COMPARISON OF EXTRACELLULAR CELLULASE ACTIVITY FROM *TRICHODERMA REESEI* AND *BACILLUS SUBTILIS* FOR POTENTIAL USE IN BIOFUEL PRODUCTION

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Abstract

Extracellular cellulase activity from *Trichoderma reesei* and *Bacillus subtilis* were compared to determine which species produces cellulases that break down cellulose at the greatest rate. Purification of extracellular cellulases was performed using acetone precipitation and DEAE-Sephadex A-50 column chromatography. *Trichoderma reesei* exhibited the greater specific activity in the crude sample while after acetone precipitation, *B. subtilis* produced a nearly four fold increase in specific activity compared to the crude specific activity of *T. reesei*. Fold purification after acetone precipitation for *T. reesei* was low at 0.0400 and *B. subtilis* yielded a fold purification of 73.0. Due to a low protein concentration, column chromatography did not result in a fold purification. Polyacrylamide gel electrophoresis produced faint banding of which are presumed to be composed of three extracellular cellulases produced from *T. reesei* and two extracellular cellulases from *B. subtilis*.

Introduction

Worldwide demands for fossil fuels are increasing each year and will result in fossil fuel shortages in the near future (Shafiee and Topal, 2009). At the current rate, scientists project that petroleum reserves will be depleted by the year 2042 (Shafiee and Topal, 2009). Currently, efforts are underway to harvest energy from renewable resources to supplement fossil fuel consumption (Kumar et. al., 2009; Pittman et. al., 2011; Sticklen, 2008). One area of focus includes the production of ethanol, through fermentation, using the linked sugars (polysaccharides) found in plants as an energy source (Brethauer and Wyman, 2010; Sticklen, 2008). Current methods used to produce ethanol from polysaccharides range from chemical acid hydrolysis and pyrolysis to enzyme hydrolysis, liberating individual sugar units (Bakare et. al., 2005). Due to economic concerns, acid hydrolysis and pyrolysis are cheaper to perform than enzyme hydrolysis and are typically used in the large-scale production of ethanol (Bakare et. al., 2005). However, undesirable byproducts are generated from acid hydrolysis and pyrolysis of polysaccharides and therefore, additional refining is required to isolated ethanol from the final mixture (Bakare et. al., 2005). Ethanol produced from the enzymatic breakdown of polysaccharides produces a more pure mixture requiring less refinement with the potential for a greater amount of ethanol to be produced once the polysaccharide is broken down (Bakare et. al., 2005).

These linked sugars in plants store energy for the plant and are very stable chemically; however, once broken down into its individual sugar units, can be universally used as an energy source by other organisms (Morgan et. al., 2013). One of the main linked sugars found in plants is cellulose and is the most abundant organic compound, estimated to comprise at least 33% of all vegetative matter (Morgan et. al., 2013; Ross et. al., 1987). Structurally, cellulose consists of linked glucose units, of which one cellulose molecule may contain over 3,000 individual glucose

units (Brown Jr., 2003). Therefore, due to the abundance of cellulose, its renewable nature, and universal ability for organisms to utilize its constituent glucose units, cellulose proves to be an ideal compound as the starting material in the fermentative generation of ethanol (Morgan et. al., 2013). However, few organisms contain enzymes capable of liberating the individual glucose molecules due to the stability of the bonds within the cellulose molecule (Pandey et. al. 2011). These few organisms capable of breaking cellulose into its constituent glucose units produce enzymes collectively referred to as cellulases (Pandey et. al. 2011).

Research investigating cellulase activity currently focuses on cellulases produced by fungi; though, bacteria also produce cellulase enzymes and may prove promising in determining the optimal source organism for the production of these cellulase enzymes (Maki et. al., 2009; Mawadza et. al., 2000). It has been established that bacteria exhibit a faster growth rate compared to that of fungi and therefore, have the potential to produce a greater amount cellulase enzymes (Ariffin et. al., 2006). However, research is lacking in the comparison of cellulase enzyme activity rates produced by fungal cellulases and bacterial cellulases. It is the goal of this study to determine if cellulases produced by a common, nonpathogenic bacteria (*Bacillus subtilis*) exhibit a greater activity rate than that of a fungus typically used in cellulase activity investigations (*Trichoderma reesei*). Specifically, extracellular cellulase enzymes between the two species will be investigated as these enzymes are secreted directly into the organism's environment and are able to act directly on the cellulose substrate without incorporation of the cellulose into the cell.

Materials and Methods

Materials

Monobasic sodium phosphate anhydrous, dibasic sodium phosphate tetrahydrate, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA disodium salt), sodium chloride, Tris-glycine buffer, 3,5-dinitrosalicylic acid (DNS), sodium hydroxide, solid phenol, sodium metabisulfite, citric acid monohydrate, Whatman No. 1 filter paper, D-glucose, acetone, 2-mercaptoethanol, monobasic potassium phosphate, calcium chloride dihydrate, magnesium sulfate tetrahydrate, ammoniumsulfate, urea, ironsulfate tetrahydrate, manganesesulfate monohydrate, zincsulfate tetrahydrate, cobalt chloride, peptone, yeast extract, thiamine, double distilled water, DEAE Sephadex A-50, Precision Plus Kaleidoscope protein standard, Mini-PROTEAN TGX gel, Coomassie stain, and carboxymethyl cellulose were obtained through Sigma Chemical Company or were on hand during the experiment. *Trichoderma reesei* and *Bacillus subtilis* were retrieved from storage and maintained on sabouraud dextrose at 27°C and nutrient agar plates at 37°C respectively.

Preparation of crude enzyme

Organisms were cultured in specific broths to induce the production of cellulase enzymes. The fungi (*T. reesei*) was incubated on a rotary shaker at 100 rpm at 27°C in a broth containing KH_2PO_4 (2 g/L), $CaCl_2 \bullet 2H_2O$ (0.4 g/L), $MgSO_4 \bullet 7H_2O$ (0.3 g/L), $(NH_4)_2SO_4$ (1.4 g/L), Urea (0.3 g/L), $FeSO_4 \bullet 7H_2O$ (5.0 mg/L), $MnSO_4 \bullet H_2O$ (1.6 mg/L); $ZnSO_4 \bullet 7H_2O$ (1.4 mg/L), $CoCl_2$ (2.0 mg/L), peptone (2.0 g/L), D-glucose (1.0 g/L), and carboxymethyl cellulose (10 g/L). *Bacillus subtilis* was

incubated on a rotary shaker at 100 rpm at 37°C in a broth containing D-glucose (0.5 g/L), peptone (0.75 g/L), $FeSO_4$ (0.01 g/L), KH_2PO_4 (0.5 g/L), and $MgSO_4$ (0.5 g/L). Each culture was centrifuged at 6,000 g for 15 minutes. The clear supernatant was retained as the sample containing the extracellular cellulase enzymes.

Enzyme purification

Acetone precipitation

Proteins in the crude collection were precipitated with an 80% acetone/ddH_2O solution. The crude extract from *T. reesei* was precipitated with a 4:1 ratio of acetone to extract while the crude extract from *B. subtilis* was precipitated with a 3:1 ratio of acetone to extract. The precipitate was allowed to form for 24 hours at -20°C. Precipitate was collected through centrifugation at 10,000 rpm, 0°C for 15 minutes. The supernatant was removed and the pellet washed and resuspended in 10 mL of 80% acetone. The resuspended pellet was collected again through centrifugation. The supernatant was removed, the pellet allowed to air-dry for five minutes to evaporate any remaining acetone, and dissolved in 0.02 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol.

DEAE-Sephadex A-50

DEAE Sephadex A-50 was packed in a vertically mounted column (1.5 cm x 30 cm) with gravity controlling the flow rate. The column was equilibrated with 3 bed volumes of 0.02 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The partially purified sample from the acetone precipitation step was loaded into the column and 5 mL fractions collected. Sodium chloride solution was added beginning with fraction number 24 and increased from 0.0 to 0.5 M at 0.1 M increments. Fractions were measured for protein content using a UV-Vis spectrophotometer at 280 nm. Fractions with the highest protein content were pooled and proteins precipitated using acetone precipitation (Black Line- Figures 1 & 2). The pellet from the acetone precipitation was resuspended in 5 mL of 0.02 M sodium phosphate buffer, pH 7.0.

Protein concentration determination

Protein concentration was determined from each stage of purification (crude, acetone, and DEAE-Sephadex A-50) using the Bradford method (Kruger, 1994) with bovine serum albumin (1 mg/mL) as the standard.

Cellulase activity measurement

Cellulase activity from each stage of purification was measured using the filter paper assay (FPA) as described by Zhang et. al. (2009) with minor modifications. One FPase unit was defined as 1 µmol glucose/(mL•min). Five samples from each stage of purification were measured for activity. Dilutions from a 10 g/L glucose solution were made in triplicate and used to develop a glucose standard curve from which cellulase activity could ultimately be determined. Color change was

measured after the addition of the DNS reagent using a UV-Vis spectrophotometer set at 540 nm.

Gel electrophoresis

Polyacrylamide gel electrophoresis was performed in a 0.5 M Tris-glycine buffer to visualize the purification achieved at each stage. A Mini-PROTEAN TGX stacking gel was loaded with the samples from each stage of purification and ran at 200 volts for 25 minutes. A Precision Plus Kaleidoscope protein standard was loaded alongside the samples. The gel was placed in a container on a rocker, stained with Coomassie blue stain for 30 minutes, destained for 60 minutes, and placed in Coomassie blue stain overnight. The gel was rehydrated for 60 minutes the following day in ddH_2O and banding photographed (Figure 3).

Results

Enzyme purification

Table 1 contains calculated activity rates for each species investigated through the purification process. *Trichoderma reesei* exhibited a statistically significant greater specific activity from the crude sample compared to that of *B. subtilis* ($p=5.28 \times 10^{-8}$; $p<0.05$). However, after acetone precipitation, *B. subtilis* yielded a statistically significant ($p=3.61 \times 10^{-10}$; $p<0.05$) greater specific activity compared to that of *T. reesei*. Fold purification for the acetone precipitation step yielded an extremely low fold purification for *T. reesei* (0.0400) while *B. subtilis* exhibited a fold purification of 73.0. The difference in specific activity between *T. reesei* and *B. subtilis* was not statistically significant for the DEAE-Sephadex A-50 column chromatography step. Additionally, fold purification from the column chromatography step was higher than in the acetone precipitation step for *T. reesei* but overall did not yield an increase in purification at the end of the experiment (Fold purification < 1). Purification did not increase between the acetone precipitation step to the column chromatography step for the *B. subtilis* sample (8.06 < 73.0).

Purity determination

Faint banding was observed for some samples along the polyacrylamide gel (Figure 3). Molecular weights were calculated from a standard curve using the banding produced from the Precision Plus Kaleidoscope protein standard. Regarding the *T. reesei* sample, no banding was produced from the crude sample, three bands were produced from the acetone precipitation samples with molecular weights of 118 kDa, 57 kDa, and 49.5 kDa, and the column chromatography step produced two bands with molecular weights of 118 kDa and 49.5 kDa. The crude sample for *B. subtilis* produced two bands with a molecular weight of 124 kDa and 87.5 kDa, the acetone precipitation yielded two bands with molecular weights of 124 kDa and 87.5 kDa, and the column chromatography produced one band with a molecular weight of 124 kDa.

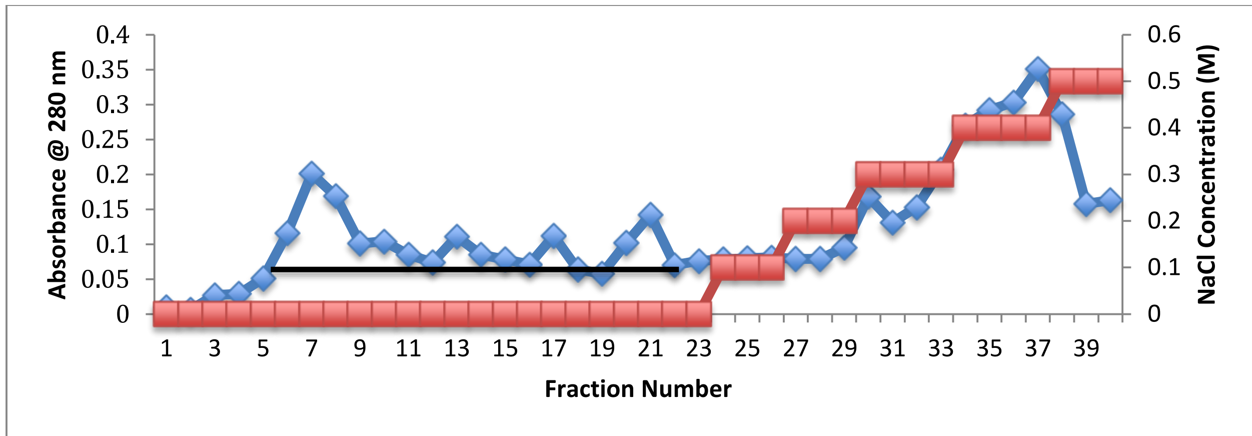

Figure 1: Protein content of each fraction collected from DEAE-Sephadex A-50 chromatography using the sample collected from the *T. reesei* acetone partial purification step. The horizontal black line indicates the fractions pooled together and later analyzed for cellulase activity.

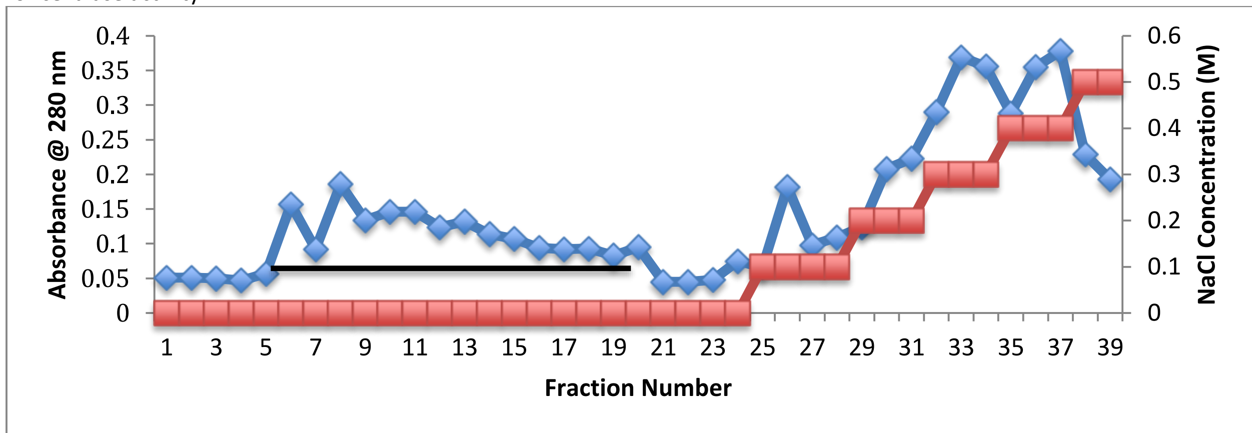

Figure 2: Protein content of each fraction collected from DEAE-Sephadex A-50 chromatography using the sample collected from the *B. subtilis* acetone partial purification step. The horizontal black line indicates the fractions pooled together and later analyzed for cellulase activity.

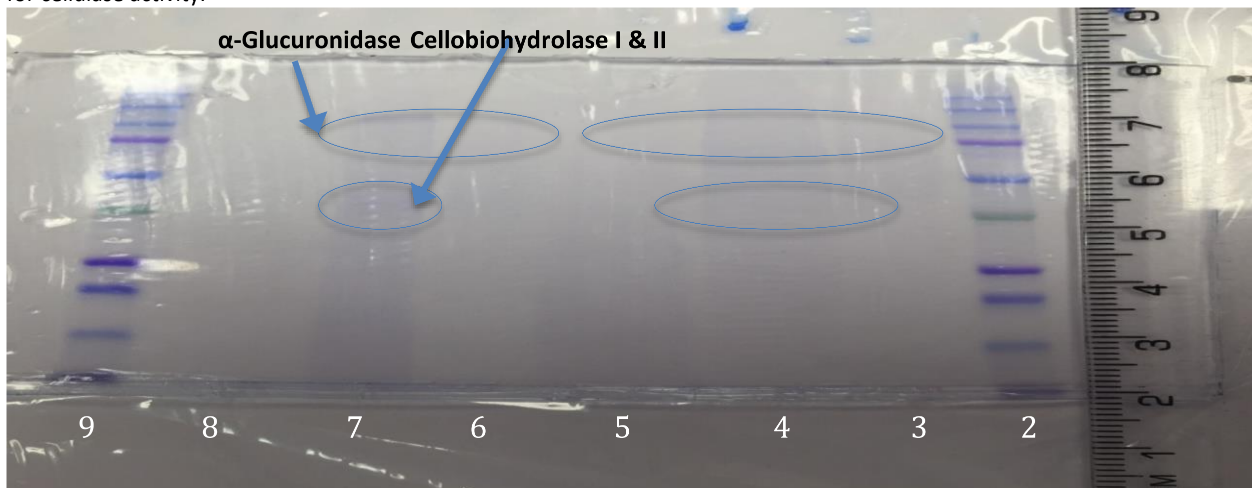

Figure 3: The faint banding observed along the gel upon completion of electrophoresis and staining. Circles are present around bands that were observed; however, are difficult to see due to lighting and photo quality. Lanes 1 and 10 were not loaded with a sample and lanes 2 and 9 contained the Precision Plus Kaleidoscope protein standard. The following samples were loaded into each well: lane 3- *B. subtilis* crude sample, lane 4- *B. subtilis* acetone precipitation sample, lane 5- *B. subtilis* column chromatography, lane 6- *T. reesei* crude sample, lane 7- *T. reesei* acetone precipitation sample, and lane 8- *T. reesei* column chromatography.

	Total Activity (umol/min)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification
***T. reesei* Crude**	175.3	1.616	108.5	100	1
***B. subtilis* Crude**	454.8	84.82	5.362	100	1
***T. reesei* Acetone**	10.66	2.456	4.342	6.08	0.0400
***B. subtilis* Acetone**	24.48	0.06255	391.4	5.38	73.0
***T. reesei* A-50**	4.496	0.3167	14.20	2.57	0.131
***B. subtilis* A-50**	7.700	0.1783	43.19	1.69	8.06

Table 1: Calculated cellulase activity for each sample analyzed ultimately derived from measured absorbance and comparison to a glucose standard.

Discussion

Enzyme purification

Extracellular cellulase activity was achieved through each round of purification for each species investigated. However, the percent yield for each purification step was lower than desired, indicating the need to further optimize the procedure, specifically the acetone precipitation step. With low percent yields for both species of the acetone precipitation step, it was difficult to achieve a further purification through the column chromatography step as little protein was present at the beginning of the column chromatography stage. Optimization of the acetone precipitation step will limit the expected loss of the protein(s) of interest with increasing purification steps and result in a sample with a higher protein concentration being loaded into the final purification step (column chromatography).

A large fold purification was obtained during the acetone precipitation step for *B. subtilis*, supporting the idea that *B. subtilis* is a potential source for cellulase enzymes with optimal activity. However, without similar purification of cellulases from *T. reesei* it is difficult to definitively state which of the two species produce cellulases capable of degrading cellulose for large-scale use. Utilizing the data obtained in making a decision on which organism to harvest cellulase enzymes from, one would have to weigh the time invested versus the activity achieved. With little time invested, *T. reesei* would be the better choice for cellulase enzymes over *B. subtilis*. On the other hand, after one round of nonspecific purification, *B. subtilis* exhibited nearly a four fold increase in specific activity over *T. reesei*, indicating the ability to greatly purify *B. subtilis* cellulases (Fold Purification = 73.0).

Gel electrophoresis

The banding along the polyacrylamide gel was very faint primarily due to the low protein concentration of the sample. In order to increase the protein concentration, the pellet from each sample during the purification process must be resuspended in a lesser amount of buffer. However, banding observed, specifically in regards to *T. reesei*, is consistent with published values of extracellular cellulase enzymes. The banding with a molecular weight of 118 kDa is consistent with that of α-Glucuronidase, the band with a molecular weight of 57 kDa is of Cellobiohydrolase II, and the band with a molecular weight of 49.5 kDa is composed of Cellobiohydrolase I. Cellobiohydrolase I and II are classified as Beta-1,4-exoglucanases, or enzymes that degrade cellulose outside of the cell. Western blotting would confirm the assumption that the banding observed is produced by the above listed extracellular cellulases.

Summary

Upon interpretation of the data (*T. reesei* or *B. subtilis*), it is apparent that extracellular cellulases from *B. subtilis* can be purified to a degree greater than that produced by *T. reesei* in the crude sample. The specific activity from the acetone precipitation for *B. subtilis* (391.4 U/mg) is nearly four times that produced by the crude collection of *T. reesei* (108.5 U/mg). Therefore, *B. subtilis* cannot be ruled out as a candidate for the producer of large-scale cellulase enzymes. Additional work is required specifically involving the optimization of the acetone precipitation step or development of a step that partially purifies the crude collection for use in column chromatography. Optimization of the acetone precipitation would involve analysis of the supernatant for cellulase activity as well as experimentation with different acetone concentrations to ensure that as much cellulase enzymes are precipitated and concentrated in the pellet. Furthermore, additional research involving the specific kinetic parameters of the cellulase enzymes must be performed to determine which species produces cellulase enzymes that act with the greatest rate in regards to substrate concentration. Finally, utilizing the information from kinetic parameters measurements, investigation of cellulase enzyme rate in the presence of varying amounts of product will allow for the determination if removal of product is required to limit the inhibitory effect some products cause on enzyme rates. Once additional investigation is performed regarding the specific kinetics of cellulase enzymes, procedures can be developed to further increase the rate at which cellulases break down cellulose.

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