



*Chemical Analysis and Testing Task
Laboratory Analytical
Procedure*

LAP-008

Procedure Title: SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation

Author(s): Tammy Kay Hayward, Nancy S. Combs,
Sherry L. Schmidt, George P. Philippidis

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SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation

Laboratory Analytical Procedure #008

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 Ethanol Project Quality Assurance Plan (QAP).

3. References

- 3.1 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #001, "Standard Method for Determination of Total Solids in Biomass".
- 3.2 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #002, "Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography".
- 3.3 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #006 "Measurement of Cellulase Activities".
- 3.4 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #011, "Determination of Ethanol Concentration in Biomass to Ethanol Fermentation Supernatants by Gas Chromatography".
- 3.5 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #013, "HPLC Analysis of Liquid Fractions of Process Samples for Soluble Sugars".
- 3.6 NREL Ethanol Project CAT Task 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°C freezer** is necessary for the storage of frozen yeast cultures.
- 5.5 A bench top **centrifuge** is required for SSF sample preparation.
- 5.6 A **shaker incubator** is necessary for the SSF's in order to keep the fermentations at 38°C +/- 2°C and 150 rpm.
- 5.7 **Bubble traps**, also called gas locks, CO₂ 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 H₂O. 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), and sterile **pipets** (disposable with tips that can be broken off conveniently to provide the wide opening needed for sampling SSF slurries).
- 5.12 **Convection oven**, with temperature control of 80 ± 3°C, **desiccator**, and **aluminum foil weighing dishes** for dry cell mass concentration measurements.

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

6. Solutions, Media, and Stock Cultures

6.14 10X YP medium (liquid)

Yeast extract	100 g/L
Peptone	200 g/L

Adjust pH to 5.0 with sulfuric acid. Autoclave for 30 minutes at 121°C.

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. Although this yeast will grow at other pH conditions, pH 5 is chosen because it is optimal compromise for SSF of most substrates when using common cellulases.

6.15 YPD Plates (solid medium)

Yeast extract	10 g/L
Peptone	20 g/L
Dextrose (glucose)	20 g/L
Agar	15 g/L

Dissolve dextrose in deionized (DI) water. Weigh agar into container, add glucose solution. Autoclave for 30 minutes at 121°C. Let cool and add sterile 10X YP medium. Mix solution gently, and aseptically pour the plates. Store plates inverted in the refrigerator.

6.16 YPD medium (liquid)

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

Adjust pH to 5.0 with sulfuric acid and then filter sterilize (do not autoclave glucose and YP together.)

6.17 Antibiotics

If desired, use 0.2 mL of penicillin and/or 0.2 mL of streptomycin filter sterilized stock solutions in the SSF mixture (stock solutions: Penicillin 5 g/L; Streptomycin 5 g/L). Use of antibiotics is not recommended because of the added ES&H risks. (See ES&H section)

6.18 Frozen stock culture of *Saccharomyces cerevisiae* D₅A

50 mL sterile 40% glycerol
50 mL inoculum from NREL-supplied plate

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°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°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 three 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 1.0×10^7 CFU/mL and 1.0×10^8 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 preparation.

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

- 6.20 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.
- 6.21 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.
- 6.22 The use of antibiotics in fermentations can lead to antibiotic resistant microbes which can cause persistent infections in researchers. In addition, prolonged exposure to antibiotics can cause allergic reactions to a variety of medications.
- 6.23 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 also recommended.
- 6.24 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)

- 6.25 The goal of this procedure is to test pretreated or raw biomass substrates by determining the initial hydrolysis (SAC) rate during saccharification catalyzed by cellulase enzymes. Microorganisms are not employed in this experiment.
- 6.26 Based on the biomass moisture content (LAP-001) and cellulose content (LAP-002) data, determine the quantity of biomass needed. Shake flasks should have stopper or Morton closure and a 2:5 medium to flask volume ratio. **All SAC flasks need to have a 1% w/w effective cellulose content. Do not dry pretreated substrates;** once dry the pores of the biomass permanently collapse and do not have the same digestibility.

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.

- 6.27 Based on the filter paper activity of the cellulase enzyme (FPU/mL) and the working weight of the flask, determine the volume of enzyme needed. **All flasks need to have an enzyme loading of 25 FPU/g cellulose based on the official NREL value for the cellulase enzyme preparation.** If all flasks in an experiment will use the same lot of enzyme and the same working weight, then the same volume of enzyme (for example 1.5 mL) will be added to each flask.

Enzyme loading is the most critical factor affecting rates and yields. More accurate addition of the enzyme can be obtained if it is diluted. Also, the new, diluted enzyme can be freshly filtered to ensure sterility. Use freshly diluted sterilized enzyme for each experiment to ensure enzyme activity has not decreased and sterility is maintained. Do not store the enzyme in diluted form for over one day.

Example calculation: 1.506 mL of 1:10 diluted enzyme is needed for an experiment where the diluted enzyme solution has an activity of 8.3 FPU/mL and a SAC flask working volume is 50 mL. Prior to starting an experiment, prepare a fresh, filter sterile (non-cellulosic filter) 1:10 dilution of enzyme in 1x media. Dilution is performed so that the volume added can be accurately measured and easily dispersed into the flask.

- 6.28 Using the substrate weight and diluted enzyme volume determined in the previous steps, calculate the amount of DI water and 10X YP medium needed. 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)
1.5 mL	1:10 diluted NREL-supplied cellulase enzyme
5.0 mL	10X YP solution
<u>39.33 mL</u>	DI water (50 - 1.5 - 5 - 4.167)
50.0 grams total	(working weight)

- 6.29 Each experiment should include an alpha-cellulose control substrate. All substrates should be tested in duplicate at a minimum. A control chart should be set up to compile the alpha-cellulose final yields over time.
- 6.30 Record the actual amount of substrate weighed into each flask to at least the nearest milligram. *For example 0.5321 grams of alpha-cellulose in Flask #1 and 0.5200 grams for Flask #2.* Percent digestibility will be based on this number. Be as accurate as possible and re-calculate the actual enzyme loading.
- 6.31 Add the DI water and 10X YP media. Gently swirl the flask and completely wet the biomass chips.

- 6.32 Check and adjust the pH of the slurry of each flask/vessel to 5.0 ± 0.2 with either lime or sulfuric acid. Add stoppers to the flasks. Weigh to the nearest milligram the entire flask set-up and record as pre-autoclave weight.
- 6.33 Since all the work up to this point has not been done aseptically, autoclave the flasks and/or vessels within two hours. Autoclave at 121°C for 30 minutes (1 hour for a fermentor containing 4 L of medium; make sure the vessel can ventilate freely). Let the flasks/vessels cool to room temperature.
- 6.34 Reweigh the flasks to the nearest milligram and record the post-autoclave weight. Add back lost weight as sterile mLs of DI water.
- 6.35 In a laminar flow hood aseptically add to the first flask or vessel, the required volume of filter sterilized cellulase enzyme, as determined in step 8.3.
- 6.36 Mix the flasks by swirling. Aseptically take a time zero slurry sample. (see step 8.15).
- 6.37 Incubate the flasks in a rotary shaker at 150 rpm and 38°C . For vessels, set the agitation speed at 150 rpm.
- 6.38 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.
- 6.39 At appropriate sampling times (for example: 0, 3, 6, 24, 48, 72, and 168 hours) take 3 mL slurry samples aseptically with sterile large mouth pipet tips or pipets for flasks, or through a port of about 0.5" internal diameter for vessels. 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.
- 6.40 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.
- 6.41 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.

- 6.42 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.
- 6.43 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.
- 6.44 Calculate the glucose yield as % of the theoretical yield (% digestibility) by using the following formula:

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

where:

[Glucose]	Residual glucose concentration (g/L)
[Cellobiose]	Residual cellobiose concentration (g/L)
[Biomass]	Dry biomass concentration at the beginning of the fermentation (g/L)
<i>f</i>	Cellulose fraction in dry biomass (g/g)

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

- 6.45 Graph and/or tabulate the collected data (glucose and cellobiose concentration vs. time) for each experiment.
- 6.46 Quality Control
- 6.46.1 *Reported significant figures:* Report % digestibility to one decimal place.
- 6.46.2 *Replicates:* At least duplicate flasks.
- 6.46.3 *Blank:* None.
- 6.46.4 *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.

- 6.46.5 *Method verification standard:* Alpha-cellulose control. The moisture content of alpha-cellulose must be measured every 3 months.
- 6.46.6 *Calibration verification standard:* None.
- 6.46.7 *Sample size:* See flask preparation instructions.
- 6.46.8 *Sample storage:* Store wet pretreated biomass in the refrigerator for no more than 3 weeks, otherwise freeze it. Store dry (88% or more total solids) biomass at room temperature. Do not use biomass that exhibits signs of spoilage.
- 6.46.9 *Standard storage:* Alpha-cellulose is considered dry biomass and can be stored at room temperature.
- 6.46.10 *Standard preparation:* None.
- 6.46.11 *Definition of a batch:* Flasks started at the same time with one set of alpha-cellulose controls.
- 6.46.12 *Control charts:* % digestibility, at time final for alpha-cellulose and enzyme activity controls. See 8.22.14.
- 6.46.13 *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.
- 6.46.14 *Enzyme activity:* The filter paper activity, (LAP-006) FPU/mL, must be measured every 6 months. Keep an activity control chart, but use the official NREL cellulase activity number for enzyme loading calculations.

9. Procedure for Simultaneous Saccharification and Fermentation (SSF)

6.47 Inoculum preparation

- 6.47.1 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.
- 6.47.2 This inoculum preparation procedure involves two growth stages. The first stage, pre-inoculum, is a flask in which the frozen stock culture, containing a standardized number of cells, is inoculated into YPD (liquid medium). This stage eases the yeast in its transition from stasis to growth phase. The growth

phase occurs in a second flask which contains YPD and is inoculated from the first.

6.47.3 To prepare the first stage flask, transfer 50 mL of sterile YPD into a 125 mL sterile baffled shake flask with a Morton closure (metal cap).

6.47.4 To prepare the second stage flask, transfer the desired amount of YPD into the appropriate sterile Morton closure flask. Base flask size on sufficient inoculum for the SSF experiment (10% v/w transfer) plus at least 20 mL for sample analysis etc. Maintain a 2:5 liquid to flask volume ratio. Account for a 10% v/v seed volume from the preinoculum.

Example: for an experiment containing six 100 g SSFs, 60 mL plus 20 mL is 80 mL needed, then to give a 2:5 ratio, we round off to 100 mL in a 250 mL flask. The inoculum recipe would be as follows: 10 mL of preinoculum into 90 mL of YPD in a sterile 250 mL baffled flask with a Morton closure.

6.47.5 Inoculate the first-stage YPD flask (pre-inoculum) with one thawed stock vial of *Saccharomyces cerevisiae* D₅A. Incubate at 38°C and 150 rpm for 6-8 hours. Before transferring to the next stage, check microscopically for contamination. Only use pure cultures.

6.47.6 Inoculate the second-stage with a 10% v/v transfer from the pre-inoculum. Incubate at 38°C and 150 rpm for 12-16 hours. 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. Check pH and perform DCM (dry cell mass) analysis. Prepare samples for HPLC and GC analysis at the end of the inoculation. Read the ethanol content and use 10% of the value as the time zero ethanol concentration in the SSF flasks. Use only pure D₅A cultures.

6.47.7 Create an inoculum control chart with the final DCM, pH, ethanol, and glucose concentrations for each inoculum.

6.48 Procedure For Dry Cell Mass Concentration

6.48.1 The goal is to measure the dry cell mass concentration.

6.48.2 Dry aluminum dishes in the oven at 80°C overnight.

6.48.3 Cool the dishes in a desiccator for 30 minutes.

- 6.48.4 Record each dish weight to four decimal places using an analytical balance.
- 6.48.5 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.
- 6.48.6 Transfer pellet by repeated vortex washes with DI water to a weighed dish.
- 6.48.7 Dry the dishes and cells in the oven at 80°C overnight.
- 6.48.8 Cool the dishes in the desiccator for 30 minutes.
- 6.48.9 Record the weight of the dishes plus dried cells.
- 6.48.10 Calculate the dry cell mass concentration of the inoculum in g/L by using the following formula:

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

6.49 Procedure for the Simultaneous Saccharification and Fermentation of Biomass

- 6.49.1 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 **3% 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.
- 6.49.2 Determine the amount of biomass needed for each SSF flask based on the biomass moisture and cellulose content. All flasks should have a **3% w/w effective cellulose concentration. Do not dry pretreated substrates.** Once dry, the pores of the biomass permanently collapse. Shake flasks should have a 2:5 medium to flask volume ratio and should be equipped with **water traps.**

- 6.49.3 Based on the filter paper activity of the cellulase enzyme (FPU/mL) and the working weight of the flask, determine the volume of enzyme needed. All flasks should use an enzyme **loading of 25 FPU/g cellulose using the official NREL FPU/mL for the enzyme preparation**. The volume of enzyme added will be the same for all SSF flasks with the same working weight. Prior to starting an experiment, prepare a fresh, filter sterile (non-cellulosic filter) 1:10 dilution of enzyme in media. Dilution is performed so that the volume added can be accurately measured and easily dispersed into the flask. Do not store the enzyme in diluted form for over one day.

Enzyme loading is the most critical factor affecting rates and yields. More accurate addition of the enzyme can be obtained if it is diluted. Also, the new, diluted enzyme can be freshly filtered to ensure sterility.

- 6.49.4 Using the above substrate weight and diluted enzyme volume, calculate the amount of DI water and 10X YP medium needed. Below is an example SSF recipe for a 250 mL water-trap flask:

For example:

3.14 g	alpha-cellulose (LAP-001, 95% moisture)
10.0 g	10X YP
10.0 mL	<i>S. cerevisiae</i> D ₅ A inoculum (second stage)
9.04 mL	NREL supplied 1:10 diluted cellulase enzyme assuming an undiluted volumetric activity of 83 FPU/mL
67.82 g	DI water

100.0	grams total

note: It is easier to add the enzyme and inoculum via a sterile pipet. The density of both ingredients is assumed to be 1 g/L.

- 6.49.5 Each experiment should include an alpha-cellulose control substrate. All SSF should be performed in duplicate at a minimum. A control chart should be set up to include the ethanol yields from a standard final time point (for example 168 hours) of alpha-cellulose SSF.
- 6.49.6 Tare the first flask, weigh in substrate and 10X YP media. Record the actual amount of substrate weighed into each flask to at least the nearest milligram. *For example 3.167 grams of alpha-cellulose in Flask #1 and 3.1743 grams for Flask #2.* Percent theoretical ethanol will be based on this number. Be as accurate as possible and re-calculate the actual enzyme loading.

- 6.49.7 Check and/or adjust the pH of the slurry to 5.0 ± 0.2 with either lime or sulfuric acid. Account for any change in weight by adding less DI water.
- 6.49.8 Add DI water. Gently swirl flask to completely wet the biomass chips in the liquid.
- 6.49.9 Add water trap, autoclave tape, label etc. to flask (do not place any water in the traps at this time.) Weigh the whole flask to the nearest milligram assembly and record this weight as pre-autoclave. Repeat for each SSF.
- 6.49.10 Since all the work up to this point has not been done aseptically, autoclave the flasks and/or vessels as soon as possible. Autoclave at 121°C for 30 minutes (1 hour for a fermentor containing 4 L of medium; make sure the vessel can ventilate freely). Let the flasks/vessels cool to room temperature. Re-weigh each flask assembly to the nearest milligram and add back lost weight as mL of sterile DI water.
- 6.49.11 In a laminar flow hood aseptically add to the first flask or vessel:
- (a) 10% v/w yeast inoculum (in this case 10 mL)
 - (b) Required mL of filtered sterilized 1:10 diluted cellulase enzyme (in this case 9.04 mL)
 - (c) Swirl to mix flask ingredients well.
- 6.49.12 Add water to the water/ CO_2 trap of the flask and incubate the flasks in a shaker at 150 rpm and 38°C . For vessels, set the agitation speed at 150 rpm.
- 6.49.13 Repeat with the other flasks. Start each flask separately and record the time of completion for first and last flasks.
- 6.49.14 At appropriate sampling times (for example 24, 48, 72, 96, 120, 144, and 168 hours) take 4 mL slurry samples aseptically with sterile large mouth pipet tips or pipets for flasks, or through a port of about 0.5" internal diameter for vessels. Store in capped tubes/vials. Chill on ice, centrifuge, collect and filter the supernatant. Analyze for glucose and cellobiose (LAP-013), glycerol, lactic acid, and acetic acid (LAP-015) by HPLC, and ethanol (LAP-011) by GC. If the analysis will be done later, freeze the filtered supernatant in HPLC/GC glass vials.

- 6.49.15 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 +/- 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.
- 6.49.16 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 biocidal solution before washing, reuse and/or disposal.
- 6.49.17 Autoclave the residual SSF material, making sure that the CO₂ 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.
- 6.49.18 Calculate the ethanol yield as % of the theoretical yield by using the following formula:

$$\%Yield = \frac{[EtOH]_f - [EtOH]_o}{0.568 f [Biomass]} \times 100\%$$

where:

$[EtOH]_f$	Ethanol concentration at the end of the fermentation (g/L)
$[EtOH]_o$	Ethanol concentration at the beginning of the fermentation (g/L)
$[Biomass]$	Dry biomass concentration at the beginning of the fermentation (g/L)
f	Cellulose fraction of dry biomass (g/g)
0.568	Conversion Factor for cellulose to ethanol based on stoichiometric biochemistry of yeast.

6.49.19 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.
- Residual and produced ethanol concentration, as well as theoretical yield vs. SSF time (expressed in hours.)
- If possible, residual concentration of glycerol and other metabolites.
- Final pH of SSF.
- Contamination assessment and description of the morphology (shape, color, size, and texture) of the colonies appearing on the plates, including *S. cerevisiae* D₅A.

6.50 Quality Control

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

6.50.2 *Replicates:* At least duplicate flasks.

6.50.3 *Blank:* None.

6.50.4 *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.

6.50.5 *Method verification standard:* Sigma alpha-cellulose control. The moisture content of alpha-cellulose needs to be measured every 3 months.

6.50.6 *Calibration verification standard:* None.

6.50.7 *Sample size:* Not applicable.

6.50.8 *Sample storage:* Store wet pretreated biomass in the refrigerator for no more than 3 weeks, otherwise freeze it. Store dry (88% or more total solids) biomass at room temperature. Do not use biomass that exhibits signs of spoilage.

6.50.9 *Standard storage:* Sigma alpha-cellulose can be stored at room temperature.

6.50.10 *Standard preparation:* None.

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

- 6.50.12 *Control charts:* Make a control chart of alpha-cellulose SSF results (final-day ethanol (g/L), final pH, and % theoretical yield) for each experiment. Make inoculum control chart, enzyme activity control chart, frozen stock viability control chart.
- 6.50.13 *Other:* Verification of a pure D₅A culture--all flasks--final pH should be 5.0 +/- 0.7, no microbes other than D₅A should be detected by microscope or plate checks. Contaminated flasks need to be repeated.
- 6.50.14 *Enzyme activity:* Measure enzyme activity using LAP-006, (Measurement of Cellulase Activities) every 6 months. 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.