CHAPTER 4 RESULT AND DISCUSSION

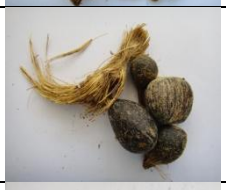
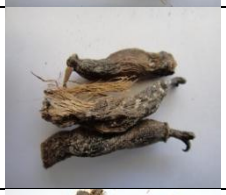
4.1 Samples for isolation of cellulolytic microorganisms

The samples that are used in this research for isolation of microorganisms were collected from the palm oil plantation and palm oil mill areas in Pechabury Province, Thailand. The samples were shown in table 4.1.

Table 4.1 Types, sources, and characteristics of samples which are selected from the palm oil plantation and palm oil mill areas.

Samples	Type of samples	Sources	Sample Characteristic	Figure
1	Soil	Plantation field	Brown color, and hard structure	
2	Leaf bamboo mix with gravel and soil	Plantation field	Dried leaves and brown color	
3	Root bamboo	Plantation field	Yellow color and easy broken	
4	Rod palm tree	Plantation field	Decomposed, dark color, easily broken, and fiber	
5	Stalk leaf palm tree	Plantation field	Stalk leaf decomposed, soil attaches at the stalk, brown color, easy broke, and no hard structure	
6	Leaf and soil	Plantation field	Leaf decomposed and dark color	

Table 4.1 Types, sources, and the characteristic of samples which are selected from the palm oil plantation and palm oil mill areas (Continuous).

Samples	Type of samples	Sources	Sample characteristic	Figure
7	Shell, fiber and seed of processing palm oil	Factory	Dark and brown color, decomposed and white spot color.	
8	Pasta palm oil processed	Factory	Smooth, clear brown color, residue pressed processing oil and oily	
9	Seed (kernel) and fiber	Factory	Dark color and decomposed	
10	Immature bunch	Factory	Dark and brown color and white spot color	
11	Residues pressed processing palm oil	Factory	Smelt, oily, easy broken, solid structure and brown color.	
12	Shell, immature bunch and fiber	Factory	After boiling process, gray color and white spot color	

The twelve (12) samples were selected from the palm oil plantation and palm oil mill areas such as soil, a rod palm tree, immature bunch, seed of the oil palm empty fruit bunch, shell, fiber of the oil palm empty fruit bunch which they contain normal flora that can decompose cellulosic material; cellulolytic microorganism. The microorganism isolated from cellulosic material should have the efficacy for breaking down the lignocellulosic materials; oil palm empty fruit bunch (OPEFB) and residue of OPEFB. According to Smruti et al., (1995) reported the cellulase producing microbes have been isolated from lignocellulosic materials such as decaying wood, forest residues and decomposed leaf in soil. In general, lignocellulotic material is content nutrient such as carbon, oxygen, phosphorus, nitrogen, mineral salt, and other nutrient that it can be used by microorganisms for living in nature.

4.2 Composition of oil palm empty fruit bunch (OPEFB) fiber and residue of acid hydrolysis of OPEFB

Table 4.2 shows compositions of OPEFB and residue based on dried weight. Residue from acid hydrolysis of OPEFB is produced (the solid part) from the acid hemicelluloses extraction process. The process is done under strong condition (121°C, for 75 min). Finally, hemicelluloses is digested form oil palm empty fruit bunch (OPEFB) fiber and pentose or and a little bit amount of glucose are by–products from hemicelluloses extraction. Pentose (xylose) is the main component in hemicelluloses which it can be used to produce sugar alcohol (xylitol); a highly valuable product. Therefore, cellulose should be the major component in residue from acid hydrolysis of OPEFB. According to Morohoshi (1991) and Sjorstrom (1993), lignocellulosic materials predominantly contain a mixture of carbohydrate polymers (cellulose and hemicellulose), lignin, extractives and ash. Hemicellulose relatively easy to hydrolyze by acids to their monomer component such as xylose, mannose, glucose, galactose, arabinose, and small amounts of rhamnose, glucuronic acid, methyl glucuronic acid and galacturonic acid. Proximate analysis revealed that OPEFB contains 9.56% protein, 6.86% lipid, 8.72% ash, 39.42% cellulose, 16.18% hemicelluloses and 13.33% lignin. After hemicelluloses extraction, protein, lipid and ash content in residue from acid hydrolysis of OPEFB decreased to 4.87%, 4.48% and 2.1%, respectively. This result indicated the main components was decreased after the hydrolysis by acid and steaming process. In contrast, the compositions of cellulose and lignin in residue from acid hydrolysis of OPEFB were higher than OPEFB due to decreasing of hemicelluloses, protein, lipid and ash component. Compositions of cellulose, hemicelloluse and lignin in residue from acid hydrolysis of OPEFB were 53.38%, 0% and 23.27%, respectively. Decreasing of hemicelluloses in residue from acid hydrolysis of OPEFB was expected because of it passed hemicelluloses extraction process. High composition of cellulose in the residue is considered as a good carbon source for saccharification process by cellulosic microorganism. According to Wyman (1994) and Sung et al., (2002), the acid hydrolysis method gives high reaction rates and significantly improves cellulose hydrolysis. Because of almost 100% hemicellulose removal is possible by acid hydrolysis (dilute-acid hydrolysis). Dilute-acid hydrolysis is not effective in dissolving lignin, but it can disrupt lignin and increases the cellulose susceptibility to enzymatic hydrolysis.

In contrast also with OPEFB, the residue of acid hydrolysis of OPEFB (table 4.2) has still a high concentration of lignin. The presence of lignin in the residue of acid hydrolysis can disrupt microorganisms to growth therefore the residue of acid hydrolysis of OPEFB is very difficult to degrade by microorganisms. According to Palmqvist et al., (2000) and Taherzardeh (1999), lignin is extremely resistant to chemical and enzymatic degradation because lignin is very complex molecule constructed of phenypropane unit linked in a three-dimensional structure.

Table 4.3 describes that preparation of residue from acid hydrolysis of OPEFB by mild sulfuric acid (H_2SO_4). A 100 g OPEFB a mix with 2% sulfuric acid 1000 mL when it was done acid hydrolysis process by using mild sulfuric acid (2%) with volume 1000 ml, after sterilization (121°C, 75 min) by autoclave and Whatman filter paper was obtained the products hydrolyzate (liquid fraction) and residue (solid fraction).

Hydrolyzate after acid hydrolysis processed was 700 mL and residue of acid hydrolysis of OPEFB was 64.6 g.

Table 4.2 Composition of OPEFB and the residue from acid hydrolysis of OPEFB

Main components (%)	**Oil palm empty fruit bunch (OPEFB)**	**Residue from acid hydrolysis of OPEFB**
Protein	9.56	4.87
Lipid	6.86	4.48
Ash	8.72	2.1
Moisture	10.85	8.61
Carbohydrate	64.01	79.94
Cellulose	39.42	53.38
Hemicellulose	16.18	0
Lignin	13.33	23.27

Table 4.3 The amount of residue was obtained after acid hydrolysis process of OPEFB

Substrate OPEFB (g)	**Chemical H_2SO_4 (2%) (mL)**	**Hydrolyzate (mL)**	**Residue (g)**
100	1,000	700	64.6

Table 4.3 also shows that the amount of the hydrolyzate was obtained only 700 mL from the initial liquor of sulfuric acid 1,000 mL. It is possible because of OPEFB fiber has been absorbed many water therefore properties of OPEFB fiber is being swelled full with water after acid hydrolysis process and when filtered was not pressed perfectly. While the residue fiber after neutralization by washing water and adding NaOH to pH normal (pH 6-7) was obtained only 64.6 g from 100 g the initial weight. It is possible because of lost when the residue is being washed by water.

4.3 Isolation of microorganisms

To obtain cellulosic microorganism, CMC, cellulose derivative which can easily dissolve in water was used as an enrichment medium to support the growth of cellulosic microorganism. The samples (plants, soil and others) were directly added into CMC broth and incubated for 10 days under shaking condition at 150 rpm, room temperature. With a long time incubation, remaining nutrients in the sample should disappear in the isolation flask. Therefore, obtained microorganism from CMC broth after incubation for 10 days should be microorganism which can utilize CMC. Target microorganisms

were obtained by streak plate technique on fresh CMC agar. Table 4.4 shows summarization of microorganisms obtained from plant and soil samples associated with oil palm the samples.

There were fifty eight (58) isolates of microorganisms isolated from the samples associated with oil palm industry. Based on colony formation and cell morphology (under microscope), those microorganisms obtained from screening was separated into a member of bacteria, actinomycetes and fungi. Thirty nine isolates (39) of microorganisms belonged to member of bacteria, while ten isolates (10) was classified to be a member of actinomycetes. The rest isolates (nine isolates) are fungi. According to Heck et al., (2002) and Semedo et al., (2000), reported many of cellulolytic aerobic and anaerobic bacteria could be isolated from decomposed soil and the lignocellulosic of residues (leaf, stalks, and stems of garden plants) for biotechnological applications. Therefore, microorganism isolated in this study would be applied and used in biological technology. Furthermore, applications and report for degradation of the lignocellulose materials by the cellulase enzyme obtained many filamentous fungi, particularly in a genus of *Trichoderma*, *Aspergillus*, *Fusariumn*, and *Penicillium* are available (Roberto et al., 2005).

Table 4.4 The fifty eight (58) isolates of microorganisms obtained from plant and soil samples associated with oil palm the samples were incubated for 10 days at 30^{o}C and agitation rate of 150 rpm.

Samples	Type of Microorganisms	Amount of Isolates	Isolates
1	Actinomycetes (A) Bacteria (B) Fungi (F)	1 3 2	1.2.A 1.1.B, 1.3.B, 1.4.B 1.5.F, 1.6.F
2	Actinomycetes (A) Bacteria (B) Fungi (F)	1 4 N.D	2.3.A 2.1.B, 2.2.B, 2.4.B, 2.5.B -
3	Actinomycetes (A) Bacteria (B) Fungi (F)	1 4 N.D	3.1.A 3.2.B, 3.3.B, 3.4.B, 3.5.B -
4	Actinomycetes (A) Bacteria (B) Fungi (F)	1 4 1	4.2.A 4.1.B, 4.3.B, 4.4.B
5	Actinomycetes (A) Bacteria (B) Fungi (F)	1 3 N.D	5.1.A 5.2.B, 5.3.B, 5.4.B -
6	Actinomycetes (A) Bacteria (B) Fungi (F)	1 3 N.D	6.2.A 6.1.B, 6.3.B, 6.4.B -
7	Actinomycetes (A) Bacteria (B) Fungi (F)	N.D 3 1	- 7.1.B, 7.2.B, 7.3.B 7.4.F

N.D = Not detected

Table 4.4 The fifty eight (58) isolates of microorganisms obtained from plant and soil samples associated with oil palm the samples were incubated for 10 days at 30^{o}C and agitation rate of 150 rpm. (Continuous)

Samples	Type of Microorganisms	Amount of Isolates	Isolates
8	Actinomycetes (A) Bacteria (B) Fungi (F)	1 3 2	8.3.A 8.1.B, 8.2.B, 8.4.B 8.5.F, 8.6.F
9	Actinomycetes (A) Bacteria (B) Fungi (F)	N.D 4 N.D	- 9.1.B, 9.2.B, 9.3.B, 9.4.B -
10	Actinomycetes (A) Bacteria (B Fungi (F)	1 4 1	10.A 10.2.B, 10.3.B, 10.4.B, 0.5.B 10.6.F
11	Actinomycetes (A) Bacteria (B) Fungi (F)	1 1 1	11.1.A 11.2.B 11.3.F
12	Actinomycetes (A) Bacteria (B) Fungi (F)	1 2 2	12.3.A 12.1.B, 12.2.B 12.4.F, 12.5.F

N.D = Not detected

4.3.1 Colony properties of microorganism

Table 4.5, showed cell and colony morphological studies of bacterial isolates. Under a light microscope, bacteria were clearly separated from fungi and actinomycetes. Colony morphological studies of bacteria can help at bacterial genus classification. Various factors influence the shape and color of the colonized bacterial colony such as type of bacterial culture medium, pH, temperature and condition (aerobic, anaerobic , anoxic etc.) for incubation. In this experiment, all microorganisms were cultured on CMC agar. There were fifty five (58) bacterial isolates obtained from plant and soil samples associated with oil palm industry. Cell morphology and colony morphology of bacterial isolates such as shape, the margins or edges of the colony, the colony's color, colony and Gram cell wall were explained to support pre-primary identification of bacterial isolates. Under a light microscope, various shapes of bacterial cell were observed such as rod, curved rod, coccus. There were 2 major types of bacterial colony shapes which could be seen after cultured on CMC agar including circular and irregular form. The color or the pigment of the colony of all bacteria were white color (opaque or translucent) and also present of cream or yellow colonies. The shape of the colony of bacteria is some circular or some irregular. The edge of bacterial colony such as low convex with entire edge and convex with undulate edge were also determined. With Gram stain technique, There were nineteen (19) isolates and twenty (20) isolates classified into a member of Gram-negative and Gram-positive bacteria, respectively.

Table 4.5 Cell, colony morphology and Gram staining of bacterial isolates

Isolates	Gram Reaction	Cell Morphology	Colony Morphology
1.1.B	Positive	Curved rod	White, circular, opaque with low convex and entire edge
1.3.B	Positive	Coccus	White, circular, opaque, with low convex and entire edge
1.4.B	Negative	Rod	White-Cream, irregular, opaque, with convex and entire edge
2.1.B	Negative	Rod	White-Cream, irregular, opaque, with low convex and undulate edge
2.2.B	Negative	Rod	White-Cream, circular, translucent, with convex and entire edge
2.4.B	Negative	Rod	White-Cream, irregular, opaque, with convex and entire edge
2.5.B	Negative	Rod	White-Cream, irregular, opaque, with low convex and undulate edge
3.2.B	Positive	Curved rod	White, circular, translucent, with low convex and undulate edge
3.3.B	Positive	Rod	White-yellow, circular, translucent, with low convex and entire edge
3.4.B	Positive	Rod	White-Cream, irregular, opaque, with convex and entire edge
3.5.B	Negative	Coccus	White-Cream, irregular, opaque, with convex and undulate edge
4.1.B	Positive	Coccus	White-Cream, circular, translucent, with convex and entire edge
4.3.B	Negative	Rod	White, irregular, translucent, with convex and undulate edge
4.4.B.	Positive	Curved rod	White-yellow, irregular, opaque, with convex and entire edge
5.2.B	Negative	Coccus	White-yellow, irregular, opaque, with low convex and undulate edge
5.3.B	Negative	Rod	White-yellow, irregular, translucent, with low convex and undulate edge
5.4.B	Positive	Curved rod	White-yellow, irregular, opaque, with convex and undulate edge
6.1.B	Negative	Rod	White-yellow, circular, opaque, with convex and entire edge
6.3.B	Positive	Curved rod	White-Cream, irregular, translucent, with convex and undulate edge
6.4.B	Negative	Rod	White-Cream, irregular, opaque, with low convex and undulate edge
7.1.B	Negative	Curved rod	White-Cream, irregular, opaque, with convex and entire edge
7.2.B	Negative	Curved rod	White-yellow, irregular, translucent, with convex and entire edge
7.3.B	Negative	Rod	White-Cream, irregular, opaque, with low convex and entire edge
8.1.B	Positive	Coccus	White-Cream, irregular, translucent, with low convex and entire edge
8.2.B	Negative	Curved rod	White-Cream, circular, translucent, with convex and entire edge
8.4.B	Negative	Curved rod	White-Cream, irregular, opaque, with low convex and entire edge
9.1.B	Positive	Curved rod	White-Cream, irregular, translucent, with low convex and undulate edge
9.2.B	Positive	Coccus	White-Cream, irregular, opaque, with convex and undulate edge
9.3.B	Negative	Curved rod	White-Cream, irregular, opaque, with low convex and undulate edge
9.4.B	Positive	Coccus	White-Cream, irregular, translucent, with convex and undulate edge
10.2.B	Positive	Rod	White, Amoeboid, translucent, with low convex and undulate edge
10.3.B	Negative	Rod	White-yellow, irregular, translucent, with convex and undulate edge
10.4.B	Positive	Coccus	White-Cream, irregular, translucent, with convex and undulate edge
10.5.B	Positive	Coccus	White-Cream, irregular, opaque, with convex and undulate edge
11.1.B	Positive	Curved rod	White-yellow, irregular, translucent, with convex and entire edge
12.1.B	Positive	Curved rod	White-Cream, irregular, opaque, with convex and entire edge
12.2.B	Negative	Curved rod	White-Cream, irregular, opaque, with convex and undulate edge

Table 4.6 Cell, colony morphology and Gram staining of actinomycetes isolates

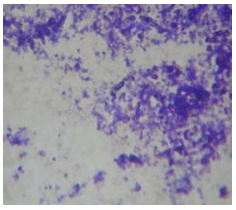
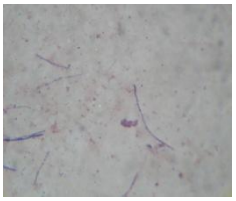
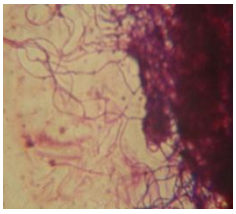
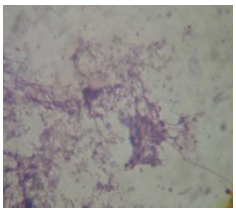
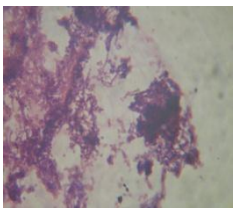
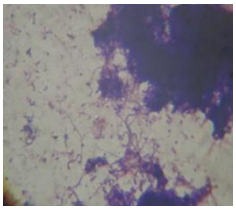
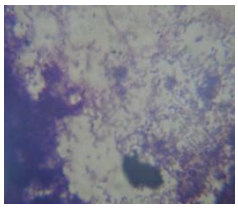
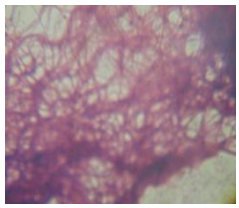
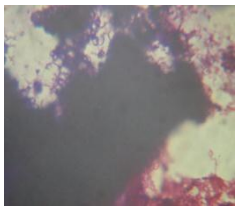
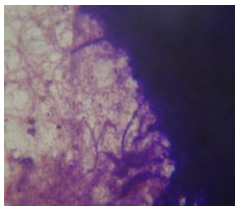
Isolates	Gram Reaction	Cell Morphology	Colony Morphology	Figure
1.2.A	Positive (+)	Filamentous	White, circular, opaque, with low convex and entire edge	
2.3.A	Positive (+)	Filamentous	White, circular, opaque, with low convex and undulate edge	
3.1.A	Positive (+)	Filamentous	White-cream, irregular, opaque, with low convex and undulate edge	
4.2.A	Positive (+)	Filamentous	White-green, circular, opaque, with low convex and undulate edge	
5.1.A	Positive (+)	Filamentous	White, irregular, opaque, with low convex and undulate edge	
6.1.A	Positive (+)	Filamentous	White, irregular, opaque, with low convex and undulate edge	

Table 4.6 Cell, colony morphology and gram staining of actinomycetes isolates (continuous).

Isolates	Gram Reaction	Cell Morphology	Colony Morphology	Figure
8.3.A	Positive (+)	Filamentous	White, irregular, opaque, with low convex and entire edge	
10.1.A	Positive (+)	Filamentous	White, circular, opaque, with low convex and entire edge	
11.2.A	Positive (+)	Filamentous	White, irregular, opaque, with low convex and entire edge	
12.3.A	Positive (+)	Filamentous	White, circular, opaque, with low convex and undulate edge	

There were ten (10) isolates of actinomycetes which could be isolated from the samples associated with oil palm industry. Colonies of actinomycetes were white color, opaque with low convex and irregular in shape (table 4.6). Under microscope and Gram-stain technique, cell morphology of actinomycetes was observed. A filamentous property and Gram stain positives were seen under light microscope (1,000×). With specific property of actinomycetes cell, all selected isolates were determined and confirmed to be actinomycetes. Characteristic of the colony and cell of actinomycetes represented in table 4.6.

Table 4.7 Cell and colony morphology of fungal isolates.

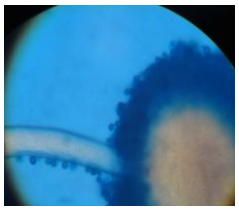
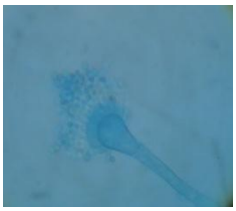
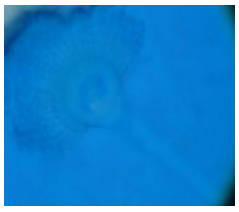
Isolate	Genus	Colony Color	Morphology	Figure
1.5.F	*Aspergillus spp.*	Black	Septate hyphae. Typical radiate conidial head	
1.6.F	*Aspergillus spp.*	Green	Septate hyphae. Typical columnar, uniseriate conidial head	
7.4.F	*Aspergillus spp.*	Green	Septate hyphae. Typical columnar, uniseriate conidial head	
8.5.F	*Aspergillus spp.*	Green	Septate hyphae. Typical columnar, uniseriate conidial head	
8.6.F	*Aspergillus spp.*	Green	Septate hyphae. Typical columnar, uniseriate conidial head	

Table 4.7 Cell and colony morphology of fungal isolates (continuous).

Isolate	Genus	Colony Color	Morphology	Figure
10.6.F	*Aspergillus spp.*	Green	Septate hyphae. Typical columnar, uniseriate conidial head	
11.3.F	*Aspergillus spp.*	Green	Septate hyphae. Typical columnar, uniseriate conidial head	
12.4.F	*Aspergillus spp.*	Green	Septate hyphae. Typical columnar, uniseriate conidial head	
12.5.F	*Aspergillus spp.*	Black	Septate hyphae. Typical columnar, uniseriate conidial head	

Fungi were important cellulosic microorganism associated with cellulose cycle. Fungi are known as the best source of cellulase enzyme and their applications in biological technology such as saccharification by fungal cellulase were reported. Table 4.7 shows the colony and cell morphological studies of fungal isolates which were isolated from the samples associated with the oil palm industry. Based on colony morphology, fungal isolates were selected. Pre-primary identification was done by investigation of arrangement of conidia under a light microscope (Lactophenol cotton blue as staining dye). All fungal isolates presented conidiosphore as a special characteristic of the genus *Aspergillus*. Therefore, nine (9) isolates of fungi were classified as member of the genus *Aspergillus*. Table 4.7 represents cell and colony morphology of fungal isolates.

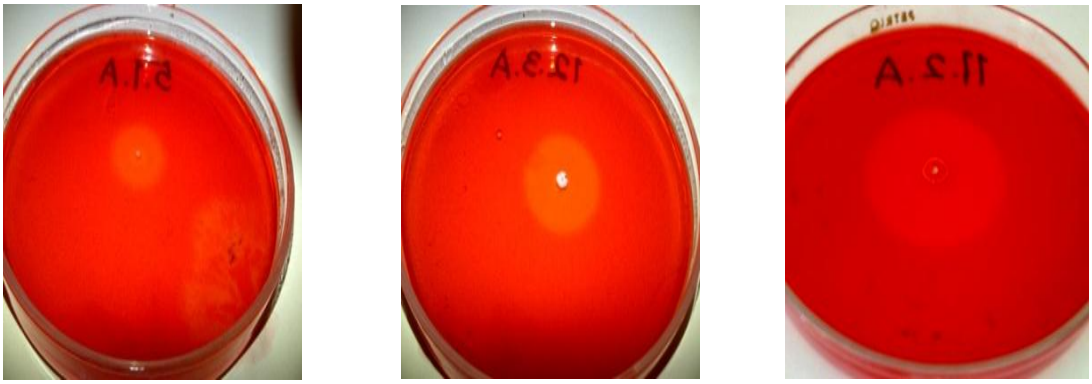
4.4 Detection of extracellular cellulase activity on CMC agar

All microorganism obtained from the screening could grow on CMC agar. However, it did not guarantee that they can produce extracellular cellulase. In this experiment, we attempt to find microorganism that can grow and produce extracellular cellulase on CMC agar but it is difficult to observe a zone of clearance around the colony (extracellular cellulase activity). According to Yoon et al., (2007), the Congo red dye has a special characteristic which is able to staining polysaccharide. Therefore, the Congo red dye is often used for the detection of extracellular enzymes in various mediums containing polysaccharide (CMC is also polysaccharide). After Congo red staining, a zone of clearance enzyme activity on CMC agar can be observed by destaining with NaCl solution.

To determine the extracellular cellulase activity on on CMC agar, all isolates were cultured on CMC agar for 3 days at room temperature. Investigation of their cellulase activity was done by Congo red staining for 30 min and destaining with 1 M NaCl solution. The formation of a clear zone of hydrolysis indicated cellulose degradation by microorganism (Ariffin et al., 2008). The ratio of a clear zone and colony size was determined. The isolates showed a high ratio between the diameter of the clear zone and the bacterial colony were also selected and used for future experimentation.

Among them, there are nine (9) isolates of microorganisms that could observe and produced extracellular cellulase by formation of a clear zone. Nine (9) isolates contained 2 (two) bacterial isolates and 7 (seven) actinomycetes isolates. This result supported that microorganisms are able to grow on CMC agar but they are not necessary to produce extracellular cellulase. Table 4.8 shows the ratio between the diameter of the clear zone and the colony of extracellular cellulase producing microorganism. Three of nine isolates from microorganisms were selected for future experiment because of they showed a large of the ratio of a clear zone and colony diameter. Three isolates included isolate 5.1.A, isolate 12.3.A and isolate 11.2.A which they were actinomycetes group.

Actinomycetes are soil microorganisms common in many types of soil. Actinomycetes population was the ranks second after bacteria, even occasionally actinomycetes were almost the same population with bacteria (Alexander, 1961 and Elberson et al., 2000). Actinomycetes life was as saprophyte and active for decomposing organic material, therefore it can improve soil fertility (Nonomura et al., 1969). Actinomycetes were one of microorganisms that can degrade cellulose in addition of bacteria, molds, and yeasts (Abe et al., 1979 and Nakase et al., 1994). According to Kanti (2005) report, actinomycetes isolated from soil in the Bukit Duabelas National Park, Jambi could produce extracellular cellulase by formation of a clear zone when it cultured in the CMC agar.



(A) **(B)** **(C)**

Figure 4.1 Cellulase activity demonstrated by a clear zone surrounded the colony of the selected actinomycetes isolates. Three isolates of actinomycetes are (A) 5.1.A, (B) 12.3.A and (C) 11.2.A.

Table 4.8 Ratio between the diameter of the clear zone and the microbial colony

Isolate codes	Diameter of colony (mm) (A)	Diameter of clearance zone (mm) (B)	Activity ratio (B/A)
3.1.A	14	26	1.86
4.2.A	13	31	2.38
5.1.A	2	16	8
8.3.A	9	37	4.11
8.4.B	5	13	2.6
9.3.B	6	12	2
10.1.A	5	18	3.6
11.2.A	8	35	4.38
12.3.A	4	24	6

There are nine (9) isolates of microorganisms that were produced cellulase activities in the formation a clear zone after staining by Congo red and 1 M NaCl solution. Microorganisms cellulase production is consisted two isolates of bacterial and seven isolates from actinomycetes. For fungi, no isolate demonstrated a clear zone on CMC medium agar. Three of nine microbial isolates were selected for high cellulase production. They are actinomycetes isolated 5.1.A, 12.3.A and 11.2.A. The ratio of clear zone has shown in table 4.3. The isolate 5.1.A demonstrated the highest cellulase

activity with the ratio 8 and followed by isolate 12.3.A and 11.2.A with the value ratio 6 and 4.38, respectively.

4.5 Determination of microbial growth and extracellular cellulase production in a liquid CMC medium

From previous experiments, the best extracellular cellulase producing actinomycetes were selected. Properties of selected isolates grown in liquid CMC medium; growth curve, cellulase activity and free-reducing sugar was also investigated in this experiment.

To find the best isolate for degradation of cellulose fiber, isolate 5.1.A, 12.3.A and 11.2.A were cultured in the CMC broth. Microbial concentration, cellulase activity and free-reducing sugar during incubation for 7 days were also determined. Microbial cell concentration (CFU/mL) and cellulase activity were determined by drop plate technique and modified Ghose's method (1987). The amount of microbial cell concentration shows in in figure 4.2. Initial cell concentration was about 3.0 Log CFU/mL. The isolate 12.3.A showed cell concentration (CFU/mL) higher than isolate 11.2.A and the isolate 5.1.A (Figure 4.2). Cell concentration (CFU/mL) of the isolate 5.1.A, 12.3.A and 11.2.A at 7 days were 5, 6.97 and 5.77 Log CFU/mL, respectively. Determination to the formation of the pellet cell during incubation, cell concentration (CFU/mL) may not be a good parameter for determination of actinomycetes growth. In addition, reduction of substrate concentration, enzyme activity or increasing of the amount of product can be used as parameter to study the growth of microorganism. In this experiment, cellulase activity and free-reducing sugar in the supernatant was also investigated.

Enzyme activity from three isolates of Actinomycetes have been measured. The result revealed that isolate 11.2.A showed cellulase activity higher than isolate 12.3.A and 5.1.A (Figure 4.3). Therefore, isolate 11.2.A should digest CMC better than the rest isolates. Cellulase activity from three isolates of Actinomycetes culturing in CMC broth were 0.62, 0.44, and 0.29 U/ml for the isolate 11.2.A, 12.3.A, and 5.1.A, respectively.

Amount of reducing sugar in CMC medium when inoculated isolate 11.2.A, 12.3.A, and 5.1.A and incubation for 7 days shows in Figure 4.4. As the same time, isolate 11.2.A could produce the highest amount of reducing sugar after incubation for 5 days, about reducing sugar of 6.66 (mg/mL) was determined. The maximum amount of reducing sugar when culturing isolate 12.3.A (reducing sugar of 4.72 mg/mL) and isolate 5.1.A (reducing sugar of 3.09 mg/mL) were obtained in 6 and 4 days, respectively.

According to result, isolate 11.2.A and isolate 12.3.A were selected due to they could produce high amount of cellulase activity and reducing sugar. However, the objective of this research is the production of ethanol from waste of palm oil industry. Therefore, isolate 11.2.A and isolate 12.3.A would be cultured in medium containing fiber from the waste of palm oil industry. Then growth, cellulase activity and amount of reducing sugar in the fiber medium should be investigated.

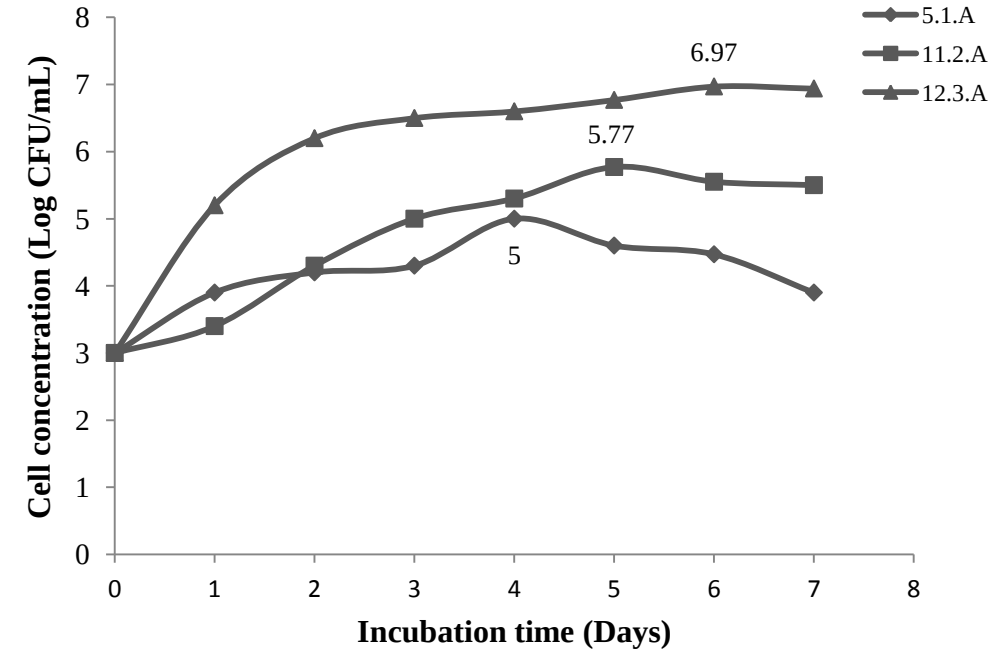


Figure 4.2 Growth of the selected cellulolytic actinomycetes in CMC broth.

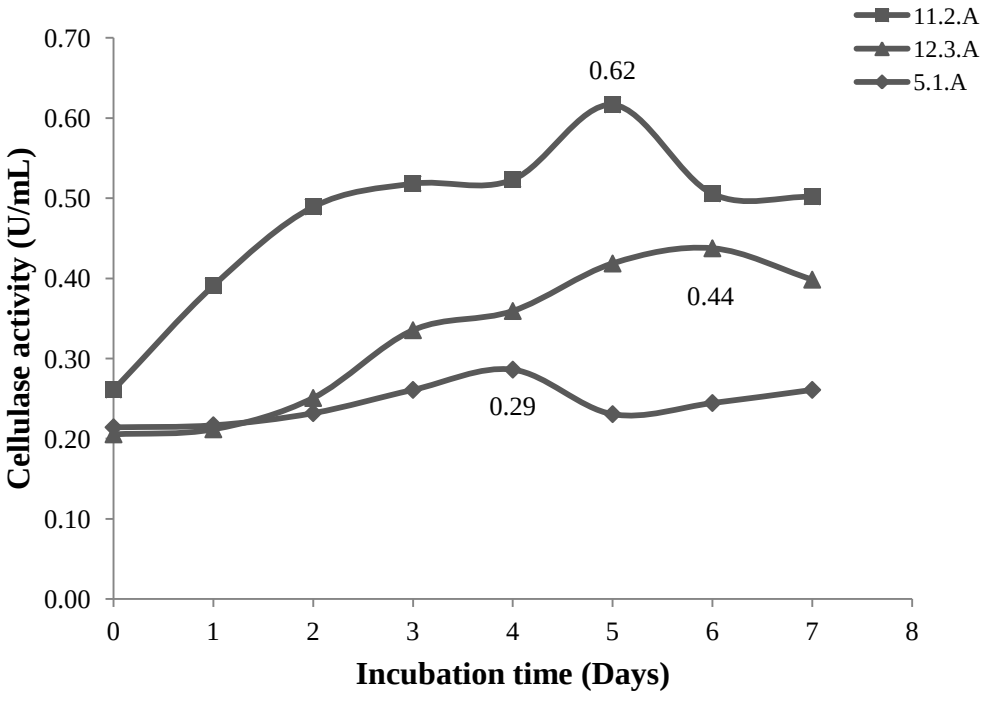


Figure 4.3 Extracellular cellulase production of the selected cellulolytic actinomycetes in CMC broth.

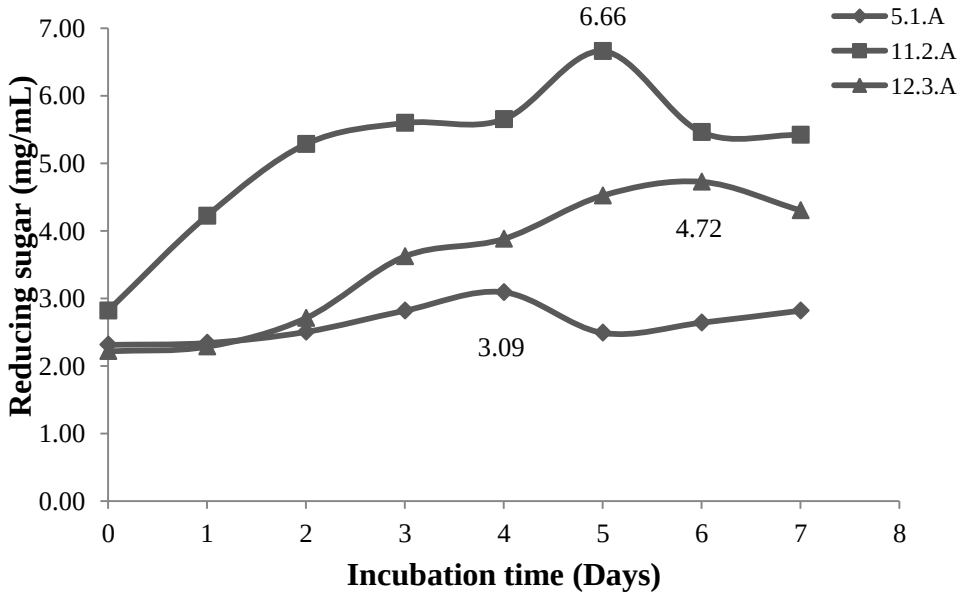


Figure 4.4 Reducing sugar in the culture supernatant of the selected cellulolytic actinomycetes growing in CMC broth.

4.6 Optimum conditions for cellulase production of the selected cellulolytic actinomycetes

From the previous section, isolate 11.2.A and isolate 12.3.A were selected based on their high cellulase and reducing sugar production. Both isolates showed high degradation efficacy of cellulose when CMC was used as sole carbon source. However, CMC is not a suitable source for large scale production of bio-fuel because of it is so expensive. Waste from the palm oil industry is considered to be a good and sustainable source for production of bio-fuel. In this section, the degradation efficacy of OPEFB and the residue of acid hydrolysis of OPEFB by the isolate 11.2.A and isolate 12.3.A were investigated.

Optimum condition for cellulase production by both selected isolates was performed in liquid medium containing OPEFB fiber and residue from acid hydrolysis as carbon sources. In preparation of inoculum, the microorganisms were transferred into CMC broth and incubation for 3 days under controlled conditions. 1% inoculum was added into fiber mediums namely OPEFB medium and residue medium and incubated for 7 days. Amount of cellulase activity and reducing sugar when culturing the isolate 11.2.A and the isolate 12.3.A in fiber mediums were determined every day. Figure 4.5 shows changing in cellulase activity in culture supernatant when culturing isolate 11.2.A and isolate 12.3.A in OPEFB medium and residue medium. The results revealed that high cellulase activity when culturing both isolates in fiber mediums was detected during treatment of OPEFB medium and the residue medium. Cellulase activity of the isolate 12.3.A in OPEFB medium and the residue medium was 0.67 and 0.54 U/mL at 6 days of incubation, while the isolate 11.2.A were 0.46 and 0.43 U/mL respectively.

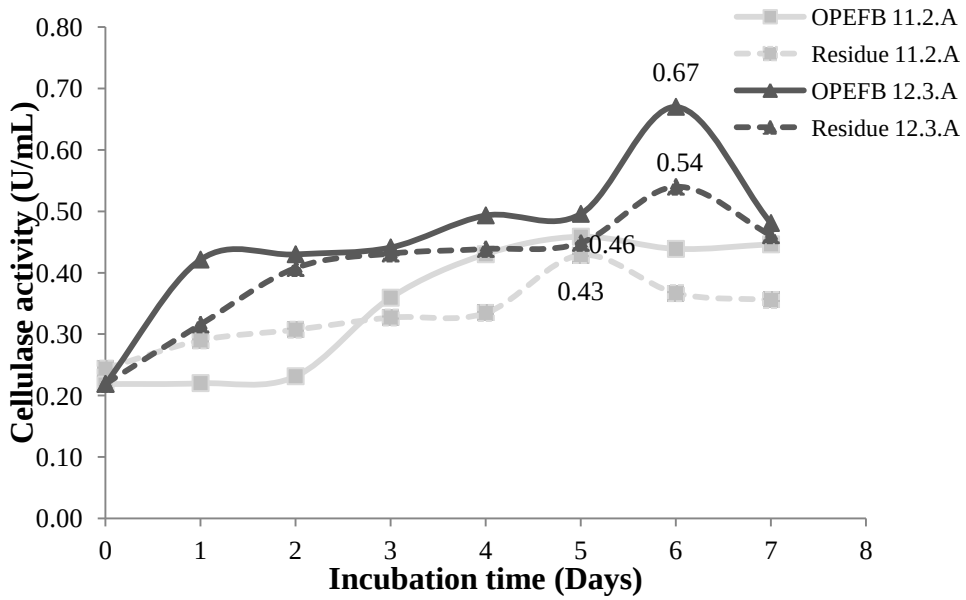


Figure 4.5 Cellulase production of the actinomycetes isolate 11.2.A and 12.3.A on OPEFB and residue culture medium, incubated at 30^{o}C and 150 rpm agitation.

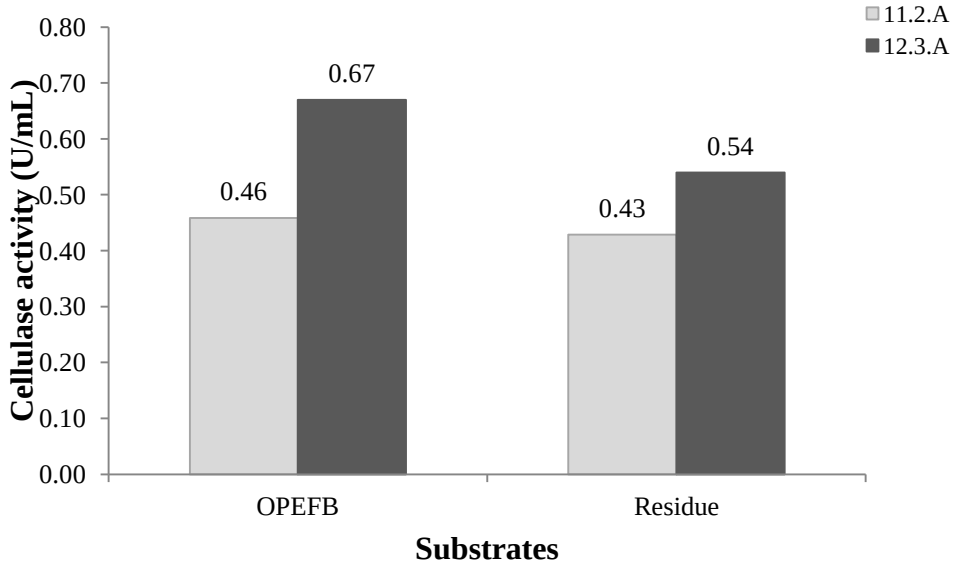


Figure 4.6 The maximum values of cellulase activity of the isolate 11.2.A and the isolate 12.3.A with OPEFB and the residue as the culture medium, growing at 30^{o}C and 150 rpm agitation.

The isolate 12.3.A was performed the high efficacy to produce reducing sugar on both media types; 3.63 mg/mL of OPEFB medium and 2.92 mg/mL of residue medium, therefore, the isolate 12.3.A was selected for decomposing OPEFB and the residue of acid hydrolysis of OPEFB.

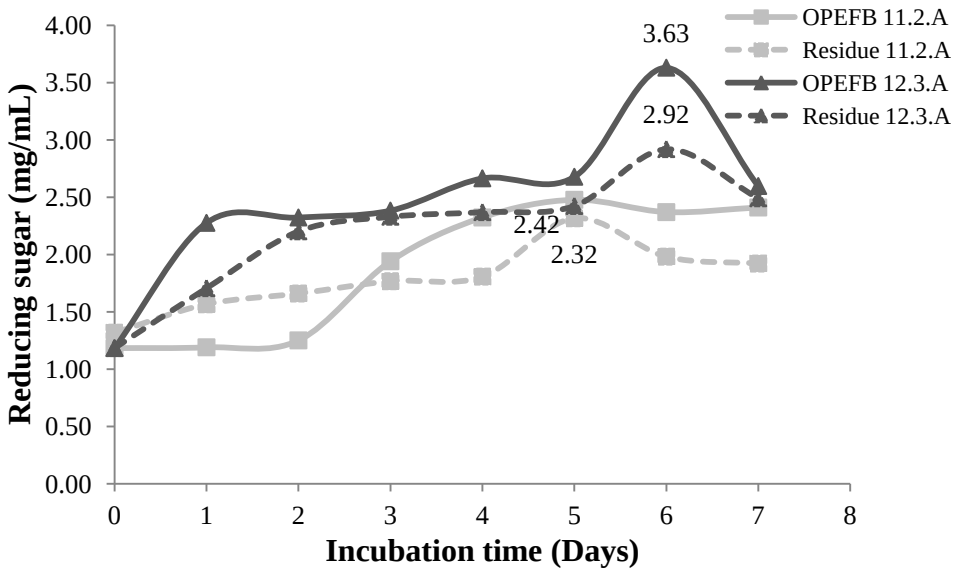


Figure 4.7 Reducing sugar in the culture supernatant of the isolate 11.2.A and the isolate 12.3.A on OPEFB and the residue as the culture medium, performing at 30^{o}C and 150 rpm agitation.

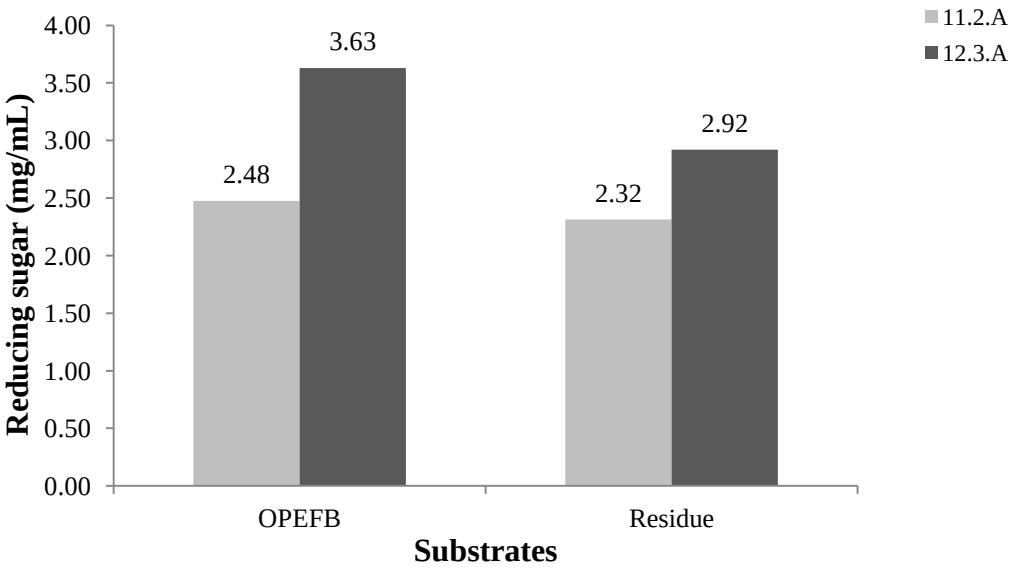


Figure 4.8 The maximum production of reducing sugar of the isolate 11.2.A and the isolate 12.3.A with OPEFB and the residue as the culture medium, culturing at 30^{o}C and 150 rpm agitation.

Base on figure 4.6 (cellulase production) and 4.8 (reducing sugar production), OPEFB medium had better support for cellulase and reducing sugar production than the residue. This could be explained by the difference in the composition of OPEFB and its acid hydrolysis residue. The acid hydrolysis extracted almost all the hemicellulose

component of the fiber (see in table 4.2). This made the residue had higher concentration of lignin (23.27 %) than OPEFB fiber (13.33%). High concentration of lignin can inhibit growth and cellulase production of the microorganism. Lignin may form a complex structure with cellulose. For this reason, the residue is becoming more difficult to degrade by the microorganism.

4.7. Optimum conditions for cellulase production of the isolate 12.3.A

4.7.1. Optimum pH

Determination of pH optimum was done in OPEFB and residue medium with various pH condition (pH 5-8). Determination of the optimum pH for cellulase production was shown in figure 4.9, 4.10 and 4.11. Cellulase production with OPEFB as a substrate of the isolate 12.3.A is shown in figure 4.9, pH 7 gave the highest production with the highest enzyme activity at 0.68 U/mL. Similar results was found when tested on the residue medium. pH 7 gave the highest enzyme production at 0.53 U/mL. This finding was supported by the work of Kanti (2005), actinomycetes produced the cellulase best at pH 7 when degrading cellulose.

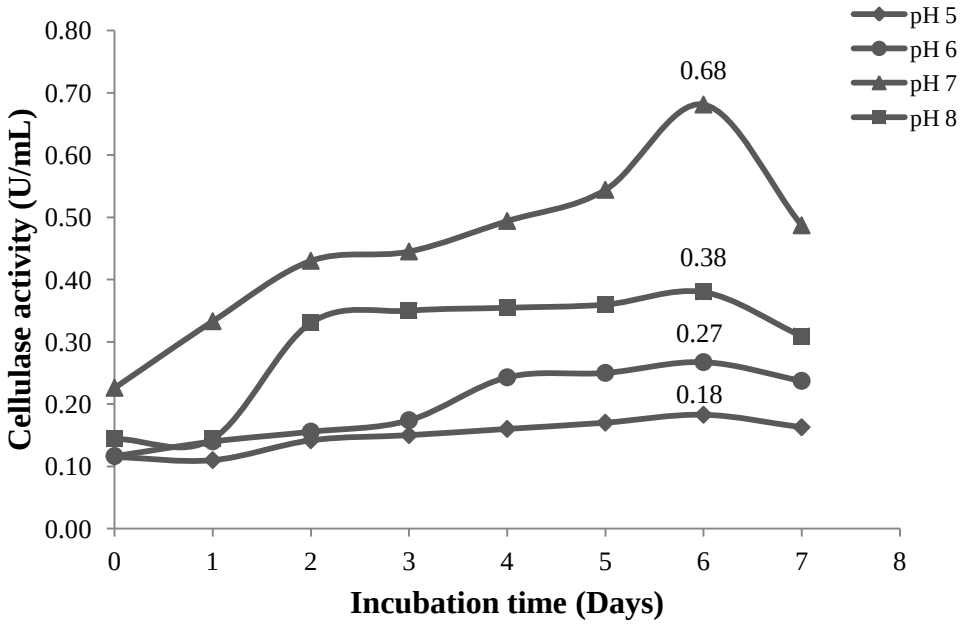


Figure 4.9 Cellulase production of the isolate 12.3.A on OPEFB medium with different pH, incubated at 30^{o}C and 150 rpm agitation.

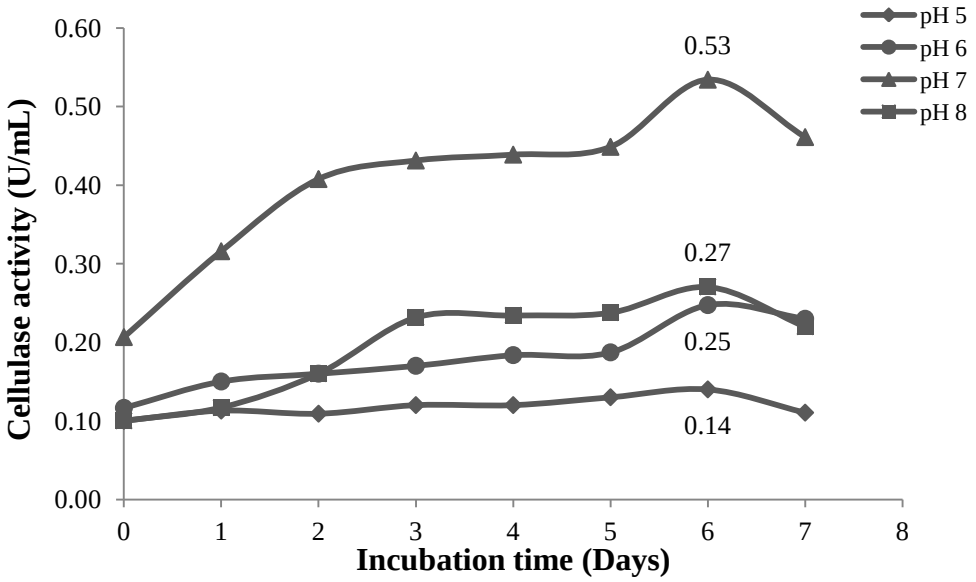


Figure 4.10 Cellulase production of the isolate 12.3.A on the residue medium with different pH, incubated at 30°C and 150 rpm agitation.

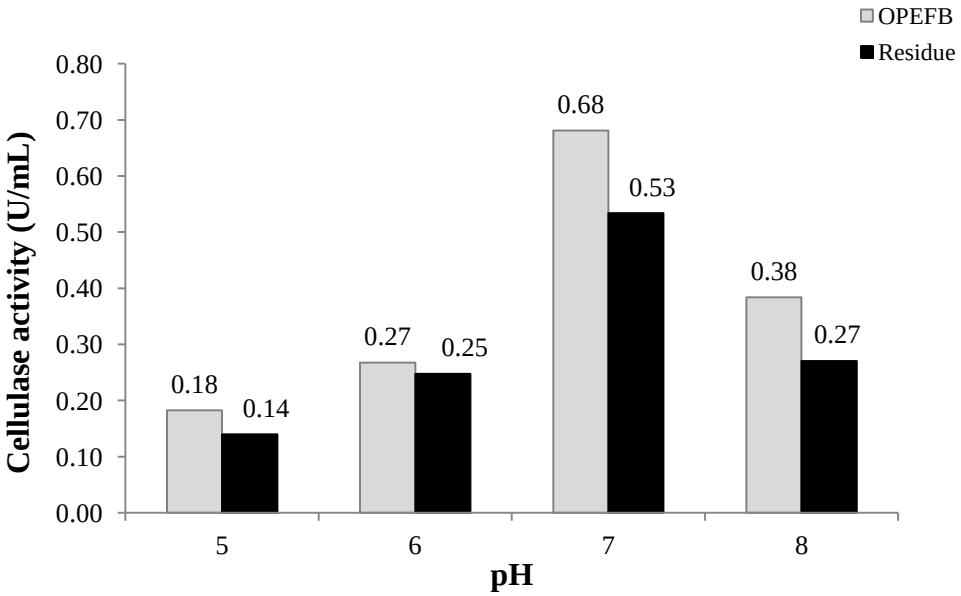


Figure 4.11 The maximum values of cellulase activity of the isolate 12.3.A in the OPEFB and the residue medium with different pH, culturing at 30°C and agitation rate of 150 rpm.

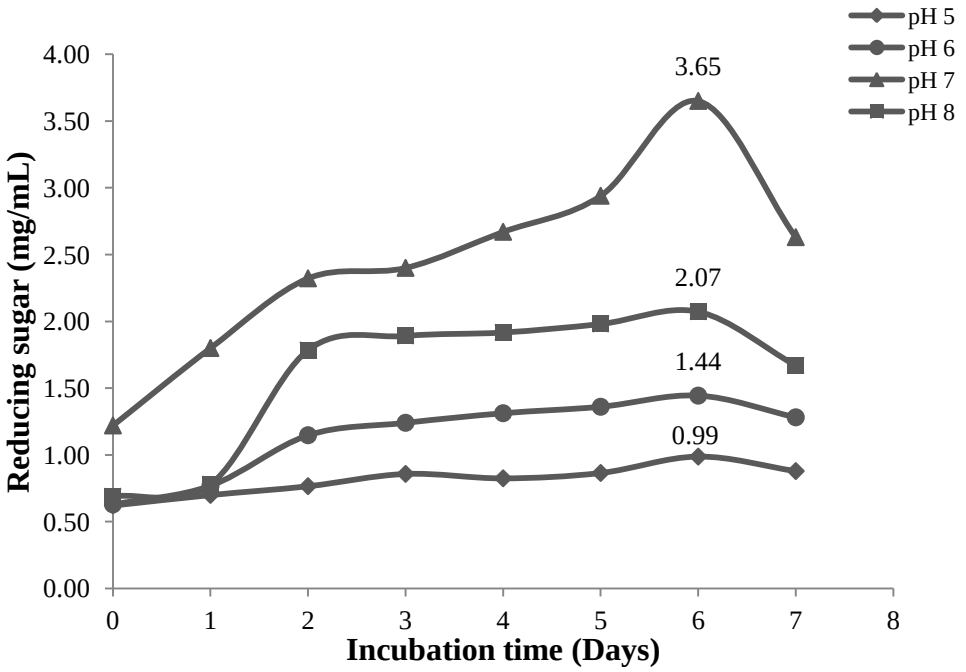


Figure 4.12 Reducing sugar in the culture supernatant of the isolate 12.3.A in OPEFB medium with different pH, performing at 30°C and 150 rpm agitation.

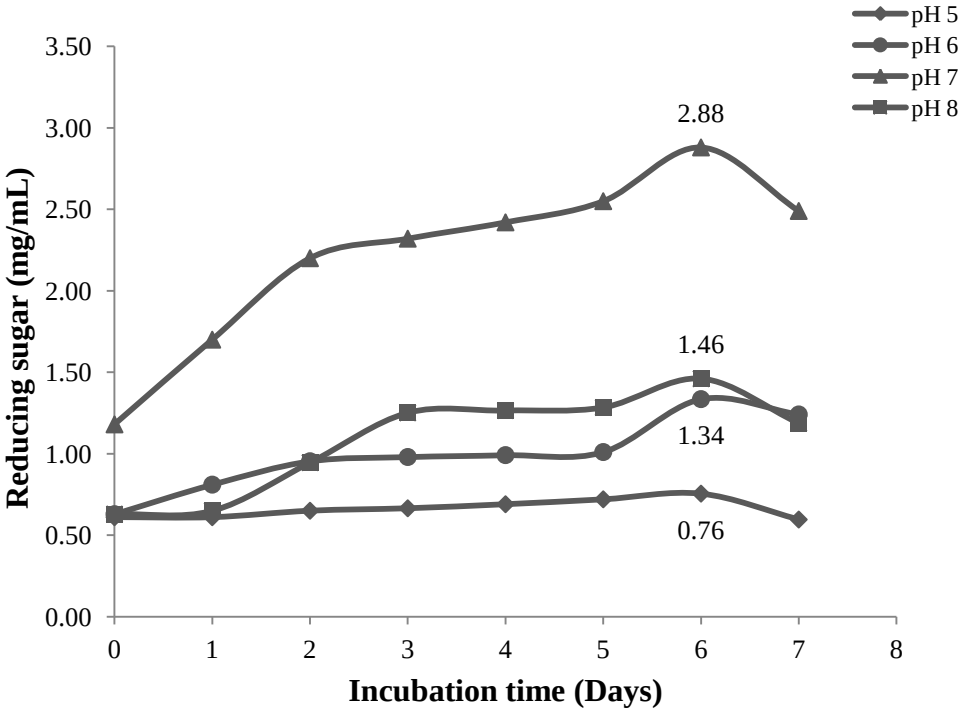


Figure 4.13 Reducing sugar in the culture supernatant of the isolate 12.3.A in the residue medium with different pH, performing at 30°C and 150 rpm agitation.

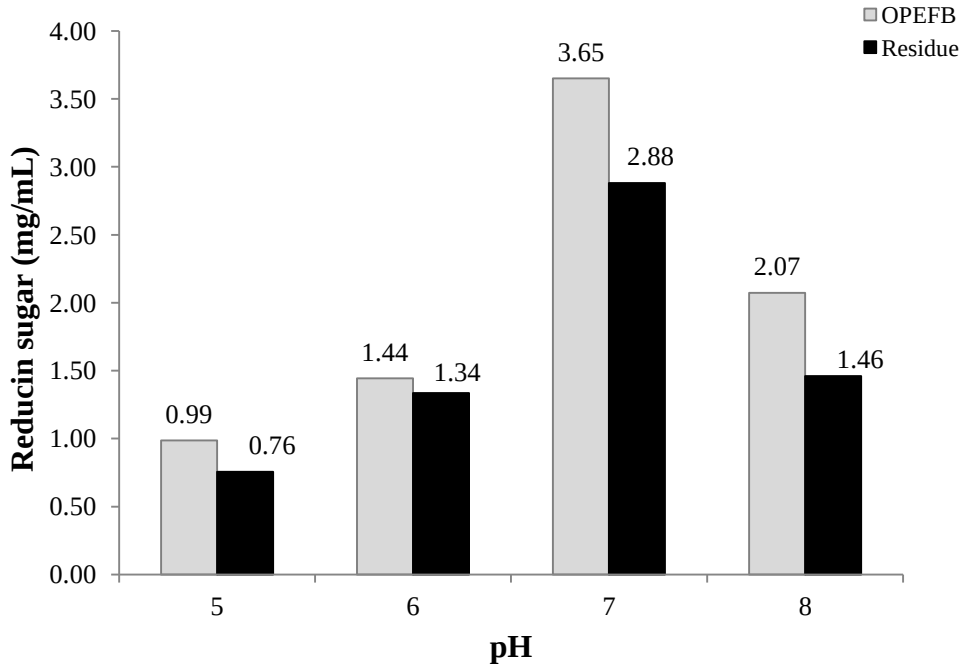


Figure 4.14 The maximum production of reducing sugar of the isolate 12.3.A in the OPEFB and the residue medium with different pH, culturing at 30^{o}C and agitation rate of 150 rpm.

Determination of pH optimum was done in OPEFB and residue medium with various pH condition (pH 5-8). Determination of the optimum pH for reducing sugar production was shown in figure 4. 12, 4.13 and 4.14. The medium with pH 7 gave the highest production with the maximum reducing sugar concentration of 3.65 mg/mL. Similar results was found when testing for the residue medium. pH 7 gave the highest reducing sugar production at 2.88 mg/mL.

According to Rochima (2000), all enzyme reactions are affected by the pH of the medium when the reaction occurred. Generally, actinomycetes were intolerant to acids and their numbers decline in the environment with a pH below 5.0 (Jiang et al., 1985 and Jiang et al., 1988). Actinomycetes prefer neutral pH with range 6.5 – 8.0. According Poejiadi and Suprianti (2005), the enzyme activity at pH describes as the giver or receiver proton groups that are important in the catalytic enzyme (ionization).

4.7.2 Optimum incubation temperature

The optimum incubation temperature for cellulase production was studied by culturing the isolate 12.3.A in 1% (w/v) OPEFB and residue medium at optimum pH obtained previous section in various incubation temperatures (30-45^{o}C). The isolate 12.3.A was cultured in OPEFB and residue medium at pH 7 and agitation rate of 150 rpm for 7 days incubation. The effect of different temperature for cellulose degradation by the isolate 12.3.A on OPEFB and the residue of acid hydrolysis of OPEFB as the culture mediums with for the production of cellulase are shown in figure 4.15, 4.16 and 4.17.

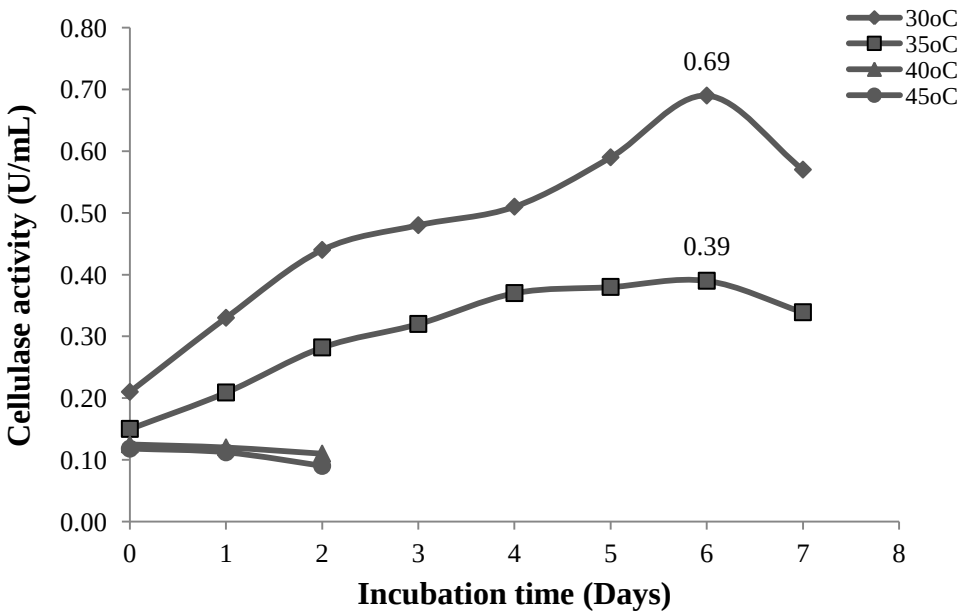


Figure 4.15 Cellulase production of the isolate 12.3.A on the OPEFB medium pH 7 incubated at various temperatures with 150 rpm agitation.

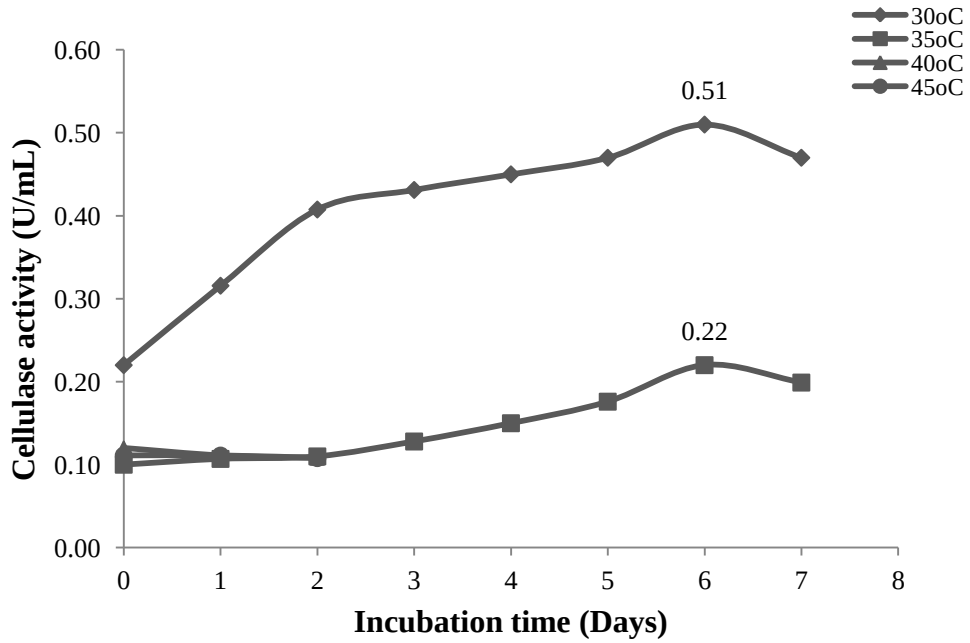


Figure 4.16 Cellulase production of the isolate 12.3.A on the residue medium pH 7 incubated at various temperatures with 150 rpm agitation.

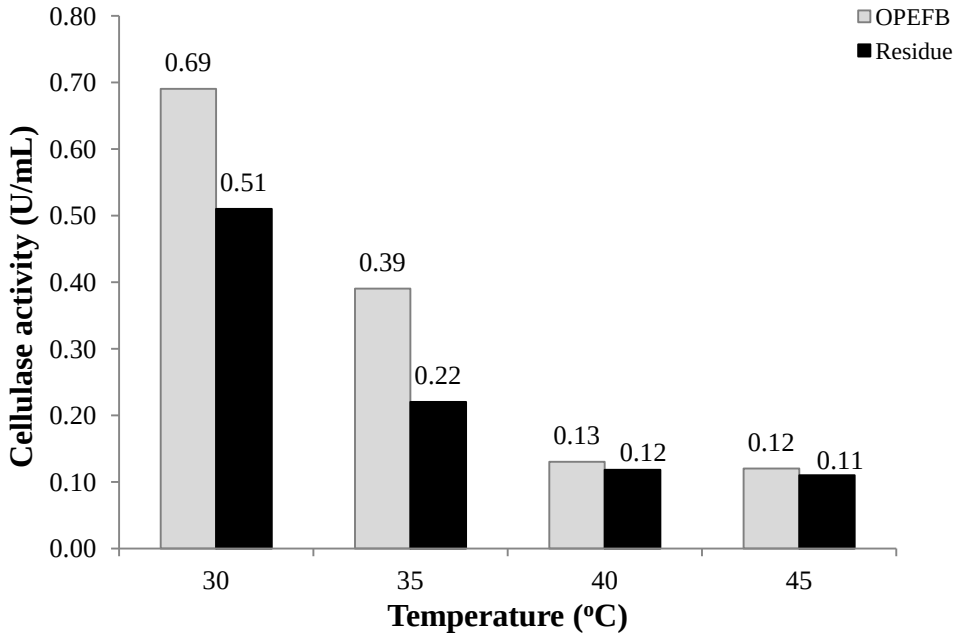


Figure 4.17 The maximum values of cellulase activity of the isolate 12.3.A in the OPEFB and the residue mediums pH 7 incubated at different temperatures with agitation rate of 150 rpm.

Base on figure 4.15, 4.16 and 4.17, the production of cellulase of the isolate 12.3.A cultured in OPEFB and the residue medium pH 7 and agitation rate of 150 rpm was maximized at 0.69 U/mL and 0.51 U/mL, respectively, with the incubation temperature at 30°C. Figure 4.17 demonstrated that OPEFB fiber was easier to degrade by the isolate 12.3.A than the residue. Tuncer et al, (2004) and Van Zyl (1985) reported that *Streptomyces* spp. produced maximum endoglucanase when cultured in the medium having pH 6.5 - 7.0 and incubated at 26 - 30°C.

Cellulase activity was decreased during the hydrolysis. The irreversible adsorption of cellulase on cellulose is partially responsible for this deactivation (Converse et al., 1988). The susceptibility of cellulosic substrates of cellulase were depending on the structural features of the substrate including cellulose crystallinity, degree of cellulose polymerization, surface area, and content of lignin. Lignin interferes with hydrolysis by blocking access of cellulases to cellulose and by irreversibly binding hydrolytic enzymes. Therefore, removal of lignin can dramatically increase the hydrolysis rate (McMillan, 1994).

The increasing temperature was lead to higher enzyme kinetic energy. The increase in temperature would decrease the activity of the enzyme, because the high temperature will accelerate damage to the conformation of the enzyme active group, therefore the enzyme have problems in interacting with the substrate and the catalytic activity of the enzyme would also decrease (Lengninger, 2000).

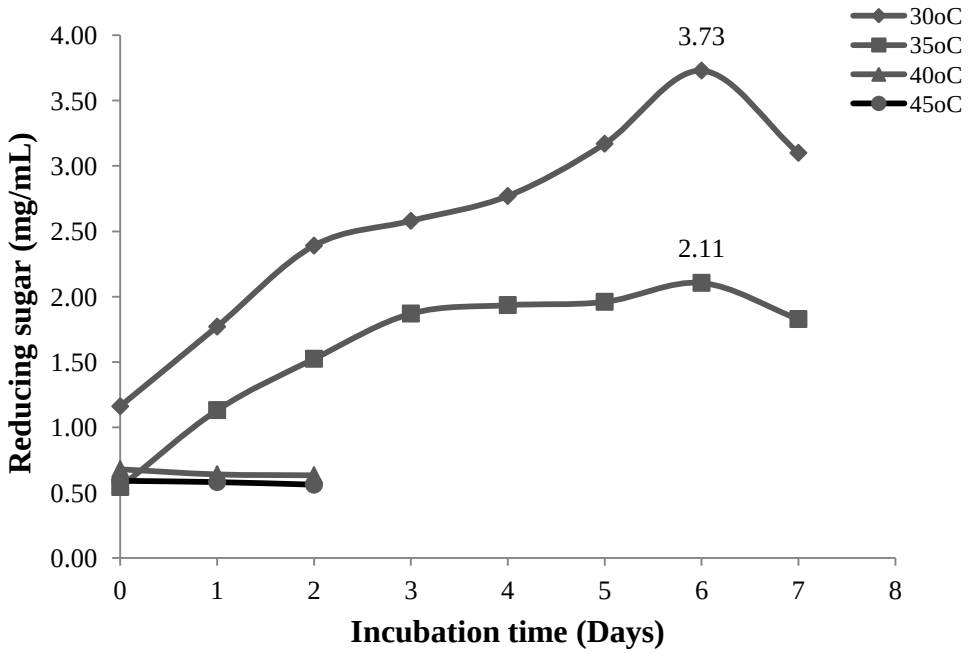


Figure 4.18 Reducing sugar production of the isolate 12.3.A in OPEFB medium pH 7 incubated at different temperatures with 150 rpm agitation.

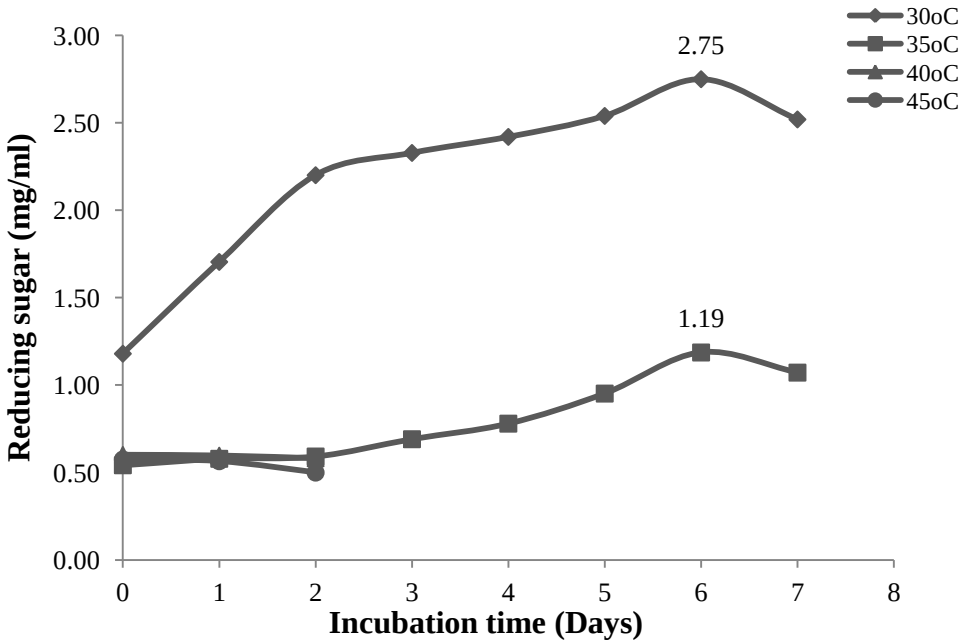


Figure 4.19 Reducing sugar production of the isolate 12.3.A in the residue medium pH 7 incubated at different temperatures with 150 rpm agitation.

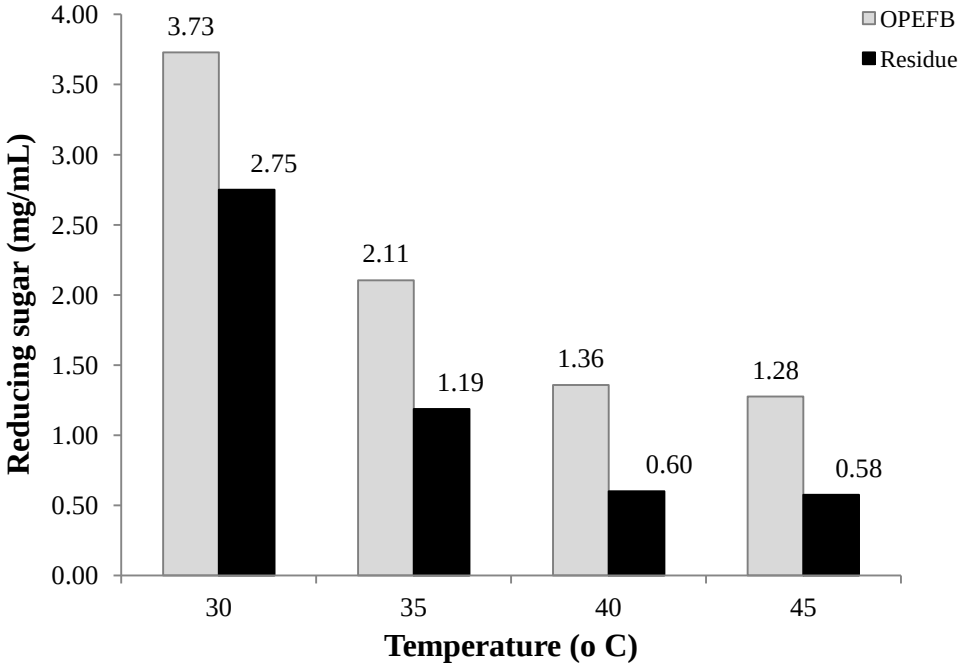


Figure 4.20 The maximum production of reducing sugar of the isolate 12.3.A in the different temperatures with OPEFB and the residue as substrates at pH 7 and agitation rate of 150 rpm.

The production of reducing sugar was measured by Miller's method. Measurement of reducing sugar was done with use different temperature incubation (30-45^{o}C) at pH 7 and agitation rate 150 rpm for 7 days incubation. The culture mediums were used in the production of reducing sugar are OPEFB and residue medium. On OPEFB medium, production of reducing sugar (figure 4.18 – 4.20) was maximized at 3.73 mg/mL with the incubation temperature at 30^{o}C. The similar result was found in the residue medium with the highest reducing sugar produced at 2.75 mg/mL. This is indicated that temperature 30^{o}C is the optimum temperature for growing the isolate 12.3.A in OPEFB and residue medium.

4.7.3 Optimum concentration of OPEFB and residue as substrate for enzyme production

The optimum substrate concentration was determined by using OPEFB and the residue medium at optimal initial pH (pH 7), incubation temperature (30^{o}C) and agitation rate 150 rpm. The substrate concentration used were 0.5, 1 and 1.5%. Production of cellulase activity was measured by using Ghose's method. Production of cellulase was shown in figure 4.21, 4.22 and 4.23 for the OPEFB and residue medium as the culture mediums. The highest cellulase production was derived from using OPEFB and the residue at 1%.

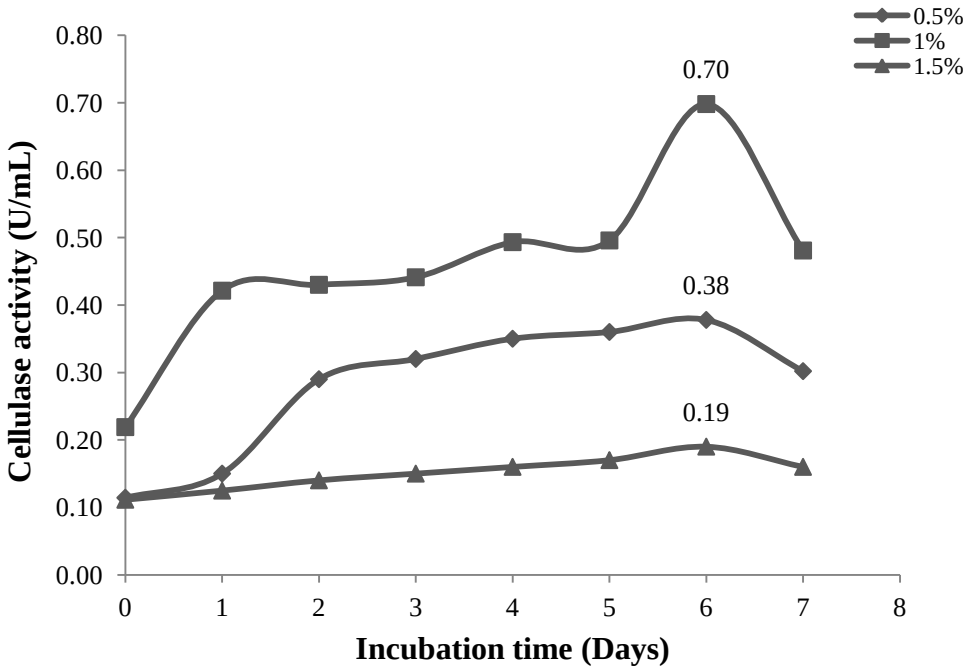


Figure 4.21 Cellulase production of the isolate 12.3.A in medium with different concentrations of OPEFB at pH 7, 30^{o}C, and agitation rate of 150 rpm.

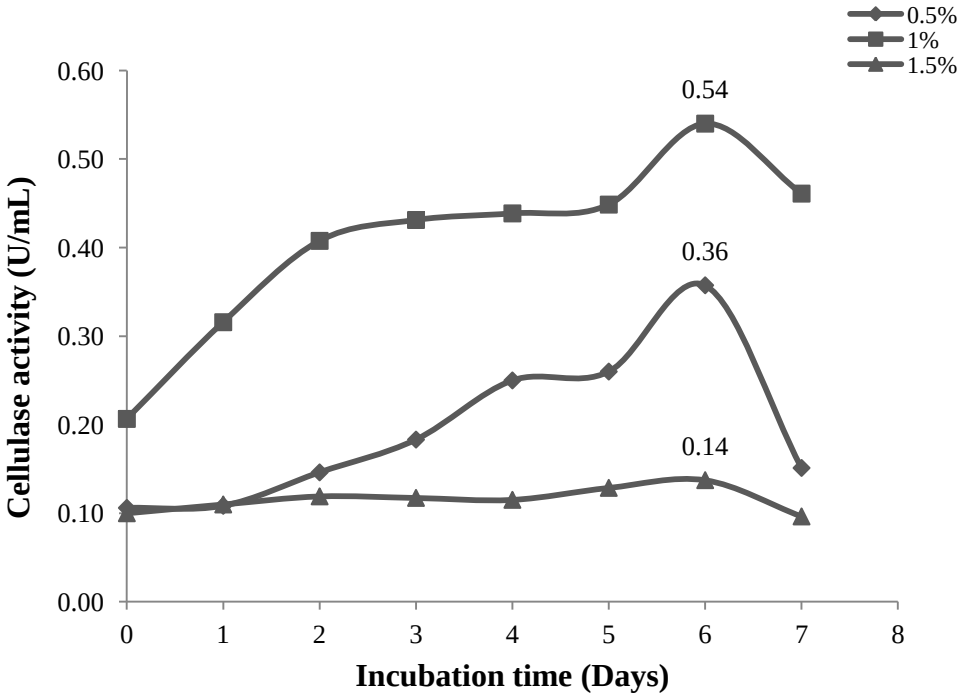


Figure 4.22 Cellulase production of the isolate 12.3.A in medium with different concentrations of the residue at pH 7, 30^{o}C, and agitation rate of 150 rpm.

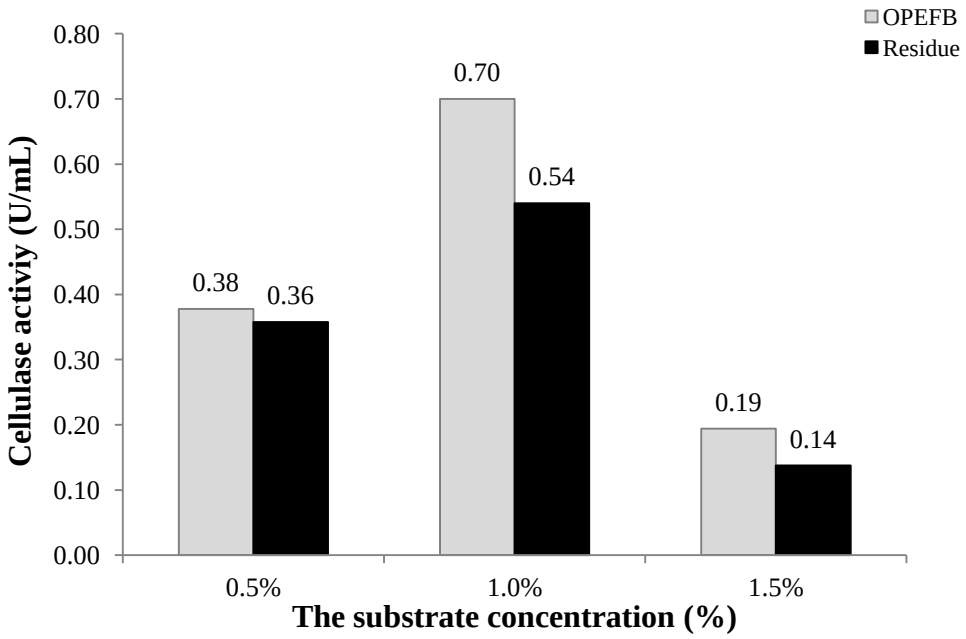


Figure 4.23 Maximum values of cellulase activity in the medium with different concentrations of OPEFB and the residue at pH 7, 30°C and agitation rate of 150 rpm.

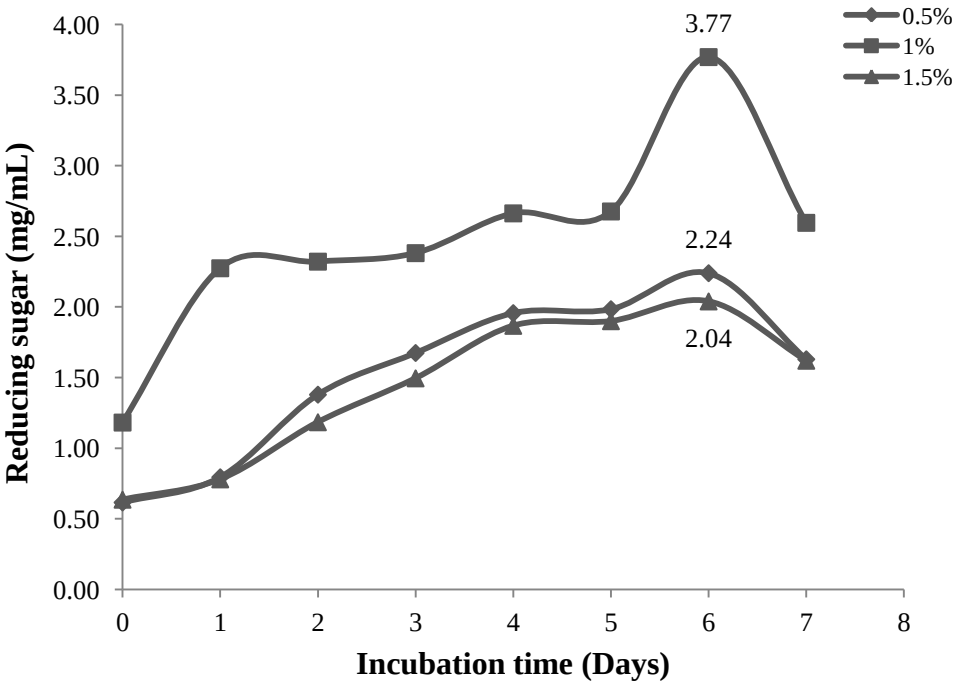


Figure 4.24 Reducing sugar production of the isolate 12.3.A in medium with different concentrations of OPEFB at pH 7, 30°C, and agitation rate of 150 rpm.

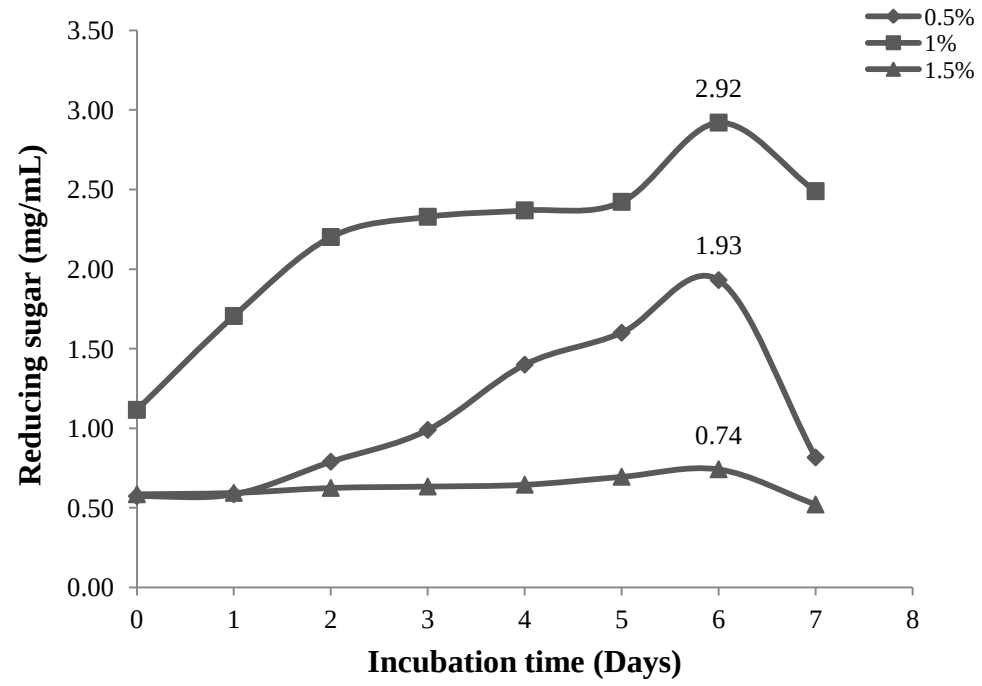


Figure 4.25 Reducing sugar production of the isolate 12.3.A in medium with different concentrations of the residue at pH 7, 30^{o}C, and agitation rate of 150 rpm.

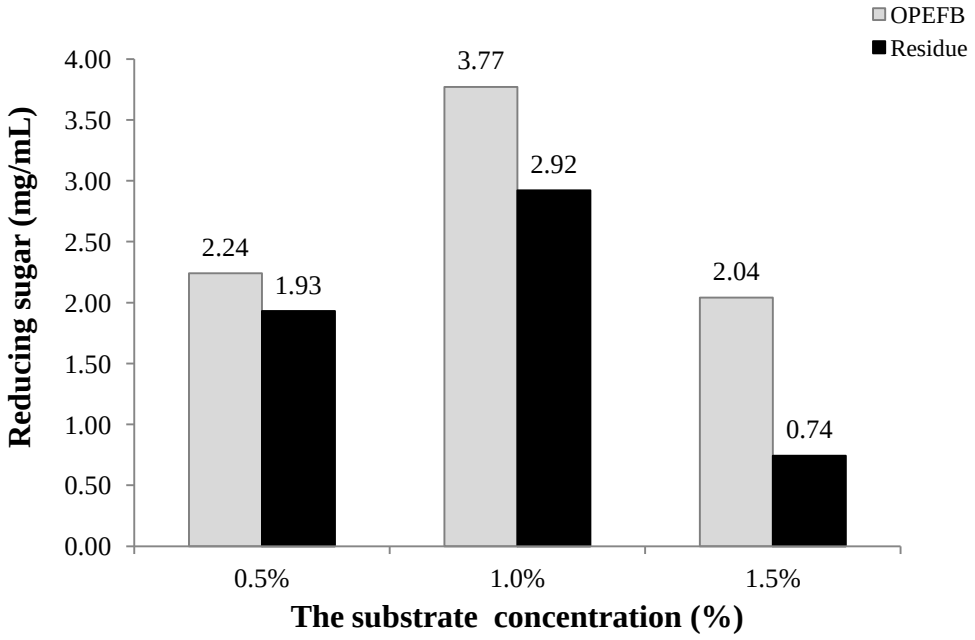


Figure 4.26 Maximum production of reducing sugar in the medium with different concentrations of OPEFB and the residue at pH 7, 30^{o}C and agitation rate of 150 rpm.

The measurement of reducing sugar was determined by using the OPEFB and the residue as the culture mediums at an initial pH (pH 7), 30^{o}C and 150 rpm agitation rate for 7 days with 1% inoculum. Reducing sugar determination was obtained from the supernatant of OPEFB and the residue medium of absorbance against glucose standard.

Results of reducing sugar production were shown in figure 4.24, 4.25 and 4.26. The result demonstrated that cellulose, which is the major component on OPEFB can be easily degraded by the isolate 12.3.A and convert to be simple sugars (monosaccharides) which can be detected by using the Dinitrosalicylic (DNS) method (Miller, 1959). 1 % of the substrate concentration were optimal for accelerating the cellulase and reducing sugar production. With this substrate concentration, microorganism can get sufficient carbon sources such as cellobiose and crystalline cellulose where carbon sources are subject to catabolite repression by soluble sugar accumulation. Too high substrate concentrations can cause substrate inhibition and thereby result in reduced efficiency (Sung et al., 2002).

4.7.4 Effect of nitrogen sources on cellulase and reducing sugar production of the isolate 12.3.A

The effect of organic nitrogen and inorganic nitrogen source in cellulase production medium of the isolate 12.3.A using 1% (v/v) inoculum, 1% substrate concentration, pH 7.0, incubated at 30^{o}C under aeration by shaking at 150 rpm. The effect of the nitrogen source was determined by the replacement of 0.05% (w/v) $(NH_4)_2SO_4$ in OPEFB and residue medium with 0.05 % (w/v) organic nitrogen and inorganic nitrogen such as peptone, yeast extract and ammonium nitrate. The effect of nitrogen sources in the OPEFB and the residue of acid hydrolysis of OPEFB medium shown in figure 4.27 - 4.32. The different nitrogen compound such as organic and inorganic for the growing of microorganism could have effects on production of cellulase. Ammonium sulfate was the most suitable nitrogen source for cellulase production of the isolate 12.3.A when using OPEFB as substrate, while peptone was for using the residue as substrate.

Base on the OPEFB and the residue composition in table 4.2, the OPEFB fiber had higher protein content (9.56%) than the residue fiber (4.87%). High protein content of OPEFB fiber might provide some organic nitrogen for the microorganism, therefore, ammonium sulfate might enough to fulfill requirement of nitrogen. The protein content of the residue fiber was very low, therefore, peptone had better support for the enzyme production. This is shown in figure 4.29 that ammonium sulfate could increase the production of cellulase in the OPEFB medium while peptone could increase cellulase production in the residue medium.

Chellapandi et al., (2008) reported that ammonium sulfate as nitrogen source was promoted glucanase production (cellulase activity) of *Streptomyces spp.* BRC2 while peptone for *Streptomyces spp.* BRC1.

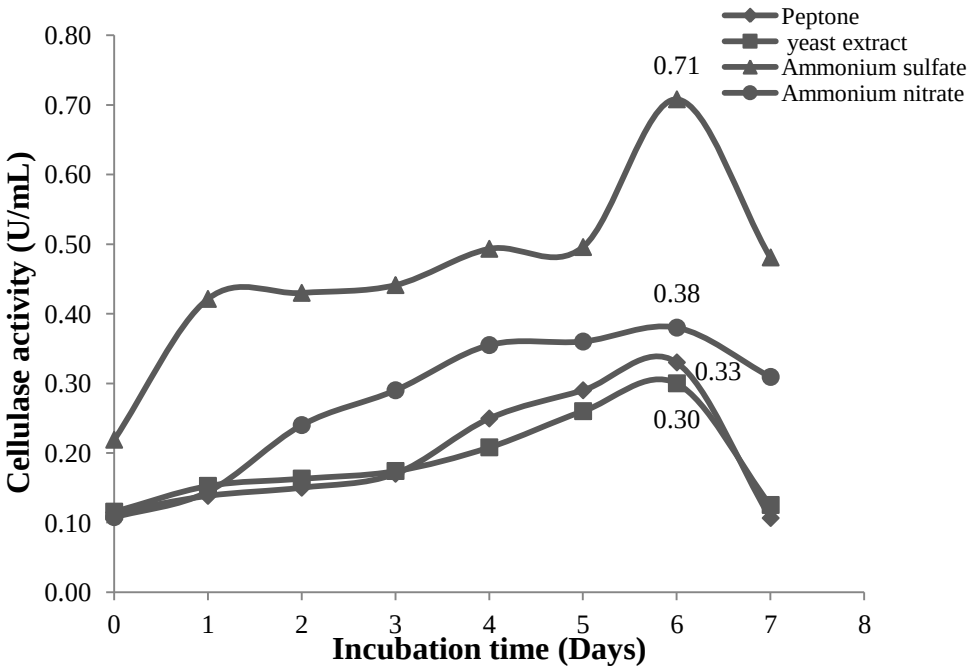


Figure 4.27 Cellulase production of the isolate 12.3.A in OPEFB medium with different nitrogen sources, cultivated at pH 7, 30^{o}C, and agitation rate of 150 rpm.

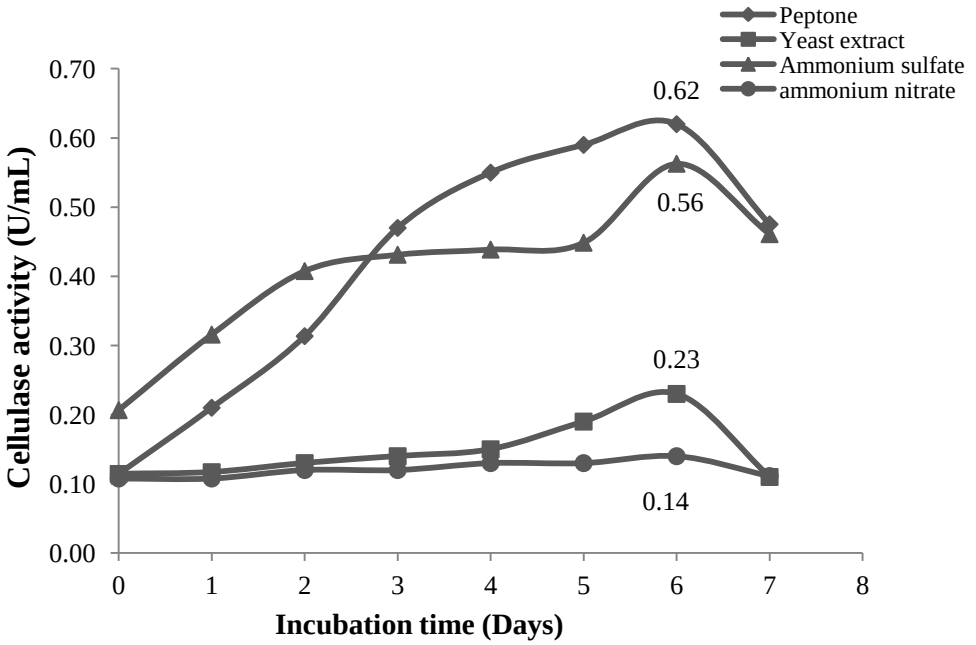


Figure 4.28 Cellulase production of the isolate 12.3.A in the residue medium with different nitrogen sources, cultivated at pH 7, 30^{o}C, and agitation rate of 150 rpm.

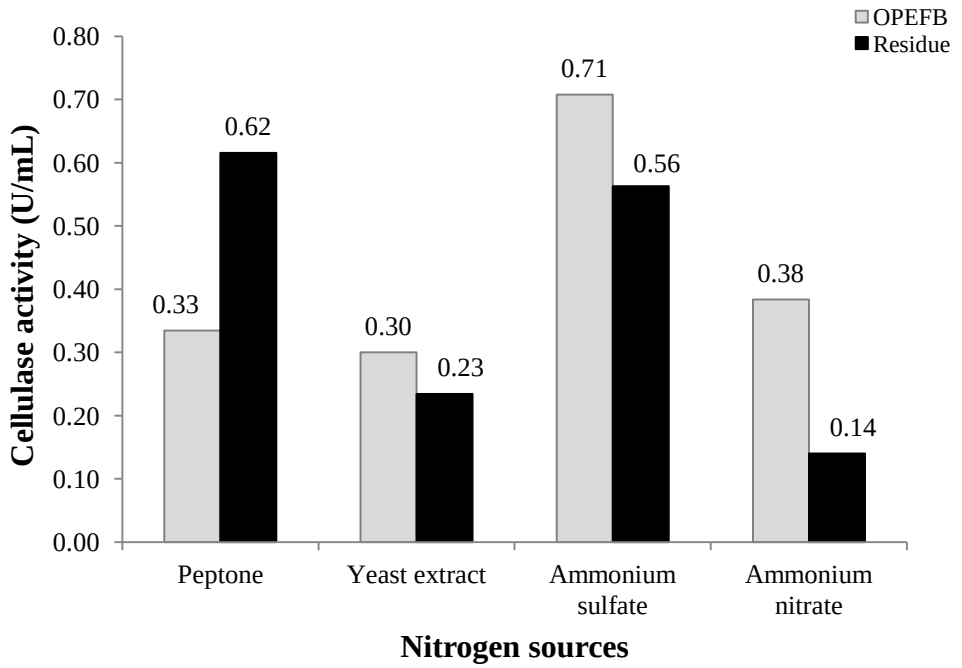


Figure 4.29 The maximum values of cellulase production in OPEFB and the residue medium with different nitrogen sources, cultivated at pH 7, 30^{o}C, and agitation rate of 150 rpm.

The production of reducing sugar was determined by using the DNS method (Miller, 1959). Measurement of the absorbance of the supernatant from OPEFB and the residue medium against the absorbance of glucose standard by a spectrophotometer. To the OPEFB medium, nitrogen sources were added in mediums such as peptone, yeast extract, ammonium sulfate and ammonium nitrate for the induce of the isolate 12.3.A to most produce cellulase enzymes and cellulase can most degrade cellulose to simple sugars (monosaccharides).

Figure 4.32, shows the production of reducing sugar in OPEFB and the residue medium. In the OPEFB medium, maximized reducing sugar production from different nitrogen sources was obtained from using ammonium sulfate. Peptone was the most support for reducing sugar production in the residue medium. This indicated that ammonium sulfate is the best nitrogen source for OPEFB medium while peptone is for the residue medium.

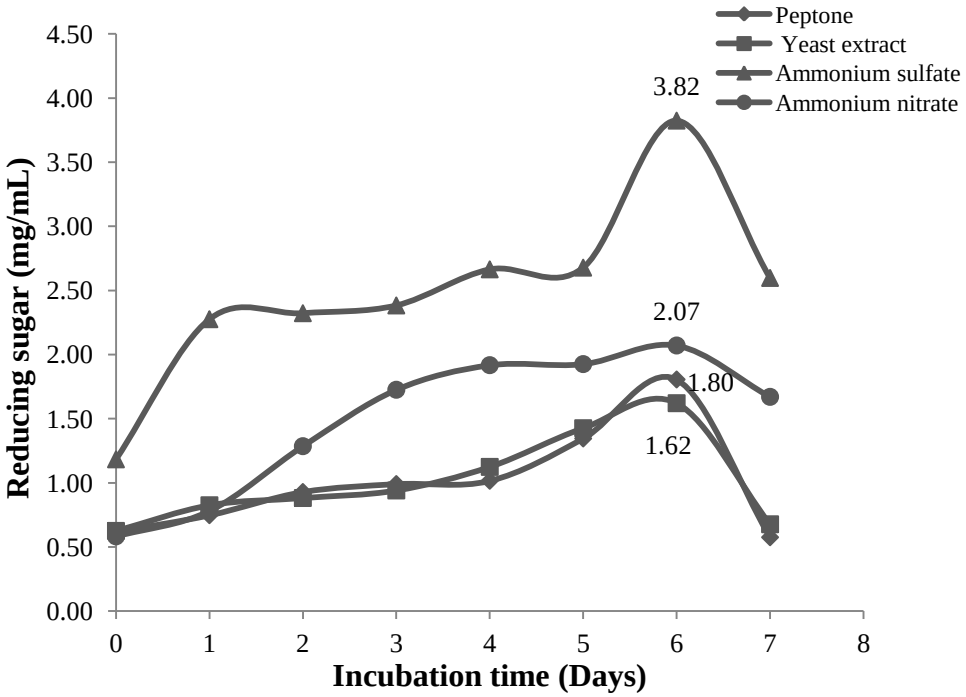


Figure 4.30 Reducing sugar production of the isolate 12.3.A in OPEFB medium with different nitrogen sources, cultivated at pH 7, 30°C, and agitation rate of 150 rpm.

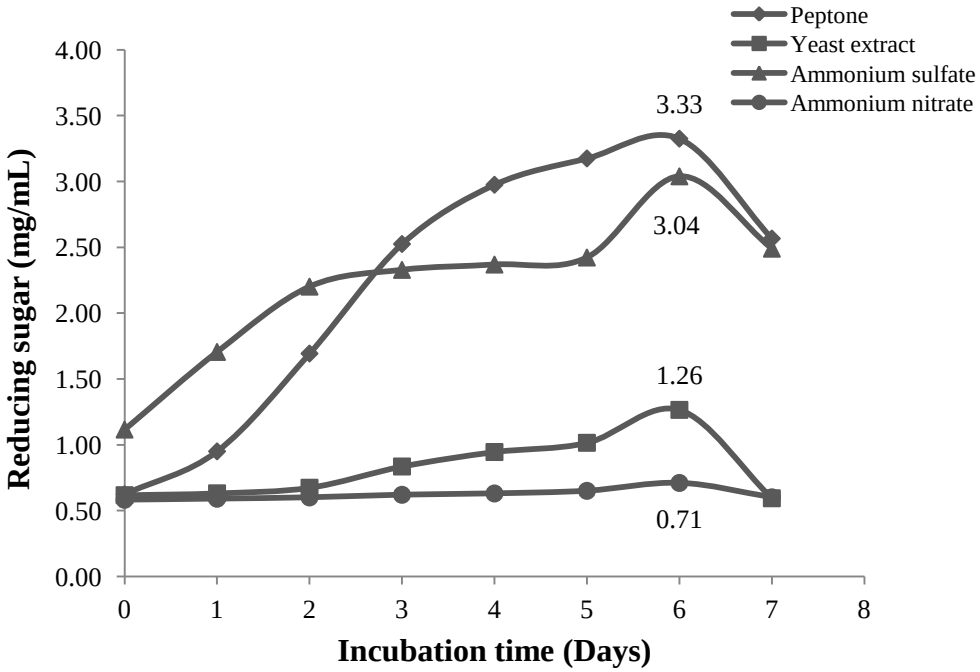


Figure 4.31 Reducing sugar production of the isolate 12.3.A in the residue medium with different nitrogen sources, cultivated at pH 7, 30°C, and agitation rate of 150 rpm.

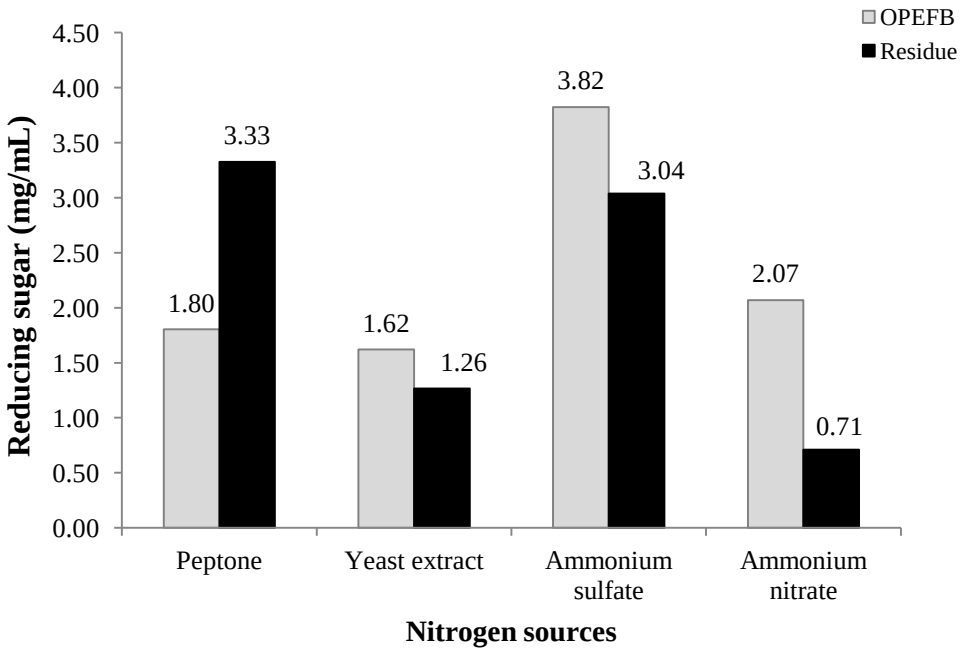


Figure 4.32 The maximum production of reducing sugar of the isolate 12.3.A in OPEFB and the residue medium with different nitrogen sources, cultivated at pH 7, 30^{o}C, and agitation rate of 150 rpm.

4.8 Cellulase partial purification

4.8.1 Crude cellulase, ammonium sulfate precipitation and dialysis

Crude cellulase production was determined from the optimum conditions of the isolate12.3.A (pH 7, 30^{o}C, 1% OPEFB and ammonium sulfate as a nitrogen source) with 150 rpm of an agitation rate for 6 days incubation. A 3 liters biorector cellulase were prepared in this study. The cell concentration, cellulase activity and reducing sugar were measured every day by using methods from Ghose's (1987) and Miller's (1959).

Cellulase is extracellular enzymes, for the isolation, extracellular enzymes was easier than intracellular enzymes because of without cell division. Enzyme isolation process implies the release of enzymes from cells that can be done mechanically, physically and chemically through the membrane and cell wall. Isolation of cellulase from the isolate 12.3.A begins by growing in broth media containing OPEFB medium for 6 days, after the incubation, the enzyme was released out of the cell or medium by a centrifuge. Production of cellulse was carried with a centrifuge at 10000 RPM, 4ºC for 15 minutes. After cellulase production is finished, purification process of cellulase will be done. In the crude cellulase (Table 4.10), total cellulase activity and specific activity were 316.62 Unit and 1.98 U/mg.

Purification conducted two stages such as ammonium sulfate precipitation and dialysis. The results of measurements of cellulase activity in each stage of the purification are shown in table 4.10.

Table 4.10 Purification of cellulase enzyme

Stages	Volume (mL)	Cellulase activity (U/mL)	Protein (mg/mL)	Total Cellulase activity (U)	Total Protein (mg)	Specific activity (U/mg)
Crude Enzyme	2,350	0.56	0.28	1,316	658	2
Ammonium sulfate precipitation (60%)	26	30.82	8.25	801.32	214.5	3.74
Dialysis	24	31.38	4.25	753.12	102	7.38

The first stages of purification were used by ammonium sulfate precipitation. A 2,350 mL of the crude cellulase (supernatant) obtained from 3 liters biorector that were collected by centrifugation at 10,000 rpm for 10 min. The crude cellulase enzyme was precipitated by the addition of ammonium sulfate $(NH_4)_2SO_4$ (60% saturated at 4^oC). A 927.87 g $(NH_4)_2SO_4$ was added in 2,350 mL of supernatant collected by centrifugation at 10,000 RPM for 10 min. Cellulase precipitation was done for overnight at a cool temperature (4^oC).

Base on table 4.10, cellulase precipitation by using ammonium sulfate (60%) can increase the specific activity of cellulase up to 3.74 U/mg. According to Wang et al. (1996), precipitation is a method using reagent additive or changing environmental conditions that cause the protein to leave the solution to form insoluble particles in the form of precipitation. The advantage using ammonium sulfate salt because it has a high solubility, moderate pH, relatively cheap, non-toxic, and does not affect the enzyme.

The enzyme suspension obtained from precipitation process was dialyzed in 0.07 M phosphate buffer solution (pH 7.0) for overnight. The phosphate buffer solution was changed every 6 hours. Remaining cellulase activity and protein content after doing dialysis were also determined. Protein content was analyzed according to Lowry's method. Cellulase enzyme after dialysis was kept in -20^oC to maintain cellulase activity. The result of the dialysis is shown in table 4.10.

In the dialysis stage, the specific activity increased cellulase activity of three-times (7.38 U/mg). An increase in the specific activity value indicates that the degree of purity of the enzyme is better. The success of a purification stage, were observed by increasing the specific activity of the enzyme after was purified enzyme (Rochima, 2009). The graph of increasing specific activity can be seen in figure 4.33.

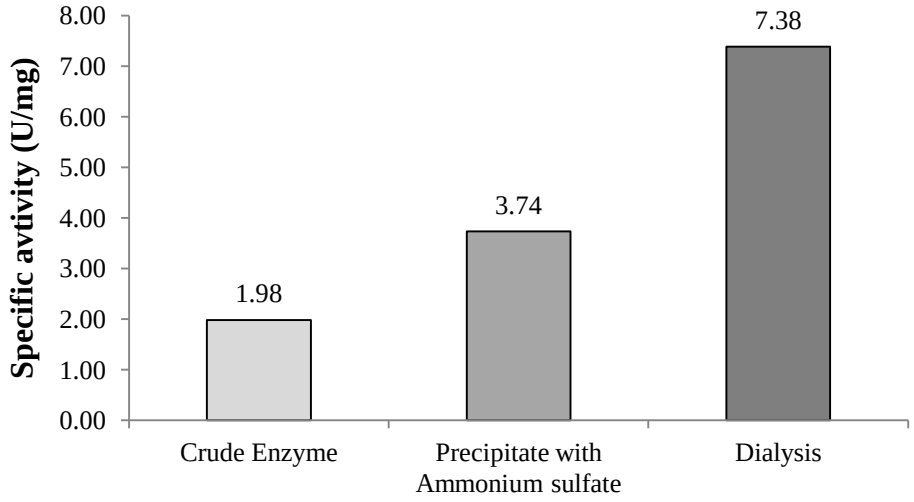


Figure 4.33 Purification stages of cellulase

Dialysis is the process of separating small molecules through semi-permeable membranes. The purpose of dialysis is to remove molecules of low molecular weight contaminants and excessive influence on the stability of the enzyme. In this process moving event ammonium sulfate salt having a molecular weight less than the sample into the buffer solution. At the time of moving salt through the membrane pores, the salt was absorbed on the membrane surface membrane subsequent move from one side to the other side of the membrane. This process is maintained by the osmotic pressure.

4.8.2 A clear zone of cellulase determination by zymogram

Zymogram was a method for the qualitative detection of cellulase activity which has several advantages that fast, not expensive and the results are simple. Zymogram gel used in this study was Polyacrylamide gel amended with 0.1% CMC. The formation of a clear zone on the gel after staining with 0.1% Congo red demonstrated CMCase activity. Cellulase enzymes in the gel were running at 120 volts for 1-1.5 hours. The gel was rinsed with distilled water for 3 times and incubated in 50 M Tris buffer at pH 7. After incubation, the gel was stained with 0.1% Congo red for 30 min and destained in 1 M NaCl to reveal clear zones of enzyme activity. There are two bands of clear zone of cellulase activity (Figure 4.34). The enzymes could be defined as CMCases or endo-ß-glucanases, since the substrate in the gel was CMC (Dobrev et al., 2012).

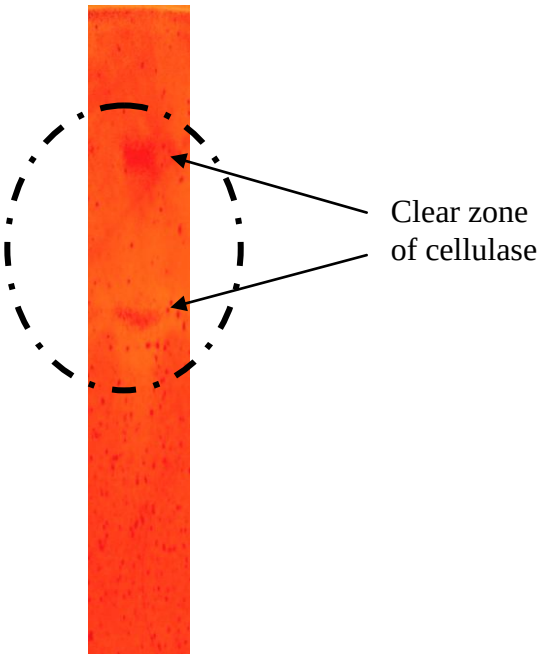


Figure 4.34 Carboxymethylcellulose (CMC) zymogram of the isolate 12.3.A cellulases.

4.9. Effect of temperature and pH on cellulase activity of the Isolate 12.3.A

4.9.1 Optimum pH

The pH optimum of the isolate 12.3.A cellulase was determined the enzyme activity on CMC in different pH buffers: 5.5, 6, 6.5, 7, 7.5 and 8. The cellulase activity was measured by using Ghose's method. The result of the effect of pH on cellulase activity is shown in Figure 4.35, where the optimal pH is 6.5 with the maximum value activity of cellulase at 41.26 U/mL. This is indicated that the activity of cellulase enzyme can perform in the acid range (5.5-6.5).

According to Rochima (2009), all enzyme reactions were affected by the pH of the medium when the reaction occurs. Generally, the enzyme is actively at neutral pH or pH range 5 - 9. To determine the optimal pH, the enzyme cellulase from isolated 12.3.A of Actinomycetes used phosphate buffer (pH 5.5 to 8), the effect of pH to activity of cellulase was reported by Bakare et al., (2005), they were studied of cellulase from wild type and two improved mutant (CRRmt24 and CRRmt4) of *Pseudomonas fluorescens* where the optimum pH for the wild-type and CRRmt4 were 7, while pH of CRRmt24 was 6.5. This is because of the active side of enzyme occurs ionization of amino acids when enzyme and substrate interact optimally and produce a larger product. But after pH 6.5 was decreased the activity of the enzyme where the interaction between the enzyme and substrate also resulted in decreasing the number of products.

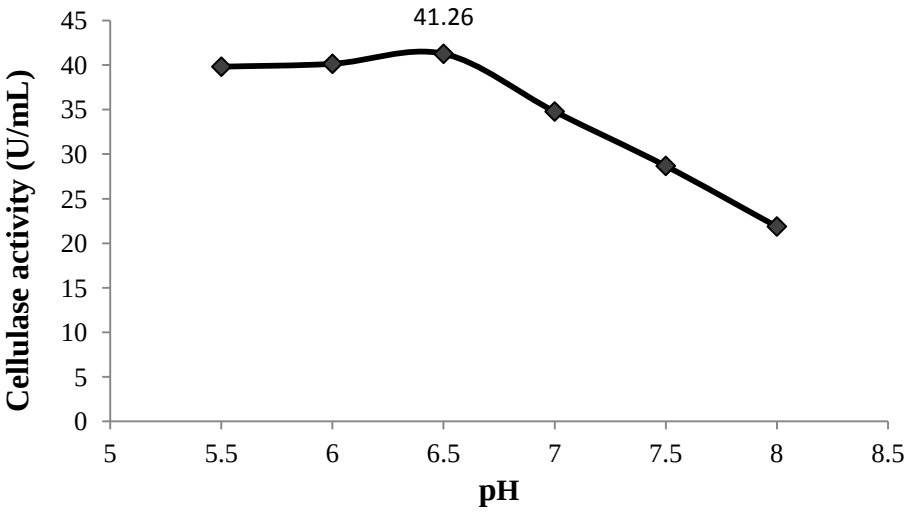


Figure 4.35 Effect of pH on cellulase activity

4.9.2 Optimum temperature

Determination of optimum temperature is done by variation of incubation temperature (30, 35, 40, 45, 50, 55 and 60^{o}C) under the optimum pH. The cellulase activity was measured by using Ghose's method. The optimum temperature for cellulase activity was obtained from the treatment showed the highest cellulase activity. Temperature effects of cellulase activity in catalytic activity. Cellulase activity will be increased along with increasing temperature to the optimum level, after that it will be declined. This is due to the enzymes undergo denaturation thus losing some of its activities. The Effect of temperature on cellulase activity of the isolate 12.3.A was shown in figure 4.36.

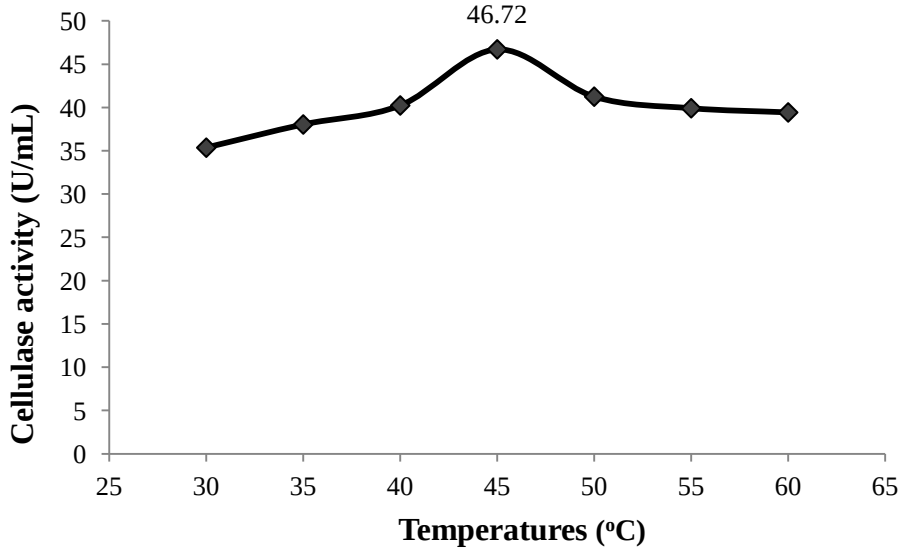


Figure 4.36 Effect of temperature on cellulase activity

Figure 4.36, cellulase enzymes from isolates 12.3.A was shown an optimum temperature at 45^{o}C with the highest cellulase activity at 46.72 U/mL. When the temperature increased from this, the enzyme activity decreased. The high temperature might damage and denature the cellulases of the isolate 12.3. Therefore, these cellulases are temperature- sensitive.

Shabeb et al., (2010) studied *Bacillus subtilis* KO strain and reported that this bacteria produced 36 Units/mL of cellulase at a temperature of 45^{o}C.The rise in temperature on enzymatic reactions can increase the kinetic energy of the molecules that react with the substrate so that the speeding collisions between molecules and facilitate the formation of enzyme-substrate complexes, and the resulting product more. The collision between the enzyme and the substrate is very effective at the optimum temperature, causing the formation of enzyme-substrate complexes increased and improved product formed (Nelson and Cox, 2000).

The increase in temperature would be decreased the activity of the enzyme, because the temperature is too high will accelerate damage to the conformation of the enzyme active group, so that the enzymes have problems in interacting with the substrate and the catalytic activity of the enzyme will be decreased (Lehninger, 2000).

4.10 Ethanol production

4.10.1 Saccharification

Enzymatic saccharification was done according to the report of Kassim et al., (2011). The crude enzyme using for saccharification was obtained from section 3.3.9.2 which total activity (unit) was measured. 5% (w/v) OPEFB and residue (saccharification medium) were prepared in 0.05 M phosphate buffer pH 6.5 (optimum pH for cellulase activity). Saccharification medium was sterilized at 121^{o}C for 15 min. 11.5 mL of The cellulase enzyme (1 mL = 31.38 U/mL) was added in 500 mL of sterilized saccharification medium (25 g substrate) with total activity unit of 360.87 units. Saccharification process was started by incubation at 45^{o}C for 48 hrs. Saccharification or enzymatic hydrolysis is prior part of ethanol production where the substrate and cellulase enzymes were mixed and incubated for generating glucose. At this stage, reducing sugar was determined every 6 hours. The result of reducing sugar development would be described in figure 4.37.

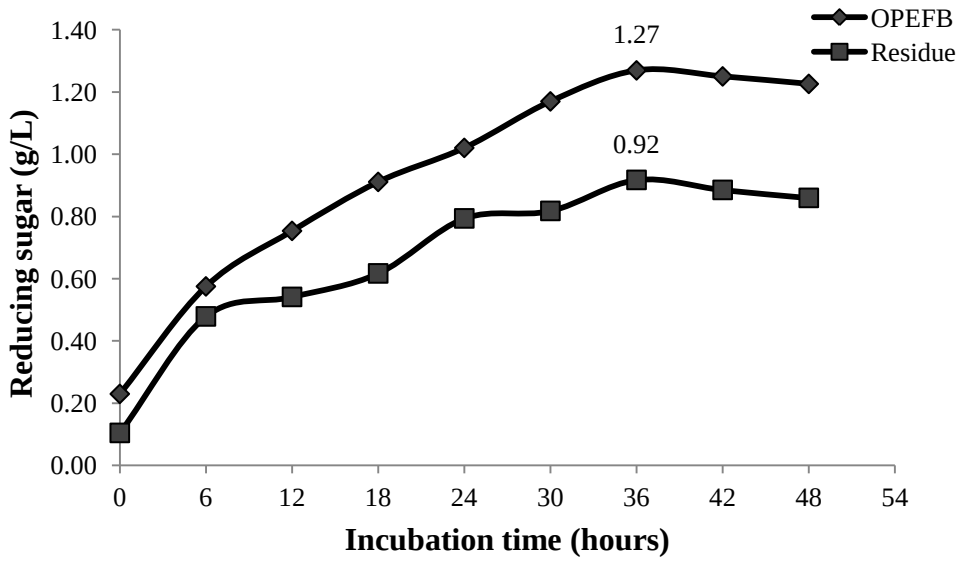


Figure 4.37 Saccharification process of 5% OPEFB and residue in buffer pH 6.5 by cellulase enzyme (31.38 U/ml) incubated at 45°C.

4.10.2. Ethanol Production

Ethanolic fermentation of the concentrated enzymatic hydrolyzate was carried out by using *Saccharomyces cerevisiae*. PH of the solution was adjusted to pH 4.5 using 5 N HCl. A total of 10% (v/v) of standardized active *S. cerevisiae* was inoculated into 100 ml of product from enzymatic saccharification the hydrolyzate in a 125 mL conical flask and incubated at temperature 30°C. The fermenting liquid was harvested every 12 hours centrifuged at 10,000 rpm for 1 min and analyzed the ethanol concentration by gas chromatography.

From figure 4.38, the production of alcohol started with initial value of reducing sugar at 1.77 g/L for OPEFB and 1.01 g/L for the residue. Sugar in the medium was decreased gradually and disappeared in 84 hours for OPEFB and 78 hours for the residue. The sugar from OPEFB and the residue was converted to ethanol by *S. cerevisiae* with the maximal ethanol production at 0.76 g/L and 0.41 g/L, respectively, for 72 hours of incubation. This indicates that *S. cerevisiae* could use sugar from OPEFB and the residue and then convert sugar to ethanol. In the ethanol production, sugar (glucose) is very important because sugar is the material to use by the yeast for ethanol production.

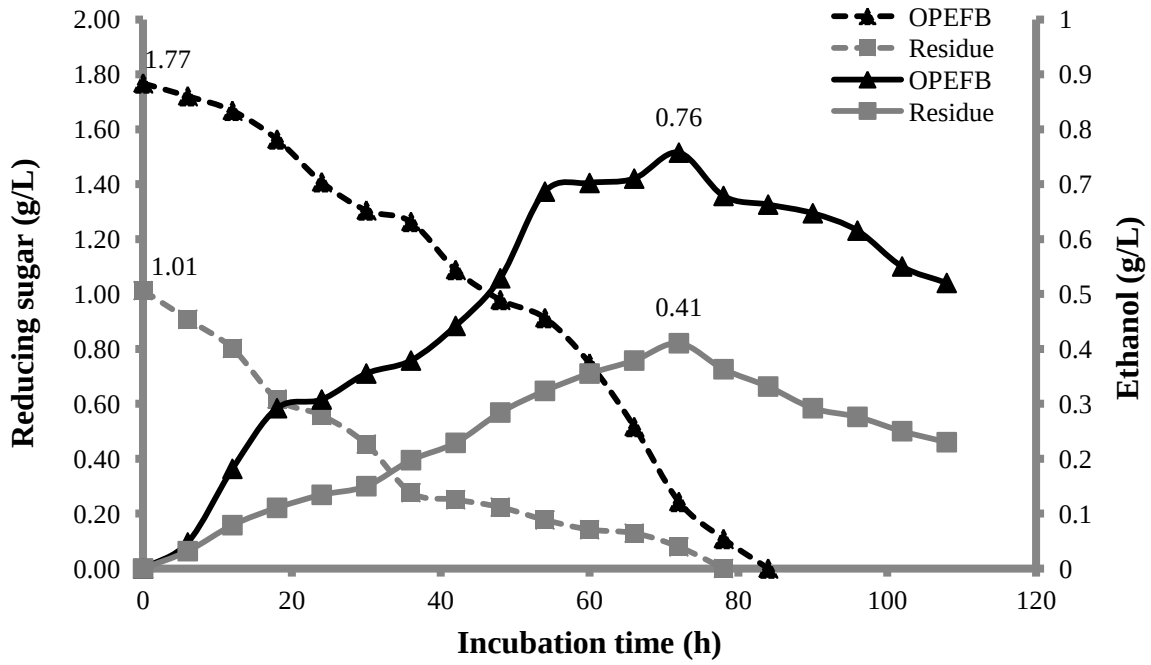


Figure 4.38 Ethanol production from OPEFB and the residue as substrates for sugar production by *Saccharomyces cerevisiae*.

4.11 Identification of cellulase producing actinomycetes by 16S rRNA sequence analysis

4.11.1 16S rRNA analysis

16S rRNA analysis was begun by isolating DNA and amplifying the gene coding for 16S-rRNA using the polymerase chain reaction. Then it is followed by purified DNA fragments are directly sequenced. Sequencing reactions are performed using DNA sequencer in order to determine the order in which the bases are arranged within the length of the sample and a computer is then used for studying the sequence for identification using phylogenetic analysis producers. However, analysis of 16S rRNA generally allows us to identify the organisms upto genus level only.

The isolates 12.3.A of actinomycetes were identified by using 16S rRNA analysis. Identification of 16S rRNA used to determine conservative base sequence and varied base sequence. Identification of 16S rRNA sequences using the conservative base and base sequences varied. Comparison of the conservative base sequences is useful for constructing phylogenetic trees universal to due relatively slow to change and reflect about the chronology of the evolution of the earth. In contrast, base sequences that are varied can be used to track the diversity and puts strain within a species (Pangastuti, 2006). The 16S rRNA gene of the selected isolate were amplified by PCR technique using 800R and 518F primers (Macrogen Inc, Seoul, Korea). The 16S rRNA sequences were compared to the public sequence databases using Basic Local Alignment Search Tool (BLAST) of The National Center for Biotechnology Information (NCBI) for supporting the bacterial identification.

4. 11.2 Construction of phylogenetic tree

A total of 1500 sequences of 16S rDNA was successfully read by using the universal primers 518F Primary 5'-CCAGCAGCCGCGGTAATACG-3' and 5'-Primary 800R TACCAGGGTATCTAATCC - 3'. The sequence of base is as follows:

AGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGC
CCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTG
CCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGA
GTCTGGGAGGCATCTCCCGGGCTGGAAAGCTCCGGCGGTGAAGGATGAGC
CCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGAC
GGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAA
AGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAA
CCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCG
CCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTT
GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGG
TGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATCCGATACGGGCAGGCT
AGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCA
GATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTG
ACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGT
CGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCA
AGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCA
TGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATAT
ACCGGAAAGCATTAGAGATAGTGCCCCCCTTGTGGTCGGTATACAGGTGG
TGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGG
ACTCACAGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGT
CAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCG
GTACAAAGAGCTGCGATGCCGCGAGGCGGAGCGAATCTCAAAAAGCCGGT
CTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTA
GTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCA
ACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACCAGCGATTGGACGATC
TACAGAGAGGGACCCCCATATAATAAAAGGGGGGTAAGAGGGGGGGCT

Table 4.11 The 15 alignment matches for the isolate 12.3.A of Actinomycetes

Code.	Accession	Description	Maximum Score	Similarity (%)
A	NR_041106.1	*Streptomyces nashvillensis* strain NBRC 13064	2619	99
B	NR_041114.1	*Streptomyces violaceorectus* strain NBRC 13102	2603	99
C	NR_043714.1	*Streptomyces gulbargensis* strain DAS131	2595	99
D	NR_041076.1	*Streptomyces roseolus* strain NBRC 12816	2591	99
E	NR_042308.1	*Streptomyces viridobrunneus* strain LMG 20317	2591	99
F	NR_041064.1	*Streptomyces filamentosus* strain NBRC 12767	2590	99
G	NR_043819.1	*Streptomyces hirsutus* strain NRRL B-2713	2582	99
H	NR_041129.1	*Streptomyces showdoensis* strain NBRC 13417	2579	99
I	NR_041120.1	*Streptomyces roseofulvus* strain NBRC 13194	2579	99
J	NR_041092.1	*Streptomyces roseoviridis* strain NBRC 12911	2579	99
K	NR_026177.1	*Streptomyces bikiniensis* strain DSM 40581	2573	99
L	NR_043345.1	*Streptomyces cinereoruber subsp. cinereoruber* strain JCM 4205	2571	99
M	NR_042299.1	*Streptomyces laurentii* strain :LMG 19959	2569	99
N	NR_044150.1	*Streptomyces omiyaensis* strain NRRL B-1587	2560	99
O	NR_044144.1	*Streptomyces zaomyceticus* strain NRRL B-2038	2556	98

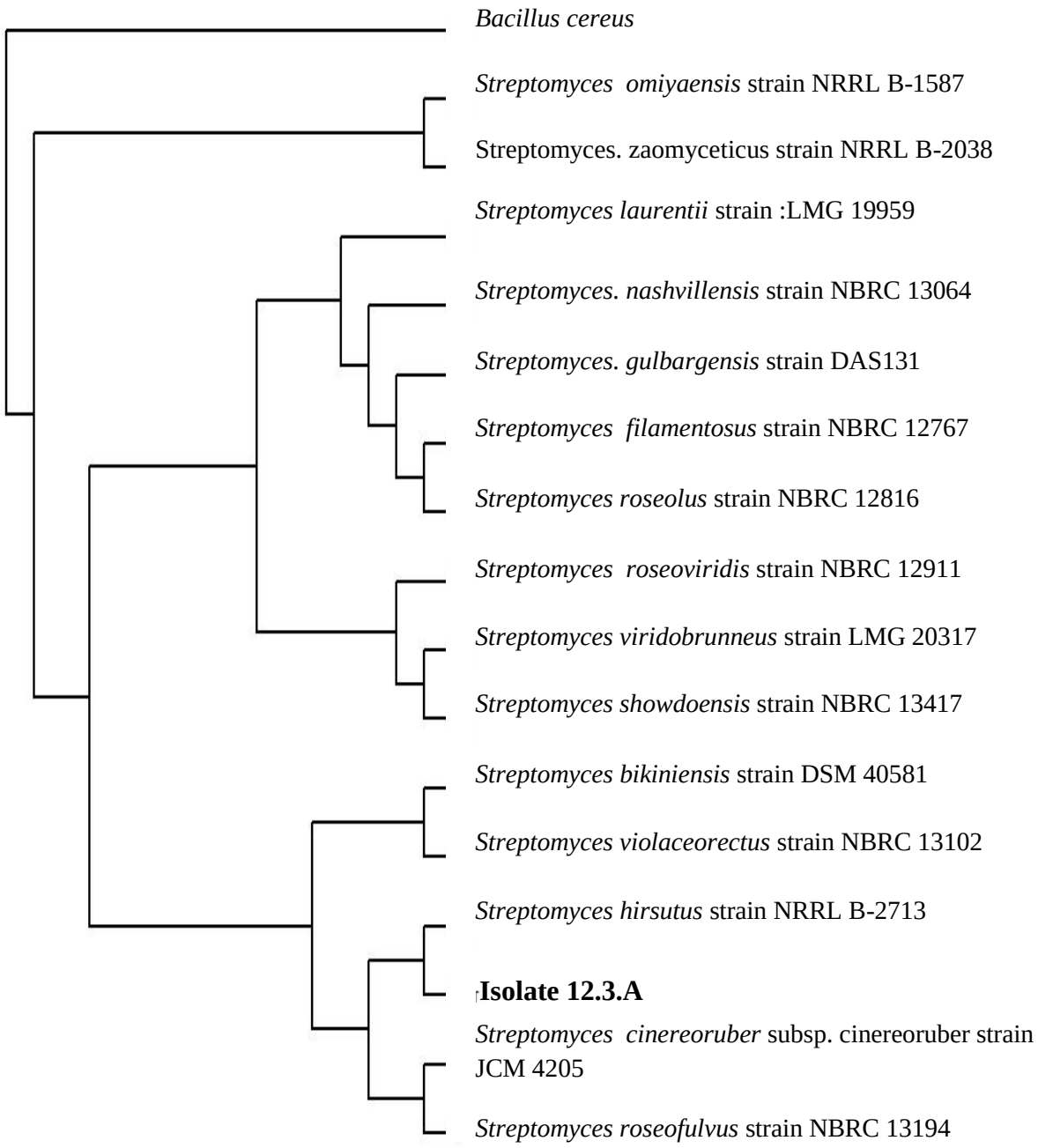


Figure 4.39 Neighbor-joining tree based on 16S rDNA gene sequences, shown the phylogenetics relationship between the isolate 12.3.A and 14 related species of the genus Streptomyces.

The result sequencing of raw data then it was entered with the program MEGA 4 and assembling the BioEdit program and subsequently converted in a FASTA format. The results of RNA sequencing in a FASTA format. For subsequent by BLAST, with searching homology on-line data base in the center of the DNA in DDBJ (http://www.ddbj.nig.ac.jp) and or NCBI (http://www.ncbi.nlm.nlh.gov/). The final stage is the analysis of kinship and the creation of a phylogenetic tree using Clustal X and TreeView software program. Based on the molecular analysis of the above it can be concluded that isolate 12.3.A of Actinomycetes have close genetic traits with *Streptomyces hirsutus* strain NRRL B-2713 with the degree similarity / homology of 99%. After having the name of the species of the isolate 12.3.A then convert to a phylogenetic tree compared with 15 species taken from the BLAST results. A phylogenetic tree would be shown in Figure 4.40. Usually if the degree of sequence base similarity of genes encoding 16S rRNA less than 95% can be considered as a new species (Mashapho, 2005).