

Fluid Dynamic Properties of Bacterial Cellulose and Application

Andrew J. Keefe
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Abstract

The purpose of this study was to develop a new use for microbial cellulose produced by the bacterium, *Acetobacter xylinum*. There is a potential application of bacterial cellulose to construct a nano-scale laminar flow reactor. Small particles (i.e. enzymes or solid phase reactants) can be embedded in a growing cellulose matrix with geographic specificity. A fluid containing a substrate can then be passed through this active region to allow a reaction to occur. The first step in developing this reactor was to analyze the fluid dynamic properties of liquid through cellulose.

Background

Cellulose is a polysaccharide made of many β -glucose monomers that is primarily used in nature for supplying structure to cells. Cellulose is the main component in cell walls of plants, algae, and fungi, making it the most abundant source of terrestrial biomass in the world today. Cotton, 94% cellulose, and wood, 50% cellulose, are the main sources for cellulose in commercial manufacturing (Brown). Cellulose can be seen as the major ingredient in production of paper, textiles, speaker diaphragms, food bulking agents, medicinal bandages, artificial skin, binding agents, and cellophane (Mormino, 2002). A new form of more pure cellulose that is only now being utilized can be found being produced by bacteria.

The bacteria most known for their cellulose producing abilities are found in the genus *Acetobacter*, and of these is *Acetobacter xylinum*. *A. xylinum* is a gram negative, aerobic bacterium that has the ability to convert up to 108 molecules of glucose an hour into cellulose (Brown). This organism is nature's most productive cellulose producing bacterium. *A. xylinum* uses cellulose as a device to raise itself to the oxygen rich air at the surface of its environment. This is also ideal for harvesting cellulose because the mechanism results in a thick pellicle (cellulose disk) of concentrated cellulose on the surface of a culture. A cellulose flotation device will increase available oxygen concentrations, but will have the negative effect of limiting the diffusion of necessary nutrients to the cell. To combat this effect, the use of a rotating disk bioreactor can be implemented.

Microbial cellulose's most appealing characteristics are its tensile strength and ability to hold hundreds of times its weight in water. Another attractive characteristic is found in the direction of growth of microbial cellulose. The majority *A. xylinum* cells are found at the top of a growing pellicle where cellulose production takes place. Since new cellulose is produced at the surface, the pellicle is grown in a downward direction. In result of this method of growth, any particles placed on the surface of the pellicle will be grown around and embedded into the cellulose matrix. These characteristics are what make cellulose subject to such innovation. (Mormino, 2002)

Experiments and Results

Rotating Disk Bioreactor:

A rotating disk bioreactor allows for aerobic cells to be exposed to air and media at manipulatable ratios for growth. As mentioned earlier, *A. xylinum* has developed a unique use for cellulose to increase its amount of accessible oxygen, but by doing this has limited its ability to attain necessary nutrients. The rotating disk bioreactor, RDB, eliminates both oxygen and the nutrient absorption limitations. The RDB works by rotating a mesh disk perpendicular to the growth media. The disk catches long cellulose strands produced by the bacteria to adhere cells to the mesh. Bacteria grow on the disk in an outward direction, only stopping once the air becomes depleted of oxygen, the growth media loses its nutrients, or the media becomes toxic with cellular waste. This method of growth was useful for producing thicker samples of cellulose for testing. In batch growth it was difficult to produce a layer of cellulose thick enough to be used in experimentation. Batch growth was still used as the main source for cellulose in the following experiments, but presented problems.

Growing Methods:

To survive and grow, *A. xylinum* requires glucose and a variety of nutrients for energy and structure. It was known what ingredients were needed, and in what amount, to produce an optimally growing culture, but it was not known what medium would produce a maximum mass of cellulose. It was hypothesized that at higher glucose concentration, larger amounts of cellulose would be produced due to the glucose dependant metabolic pathway of cellulose production, seen below.

glucose → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose → cellulose

This hypothesis was tested by growing *A. xylinum* in different concentrations of glucose.

Table 1 - The mass of cellulose produced in varying concentrations of glucose at the nominal glucose free media concentration

Glucose Concentration	10 g/L	20 g/L	30 g/L	40 g/L	50 g/L
Mass	17.82 g	17.71 g	15.90 g	16.18 g	17.30 g

These results showed no increase between glucose concentration and mass of cellulose, therefore the hypothesis failed. This data does not disprove the production pathway, but demonstrates that the cells were unable to survive long enough, or well enough, to carry through with producing excess cellulose. The mass measurements of cellulose for the following experiments were taken before water was removed. This was necessary because mass of dried cellulose would have been too small to measure accurately. This experiment proved glucose was not the limiting factor in concentrations over 10 g/L.

The next experiment tested cellulose growth in response to varying concentrations of glucose free media, GFM. GFM consists of the necessary nutrients required in construction of *A. xylinum* cells. The nominal solution of GFM can be seen in appendix 1. This media was what glucose was added to to make a medium capable of cellular growth. Since glucose was not the limiting factor above a concentration of 10 g/L, it was hypothesised that alterations in GFM must generate some change in cellulose production.

Table 2 - The mass of cellulose produced in varying concentrations of GFM with a glucose concentration of 50g/L

Glucose Free Media Concentration (x nominal concentration)	x0.5	x1.0	x1.5	x2.0
Mass	11.63 g	14.93 g	19.58 g	25.54 g

This data shows that variations in GFM did have an effect on the amount of cellulose produced. The data was also seen to take on a positive linear relationship. This linearity cannot be assumed to exist throughout the whole spectrum of possible concentrations of GFM because it is unrealistic to believe growth can occur when the GFM concentration is zero. What can be said, in excess of glucose and above x0.5 nominal GFM concentrations, is mass of cellulose produced increases linearly with GFM concentration. A comparison between the effects of glucose concentration and GFM concentration on cellulose produced can be seen in figure 1. Oxygen availability was held constant during these experiments by using the same size Erlenmeyer flasks and filtering resistances. This made creating a linear equation between GFM and the mass cellulose produced useless outside of these constraints.

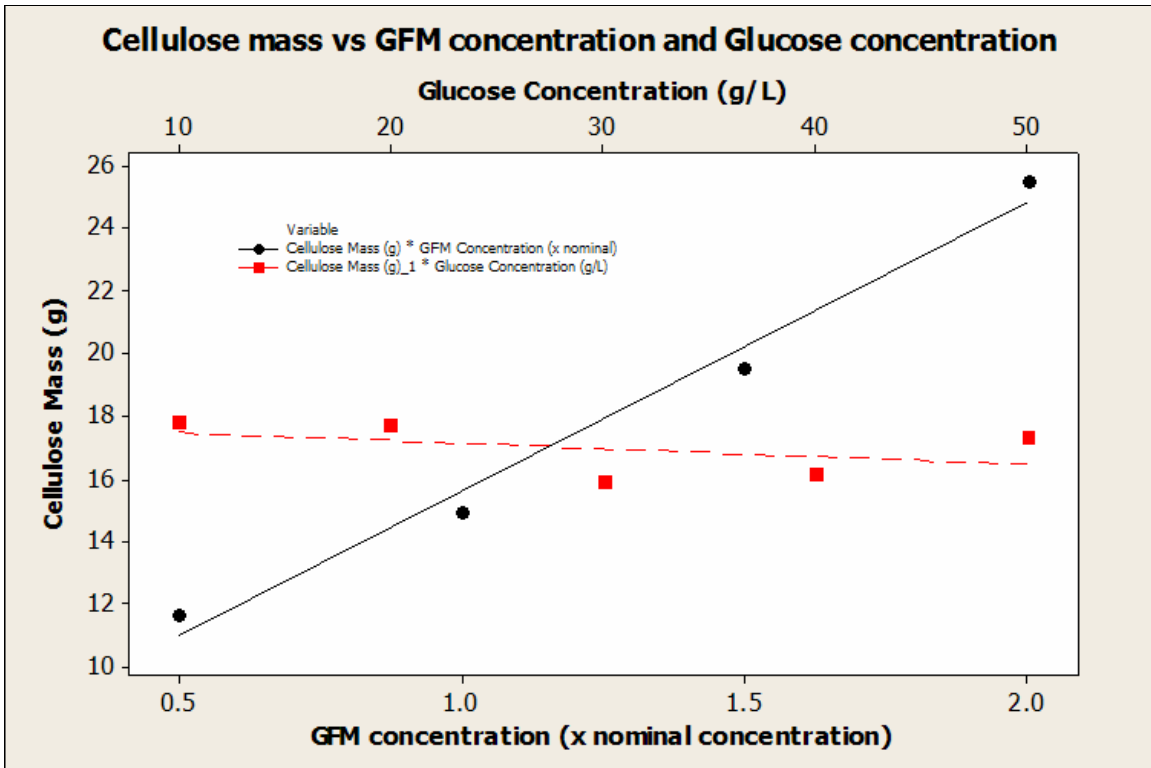


Figure 1 – The compared masses of cellulose under varying glucose and GFM concentrations.

During first experiment testing the concentration of glucose on cellulose production, random large differences in cellulose mass were observed between cultures. After disassembly, it was discovered the amount of cellulose produced correlated directly with the degree of packed cotton used as a filter. Since it was later discovered that glucose concentration has no effect on cellulose production, the data from this experiment was in direct result of oxygen concentration. The results from this experiment can be seen in table 3 and figure 2. The outcome shows how higher filtration of air negatively effects the production of cellulose. Cultures from this point on were exposed to the same level of packed cotton.

Table 3 - The mass of cellulose produced in varying concentrations of glucose and degrees of packing at the nominal glucose free media concentration

Glucose Concentration	10 g/L	20 g/L	30 g/L	40 g/L	50 g/L
Mass	16.25 g	27.17 g	15.87 g	31.03 g	33.21 g
Degree of Cotton Packing	Heavy	Medium	Heavy	Light	Light

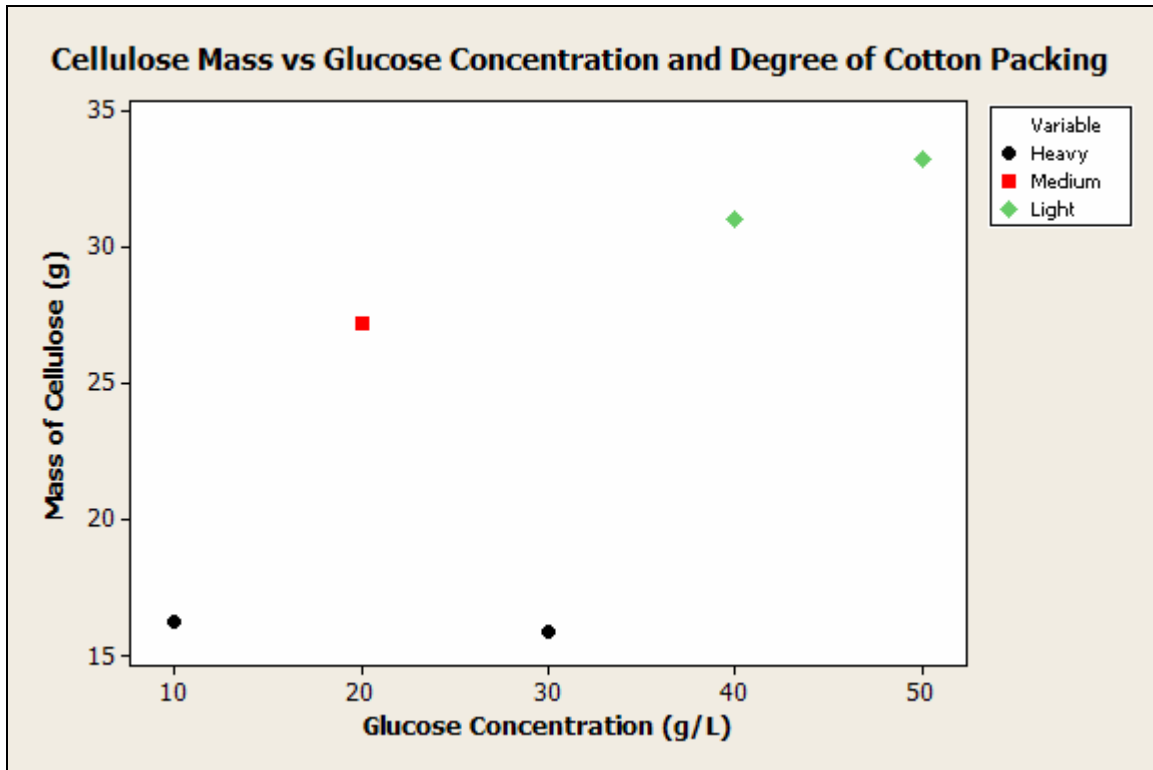


Figure 2 - This figure shows the effect degree of cotton packing had on cellulose growth

Thickness and Treatment Experiment:

This experiment tested the capillary action of dried cellulose at different thicknesses. When sanitizing the cellulose pellicles for testing, half the disks were boiled in 1M sodium hydroxide, when the other half were boiled in distilled water. Microbial cellulose contains a large number of cells within its matrix. A popular method to remove these cells from cellulose requires boiling pellicles in a solution of sodium hydroxide. The strong base, with the aid of heat, loosens the cellulose filaments to allow the embedded cells to be removed (Marx-Figini, 1974). After sanitation, the disks were dried on circular rubber tubing. Once the pellicles dried they were attached to a device capable of holding the membranes at equal heights from the ground. Horizontal cuts were made along the bottom to limit the amount of error created by varying lengths of submerged cellulose. After anchoring, the dried membranes were vertically submerged in dyed distilled water, allowing only the bottom ends to break the surface. After a significant amount of time, equilibrium was reached. The degree of absorbance was determined by measuring the distance dye traveled up the cellulose from the water's surface. The results from this experiment are seen in table 4 and figure 3.

Table 4 – This table shows the absorbance results from the thickness and treatment experiment

Relative Pellicle Thickness	Treatment Method	
	Sodium Hydroxide (NaOH)	Water (H ₂ O)
Light	3.8 mm	1.75 mm
	4.0 mm	1.8 mm
Regular	3.9 mm	1.8 mm
	3.9 mm	1.7 mm
Thick	4.1 mm	1.9 mm
	4.1 mm	1.9 mm

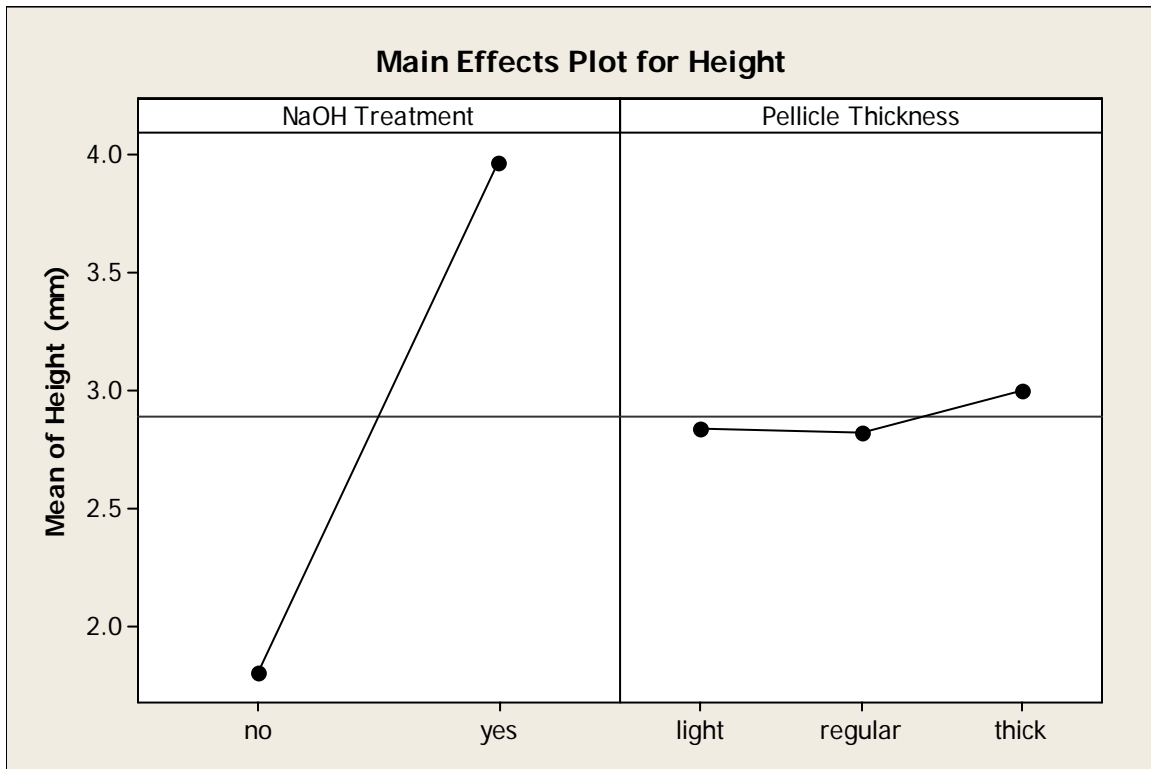


Figure 3 - This mean effect plot shows the difference sodium hydroxide treatment and thickness had on absorbance

The difference in absorbance between treated and untreated cells was tested to discover if the presence of cells had an effect on its absorptive characteristics. This was proven to be true by the doubling of absorptive height in treated pellicles. The lack of response between cellulose thickness and absorptive height shows how thickness has little to no effect on cellulose’s absorptive properties.

The results clearly show how treatment has a more dramatic effect on absorbance than pellicle thickness. To quantify these results, statistical analysis was performed on two null hypotheses: one to test the data’s total similarity, the other to test their directional similarity. The null hypotheses tested for a significance level of 0.01 in both cases. The calculations for these analyses can be seen in appendix 2. The results of the

test showed that the effects of thickness were masked by whether or not cellulose was treated.

Convergence Experiment:

The convergence experiment was performed to test the downward flow of liquid through dried cellulose and to see how converging flows are mixed. The setup, as seen in figure 4, consists of a single film of cellulose formed in the shape of a “T”. Activation height, H , is the height the liquid needed to achieve before the flow directed downward. The mixing region, M , was the area where the reactants mixed before entering the reactive region. The reactive region, R , is where reactive particles would be embedded to catalyze a reaction. In these experiments the reactive region was not incorporated because it was more important to understand the properties of flow before implementing a reaction. This unique arrangement of cellulose allows for two different streams to enter the same membrane through different cellulose films. Qualitative observations were made to obtain results. An example of such an experiment can be seen in figure 5.

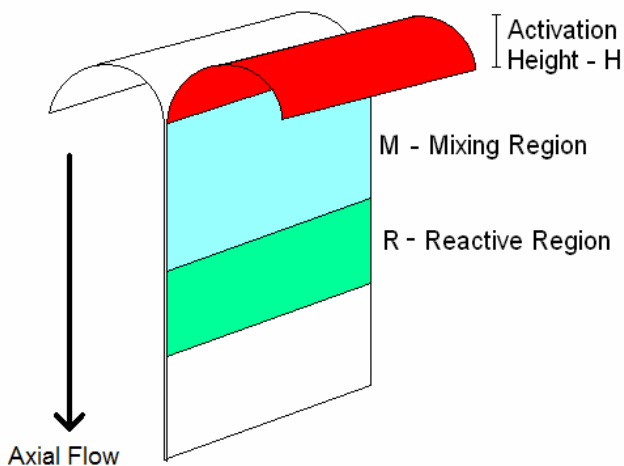


Figure 4 - A diagram of an axial nano-scale laminar flow reactor

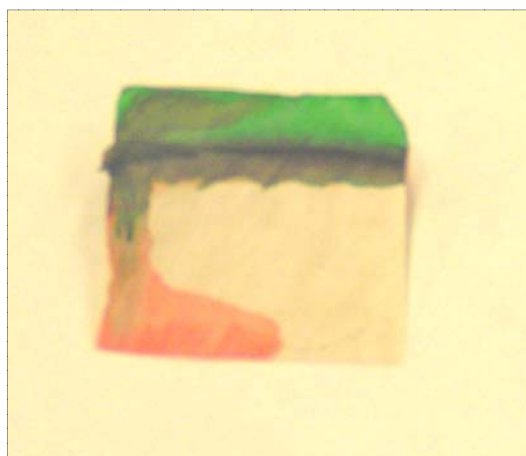


Figure 5 – A picture showing the result of a test of mixing red and green dyed water cellulose streams. The red fin is in front, perpendicular to the photograph. The green fin can be seen flat on

the surface. The mixing region can be seen in a brown color below both fins. Leaking occurred on the left side of the test, but had no effect on the rest of the experiment.

Microbial cellulose does have the capacity to hold over a hundred times its weight in water, but its ability to flow water axially through its matrix is somewhat hindered. The results of this experiment disprove the idea of having an axial laminar flow reactor, but give promise to a reactor that flows in the lateral direction. A diagram of this is seen in Figure 6. What was taken from this experiment was the high degree of mixing that occurred within the mixing region of the reactor. Mixing occurred so immediately that a distance measurement could not be taken. In a lateral nano-scale laminar flow reactor there would be little cellulose the reactant fluid would flow through. The rapid mixing seen in the axial reactor gave evidence to ensure a well mixing lateral flow reactor.

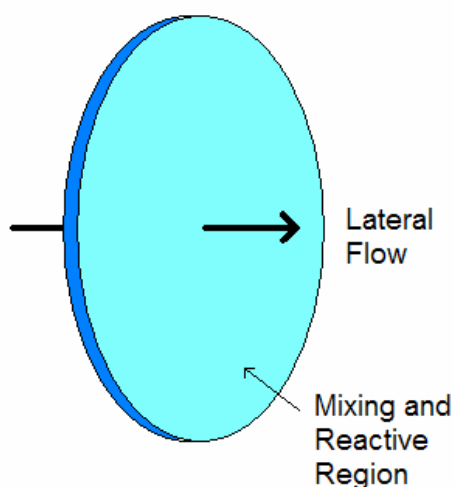


Figure 6 - A lateral flow reactor with no activation height and no discrepancy between the mixing and reactive region

Conclusion

In conclusion, microbial cellulose was seen to have the absorptive properties needed to produce a nano-scale laminar flow reactor. When growing cellulose, it was necessary to produce the thickest pellicles possible. Thick pellicles were needed so slices could be made to form fins for experiments such as the convergence experiment. For optimal thickness, tests were performed on altering glucose and glucose free media concentrations. Capillary action took place axially through a cellulose membrane, but not to the extent of becoming a well functioning reactor. The results of the convergence experiment can be applied to creating a better functioning lateral flow reactor. It was also determined that cellulose thickness perpendicular to fluid flow has no measurable effect on the rate of flow through a cellulose membrane. Through experiment, cellulose purification was also determined to have a large positive effect on cellulose's absorptive characteristics.

Dealing with a living system, uniformity was difficult to maintain. Finding similar pellicle thicknesses for testing usually resulted in a large margin of error. An inefficient

solution to this problem would be to increase batch production. This would enlarge the pool from where cellulose disks could be grouped. There is no ultimate solution to enforce equal growth between living systems. Another major source of error was in the lack of data. More data points would have provided more accurate results. This was the cause of not manufacture enough cellulose samples. At times the incubator was operating at maximum capacity, and with the contribution of contamination or lack of growth, usable pellicles were lacking. Contamination was a substantial when dealing with containers having large openings. During physical manipulation, or sanitation through boiling, several samples of were also lost. This was due to inexperience and improper technique and was solved over time.

By understanding the basic fluid mechanics of flow through bacterial cellulose, further steps can be taken to develop a functional enzymatic cellulose membrane. With more testing, proper differential equations can be made to describe fluid flow through cellulose. Due to the growing properties of *Acetobacter xylinum*, there is ease in embedding particles, such as enzymes or other reactive molecules, into a cellulose membrane. After an enzymatic membrane is constructed, differential equations can be also made to model reactivity. Future plans for this project would involve implementing these new ideas and testing their effect on permeable substrate solutions. Fed batch growth with multiple media additions is a method being developed to automate cellulose growth in a bioreactor. This growing method, coupled with enzymatic cellulose testing, will show promise towards the development of a nano-sacle laminar flow reactor.

Literature Cited

Brown, Malcolm R. "Microbial Cellulose: A New Resource for Wood, Paper, Textiles, Food and Specialty Products" Position Paper, University of Texas at Austin, Austin, Texas

Marx-Figini, M., and Pion, B.G. "Kinetic Investigations on Biosynthesis of Cellulose by *Acetobacter xylinum*", *Biochimica et Biophysica Acta*, Vol. 338, pp. 382-393. (1974)

Mormino, R. Gostomski, P., and Bungay, H. "Plate and disk bioreactors for making bacterial cellulose" in *Biological Systems Engineering* M. R. Marten, T. H. Park and T. Nagamune ACS Symposium Series 830, (2002).

Appendices

Appendix 1 – The ingredients used to produce the nominal glucose free media (GFM) concentration.

Component:	Amount (g, ml):
Ammonium Sulfate	5.0 g
Sodium Phosphate (monobasic)	2.7 g
Magnesium Sulfate	0.1 g
Yeast Extract (Sigma)	0.5 g
Bactopeptone	0.5 g
Citric Acid	1.2 g
Trace Element Solution	2.0 ml
Cycloheximide Solution	10.0 ml
DI Water	900.0 ml

Appendix 2 – The testing of the null hypotheses to a 0.01 significance level

	Number of test samples	Average Value	Standard Deviation
Treated	$m = 6$	$\bar{x} = 3.9667$	$\sigma_m = 0.1211$
Untreated	$n = 6$	$\bar{y} = 1.8083$	$\sigma_n = 0.0801$

Total Similarity Test:

Null hypothesis $H_0 : \mu_m - \mu_n = 0$

Alternative hypothesis $H_a : \mu_m - \mu_n \neq 0$

For a significance level $\alpha = 0.01$, H_0 will be rejected if $z \geq 2.58$ or $-2.58 \geq z$

Directional Similarity Test:

Null hypothesis $H_0 : \mu_m - \mu_n = 0$

Alternative hypothesis $H_a : \mu_m - \mu_n > 0$

For a significance level $\alpha = 0.01$, H_0 will be rejected if $z \geq 2.33$

$$z = \frac{\bar{x} - \bar{y}}{\sqrt{\frac{\sigma_m^2}{m} + \frac{\sigma_n^2}{n}}} \quad z = \frac{3.9667 - 1.8083}{\sqrt{\frac{0.1211^2}{6} + \frac{0.0801^2}{6}}} = 36.413$$

With $z = 36.413$ calculated, the P value ≤ 0.001 in both cases. The P-value being the probability of rejecting the null hypothesis when it is true (Type I error).