African Journal of Biotechnology Vol. 8 (13), pp. 3018-3022, 6 July, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Production of bio-ethanol from corncobs using *Aspergillus niger* and *Saccharomyces cerevisae* in simultaneous saccharification and fermentation

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Accepted 17 April, 2009

Maize is the most abundant cereal grown in Ghana and is accompanied by enormous amount of agrowastes of which corncobs form 30%. This agrowaste which is currently under utilized was used to produce bio-ethanol. *Aspergillus niger* isolated from soil sampled from Ejura farms was used to hydrolyze the corncobs into simple sugars. Filtrate obtained from corncobs broth fermented by *A. niger* was used as crude enzyme in optimization tests on corncobs powder suspended in 50 mM citrate buffer pH 5.0. Optimum temperature, pH and substrate concentration for saccharification were 40°C, 4.0 and 6% respectively. *Saccharomyces cerevisae* was added to *A. niger* filtrate to cause fermentation of the corncobs. The highest ethanol concentration of 0.64 g/l was recorded over the 24 h fermentation period.

Key words: *Aspergillus niger*, saccharification, simultaneous saccharification, fermmentation.

INTRODUCTION

Production of bio-ethanol from lignocellulosic materials such as agricultural wastes though faces challenges, can substitute bio-ethanol production from edible food substances. Maize (*Zea mays*) is the most abundant cereal produced in Ghana (Asante, 2004). This is accompanied by large quantities of maize agrowastes which is under utilized. Corncobs form about 30% of maize agro-wastes (Rangkuti and Djajanegara, 1983). Currently the corncobs are burnt as fuel in households of peasant rural farmers. Production of bio-ethanol from maize agrowaste has been attempted with enzymes from different sources for hydrolysis of lignocellulose and with different organisms for fermentation (Ohgren et al., 2006; Eken-Saracoglu and Arslan, 2000; Cao et al., 1996 and Wyman et al., 1992). This work aimed at producing bio-ethanol from corncobs in a batch simultaneous saccharification and fermentation. *Aspergillus niger* isolated from soil samples from Ejura farms was used to hydrolyze corncobs into simple sugars. *Saccharomyces cerevisae* was used to ferment the simple sugars into ethanol.

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MATERIALS AND METHODS

Cellulase production by *Aspergillus niger*

Cellulase was produced in corncobs based broth medium (CBB). CBB is a modified Mandel broth medium as described by Jeffries (1987) and consisted of the following per liter: $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.04 g, $CaCl_2$ 0.3 g, $MgSO_4.7H_2O$ 0.3 g, Citric acid 0.25 g, Tween 80 2 ml, Avicel cellulose 10 g, yeast 1 g and Trace metal stock solution 1 ml. The trace metal stock solution consisted of the following chemicals per 500 ml: $FeSO_4$ 2.55 g, $MnSO_4.H_2O$ 0.93 g, $ZnSO_4.H_20$ 1.78 g, $Co(NO_3)_2.6H_2O$ 1.25 g, and Conc. HCl 5 ml. Two hundred and fifty milliliters of CBB was inoculated with 3.02 $\times 10^7$ spores of *A. niger* and incubated on shaker (G24 Environmental incubator shaker, New Brumswick Co. USA) at 120 rpm at 25°C for 6 days period. The broth was centrifuged at 10000 g for 10 min and the supernatant was used as crude enzyme in saccharification tests. To determine enzyme activity over the period, 5 ml aliquots were collected at 24 h interval.

Optimization tests

The method of Baig et al. (2004) was adopted. One percent corncobs suspension was prepared by suspending 10 g of the substrate in 1 liter 50 mM citrate buffer pH 5.5 and autoclaved at 121°C for 20 min. Exactly 15 ml of the substrate suspension were put in each stopped 100 ml Erlenmeyer flasks and 5 ml of culture filtrate obtained from *A. niger* culture were added to each. Saccharification

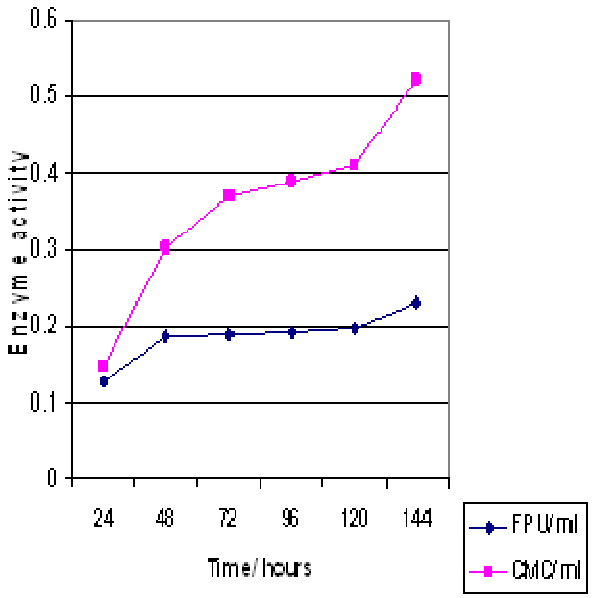

Figure 1. Enzyme activity of *A. niger* in CBB over 144 h period. FPU = Filter paper unit, and CMC = carboxymethyl cellulose unit.

was performed in a water bath shaker at 27±2°C for 24 h. The resultant supernatants following centrifugation (2500 g, 15 min) were assayed for total reducing sugars by DNS method (www.glue.umd.edu/~nsw/ench485/lab4a.htm). The sugars released were expressed as equivalent of glucose.

The optimum temperature of saccharification was determined by incubating the reaction mixture at different temperature ranging from 25 to 60°C. The optimum pH was determined by adjusting the pH of the reaction mixture from 3.5 to 6.5. The optimum substrate concentration was determined by preparing substrate suspensions 1 to 6%.

Yeast inoculum preparation

The yeast inoculum was prepared as described by Scholar and Benedikte (1999) and Suh et al. (2007). One gram of dry baker's yeast purchased from Kumasi central market was grown on Yeast Peptone Dextrose (YPD) agar plate at 30°C for 48 h to activate the yeast and check for contaminates. A loopful of the yeast colony was transferred from the agar plate into 100 ml of 5% YPD broth and incubated at room temperature on a shaker at 130 rpm for 48 h. Seven milliliters of the broth (from calculation) was centrifuged at 4500 rpm for 5 min. The supernatant was decanted, and the pellet was resuspended in 10 ml of sterile distilled water twice, centrifuged and the supernatant decanted. The pellet was resuspended in $1/10^{th}$ of 50 mM citrate buffer of working for each flask and was used as its inoculum.

Simultaneous saccharification and fermentation (SSF)

The SSF was carried out as described by Dowe and McMillian (2008). Six grams of corncobs powder, 1.0 g yeast extract and 2.0 g peptone were placed in each 250 ml Erlenmeyer flask, 50 mM citrate buffer, pH 5.0 added to form 80 ml suspensions and fitted with two-hole silicone bungs. The flasks and their accessories were autoclaved at 121°C for 20 min. After cooling to room temperature, 10 ml of the *A. niger* culture filtrate and 10 ml yeast inoculums were also introduced into each flask. For each flask sterile syringe was inserted into a tube fixed in one hole and fermentation lock device fixed to the tube in other hole of the bung. The flasks were incubated at room temperature on shaker at 110 rpm for 24 h. Five ml aliquots were drawn from the flasks at 4, 6, 8, 12 and 24 h with the inserted sterile syringes. The syringes were disposed off after every sampling. The aliquots were centrifuged at 10000 g for 10 min and the supernatant kept frozen for gas chromatographic analysis for ethanol.

Determination of ethanol concentration by gas chromatography

Ethanol concentration was determined using a Perkin Elmer, Autosystem XL, Gas Chromatograph (USA) equipped with a flame ionization detector (FID), coupled to a Yokogawa 3021 Pen recorder. A chromopak K 80/100 CRS column was used. The flow rate of the carrier gas, N_2, was 42 ml/min. H_2 and air were the fuel used. The oven temperature, injector temperature and detector temperature were 130, 200 and 200°C respectively. The injected volume was 1 µl and the retention time was 8.5 min. Identification and quantification was based on direct comparison of the gas chromatogram response to ethanol standards.

All the tests were laid in complete randomized design and each treatment was tested in triplicate. ANOVA analyses were carried out with Assistat 7.5 beta (Statistical Assistant, 2008). Graphs were plotted with Microsoft excel.

RESULTS AND DISCUSSION

A niger, the best cellulolytic fungi isolated from soil sampled from Ejura farms was cultured on CBB for 6 days on shaker at 120 rpm. Aliquots of 5 ml were sampled at 24 h interval and assayed for enzyme activities. Figure 1 illustrates the enzyme activities over the 144 h period.

There was progressive increase in enzyme activity from 24 to 144th h after incubation. Cellulase is an induced enzyme and its production increased with increase in fungal biomass over the incubation period and as simple sugar in the substrate diminished (Lynd et al., 2002).

Saccharification of corncob powder over the 24 h is illustrated in Figure 2. There was increase in saccharification from 0 to 24^{th} h. The increase was steeper up to 9^{th} h (phase 1) than from 9 to the 24^{th} h (phase 2). The slow down in rate for hydrolysis must be due to the action of the enzymes been slowed down by obstacles that interfere with their path or a loss in activity and/or processivity making them less effective (Yang et al., 2006).

The effects of substrate concentration, temperature and pH on release of reducing sugars were also carried out.

The rate of sacccharifficatiion is directly proportional to substrate concentration up to the optimal substrate concentration. This is because random collisions between the substrate and enzyme active sites happen more frequently. Beyond the optimum, the active sites are saturated so higher substrate concentration has no effect on rate of saccharification (www.canacad.ac.jp:3445/BiologyIBHL/541). Saccharification increased with substrate concentration as shown in Figure 3. There was

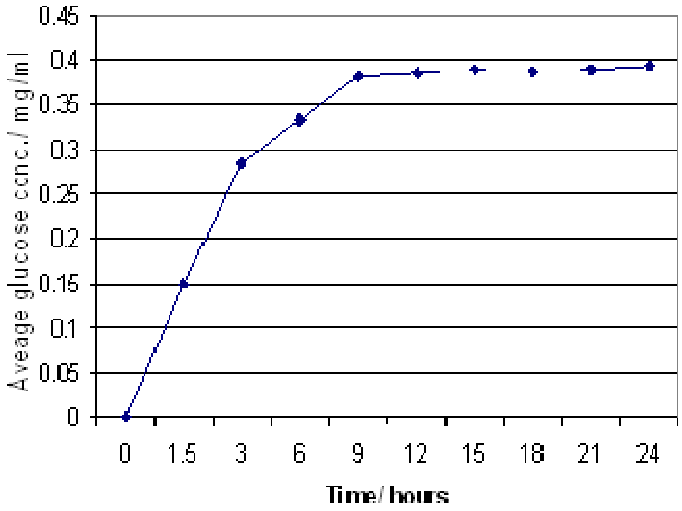

Figure 2. Time course for saccharification of corncob powder by *A. niger*.

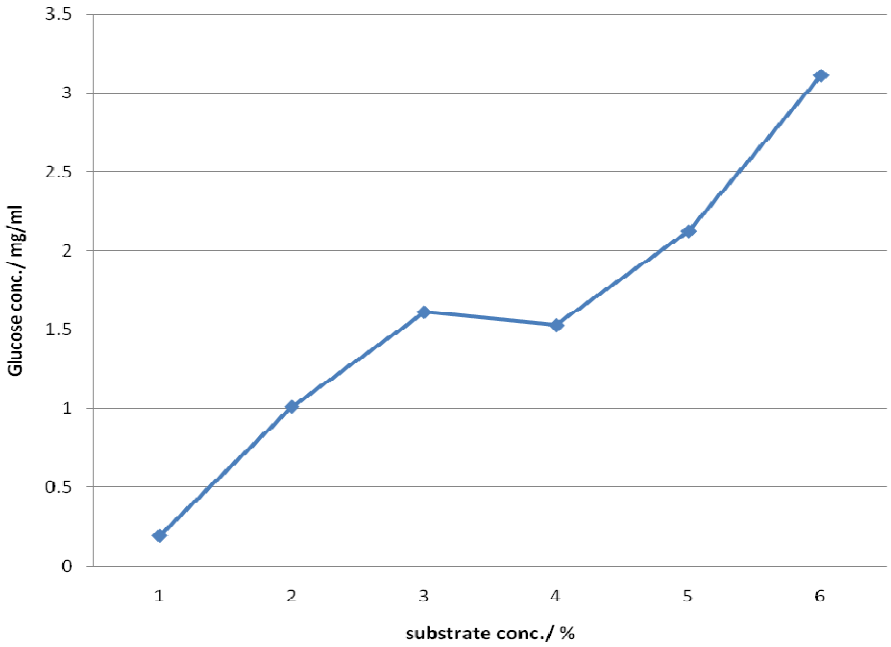

Figure 3. Effect of corncob powder substrate concentration on saccharification by *A. niger*.

increase in reducing sugars with increase in substrate concentration. The highest mean glucose concentration of 3.1105 mg/ml was recorded for substrate concentration of 6% and was significantly different ($p<0.05$). Substrate concentration of 1% released the least reducing sugars concentration. The glucose concentration for 6% substrate concentration was higher and significantly different from 5% substrate concentration, which suggests that 6% is or below optimum substrate concentration.

Temperature has complex effect on enzyme activity and hence saccharification. It affects the speed of molecules; the activation energy of the catalytic reaction and thermal stability of the enzyme. Generally saccharification increased with temperature up to the optimum after which it declines. The increase with temperature is due to corresponding increase in kinetic energy and the decline after the optimum due enzyme denaturation (Shuler and Kargi, 1997; Chaplin and Bucke, 1990a; www.blurtit.com/science/chemistry). Effect of temperature on saccharification is shown on Figure 4. Saccharification increased from 25°C to maximum at 40°C after which it decreased up to 60°C. Temperature (40°C) recorded the

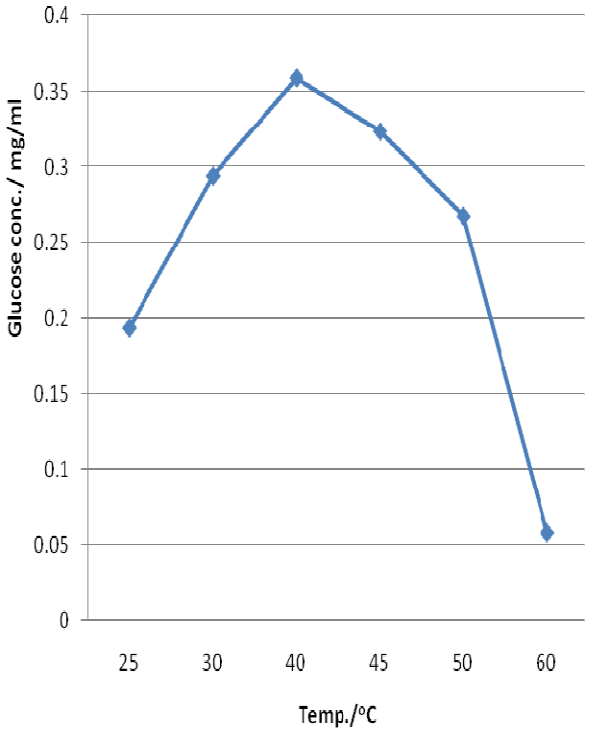

Figure 4. Effect of temperature on saccharification of corncob powder by *A. niger*.

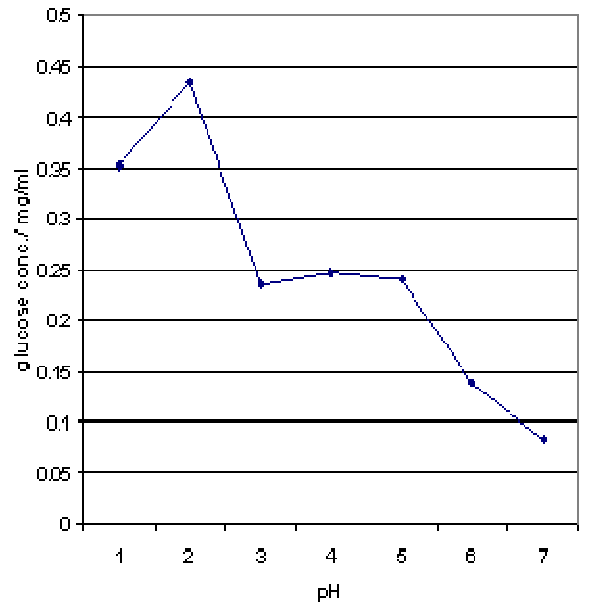

Figure 5. Effect of pH on saccharification of corncob powder by *A. niger*.

highest release of reducing sugars and it was significantly different ($p<0.05$). Thus the optimum temperature is 40°C, implying that the isolate is mesophile (Shuler and et al. (2007) reported of *A. niger* with optimum temperature of 40°C on coir waste and sawdust. Fewer studies of enzymes from mesophilic fungi are available (Baig et al., 2003). Saccharification was least significant ($p<0.05$) at 60°C. The decrease from 50 to 60°C was sharp due to the fact that enzyme denaturation is much faster (Shuler and Kargi, 1997; Chaplin and Bucke, 1990a; www.blurtit.com/science/chemistry).

The pH of a solution has several effects on the structure and activity of enzymes and hence saccharification. Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups vary, according to their acid dissociation constants, with the pH of the solution. Thus pH affects the reactivity of the catalytically active groups (Chaplin and Bucke, 1990b).

Figure 5 illustrates the effect of pH on release of reducing sugars from the substrate. Saccharification increased from pH 3.5 to a maximum of 4.0 after which it decreased up to 6.5. The highest saccharification which was significantly different ($p<0.05$) was recorded at pH 4.0. This makes the isolate acidophile (Nester et al., 2001). Saccharification was least significant ($p<0.05$) at pH 6.5, thus the optimum pH was 4.0. The relative low pH provides acidic condition which prevents bacterial contamination during fermentation (Olofsson et al., 2008).

Figure 6 shows the trend of ethanol production over 24 h period at 25°C and 110 rpm. SSF was carried out with 10 ml of *A. niger* filtrate. Ethanol concentrations of the fermentations were determined at 4, 6, 8, 12 and 24 h after inoculation. The mean ethanol concentration was 0.4326 g/l. There was increase in ethanol production over the period. There might be increase in saccharification over the period making glucose available to *S. cerevisae* for fermentation. Olofsson et al. (2008) reported that enzymatic hydrolysis of the solid fraction has a large control over the total rate of ethanol production in SSF. Also the ethanol concentration and other by-products in the broth might not have reached the inhibitory level of the yeast over the period. The highest concentration of 0.642 g/l was recorded at 24th h. Comparison to similar works in literature is difficult because ethanol concentration was not cited and they differ in either in type of pretreatment if any and detoxification, substrate concentration, fermentation strain, temperature or mode of operation which affects the final ethanol concentration as reported in work by Olofsson et al. (2008).

Conclusion

The optimization test has shown that the enzyme is mesophilic and acidophilic. The former makes it possible to find suitable temperature for SSF whilst the latter provides condition that controls contamination. Bioethanol was produced from corncobs. The highest mean ethanol concentration of 0.642 g/l from shake flask is appreciable.

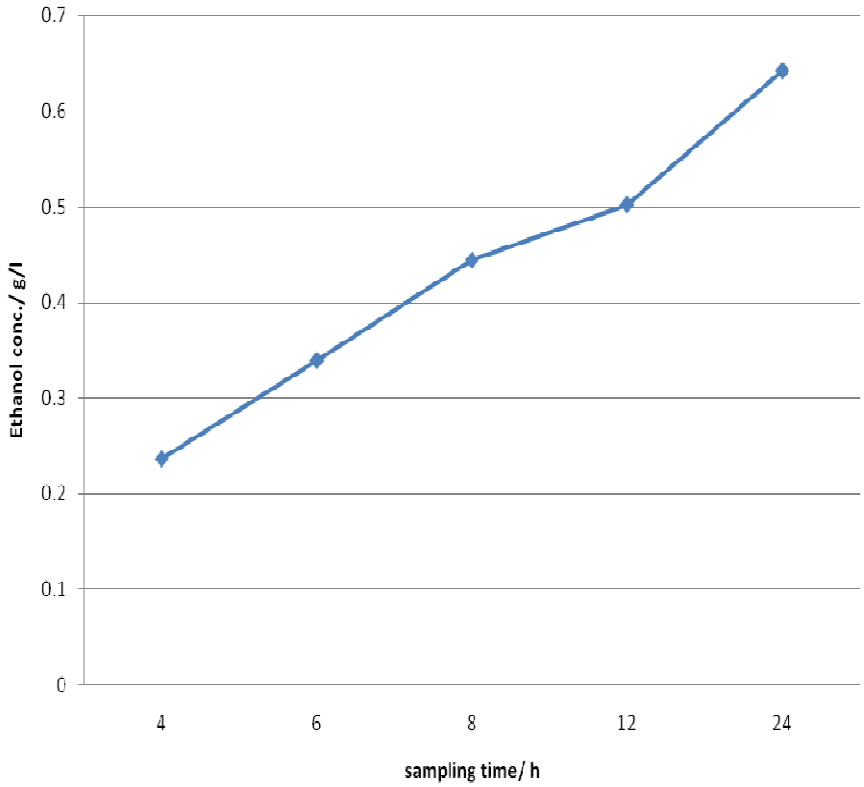

Figure 6. Time trend for ethanol concentration over 24 h fermentation period.

ACKNOWLEDGEMENT

We are grateful to the management of Ejura farms, Ejura for allowing us to take soil samples from their farms.

REFERENCES

Asante A (2004). Assessment of food import and food aid against support for agricultural development, the case of Ghana. Draft report for FAO Regional Office, p.10.

Baig MM, Mane VP, More DR, Shinde LP, Baig MI (2003). Utilization of banana agricultural waste: production of cellulases by soil fungi. J Environ. Biol. 24(2): 173-176.

Baig MV, Baig MLB, Baig MIA, Yasmeen M (2004). Saccharification of banana agrowaste by cellulolytic enzymes. Afr. J. Biotechnol. 3: 447-450.

Cao NJ, Krishnan MS, Du JX, Gong CS, Ho NWY, Chen ZD, Tsao GT (1996). Ethanol production from corn cob pretreated by ammonia steeping process using genetically engineered yeast. Biotechnol. Lett. 18(9): 1013-1018.

Chaplin M, Bucke C (1990a). Effect of pH and ionic strength on enzyme catalysis In: Enzyme technology. Cambridge University Press. www.lshu.ac.uk/biology/enztech/temperature.html.

Chaplin M, Bucke C (1990b). Effect of pH and ionic strength on enzyme catalysis In: Enzyme technology. Cambridge University Press. www.lshu.ac.uk/biology/enztech/ph.html.

Dowe N, McMillan J (2008). SSF Experimental protocols- lignocellulosic biomass hydrolysis and fermentation. Laboratory analytical procedure. NREL technical report. NREL/TP-510-42630.

Eken-Saracoglu N, Arslan Y (2000). Comparison of different pretreatments in ethanol fermentation using corn cob hemicellulosic hydrolysate with *Pichia stipitis* and *Candida shehatae*. Biotechnol. Lett. 22: 855-858.

Lynd LR, Weimer PJ, Zyl WH, Pretorius IS (2002). Microbial cellulose utilization: fundamentals and biotechnology. MMBR, 66(3): 506-577.

Nester EW, Anderson DG, Roberts CE, Pearsall NN, Nester MT (2001). Dynamics of prokaryotic growth In: Microbiology: A human perspective.3rd ed. McGraw-Hill, New York. pp. 87-108.

Ohgren K, Bengtsson O, Gorwa-Grauslund MF, Galbe M, Hahn-Hagerdal B, Zacchi G (2006). Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with *Saccharomyces cerevisae* TMB3400. J. Biotechnol. 126(4): 488-498.

Olofsson K, Bertilsson M, Liden G (2008). A short review on SSF-an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol. for Biofuels. 1(7): 1-14.

Rangkuti M, Djajanegara A (1983). The utilization of agricultural by-products and wastes (as animal feeds) in Indonesia. In: The use of organic residues in rural communities animal feeds in South East Asia. Proceedings, Workshop on Organic Residues in Rural Communities. Denpasar (Indonesia), 11 Dec 1979, pp.11-25.

Scholar J, Benedikte W (1999). Practical fermentation: a guide for schools and colleges. The society for general microbiology. www.ncbe.reading.ac.uk.

Shuler ML, Kargi F (1997). Bioprocess engineering: Basic concepts. 1st ed. Prentice-Hall Inc. New Jersey. pp. 49-78, 232-239.

Suh S, Zhang N, Nguyen N, Gross S, Blackwell M (2007). Lab manual for yeast study. Mycology lab. Louisiana state university.
Statistical assistance (2008). Assistat 7.5 beta. www.assistat.com.

Wyman CE, Spindler DD, Grohmann K (1992). Simultaneous saccharification and fermentation of several lignocellulosic feedstocks to fuel ethanol. Biomass Bioenergy, 3(5): 301-307.

Yang B, Willies DM, Wyman CE (2006). Changes in the enzymatic hydrolysis rate of avicel cellulose with conversion. Biotechnol.
Bioeng. 94(6): 1122-1128. www.blurtit.com/science/chemistry.
www.canacad.ac.jp:3445/BiologyIBHL/541.
www.glue.umd.edu/~nsw/ench485/lab4a.htm.