Utility of the Anaerobic Fungus Orpinomyces sp. C1A for Biofuel Production

Abstract

Anaerobic fungi belonging to Phylum Neocallimastigomycota are recognized by their efficiency in biomass degradation, which has led to considerable research into their potential role in biofuel production. I am evaluating the ability of an anaerobic fungal isolate Orpinomyces sp. strain C1A to degrade lignocellulosic biomass, with special interest in the function of the cellulose- and hemicellulose-degrading enzymes released by the fungi. The experimental design involved three steps of testing. First, the C1A was grown on sodium hydroxide-pretreated corn stover, in order to quantify fermentable sugar release into the culture medium. Once multiple lignocellulolytic enzyme activities were identified in the C1A culture media, detected by separate enzyme assays, the extent of biomass degradation, sugar release, and ethanol conversion was compared based on varying time of sampling from inoculation and time of E. coli addition. In the final step, this process was repeated, but on varying plant sources. The goal is to maximize biofuel production from this fungus by determining the optimal time for adding the organisms and retrieving their products, as well as the ideal lignocellulosic plant matter. The results suggest potential utility of strain C1A and other anaerobic fungi in biofuel and biochemical production.

Introduction

Ligonocellulosic biomass is organic material originating from dry plant matter, which is composed of high levels of cellulose and hemicellulose and lignin, which is bound between these carbohydrate polymers. Lignocellulosic feedstock is the most common and abundant nonfood biomaterial available from plants to be used for conversion into biofuel, so second generation biofuels are highly likely to have been made from this source (Naik et al, 2009). Grasses make

up the majority of plants that qualify as lignocellulosic biomass. Being perennial and quick-growing, they require only a small investment of energy in planting and harvesting, which combines nicely with their ability to grow on marginal land. The most common way that lignocellulosic biomass is converted into ethanol and other fuel forms is through a relatively basic process of biochemical conversion. Two methods are possible: anaerobic digestion and fermentation. Before anaerobic digestion or fermentation can occur, the lignocellulosic matrix must undergo a pretreatment. This allows for the deconstruction of the complexes and separation of the hemicellulose, cellulose, and lignin, making them available for enzymatic attack. These treatments can be physical such as grinding to increase surface area of the biomass, chemical such as adding dilute acid to add or remove certain substituents from the polymers, or most commonly, biological such as using certain bacteria or fungi to degrade the lignocellulosic biomass and break down the sugars. Then the rest of the conversion can take place (Zhao, 2011).

Members of anaerobic gut fungi from sheep, cows, and other ruminants are versatile biomass degraders with fermentative metabolism. With their lignocellulolytic capabilities and ethanol-producing metabolism, they could potentially serve roles in biofuel production. Anaerobic fungi belong to the phylum Neocallimastogomycota, which shares qualities of aerobic fungi (hyphal growth) and anaerobic bacteria (sugar fermentation). These microorganisms are highly fibrolytic; they produce a large variety of enzymes that degrade the cellulosic, hemicellulosic, and glycolytic fractions of the plants that serve as their substrates. They can also ferment some of the sugar monomers to fatty acids and fuel in the form of ethanol (Struchtemeyer, 2014). Because anaerobic fungi secrete these enzymes into their environment, these proteins can continue to work on the plant material whether or not their parent cells are still active. The anaerobic fungal species Orpinomyces is normally found in the rumen chamber,

hindgut, and feces of ruminant herbivores. Orpinomyces strain C1A, extracted from Angus steer feces, was selected because it is easy to maintain and has adapted to metabolizing common lignocellulosic plant matter that the animals consume and digest. The typical plant substrates include a variety of bioenergy crops and crop residues, such as corn stover, switchgrass, energy cane, and more (Nicholson, 2005). The goal of this project is to evaluate the suitability of C1A for biofuel production from lignocellulosic plants, as well as determining the steps to optimize the quantities of ethanol produced over sugars and organic acids.

Materials and Methods

All experiments were conducted in triplicate, and unless otherwise specified.

Part 1A.

i) Subculturing.

Orpinomyces strain C1A was grown under anaerobic conditions in a rumen fluid basal medium with cellobiose as the substrate. For regular subculturing, two Balch tubes were inoculated every 2-3 days. More tubes or serum bottles were inoculated as needed on days prior to experimentation.

ii.) Plant Mass Pretreatment.

Samples of corn stover were obtained from local sources. These samples were dried overnight and sieved to get smaller particle size. Then they were pretreated with 1% sodium hydroxide (NaOH) solution inside a sealed serum bottle. The samples were washed with deionized water and dried for at least 48 hours before being used in the experiments.

iii.) Growth of Strain C1A on Pretreated Plant Material.

In an anaerobic hood, 0.45g of NaOH-treated corn stover substrate was added to each serum bottle, already containing 45ml of rumen fluid-free medium. The bottles were re-sealed and transferred to a CO2 gassing station. Here 5ml of fluid was anaerobically removed from either a Balch tube or serum bottle containing log-phase C1A in culture medium. This amount of inoculum was transferred to each bottle via CO2-gassed syringes with needles. The inoculated bottles were incubated in a nonshaking incubator set at 39°C.

iv.) Inhibition of Strain C1A and Supernatant Sampling

Bottles were sacrificed, in triplicate, at different time intervals to quantify sugar production and substrate loss, so that optimal time of inhibition could be determined. Group A was inhibited 24 hours after inoculation, Group B after 36 hours, and Group C after 48 hours. To inhibit the cultures, 5ml of cyclohexamide was anaerobically added to each serum bottle.

v.) Extraction of Growth and Supernatant.

Samples were collected from every bottle on Days 0, 2, 4, 7, and 14 after initial inoculation. Using CO2-gassed syringes, 1ml of supernatant was removed from each bottle and placed in a 1.7ml centrifuge tube. Samples were stored at -20°C until all had been collected for experimentation. On the final day, the bottles were opened so that the plant matter could be collected and supernatant extracted. Supernatant was stored in 50ml falcon tubes at -20°C. The plant material was dried in individual aluminum dishes overnight at 37°C then weighed. The final plant weight was subtracted from the time zero dry weights of each plant material to give the amount of biomaterial consumed by the C1A in each bottle.

Part 1B

The results of step 1A showed that the highest levels of fermentable sugars were produced in the bottles inhibited 48 hours after inoculation. New bottles were inoculated with C1A, and all were

inhibited after 48 hours. Regular samples were collected using the same methods above, at days 0, 2, 4, 7, 8, and 14. A collection of enzyme assays were performed on all the samples to verify the presence of enzymatic activity continuing after fungal cell inhibition. Samples were tested on assays to detect both cellulose and hemicellulose acting enzymes, since both types are needed to break down lignocellulosic plant matter.

i.) Cellulase Assays.

1. Endoglucanase. The Megazyme cellulase assay kit was used. The enzyme activity is expressed as U per g protein.

2. Exoglucanase. Avicel was used as the substrate. This enzyme cleaves the end of cellulose chains into cellobiose units; released cellobiose was quantified using DNS assay. The enzyme activity is expressed as mmoles of cellobiose per g protein.

3. Cellobiohydrolase (CBH). The substrate was 4-nitrophenyl-cellobioside as substrate and the activity is expressed as μmoles of p-nitrophenol released per g protein.

4. β-glucosidase. The Sigma-Aldrich β-Glucosidase Activity Assay Kit was used. The enzyme activity is expressed as U per g protein.

ii.) Hemicellulase Assays:

1. Xylanase. Birchwood xylan was used as a substrate. Released xylose was quantified by DNS assay. The enzyme activity is expressed as μmoles of xylose per g protein.

2. α-glucuronidase. The Megazyme α-D-glucuronidase kit (K-AGLUA) was used. The enzyme activity is expressed as U per g protein.

3. Mannosidase. The substrate was 4-nitrophenyl-mannoside and the activity is expressed as μmoles of p-nitrophenol released per g protein.

4. Xylosidase. The substrate was 4-nitrophenyl-xylopyranoside and the activity is expressed as μmoles of p-nitrophenol released per g protein.

5. Mannanase. The substrate was locust bean gum. Released mannose units were quantified using a DNS assay. The enzyme activity is expressed as μmoles of mannose per g protein.

Part 2

Following the methods above, new serum bottles were inoculated with C1A on corn stover substrate and inhibited after 48 hours.

i.) Addition of E. coli

Two days prior to addition, E. coli was cultured on an Agar plate. After incubation, cells were removed from the plate and diluted into a 10ml solution in a 15ml falcon tube. Four groups of bottles, in triplicate, received 0.5ml of the E. coli solution at different times after inoculation. Group A served as the control group and did not receive any of the bacteria. E. coli was added to Group B on day 2, Group C on day 6, and Group D on day 14.

ii.) Sampling Scheme.

Following the methods from Part 1, samples were collected from all bottles on days 0 (inoculation), 2, 3, 6, 9, 12, and 14. On the final day, the biomass was removed for dry weight measurement and supernatant was separated into a 50ml falcon tube.

Group D was sampled more extensively following the addition of E. coli on day 14. Samples were taken at the time of addition, and then 1, 2, 4, 8, 12, 24, 31, 35, 43, and 51 hours following.

iii.) Product Analysis

Dry weights for taken for the biomass from each bottle and subtracted from the original 0.5g of each plant. As before, glucose and xylose levels were measured from all samples. Also, ethanol and fatty acid levels were measured using high-pressure liquid chromatography (HPLC).

Part 3

i.) Preparation of Bottles.

Corn stover, switch grass, energy cane, sorghum, mixed prairie grass, and alfalfa were treated with NaOH according to directions in Part 1A, step ii. Bottles were prepared with media. In an anaerobic chamber, each bottle received 0.5g of one of the plants. This time, there were five bottles per plant type. The bottles were inoculated with C1A and inhibited after 48 hours.

ii.) Sample Collection

Rather than collecting regular 1ml samples from each bottle, an entire bottle from each plant group was sacrificed at different time points. One bottle from each group was opened on days 0, 2, and 7. The final three bottles from each group were opened on day 14. From each sacrificed bottle, supernatant was separated into a 50ml falcon tube and stored at -20°C until all were collected. Glucose was measured for each sample. The plant matter from each sacrificed bottle was collected for dry weight measurement, to be subtracted from the original 0.5g as before.

Results

Part One

The longer the C1A grew before inhibition, the more plant mass was used and more sugars produced. After two weeks, the highest yield (86.82mgs) of fermentable sugars was produced when the C1A was inhibited 48 hours after inoculation. This part of the study showed a direct correlation between the percentage of plant mass lost and the milligrams of sugars produced. Since the same weight (0.45g) of corn stover plant matter was added to each bottle, a

percentage was calculated based on the grams of fermentable sugar per gram of the original dry weight. This ratio more than doubled from T24 to T48. Interestingly, the amount of xylose produced after T48 was only about a milligram more than xylose produced after T36. Meanwhile, glucose production nearly doubled after both 12 hour periods from T24 to T36, then T36 to T48. See Table 1 for exact values.

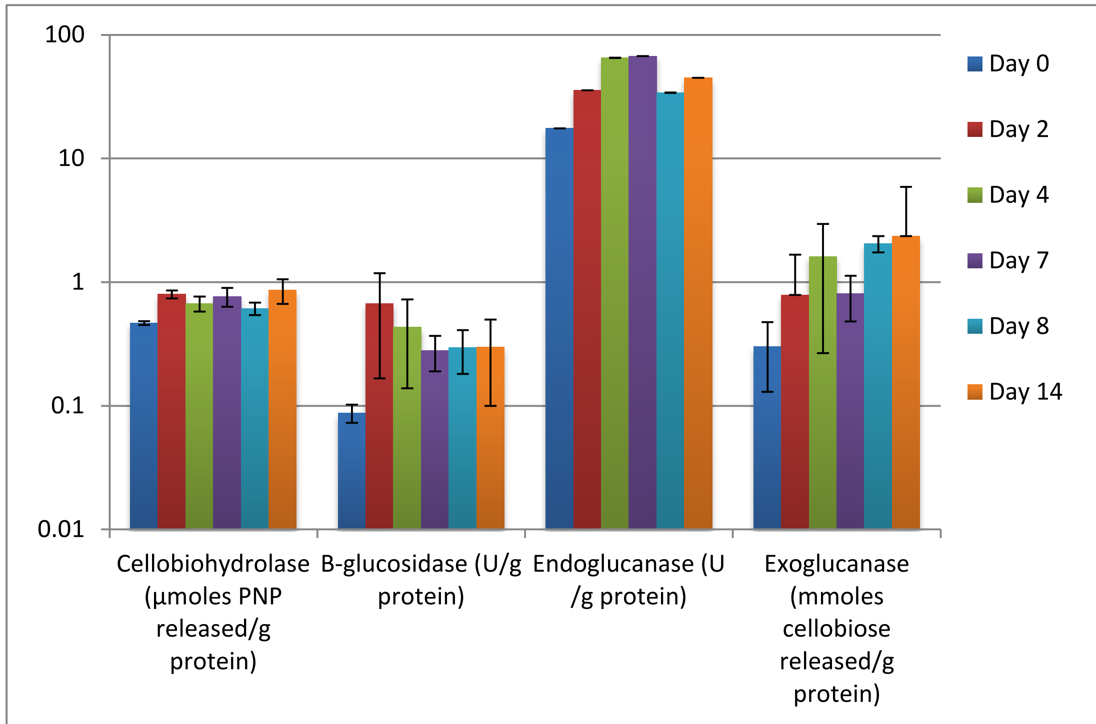
Time of inhibition (hours after inoculation)	Dry Weight at T_o (g)	Dry Weight at T_f (g)	Used Dry Weight (g)	Percentage Lost (%)	Glucose Produced (mg)	Xylose Produced (mg)	Total Fermentable Sugars (mg)	g of Fermentable Sugar / g Original Dry Weight
24	0.45	0.41	0.04	7.93	12.43	18.87	31.30	0.07
36	0.45	0.38	0.07	16.30	28.07	31.62	59.69	0.13
48	0.45	0.31	0.14	31.78	54.24	32.58	86.82	0.19

Table 1. Comparison of utilization of plant weight versus sugar production after inhibition at 24, 36, and 48 hours post-inoculation.

After these results, every bottle was inhibited at 48 hours. Next, trends were detected in the enzyme analysis. The overall trend shown by the enzyme assays is a general increase in enzymatic activity as the time until sacrifice increased. Although a few enzymes peaked in activity around day 7, the activity after two weeks was always higher than the initial activity. This indicates that despite the inhibition of the C1A cells, the enzymes already released continue to function.

When comparing the enzymatic activity in the pellet versus the supernatant, no major differences were apparent, with the exception of endoglucanase. Of the cellulose-degrading enzymes, endoglucanase had the highest activity. Xylanase had the highest activity of the hemicellulose enzymes. Most of the enzymes maintained activity between 0.1 – 1.0 units. See Figures 1a – 1d for specific units, varying based on enzyme and product released.

Figure 1a. Activity of cellulose-degrading enzymes from supernatant on days 0, 2, 4, 7, 8, 14.



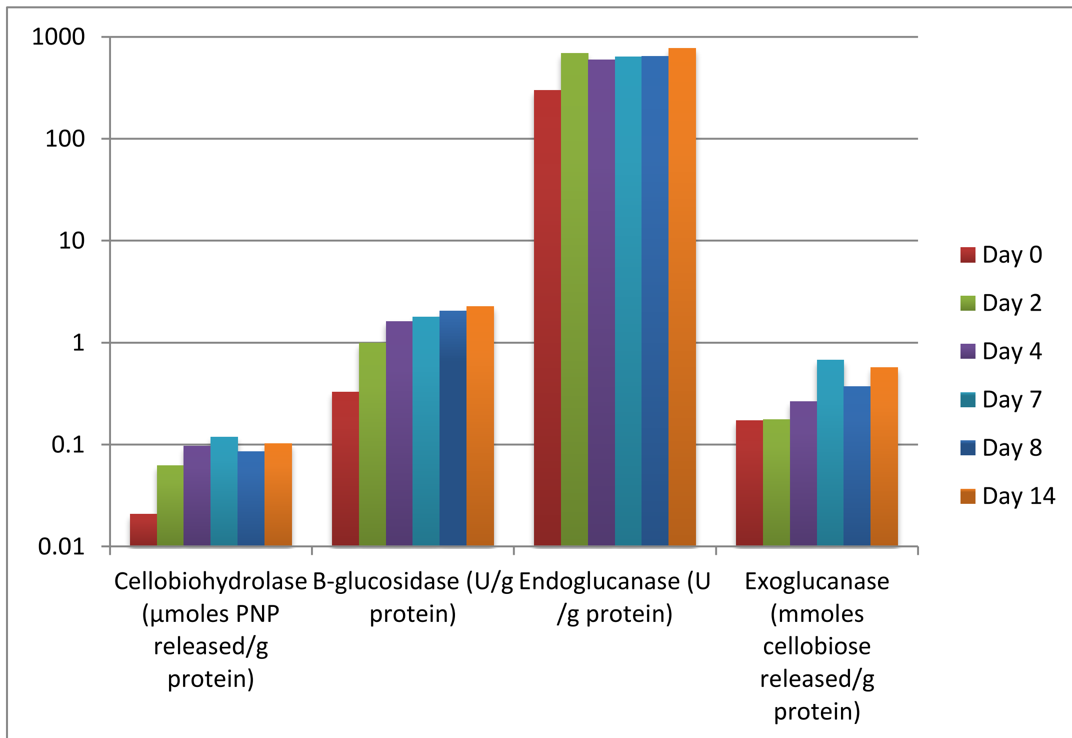


Figure 1b. Activity of cellulose-degrading enzymes from pellet on days 0, 2, 4, 7, 8, 14.

Figure 1c. Activity of hemicellulose-degrading enzymes from supernatant on days 0, 2, 4, 7, 8, 14.

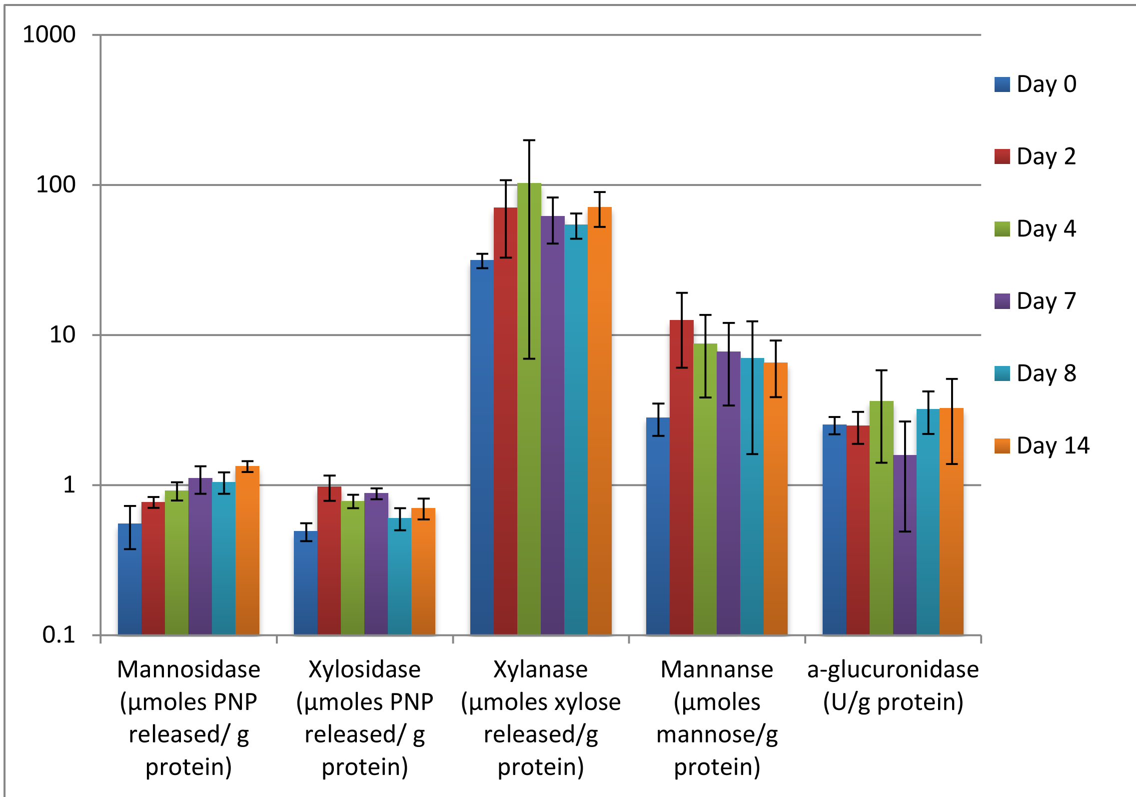
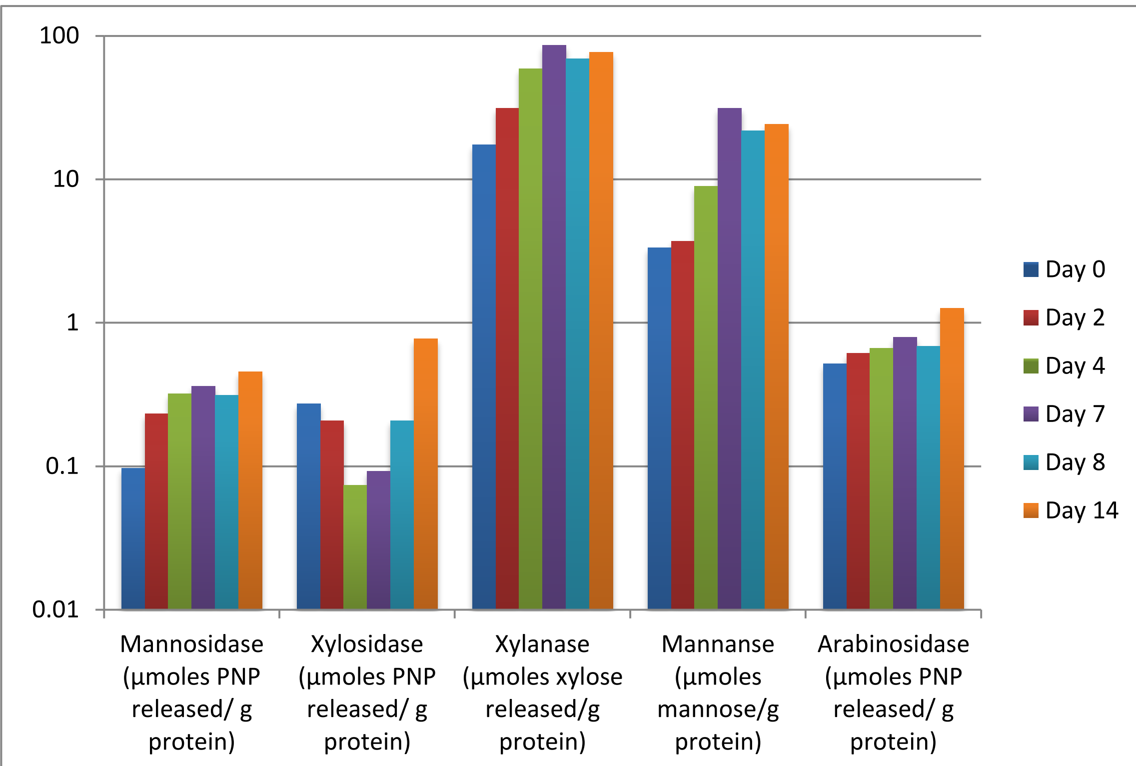


Figure 1d. Activity of hemicellulose-degrading enzymes from pellet on days 0, 2, 4, 7, 8, 14.



Part Two

The relationship between all of the products can be seen in figure 2A. This is without E. coli fermenting the sugars yet. For the first two days, the fungal mass increases and the weight of the plant matter decreases inversely, as the C1A grows using corn stover substrate. The fungal biomass stops increasing and plateaus on day two due to inhibition at 48 hours. After this point of inhibition, the amount of organic acids rapidly increases and sugar production begins. The weight of corn stover decreases as the plant is broken down into glucose and xylose fractions. Glucose is the dominant sugar, with production over doubling that of xylose. At this point, the ethanol levels stay low because fermentative metabolism has not occurred yet. Upon E. coli addition, all of the glucose was rapidly converted into ethanol, while xylose was more gradually consumed. As expected, there is an inverse correlation between the amount of sugar and amount of ethanol as time progressed, as seen in Figure 2b. All of the sugars in a bottle were converted by around 22 hours post E. coli addition. Ethanol levels declined after this point.

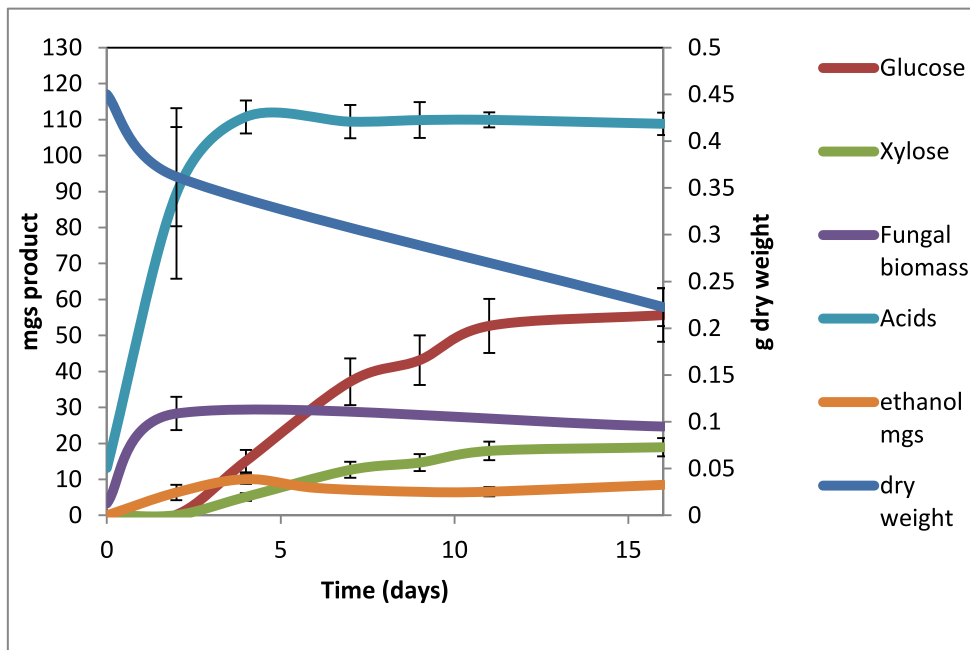


Figure 2a. Levels of substrates and products after a two week period (prior to E. coli addition).

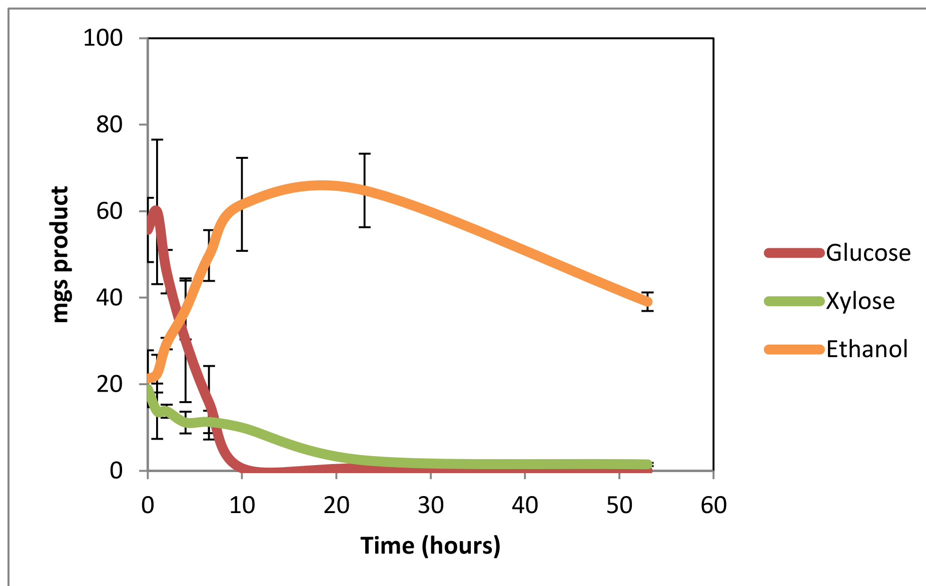


Figure 2b. Levels of glucose and xylose compared to levels of ethanol during anaerobic fermentation via E. coli.

Part Three

Sugars continued to accumulate from all six plants tested. A range of 28-40% of each plant was consumed, excluding Alfalfa which had only 11% loss and consequently produced the lowest amount of sugar. Corn stover produced the best yield of sugar per gram of original weight (19.29%). Energy cane was the next best plant source, offering 80.8mg of fermentable sugars from its original dry weight of 0.541g (15% yield). Switchgrass, sorghum, and mixed prairie grass all produced similar results that indicated decent breakdown to sugars, but only about half as much as the energy cane. See Table 2 for specific values.

Plant (All NaOH-treated)	Original Dry Weight (g)	Final Dry Weight (g)	Percent Loss (%)	Total Fermentable Sugars (mg)	g Sugar/ g Original Dry Weight
Switchgrass	0.533	0.319	40.1500938	47.26	0.08866792
Alfalfa	0.515	0.45733333	11.197411	19.85	0.03854369
Energy Cane	0.541	0.37666667	30.3758472	80.8	0.14935305
Sorghum	0.51	0.36466667	28.496732	36.37	0.07131373
Mixed Prairie	0.543	0.35166667	35.2363413	47.7	0.0878453
Corn Stover	0.45	0.31	31.78	86.82	0.19293333

Table 2. Comparison of plant matter usage versus sugar production for six types of lignocellulosic plants.

Discussion

With increasing global interest in biofuels, new strategies are being developed to make the manufacturing process more efficient and practical. Since anaerobic fungal isolate Orpinomyces strain C1A is a versatile biomass degrader with natural fermentative mechanisms, it is a promising resource for secondary biofuel production. Analysis of the C1A cellulose and hemicellulose degrading enzymes, E. coli fermentation on these sugar monomers, and the differences in product yield based on varying plants lead to better optimization of the process. The results of this study were very positive – almost all of the plant material was broken down into sugars, and the sugars were successfully fermented into ethanol.

Now that it has been established that C1A has enzymes that work efficiently on breaking down the sugars of corn stover and that E. coli can be added to successfully convert the sugar products to ethanol, the methods in this study need to be further reconstructed for better optimization of the fuel product. The next trial to run could test the amount of plant matter to add to each bottle to maximize enzyme productivity. Other experiments could go more in depth into determining the best time to add the E. coli or to sacrifice the bottles. Once the concerns in the C1A project have been worked out, the experiments can be repeated with additional anaerobic organisms with similar traits in order to find the most ideal specimen for this task.

In conclusion, this study demonstrates that C1A is capable of quickly and easily initiating a simple chain of events for effective lignocellulosic biofuel production. It also suggests that corn stover is the best plant to serve as the substrate for this fungus. Since this strain is common and easy to isolate and corn stover is easily obtainable this combination is highly promising for utilization in lignocellulolytic biofuel systems.

References

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