Cellulase production

Introduction

With an increase demand for cheap and renewable fuel, bioethanol appears to be the appropriate answer. Currently, the production of bioethanol can be divided into three generations. First generation of bioethanol production from sugar or molasses. Although sugar which come from sugar cane, beet root, corn or cassava is renewable, the utilization of such raw materials affects demand for food and feed. Production of bioethanol from cellulosic materials such as grass, wood or rice husk therefore, is appealing. This is classified as 2nd generation bioethanol production. The 3rd generation bioethanol production is produced from algae. The last generation uses large area of land as well as water for cultivation. Consequently, the 2nd generation seems to be appropriate. This is because the grasses or other cellulosic biomasses are the most abundant organic substance on earth.

Cellulosic biomasses compose of three components including cellulose, hemicellulose and lignin. Cellulose is the major constitute. It is a linear polymer of glucose with β(1-4) linked D-glucose unit with hydrgen bond linked between side chain causing crystalline. Hemicellulose composes of a mixture of pentose and hexose sugars (xyloase, arabinose, glucose, mannose, galactose, glucuronic acid, galacturonic acid and methylgalacturonic acid). It has amorphous, branched structure with xylose as the major component in backbone with β(1-4) linkage. Lignin is polyphenolic compounds. It links to hemicellulose protecting it form degradation by cellulase. Therefore, cellulosic biomasses are commonly pre-treated to remove lignin prior to saccharification and alcoholic fermentation by physical, chemical or microbiological method.

Cellulase is a group of enzymes including exoglucanase, endoglucanase and β-glucosidase worked collectively to hydrolyze cellulose as shown in Figure 1. Endoglucanase cleaves β(1-4) bond on the middle of polymer chain producing cello-oligosaccharides. Exoglucanse hydrolyses bond at the polymer end resulting in molecules of cellobiose. Then β-glucosidase convert a molecule of cellobiose to two molecules of glucose.

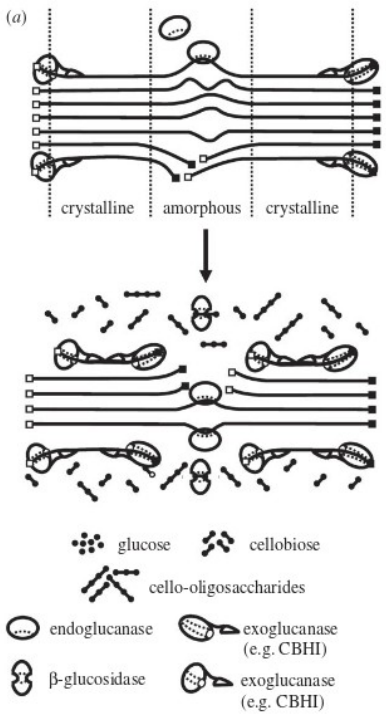

Figure 1. Cellulase enzyme and degradation of lignocellulosic biomass (van Zyl *et al.*, 2011)

Microorganisms can be used to produce cellulase. Table 1 shows a list of microorganisms with cellulase producing ability. Fungi is commonly used especially *Trichoderma* sp., *Aspergillus* sp. and other white rot fungi. Each strains of fungi may principally produce only one kind of the enzyme. Production of fungal enzymes is commonly carried out by either submerge or solid state fermentation. As cellulase is a multi-enzymes, optimization of cellulase production by either mixing of crude enzyme extract from each fungal strain or production of cellulase from mixed fungal strains should increase cellulase activity compared with that of single strain.

Table 1: Microorganisms having cellulolytic abilities.

Fungi	Soft rot fungi *Aspergillus niger; A. nidulans; A. oryzae; A. terreus; Fusarium solani; F. oxysporum; Humicola insolens; H. grisea; Melanocarpus albomyces; Penicillium brasilianum; P. occitanis; P. decumbans; Trichoderma reesei; T. longibrachiatum; T. harzianum; Chaetomium cellulyticum; C. thermophilum; Neurospora crassa; P. fumigosum; Thermoascus aurantiacus; Mucor circinelloides; P. janthinellum; Paecilomyces inflatus; P. echinulatum; Trichoderma atroviride* Brown rot fungi *Coniophora puteana; Lanzites trabeum; Poria placenta; Tyromyces palustris; Fomitopsis sp.* White rot fungi *Phanerochaete chrysosporium; Sporotrichum thermophile; Trametes versicolor; Agaricus arvensis; Pleurotus ostreatus; Phlebia gigantea*
Bacteria	Aerobic bacteria *Acinetobacter junii; A. amitratus; Acidothermus cellulolyticus; Anoxybacillus sp.; Bacillus subtilis; B. pumilus; B. amyloliquefaciens; B. licheniformis; B. circulan; B. flexus; Bacteriodes sp.; Cellulomonas biazotea; Cellvibrio gilvus; Eubacterium cellulosolvens; Geobacillus sp.; Microbispora bispora; Paenibacillus curdlanolyticus; Pseudomonas cellulosa; Salinivibrio sp.; Rhodothermus marinus* Anaerobic bacteria *Acetivibrio cellulolyticus; Butyrivibrio fibrisolvens; Clostridium thermocellum; C. cellulolyticum; C. acetobutylium; C. papyrosolvens; Fibrobacter succinogenes; Ruminococcus albus*
Actinomycetes	*Cellulomonas fimi; C. bioazotea; C. uda; Streptomyces drozdowiczii; S. lividans; Thermomonospora fusca; T. curvata*

(from: Chander Kuhad, Gupta, Singh, 2011)

Objective

1. to study production of cellulase by mixing of crude enzyme extract from each fungal strain or production of cellulase from mixed fungal strains
2. to use agricultural waste such as banana pseudostem as substrate for cellulase production

Materials and Methods

Experimental plan

Each strains of fungi is used to produce crude cellulase enzyme under solid state fermentation. Banana pseudostem will be used as substrate without any pretreatment. The fermentation will be carried out for 7 days in an Erlenmeyer flask at room temperature. Crude cellulase enzyme will be extracted afterward and examined for cellulase activity.

Crude enzyme production

Five grams of banana pseudostem (dried) will be mixed with 20 mL of salt solution as shown in **Table 2** prior to sterilization at 121°C for 15 min. After the temperature of culturing medium is at room temperature, three pieces of 7 days fungi grown on PDA at room temperature will be added to the sterile solid state media. The solid state fermentation will be carried out for 7 days at room temperature.

Table 2: Composition (g/L) of **salt solution** used for preparing culturing media for crude cellulase production (Ingale et al., 2014)

$(NH_4)_2SO_4$	1.4
KH_2PO_4	2.0
$CaCl_2*2H_2O$	0.3
$MgSO_4*7H_2O$	0.3
$FeSO_4*7H_2O$	0.005
$MnSO_4*H_2O$	0.0016
$ZnSO4*7H_2O$	0.0014
$CoCl2*6H_2O$	0.002
Peptone	0.1
Tween-80	0.1

Table 3. List of spore of fungi used in this experiment

Aspergillus niger TISTR 3240
Trichiderma reesei TISTR 3080
Trichoderma viride TISTR 3167
Volvariella volvacea (straw mushroom)

To extract crude enzyme, 20 mL of citrate buffer (50 mM, pH 5.0) is mixed with the fermented medium and brought for shaking at 150 rpm for 30 min before filtered through a filter paper. The filtrate is then centrifuge at 5000 rpm for 15 min. The supernatant is collected and kept at 4°C for further analysis of cellulase activity.

Determination of Cellulase activity by filter paper assay

One milliliter of appropriately diluted crude enzyme is mixed with 1 ml of citrate buffer (50

mM, pH 4.8) and 50 mg of Whatman filter paper #1 (1 x 6 cm strip that has been curled around a glass rod) in a test tube. Then, the test tube is incubate at 50°C for 60 min. The reducing sugar is measured using DNS method at 540 nm. One filter paper unit (FPU) was defined as the amount of enzyme releasing 1 mole of reducing sugar from filter paper /ml /h.

Results & Discussion

1. Please draw flow chart of this experiments
2. Cellulase activity

Fungi	OD	Dilution factor	FPU (1 mole of reducing sugar from filter paper /ml /h)
Aspergillus niger TISTR 3240			
Trichiderma reesei TISTR 3080			
Trichoderma viride TISTR 3167			
Volvariella volvacea (straw mushroom)			

3. Please explain results and give discussion accompanied with 2-3 references

Determination of reducing sugar (glucose) by DNS method

1. Mix sample or standard glucose solution with DNS reagent (0.5 mL each)
2. Heat the mixture in a boiling bath for 10 min
3. Cool the mixture in an ice bath or cold water bath and dilute the mixture with 10 volume of distilled water (5 mL)
4. Measure absorbance at 540 nm using distilled water as blank
5. Construct standard curve and concentration of glucose in the sample

Note: DNS reagent can be prepared by mixing 1% 3,5-dinitrosalicylic acid (DNSA), 30% sodium potassium tartrate, and 0.4 M NaOH. For preparing 500 mL solution, this can be done by mixing 5 g of 3,5-dinitrosalicylic acid (slowly added) into 400 mL solution containing 8 g of NaOH at 70°C. Then, adding 150 g of sodium potassium tartrate (calculate with no $4H_20$).
(http://faculty.mansfield.edu/bganong/biochemistry/reagents.html)

References

Chander Kuhad, R., Gupta, R. & Singh, A. 2011. Microbial Cellulases and Their Industrial Applications. Enzyme Research, Volume 2011 (2011), Article ID 280696, 10 pages

van Zyl, W. H., Chimphango, A. F. A., den Haan, R., Görgens, J. F. and Chirwa, P. W. C. 2011. Next-generation cellulosic ethanol technologies and their contribution to a sustainable Africa. Interface Focus, 1, 196–211