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PRODUCTION OF CELLULASE FROM PALM OIL INDUSTRIAL SOLID WASTE BY ACTINOMYCETES ISOLATE 12.3.A

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Key words : *Acid hydrolysis pretreatment, Oil palm empty fruit bunch, Palm oil industrial waste, Streptomyces hirsutus,*

Abstract - This research aimed at utilization of palm oil industrial solid waste for enzyme cellulase production. The abundant waste of palm oil industry is oil palm empty fruit bunch (OPEFB) which composed of lignocellulosic material. A potential of using OPEFB and its acid pretreatment residue to accelerate cellulase production by cellulolytic microorganism was tested. The high cellulase producing actinomycetes isolate 12.3.A that was isolated from the oil palm plantation area in Phetchaburi, Thailand was used in this study. The isolate 12.3.A was preliminary appointed to be *Streptomyces hirsutus* from the result of 16S-rRNA gene analysis. The optimal conditions for cellulase production of *S. hirsutus* isolate 12.3.A were similar for both substrates. The best yields were derived from culturing the cells at pH 7, 30°C, and substrate concentration at 1% for 6 days. The highest cellulase activities from OPEFB and the acid residue were 0.71 U/mL and 0.56 U/mL, respectively. The suitable nitrogen sources for the culture medium made of OPEFB was ammonium sulfate, while peptone was for the residue. The zymogram assay with polyacrylamide amended with carboxymethylcellulose (CMC) demonstrated that the isolate 12.3. A produced 2 CMCases (endo-β-glucanases) which had optimal conditions for the activity at pH 6.5 and 45°C. The pretreatment should loosen the complex structure of lignocellulosic component of OPEFB for facilitating the attack of cellulose by microbial cellulase. Acid hydrolysis pretreatment extracted all hemicellulose out and increased the percentage of cellulose of the residue. However, this increased the percentage of lignin as well. Therefore, the acid hydrolysis pretreatment of the OPEFB did not assist the acceleration of the microbial cellulase production. However, OPEFB and its acid hydrolysis residue may act as good substrates for cellulase production of *S. hirsutus* isolate 12.3.A.

INTRODUCTION

Cellulose is the main constituent component in the plant biomass. Cellulose is a linear polymer consisting of D-anhydroglucopyranose molecules which are joined together by the bond of β-1,4 glycosidic of the degree of polymerization (Zhang, *et al*., 2007). According to Schwarz (2001), cellulose is difficult to degrade because it is crystalline and insoluble in water. Moreover, cellulose is rarely found in pure form but together with lignin and hemicellulose in nature. As plant biomass, cellulose often found in the biomass lignocellulosic materials such as biomass agricultural, forestry and agro-industrial wastes that are abundant, renewable and inexpensive energy sources. Lignocellulosic wastes are accumulated every year in large quantities, causing environmental problems. However, due to their chemical composition based on sugars and other compounds of interest, they could be used for production of by-product such as:

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ethanol, food additives, organic acids, enzymes and others.

Cellulose degrading organisms have been used to convert cellulose to sugar dissolved components, some of which have been applied in biotechnology and industry (Cherry and Fidantsef, 2003; Kotchoni, *et al*., 2003). Cellulose can be degraded easily and quickly by certain organisms such as bacteria, fungi, Actinomycetes. Expert classified cellulose decomposers organisms to be aerobic bacterial, myxobacteria, anaerobic bacteria, thermophilic group, actinomycetes, filamentous fungi, mushroom, protozoa and insects. Success in breaking down cellulose depends on the nature or circumstances of cellulose degrading micro-organisms and environmental factors such as humidity, aeration, temperature, and nitrogen availability of adequate and nutritional elements (Sutedja, *et al*., 1991). More recently, some bacteria such as *Clostridium thermocellum, Clostridium cellulolyticum, Clostridium cellulovorans, Clostridium josui, Ruminocoocusalbus,* while the other group of actinomycetes is *Thermoactinomycetes* sp., *Thermonosporacurvata* and *Streptomyces* sp. have been reported as a producer of cellulose, using a different substrate such as cellulose, carboxymethyl-cellulose, starch, and glucose as the carbon source (Belaich, *et al*., 2002; Keller, *et al*., 2005).

When degrading cellulose, microorganisms produce an enzyme called cellulase. Cellulase (1,4-β-D-glucan glucanohydrolase) is a multienzyme complex consisting of three main components: endo-β-glucanase, exo-β-glucanase and β-glucosidase which has demonstrated synergistic activity in hydrolyzing cellulose (Emert, *et al*., 1974). The first cellulase was used as animal feed (Ishikuro, 1993). In industry, cellulase was used to produce organic acids (Luo, *et al*., 1997), detergents and chemicals. Cellulases were also used in the pulp and paper industry Oksanen, *et al*., 2000), in the fermentation of sugar and ethanol products (Levy, *et al*., 2002), in the textile industry (Miettinen, *et al*., 2004; Nierstrasz and Warmoeskerken, 2003), and in the food industry (Urlaub, 2002; Penttila and Limon, 2004). Cellulase was used to transform cellulosic waste paper to ferment sugars (Van Wyk and Mohulatsi, 2003).

Oil palm empty fruit bunch (OPEFB), a lignocellulosic material is generated as an abundant waste product from oil palm industry. This waste material has a low cost value which could be converted tobe high-value products. The process requires microorganisms that can degrade lignocellulose from OPEFB induction using extra-cellular enzymes such as cellulase, hemicellulase (xylanase), and lignin peroxidase. Chemical and enzymatic methods are the most commonly applied methods for hydrolyzing cellulosic materials. In the chemical method, the hydrolysis of sugar polymers in lignocellulose is catalyzed by an acid, whereas in the enzymatic process, enzymes are used for hydrolyzing cellulose and hemi-cellulose to monomeric sugars. In this study, OPEFB was pretreated using dilute sulfuric acid and steaming under pressure. OPEFB and its acid hydrolysis residue were testified for acceleration of cellulase production of a selected high cellulolytic microorganism.

MATERIALS AND METHODS

Microorganism

Cellulolytic Actinomycetes isolate 12.3.A isolated from soil sample of oil palm plantation area in Phetchaburi, Thailand.

Characterization of the cellulolytic actinomycetes

Colony morphology of the isolate 12.3.A was characterized on CMC agar. Cell and filament morphology were Gram stained and observed under light microscope. Extracellular cellulase activity was demonstrated on CMC agar (pH 7) at 30°C. After 3 days of incubation, the cultured plate was flooded with an aqueous solution of Congo red (1% w/v) for 15 min and destained with 1 M NaCl. The unstained zone indicated cellulose degradation.

The 16S rRNA gene analysis

The 16S rRNA gene of the isolate12.3.A was amplified by PCR technique using 800R and 518F primers (Macrogen Inc, Seoul, Korea). The 16S rDNA 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.

Preparation OPEFB fiber

The oil palm empty fruit bunches (OPEFB) was obtained from a palm oil industry. The bunch was sterilized at 121 °C for 15 min, dried at 105 °C, and cut into small pieces. The OPEFB was digested into small size (1-2 mm) by a crusher machine

(Nurkaya,2013).

Preparation of acid hydrolysis residue of OPEFB

The OPEFB fiber was added into 250 mL Erlenmeyer flask containing 2% sulfuric acid at a ratio of 1:10 (w/v) (1g fiberand 10 mL of sulfuric acid). The acid hydrolysis was done under strong condition (121 °C, 75 min)(Putthida, *et al*., 2011). Liquid fraction was separated from solid by filtration with Whatman No. 1. The composition of liquid hydrolyzate was determined. Solid fraction was neutralized by rinsed with distilled water until pH of rinsed water became neutral.

Determination of the composition of OPEFB and acid hydrolysis residue

Measurement of the main component (protein, lipid, ash, moisture, carbohydrates, cellulose, hemicellulose and lignin) of OPEFB and residue of acid hydrolysis was used proximate analysis (A.O.A.C., 1984) and cellulose, hemicellulose, and lignin were determined by Van Soest method (Van Soest, *et al*., 1991).

Measurement of cellulase enzyme activity

Cellulase enzyme activity was assayed using Dinitrosalicylic (DNS) by Ghose method (Ghose, 1987), with some modifications. 0.5 mL of culture supernatant was added into 0.5 mL of 1% CMC in 0.05M phosphate buffer (pH 7) and incubated at 50°C for 60 min. The reaction was stopped by adding of 3.0 mL of DNS reagent and subsequently placing the reagent tubes in a water bath at 100 °C for 5 min. After boiling, the tubes were transferred immediately into a cold water bath. A 10 mL distilled water was added andthe absorbance of the reaction liquid was measured at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme (mL) that released 1 μmole of glucose per min.

Factors affecting cellulase production from OPEFB and acid hydrolysis residue of the isolate 12.3.A

The effect of pH (5, 6, 7 and 8), temperature (30, 35, 40 and 45 °C), substrate concentration (0.5, 1 and 1.5% (w/v)), and nitrogen source (peptone, yeast extract, ammonium sulfate and ammonium nitrate at 0.5 g/L) on enzyme cellulase production of the isolate 12.3.A using OPEFB and its hydrolysis residue as sole carbon source in the culture medium were explored.

Partial purification of cellulase

The crude cellulase enzyme in culture supernatant was partially purified with the addition of 60% saturated ammonium sulfate (at 4°C) and setting overnight. The pellet enzyme was collected by centrifugation at 10,000 rpm, 4°C for 10 min and dissolved in 5 mL of 0.2 Mphosphate buffer pH 7. Then, the enzyme suspension was dialyzed overnight in 0.07 M phosphate buffer (pH 7). The final cellulase activity and protein content of the partially purified cellulase were determined.

Zymogram for demonstration of cellulase activity

Polyacrylamide gel amended with 0.1% CMC was loaded with the isolate 12.3.A cellulase and the gel was electrophoresed at 120 volts for 1-1.5 hours in electrophoresis chamber. The gel was rinsed with distilled water 3 times and incubated in 50 mM Tris buffer pH 7 for 1.5 hours. Then, the gel was stained with 0.1% Congo red and destained with 1 M NaCl. The unstained zone revealed the position of cellulase enzyme.

Optimal conditions for cellulase activity

The optimum conditions for the cellulase activity of the isolate 12.3.A were investigated at different pH (5.5, 6, 6.5, 7, 7.5 and 8) and different temperature (30, 35, 40, 45, 50, 55 and 60 °C).

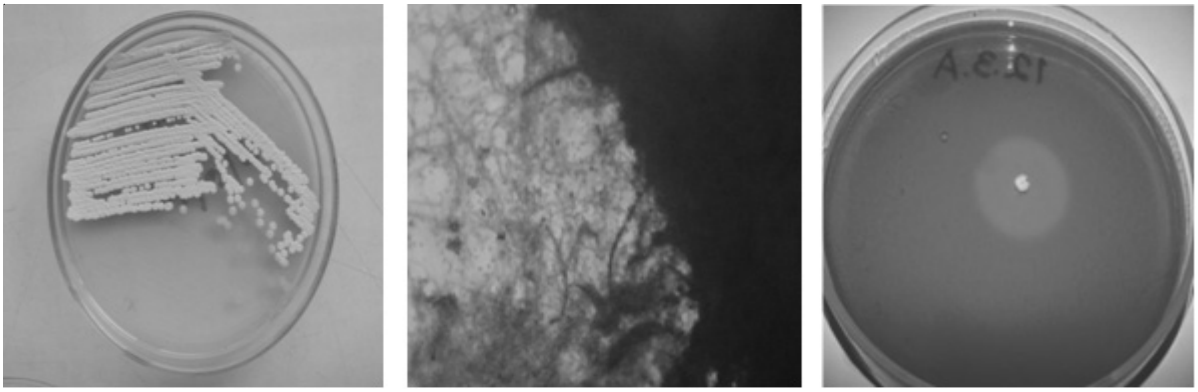
RESULTS AND DISCUSSION

Characteristics and cellulase activity of actinomycetes isolate 12.3.A

The colony of actinomycetes isolate 12.3.A was white, circular and opaque, with low convex and undulate edge. Cells were filamentous shape and stained Gram-positive. The hooked spore-chains at the end of aerial mycelia had been observed.

Identification of the isolate 12.3.A by 16S- rRNA gene analysis

Based on the molecular analysis of the 16S- rRNA geneit 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,the sequence was used to make a phylogenetic tree compared with 15 species taken from the BLAST



(a) (b) (c)

Fig 1 (a) colony shape of the isolate 12.3.A. (b) Staining Gram and filamentous shape of the isolate 12.3.A. (Nurkaya, 2013) (c) a clear zone surrounded the colony of the isolate 12.3.A (Nurkaya and Chaiyanan, 2012)

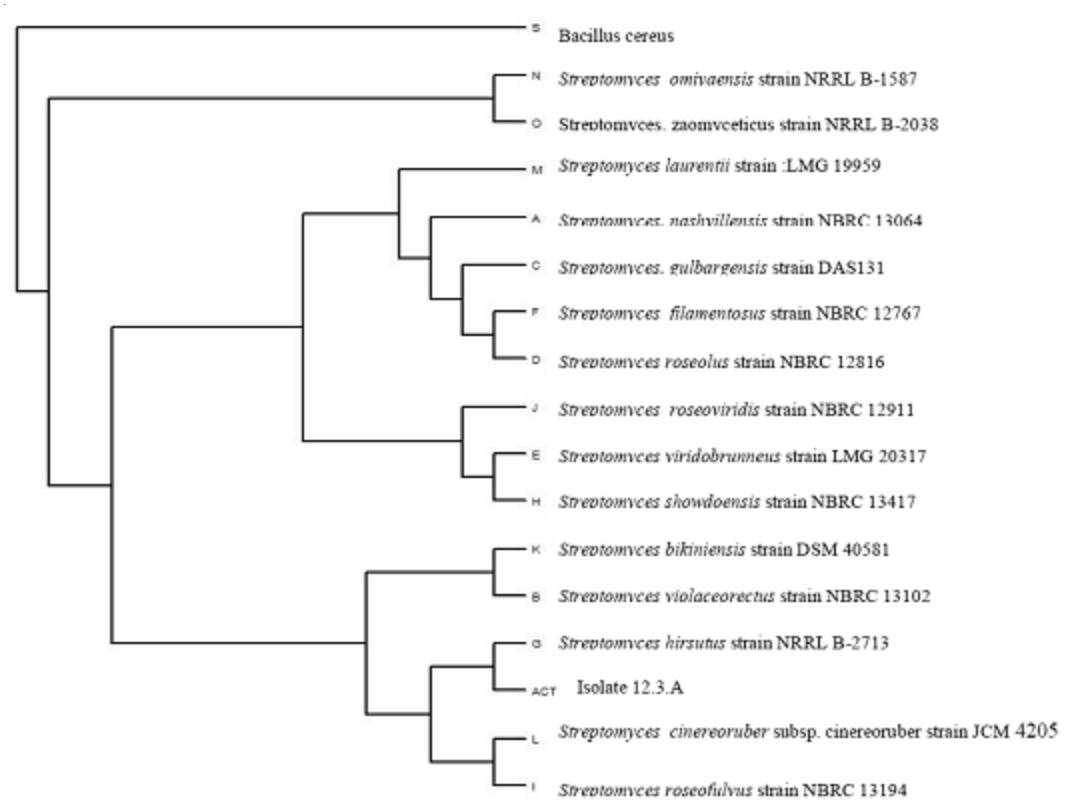


Fig 2 Neighbor-joining tree based on 16S- rRNA gene sequences, shown the phylogenetics relationship between the isolate 12.3.A and 15 related species of the genus Streptomyces (Nurkaya, 2013)

results (Fig. 2). Therefore, the isolate 12.3.A was preliminary appointed to be *Streptomyces hirsutus*

Composition of OPEFB and acid hydrolysis residue of OPEFB

The chemical components of OPEFB and residue of acid hydrolysis (Table 1) revealed that hemicellulose was taken out by the acid hydrolysis. After hydrolysis, the cellulose and lignin components of the residue were higher than the OPEFB. Protein, lipid, and ash of the OPEFB fiber were higher than the acid hydrolysis residue.

According to Wyman (1994), Sung and Cheng (2002),the acid hydrolysis method gives high

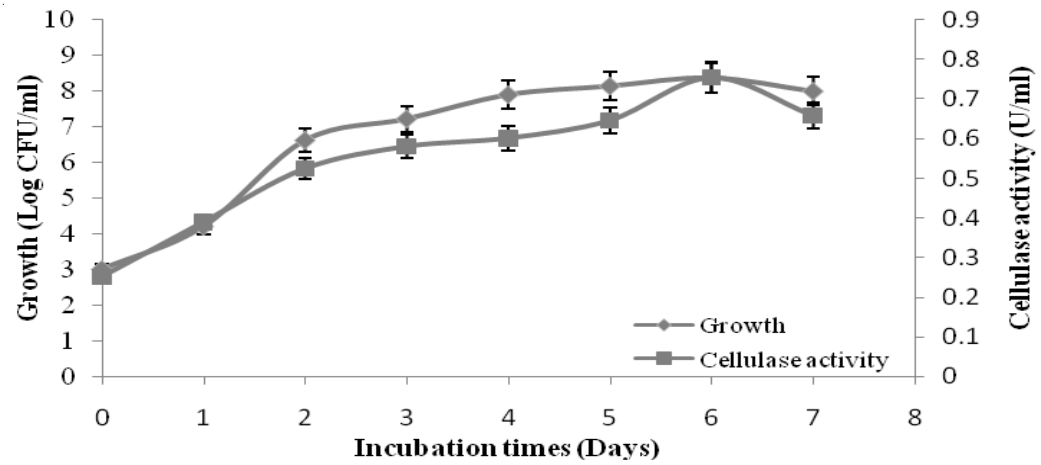


Fig. 3 Growth and cellulase production of *Streptomyces hirsutus* isolate 12.3.A in CMC broth.

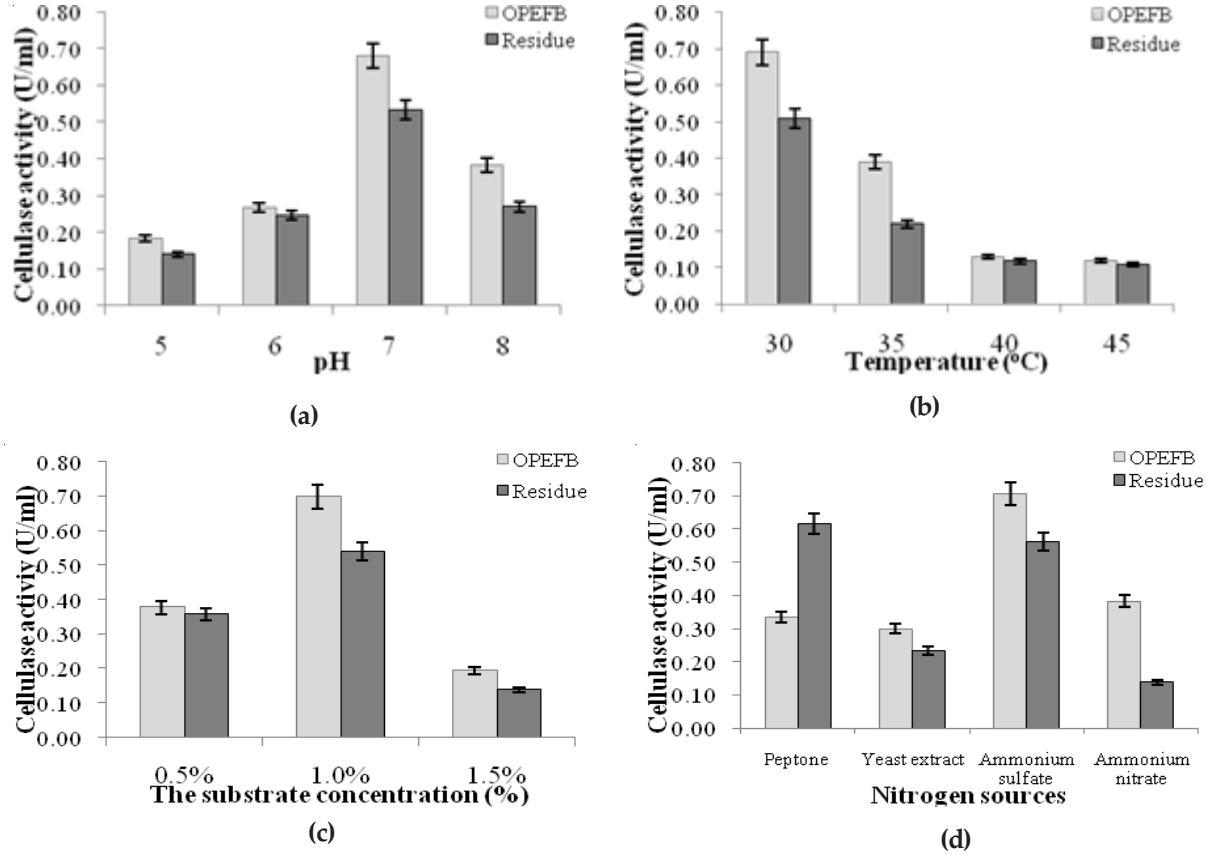


Fig. 4 Optimal conditions for cellulase production from OPEFB and acid hydrolysis residue of *Streptomyces hirsutus* isolate 12.3.A. (a) pH, (b) Temperature, (c) the substrate concentration and (d) Nitrogen sources. All treatment were used 150 rpm agitation.

reaction rates and significantly improves cellulose hydrolysis.100% hemicellulose could be removed by using acid hydrolysis (dilute-acid hydrolysis). Dilute-acid hydrolysis is not effective in dissolving lignin, but it can disrupt lignin and increases the celluloses susceptibility to enzymatic hydrolysis.

Growth and cellulase production *Streptomyces hirsutus* isolate 12.3.A in CMC medium

Figure 3 shows growth and cellulase production *S.*

Table 1. Composition of OPEFB and residue of acid hydrolysis of OPEFB

Main component	OPEFB (%)	Residue (Acid hydrolysis)(%)
Moisture	10.85	8.61
Protein	9.56	4.87
Lipid	6.86	4.48
Ash	8.72	2.09
Carbohydrate	74.86	88.56
Cellulose	39.42	53.38
Hemicellulose	16.18	0
Lignin	13.33	23.27

Source: Nurkaya, 2014

hirsutus isolate 12.3.A in CMC medium.

The isolate 12.3.A could use CMC as a sole carbon source and reached maximum growth in 6 days at 8.38 Log CFU/mL. CMC was a good accelerator for the microbial cellulase production. The highest cellulase was obtained 0.75 U/mL in 6 days.

Optimal conditions for cellulase production from OPEFB and acid hydrolysis residue of *S. hirsutus* isolate 12.3.A

From the Figure 4, the optimal conditions for cellulase production from OPEFB of *S. hirsutus* isolate 12.3.A were pH 7, temperature at 30°C, OPEFB fiber concentration at 1%, and the best nitrogen source was ammonium sulfate. The results were similar for the acid hydrolysis residue except peptone was the most supporting nitrogen source. The highest cellulase activitiesfrom OPEFB and the acid residue were 0.71 U/mL and 0.56 U/mL, respectively.

Partial purification of cellulase produced by *S. hirsutus* isolate 12.3.A

From Table 2, protein precipitation by using saturated ammonium sulfate (60%) and following with dialysis could increase the specific activity of cellulase from 2.0U/mg to 7.38 U/mg.

Zymogram of *S. hirsutus* isolate 12.3.A

Zymogram gel used in this study was polyacrylamide gel amended with 0.1% CMC. Zymo gram of the partial purified enzyme of *S. hirsutus* isolate 12.3.A revealed two bands of clear zone of cellulase activity after staining with 0.1% Congo red and destained with 1 MNaCl (Figure 5). The enzymes could be defined as CMCases or endo-β-

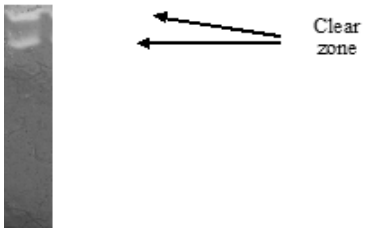


Fig. 5 Two bands of clear zone of cellulase enzyme were shown after running by electrophoresis.

glucanases, since the substrate in the gel was CMC (Dobrev and Zhekova, 2012).

Optimal conditions for *S. hirsutus* isolate 12.3. A endo-β-glucanases

The pH optimum of endo-β-glucanases was determined by changing pH of incubation buffer from pH 7 to 5.5, 6, 6.5, 7.5 and 8 (Figure 6). Determination of optimum temperature for *S. hirsutus* isolate 12.3. A endo - β glucanases was done by variation of incubation temperature (30, 35,40,45,50, 55and 60 °C) (Figure 7). The endo-β-glucanases of *S. hirsutus* isolate 12.3.A was shown the optimum pH and temperature at 6.5 and 45°C, respectively.

Comparison of cellulase production of *S. hirsutus* isolate 12.3.A between using OPEFB and its acid hydrolysis residue

The acid pretreatment should aid the microbial

Table 2. Purification of Cellulase from *Streptomyces hirsutus* isolate 12.3.A.

Stages	Volume (mL)	Cellulase activity (U/mg)	Protein (mg/mL)	Total Cellulase activity (U)	Total Protein (mg)	Specific activity (U/mL)
Crude Enzyme	2,350	0.56	0.28	1,316	658	2.0
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

Source: Nurkaya, 2014

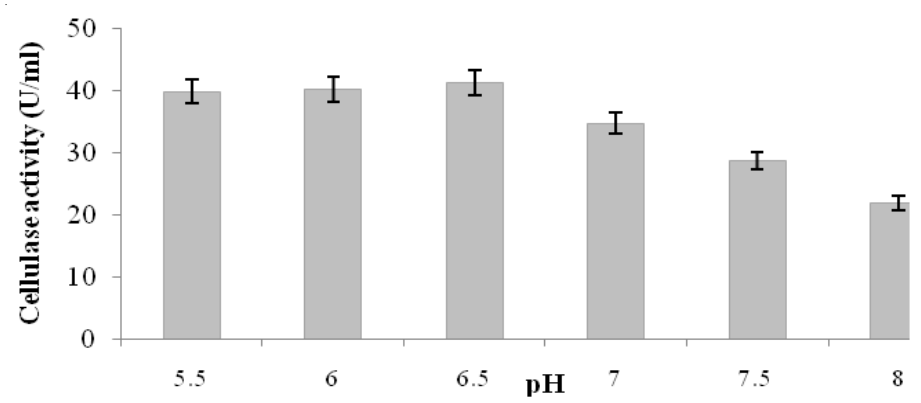


Fig. 6 Effect of pH on cellulase activity

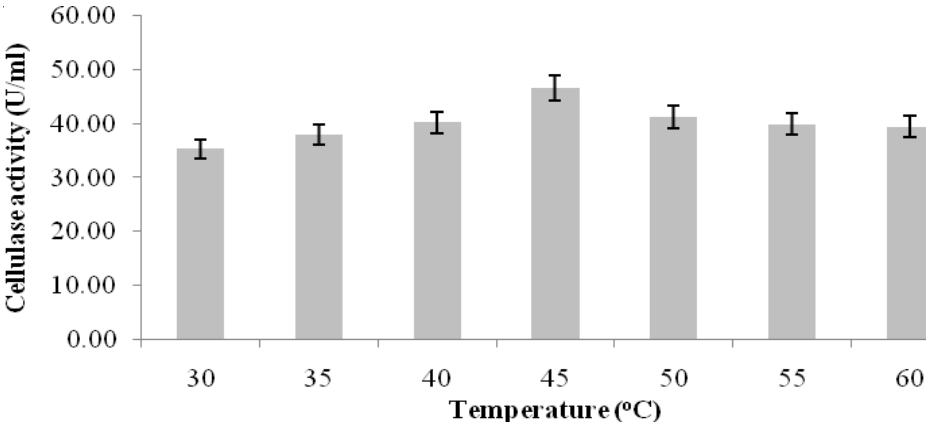


Fig. 7 Effect of temperature on cellulase activity

cellulase to attack the cellulose of the OPEFB. On the contrary, the cellulase production from the residue was lesser than from the OPEFB. This could be explained by the difference between the composition of OPEFB and the residue (Table 1). After acid hydrolysis pretreatment extracted all hemicellulose out, not only the percentage of cellulose of the residue was increased but also the percentage of lignin. The higher in percentage of lignin might retard the enzyme activity. The residue had organic components less than the OPEFB, therefore, the cellulase production from the residue required organic nitrogen source while OPEFB need only inorganic nitrogen source. However, both OPEFB and its acid hydrolysis residue could be good accelerators for cellulase production of *S. hirsutus* isolate 12.3.A.

CONCLUSION

The high cellulase producing actinomycetes isolate 12.3. A isolated from the oil palm plantation area in Phetchaburi was identified to be *Streptomyces hirsutus* with 99% similarity to *Streptomyces hirsutus* strain NRRL B-2713. The optimal conditions for cellulase production of *S. hirsutus* isolate 12.3.A from OPEFB and its acid hydrolysis residue were pH 7, 30 °C, and 1% the substrate concentration. Ammonium sulfate was the best nitrogen source for OPEFB medium and peptone was for the residue medium. A partial purification of cellulase by 60% ammonium sulfate precipitation and subsequently dialysis had the cellulase activity at 7.38 U/mg. The zymogram assay with poly-acrylamide amended with CMC demonstrated that the isolate 12.3.A produced 2 CMCases (endo-β-glucanases). The optimal conditions for the cellulase activity were at pH 6.5 and 45 °C.

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