The Use of Glucoamylase- Bioconjugated Nanoparticles to Facilitate Starch Fermentation

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Abstract:

This project concerns the cloning and synthesis of an enzyme, glucoamylase, for bioconjugation to magnetic particles, which could potentially lead to a more efficient process for biofuel production. There are many methods currently utilized for biomass pre-processing; however, this project particularly focuses on the creation of glucoamylase-conjugated magnetic nanoparticles, which allows for the enzymes to be retrievable enabling novel reuse. Glucoamylase is an enzyme that breaks the bonds of large carbohydrates (starches). Particularly, glucoamylase is an amylase that cleaves the last alpha-1,4-glycosidic linkage at the nonreducing end of amylose and amylopectin to yield glucose. The resulting monomeric glucose molecules can then be fermented by organisms such as yeast into biofuels of interest such as ethanol. The glucoamylase from this project is specifically targeted from *Trichoderma* reesei, an organism commonly used in industry for the production of cellulytic enzymes. Glucoamylase has not been widely isolated from this organism before, so the development of a method for isolating and cloning this enzyme has been formulated and tested for this project. Various methods have been tested in order to create a cDNA library containing the gene for this enzyme from *Trichoderma reesei*; however, none have extremely high and successful yields. These low yields from *Trichoderma reesei* have led to the production of glucoamylase similar to that of *Trichoderma reesei* from two organisms, *Pichia stipitis* and *Debaryomycese hansenii*. In addition, experiments have been performed with fluorescent silica-coated iron oxide particles to test for separation capabilities. Based on preliminary data, it is hopeful that when these enzymes are conjugated to the particles, a successful, high-yielding magnetic recovery will be observed.

I. Introduction:

Sources of energy, especially fuel, are particularly important in today's society. Finding new supplies and novel methods for obtaining these fuels is of the upmost importance in the scientific community. One of the commonly utilized methods for biofuel production is with yeast cells. They can convert biomass to fuels such as ethanol with relatively high yields; however, this is a difficult procedure because the biomass has to be pre-processed into sugar monomers before it can be fermented by the yeast. This pre-processing involves first physically breaking apart the biomass polymers and then using enzymes to digest these fragments into their sugar monomers so that they can be used for fermentation by various organisms. This pre-processing procedure is very expensive and can account for a significant portion of the biofuel operating cost because enzymes are expensive when they cannot be efficiently recovered and reused (Galbe et al. 2007; Wyman 2007; Aden et al. June 2002). If these enzymes were able to be recovered and reused, this could potentially reduce the cost needed for biofuel synthesis.

Traditionally, enzyme reuse has been accomplished through immobilization techniques. However, these methods suffer from diffusion limitations and the requirement of soluble substrates. For the case of biomass degradation, enzymes must be able to access the small pore sizes in cellulose and hemicelluloses. Thus, one of the modern methods currently being investigated in the scientific community for the recovery and reuse of enzymes is through the creation of metallic particles. These particles can be synthesized and proteins can be tethered to their surface using genetically modified enzymes. Once

this is successful, these particles can be placed in a reactor to allow for homogeneous catalysis to occur. Homogenous catalysis is a type of catalysis where the catalyst is in the same state as the reactant. After the completion of the reaction, a magnet of sufficient strength can be used to retrieve these metallic particles from solution. This allows for these bioconjugated particles to be reused in the future. This method is currently very novel and thus required a significant amount of preliminary research to determine its feasibility in industry. If successful, this method can potentially save money on industrial processing as it reduces the amount of material required for each batch run.

In this project, the main reaction that will be focused on is the conversion of starches into glucose for eventual ethanol production. Ethanol is an important product because it is currently added to gasoline to reduce emissions and reduce the use of petroleum. In addition, ethanol can be used as a sole fuel for some vehicles after minor engine modifications. Yeasts are quite adept at efficient conversion of sugars into ethanol. However, these cells must have the enzymes necessary to initially break polymers down into their sugar monomers. Yeasts can thus take advantage of enzymes such as glucoamylase to first break down the starch into glucose before fermentation proceeds. To accomplish this task, current approaches include using cell extracts containing various enzymes (Singh et al. 1995) as well as enzymes from co-cultures with other secreting organisms (Kurosawa et al. 1989; Jeon et al. 2008) along with recombinant gene expression are utilized. While successful, these methods are not fully efficient, thus require a novel approach to improving overall performance of the conversion process. Beyond starches, another possible source of biomass is cellulose. There are three major enzymes required for the conversion of cellulose to monomeric sugars: cellobiohydrolase, endoglucanase, and beta-glucosidase (Kumar et al. 2008). These three enzymes can be naturally produced by a fungus, *Trichoderma reesei.*

II. Background

Glucoamylase from Trichoderma reesei

Trichoderma reesei is a soil mesophilic soft-rot ascomycete fungus. It is commonly used as a source of enzymes such as cellulases and hemicellulases because it produces them easily (Martinez) in both a laboratory setting and in an industrial setting. In particular, the enzyme of most interest for this project is glucoamylase, and from the recently sequenced full genome of *Trichoderma reesei*, it can be seen that the gene for glucoamylase contains introns. This makes cloning this gene difficult from simply genomic DNA. Introns are non-coding portions of DNA that do not get translated into protein. The coding portions of DNA are exons. When DNA is transcribed and translated, the introns eventually get spliced out of the sequence so that a fully functioning protein can be made. Proteins need to be in a specific conformation resulting from the interactions between amino acids and other factors. If there are additional amino acids, then the correct protein cannot be made. This protein will be expressed and purified in another host, the bacteria *Escherichia coli*. As a result, to obtain a working copy of the protein, an intronless version of the gene has to be obtained using a cDNA library from *Trichoderma reesei* RNA.

Metallic nanoparticles

Nanotechnology is an innovative field that has proven to be very important. It has widespread use in applications such as drug delivery, biofuel synthesis, fuel cell reactors, and many other important fields. Particularly within the realm of nanotechnology, metallic nanoparticles have long been synthesized for various purposes. One of their main uses is in catalysis. The world is made up of chemical reactions. These chemical reactions can occur very slowly naturally; however, the kinetics of the reaction can be greater if enzymes specific to the reaction are present, in other words, if enzymes are present to catalyze or speed up the reaction. Currently there are many metallic nanoparticles that have been synthesized in a variety of methods using a great number of metals. These nanoparticles have unique properties that can be utilized in various reactions. The most common one is that it provides an increased surface for the reaction to occur in. One of the other properties that is getting increased focus is the magnetic capabilities of metallic nanoparticles. There have been some preliminary publications about the ability to retrieve nanoparticles from solution in order to reuse them as metal catalysts in additional reactions (Zeng et al.2010, Luo et al. 2010). There has not, however, been a significant amount of work conducted using metallic nanoparticles with tethered enzymes for retrievable and reusable catalysts. In this system, the metallic nanoparticles would serve as the metallic element for magnetic retrieval and the enzyme on which it is tethered onto would serve as the catalyst. The nanoparticles and a depiction of their magnetic separation are illustrated in figure 1. This method would prove to be very important in the field of nanotechnology, not only as an advancement in the synthesis of metallic and magnetic nanoparticles but also an advancement in enzyme production.

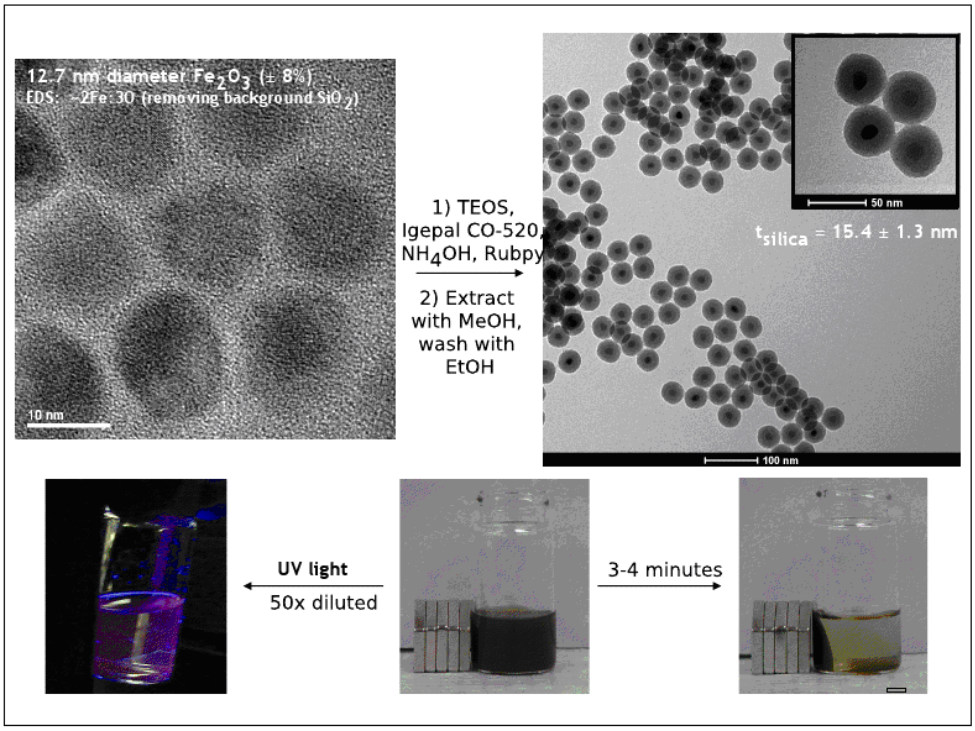

Figure 1. *Fluorescent Silica- coated Iron Oxide particles and their magnetic separation. Adapted from the Korgel Lab 2009.*

III. Materials and Methods

Trichoderma reesei (QM9914), *Saccharomyces cervisiae*, *Pichia stipitus*, and *Debaryomycese hansenii* cells were obtained and used throughout this project. In addition various kits were also used. These kits and solutions included the Promega SV Total RNA Isolation System, Ambion TRI Reagent solution for RNA extraction, Invitrogen SuperScript III First-Strand Synthesis SuperMix, PCR reagents, Qiagen PCR clean up kit, and restriction enzyme digestion reagents. The plasmids used for the ligation step to the glucoamylase DNA fragments are Invitrogen pRSET A, B, and C. NEB DH10β competent cells were used for transformation. For cDNA production and specific gene amplification, there were many primers used. The primers and their melting temperatures are listed in Table 1.

Primer Name	**Strand**	**Primer Sequence (5'→3')**	**T_m (°C)**
Trichoderma reesei Glucoamylase	Forward	CGGGGTACCATGCACGTCCTGTCGACTGCGGTGC	88.9
Trichoderma reesei Glucoamylase	Reverse	CCCAAGCTTTTACGACTGCCAGGTGTCCTCCTTG	81.0
Trichoderma reesei Ribosome	Forward	TACCGCGAAACTGCGAATGGCTCA	63.0
Trichoderma reesei Ribosome	Reverse	TACGACTTTTACTTCCTCTAAATG	50.3
Trichoderma reesei Ribosome (II)	Reverse	TACGACTTTTACTTCCTCTAAATGACCGAGTTTGG	72.3
Trichoderma reesei GNA3	Forward	AACGTTGTCACTCTCGTCAAGGAATCG	63.1
Trichoderma reesei GNA3	Reverse	TGGGAAAGAACGTGATGGTTTGAGAG	59.9
Trichoderma reesei Mid ribosome gene section	Forward	GAACCTTGGGCCTGGCTGGCCGGTC	71.1
Trichoderma reesei Mid ribosome gene section	Reverse	GCCGCAGGCTCCACCCCTGGTGG	71.0
Trichoderma reesei sar1	Forward	TGGATCGTCAACTGGTTCTACGA	57.6
Trichoderma reesei sar1	Reverse	GCATGTGTAGCAACGTGGTCTTT	58.1
Pichia Stipitus Glucoamylase	Forward	CCGTATCCGGATCCATGAAGTTGCAATTACTATT	63.6
Pichia Stipitus Glucoamylase	Reverse	CCGTATCCAAGCTTCTAGAATTGGTCTAAAATATCTA	60.7
Debaryomycese hansenii Glucoamylase	Forward	CCGTATCCGGATCCATGATGAAGTTAAATTTGAT	62.6
Debaryomycese hansenii Glucoamylase	Reverse	CCGTATCCAAGCTTCTAATTATGATTATTATTGTGATTG	58.6

Table 1. *Primers used throughout the project.*

For preliminary experiments with magnetic nanoparticles, fluorescent silica iron oxide particles were obtained from the Korgel group. These particles were characterized with a fluorescent spectrophotometer and a transmission electron microscope. The magnets used were neodymium magnets with a field strength of around 0.2 Tesla and samarium cobalt magnets with a field strength of around 1 Tesla.

A. Experiments with Iron Oxide Particles

Saccharomyces cerevisiae cells were inoculated and grown. The cell density at OD_{600} was measured after each day in order to determine when was the best time to obtain the desired number of cells for the experiments. After this has been determined, these cells were centrifuged down and then resuspended to be mixed with fluorescent silica coated iron oxide particles later. These particles are made from two different methods, one with a thin shell and one with a thicker shell. The method of making the particles was observed to determine which type was the easiest to separate out in solution with cells using magnets. In addition, different ratios of cells to nanoparticles were used to determine the separation efficiency. All experiments were performed in glass vials, and the cells were incubated with the nanoparticles for a certain period of time prior to the start of magnetic separation. The separation efficiency was determined by measuring the fluorescent absorbance of the solution on the side of the bottle opposite that of the magnet after certain amounts of times.

B. RNA Isolation

RNA must be isolated from *Trichoderma reesei* to create the cDNA library. There are many techniques that have been used to isolate RNA. Since this is not a commonly used organism, the best method for RNA extraction must be determined. For RNA extraction, a Promega SV total RNA isolation system was used according to the procedural instructions that came with the kit. After some experiments, another method was used for RNA extraction. It involves the usage of the Ambion TRI Reagent to extract RNA from *Trichoderma reesei* cells. This kit required approximately 5×10^5 cultured cells. These cells were then lysed by the TRI reagent, and the homogenate is then separated into different phases through the addition of bromochloropropane, BCP, and centrifuging. The RNA is in the aqueous phase while the DNA and proteins are in the interphase and organic phases respectively. After, the RNA is precipitated out with isopropanol and is washed with ethanol to be resuspended in a certain amount of nuclease free water. The effectiveness of the RNA isolation from these two kits was determined by running the product on a gel. A MOPS/formaldehyde gel was used to minimize RNA degradation.

C. cDNA Library

In order to make a cDNA library from RNA, the Invitrogen SuperScript III First-Strand Synthesis SuperMix Kit was used to create the 1^{st} cDNA strand. Everything was preformed according to the kit procedure, and then more steps were added afterwards to create the 2^{nd} strand of cDNA. The steps for creating the 2^{nd} strand cDNA were to take 1^{st} strand cDNA, water, RNAase I, and DNA polymerase and incubate that mixture for two hours at 15 °C. This reaction was terminated with 0.5 M EDTA at a pH of 8.0. After this process, polymerase chain reaction was performed using the primers specific to the gene and organism. The protocol was a standard

polymerase chain reaction protocol with buffer, dNTP, Taq DNA polymerase, template (in this case double stranded cDNA), primers (both forward and reverse strands), and water. The annealing temperature for all of these was just 5 °C under the melting temperature of the primers. After the polymerase chain reaction, the product was run on a standard 1% agarose gel to determine if the gene was successfully produced.

D. Testing the efficacy of the cDNA procedure

Due to some difficulties in the lab with respect to the cDNA library procedure, a test was performed with another lab to make sure that the procedures were correctly carried out and did not need any modifications. The whole procedure was performed with frog RNA obtained from the lab of John Wallingford. The primer used for this was for EF1α which is a common elongation factor found in many organisms. The annealing temperature for these primers was 58 °C, and the PCR was run for 30 cycles. After PCR was complete, the resulting product was run on an agarose gel to determine if the cDNA library procedure was correct.

E. Glucoamylase from Pichia stipitus and Debaryomycese hansenii

Glucoamylases from *Pichia stipitus* and *Debaryomycese hansenii* were obtained using standard genetic procedures. This includes first designing primers specific to the glucoamylase in each of these organisms. These primers like the other primers were designed to include specific restriction enzyme cut sites on them for eventual ligation to the pRSET B vector. These primers were used for PCR to amplify out the glucoamylase genes for both of these organisms. Once this was complete, a 1% agarose gel was again used to determine if product was successfully obtained. Afterwards, the product was cleaned up using a Qiagen PCR clean up kit and was measured with a nanodrop to determine the exact concentration of DNA present in the solution. Once that has been performed, a sequential digest was done to the DNA and plasmid. The two restriction enzymes used for these organisms were BamH1 and HindIII. A sequential digest was used instead of a double digest because the chart suggested that this was more optimal in terms of preventing any star activity from occurring. Star activity, when there isn't perfect cutting specificity from the restriction enzymes, causes problems with running a perfect digest. The BamH1 was then determined to have the best activity in NEB buffer 3, and HindIII was then determined to have the best activity in NEB buffer 2. Between the digest and after the 2nd digest, the product was cleaned up using the Qiagen cleanup kit again. This was to ensure that there were no remaining active enzymes left in the solution. After the digests, a ligation step was performed in order to ligate the DNA and the vector together since they have both been digested by the same restriction enzymes and should have the same sticky ends. Once this step was complete, a transformation using competent cells was performed via the heat shock method. The product from the transformation was then plated on plates that contained LB and ampicillin in order to select for cells that have successfully transformed the plasmid. The ampicillin resistance is part of the pRSET B vector genome. pRSET B vector was used because it was designed to have histidine tagged regions in the beginning portion of the plasmid so that it can be transcribed on to the protein. This would allow for the metallic nanoparticles to interact with this

reaction so that it can successfully bind to each other and form a bioconjugated particle. Also, the restriction enzymes picked for this experiment cut the plasmid so that it does not interrupt the reading frame. This is very important for the correct translation of glucoamylase. Once living colonies have been picked off the plate and grown up, a miniprep was performed on the product. This allows for just the plasmid to be isolated from the product. In order to determine if the plasmid does indeed have the gene of interest, another digest was performed either with BamH1 or HindIII to see the length of the DNA segment when it was run on an agarose gel. If it was around 2.9kb, then it is just the plasmid; however, if it was around 3.5kb (since the glucoamylase genes are about 1,500 bp), then the ligation and transformation was successful. In order to obtain more products, another method was used for the PCR cleanup. A gel was run and the original PCR products were extracted and precipitated in order to obtain greater yields. After that, everything else was run like it was before.

F. Experiments with glucoamylase and fluorescent silica iron oxide particles

A few preliminary tests were conducted with both glucoamylase and fluorescent silica iron oxide particles. The main one currently conducted was to observe the separation efficiency of the nanoparticles when in solution with commercial glucoamylase. This gives a good measure of how beneficial this project will be and how effective it will be as well. Nanoparticles were made and place in different concentrations of commercial glucoamylase to see if it separated out well.

IV. Results and Discussion

A. Experiments with Iron Oxide Particles

From the results of the experiments with the iron oxide particles and *Sarccharomyces cerevisiae* shown in tables 2 and 3, it can be determined that there was no difference between the thin shelled nanoparticles and the thicker shelled nanoparticles. This can be seen in the data below. The proportions between the two types of nanoparticles are roughly the same which indicates that the same amount of nanoparticles was magnetically separated out after 3 hours.

	Intensity (A.U.)		
Solution Sample	**0 hr**	**3 hr**	**0hr/3hr**
Nanoparticles with H_2O only	1.87	0.8	2.3375
Nanoparticles with cells of OD_{600}: 0.1	1.65	0.91	1.8132
Nanoparticles with cells of OD_{600}: 1.0	1.27	1.24	1.0242

Table 2. *Fluorescent absorption of the solutions before and 3 hours after magnetic separation for thin shelled nanoparticles. The measurement is the intensity in A.U. of the solution measured with a fluorescent spectrophotometer. Then a ratio was calculated to determine the relative change in intensity in order to compare it with the data from the thick shelled nanoparticles.*

	Intensity (A.U.)		
Solution Sample	**0 hr**	**3 hr**	**0hr/3hr**
Nanoparticles with H_2O only	9.47	4.5	2.1044
Nanoparticles with cells of OD_{600}: 0.1	7.66	6.14	1.2476
Nanoparticles with cells of OD_{600}: 1.0	4.71	4.61	1.0217

Table 3. *Fluorescent absorption of the solutions before and 3 hours after magnetic separation for thick shelled nanoparticles. The measurement is the intensity in A.U. of the solution measured with a fluorescent spectrophotometer. Then a ratio was calculated to determine the relative change in intensity in order to compare it with the data from the thin shelled nanoparticles.*

This data was obtained by placing the different solutions in glass vials. A fluorescent spectrophotomer was used to

excite the solution at 459 nm, and the fluorescent silica nanoparticle emission at 600 nm was measured. (Heitsch et al. 2008). This measurement indicates the amount of nanoparticles present in the solution since concentration is directly related to absorbance. The fluorescence was measured before magnetic separation and after 3 hours of separation to determine if there was difference in the fluorescence absorption. Separation is performed by placing magnets next to vials containing the solution for three hours. After that time, nanoparticles should be attracted to the magnet on one side, so the solution on the other side can be pulled out and measured for the presence of nanoparticles with a fluorescent spectrophotometer. The data shows a decrease in the absorption after three hours for each solution indicating that magnetic separation was successful; however, separation was best for the solution containing just nanoparticles and water than for the solutions containing cells. There are fewer cells in solution to block the nanoparticles from reaching the magnets

Based on the conclusions from the data, it can be determined that magnetic separation will be more difficult the more cells that are present. It can also be concluded that it does not matter what type of nanoparticle is used, whether that is thin shelled or thick shelled fluorescent silica oxide nanoparticles.

B. RNA isolation and cDNA library synthesis of Trichoderma reesei

There were a few problems pertaining to the RNA isolation and cDNA library synthesis of *Trichoderma reesei*. After a considerable amount of time spent on trying to successfully extract RNA, it has been determined that RNA was successfully extracted using the Ambion TRI Reagents. This was determined based on the usage of a bioanalyzer. After accomplishing this initial step towards making a cDNA library of the *Trichoderma reesei* glucoamylase, the next hurdle proved to be significantly greater. After repeated experimentation, no band can be found in the gel after the cDNA library underwent PCR. The problematic step could not be determined because there were many steps between RNA extraction and PCR. After conducting various experiments altering the reaction temperatures and reactant quantities, frog RNA was obtained from another lab to test out the whole procedure. It was determined that the second strand cDNA process was not necessary for the creation of double stranded cDNA. The gel in figure 2 indicates that there is no DNA band for the cDNA procedure that included the second strand cDNA synthesis but a band did show up for the cDNA procedure without the second strand cDNA synthesis part.

← Elongation Factor Band

Figure 2. *Gel of the cDNA products. Lane 1- ladder, lane 2- without 2nd strand cDNA procedures, and lane 3- with 2nd strand cDNA procedures.*

This was an important step towards making the glucoamylase from *Trichoderma reesei*. Despite this progress, the glucoamylase gene still proved very difficult to obtain. Different

Trichoderma reesei genes were then tested to see if it was just the glucoamylase gene that was not producing any products. These genes were common in glucoamylase, and their sequences did not involve the cDNA library part. These primers, however, still did not yield any results on the gel. This leads one to conclude that perhaps it is not *Trichoderma reesei* in the vials that were purchased. Alternatively, the RNA prep may not have been complete enough to obtain proper coverage of all RNA molecules in the cell. Finally, it is possible that there was also contamination in the cell cultures which would cause the isolation of the gene more difficult. Either way, further testing is needed to be conducted to determine if glucoamylase can be successfully made from the *Trichoderma reesei* genome.

C. Glucoamylase from Pichia stipitus and Debaryomyces hansenii

Based on the gels run on the PCR products from these cells, it can be determined that the glucoamylase gene was successfully amplified from these two organisms. Digestion and transformation of the products using the Qiagen PCR cleanup kit has thus far proven to be unsuccessful because the kit significantly reduces DNA concentration of each sample. This problem, however, was fixed by using the precipitation technique after gel extraction. This method produced cells that grew on ampilcilin plates which indicates that at the very least the plasmid has been transformed. Based on the gel of the digestion of the products, it can be seen that the desired glucoamylase indeed has been successfully cloned. This can be seen by running a single digest using either BamH1 or HindIII and then running a gel. A band at around 4,300 bp would indicate the presence of the glucoamylase gene in the plasmid whereas a band at 2,900 would indicate that only plasmid got transformed into the competent cells. From figure 3, since there is a band around the 4,000 bp ladder lane for six of the samples, 4 from *Pichia stipitus* and 2 from *Debaryomyces hansenii*, it can be concluded that transformation was successful. The samples are just digests of the minipreps of the cells from different colonies picked off after transformation. This allows for further advancement of the process which is to produce glucoamylase enzymes from the cells.

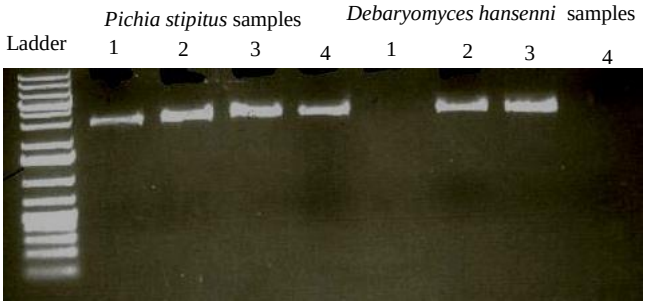

Figure 3*. Gel of Pichia stipitus and Debaryomyces hansenii glucoamylase plasmid digests. Lane 1 is the ladder. Lanes 2-5 are samples of digests of the different Pichia stipitus colonies after transformation and miniprep. Lanes 6-9 are samples of digests of the different Debaryomyces hansenni colonies after transformation and miniprep.*

D. Experiments with glucoamylase and fluorescent silica iron oxide particles

The preliminary experiments with the commercial glucoamylase and the nanoparticles indicate that the presence of glucoamylase in solution does not affect the separation abilities of the nanoparticles. This can be seen in the figure 4 showing the difference in fluorescence absorption before and after 3 days of separation. The solutions were placed next to a magnet for three days in order to allow for separation to occur. The magnet held the nanoparticles on one side while the remaining solution was taken to

measure the fluorescence. The fluorescence absorption measures the presence of the nanoparticles in solution since these nanoparticles are dyed with Rubidium. The intensity is directly related to the concentration of nanoparticles present: the more nanoparticles there are, the higher the intensity. The data in figure 4 shows that the peaks for the two solutions before and after are very close to each other indicating that the presence of glucoamylase in solution does not affect the separation abilities of the nanoparticles. If the difference between before and after for the two solutions is large, then there is a difference in separation ability; however, since the before peaks and the after peaks were very similar, it can be concluded that there is not a significant difference between the two solutions. This is very important for future large scale experiments because there will inevitably be extra free floating glucoamylase in solution and knowing that it will not affect the separation will enable the process to work at its optimal efficiency.

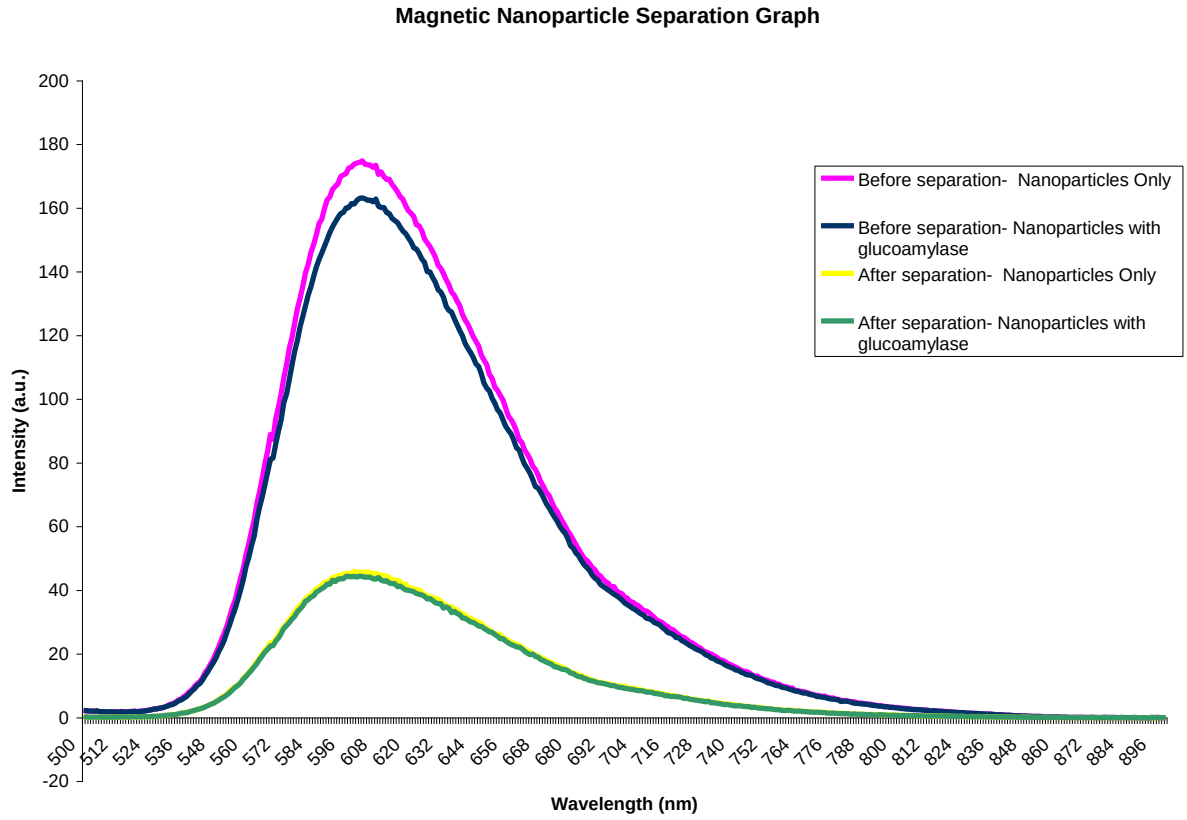

Figure 4. *Intensities before and after magnetic separation for the two different solutions: nanoparticles only and nanoparticles with glucoamylase. This is measured by a fluorescent spectrophotometer of the solutions at the start of the experiment and after allowing for the nanoparticles to be separated by a magnet after three days. The wavelength of interest is 600nm, which is where the fluorescent dye coated on the nanoparticles can be detected.*

V. Conclusion

There is currently insufficient data to conclusively determine the feasibility of bioconjugating glucoamylase to nanoparticles. This project has, however, made advances towards achieving the overall goal. This project has shown that it is possible to separate the nanoparticles when they are in solution with *Sarrcharomyces cerevisiae*, which is important because these cells are what are going to be used for the eventual fermentation step of ethanol production from glucose. In addition, this project has also worked out how to successfully extract RNA and convert that into a cDNA library. This is critical for the production of glucoamylase from *Trichoderma reesei* since that is not an intronless gene naturally in the cell. In addition, the successful extraction of glucoamylase from *Pichia stipitus* and *Debaryomycese hansenii* can be used to further test the feasibility of this project. These glucoamylases can be used to conjugate with nanoparticles to test the separation abilities of these particles in solution. Since the glucoamylases from *Pichia stipitus* and *Debaryomyces hansenni* are similar to the glucoamylase from *Trichoderma reesei*, the results from these glucoamylases can be applied to that of the glucoamylase from *Trichoderma reesei*. Further experiments need to be performed to determine how efficient these bioconjuaged particles are in solution. If they are proven to be highly effective, then this could be really beneficial to industry. The next step of this project would be to place it in a reactor system to analyze the costs and overall large-scale efficiency of the particles.

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