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# SSF Experimental Protocols — Lignocellulosic Biomass Hydrolysis and Fermentation

## Laboratory Analytical Procedure (LAP)

## Issue Date: 10/30/2001

N. Dowe and J. McMillan

*Technical Report*
NREL/TP-510-42630
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# SSF Experimental Protocols:
# Lignocellulosic Biomass Hydrolysis and Fermentation

## Laboratory Analytical Procedure

### 1. Introduction

1.1 Ethanol is a promising alternative fuel which can be produced biologically from a variety of waste materials such as paper products, corn fiber, sawmill waste, straw, and rice. Ethanol has been made from grapes, barley and potatoes for thousands of years. The production of ethanol from non-starch, lignocellulosic materials is, however, a fairly recent development. There are many ways to produce ethanol from lignocellulosic material. The method discussed here is known as simultaneous saccharification and fermentation (SSF). It utilizes cellulase enzyme to break down the cellulose and yeast to ferment the resulting glucose. The ethanol can be blended with gasoline or used neat in combustion engines. As a fuel, ethanol burns cleaner than gasoline, is completely renewable, and relatively less toxic to the environment.

### 2. Scope

2.1 The described protocols have been developed based on the personal experience of NREL researchers with biomass conversion and may be revised periodically. It is the sole responsibility of the user of the protocols to obtain updated versions from the NREL technical monitor. These procedures and their revisions **by no means** represent optimal conditions for the described experimentation and are proposed simply as a means of maintaining consistency. Furthermore, the results may vary depending on the expertise of the researcher and the quality of the materials employed in the studies.

2.2 This LAP consists of two separate sub-procedures. The first is "Hydrolysis of Lignocellulosic Biomass". The second is "Simultaneous Saccharification and Fermentation of Biomass". This procedure is intended to test a variety of lignocellulosic substrates and provide a consistent method for their evaluation among NREL subcontractors. **The procedures are intended for raw biomass substrates or washed, pretreated substrates only** i.e. pretreated substrates containing acetic acid, furfural, and/or other inhibitors of yeast metabolism must be extensively washed with water to remove these inhibitors prior to the experiments.

2.3 All analyses shall be performed according to the Biofuels Program Quality Assurance Plan (QAP).

### 3. References

3.1 NREL Biofuels Program BAT Team Laboratory Analytical Procedure #001, "Standard Method for Determination of Total Solids in Biomass".

3.2 NREL Biofuels Program BAT Team Laboratory Analytical Procedure #002, "Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography".

3.3 NREL Biofuels Program BAT Team Laboratory Analytical Procedure #006 "Measurement of Cellulase Activities".

3.4 NREL Biofuels Program BAT Team Laboratory Analytical Procedure #011, "Determination of Ethanol Concentration in Biomass to Ethanol Fermentation Supernatants by Gas Chromatography".

3.5 NREL Biofuels Program BAT Team Laboratory Analytical Procedure #013, "HPLC Analysis of Liquid Fractions of Process Samples for Soluble Sugars".

3.6 NREL Biofuels Program BAT Team Laboratory Analytical Procedure #015, "HPLC Analysis of the Liquid Fractions of Process Samples for Organic Acids, Glycerol, HMF, and Furfural".

3.7 T. Vinzant, L. Ponfick, N. Nagle, C. Ehrman, J. Reynolds, and M. Himmel, "SSF Comparison of Selected Woods from Southern Sawmills." 1994. Applied Biochemistry and Biotechnology Vol. 45/46 pp 611-626.

3.8 G. Philippidis, T.K. Smith and C. Wyman, "Study of the Enzymatic Hydrolysis of Cellulose for Production of Fuel Ethanol by Simultaneous Saccharification and Fermentation Process." 1993. Biotechnology and Bioengineering Vol. 41 pg 846-853.

## 4. Terminology

4.1 Saccharification (SAC) or hydrolysis of lignocellulosic biomass: the addition of enzyme to lignocellulosic biomass which results in the formation of oligomers, cellobiose and glucose. It is performed under sterile conditions and is also referred to as digestibility.

4.2 Simultaneous saccharification and fermentation (SSF): a method for producing ethanol from lignocellulosic biomass in which both enzymatic saccharification of cellulose by enzymes and fermentation of the resulting sugars by yeast occur at the same time in the same vessel.

## 5. Apparatus

5.1 In addition to the equipment described in LAPs 01, 02, 06, 011, 013, and 015 the following are required for this work.

5.2 An **autoclave** is necessary for the sterilization of media and flasks both prior to and after experiments.

5.3 A **laminar flow hood or biosafety cabinet** is necessary for sterile sampling.

5.4 A **-70$^{o}$C freezer** is necessary for the storage of frozen yeast cultures.

5.5 A bench top **centrifuge** is required for SSF inoculum and sample preparation.

5.6 A **shaker incubator** is necessary for the SSF's in order to keep the fermentations at 32$^{o}$C +/- 2$^{o}$C and 130 rpm.

5.7 **Bubble traps**, also called gas locks, $CO_2$ traps and water traps, are devices which prevent air from entering the shake flask and at the same time allow carbon dioxide to escape. They must be autoclavable. One such device is a rubber stopper through which a glass tube is inserted. A cotton plug is placed in the tube and the tube is connected to silicone tubing the end of which is submerged in a test tube with $H_2O$. The test tube is taped to the side of the flask. Another device that can be inserted into a rubber stopper is all glass and has a u-tube filled with water. The carbon

dioxide can bubble out, but the water prevents the air from entering. SSF's require a bubble trap.

5.8 An **analytical balance** is necessary for accurately measuring out biomass samples and preparing SSF flasks.

5.9 **Cell counting chamber slide** (for ex. hemocytometer) for yeast cell counts.

5.10 **Microscope** capable of 1000 times magnification.

5.11 Autoclavable **shake flasks**, **Morton closures** (metal caps), sterile **pipets** (disposable with tips that can be broken off conveniently to provide the wide opening needed for sampling SSF slurries) or 5 mL sterile pipet tips with the ends cut off.

5.12 **Convection oven**, with temperature control of 80 ± 3°C, **desiccator**, and **aluminum foil weighing dishes** for inoculum dry cell mass concentration measurements.

5.13 A **glucose analyzer** is suggested for rapid analysis of glucose. Manufacturers include Yellow Springs Instruments.

5.14 **IR oven** for determining percent dry solids.

5.15 **Spectrophotometer** for reading optical density at 600 nm.

## 6. Solutions, Media, and Stock Cultures

6.14 10X YP medium (liquid)

| | |
|---|---|
| Yeast extract | 100 g/L |
| Peptone | 200 g/L |

Autoclave for 30 minutes at 121$^{o}$C or filter sterilize through a 0.45 μm or smaller sterile filter into a sterile receiver.

Yeast Extract, Peptone, Dextrose (YPD) media is a common growth medium for yeast. It is rich in amino acids, vitamins, and minerals necessary for yeast growth and fermentation. This complex medium is supplied in excess so that nutrients are not a limiting factor.

6.15 YPD plates (solid medium)

| | |
|---|---|
| Yeast extract | 10 g/L |
| Peptone | 20 g/L |
| Dextrose (glucose) | 20 g/L |
| Agar | 20 g/L |

Heat to dissolve yeast extract, peptone, dextrose and agar in deionized (DI) water. Adjust to the desired final volume. Autoclave for 30 minutes at 121$^{o}$C. Cool agar medium in a water bath set at 50 $^{o}$C. Aseptically pour the plates. Let cool. Store plates inverted in the refrigerator.

6.16 YP with 5% dextrose medium (liquid)

| | |
|---|---|
| Yeast extract | 10 g/L |
| Peptone | 20 g/L |
| Dextrose (glucose) | 50 g/L |

Dissolve the components in DI water. Adjust to the desired final volume. Filter sterilize through a 0.45 μm or smaller sterile filter into a sterile receiver. You can also make this medium with 10X YP stock solution and sterile 500 g/L stock solution glucose.

| | |
|---|---|
| 10X YP stock solution | 100 mL/L |
| 500 g/L glucose stock solution | 100 mL/L |

Using sterile technique, pipet the appropriate amount of each stock solution into a sterile flask. Make up the remaining volume with sterile DI water.

6.17 1.0 M citrate buffer pH 4.8

| | |
|---|---|
| Citric acid monohydrate | 210 g |
| DI water | 750 mL |
| NaOH - add until pH equals 4.3 | 50 to 60 g |

Dilute to 1 L and check pH. If necessary add NaOH until the pH is 4.5. The 1 M stock can be frozen in aliquots to store. When the 1 M stock citrate buffer stock is diluted with water to 50 mM the pH should be 4.8. After diluting the citrate buffer check and adjust the pH if necessary to pH 4.8.

6.18 Frozen stock culture of *Saccharomyces cerevisiae* $D_5A$

50 mL sterile 40% glycerol
50 mL culture plate supplied by NREL

Autoclave or filter sterilize a 40% solution of glycerol in DI water. Let cool to room temperature. Prepare initial inoculum from the plate by transferring culture into 100 mL of YPD media in a sterile 250 mL flask. Incubate in a rotary shaker at 38$^{o}$C for 24 hours. Test for pH, glucose, and ethanol. The pH should be between 4.5 and 5.0, glucose should be between 0 and 5 g/L, and ethanol between 8 and 10 g/L. Observe the culture under the microscope for bacterial contamination and culture purity. Mix the glycerol and inoculum aseptically. Dispense one milliliter aliquots into sterile cryovials. Place in a -70$^{o}$C freezer. Each cryovial will have a standardized number of yeast cells per vial and subsequent SSF inocula should be prepared using a frozen vial..

Once every six months perform a viability check on the frozen stock. Thaw one vial. Vortex to resuspend cells. Perform a cell count with a hemacytometer under the microscope. Then, perform colony forming unit (CFU) tests using YPD plates by diluting the culture with sterile saline (0.89% NaCl solution) to obtain a spread plate cell count of 30-300 cells/plate.

The number of cells on the plate multiplied by the dilution factor gives you the CFU's/mL. Percent viability is CFUs/mL divided by the hemacytometer count/mL. For example 1.0 x $10^7$ CFU/mL and 1.0 x $10^8$ cells counted/mL gives a viability of 10%. Make a new frozen stock when the % viability drops below 50%. Maintain a control chart on the viability.

6.19 Liquid cellulase enzyme

Filter sterilize all enzyme upon arrival. Use non-cellulosic based filters such as the 0.45 mm VacuCap 90 from Gelman, product number 4624 made of polyethersulfone. Nylon and glass pre-filters are also suggested. Store enzyme in the refrigerator in sterile containers. The activity should be monitored using the LAP-006, "Measurement of Cellulase Activities". Cellulase activity values should be used to track enzyme stability over time. A control chart should be created for this purpose. However, the SSF and SAC loadings need to be based on a consistent and standardized number, that being the official NREL Filter Paper Units per milliliter number for that preparation. This standardization allows us to more easily compare SSF and SAC data obtained from different subcontracts.

## 7. ES&H Considerations and Hazards

7.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

7.2 Treat all biological growths with caution. Do not smell flasks as a method of checking for contamination. Any contaminant microorganism has the potential of being a health hazard.

7.3 Avoid breathing dusts of yeast extract by weighing and transferring the solid in a chemical fume hood. Yeast extract dust can coat the lungs and cause allergic reactions and/or breathing problems. Dust masks are recommended.

7.4 Autoclave all samples from SSF or SAC or inoculum prior to disposal. Treat unautoclaved glassware, etc., with a 1% Chlorox or 30% hydrogen peroxide solution to kill organisms prior to washing.

## 8. Procedure for the Hydrolysis of Lignocellulosic Biomass (SAC)

8.1 The goal of this procedure is to test pretreated or raw biomass substrates by determining the initial hydrolysis rate during saccharification (SAC) catalyzed by cellulase enzymes. Microorganisms are not employed in this experiment.

8.2 The pretreated substrate must be washed to remove the residual glucose and inhibitors before loading the flasks. Generally, it takes 12 wash volumes to remove the glucose to less than 0.1 g/L glucose. A typical way of washing the solids is to centrifuge in two weight volumes. Decant the water and repeat five more times. Monitor the glucose in the wash water with the YSI analyzer until the glucose falls below 0.1 g/L.

8.3 Determine the total solids of the washed substrate using the IR oven. This value will be used to calculate the amount of washed solids to add to each flask.

8.4 Load each SAC flask with 1% w/w cellulose, 1% w/v yeast extract. 2% w/v peptone, 0.05 M citrate buffer (pH 4.8) and cellulase enzyme at the desired loading. Shake flasks should have a 2:5 medium to flask volume ratio and a stopper or

Morton closure. **It is recommended that all SAC flasks have a 1% w/w effective cellulose content but other cellulose loadings can be tested.** Lower cellulose loadings minimize feedback inhibition from the glucose released.

8.5 Based on the washed biomass moisture content (LAP-001) and cellulose content (LAP-002) data, determine the quantity of biomass needed. **Do not dry pretreated biomass that will be enzymatically saccharified.** Once dry the pores of the biomass may irreversibily collapse**.** Weigh the correct amount of biomass and DI water into the flask. Record the actual amount of substrate weighed into each flask to at least the nearest one-hundredth of a gram. Depending on the amount of biomass added, the volume of enzyme might need to be adjusted.

Example: 0.5263 g of alpha-cellulose is the weight needed based on the calculation for a 50 gram working weight and 95% total solids. 4.167 grams is the weight needed of a pretreated wood with a 60% cellulose content (LAP-002) and a 20% total solids content (LAP-001) in a final working weight of 50 grams.

8.6 Add the cap, autoclave tape, label, etc. to the flask. Weigh the filled and capped flask to the nearest one-hundredth of a gram and record this weight as pre-autoclave. Repeat for each flask.

8.7 Since all the work up to this point has not been done aseptically, autoclave the flasks as soon as possible. Autoclave at 121$^{o}$C for 30 minutes. Let flasks cool to room temperature. Re-weigh each flask to the nearest one-hundredth of a gram and add back lost weight as mL of sterile DI water.

8.8 Based on the filter paper activity of the cellulase enzyme (FPU/mL), the desired enzyme loading and the amount of cellulose added, calculate the amount of enzyme needed for each flask Refer to LAP 006 for measuring cellulase activities. **Use the official NREL FPU/mL for the reference cellulase enzyme preparation**.

Enzyme loading is the most critical factor affecting rates and yields. More accurate addition of the enzyme can be obtained if it is diluted in YP and citrate buffer. Do not store the enzyme in diluted form for over one day.

8.9 Each experiment should include an appropriate control using a reference pretreated substrate and reference cellulase enzyme loaded at a standard level. If a reference pretreated biomass substrate is not available, use alpha-cellulose or another commercially available form of cellulose. (NREL's current reference pretreated substrate is a washed pretreated yellow poplar sawdust from PDU run # P980302SD condition #7 or #2 and the reference cellulase enzyme preparation is commercial CPN cellulase loaded at a level of 10 or 15 FPU/g cellulose.) All SACs should be performed in duplicate. Control charts should be set up to plot the cellulose conversion (or glucose production) yields from a standardized final time point (typically 168 hours).

8.10 An example of a SAC flask recipe is as follows:

| | |
|---|---|
| 4.167 g | pretreated wood (1% w/w cellulose, substrate has a 60% cellulose content and a 20% total solids content) |
| 0.23 mL | NREL-supplied cellulase enzyme 25 FPU/g cellulose loading (55 FPU/mL) |
| 5.0 mL | 10X YP solution |
| 2.5 mL | 1 M citrate buffer (pH 4.8) |
| 38.13 mL | DI water (50 - 0.23- 5 – 2.5-4.167) |

50.0 grams total (working weight)

8.11 In a laminar flow hood aseptically add the YP, citrate and enzyme mixture.

8.12 Mix the flasks by swirling. Aseptically take a time zero slurry sample. (see step 8.15).

8.13 Incubate the flasks in a rotary shaker at 130 rpm (agitation for other vessels will depend on the working and total volume of the SAC flask. The process being evaluated, including the cellulase enzyme(s) being used, should guide the selection of the incubation temperature. When using CPN enzyme, it is not recommended to use incubation temperatures above 50$^{o}$C.

8.14 Repeat steps 8.11-8.13 with the other flasks. Start each flask individually and note the time of completion for first and last flasks.

8.15 Appropriate sampling times are 0, 3, 6, 24, 48, 72, and 168 hours. Break off the end of a sterile 5 mL pipet and take 3 mL slurry samples aseptically. Store in capped tubes/vials. Place the samples on ice until all the samples of that specific time point have been collected. Place the capped tubes/vials in a boiling water bath for exactly 5 minutes to inactivate the enzyme. Chill on ice.

8.16 Centrifuge and filter to remove denatured enzyme and lignocellulosic biomass. Determine the amount of glucose present in each supernatant sample by YSI or HPLC (LAP-013). Measure the concentration of cellobiose by HPLC (LAP-013) for at least 3 of the time-points. If the analysis will be done later, freeze the supernatant in sealed HPLC glass vials.

8.17 For the **last time point** make samples for YSI and HPLC as in step 8.15-8.16. In addition, streak a sample from each flask or vessel on a YPD plate to check for contamination by any microorganism. Observe, under the microscope, a sample of the slurry for the presence of contaminants. Report the final slurry pH of each flask or vessel.

8.18 All test tubes and flasks containing cultures or samples should be autoclaved prior to disposal. All other items (e.g. pipettes, syringes) that come into contact with the culture should be placed in containers of biocidal solutions before sterilization and reuse or disposal. Contaminated pipette tips and microcentrifuge tubes should be autoclaved before disposal.

8.19 Autoclave the residual hydrolysis material, making sure that the stoppers are loose enough to allow ventilation. Ensure that fermentors can ventilate freely. Sterilized liquids may be discharged down the drain after the solids are removed and thrown in the trash.

8.20 Calculate the glucose yield as % of the theoretical yield (% digestibility) by using the following formula:

$$\%Yield = \frac{[Glucose] + 1.053\,[Cellobiose]}{1.111\,f\,[Biomass]}\,X\,100\%$$

where:

| | |
|---|---|
| [*Glucose*] | Residual glucose concentration (g/L) (Make sure any glucose carried over with the enzyme is subtracted from the residual glucose) |
| [*Cellobiose*] | Residual cellobiose concentration (g/L) |
| [*Biomass*] | Dry biomass concentration at the beginning of the fermentation (g/L) |
| *f* | Cellulose fraction in dry biomass (g/g) |

The multiplication factor, 1.053, converts cellobiose to equivalent glucose.

8.21 Graph and/or tabulate the collected data (glucose and cellobiose concentration vs. time) for each experiment.

8.22 Quality Control

8.22.1 *Reported significant figures:* Report % digestibility to one decimal place.

8.22.2 *Replicates:* At least duplicate flasks.

8.22.3 *Blank:* Include a flask with only the medium and enzyme to determine how much, if any, glucose comes from the enzyme and medium.

8.22.4 *Relative percent difference criteria:* 5% digestibility within one set of flasks run at the same time, by the same person, in the same shaker, with the same analytical instrument.

8.22.5 *Quality Assurance Standard:* Use a well-characterized standard material (i.e., one which has known digestibility characteristics) to confirm (or quantify) the degree of experiment-to-experiment reproducibility. Currently NREL is using washed pretreated yellow poplar sawdust from P980302SD run #7 or #2 with CPN. Sigma alpha-cellulose can also be used as a control. The moisture content of alpha-cellulose needs to be measured every 3 months because it is hydroscopic.

8.22.6 *Calibration verification standard:* None.

8.22.7 *Sample size:* See flask preparation instructions.

8.22.8 *Sample storage:* Store liquor samples in the freezer if the analysis is not going to be carried out within a day.

8.22.9 *Standard storage:* Pretreated sawdust is refrigerated. Washed pretreated sawdust can also be stored for several months at 4$^{o}$C. A dry solids analysis should be done on stored sawdust prior to use. Alpha-cellulose is considered dry biomass and can be stored at room temperature.

8.22.10 *Standard preparation:* None.

8.22.11 *Definition of a batch*: Flasks started at the same time with one set of alpha-cellulose controls.

8.22.12 *Control charts:* % digestibility and rate of digestibility from controls. See 8.22.14.

8.22.13 *Sterility verification:* In all flasks, the final pH should be 5.0 ± 0.2, no microbes should be detected by microscope or plate checks. Flasks that did not remain sterile must be repeated.

8.22.14 *Enzyme activity:* Measure enzyme activity using LAP-006, (Measurement of Cellulase Activities) every 6 months. Include the 1:20 diluted CPN as a positive control when measuring FPU activity. Tabulate and make a control chart. Again, use the official NREL number for the SAC.

## 9. Procedure for Simultaneous Saccharification and Fermentation (SSF)

9.1 Inoculum preparation

9.1.1 The goal of this procedure is to prepare a seed culture for SSF. An aerobic fermentation of glucose is used to produce yeast cell mass.

9.1.2 Prepare the sterile inoculum flask with YP and 5% w/v glucose. Keep a 1:5 working volume to total flask volume ratio. Use a baffled flask with a Morten cap.

9.1.3 Inoculate the YPD flask with one thawed stock vial of *Saccharomyces cerevisiae* $D_5A$. Incubate for 10-14 hours in a rotary incubator shaker operating at the SSF operating temperature and 130 rpm. The required incubation time depends on the incubation temperature, with shorter incubation times permissible at higher incubation temperatures.

9.1.4 Before transferring, check microscopically for contamination and analyze for residual glucose concentration. The culture can be transferred once the glucose falls below 2 g/L. Optimally, there should be some residual glucose to ensure cells are still in the growth phase. Take an optical density (O.D.) at 600 nm. Keep the O.D. value below 0.800 by diluting with water when appropriate. Multiply O.D. reading by any dilution factor. Check pH and perform DCM (dry cell mass) analysis (optional). Prepare samples from the inoculum flask for HPLC and GC analysis before transferring to the SSF flasks.

9.1.5 Create an inoculum control chart with the final O.D., pH, ethanol, DCM (optional), and glucose concentrations for each inoculum.

9.1.6 At time of transfer determine the amount of culture needed to inoculate

$$Total\ vol.\ from\ inoc.\ flask\ (mL) = \left( \frac{SSF\ vol.\ (mL) \times 0.5}{inoc.\ O.D.\ t_f} \right) \times \left( number\ of\ SSF\ flasks + 1 \right)$$

your SSF flasks for a starting O.D. of 0.5.

Spin the inoculum down in a table top centrifuge (an example of conditions is 4500 rpm for 5 minutes). Decant supernatant. Resuspend in sterile deionized water and spin again. Resuspend cells in $1/10^{th}$ of the volume of water. This will be the inoculum for the SSF flasks concentrated 10X.

9.2 Determine the Inoculum Dry Cell Mass Concentration (optional)

9.2.1 The goal is to measure the dry cell mass concentration from the inoculum.

9.2.2 Dry aluminum dishes in the oven at 80$^{o}$C overnight.

9.2.3 Cool the dishes in a desiccator for 30 minutes.

9.2.4 Record each dish weight to four decimal places using an analytical balance.

9.2.5 Using a sterile pipet, take a 10-mL inoculum sample, centrifuge, and wash the cell pellet twice with 10 mL of DI water (2 volume wash). After the second wash and centrifuge cycle, resuspend the pellet in 5 mL of DI water.

9.2.6 Transfer pellet by repeated vortex washes with DI water to a weighed dish.

9.2.7 Dry the dishes and cells in the oven at 80$^{o}$C overnight.

9.2.8 Cool the dishes in the desiccator for 30 minutes.

9.2.9 Record the weight of the dishes plus dried cells.

9.2.10 Calculate the dry cell mass concentration of the inoculum in *g/L* by using the following formula:

$$DCM = \frac{weight\ of\ dish\ plus\ dried\ cells - weight\ of\ dish}{0.01\ L}$$

9.3 Procedure for the Simultaneous Saccharification and Fermentation of Biomass

9.3.1 The goal of this procedure is to assess the conversion of lignocellulosic biomass into ethanol using the SSF process. This procedure is almost identical to the saccharification protocol differing only in the following ways: (1) **yeast** is used to convert the glucose into ethanol, (2) cellulose content is higher at **6% w/w,** (3) **bubble traps** are used to maintain anaerobic conditions and (4) carbon dioxide, ethanol, glycerol, lactic and acetic acid are formed and residual glucose and cellobiose levels remain low.

9.3.2. The pretreated substrate must be washed to remove the residual glucose and inhibitors before loading the flasks. Generally, it takes 12 wash volumes to remove the glucose to less than 0.1 g/L glucose. A typical method of washing the solids is to weigh the wet (but dewatered) solids, then add water at twice the weight of the wet solids. Mix the water and solids well, then centrifuge at settings that will create a packed pellet. Decant the water and repeat five more times. Monitor the glucose in the wash water with the YSI analyzer until the glucose falls below 0.1 g/L.

9.3.3 Determine the total solids of the washed substrate using the IR oven. This value will be used to calculate the amount of washed solids to add to each flask.

9.3.4 Load each SSF flask with 6% w/w cellulose, 1% w/v yeast extract, 2% w/v peptone, 0.05 M citrate buffer (pH 4.8), cellulase enzyme at the desired enzyme loading and $D_5A$ inoculum (starting O.D. 0.5). . Shake flasks should have a 2:5 culture volume to flask volume ratio and should be equipped with **water traps**. If the flasks do not mix well at 6% w/w

cellulose then lower the cellulose to a point where there is good mixing (3-5% w/w cellulose). A spreadsheet is available to simply the calculation of flask addition amounts.

9.3.5 Determine the amount of biomass needed for each SSF flask based on the biomass moisture and cellulose content. **Do not dry pretreated biomass that will be enzymatically saccharified.** Once dry, the pores within the biomass may irreversibly collapse. Weigh the correct amount of biomass and DI water into the flask. Record the actual amount of substrate weighed into each flask to at least the nearest one-hundredth of a gram. Depending on the amount of biomass added, the volume of enzyme might need to be adjusted. Use the spreadsheet for calculating the enzyme loading.

9.3.6 Add a water trap, autoclave tape, label etc. to flask (do not place any water in the traps at this time.) Weigh the whole flask assembly to the nearest one-hundredth of a gram and record this weight as pre-autoclave. Repeat for each flask.

9.3.7 Since all the work up to this point has not been done aseptically, autoclave the flasks or vessels as soon as possible. Autoclave at 121$^{o}$C for 30 minutes. Increase autoclave times to accommodate large loads or large volumes of media. Make sure the vessels can ventilate freely. After autoclaving, let the flasks cool to room temperature. Then, re-weigh each flask assembly to the nearest one-hundredth of a gram and add back lost weight as mL of sterile DI water.

9.3.8 Based on the filter paper activity of the cellulase enzyme (FPU/mL), the desired enzyme loading and the amount of cellulose added, calculate the amount of enzyme needed for each flask. Refer to LAP 006 for measuring cellulase activities. **Use the official NREL FPU/mL for the NREL reference enzyme preparation**. Enzyme loading is the most critical factor affecting rates and yields. More accurate addition of concentrated commercial enzymes can be obtained if the enzyme is first diluted in the YP and citrate buffer before adding to the flasks.

9.3.9 Each experiment should include an appropriate control using a reference pretreated substrate and reference cellulase enzyme loaded at a standard level. If a reference pretreated biomass substrate is not available, use alpha-cellulose or another commercially available form of cellulose. (NREL's current reference pretreated substrate is a washed pretreated yellow poplar sawdust from PDU run # P980302SD condition #7 or #2 and the reference cellulase enzyme preparation is commercial CPN cellulase loaded at a level of 10 or 15 FPU/g cellulose.) All SSF should be performed in duplicate. A control chart should be set up to plot the cellulose conversion (or ethanol production) yields from a standardized final time point (typically 168 hours).

9.3.10 Often, commercial enzyme preparations contain stabilizers like sucrose that can be used by the yeast to produce ethanol. To determine how much ethanol is made from just the enzyme and medium, a control with each enzyme preparation to be tested and all the medium components except the pretreated substrate is included. Usually the flask is loaded with the

highest amount of enzyme used in the tests. The amount of ethanol produced per mL of enzyme is determined then subtracted from the ethanol produced in the SSF flasks to estimate the amount of ethanol produced from the hydrolyzed cellulose.

9.3.11 Below is an example of how an excel spreadsheet can be used to calculate flask components and volume additions.

| Description | Flask Number | Sample Size (g) | Desired Glucan Loading (%) | Desired Glucan Loading (g) | Total Solids (%) | Glucan (dwt %) | Sample Needed (g) | Actual Sample Loaded (g) | Actual Glucan Loaded (g) | Desired Cellulase Loading (FPU/g) |
|---|---|---|---|---|---|---|---|---|---|---|
| #2 Solids 5 IU's | 1 | 100 | 6.00 | 6.00 | 24.90 | 60.73 | 39.68 | 39.68 | 6.00 | 5.00 |
| #2 Solids 5 IU's | 2 | 100 | 6.00 | 6.00 | 24.90 | 60.73 | 39.68 | 39.68 | 6.00 | 5.00 |
| #2 Solids 10 IU's | 3 | 100 | 6.00 | 6.00 | 24.90 | 60.73 | 39.68 | 39.68 | 6.00 | 10.00 |
| #2 Solids 10 IU's | 4 | 100 | 6.00 | 6.00 | 24.90 | 60.73 | 39.68 | 39.68 | 6.00 | 10.00 |
| #2 Solids 15 Iu's | 5 | 100 | 6.00 | 6.00 | 24.90 | 60.73 | 39.68 | 39.68 | 6.00 | 15.00 |
| #2 Solids 15 IU's | 6 | 100 | 6.00 | 6.00 | 24.90 | 60.73 | 39.68 | 39.68 | 6.00 | 15.00 |
| #2 Solids 20 IU's | 7 | 100 | 6.00 | 6.00 | 24.90 | 60.73 | 39.68 | 39.68 | 6.00 | 20.00 |
| #2 Solids 20 IU's | 8 | 50.00 | 6.00 | 3.00 | 24.90 | 60.73 | 19.84 | 19.84 | 3.00 | 20.00 |
| #2 Solids 25 IU's | 9 | 100 | 6.00 | 6.00 | 24.90 | 60.73 | 39.68 | 39.68 | 6.00 | 25.00 |
| #2 Solids 25 IU's | 10 | 100 | 6.00 | 6.00 | 24.90 | 60.73 | 39.68 | 39.68 | 6.00 | 25.00 |
| #7 Solids 10IU's | 11 | 100 | 6.00 | 6.00 | 24.31 | 60.84 | 40.57 | 40.57 | 6.00 | 10.00 |
| #7 Solids 25 IU's | 12 | 100 | 6.00 | 6.00 | 24.31 | 60.84 | 40.57 | 40.57 | 6.00 | 25.00 |
| Control | 13 | 100 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 25.00 |

| Description | Flask Number | Cellulase Added (mL) | 1M Citrate Buffer (mL) | YP 10x Stock (mL) | Inoculum 10x (mL) | Water to Add (mL) | Weight Before Autoclave (g) | Error In Water Volume (+ / -)(g) | Weight After Autoclave (g) | Water Correction (g) |
|---|---|---|---|---|---|---|---|---|---|---|
| #2 Solids 5 IU's | 1 | 0.55 | 5.00 | 10.00 | 1.00 | 43.78 | 244.79 | 0.06 | 241.87 | 2.86 |
| #2 Solids 5 IU's | 2 | 0.55 | 5.00 | 10.00 | 1.00 | 43.78 | 236.41 | 0.00 | 233.25 | 3.16 |
| #2 Solids 10 IU's | 3 | 1.09 | 5.00 | 10.00 | 1.00 | 43.23 | 228.90 | 0.10 | 225.65 | 3.15 |
| #2 Solids 10 IU's | 4 | 1.09 | 5.00 | 10.00 | 1.00 | 43.23 | 263.45 | 0.04 | 259.86 | 3.55 |
| #2 Solids 15 Iu's | 5 | 1.64 | 5.00 | 10.00 | 1.00 | 42.69 | 254.12 | 1.62 | 250.84 | 1.66 |
| #2 Solids 15 IU's | 6 | 1.64 | 5.00 | 10.00 | 1.00 | 42.69 | 253.68 | 0.12 | 250.60 | 2.96 |
| #2 Solids 20 IU's | 7 | 2.18 | 5.00 | 10.00 | 1.00 | 42.14 | 218.42 | 0.00 | 215.15 | 3.27 |
| #2 Solids 20 IU's | 8 | 1.09 | 2.50 | 5.00 | 0.50 | 21.07 | 223.32 | 0.06 | 221.03 | 2.23 |
| #2 Solids 25 IU's | 9 | 2.73 | 5.00 | 10.00 | 1.00 | 41.59 | 247.48 | -0.03 | 244.27 | 3.24 |
| #2 Solids 25 IU's | 10 | 2.73 | 5.00 | 10.00 | 1.00 | 41.59 | 247.71 | 0.04 | 244.90 | 2.77 |
| #7 Solids 10IU's | 11 | 1.09 | 5.00 | 10.00 | 1.00 | 42.34 | 241.05 | 0.21 | 237.82 | 3.02 |
| #7 Solids 25 IU's | 12 | 2.73 | 5.00 | 10.00 | 1.00 | 40.70 | 245.92 | 0.30 | 242.72 | 2.90 |
| Control | 13 | 2.73 | 5.00 | 10.00 | 1.00 | 81.27 | 226.85 | 0.00 | 222.39 | 4.46 |

9.3.12 In a laminar flow hood aseptically add to the first flask or vessel:

(a) Water lost from autoclaving the flasks.

(b) YP and citrate buffer if enzyme is not going to be mixed with medium components

(c) 10X concentrated inoculum to achieve O.D. of 0.5 @ 600nm

(d) **Final addition is the enzyme, either alone or diluted, in the YP and citrate buffer.**

9.3.13 Mix contents well. Break off the end of a 5 mL pipet and take at least a 4 mL sample of the slurry. Store in capped tubes/vials and chill on ice. Take 0.5 mL for a pH sample. Centrifuge, collect and filter the supernatant from the rest of the sample. Analyze for glucose and cellobiose (LAP-013), glycerol, lactic acid, and acetic acid (LAP-015) by HPLC, and ethanol (LAP-011) by GC. YSI glucose readings are also taken at each sample point. If the analysis will be done later, freeze the filtered supernatant in HPLC/GC glass vials.

9.3.14 Add water to the water/bubble traps of the flasks and incubate the flasks in a shaker at 130 rpm (agitation for other vessels will depend on the volume of the SSF). The incubation temperature will depend upon your process. However, the upper temperature limit for $D_5A$ yeast in an SSF is 38$^o$C. (Note: NREL has often operated the SSF tests at 32$^o$C to estimate the level of integrated process performance that would be achieved using an adapted variant of *Zymomonas mobilis* exhibiting a temperature optimum of 32$^o$C.)

9.3.15 Repeat with the other flasks. Start each flask separately and record the time of completion for first and last flasks.

9.3.16 Appropriate sampling times are 0, 4-8, 24, 48, 72, 96, 120, 144, and 168 hours. Break off the end of a 5 mL pipet and take at least a 4 mL sample of the slurry. Store in capped tubes/vials and chill on ice. Take 0.5 mL of the sample for pH analysis. Centrifuge, collect and filter the supernatant from the rest of the sample. Analyze for glucose and cellobiose (LAP-013), glycerol, lactic acid, and acetic acid (LAP-015) by HPLC, and ethanol (LAP-011) by GC. YSI glucose readings are also taken at each sample point. If the analysis will be done later, freeze the filtered supernatant in HPLC/GC glass vials.

9.3.17 For the **last time point** make samples for YSI, GC and HPLC. In addition, streak a sample from each SSF flask or vessel on a YPD plate. Plates should show viable yeast with no contaminant organisms. Observe slurry under the microscope for presence of biomass fibers and yeast cells. The presence of foreign organisms is cause for repeating the SSF. Read and record the slurry pH of each flask or vessel. Final pH should be 5.0 $\pm$ 0.7. Drop in pH to less than 4.0 is cause for repeating the SSF. If possible, perform compositional analysis (LAP-002) of the SSF residue and close mass balance.

9.3.18 All test tubes and flasks containing cultures or samples should be autoclaved prior to disposal. All other items (e.g. pipettes, syringes) that

come into contact with the culture should be placed in biocide solution before washing, reuse and/or disposal.

9.3.19 Autoclave the residual SSF material, making sure that the bubble traps are dry, so that the flasks can ventilate. For fermentors, ensure that they can ventilate freely. Sterilized liquids may be discharged down the drain after the solids (biomass, dead yeast cells, and denatured enzyme) are removed and thrown in the trash.

9.3.20 Calculate the % theoretical ethanol yield or % cellulose conversion by using the following formula:

$$\%\ Cellulose\ \ Conversion\ = \frac{[EtOH\ \ ]_f - [EtOH\ \ ]_o}{0.51\left(f\ \ [Biomass]\ \ 1.111\right)} X\ 100\%$$

where:

| | |
|---|---|
| $[EtOH]_f$ | Ethanol concentration at the end of the fermentation (g/L) minus any ethanol produced from the enzyme and medium |
| $[EtOH]_o$ | Ethanol concentration at the beginning of the fermentation (g/L) which should be zero |
| [*Biomass*] | Dry biomass concentration at the beginning of the fermentation (g/L) |
| *f* | Cellulose fraction of dry biomass (g/g) |
| *0.51* | Conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast |
| *1.111* | Converts cellulose to equivalent glucose |

9.3.21 Report, graph, and/or tabulate information about each experiment including:

- Any observations about the experiment (e.g. foaming, color, etc.).
- Any deviations from the standard protocol.
- Rate of % theoretical cellulose conversion
- 7-day cellulose conversion as a function of enzyme loading
- If possible, residual concentration of glycerol and other metabolites.
- Final pH of the SSF.
- Contamination assessment and description of the morphology (shape, color, size, and texture) of the colonies appearing on the plates, including *S. cerevisiae* $D_5A$.

9.4 Quality Control

9.4.1 *Reported significant figures:* Report % theoretical yield to one decimal place.

9.4.2 *Replicates:* At least duplicate flasks.

9.4.3 *Blank:* Enzyme control flasks to determine the amount of ethanol produced from the various enzyme preparations.

9.4.4 *Relative percent difference criteria:* 5% yield within one set of flasks run at the same time, by the same person, in the same shaker, with the same analytical instrument.

9.4.5 *Quality Assurance Standard:* Use a well-characterized standard material (i.e., one which has known digestibility characteristics) to confirm (or quantify) the degree of experiment-to-experiment reproducibility. Currently NREL is using washed pretreated yellow poplar sawdust from P980302SD run #7 or #2 with CPN at 10 or 15 IU's. Sigma alpha-cellulose can also be used as a control. The moisture content of alpha-cellulose needs to be measured every 3 months because it is hydroscopic.

9.4.6 *Calibration verification standard:* None.

9.4.7 *Sample size:* Not applicable.

9.4.8 *Sample storage:* Store liquor samples in the freezer if the analysis is not going to be carried out within a day.

9.4.9 *Standard storage:* Pretreated sawdust is refrigerated. Washed pretreated sawdust can also be stored for several months at 4 $^{o}$C. A dry solids analysis should be done on stored sawdust prior to use. Sigma alpha-cellulose can be stored at room temperature.

9.4.10 *Standard preparation:* None.

9.4.11 *Definition of a batch*: Flasks started at the same time by the same researcher.

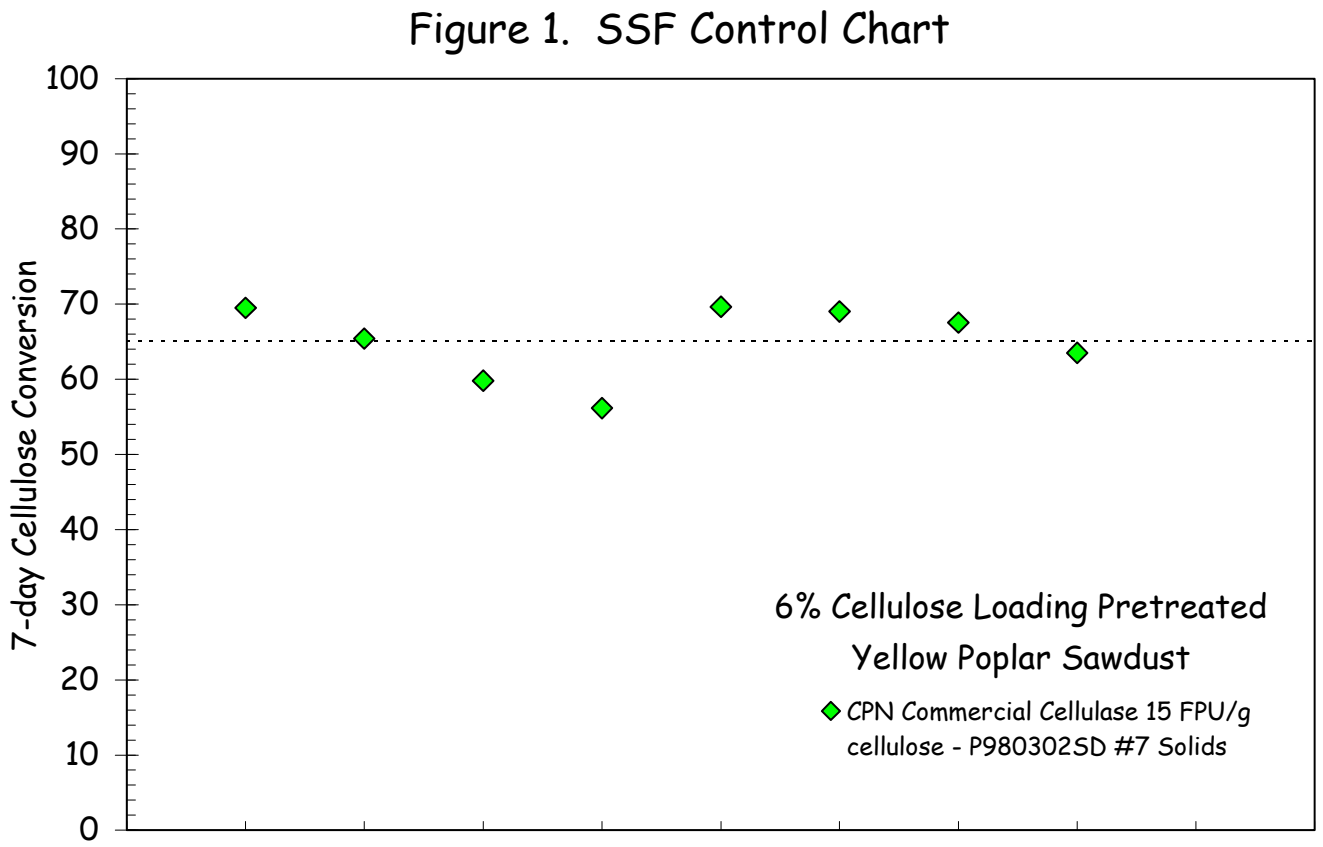
9.4.12 *Control charts:* Make a control chart of 7-day cellulose conversion as a function of CPN enzyme loading for the washed pretreated yellow poplar and compare to database results. Chart the rate of digestibility over the seven days at the targeted CPN enzyme loading and also compare to database results. If alpha-cellulose is to be used, create a separate control chart of the SSF results. Make inoculum control chart, enzyme activity control chart, frozen stock viability control chart.

9.4.13 *Other:* Verification of a pure $D_5A$ culture--all flasks--final pH should be 5.0 +/- 0.7, no microbes other than $D_5A$ should be detected by microscope or plate checks. Contaminated flasks need to be repeated.

9.4.14 *Enzyme activity:* Measure enzyme activity using LAP-006, (Measurement of Cellulase Activities) every 6 months. Include the 1:20 diluted CPN as a positive control when measuring FPU activity. Tabulate and make a control chart. Again, use the official NREL number for the SSF. Enzyme loading is the most critical factor in rates and yields of ethanol production via SSF.

## Appendix 1

Control chart and data for NREL's reference pretreated substrate, washed pretreated yellow poplar sawdust from PDU run # P980302SD condition #7 and CPN commercial cellulase loaded at 15 FPU/g cellulose.

**Table 1. Compilation of SSF Data - CPN at 15 FPU/g cellulose - Pretreated Yellow Poplar from P980302SD Condition #7**

| Exp. # | Flask # | Enzyme Type | Wood Solids Loading (% w/w cellulosic solids) | FPU Loading (IU/g cellulose) | Soluble Protein (g/L >30kD) | Protein Loading (mg>30kD/g cellulose) | Source | Beta-G | Processing | Enzyme lot or Solids Lot | % ethanol yield (cellulose digestibility) 7 Day % Theoret. EtOH Yield | Average 7 Day % Theoret. EtOH Yield | Std. Dev. 7 Day % Theoret. EtOH Yield |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9902 | 7 | Commercial Enzyme CPN | 6 | 15.0 | 2.6 | 43.33 | commercial | none | filtered | Solids 7 | 69.52 | | |
| 9903 | 10 | Commercial Enzyme CPN | 6 | 15.0 | 2.6 | 43.33 | commercial | none | filtered | Solids 7 | 65.42 | | |
| 0008 | 7 | Commercial Enzyme CPN | 6 | 15.0 | 2.6 | 43.33 | commercial | none | filtered | Solids 7 | 59.80 | | |
| 0014 | 6 | Commercial Enzyme CPN | 6 | 15.0 | 2.6 | 43.33 | commercial | none | filtered | Solids 7 | 56.16 | | |
| 0021 | 13 | Commercial Enzyme CPN | 6 | 15.0 | 2.6 | 43.33 | commercial | no | filtered | Solids 7 | 69.65 | | |
| 0021 | 14 | Commercial Enzyme CPN | 6 | 15.0 | 2.6 | 43.33 | commercial | no | filtered | Solids 7 | 69.00 | | |
| 0021 | 15 | Commercial Enzyme CPN | 6 | 15.0 | 2.6 | 43.33 | commercial | no | filtered | Solids 7 | 67.55 | | |
| 0024 | 7 | Commercial Enzyme CPN | 6 | 15.0 | 2.6 | 43.33 | commercial | no | filtered | Solids 7 | 63.50 | 65.07 | 4.96 |