CHAPTER 3 MATERIALS AND METHODS

3.1. Raw materials and chemicals

3.1.1 Raw materials

All of the samples for the isolation of cellulolytic microorganisms (soil, leaf bamboo, rod of palm tree, seed of the palm tree, fiber of the oil palm empty fruit bunch, etc.) were collected from the palm oil plantation and palm mill area in Pechabury Province, Thailand

3.1.2 Chemical

3.1.2.1 H_2SO_4
3.1.2.2 NaOH
3.1.2.3 Phenol
3.1.2.4 Dinitrosalicylic acid
3.1.2.5 $CuSO_4$
3.1.2.6 Rochelle salt
3.1.2.7 Sodium carbonate
3.1.2.8 Other the chemical compounds

3.1.3 Microbial culture medium

3.1.3.1 Nutrient agar (NA)
3.1.3.2 Potato dextrose agar (PDA)
3.1.3.3 Carboxymethyl cellulose agar (CMC agar)
3.1.3.4 Yeast peptone glucose agar (YPG agar)
3.1.3.5 Nutrient Broth
3.1.3.6 Potato dextrose broth (PDB)
3.1.3.7 Other microbial culture mediums

3.2 Equipment for analysis

3.2.1 Machinery and equipment

3.2.1.1 Digital balance 6 digits (Mettler Toledo, Thailand)
3.2.1.2 Laminar air flow (Astec Microflow Limited, Model ABS1200)
3.2.1.3 Microwave (Light Up Dial, Sharp, Model R 241)
3.2.1.4 Refrigerator (Expresso Cool, LG)
3.2.1.5 Freezer (Frezeer Cool, Sanyo)
3.2.1.6 Rotary vacuum evaporator (Buchi rotavapor R200, Switzerland)
3.2.1.7 Gas chromatography mass spectrophotometer completed with capillary column (Autosystem XL, Perkinelmer Instruments, Perkin Elmer Ltd)
3.2.1.8 Light microscope (Olympus, type CH-2, Olympus Optical Co., Ltd, Japan)
3.2.1.9 Electrophoresis (Power PAC 200, Bio-Rad)
3.2.1 10 Hot plate and Stirrer (IKA , Model C-MAG HS7, made in China)
3.2.1.11 Vortex mixture (Vortex 2, Scientific Industries, New York, USA)
3.2.1.12 UV-visible spectrophotometer (U-1800 spectrophotometer)

3.2.1.13 Digestion unit (Model K-424, Buchi)
3.2.1.14 Vortex mixture (Vortex genie-z of scientific industries, USA)
3.2.1.15 Autoclave (SA300VL, Japan).
3.2.1.16 Oven dry Unit (Carbolite)
3.2.1.17 Micropippete (100-1000µL), micropippete (20-200µL), micropippete (1–5 mL) and micropippete (2-20µL) (Boeco, Germany)
3.2.1.18 Incubator (EcoCell 111, Germany)
3.2.1.19 Solvent extractor (Velp scientifica, Model SER 14-B)
3.2.1.20 Distillation unit (UDK 132, Semiautomatic distillation, Velp Scientifica)
3.2.1.21 High performance liquid chromatography (Agis)
3.2.1.22 Fermentor (Volume 2 liter, Biostat-B, Biotech International)
3.2.1.23 Fermentor (Volume 4 liter, Biostat-B, Biotech International)
3.2.1.24 pH Meter (Model 713, Metrohm)
3.2.1.25 Centrifuge (Avanty centrifuge, Model J26-XPI, Beckman Coulter)
3.2.1.26 Small centrifuge (IEC Centra-M2 centrifuge, made in USA)
3.2.1.27 Mini spin (Eppendorf)
3.2.1.28 Orbital shaker (Thermo Forma)
3.2.1.29 Shaking water bath (Model VS-1205SWL, Vision)
3.2.1.30 Digital camera (Sony Cybershot D D33021)

3.2.2 Glass equipment

3.2.2.1 Beaker
3.2.2.2 Glass rod
3.2.2.3 Burette
3.2.2.4 Glass funnels
3.2.2.5 Flask; 125, 250, 500 and 1000 mL
3.2.2.6 Volumetric cylinder
3.2.2.7 Loop
3.2.2.8 Needle
3.2.2.9 Slide
3.2.2.10 Cover glass
3.2.2.11 Glass tube

3.3 Method

3.3.1 Source of microorganisms

Samples for isolation of microorganisms used in this study would be collected from several places associated with the oil palm Industry. Sample properties were also recorded.

3.3.2 Preparation of oil palm empty fruit bunch (OPEFB)

The oil palm empty fruit bunches (OPEFB) were obtained from the palm oil company in Pechabury Province, Thailand. The OPEFB was digested into small size of a crusher machine. Compositions of OPEFB fiber were determined (Appendix B and C).

3.3.3 Preparation of residue from acid hydrolysis of OPEFB

The OPEFB fiber was added into 250 ml Erlenmeyer flask containing 2% sulfuric acid at a ratio of 1:10 (w/v) (1 gram fiber and 10 mL of sulfuric acid). The acid hydrolysis were done under strong condition (121^{o}C, 75 min). 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 to a pH of rinsed water became neutral. Solid residue was dried at 80^{o}C for overnight and kept in a closed chamber until used. Compositions of solid residue were also determined (Appendix B and C).

3.3.4 Isolation of microorganisms

Liquid carboxymethyl cellulose medium (CMC medium); 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgS0_4.7H_2O$, 0.01 g $FeSO_4.7H_20$, 0.01 g $MnSO_4.H_2O$, 0.5 g $(NH_4)_2SO_4$ and 10 g CMC in 1 liter of distilled water was prepared and sterilized at 121^{o}C for 15 minutes. It was used to enrich microorganism from samples according to section 3.3.1. 1 g each sample were added into 100 ml sterilized CMC medium and incubated at room temperature (30-32^{o}C) under shaking condition (150 rpm) for 10 days. After incubation, microorganism; bacteria, fungi and actinomycetes were collected by a streak plate technique on CMC agar (1.5 % agar was added into the CMC medium). Cultured petri dishes were incubated at room temperature (30-32^{o}C) for 3 days. After incubation, colonized microorganisms were subsequently transferred on fresh CMC agar and incubated for 3 days to obtain pure cultures. All strains were kept in a deep freezer at -80^{o}C and used for study in the next section (3.3.5).

3.3.5 Pre–primary identification and characterization of cellulolytic microorganisms

Pre–primary identification was done to separate and classify selected strains into the member of bacteria, actinomycetes and fungi.

3.3.5.1 Colony properties of microorganism

Colony properties of microorganism were observed based on a single colony of microbes grown on a CMC medium. Cultures were incubated for 3 - 5 days at room temperature and then the shape, color, surface, internal structure and the structure of the edge of the colony were also observed. Based on colony properties, bacteria, actinomycetes and fungi were also separated. Cell morphologies and spore formulation of selected isolates were also determined.

3.3.5.2 Slide culture technique for fungi

In order to accurately identify fungus, it is essential to observe the precise arrangement of the conidiophores (conidial ontogeny). A simple modification of fungal slide culture technique was used to observe conidiophores of selected fungal isolates (Riddle's, 1950). A method is described in section of Appendix D and conidiophores picture was recorded and used to identify the genus of fungal isolates.

3.3.5.3 Gram staining technique

The Gram stain is the most important and universally used stained technique in the bacteriology laboratory. It is used to distinguish between gram-positive and gram-negative bacteria, which have distinct and consistent differences in their cell walls. Gram staining of bacteria is described in Appendix D. Consequently, Gram-positive and Gram-negative bacteria were classified.

3.3.6 Detection of extracellular cellulases activity on CMC agar

To determine the efficacy of cellulose degradation by selecting isolates, extracellular cellulases activity of all isolates was investigated on CMC agar (pH 7.0). The pure culture of all isolates cultured on CMC agar at room temperature for 3 days. After incubation, cultured plates were flooded with an aqueous solution of Congo red (1% w/v) for 15 min. To detect extracellular cellulases activity, the Congo red solution was then poured off and the plates were destained three times with 1 M NaCl for 15 min (until the clear zone was observed). The formation of a clear zone of hydrolysis indicated cellulose degradation by microorganism (Ariffin et al., 2008). Ratio of the 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.

3.3.7 Determination of extracellular cellulases activity and cell growth in a liquid CMC medium

According to section 3.3.6, the best extracellular cellulases isolates were selected. Properties of selected isolated grown in liquid CMC medium; growth curve, cellulase activity and free-reducing sugar were also investigated.

3.3.7.1 Inoculum preparation

The selected isolates were re-streaked on CMC agar and incubated for 3 days. A single colony of selected isolates was directly transferred into 100 mL CMC medium and incubated for 3 days on shaker incubator at 150 rpm (room temperature). After incubation, cell suspension in the flask was used as inoculum.

3.3.7.2 Determination of the growth curve of selected isolates

1% inoculum was added into a 250 mL flask containing 100 ml fresh CMC medium. Sample flask was incubated at room temperature for 7 days under shaking condition at 150 rpm. Initial cell concentration and amount of cell in every day were determined by drop plate technique (see in Appendix F). Cell concentration (CFU/mL) and time (day) was plotted.

3.3.7.3 Determination of cellulase activity

The extracellular cellular activity of select isolates was determined according to modified Ghose's method in 1987 (see in Appendix G). Supernatant was collected by centrifugation at 10.000 rpm for 4 min. A 0.5 mL of culture supernatant was added in 0.5 mL of 1% (w/v) carboxymethylcellulose (CMC) dissolved in 0.05M phosphate buffer (pH 7) in a test tube and incubated at 50 °C for 60 min. The reaction was stopped by the addition of 3.0 ml of dinitrosalicylic acid (DNS) reagent and by 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. 10 mL distilled water was added and reducing sugar in the sample was measured by spectrophotometric technique with absorbance at 540 nm against the blank (1% CMC in 0.05 M phosphate buffer). Amount of total reducing sugar (mg/ml) in the reaction was determined by comparing to a standard curve of glucose generated by Miller's method in 1959 (Appendix H). Cellulase activity (U/mL) was determined by equation (3.1).

$$\text{Cellulase activity (U/mL)} = \frac{(X)D}{0.18 \text{ x } 0.5 \text{ x } 60} \qquad (3.1)$$

Where :

(X)	=	The amount of glucose was produced in the CMC reaction (mg/mL)
D	=	Dilution factor
0.18	=	Molecular weight of glucose
60	=	Incubation time (minute)
0.5	=	Supernatant (mL)

Unit of cellulase activity was defined as the amount of enzyme that released 1 µmole of glucose per min. A good time for obtaining the highest cellulase activity was also determined by plotting graphs of extracellular cellulase activity (U/mL) and duration (day).

3.3.7.4 Determination of reducing sugar

In addition, reducing sugar in CMC solution during incubation was also determined by Miller's method in 1959. Supernatant was collected by centrifugation at 10.000 rpm for 4 min. A 0.5 mL of supernatant (enzyme) was added 0.5 mL of distilled water. The reaction was stopped by the addition of 3.0 ml of dinitrosalicylic acid (DNS) reagent and by 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. 10 mL distilled water was added and measure the absorbance at 540 nm against the blank (1% CMC in 0.05 M phosphate buffer). Amount of total reducing sugar (mg/mL) in the reaction was determined by comparing to a standard curve of glucose generated by Miller's method in 1959 (Appendix I). Reducing sugar (mg/mL) was determined by equation (3.2).

$$\text{Reducing sugar concentration (mg/mL)} = \frac{A_{540}}{0.9922} \times D \qquad (3.2)$$

Where:

D	=	Dilution factor
A_{540}	=	absorbance value at 540 nm
0.9922	=	equation from glucose standard (see in appendix I)

3.3.8 Investigation of optimum conditions of cellulase production medium

Optimum condition for cellulase production by selecting isolates was performed in liquid medium containing dried OPEFB and residue as carbon source. The composition of liquid medium used in this study was 1.0 g/L KH_2SO_4, 0.5 g /L $MgSO_4.7H_2O$, 0.5 g/L NaCl, 0.02 g/L $FeSO_4.7H_20$, 0.01 g/L $MnSO_4.H_20$, 0.5 g/L $(NH_4)_2SO_4$ and 10 g dried OPEFB or residue. Liquid medium contains 1 % (w/v) OPEFB as sole carbon source called OPEFB medium and residue medium means replacement of 1 % (w/v) OPEFB with 1 % (w/v) residue.

3.3.8.1 Inoculum preparation

The selected isolates were re-streaked on CMC agar and incubated for 3 days. A single colony of selected isolates was directly transferred into 100 mL CMC medium and incubated for 3 days on shaker incubator at 150 rpm (room temperature). After incubation, cell suspension in the flask was used as inoculum.

3.3.8.2 Determination of optimum pH

Determination of pH optimum was done in OPEFB and the residue medium with various pH condition (pH 5-8). 1% (v/v) inoculum was applied into 100 ml sterilized OPEFB and residue medium and incubated at room temperature for 7 days under shaking condition at 150 rpm. Cellulase activity was measured every day by using the method described in section 3.3.7.3. The optimum pH condition was determined as treatment showed the highest cellulose activity. Consequently, initial pH for cellulase production when 1 % (w/v) OPEFB and residue medium used as culturing medium was determined.

3.3.8.3 Determination of optimum incubation temperature

The optimum incubation temperature for cellulase production was studied by culturing selected isolates in 1% (w/v) OPEFB and residue medium at optimum pH obtained previous section in various incubation temperatures (30-45 ^oC). 1% (v/v) inoculum was applied into 100 ml sterilized 1 % (w/v) OPEFB and residue medium and incubated at various temperatures (30, 35, 40, and 45°C) for 7 days under shaking condition at 150 rpm. Cellulase activity was measured every day by using method described in section 3.3.7.3. Optimum temperature was selected as treatment showed the highest cellulase activity.

3.3.8.4 Optimum concentration of OPEFB and residue

The optimum substrate concentration was determined by using OPEFB and residue medium which initial pH value and incubation temperatures of OPEFB and residue mediums were set according to results in section 3.3.8.2 and 3.3.8.3. 1% (v/v) inoculum was applied into 100 ml sterilized 0.5%, 1% and 1.5% (w/v) OPEFB medium and 0.5%, 1% and 1.5% (w/v) residue medium and incubated for 7 days under shaking condition at 150 rpm. Cellulase activity was measured every day by using the method described in section 3.3.7.3. Optimum substrate concentration in cellulase production by using OPEFB and residue medium as treatment showed the highest cellulase activity in both substrates.

3.3.8.5 Effect of nitrogen sources

The effect of organic nitrogen and inorganic nitrogen source in cellulase production medium was also investigated. 1% (v/v) inoculum was applied into 100 ml sterilized and optimized OPEFB and residue medium obtained from section 3.3.8.2, 3.3.8.3 and 3.3.8.4 and incubated for 7 days under shaking condition at 150 rpm. The effect of the nitrogen sources were determined by the replacement of 0.05% (w/v) $(NH_4)_2SO_4$ with 0.05 % (w/v) organic nitrogen and inorganic nitrogen such as peptone, yeast extract and ammonium nitrate in OPEFB and residue medium. Cellulase activity was measured every day by using the method described in section 3.3.7.3. An optimum nitrogen source for cellulase production medium was determined as treatment showed the highest cellulase activity.

3.3.9 Cellulase purification

3.3.9.1 Crude cellulase production

To obtain a large amount of cellulase protein, 3 liters bioreactor was used in this study. The medium composition and suitable condition for production of enzyme cellulase was obtained from section 3.3.8.2, 3.3.8.3, 3.3.8.4 and 3.3.8.5. From the previous experiment, the highest cellulase activity was obtained in the treatment using OPEFB and $(NH_4)_2SO_4$ as carbon and nitrogen source. Therefore, the medium composition used in the study was 1.0 g/L KH_2SO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L NaCl, 0.02 g/L $FeSO_4.7H_20$, 0.01 g/L $MnSO_4.H_20$, 0.5 g/L $(NH_4)_2SO_4$ and 10 g dried OPEFB pH 7.0. Method to prepare inoculum was identical to section 3.3.8.1. Condition of bioreactor was set at the agitation rate of 150 rpm, the incubation temperature of 30^oC and aeration rate of 150 rpm. Cell concentration, cellulase activity and reducing sugar were measured every day by using methods described in section 3.3.7.2, 3.3.7.3 and 3.3.7.4.

3.3.9.2 Ammonium sulfate precipitation

The crude cellulase enzyme obtained from section 3.3.9.1 was precipitated by the addition of ammonium sulfate $(NH_4)_2SO_4$ (60% saturated at 4^oC). 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. The complete enzyme precipitation was obtained by stirring mixing solution for overnight in cool temperature (4^oC). The pellet enzyme was collected by centrifugation at 10,000 rpm, 4^oC for 10 min and dissolved by the addition of 5 ml of 0.2 M phosphate buffer pH 7. Remaining cellulase activity and protein content were determined. Protein content was analyzed according to Lowry's method (see in Appendix I).

3.3.9.3 Dialysis cellulase enzyme

The enzyme suspension obtained from section 3.3.9.1 was dialyzed in 0.07 M phosphate buffer solution (pH 7) 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 (see in Appendix J). Crude enzyme was kept in -20^oC to maintain cellulase activity.

3.3.9.4 Detection cellulase activity by zymogram

For the CMC zymogram, a 12% PAGE separation gel containing 0.1% carboxymethylcellulose (CMC) was prepared (see in Appendix L). A suitable diluted crude enzymes obtained from section 3.3.9.2 were loaded into of the gel (15 μm/lane). After electrophoresis, cellulase enzyme was re- activated by dipping in 50 M Tris buffer at pH 7 containing 1% Triton X-100 for 1 hour. 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 zones of clearing. (See in Appendix M)

3.3.10 Effect of temperature and pH on cellulase activity

3.3.10.1 Optimum pH

The pH optimum of cellulase was determined by changing pH of incubation buffer from pH 7 to 5.5, 6, 6.5, 7, 7.5 and 8, respectively. The residue activity was measured by using the method described in section 3.3.7.3. The pH optimum for cellulase activity was obtained from the treatment showed the highest cellulase activity.

3.3.10.2 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 residue activity was measured by using the method described in section 3.3.7.3. The optimum temperature for cellulase activity was obtained from the treatment showed the highest cellulase activity.

3.3.11 Ethanol production

3.3.11.1 Saccharification (enzymatic hydrolysis)

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. The crude enzyme was added into the sterilized saccharification medium with total activity unit of 350 units. Saccharification process was started by incubation at 45^{o}C for 48 hrs. Reducing sugar (section 3.3.7.4) in saccharification solution was determined every 6 hours. After saccharification, reducing sugar in residues enzymatic hydrolyzate was concentrated about 10 times by using rotary evaporator (see in Appendix N). Concentrated residues enzymatic hydrolyzate was used for ethanol fermentation.

3.3.11.2 Inoculums preparation

Saccharomyces cerevisiae was initially grown on yeast-peptone-glucose (YPG) and incubated at a temperature of 30^{o}C and a rate of agitation 150 rpm for 18 to 24 hours. In this study, YPG medium consisted of (g/L); 10 g yeast extract, 20 g peptone and 20 g glucose. After the incubation, the cells were then harvested by centrifugation at 10,000 rpm at 4^{o}C for 15 minutes. The pellet was then rinsed twice with sterilized saline solution before being re-suspended in sterilized saline solution to obtain yield an Optical Density (OD) of 1.0 at 600 nm (Kassim et al., 2011). The standardized *Saccharomyces cerevisiae* was used for subsequent study**.**

3.3.11.3 Ethanol production

Fermentation of concentrated residues enzymatic hydrolyzate was carried out by using *S. cerevisiae*. pH of solution was adjusted to pH 4.5 with 5 N HCl. A total of 100 mL of residues from enzymatic saccharification was prepared in a 125 mL conical flask. A total of 10% (v/v) of standardized active *S. cerevisiae* was added to the hydrolyzate and incubation at temperature 30^{o}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. Condition for ethanol analysis by gas chromatography is described in Appendix O.

3.3.12 Identification of cellulase producing actinomycetes by 16S rRNA sequence

3.3.12.1 16S rRNA analysis

The 16S rRNA gene of the selected isolates were 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.

3.3.12.2 Construction of phylogenetic tree

Phylogenetic tree was constructed by using PHYLIP program (Felsenstein, 1993). Genus and species were determined based on position of selected isolates on phylogenetic tree.

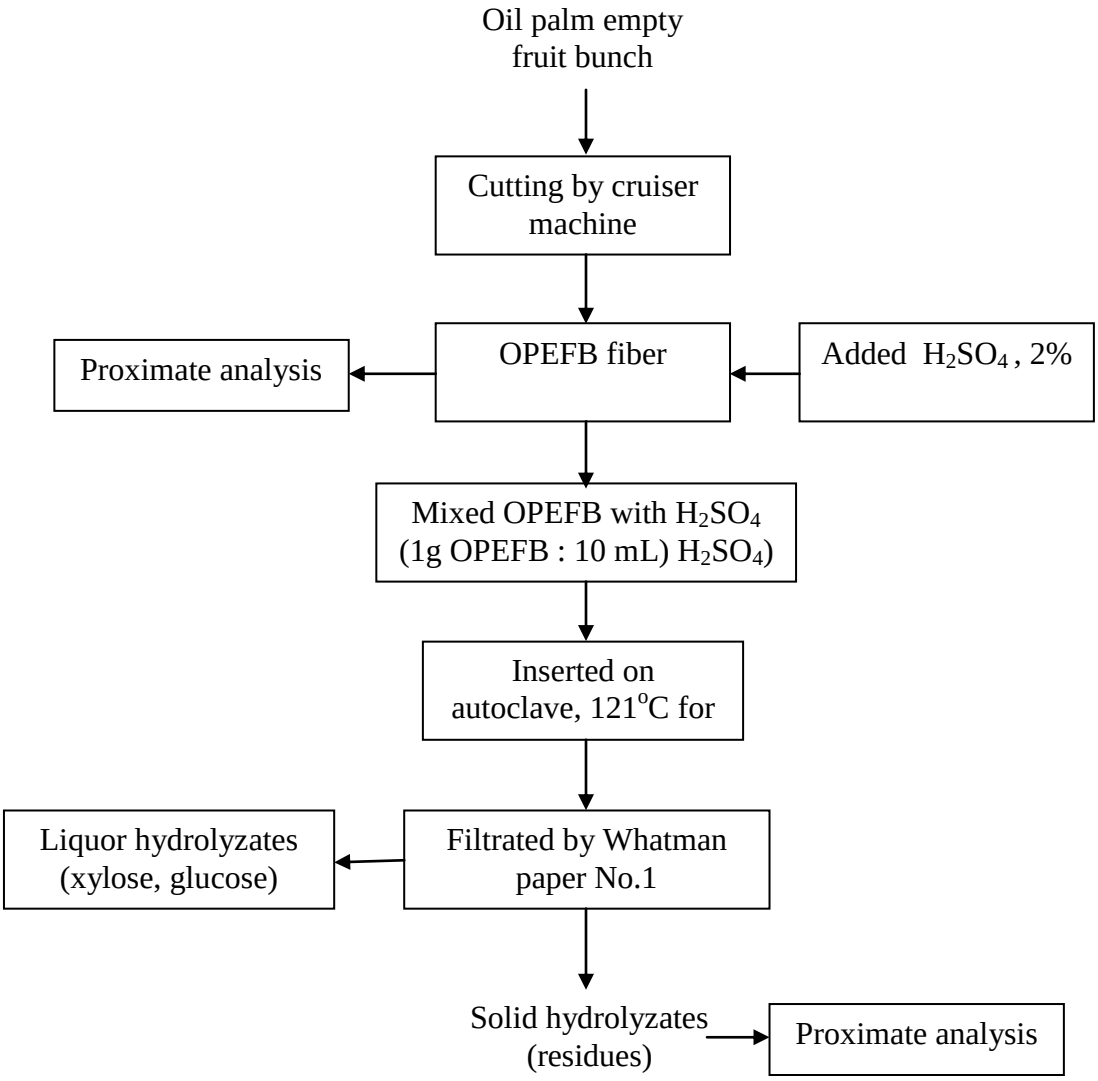

Figure 3.1 Flow chart preparation of residue from acid hydrolysis of OPEFB

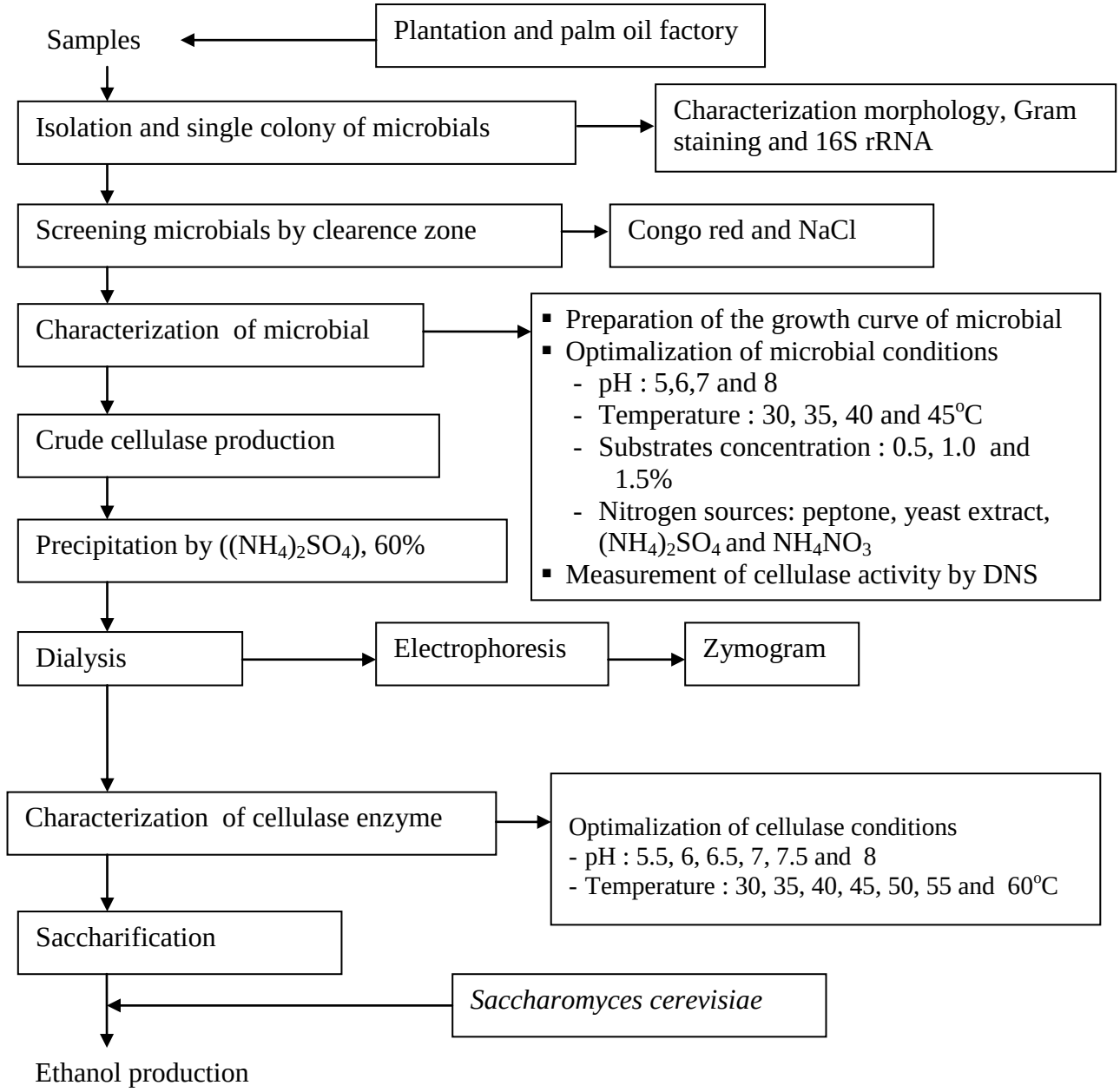

Figure 3.2 Flow chart of the ethanol production from microbial cellulase associated with the palm oil industry.

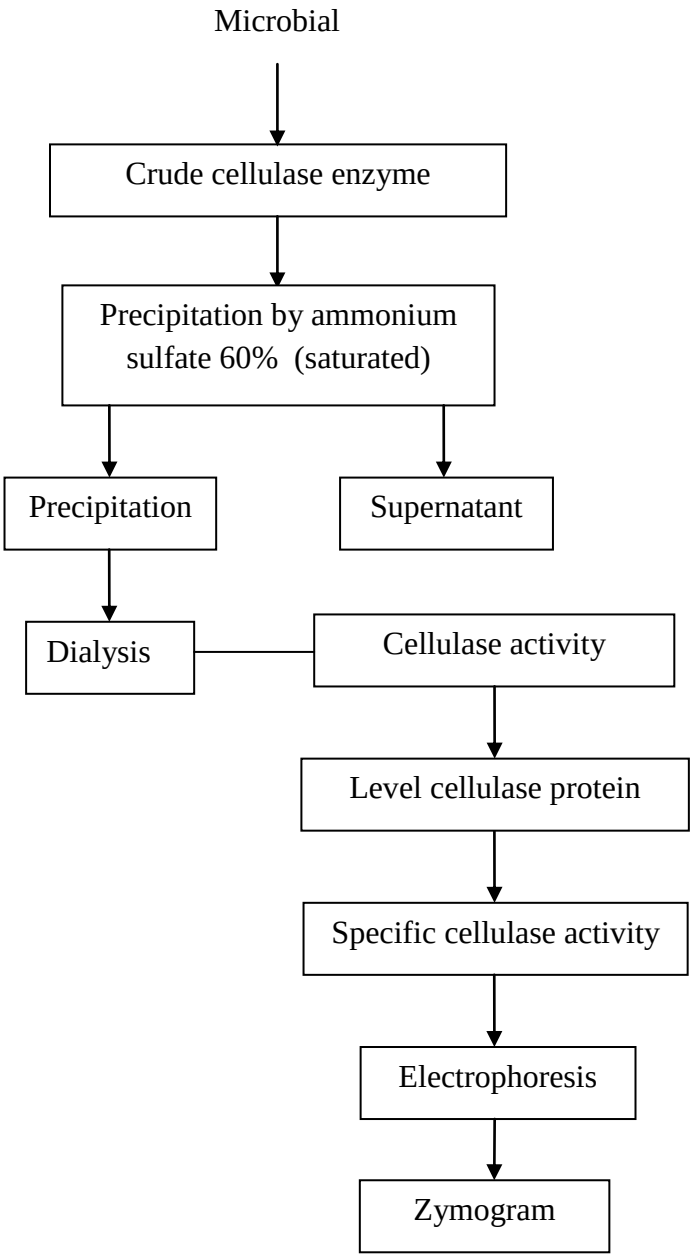

Figure 3.3 Flow chart zymogram of cellulase enzyme.

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