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Research Article

Isolation of Cellulose Degradation Bacteria (CDB) from acid soil as a potential candidate of organic waste degradation

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Abstract

Background and Objective: The study aimed to obtain CDB with high degraded activities, determined growth curve, protein content, and cellulase maximum activity (exoglucanase and endoglucanase). **Materials and Methods:** The cellulose activity calculated according to Miller (1959), protein content was measured by Bradford method with bovine serum albumin (BSA) as a standardize protein. **Results:** Six isolates of CDB were found as potential degradation of organic waste (Km25, Sr75, Jm, U6, G8, and Km13). Growth curve, protein level, and protein maximum activity occurred on day-3. The largest diameter of clear zone of six isolates was Km25, Sr75, Jm, U6, G8, and Km13 (3.32, 3.31, 2.41, 2.36, 2.19, and 2.04 mm, respectively). Endoglucanase and exoglucanase maximum activity were 0.011-0.402 IU/mL and 0.0028-0.155 IU/mL, respectively. Six isolates showed high activities of cellulase with diameter of clear zone ≥ 2 cm (Km25, Sr75, Jm, U6, G8, and Km13). Growth maximum curve was on day-3. Highest endo- and exoglucanase activities were on day-3 (0.402 IU/mL and 0.155 IU/mL, respectively) in Jm isolate.

Key words: Identification, degradation, clear zones, cellulase.

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Introduction

Indonesia has high biodiversity, especially animals, plants, and microorganisms. The diversity of microorganisms was larger than animals and plants. Lack exploration of microorganisms, and most of natural scientist unrealized the role of microorganisms in many fields of human life. Soil is a habitat which dominated by microorganisms such as bacteria, fungi, algae, and protozoa (Subba, 1995).

CDB is commonly found in soil as decomposing organic waste, producing cellulase for degrading cellulose (a polysaccharide of glucose formation). Cellulase is an extracellular enzyme consisting of endo β-1.4-gluconase (CMCase, Cx cellulase exocellulase, or carboxymethyl cellulose), exsocellulolytic β-1.4-gluconase (aviselase, cellobiohydrase cellulose, C1), and β-1.4-gluconase or cellobiose (Crueger cit Brock, 1984). In general, cellulase activity of each isolate is higher on agricultural waste is given substrate than pure cellulose substrate, it's due to hemicellulolytic enzyme produced by bacteria (Meryandini et al, 2009). Clostridium cellulorans synthesizes hemicellulolytic enzyme (xyiA) when grown on a substrate of cellulose, such as cellobiose, and the expression of cellulase is associated with the expression hemicellulase (Subba, 1995).

Cellulase including to hydrolases, catalyzes hydrolysis reaction of β-1.4-glycosides break contained in cellulose molecules. All cellulase is produced in aerobic conditions, on extracellular enzymes that work synergistically against cellulose (Lynd et al, 2002). Cellulose is a common name or trivial's name, while the system name is β-1.4-glucan-4-glucanohydroxyl. Cellulase is a complex enzyme that consists of three main components namely endo- β -glucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and cellobiose or β -glucosidase (EC 3.2.1.21), works synergically or together to decompose cellulose into glucose (Gerhartz, 1990; Wood and Bhat, 1988). Reese et al (1972) suggested the works of each component of enzymes are Endo-1) β-glucanase, β -1.4-glucan glucanohydrolase, D-CMCase, Cx: disconnect random chain of cellulose that consists of glucose and cello- oligo-saccharide. 2 Exo-β)-1.4-glucanase, β -D-glucan cellobiohydrolase, Avicelase, C1: attacking the outside of cellulose on the ends of the nonreduction with cellobiose as the main structure. 3). β -glucosidase, cellobiase: cellobiose hydrolysis into glucose. In general, the total cellulase activity is solvency cotton, filter paper or Avicel exoglucanase, endoglucanase and β -glucosidase, and the third substrate is the high content of cellulose crystals so it is very good for determining the activity of a high cellulose content of cellulose crystals (Wood and Bhat, 1988).

Bacteria have a fairy complex pattern to degrade cellulose (Hou et al, 2004). Figure two showed the activity of cell, begin with cell reproduction by snapping cell along the cellulose fibrils (Fig. 2A), a few buds (0.1-0.2 μm) appears on the surface of cells, directly contact to fibrils (Fig. 2B), cellulose is degraded and result sugar reduction which is used for cell reproduction and form cell colonies (Fig. 2C). The degradation occurs by attaching cell into fibril thread, whereas cells which only adjacent to fibrils, the cellulose unbrreak (Fig. 2D). Cell death occurs when run out of cellulose or cellulolytic product (Fig. 2E), Buds only appear (0.1-0.2 μm) on the surface of Sorangium when cell in growth process (Fig. 2F).

Materials and Methods

Sample Collection

Some kinds of soil acid like ultisol, peat, garbage dump, soil litter, and sap wood were collected for isolation of cellulose degrading bacteria. Soil was taken 10-20 cm in depth subsurface, transferred into dark labeled plastic, and stored for next analysis.

Isolation and selection of CDB

CDB strains were isolated from soil by using serial dilutions and pour plate technique. The medium used for isolation of CDB contains 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K2HPO4, 1 % agar, 0.03 % MgSO4.7H2O, 0.25 % (NH4)2SO4 and 0.2 % gelatin at pH 7 for 48 hours of incubation at 30˚C. CDB colonies were purified by repeated streaking. The purified colonies were preserved at 4oC for further identification and selection for cellulase production (Yin et al, 2010).

Pure cultures of CDB isolates were individually transferred in CMC agar plates by picking a colony with toothpick, repeated for three times and incubated for a week. After incubation, CMC agar plates were flooded with 1% Congo-Red and allowed to stand for 15 min at room temperature. One molar NaCl was thoroughly used for counterstaining the plates. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for CDB. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies (Lu et al, 2002), and only these were taken for further study. Cellulose-degrading potential of the positive isolates was also qualitatively estimated by calculating hydrolysis capacity (HC), that is, the ratio of diameter of clearing zone and colony (Hendricks, 1995). Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis (Andro et al, 1984). The bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged system.

Determine curve of cell growth and cellulase enzyme activity

Two full loops of bacteria colonies were transferred into 100 ml 1% CMC broth, incubated in shaking incubator at room temperature. Every 24 hours, the turbidity of broth and the activity of the cellulase were measured. Crude cellulose was precipitated on 12.000 rpm for 10 minutes at 4oC. One unit cellulase activity is defined as 1 μmol glucose yield in a minute. One unit of activity is equivalent to 16.67 nkat Dybkaer (Miller, 1959).

Protein level measurement

Bovine serum albumin with Bradford (BSA) was used as standard protein, with concentration: 0.0 - 0.625 - 1.25 - 2.5 - 5.0 - 10.0 - 20 - 12 μg/ml, and 200 ul BSA was added, shaken, and allowed to stand for 10 minutes. Samples were absorbance. Absorbency break was performed by 595nm wavelength spectrophotometer UV-VIS. The levels of protein were determined based on standard protein curves (Bradford 1976).

Cellulase activity assay

Cellulase activity (endoglucanase and exoglucanase) was assayed using dinitrosalisic acid (DNS) reagent by estimation of reducing sugars released from solubilized-CMC in 0.05 M phosphate buffer. Crude cellulose was added to 0.5 ml of 2% CMC in 0.05 M phosphate buffer and incubated at 37˚C for 60 min. After incubation, reaction was stopped by the addition of 3 ml of DNS reagent and boiled at 100˚C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using glucose calibration curve (Miller, 1959). One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1µmol of glucose per minute under standard assay conditions.

Results

Isolation of cellulolytic degradation bacteria from acid soil

A total of 24 bacterial isolates were able to grow on carboxy-methyl-cellulose specific media, found to be positive on selection media (cellulose Congo-Red agar) producing clear zone, six isolates with clear zone index ≥ 2.0 cm, (Fig. 1), the greater the clear zona index the higher degraded-cellulase activity. Clear zone test is a qualitative method to show the activity of cellulolytic, which grow around the bacteria colony (Ponnambalam et al., 2011).

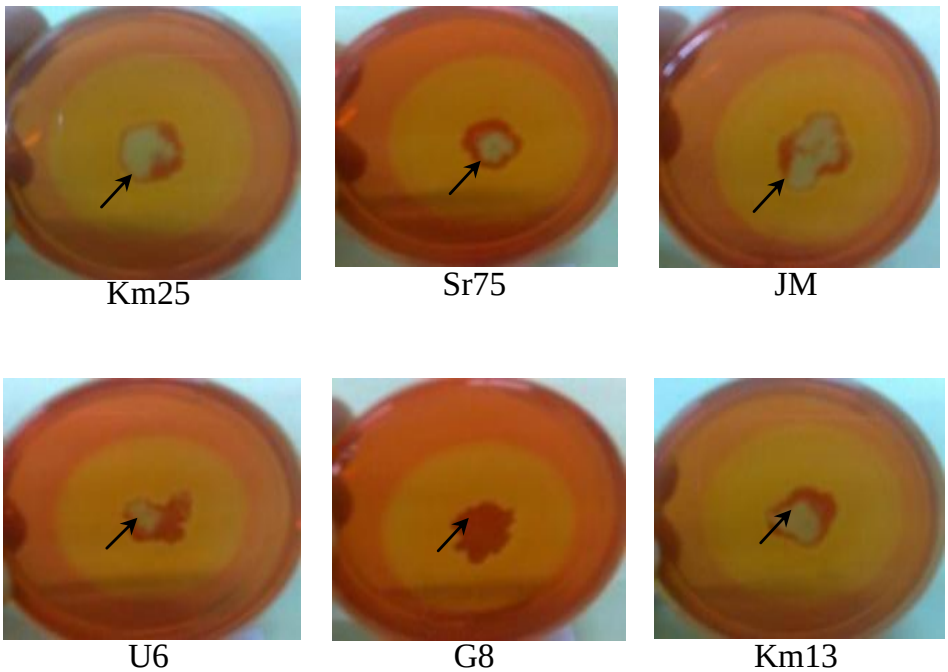

Figure 1. The clear zone of six degraded-cellulolytic isolates incubated for four days in CMC, formed after tested by Congo-red. Black arrows showed the area of clear zone for each isolate.

Figure 2 showed that the size of clear zone is greater than bacterial colony for all six isolates because cellulose was secreted outside of bacterial colony, thus the size of secreted cellulose is greater than colony. Clear zone is formed which related to the solubility of enzyme. The higher of solubility level the greater clear zone diameter formed. The diameter of clear zone is generally greater than diameter of colony, it's due

to cellulase was secreted surrounding cellulose by bacteria. Bacteria are unable to put cellulose into cell, because the size of cellulose molecule is larger than the size of bacterial cell (Zverlova et al., 2003).

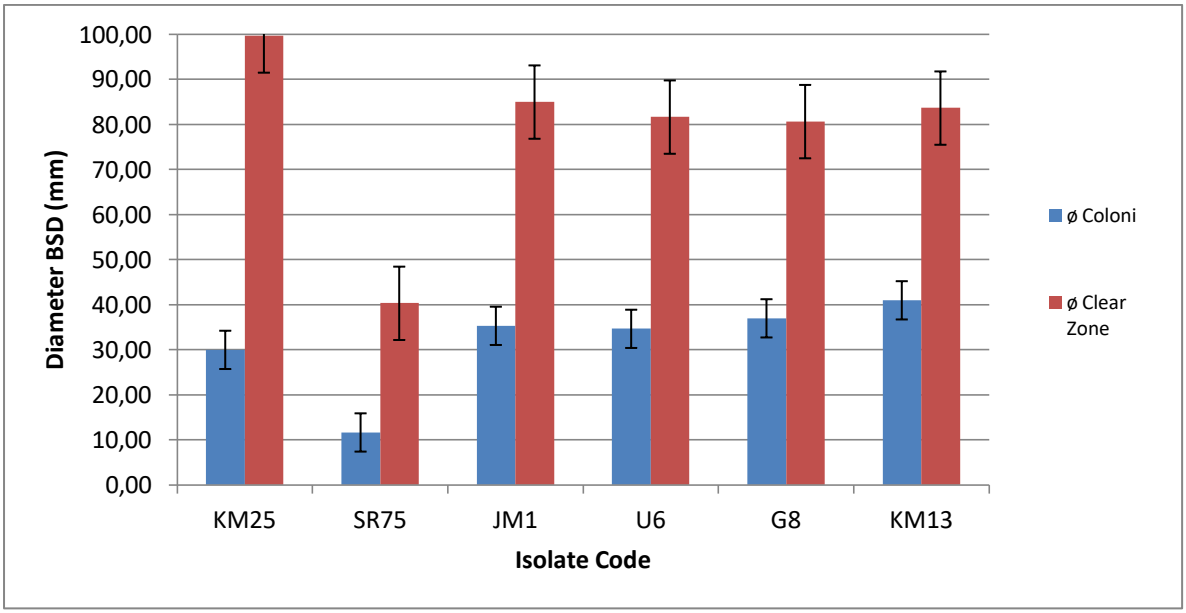

Figure 2. The comparison of colony diameter with clear zone diameter of six isolates after four days incubation. The size of clear zone is greater than bacterial colony for all six isolates.

Degraded-celulolytic bacterial growth assay

Degraded-celulolytic bacterial growth is determined by propagating the inoculums in CMC liquid with seven days incubation period. The growth curve is used as the optimum time for determining the production of cellulase. Bacterial growth was defined as an increase of cell weight, because cell weight relative is equal to cell cycle, then bacterial growth was defined as the increase number of cells (Purwoko, 2007). The growth curve was obtained with turbidimetric method, that is to determine the number of bacteria by measuring the optical density of 595 nm (OD 595) (Alpha, 1998).

Figure 3 showed that adaptation period of bacteria occurred on day-1 to day-2, bacterial cells undergo microbial adjustment towards its surroundings particularly the adjustment to the media for its growth. Exponential time occurred on day 3-4, bacteria are experiencing rapid growth, much energy is needed to survive by producing metabolic substances such as cellulase to degrade complex carbohydrates into simple sugar. Bacterial stationary period occurred on day 5-7, which is live bacteria as many as dead bacteria. While the phase of death occurred when dead bacteria was more than live bacteria during day 5-6. It's due to lack nutrition of medium, and cells were destroyed by enzymes secreted by bacteria itself (Waluyo, 2007).

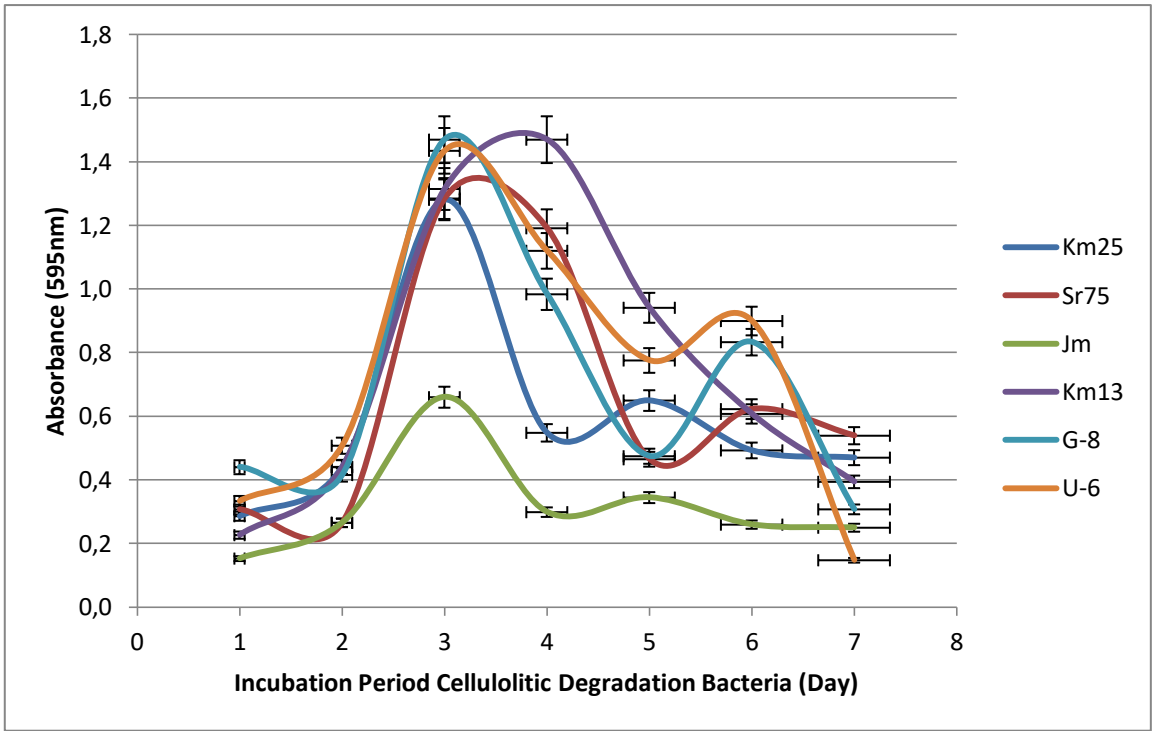

Figure 3. Curve of CDB growth on day-7 incubation period

Protein content in crude extract of degraded-CDB

Protein content in crude extract describes quantity of BSD enzymes content, and serves to determine the unit of enzyme activity. Dissolved protein was setted based on bovine serum albumin standard curve (BSA). Standard curve made 0.0-0.625-1.25-2.5-5.0-10.0-20-12 µ g/ml. Add 200ug Bradford. This method is based on the reaction of reagent Bradford it was so that the blue complex compound is formed according to the following reaction:

Figure five presented the maximum protein content of CDB was produced day-3 and started to decrease on day-4. The highest protein content was on Sr75 isolate (8.00 ng/ml), Km25 (7.5 ng/ml), Jm (7.3 ng/ml), U6 (6.9 ng/ml), G8 (6,7 ng/ml), and Km13 (6.1 ng/ml). Protein content was drastically decreased om day-7, ranged from 0.8 – 2.9 ng/ml. This protein content is much lower than previous results, Saryono (1991) reported that protein content of cellulase extracted from snail (Achatina fulica) was 4.4 mg/ml extract. It may be due to the crude extract of enzyme in this study was too dilute (Fig.4), and the solidity process was not done as Saryono (1991).

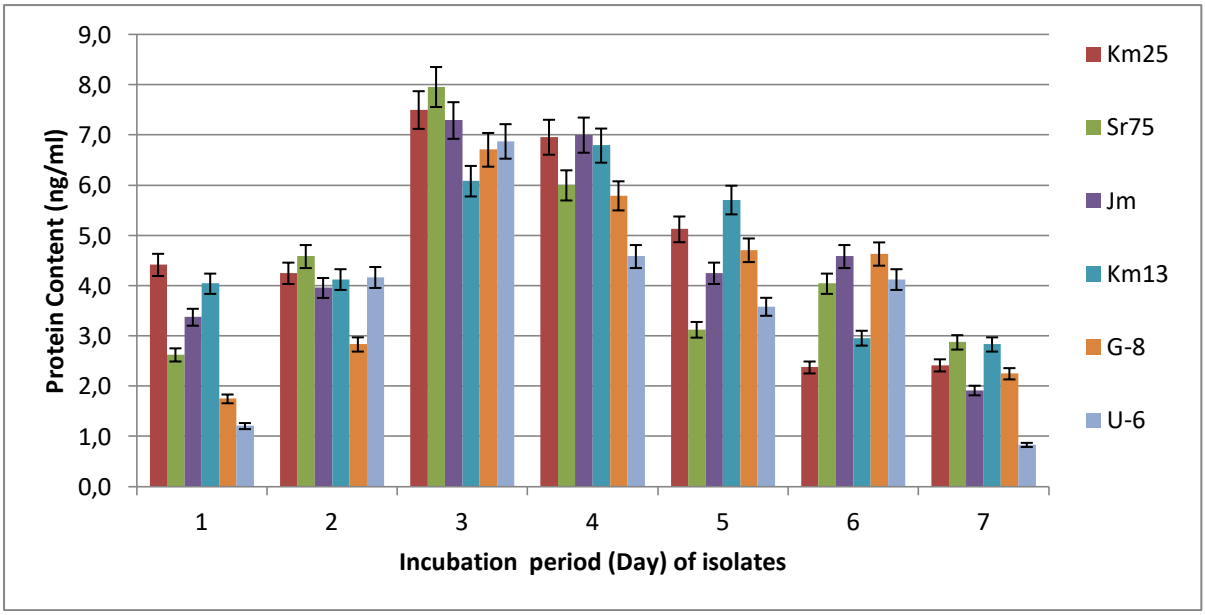

Figure 4. Protein production activity of CDB during incubation period for seven days. The peak of maximum protein content was at day-3, and Sr75 isolate yielded the highest protein content.

The cellulase activity

The activity of endoglucanase on CDB ranged from 0.337 – 0.402 IU/ml during incubation period of seven days. The maximum activity of endoglucanase was on day-3 incubation period. On day-3, the endoglucanase maximum activity was on Jm (0.402 IU/ml), Sr75 (0.377 IU/ml), U6 (0.356 IU/ml), Km25 (0.341 IU/ml), and Km13 and G8 isolate (0.337 IU/ml). On day-7, the activity of endoglucanase on CDB decreased down to 0.011-0.031 IU/ml.

Fig.5 and Fig.6 showed that cellulase activities increased along with cell growth. of the cells. The maximum activity was on exponential phase, but when CDB experienced to stationary phase, endoglucanase activity declined. On the stationary phase, speed of cell division is equal to cell death and lysis of cell. In addition to cellulose, protease is also resulted (Grindra, 1993). Optimum time of enzyme production is used as harvest time of enzymes to degrade substrate of waste. Meryandini (2009) suggested that peak of activity and the optimum time for cellulase production of C5-3 and C4-4 isolate is on the second day (0.026 nkat/ml and 0.390 nkat/ml). Gupta et al. (2011) found that endoglucanase activity on CBD derived from some invertebrates ranged from 0.1622 – 0.400 IU/ml, the highest endoglucanase activity was on CDB-8 isolate (0.400 IU/ml).

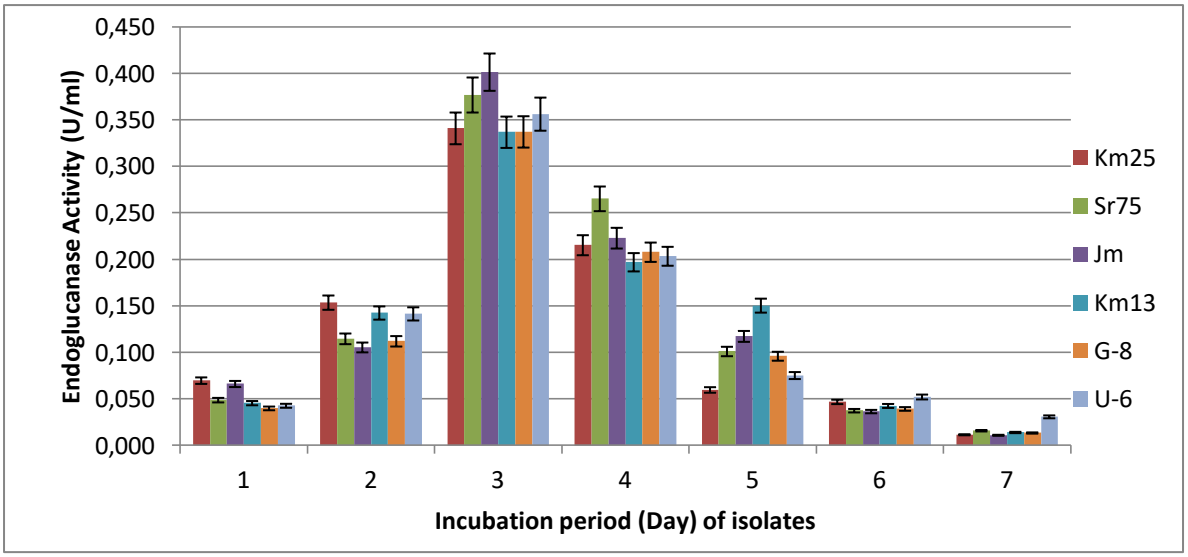

Figure 5. Endoglucanase activity of six BSD isolates during incubation period (seven days). The endoglucanase maximum activity was on day-3, and drastically decreased on day-7.

The activity of exoglucanase on CDB ranged from 0.0032 – 0.155 IU/ml during incubation period of seven days. The maximum activity of exoglucanase was on day-3 incubation period. The exoglucanase maximum activity was on Jm (0.1550IU/ml), G8 (0.1475 IU/ml), U6 (0.1275 IU/ml), Km25 (0.1260 IU/ml), Km13 (0.1228), and Sr75 (0.1173 IU/ml), and isolate. On day-7, the activity of endoglucanase on CDB was almost little or nothing. It may be due to the supply food of CDB is already diminished, the stock of carbon that will be overhauled into a simple sugar for bacterial life already decreased, where the number of alive CDB as many as died CDB (Waluyo, 2007).

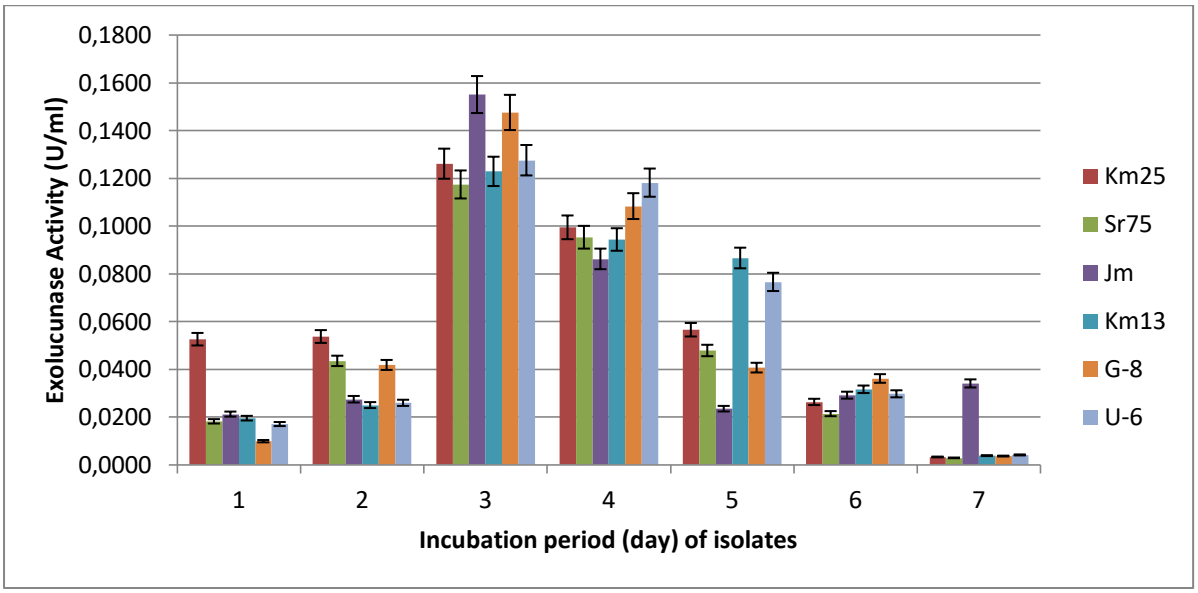

Figure 6. Exoglucanase activity of six BSD isolates during incubation period (seven days). The exoglucanase maximum activity was on day-3, and drastically decreased on day-7.

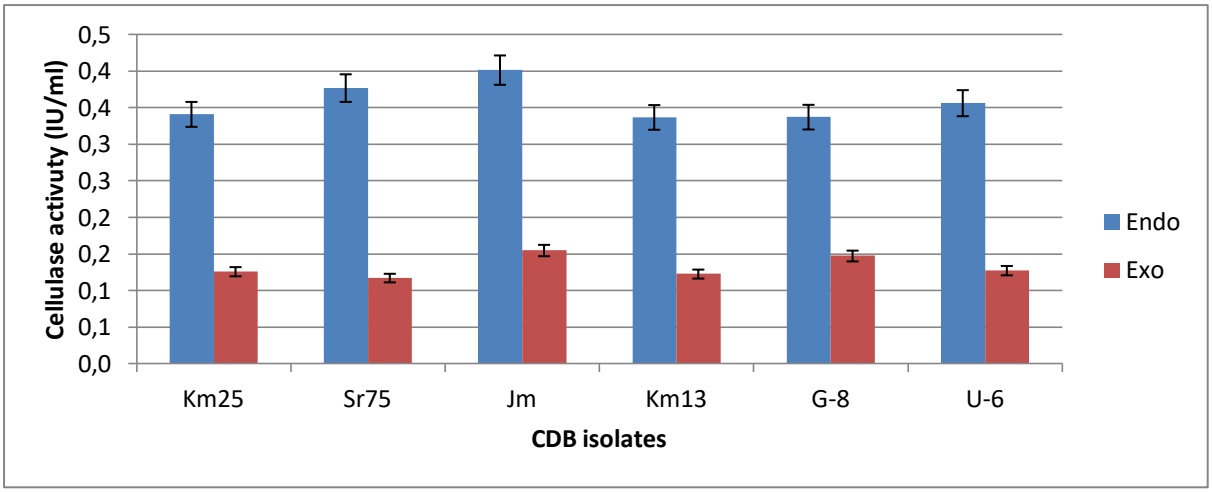

Figure 7. Comparison of endoglucanase and exoglucanase activity of six isolates on day-3 incubation period.

Fig.7 showed the activity of endoglucanase was greater than exoglucanase, it was due to endoglucanase played role as degrading enzyme. Activity of endoglucanase on six isolates ranged from 0.337 – 0.402 IU/ml, and the highest activity was on Jm (0.402 IU/ml), whereas exoglucanase activity ranged from 0.123 – 0.155 IU/ml and the highest activity was also on Jm isolate (0.155 IU/ml). Gupta (2011) reported that the highest activity of endoglucanase on CDB in some invertebrates is CDB8 (0.400 IU/ml), and the highest activity of exoglucanase is on CDB10 isolates (0.166 IU/ml).

Conclusion

There were six isolates with index clear zone ≥ 2.0 i.e. Km25 = 3.32 mm; Sr75 = 3.31 mm; JM = 2.41 mm; U-6 = 2.36mm; G-8 = 2.19 mm, and Km13= 2.04 mm. The maximum growth of CDB was on day-3 at basal salt media, incubated for seven days, shaking at 160 rpm at 33oC. The maximum protein content of six isolates was on day-3, the highest protein content was on Sr75 isolate (8.00 ng/ml). Whereas, the activity of endoglucanase ranged from 0.337 – 0.402 IU/ml, the highest activity was on Jm (0.402 IU/ml), exoglucanase activity ranged from 0.123 – 0.155 IU/ml, the highest activity was also on Jm isolate (0.155 IU/ml).

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