms from agricultural waste from the Lumbang village and its potential as a biocatalytic candidate for making liquid fertilizer by measuring the total nitrogen content of the liquid fertilizer.

2. Materials and Methods

2.1. Collection of Samples

The sampling method of soil was conducted at the open agricultural waste location (Lumbang village, Lumbang sub-district, Probolinggo regency, East Java, Indonesia) so that sunlight can be accessed and the taking is carried out at a depth of ± 10 cm from the surface.

The materials are used in making liquid fertilizer were rice brans, molasses, cow dungs and cow rumens. Rice brans were obtained from a rice mill located in Tandon Sentul, Lumbang, Probolinggo. Molasses were obtained from the village of Sidoarjo. Cow dungs were obtained from cattle farms in the village of Tandon Sentul, Lumbang, Probolinggo. Whereas the cow rumens were obtained from the slaughterhouse of beef cattle in the village of Lumbang, Probolinggo.

2.2. Isolation and selection of bacteria that produces cellulase

One gram of soil sample was put into 10 mL of fresh liquid media on a 50 mL erlenmeyer containing 0.2% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4 , 0.1% (w/v) NaCl, 0.24% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% (w/v) CaCl_2 , 0.5% (w/v) bacto tryptone, 0.5% (w/v) glucose and 1% (w/v) CMC. Samples were incubated at 30°C and 150 rpm for 24 hours. After enrichment in the same medium for three times, the results of inoculation were carried out in series dilution.

The result of dilution (10^4) was spread in solid media (containing 0.2% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4 , 0.1% (w/v) NaCl, 0.24% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% (w/v) CaCl_2 , 0.5% (w/v) bacto tryptone, glucose 0.5% (w/v) and 1% (w/v) CMC) and incubated at 30°C for 24 hours.

Different colonies were selected from the growing colonies. Then the colonies selected were transferred into two plates containing 1% (w/v) CMC, namely a replica plate and master plate. The two plates were incubated at 30°C for 36 hours. At the end of the incubation period, 0.1% congo red was stained on a replica plate to clarify the clear zone (halo) formed, after 30 minutes the excess of congo red was washed with 0.9% NaCl [12]. Cellulase index value of colonies which show *halo* on solid media was determined by comparing *halo* diameter and colony diameter.

2.3. Measurement of cellulolytic microorganism growth curve and cellulase activity

As many as one full-loop microorganism were inoculated into 50 mL of liquid medium containing 1% (w/v) CMC at 30°C and 150 rpm for 24 hours. Measurements of cell turbidity were carried out every two hours. Crude extracts of cellulases were obtained by centrifugation of the isolate culture at 6,000 rpm for 15 minutes at room temperature. The cellulase activity was measured by using the DNS (3,5-dinitrosalicylic acid) method [13]. A unit of cellulase activity was defined as a number of enzymes that produce 1 μmol of glucose in one minute. Pellet cells were degraded by sonication to optimize yield. For each 0.5 mL cell pellet was added 500 μL phosphate citrate buffer pH 7. Sonication was done 10 minutes at intervals per minute for cooling.

2.4. Characterization of cellulases

Characterization of cellulase includes determination of optimum pH and optimum temperature. The optimum pH of cellulase activity was determined using CMC as substrate at 37°C, in buffers of various pHs (phosphate citrate buffer, pH 3-7; phosphate buffer, pH 8; boric-borax acid buffer, pH 9; borax-NaOH buffer, pH 10). The optimum temperature for cellulase activity was determined by performing the enzyme reaction at temperature between 30 – 100°C.

2.5. Production of liquid fertilizer

There are variations of components in making liquid fertilizer (Table 1.1.). Each type of liquid fertilizer is processed in 5 L sized containers for 5 and 15 days. All ingredients were solubilized in 1 L of water in a 5 L barrel and stirring evenly until dissolved. Then the barrel was tightly closed until the air cannot enter, the barrel was opened and closed once a day for 15 days. After 5 and 15 days, liquid fertilizer was filtered until a clean solution is free from solids.

Table 1. Component variations of materials for making liquid fertilizer

Types of Liquid Fertilizer	Components				
	Molasses (v/v%)	Crude extract of cellulases (v/v%)	Rice brans (b/v%)	Cow dung (b/v%)	Cow rumens (v/v%)
I	5	0	6	15	15
II	5	10	6	15	15
III	0	10	6	15	15
IV	5	10	12	15	15

2.6. Observation of liquid fertilizer

Temperature, pH and total nitrogen level were determined during the fermentation process. Measurement of temperature and pH was carried out every day in the afternoon (17.00 to 18.00 WIB) for 15 days. Temperature measurements were carried out using a thermometer, and pH measurements were carried out using pH indicator (pH-Indicator strips pH 0-14 Universal Indicator, Merck). Measurement of total nitrogen levels (%) was carried out on the 5th and 15th days using the Mkrokjeldahl method.

3. Results and Discussion

3.1. Isolation and selection of microorganisms that produce cellulases

In this study, we obtained four isolates of cellulolytic microorganisms (**Figure 1**). The four colonies were selected based on clear zones produced after staining with congo red on solid media containing 1% (w/ v)CMC [12]. Muthuvelayudham & Viruhagiri suggested that cellulases achieved maximum activity using synthetic cellulose carbon sources such as CMC [14]. Based on previous research, 1% cellulose concentration was the optimum concentration for cellulase production [15]. Cellulolytic activity could be expressed qualitatively by measuring clear zone diameter (*halo*). Selection one isolate to be explored base on cellulase index, i.e. the difference in diameter of the clear zone with the diameter of the colony divided by the diameter of the colony. Based on the results of the qualitative test, it was found that NH1 isolate had the highest activity ($19,580 \pm 0.3$ mm) (**Table 2**).

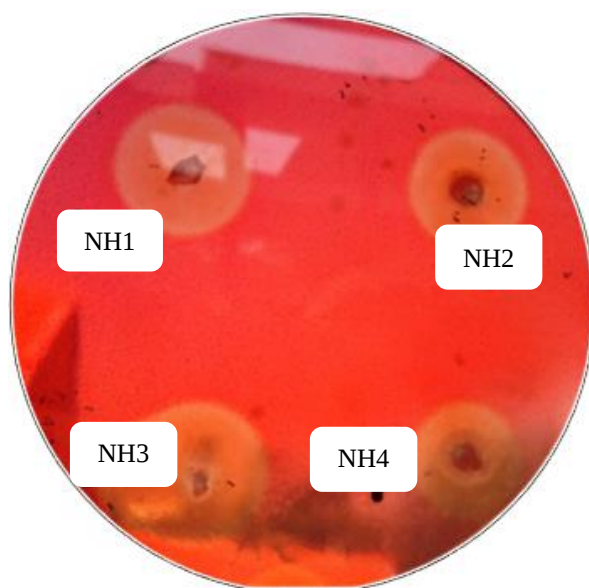


Figure 1. Clear zones (*halo*) that were produced by soil microorganisms on solid media containing 1% (w/ v) CMC after coloring using Congo-red.

Table 2. Cellulase index of microorganisms that have been isolated from agricultural waste.

Isolate	Cellulase index (mm)
NH1	19.686 ± 0.3
NH2	15.477 ± 0.5
NH3	11.191 ± 0.6
NH4	6.777 ± 0.07

Determination of the cellulase index was used as a basis for selecting microorganisms that had the ability to produce cellulases. Furthermore, we selected NH1 isolate to be further characterized, because the cellulase index values produced were greater than other isolates.

3.2. The growth curve and cellulase activity of NH1 isolate

Based on the growth curve in **Figure 2**, it show that NH1 isolate undergo four different phases. In the first phase, called the adaptation phase (lag phase), occurred in the zero to fourth hour. During this time the bacteria adapted to the new environment, and the cell has not cleaved. The second phase, called exponential (log phase), occurred in the 4th hour to 12th hour, where the active cell divided. The third phase, namely the stationary phase, occurred around the 12th to 18th hour, where cell death begins. The number of dead cells was approximately the same as the number of living cells in this phase. Cell death was caused by reduced nutrition in the media. The fourth phase, the phase of death, starts at the 18th hour and continues to decline in the next hour. In this phase, the number of cells that died exceeds the number of living cells. This occurred when the substrate concentration was below the concentration needed to maintain cell resistance, so that the cell undergoes lysis and death [14].

Determination of cellulase production patterns produced by NH1 isolates used the DNS method for measuring hydrolysis products which are calculated as glucose. One unit of cellulase activity was defined as the amount of cellulase in 1 mL of cellulase preparations which released 1 μ m of reducing sugar which was calculated as glucose per minute in experimental conditions. The results of cellulase activity test in liquid culture supernatant are shown in **Figure 2**. Cellulase activity was detected, but at very low levels, up to 24 hours. In the following hours (starting at 27 hours), where the OD value decreased significantly on the growth curve, cellulase activity increased sharply. At this time the cell undergoes lysis, the cellulase which was originally inside the cell would be released into the medium, so that the cellulase activity test in the supernatant shows a high value.

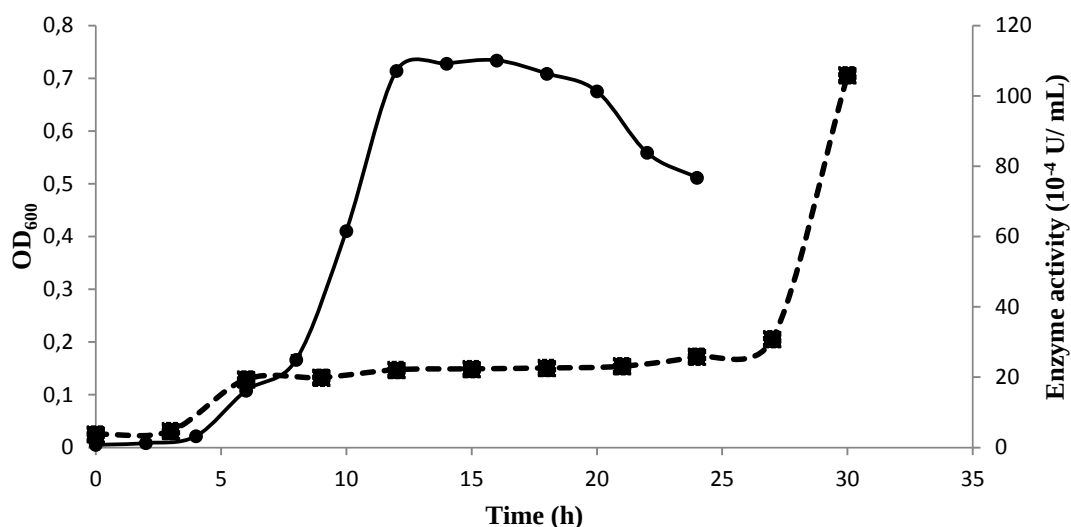


Figure 2. The growth curve of isolate LP1 (---) and the production curve of cellulase (-■-) during the fermentation process.

In general, the production curve was made to serve as a reference for harvest time in cellulases production. However, based on the data in **Figure 2** and the previous discussion shows that the optimum cell number was at 12 hours, where cellulases are still bound to the cell. Therefore for cellulases production, cells were harvested at 12 o'clock, and cellulases were released from the cell through sonication of cell pellets. Cell lysate cellulase activity showed a higher increase compared to the cellulase activity found in the liquid supernatant (**Figure 3**).

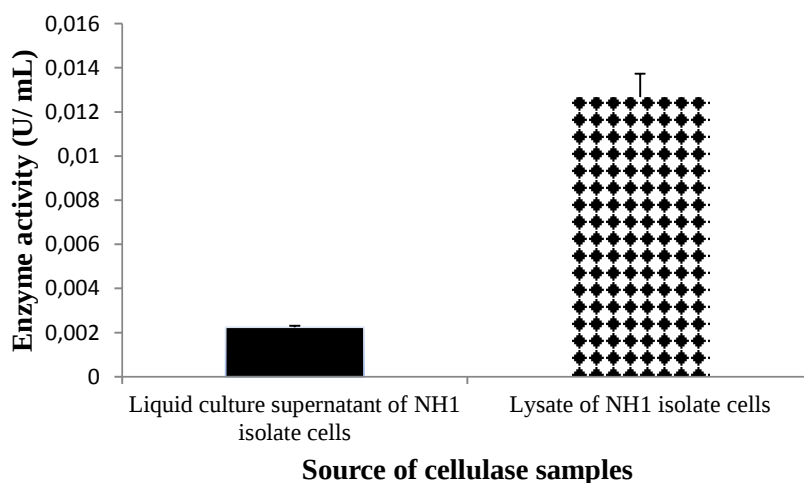


Figure 3. Cellulase activity of liquid culture supernatant and lysate of NH1 isolate cells

The same phenomenon was explained by Begum and Absar [16], which stated that the β -D-glucosidase enzyme that hydrolyzed cellulose and several large molecules that had low periplasmic solubility. Some cellulases were released in a liquid medium, and some others were still tied to the cell surface. Based on this report, most of the cellulase produced by NH1 isolates was periplasmic. For the production of this enzyme, the sonication of cell pellets was needed.

3.3. Characterization of cellulases from NH1 isolates

One of the factors that influence cellulase enzymes activity is temperature. The highest cellulase enzyme activity was obtained at 60°C with activity 0.0185 U/ mL (**Figure 4**). This indicates that the enzymes of the isolate of NH1 in this study were thermostable, because this enzymes still had activity at temperature 60°C. The activity of cellulases began to decrease at 70°C. The decrease in enzyme activity was caused by atoms in enzyme molecules that had considerable energy to move. It was caused by changes in the shape of the enzyme structure due to the increased thermal vibrations of the components of the atoms. Hence, protein-forming enzyme was denatured [17].

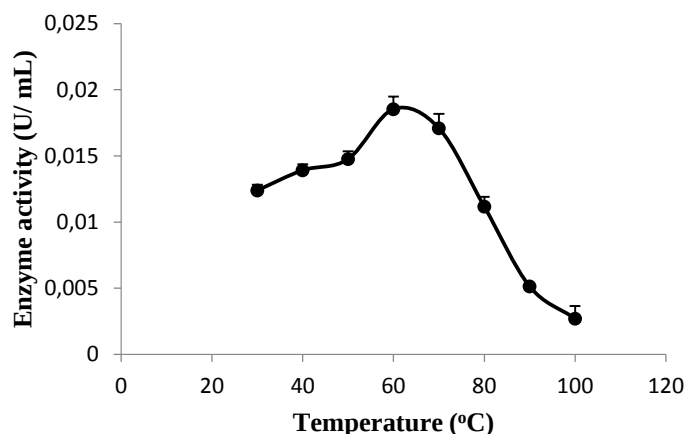


Figure 4. The optimum temperature curve of cellulases

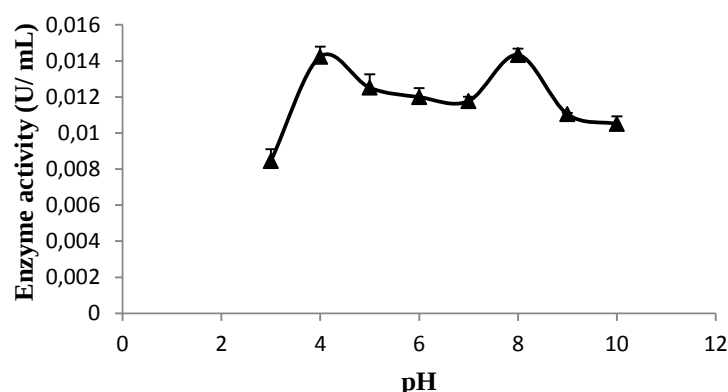


Figure 5. The optimum pH curve of cellulases

Another factor that affects enzyme activity is pH. Each enzyme had an optimum pH and the optimum pH causes maximum enzyme activity. The cellulase enzyme in this study had two optimum pH points which is not much different (**figure 5**). The first optimum point showed an idea that cellulases were acidophilic or acid-resistant (pH 4) with an activity of 14.233×10^{-3} U/ mL. The second optimum point illustrated that the cellulases were alkalophilic or alkaline-resistant (pH 8) with an activity of 14.332×10^{-3} U/ mL. We concluded that cellulases produced by NH1 isolates had the highest activity at pH 8. One of the factors influencing the presence of two optimum pH points of cellulases from LP1 was the initial environment where microbes were taken. The degree of pH of the agricultural soil environment is indicated by the presence of chemical elements. Hence, the environment could be acidic and alkaline. Microorganisms that lived in this environment would adjust the condition. Therefore, the microorganisms that are resistant to both acidic and alkaline pH. Cellulases in this research were very unique.

Relevant reports on optimum temperature and pH of cellulase were very diverse. Liang, et al. has successfully determined the optimum temperature and pH of cellulase produced by bacteria from the Nature Reserve in Subtropics Region of China (ME27-1) of 50°C and 5.5, respectively [18]. *P. curdlanolyticus* B-6 had an optimum pH of 7.0 and an optimum temperature of 37 °C [19]. Kumar et al. reported that the optimum pH and temperature of *P. polymyxa* were 5.5 and 37°C, respectively [20]. Imran, et al. reported that the maximum activity of cellulase produced by *Aspergillus niger* from agricultural waste was at 4.0 of pH and 60°C of temperature [11]. In addition, Imran, et al. mentioned the optimum pH and temperature of several other species such as *Eichhornia crassipes* (5.0 of pH and 40°C of temperature) and *Aspergillus japonicas* C03 (4.0 of pH and 50-55°C of temperature) [11]. Cellulases that were produced in this study were resistant to high temperatures, acidic pH, and alkaline pH. So that it has a very good ability to be applied in the industry and could be used as a catalyst candidate in the production of liquid organic fertilizer, especially in the face of changes in fermentation conditions during the changing production process.

3.4. Liquid organic fertilizer

The materials that were used in making liquid fertilizer were rice brans, molasses, cow dungs, and cow rumens. Cellulases in the production of this fertilizer were obtained from NH1 isolates in the form of crude enzyme extracts. The crude enzyme extract was obtained from NH1 isolate fermentation in production media containing 1% CMC for 30 hours and optimized by sonication of cells. Cellulase that was produced by cellulolytic microorganisms could degrade carbohydrates [11]. Rice brans and cow dungs were carbon sources and provide the elements that were needed by plants. Molasses were given in this study to accelerate the growth of microorganisms so that they grew rapidly. The fermentation process in the production of liquid fertilizer required microorganisms. The source of microorganisms that was used in this study was microorganisms from cow rumens and cow dungs.

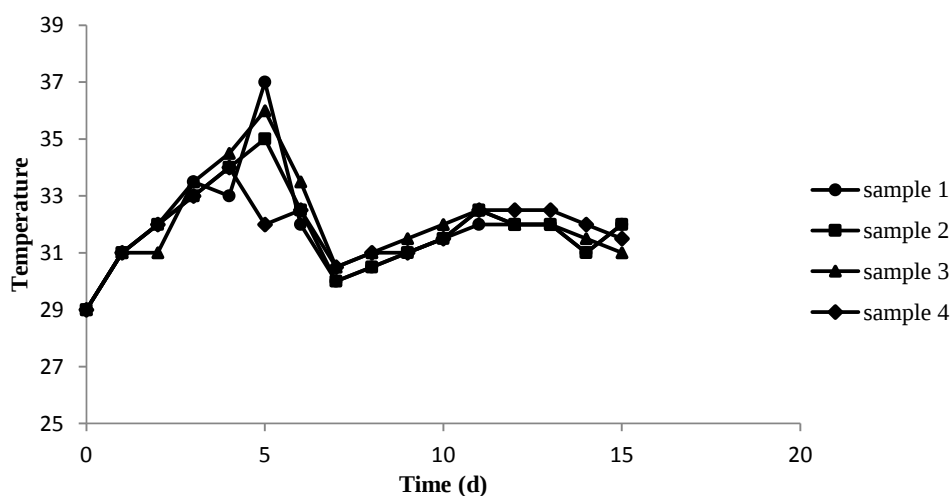


Figure 6. Curve of temperature changes during the fermentation process

In this study, we observed temperature and pH during the fermentation process (Figure 6 and 7). The highest temperature during the fermentation process was on the 5th day and then the temperature is relatively the same with ambient temperature conditions. The initial pH of liquid fertilizer was 7.0, this showed that there was no microorganism activity, so the pH of the solution tended to be neutral. In previous studies, the initial pH of fermentation was 7.05 and after five days the fermentation pH was 5.70 [21]. these results were not much different from the results of our research.

Nitrogen was a key nutrient that had an important role in most plants [22]. In addition, it was very necessary for the development or growth of vegetative parts such as leaves, stems, and roots. The best treatment of liquid fertilizer with the highest total nitrogen content was 0.2073% which contains 5% (v/v) molasses, 10% (v/v) crude enzyme extracts, 12% (w/v) rice brans, 15% (w/v) cow dungs and 15% (w/v) cow rumens (Figure 8, Table 1). The results of the measurement of total nitrogen levels in this

study were cooperative results between two enzymes produced by the crude extract of cellulase isolated from NH1 isolates and also from cellulase produced by microbes in the cow rumens. The rumen in ruminants animals contains cellulosic bacteria [23], so that the presence of cellulase in the cow rumen in this study also affects the degradation process of cellulose components. But in this study only variations on cellulase from NH1 isolates were carried out. The total nitrogen level in the production of liquid fertilizer with and without crude extracts of cellulases are 0.2073% and 0.1104%, respectively. Based on these results, it could be seen that the presence of cellulase had a role in the process of making liquid organic fertilizer. Addition of crude cellulase extract from NH1 isolates was able to increase the total nitrogen content by 87.77%.

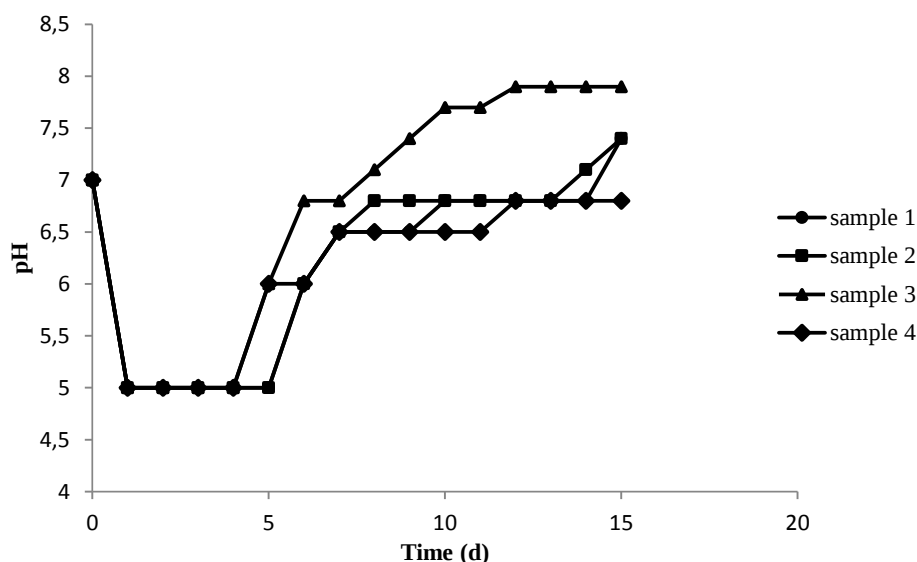


Figure 7. Curve of pH changes during the fermentation process

The value of nitrogen content from the two days that were selected had not met the standard quality of liquid fertilizer (SNI 19-7030 2004). The low total nitrogen content in organic liquid fertilizer was the lifting of nitrogen in the form of nitrogen gas or in the form of ammonia gas which was formed during the process of making organic fertilizer. Previous research states that bacteria isolated from earthworm viscera (*Brevibacillus agri*, *Bacillus cereus*, *Bacillus licheniformis*, and *Brevibacillus parabrevis*) could convert fish waste and conversion results were used as liquid fertilizer that had a total Nitrogen content of 1.57% [26]. This level was smaller than the results of our study. Our research is a new study and shows the role of cellulase in the process of making liquid fertilizer. However, further research regarding better combinations needs to be done, so that they are in accordance with the quality standards of liquid fertilizers. In addition, it is necessary to measure P and K levels of liquid organic fertilizer that is produced.

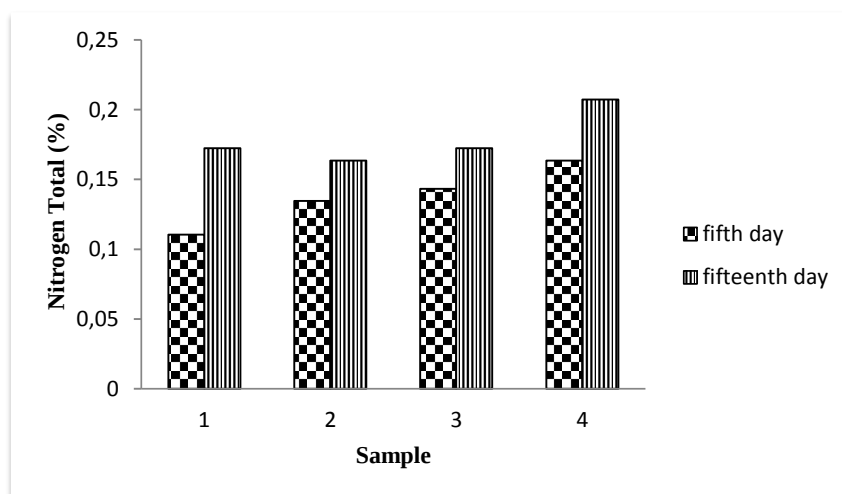


Figure 8. Total nitrogen content of liquid organic fertilizer on different days. Sample 1, without using crude cellulase extracts. Samples 2, 3, 4, using crude cellulase extracts by comparison of different rice brans (Table 1.1.).

4. Conclusion

Four cellulolytic microorganisms have been isolated. The widest clear zone produced by NH1 isolate had diameter of 19.69 ± 0.3 mm. The optimum pH and temperature of crude extracts of cellulases were 8.0 and 60°C, respectively. The total nitrogen level in the production of liquid fertilizer with and without crude extracts of cellulases are 0.2073% and 0.1104%, respectively. So this crude extracts of cellulases increase 87.77 % of total nitrogen level. Thus, this study revealed the cellulases produced by NH1 isolate from agricultural waste can be used as biocatalyst for the production of liquid fertilizer.

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