COMBINED FERMENTATION AND RECOVERY USING EXPANDED BED CHROMATOGRAPHY

A Thesis

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by

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ABSTRACT

Expanded Bed Chromatography (EBC) is rapidly becoming the preferred choice for initial product recovery from crude process streams as it enables direct protein recovery from culture broths after appropriate dilution. However, the process is time intensive, and there are still some difficulties with very high cell density cultures in the 500 g/L range. Problems include in-column clogging and poor column efficiency. With the development of a new prototype EBC column capable of product recovery from undiluted culture broth, it is proposed in this study to combine the fermentation with EBC recovery. This strategy was tested using a wild type, non-producing strain of *Pichia pastoris*. Culture broths were spiked with 200 mg/L lysozyme to mimic actual production fermentation. Key parameters for the process were identified and tested independently to better assess system performance: potential toxic effects of the resin on the culture, nutrient deprivation of the cells as they pass through the column and binding of the target protein from whole broth. The cation exchanger had a negligible effect on cell proliferation in shake flask studies using YNB Medium. Isolation of the culture from the fermenter for up to two hours appeared to have minimal effect on overall cell viability and the ability to metabolize methanol. The dynamic binding capacity for lysozyme was 50 mg/mL in buffer, and 20 mg/mL in undiluted fermentation broth containing 500 g/L cells. When harvested undiluted fermentation broth was allowed to recirculate through the EBC column, the binding capacity was increased to 30 mg/mL. The combination of the fermentation and recovery process allowed for a binding capacity of 30-40 mg/mL, with no dramatic effects on biomass accumulation or metabolic rate.

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LIST OF ABBREVIATIONS

ΔA	Change in Absorbance
AF	Anti-foam
AOX1	Alcohol Oxidase 1
BMGY	Buffered Minimal Glycerol Complex Medium
BSM	Basal Salt Media
CHO	Chinese Hamster Ovary
CMHZ	CM Hyper-Z
DBC	Dynamic Binding Capacity
DO	Dissolved Oxygen
EBC	Expanded Bed Chromatography
EFT	Estimated Fermentation Time
OD	Optical Density
PI	Isoelectric Point
PSI	Pounds per Square Inch
RPM	Rotations Per Minute
TFF	Tangential Flow Filtration
YEP	Yeast Extract Peptone
YNB	Yeast Nitrogen Base

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INTRODUCTION

Protein manufacturing via transgenic organisms has become an important and growing business in today's society. As the demand for additional pharmaceutical and industrial proteins increases, new technology must be developed to make the recovery of these proteins both more economical and efficient

Pichia pastoris, a methylotrophic yeast, has been developed into a heterologous protein expression system (Higgins and Cregg, 1998). *Pichia* combines many of the benefits of *E. coli* expression, such as high biomass and yield, with the advantages of expression in a eukaryotic system (Wegner, 1990). Eukaryotic systems allow for more advanced post-translational modifications, such as glycosylation, disulfide-bond formation and proteolytic processing (Cregg *et al*., 2000). Also, protein expression levels can be high, with levels as high as 14.8 g/L of clarified supernatant reported (Werten *et al*., 1999). *Pichia* may be grown at a pH range of 3-6.

One of the two genes which encode alcohol oxidase in *P. pastoris* is the AOX1 gene, the expression of which is controlled at the level of transcription (Lin Cereghino and Cregg, 1999). When the cells are grown on methanol, approximately 5% of poly(A)$^+$ RNA is from AOX1, however the AOX1 message is not detectable when grown on other carbon sources (Cregg and Madden, 1988). When the yeast are grown on methanol as the sole carbon source, alcohol oxidase may contribute up to 35% of the total cellular protein (Couderc and Baratti, 1980). The *Pichia pastoris* system has been genetically engineered so that an upregulation in the AOX1 promoter causes an induction of a target foreign protein.

Despite the fact that methanol serves as a carbon source for *P. pastoris*, if allowed to accumulate it is toxic to the cells. For this reason the methanol feeds are well defined, so as not to cause significant stress to the yeast. Studies have shown that 10 g/L methanol causes only a slight lessening in specific growth rates; growth is not fully inhibited until 30 g/L (Katakura *et al.,* 1998; Charoenrat *et al.,* 2005). Methanol levels are generally maintained at a nearly undetectable level such that as a drop of methanol is added to the culture, it is immediately consumed. A simple test for this can be done using what is referred to as a DO (dissolved oxygen) spike test. If the culture is truly limited on methanol, i.e. there is no residual methanol in the fermenter, than an interruption of the methanol fed will cause a rapid and immediate spike in DO since no carbon source is available. If residual methanol is present, than an interruption of the feed will have no effect on DO.

In addition, the presence of too much molecular oxygen in culture can lead to significant stress on the cells. In culture medium, molecular oxygen may form several reactive oxygen species: superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), or hydroxyl radicals ($HO^{\bullet}$) (Tran *et al*., 1993). These reactive oxygen species attack nearly all cell parts and cause DNA strand breaks, as well (Cantoni *et al*., 1986; Tran *et al*., 1993). Other stressing factors which may also lead to the formation of reactive oxygen species within the cell (Hristozova *et al.,* 2002) include osmotic stress, hypoxic conditions, excessive transcription of the target protein, carbon starvation, change of carbon sources, temperature and pH changes, or toxic chemicals (Gasser *et al*., 2006; Sinha *et al.,* 2005; Shi *et al*., 2003; Vijayasankaran *et al.,* 2005). The perennial difficulty when working with *P. pastoris*, or any other recombinant microorganism, is to moderate

the negative effects caused by culture conditions with the positive effects those conditions have on the production and quality of the target protein. Consequently, specific protein production per cell is often at odds with an increased cell count within the fermenter; usually neither are at their maximal level, since the two aspects must be balanced to create a maximal protein titer.

Pichia pastoris fermentations can reach very high cell densities, with wet weights nearing 500 g/L and viscosities higher than 40 mPas (Anspach et al, 1999). Traditional recovery methods which have been used include tangential flow filtration (TFF) and centrifugation. Because of the high solids concentration and ionic strength of the fermentation broth, the broth must be diluted and run at a low flow rate; consequently these processes are relatively slow and inefficient. In addition, during centrifugation a significant amount of broth is lost with subsequent loss of product; with TFF the membranes are easily plugged by a layer of cells and cell debris, leading to a decrease in trans-membrane flux, and thus low flow rates and high backpressures.

It has been proposed that replacing the traditional steps involved in cell separation and product recovery with expanded bed chromatography (EBC) will increase the efficiency of purification. While traditional chromatography generally uses a packed bed design, EBC employs a specialized resin to adsorb the target protein while in a fluidized bed (**Figure 1**). The resin is designed to have a high density and smaller radius than many resins used in other chromatography applications. This alteration of physical properties allows for the beads to remain suspended and uniformly distributed in the media, without sinking or being washed away.

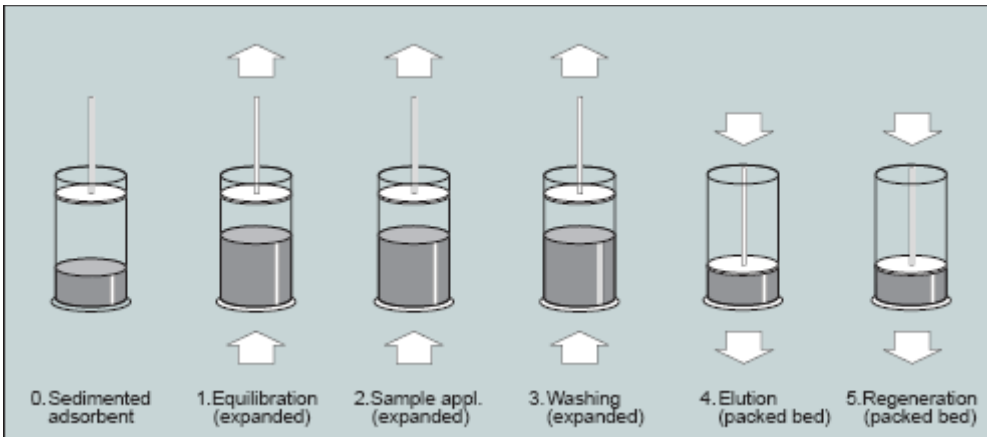

Figure 1 - Expanded Bed Chromatography Steps (Amersham Biosciences)

The high cell densities achieved in *Pichia pastoris* fermentations also have the potential to cause adverse effects in an EBC column. The high cell densities can cause clogging of the distributor and frit entering the EBC column, leading to uneven flow and poor adsorption in the column.

Numerous techniques and alterations in design have been used to address the difficulties noted in EBC operation. One of the most common and successful methods has been to increase the density of the chromatography resin by using zirconium oxide (bead density of 3.2 g/ml) as a backbone which allows for an increased feedstock density (Charoenrat *et al*., 2006). This resin also utilizes a higher charge density as shown in **Figure 2**, by creating a hydrogen matrix containing the binding sites, thereby improving mass transfer and allowing for a high fluidization velocity (Shiloach et al, 2003). This resin, which was used throughout this study, is currently marketed as CM-Hyper-Z and is available from Pall Corp. Another common approach has been to alter the basic design of the EBC column from having the entire incoming stream pass up through the distributor and into the column, to using a tangential flow type design which circulates broth under the distributor, causing a scrubbing effect and preventing debris from accumulating under the distributor. This column will be referred to as the "T-column"

(Biseps Inc., Monroe, WA) and was used throughout this study. Operation of the T-column is as described in **Figure 1**.

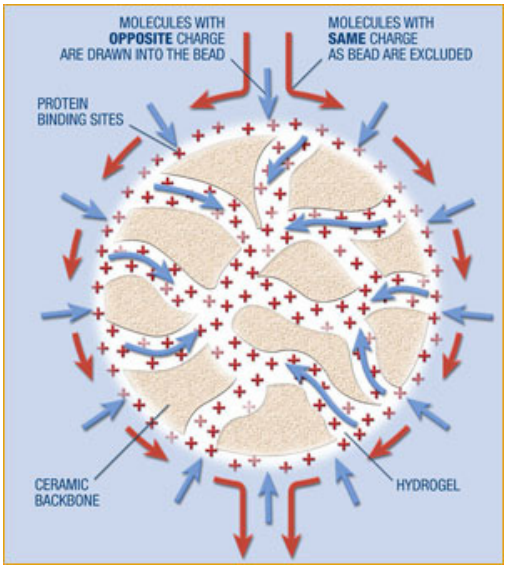

Figure 2 - Expanded Bed Chromatography Bead (Pall Corporation)

The concept of coupling a fermentation with a recovery column is not new, however, coupling a column with a high cell density fermentation in which the cells are recycled has never been recorded in the literature. Other systems, where the cells are not recycled, are more common. One of these systems was used by Charoenrat *et al*., (2006) where the EBC Streamline^{tm} column, from GE EBC was used to compare a newer, denser, resin to an older resin, and were able to improve their binding efficiency using unclarified broth by 25%. They were unable to run undiluted whole broth however and instead diluted 450 g/L fermentation broth to 246 g/L. Brobjer *et al*., (1999) performed an EBC separation on a dense culture of crude *Escherichia coli* homogenate. In other systems where the cells and products are considered to be more valuable, such as mammalian cell culture, undiluted hybridoma cells have been passed through a fluidized bed to absorb monoclonal antibodies but these were not recycled back to the production fermenter (Erickson *et al.,* 1994; Lütkemeyer *et al*., 1999). Another system used in

mammalian cell culture is the perfusion reactor, where the cells are actively retained in the bioreactor using a membrane separation, while cell-free product is removed. A separate stream is typically drawn off to help remove any dead cells. Perfusion reactors are similar to a combined fermentation and recovery system, but do not allow for cells to pass through the adsorbent, and instead only pass spent media through. A great deal of research has been performed to increase the perfusion system (Gray *et al*., 1996; Hu and Adams, 1997).

Even with the advantages EBC provides for recovery of fermentation products, one of the main issues which must be addressed is the extended time required to perform a single EBC process (up to 24 hrs). Since the T-column allows for capture and recovery from undiluted cell broth, the fermentation step may be combined with an EBC cation exchange step without adverse effects on the culture.

The purpose of this study was to determine the feasibility of combining an active fermentation with product recovery by recycling cells back to the fermenter after passing through the EBC column. A non-producing strain of *P. pastoris* was used and during methanol induction, the culture was spiked with a known amount of lysozyme. Lysozyme was used due to the high isoelectric point, and that it has a well-characterized assay. Studies were performed to isolate and test individual parameters which were expected to cause adverse effects on the culture. The individual parameters examined were (1) the effect of the CM-Hyper-Z resin on the cells; (2) the effect on the culture of nutrient deprivation caused by passing out of the fermenter and through the EBC column; and (3) the determination of the binding capacity for lysozyme under test-tube or static, dynamic or flow-through, and recirculating binding conditions.

MATERIALS AND METHODS

The chemicals and gasses used were of analytical grade whenever available and were obtained from Sigma, Fisher Scientific, or Merriam Graves. CM-Hyper-Z resin (CMHZ), manufactured by Pall Corporation, was the sole resin used throughout these experiments. Reagent grade, 18 Mohm, de-ionized water was used for all experiments.

1. *Pichia pastoris*

An empty-vector parent strain of *Pichia pastoris*, X-33, was obtained from Blue Sky Biotech, Inc. (Worcester, MA). Cryostocks were generated by taking the original sample and inoculating into 40 mL of BMGY media, in a 250 mL Erlenmeyer flask for 11.5 hours at 28°C and 200 RPM. The 40mL of liquid was then divided equally into two 2L Erlenmeyer flasks, with 500 mL BMGY media in each of them, and grown at 26°C for 15 hours, until the OD_{600} reached 11.48. The cells were harvested aseptically and centrifuged at 4,000 RPM for 20 minutes in pre-sterilized 500 mL centrifuge bottles in a Beckman J2-21 centrifuge, with a JA-10 rotor at 4°C. The cell pellet was resuspended in an equal volume of sterile 80% glycerol, and divided into sixty cryovials each containing 0.5mL apiece. The cryovials were stored until used in a -80°C chest freezer (Thermo Electron Corp, model #5472, Waltham, MA).

2. Fermentation Procedure

2.1. Operation of the Bioflo 3000 Fermenter

A typical *P. pastoris* fed-batch fermentation was performed as follows:

1. The desired volume of BSM was prepared and introduced into the reactor. DO and pH probes, a nutrient feed port, a line for air and oxygen feeding, a harvest line, a sampling port, as well an agitator motor of the correct size for the fermentation vessel were attached to the fermenter.
2. The reactor was sterilized at 15 PSI for 35 minutes. Any lines which might allow air in or out were clamped.
3. The night prior to the experiment the pH of the medium was adjusted to 3.0 with concentrated ammonium hydroxide (NH_4OH), the DO probe was connected to the instrument power in order to polarize and stabilize the signal, and the temperature was set to 30°C.
4. The following morning the pH was adjusted to 4.5 using concentrated NH_4OH.
5. With the aeration to the vessel activated, a 20 min. time interval was allowed to pass for the NH_3 fumes to clear from the vessel headspace. After this time, the trace metal solution was added (PTM1 salts) at 4.35 mL/L and 0.435 mL/L of 0.4 g/L D-biotin was also added to the fermenter(s).
6. The DO probe was calibrated to 100% once the DO signal stabilized.
7. When applicable, the resin was placed aseptically into the fermenter.
8. The fermenter was inoculated.
    a. *P. pastoris* was grown in batch culture until all of the fixed-carbon (glycerol) in the fermenter was consumed, which was usually 20-24 hours. The pH was maintained at 4.5 using concentrated NH_4OH and the Bioflo

3000's control system, and the DO concentration was maintained at 40% - 50% saturation.

b. KFO™ 880 anti-foam (Kabo Chemicals, Inc., Cheyenne, WY) was used to control the foaming within the fermenter.

9. A 50% glycerol solution was fed at $18.15\frac{mL/hr}{L_{InitialVolume}}$ for four hours. The glycerol feed contained 12 mL/L PTM1 and 1.2 mL/L of 0.4 g/L biotin.

10. After glycerol feeding, methanol feed was introduced to the culture and the pH set point was decreased to 3.0, mimicking a typical Pichia process. In this way, the metabolism of the culture would drop the pH gradually to the set point of 3.0. The methanol feed also contained 12 mL/L PTM1 and 1.2 mL/L of 0.4 g/L biotin.

    a. Methanol feeding was introduced at a low feed rate initially to allow the culture to adapt. $3.63\frac{mL/hr}{L_{InitialVolume}}$ of pure methanol for a minimum of two hours was fed, or until a successful dissolved oxygen (DO) spike is obtained. A DO spike is determined by stopping the nutrient feed, in this case methanol, and measuring the time required for the DO to rise at least 20%. If the DO rises by at least 20% in under a minute, the spike is considered successful.

    b. The flow rate was the increased to $7.26\frac{mL/hr}{L_{InitialVolume}}$ methanol for at least one hour, or until a successful DO spike was obtained.

c. The flow rate was increased again to the maximum feed rate of $10.89 \frac{mL/hr}{L_{InitialVolume}}$ methanol for the remainder of the experiment.

11. In the recirculation, and combined fermentation and recovery experiments, 46 hours after beginning methanol feeding, an Expanded Bed Chromatography separation was performed using the Bioseps, Inc. 1.1 cm EBC T-column. See Methods section (3.1) for the operational procedure to run the EBC.

3. Expanded Bed Chromatography (EBC)

3.1. Operational Procedure

A 1.1 cm diameter T-column, manufactured by Biseps Inc. (Monroe, WA), was used for all EBC studies. The column was cleaned by passing at least 10 column volumes of 1 M NaOH through in up-flow mode, followed by sufficient deionized water to remove all NaOH until the pH at the column outlet was less than 9.

Prior to the introduction of culture broth, the column was equilibrated with 20 mM sodium citrate buffer, pH 3.0 in up-flow mode until the resin bed height had equilibrated and the effluent pH was 3.0. The sample (containing lysozyme) was then applied by switching the feed tube from the equilibrating solution to the sample solution. For samples with a high density, the flow rate through the column was lowered in order to prevent overexpansion and maintain column expansion at approximately 2X. After the sample load was complete, the column was washed with 20 bed volumes of 20 mM sodium citrate buffer, pH 3.5 in up-flow mode to remove solids and weakly bound proteins. The resin was then allowed to settle, and the column was further washed with 7

bed volumes of 20 mM sodium citrate buffer, pH 3.5 in down-flow mode. Early EBC runs utilized an application of 10 bed volumes of 20 mM tris-acetate buffer, pH 7.5 in down-flow mode in order to remove undesired proteins from the resin. In those runs, to recover the lysozyme, 20 mM tris-acetate buffer with 1 M NaCl, pH 9.5 was applied followed by 20 mM tris-acetate buffer with 2 M NaCl, pH 9.5 in order to collect all of the lysozyme from the resin. Later EBC runs used 40 mM, pH 7.5 tris-acetate buffer in order to remove the undesired proteins, and 40 mM tris-acetate buffer with 1 M and 2 M NaCl, pH 8.5 in order to collect the lysozyme from the resin since it was found that lysozyme stability was affected above pH 9.

Following the removal of the lysozyme from the resin, the column was washed employing the original cleaning procedure. When not in use the resin was stored in 20% ethanol.

4. Determination of Resin Effects upon Cells

4.1. Sterilization Procedure

The CM Hyper-Z resin was sterilized by rinsing in several changes of sterile dH_2O in a pre-sterilized flask, followed by incubation in 1 M NaOH for one hour, and then rinsed with sterile dH_2O until a pH measurement of less than 9 verified removal of the NaOH.

To sterilize the 0.5 mm glass beads, the beads were rinsed, placed into a sealed Erlenmeyer flask, and autoclaved for a minimum of 30 minutes at 121°C.

All flasks containing media were autoclaved for a minimum of 30 minutes at 121°C prior to the addition of resin or glass beads.

4.2. Maceration Effects of the Resin on *P. pastoris* in a Bioflo 3000 Fermenter

To determine the effect of the resin on cell growth, an experiment was performed in two 5 L Bioflo 3000 fermenters (New Brunswick Scientific, Edison, NJ). One reactor was used as a control, and run as previously described for 72 hours. Into the other fermenter, was placed 250 mL of resin just prior to inoculation. Later, additional PTM1 solution was added to the fermenter containing the resin to determine if the resin was adsorbing the heavy metals.

4.3. Maceration Effects of the Resin on *P. pastoris* in Shake Flasks

The seven flasks prepared contained the following in 500 mL Erlenmeyer flasks:

1. 85 mL YNB media.
2. 115 mL YNB media.
3. 100 mL YNB media; 15mL CMHZ resin.
4. 100 mL YNB media; 15mL CMHZ resin; 20mg lysozyme.
5. 100 mL YNB media; 15mL CMHZ resin; 20mg lysozyme.
6. 100 mL YNB media; 15mL glass beads.
7. 100 mL YNB media; 15mL glass beads.

A 10 mg/mL stock solution of lysozyme was made in 100 mM phosphate buffer, pH 7.0 and filter-sterilized (Cameo 25GAS, 0.22 μm pore size), and 2 mL of this stock solution was aseptically pipetted into flasks #4 and 5. Flask #1 was inoculated the night before with *P. pastoris* and grown up for 18.5 hours. It was used to inoculate flasks #2, 3, 4 and 6 with 10 mL each. Flasks #5 and 7 were not inoculated and were left sterile to

act as a sterility control. The 0.5 mm glass beads were used to test the maceration effects on the cells, since glass beads are approximately the same size, but unlike the resin, will not absorb nutrients under the experimental conditions used. The flasks were incubated for two days at 28°C and 200 RPM. Cell counts were performed using a Reichert hemocytometer (Bright-Line, Buffalo, NY). A 0.1 mL droplet of medium was placed onto the hemocytometer, at a dilution of $1:10^5$, and the number of counted cells was multiplied by 10^4 in order to calculate the cells/mL concentration. All counts were performed in at least triplicate.

Separate experiments were also performed using YEP plates. In these experiments, an additional flask was prepared in the same manner as flask #3. This flask was inoculated at the same time as the other flasks, but the resin was added 10 hours after flask inoculation. The samples were plated on YEP plates at dilutions of $1:10^{-7}$, 10^{-8} and 10^{-9}. All platings occurred in duplicate.

5. Determining the Effect of Nutrient Deprivation on *P. pastoris*

5.1. Operational Setup of the System

Nutrient deprivation while the cells were away from the primary fermenter had the potential to have a large negative effect on cell viability. To isolate the possible effect of nutrient deprivation on *P. pastoris*, another, smaller, fermenter was added into a loop together with the main fermenter. The main fermenter was a 5 L Bioflo 3000 fermenter containing 3 L of BSM media initially. The second fermenter was a 1 L Bioflo 3000 fermenter which was maintained at a fluid volume of 1 L. The fermenters were connected by #16 Masterflex tubing and fluid was circulated between the fermenters using a Cole-Parmer Instrument Company Masterflex pump (Vernon Hills, Illinois) at

140 mL/min between the fermenters, in order to ensure that the DO and pH in both fermenters were maintained at similar levels. Both fermenters were fed, aerated, and controlled at the DO, pH and temperature set points until 20 hours after the start of methanol. At this point, all controls other than agitation and temperature, including aeration and the methanol feed, to the 1 L fermenter were terminated. Residence time studies were performed where the average time fermentation broth spent away from the 5 L fermenter was 7.5, 60, or 130 minutes. A control was also run where the 1 L fermenter was aerated and fed methanol throughout the entire experiment.

During the course of the residence time studies, both the wet weight and CO_2 outputs were monitored in order to determine if the yeast were negatively affected. The CO_2 was monitored using an EX-2000 on-line gas analyzer (New Brunswick Scientific, Edison, NJ).

6. Determination of the Lysozyme Binding Capacity

6.1. Protocol for Determination of Lysozyme Concentration

A 0.3 g/mL *Micrococcus lysodeikticus* substrate solution was prepared in 0.1 M phosphate buffer, pH 7.0. A Beckman DU530 spectrophotometer was set to 450 nm and was zeroed using a macro (1 cm path) visible light transmitting cuvette containing 3 mL phosphate buffer.

To test samples, 2.9 mL of substrate solution was placed into a macro visible-light cuvette and allowed to equilibrate at ambient temperature. The reaction was begun by adding 100 μL of the enzyme solution to the cuvette and mixing gently, but thoroughly, with the pipette tip. The spectrophotometer was then closed and the timer started. Once the timer was running the OD_{450} was recorded every 15 seconds for a total of 2 minutes.

The assay tests for a decrease in absorbance as the lysozyme, an enzyme, breaks down the cell walls of *Micrococcus lysodeikticus*.

If the reaction velocity was not between 0.02 and 0.04 absorbance units/minute the solution was diluted until it fell within that range and the assay run again. The reaction velocity (ΔA/min) was calculated for all the possible one-minute intervals, and the initial reaction velocity, the largest ΔA, was used for further calculations. The units/mL of the original sample was calculated using the following formula, where the volume assayed was 100μL, and by definition, 1 unit is a ΔA of 0.001/minute:

$$UnitsAssayed = \frac{InitialVelocity}{0.001}$$

$$\frac{Units_{Undiluted}}{mL} = \frac{\left(Units_{Assayed}\right)\left(DilutionFactor\right)}{Vol_{Assayed}}$$

Equation 1 - Activity of Lysozyme

6.2. Effect of Basal Salt Media on the Binding Capacity of the Resin

In order to determine the effect of the basal salt media on the binding capacity of the CMHZ resin, an experiment was performed testing each of the individual media components separately. Two sets of seven 100 mL samples were prepared, each at the same concentrations as in the BSM media:

1) H_3PO_4 (392 mM), pH adjusted to pH 3.0 with KOH.
2) H_3PO_4 (392 mM) and $MgSO_4 \cdot 7H_2O$ (153 mM), pH adjusted to pH 3.0 with KOH.
3) H_3PO_4 (392 mM) and $CaSO_4$ (392 mM), pH adjusted to pH 3.0 with KOH.
4) H_3PO_4 (392 mM) and K_2SO_4 (104 mM), pH adjusted to pH 3.0 with KOH.

5) H_3PO_4 (392 mM) and trace salts to 10 μL/mL, pH adjusted to pH 3.0 with KOH.
6) Basal salt media without glycerol.
7) 20 mM sodium citrate buffer (control).

With the exception of trial 7, 1 mL of each solution was pipetted into a 2 mL Eppendorf microfuge tube containing 0.5 mL of settled resin and 15 mg of lysozyme. Trial 7 did not include resin and served as a control. The tubes were inverted every half hour for two hours, and then assayed for lysozyme as previously described.

6.3. Test-tube Binding Capacity

A stock solution of 5 mg/mL lysozyme was prepared in 20 mM sodium citrate buffer, pH 3.3. A combination of the stock solution and 20 mM sodium citrate buffer, pH 3.3 were put into 2 mL Eppendorf tubes with 100 μL of settled resin, and put on a Clay Adams Nutator Mixer (BD Diagnostic Systems, Parsippany, NJ) for two hours. Tubes were adjusted so that they had 0, 5, 10, 25, 50, or 75 milligrams of lysozyme per milliliter of resin. After allowing the resin to settle, the supernatant in the tube was tested to determine binding capacity.

6.4. Dynamic Binding Capacity in Whole Fermentation Buffer

The dynamic binding capacity of a system is the amount of target protein the system will bind under actual flow conditions before significant breakthrough of unbound protein occurs. It is considered more reliable for predicting real process performance than static binding capacity. Significant breakthrough is defined on a case-by-case basis, and thus dynamic binding capacities are often accompanied with a context value, such as

stating it is at 2% breakthrough, signifying that 2% of the target protein entering the system is leaving unbound.

To determine the binding capacity in buffer, after setting up the EBC column with a resin bed depth of 19 cm, a stock solution of 5 mg/mL lysozyme in pH 3.1, 20 mM sodium citrate buffer was passed through the column at multiple flow rates in order to determine the dynamic binding capacity. Stock solution was passed through the column at 250 and 347 cm/hr, which corresponds to 3.9 and 5.4 mL/minute, respectively. These flow rates resulted in overall bed expansion of 1.6 and 2.0-fold, for the 250 and 347 cm/hr trials, respectively. Binding experiments were performed until significant breakthrough was detected.

6.5. Dynamic Binding Capacity in Whole Fermentation Broth

To determine the binding capacity using fermentation broth, the EBC column was set up with a resin bed depth of 15 cm, and fresh fermentation broth was harvested and spiked to 200 mg/L with lyophilized commercial lysozyme. The fermentation broth had a wet weight of 476 g/L. The spiked fermentation broth was applied to the EBC column, which had been pre-expanded with pH 3, 20 mM sodium citrate buffer. Initially, the sample was applied at 6 mL/min, but this was later reduced to 4.9 mL/min, to maintain the bed expansion between 2-3X. Binding experiments were performed until significant breakthrough was detected.

6.6. Recirculating Binding Capacity in Fermentation Broth

The binding capacity in recirculation mode was tested in order to determine the saturation point of the resin, as well as to simulate and prepare for conditions to be tested

in later experiments when the active fermentation was coupled to the recovery column. A 3 L batch of *Pichia pastoris* was grown under normal conditions in a 5 L fermenter, and attained a final wet weight of 424 g/L before the run was ended, and all controls other than temperature and agitation were terminated. Prior to terminating the fermentation, the EBC column was prepared by equilibrating and expanding the resin in upflow mode with 20 mM sodium citrate buffer, pH 3.0 flowing at 10 mL/min. The fermentation broth was then applied from the fermenter to the column at 3 mL/min. All fluid leaving the EBC column, prior to cells exiting the column, was discarded. Once cells began exiting the column, the EBC exit tube was reattached to the fermenter and all material leaving the EBC was allowed to return to the fermenter.

A sample was removed from the experiment approximately every 30-60 minutes and assayed for lysozyme activity as described in Materials section 6.1 on page 14.

Originally the temperature was maintained at 30°C; however the temperature was lowered to 20.5° approximately 6 hours into the binding experiment because it was thought that lowering the temperature could possibly increase binding. The binding experiment was allowed to run a total of 30 hours or until the lysozyme activity in the fermenter remained constant, signifying that no more lysozyme was being removed from the broth by the resin.

7. The Combined Fermentation and Recovery System

7.1. System Set-up and Operation

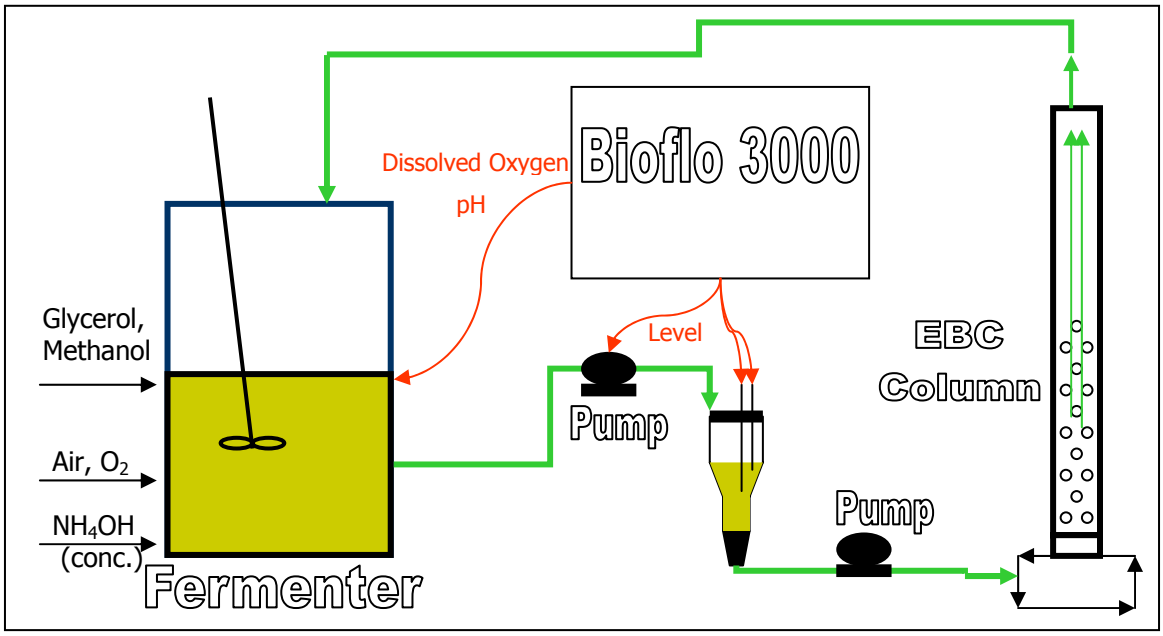

Figure 3 - Schematic of Operating System

The fermenter was set up, inoculated, and run as described in Methods section 2.1 throughout batch phase, and glycerol fed-batch. Forty six hours after starting the low feed-rate of methanol, lysozyme was added to a concentration of approximately 200 mg/L.

The 1.1cm EBC column was prepared by equilibrating and expanding the resin in up-flow mode with pH 3, 20 mM sodium citrate buffer flowing at 10 mL/min, which maintained a bed expansion of 2-2.5-fold.

Figure 3 shows the schematic of the system used in these experiments. The fermentation broth was pumped out of the fermenter (indicated by green line) into a small container, which had a holding volume of 20 mL. The holding container was used to allow the entrained gasses in fermentation broth to escape so as not to introduce air into the EBC column. Introduction of bubbles into the EBC would pose a significant bed

stability problem. A level controller, controlled by the Bioflo 3000, was placed into the holding container to ensure that the liquid level was maintained. A Cole-Parmer peristaltic pump was used to recirculate the fermentation broth through the EBC column. This loop served to keep the mesh at the bottom of the column from clogging up. As the broth was then pumped into the EBC, and as soon as cells began to enter the column, the feed rate was reduced immediately to 3 mL/min, a previously determined flow rate which would give a 2-2.5-fold expansion. All fluid leaving the EBC column, prior to cells exiting the column, was not recirculated to the fermenter. Once cells began leaving the column, the EBC exit tube was reattached to the fermenter and all material leaving the EBC was allowed to return to the fermenter. The fluid exiting the EBC column was returned to the fermenter via gravity. The residence time outside of the bioreactor (including the column) was reduced from 60 minutes, to 30 minutes, due to improvements made on the design of the air removal trap. Throughout the entire experiment, the fermenter was maintained under optimal conditions as described in Methods section 2.1.

Samples were taken every 1-3 hours and centrifuged 4,000 RPM for 20 minutes in a Beckman J2-21 centrifuge, with a JA-18 rotor at 4°C. The pellet and supernatant were separated, and the wet weight was determined. In several experiments, the CO_2 concentration in the off-gas was also determined as described in Methods section 5.1. The experiment was conducted for 24 hours, and then all feeds and controls, with the exception of temperature and agitation, were terminated. The temperature control was lowered to 18°C, and the agitation was lowered to 200 rpm. The culture was recirculated

for an additional 20 hours, in order to determine if the resin had been saturated, as well as to monitor the fermentation.

7.2. Lysozyme Recovery

To recover the lysozyme from the resin, the resin was first washed in up-flow mode with 20 mM sodium citrate, pH 3.5 until all traces of cell debris were removed from the column. The resin was then allowed to settle, and was washed in down-flow mode with 250 mL of 20 mM sodium citrate buffer, pH 3.5 and sequentially in down-flow mode, the resin was washed with 20 mM tris-acetate buffer, pH 7.5, to remove loosely bound undesired proteins. In run #1, the target protein, lysozyme, was then eluted using 20 mM tris-acetate buffer, pH 9.5 with 1 M sodium chloride, followed by 20 mM tris-acetate buffer, pH 9.5 with 2 M sodium chloride. In run #2 the lysozyme was eluted using 20 mM tris-acetate buffer, pH 8.5 with 1 M sodium chloride, followed by 20 mM tris-acetate buffer, pH 8.5 with 2 M sodium chloride. Each of the tris-acetate elutions were performed until an OD_{280} reading upon them showed no additional protein being eluted.

A benchtop experiment was also performed to determine whether lysozyme activity was degraded in fermentation broth under ambient conditions. This was performed by removing a sample from the fermentation, and placing it in a microfuge tube, and leaving it on the bench for 24 hours, and then centrifuging and assaying the supernatant for lysozyme activity.

RESULTS

1. Determination of Resin Effects upon Cells

1.1. Effects of the Resin on *P. pastoris* in a Bioflo 3000 Fermenter

The comparison between cell density increases in single runs of fermenters with and without resin appears in **Figure 4**. In the control, the glycerol feed began at 47:23 EFT (Elapsed Fermentation Time, in hours), and the low methanol feed began at 51:48 EFT.

The experimental fermenter with resin was terminated early due to the unusually slow growth rate, and that the fermentation had not gone limited on the batch-phase carbon, even eight hours after the control fermentation had. In order to isolate the cause of the slow growth, at 12:35 EFT a dose of 1 mL of PTM1 salts were added to the fermenter. The subsequent decrease in dissolved oxygen (DO) indicated a high demand for oxygen, most likely due to an increase in culture metabolism. To ensure this decrease was not due to the metals actually affecting the probe itself, a trial was run where metals were added to an operating probe without culture and the DO was found to not be affected. The increased metabolism noted by the decreasing DO lasted for approximately fifteen minutes, and then the DO returned to the original level. The addition of PTM1 salts was tested at other times during the fermentation, and a similar effect occurred each time. The addition of glycerol or ammonium, a nitrogen source, had no effect on DO. A similar dose of PTM1 salts to the control fermentation had no similar effect (data not shown).

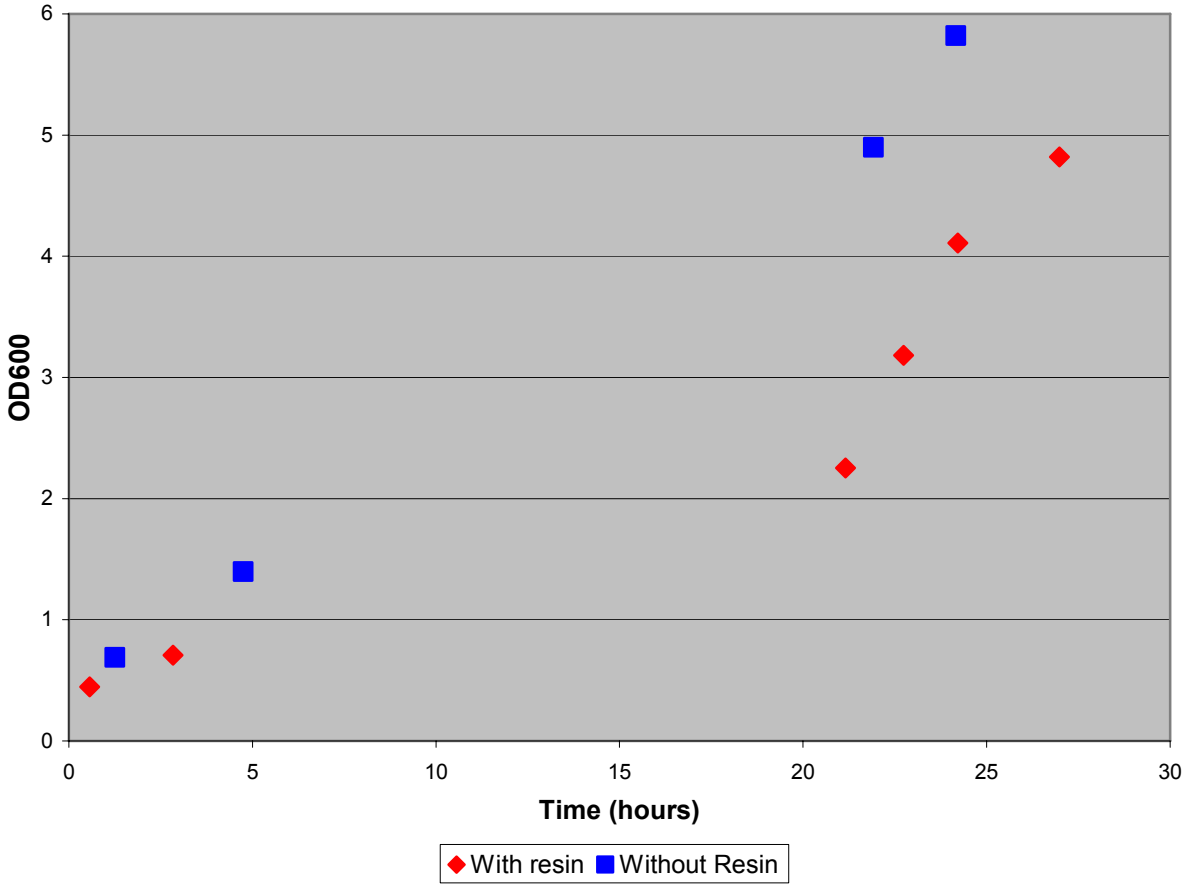

Figure 4 - Cell Density Comparison Between Fermenters With and Without Resin Present.

1.2. Maceration Effects of the Resin on *P. pastoris* in Shake Flasks

Similar cell densities were found between all medium formulations containing glass beads and resin as in the control (**Table 1**). Each value is the average of at least three readings. Similar results were found in a later study (**Table 2**), where the cell densities determined were comparable to the control among the flasks containing resin and glass beads. Interestingly, the flask which had the resin added many hours after inoculation had a higher level of cell growth than any of the other flasks. Several repetitions of this experiment showed that this occurred regularly and was not an isolated incident (data not shown). The 10^{-9} dilution is not shown due to non-growth.

	Cell Counts (cell/mL)
115 mL media, no resin (control), inoculated	**6.50×10^{10}**
100 mL media, 15 mL glass beads, inoculated	**2.63×10^{10}**
100 mL media, 15 mL resin, Lysozyme, inoculated	**3.00×10^{10}**
100 mL media, 15 mL resin, inoculated	**2.66×10^{10}**

Table 1 – Cell Counts in Shake Flask Study

	Colony count (10^{-7} dilution)	Colony count (10^{-8} dilution)	Average cell density (cells/mL)
100 mL media, 15 mL glass beads Not inoculated	Both plates sterile		
100 mL media, 15 mL resin Not inoculated	Both plates sterile		
100 mL media, Inoculated (Control)	69	7	7.4×10^9
	100	6	
100 mL media, 15 mL resin, Inoculated	61	9	8.0×10^9
	79	9	
100 mL media, 15 mL resin, Lysozyme, Inoculated	70	11	8.3×10^9
	92	6	
100 mL media, 15 mL resin (Late), inoculated	154	18	15.8×10^9
	129	14	
100 mL media, 15mL glass beads	79	8	7.8×10^9
	81	7	

Table 2 - Plate Counts From a Shake Flask Maceration Study

2. Determining the Effect of Nutrient Deprivation on *P. Pastoris*

Figure 5 shows the effect of residence time biomass accumulation, measured as wet weight. The residence time is defined in this study as the average amount of time which fermentation culture spends away from both a carbon and oxygen source, i.e., the primary or 5 L fermenter. The control fermentation, however was fed carbon and supplied with oxygenation continuously, even while in the secondary, or 1 L fermenter. The results of the residence time experiments, especially at high residence times were

unexpected. Even for a 130 minute residence time in the 1 L fermenter, the wet weight and CO_2 output did not appear to decrease to the extent expected (**Figure 5** and **Figure 6**) and were very similar to those values obtained for the control and the other residence times. The units used in **Figure 5** are the wet weight of biomass, in grams per liter. Since the control was in effect fed proportionally more carbon (4/3 more carbon) the corresponding wet weights must be adjusted accordingly. For this reason, the rate of change for the wet weight in the control system was lowered by 25%. The data from the experimental system was not altered. Since the increase in feed rates is directly proportional to the increase in volume, this adjustment is justified. The units used in **Figure 6** are milliliters of CO_2 evolved from the 5 L fermenter per minute, and were not adjusted since the CO_2 evolved in the small fermenter was allowed to exit through the condenser attached to the small fermenter.

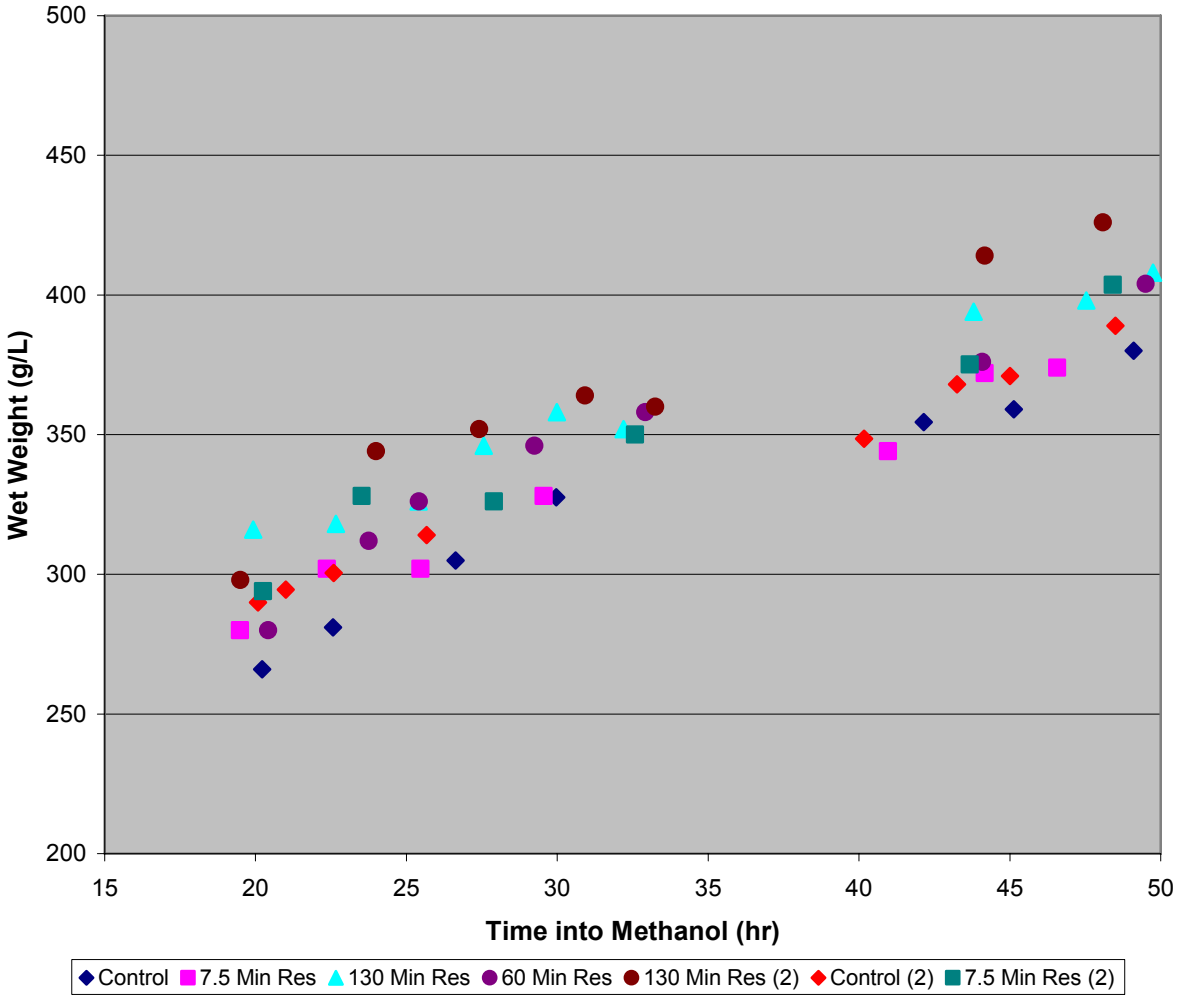

Figure 5 - Wet Weight as a Function of Residence Time, Adjusted for Feed Volume

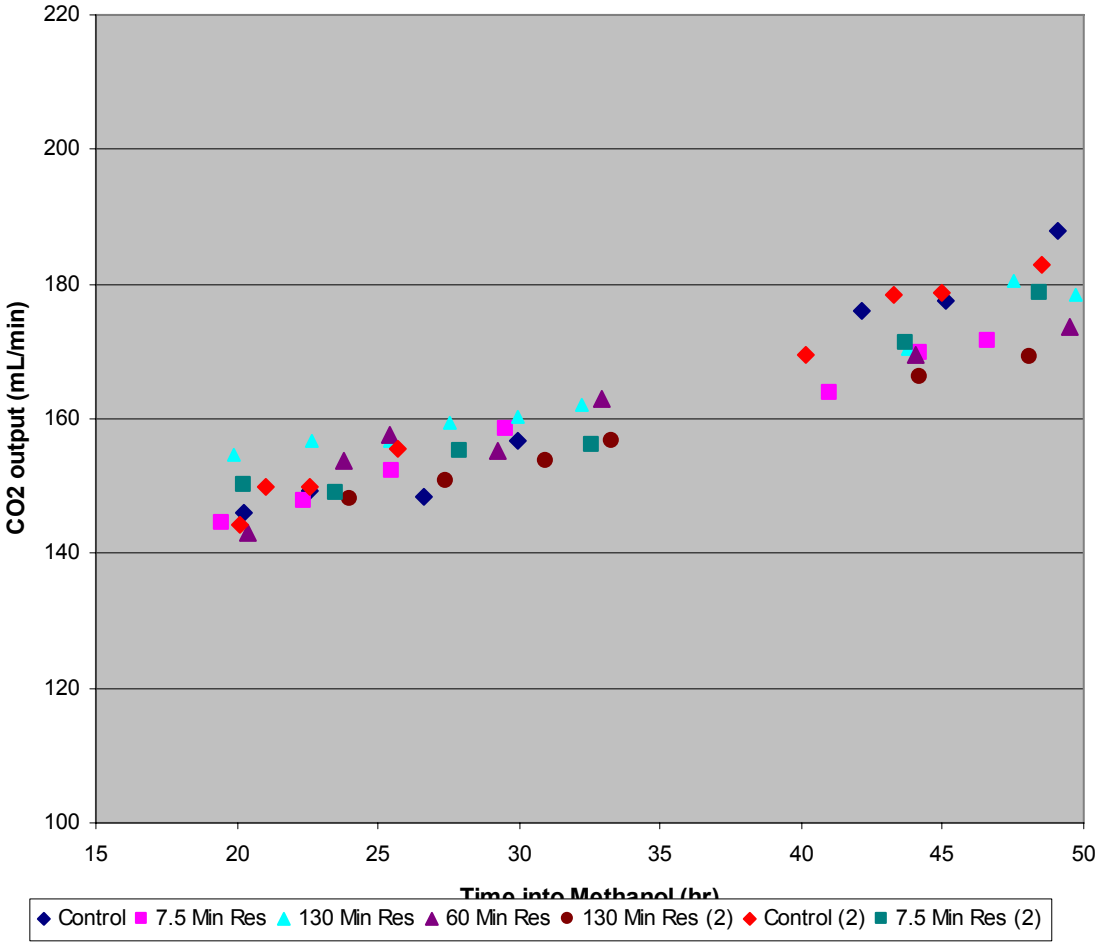

Figure 6 - Milliliters Carbon Dioxide per Minute Evolved from the 5 L Fermenter as a Function of Time

3. Determination of the Lysozyme Binding Capacity

3.1. Effect of Basal Salt Media on the Binding Capacity of the Resin

Table 3 shows the effect of Basal Salt Media (BSM), and its components, on the binding capacity of the CM Hyper-Z resin for lysozyme. The samples tested were supernatants after exposure to the resin for a period of 2 hours. The average activity shown in **Table 3** is the average of a minimum of two assays performed in multiple independent microfuge tubes.

	Residual activity (U/mL)	Percentage Bound
Control with resin (20 mM citrate buffer, pH 3)	ND	100%
Control without resin (20 mM citrate buffer, pH 3)	137,000	0%
H_3PO_4 (392 mM) + KOH	2,383	98.3%
H_3PO_4 (392 mM) + $MgSO_4 \cdot 7H_2O$ (153 mM) + KOH	3,675	97.3%
H_3PO_4 (392 mM) + $CaSO_4$ (392 mM) + KOH	3,548	97.4%
H_3PO_4 (392 mM) + K_2SO_4 (104 mM) + KOH	3,868	97.2%
H_3PO_4 (392 mM) + PTM1 trace salts	2,432	98.2%
BSM w/o glycerol	7,765	94.3%

Table 3 - - The Effect of Basal Salt Media, and its Components, on CM Hyper-Z Binding Capacity, and Binding Percentages

3.2. Test-Tube Binding Capacity

The data presented in **Table 4** show the static binding capacity of the CM Hyper-Z resin in microfuge tubes. From these data, it appears that breakthrough occurs at just under 25 mg/mL.

	Units/mL of lysozyme activity	% of lysozyme bound
0 mg/mL starting concentration	0	—
10 mg/mL starting concentration	0	100%
25 mg/mL starting concentration	510	80%
50 mg/mL starting concentration	8320	20%
75 mg/mL starting concentration	9330	10%

Table 4 - Static Binding Capacity of Lysozyme With CM Hyper-Z

3.3. Dynamic Binding Capacity in Buffer

The dynamic binding capacity of lysozyme in 20 mM sodium citrate buffer, pH 3.1, was determined at flow rates of 250 and 350 cm/hr in a 1.1 cm column (**Figure 7**). The 2% breakthrough points for 250 and 350 cm/hr with 20 mL of CM Hyper-Z resin at

1.6-2.0-fold expansion were determined to be 50 and 75 mg/mL, respectively. C/C_0 represents the ratio of the lysozyme concentration leaving the column, to that entering the expanded bed chromatography (EBC) column. A ratio of 1.0 signifies that no material entering the EBC column is being bound to the resin before leaving the column. These values indicate individual runs.

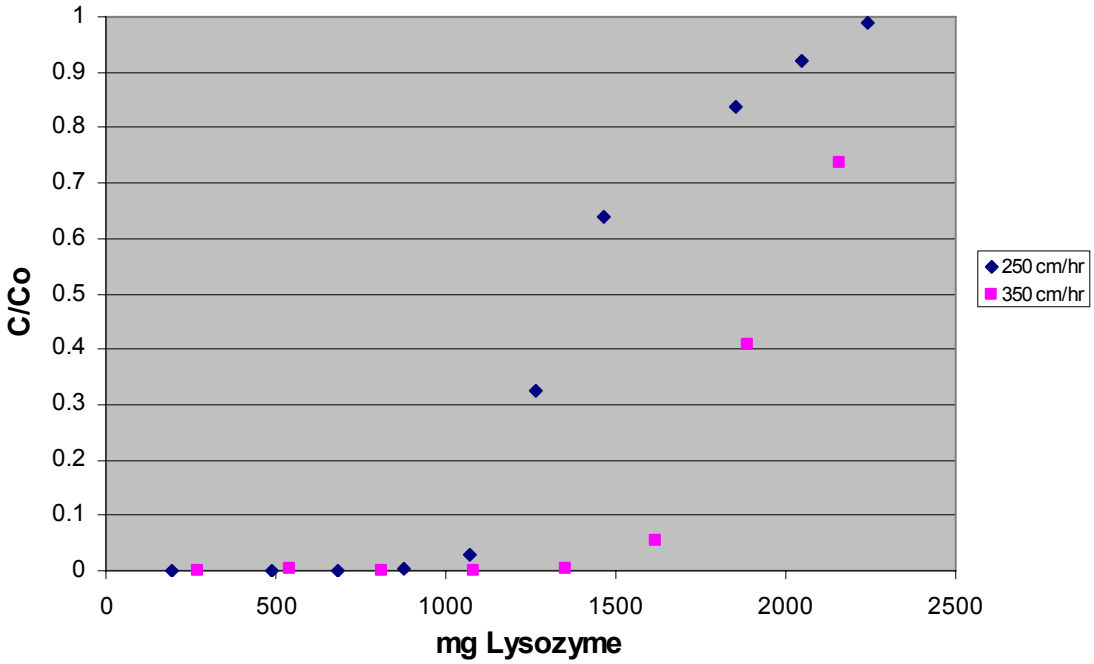

Figure 7 - The Dynamic Binding Capacity of Lysozyme in Citrate Buffer

3.4. Dynamic Binding Capacity in Whole Fermentation Broth

The dynamic binding capacity (DBC) of hen egg lysozyme in whole fermentation broth was determined by harvesting the culture, then passing the undiluted fermentation broth, spiked with 200 mg/L lysozyme, through the EBC column. The initial linear velocity was 6.31 cm/min or 6 mL/min, but was lowered to 5.16 cm/min (4.9 mL/min) after 207 mg of lysozyme had passed through the column, due to the bed being over-expanded. When 300 mg of lysozyme had passed through the column, 2% breakthrough

was achieved (**Figure 8**). Since there was 15 mL of resin in the column, this translated to a 2% breakthrough at 20 mg/mL resin. This level of lysozyme was chosen in order to provide resin saturation; despite that strains of *P. pastoris* producing 1 - 3 g/L of product are common in industrial settings.

Despite the higher initial flow rate, the 2% breakthrough determined is considered to be applicable for the 4.9 mL/min flow rate, because the bed had come to equilibrium at 4.9 mL/min. Breakthrough occurred during the 4.9 mL/min flow rate after having been at equilibrium for 100 minutes.

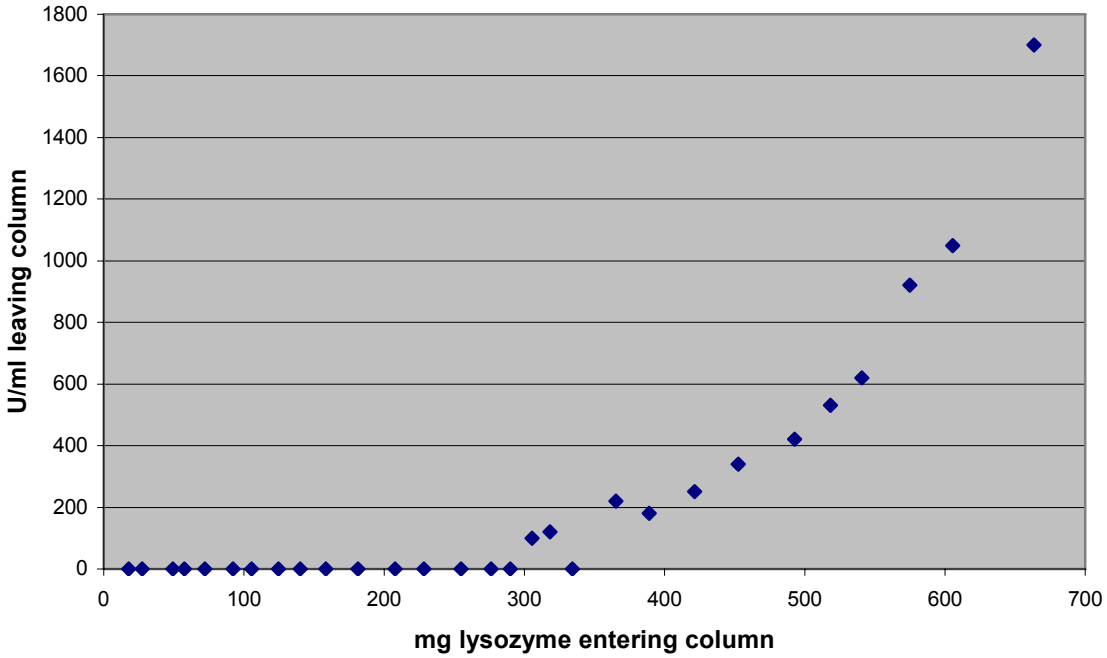

Figure 8 - Binding Capacity of Lysozyme in Whole *Pichia pastoris* Fermentation Broth

3.5. Recirculating Binding Capacity in Whole Fermentation Broth

To determine the saturation point of the CM Hyper-Z resin under conditions similar to the complete system, a recirculation binding study was performed. **Figure 9** shows the lysozyme activity within the fermenter for one run. The long running time was

specified in order to ensure resin saturation, which was determined to be approximately 30 mg/mL.

The conditions in the fermenter were changed during the experiment, to lower the temperature from 30 to 20.5°C just less than 6 hours into the experiment, to determine if temperature had an effect on binding. In order to maintain 2-2.5-fold bed expansion, the experiment was run at 3 mL/min.

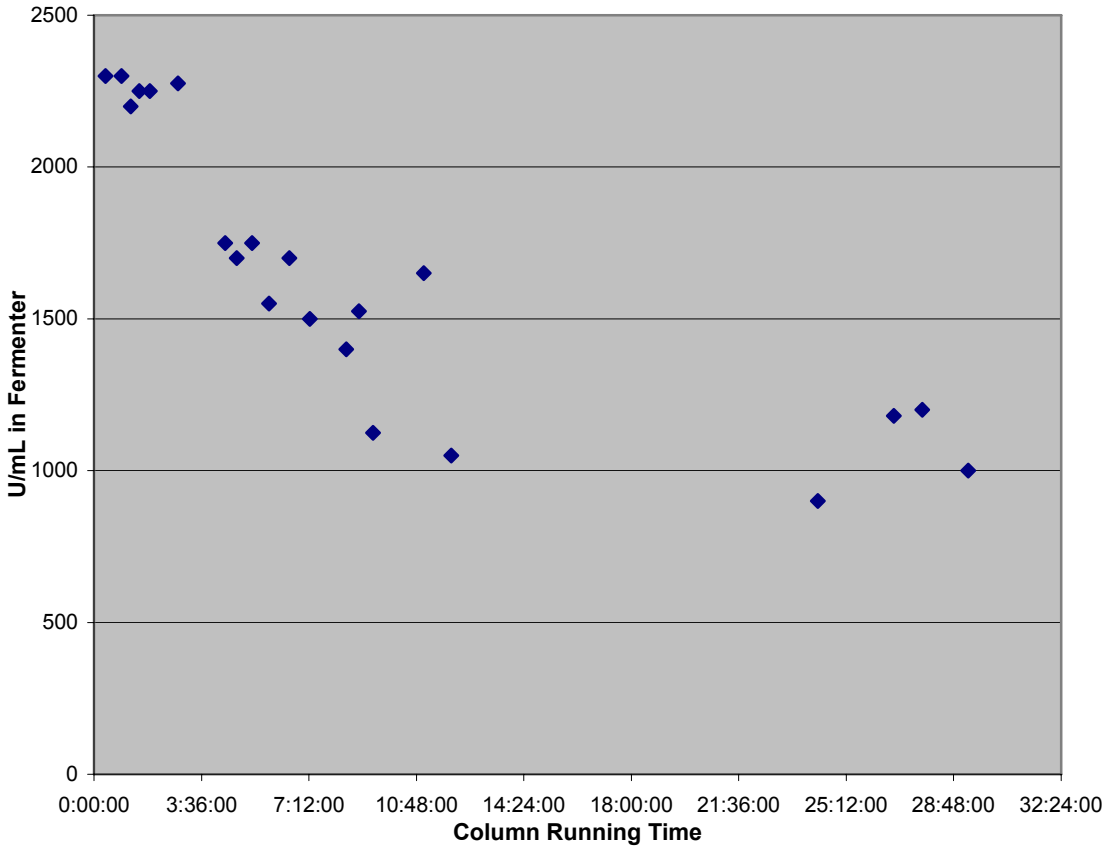

Figure 9 - Recirculating Binding Capacity Study in Whole Broth

4. The Combined Fermentation and Recovery System

In the combined fermentation and recovery apparatus, the activity within the fermenter was measured and plotted against the running time of the EBC column. **Figure 10** and **Figure 11** show the results from two separate sets of experiments, but had

similar behaviors. In both experiments there was little additional binding after 24 hours. The binding capacities of the experiments were different though; the first run demonstrated a binding capacity of 43 mg/mL at 496 g/L wet weight, whereas the second experiment showed a 31 mg/mL binding capacity at 478 g/L wet weight.

The total activities within the reactors were 10 x 10^6 and 5.5 x 10^6 units for runs #1 and #2, respectively, as calculated by the starting lysozyme concentration within the fermenter and the initial volume within the fermenter. Using the final lysozyme activities and reactor volumes, there were 3.2 x 10^6 and 2.5 x 10^6 units of activity remaining in the reactors at the ends of Runs #1 and #2, respectively. As shown in **Table 5**, 5.02 x 10^6 units were eluted from the resin, leaving 1.8 x 10^6 units unaccounted for in run #1. In run #2, 1.77 x 10^6 units were recovered from the resin (**Table 6**), which leaves 1.23 x 10^6 units unaccounted for.

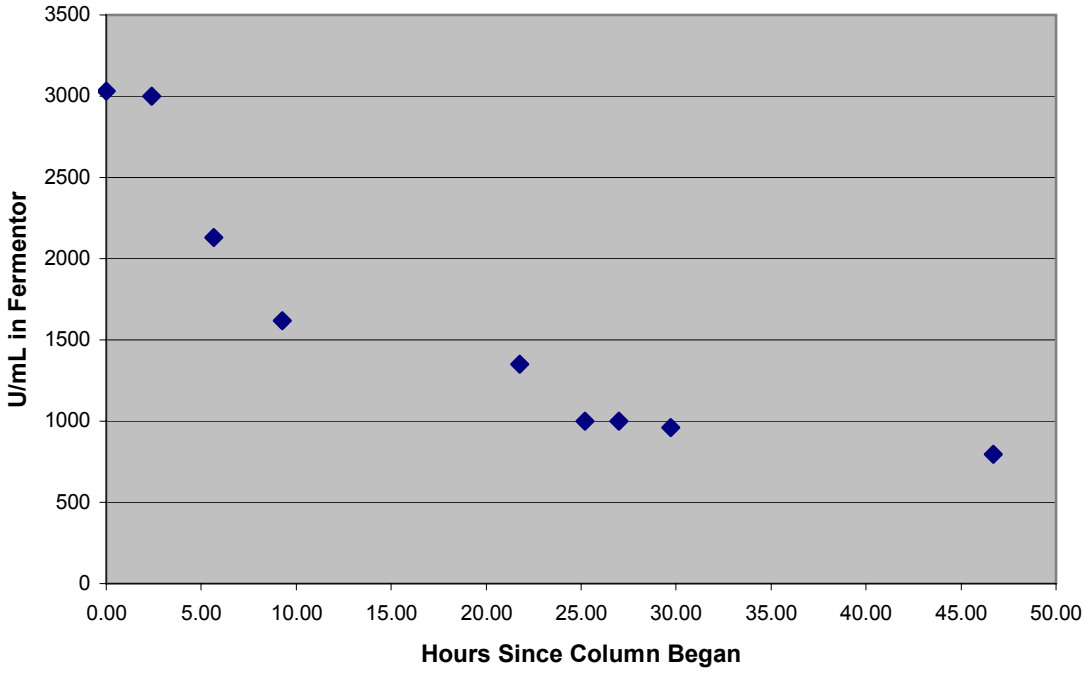

Figure 10 - Whole Broth Binding Study While Recirculating and Growing *Pichia pastoris*; Run 1

	Average activity (U/mL undiluted)	Amount applied	Total Units
Wash (up-flow with 20 mM sodium citrate buffer, pH 3.5)	ND	>1 L	0
DW (Down-flow with 20 mM sodium citrate buffer, pH 3.5)	ND	150 mL	0
Down-flow with tris-acetate 20 mM, pH 7.5	ND	100 mL	0
Down-flow with tris-acetate 20 mM + 1 M NaCl, pH 9.5	24,700	100 mL	2.47×10^6
Down-flow with tris-acetate 20 mM + 2 M NaCl, pH 9.5	17,000	150 mL	2.55×10^6

Table 5 - Elution Volumes and Enzyme Activity for Run #1

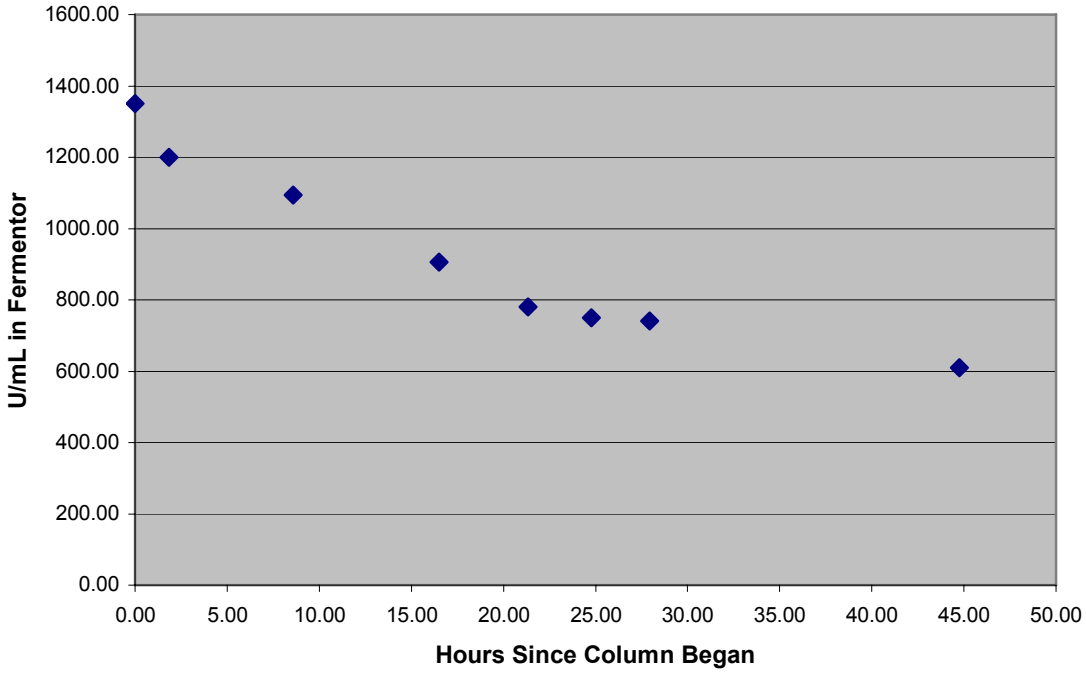

Figure 11 - Whole Broth Binding Study While Recirculating and Growing *Pichia pastoris*; Run 2

	Average activity (U/mL undiluted)	Amount applied	Total Units
Wash (up-flow with 20 mM sodium citrate buffer, pH 3.5)	NQ	>1 L	0
DW (Down-flow with 20 mM sodium citrate buffer, pH 3.5)	NQ	150 mL	0
Down-flow with tris-acetate 40 mM, pH 7.5	NQ	200 mL	0
Down-flow with tris-acetate 40 mM + 1 M NaCl, pH 8.5	5,800	200 mL	1.16×10^6
Down-flow with tris-acetate 40 mM + 2 M NaCl, pH 8.5	3,040	200 mL	0.608×10^6

Table 6 - Elution Volumes and Enzyme Activity for Run #2

DISCUSSION

The model protein used in these trials, hen egg lysozyme, has an isoelectric point of 11.0, and a molecular weight of 14,400 Da. Lysozyme is responsible for breaking down the polysaccharide walls of many kinds of bacteria and thus it provides some protection against infection. Lysozyme functions by serving as an unspecific innate opsonin by binding to the bacterial surface to reduce the negative charge and facilitate phagocytosis of the bacterium before opsonins from the acquired immune systems attack the bacterium. Lysozyme attacks peptidoglycans and hydrolyzes the bond that connects N-acetyl muramic acid with the fourth carbon atom of N-acetylglucosamine. Hen egg-white lysozyme is a single chain of 129 residues. It has an alpha+beta fold, consisting of five to seven alpha helices and a three-stranded antiparallel beta sheet. The enzyme is approximately ellipsoidal in shape, with a large cleft in one side forming the active site (**Figure 12**). Lysozyme is considered to be a stable protein under most conditions, although low temperatures and low pH further enhance stability. Lysozyme was chosen for this experiment due to its apparent stability and high isoelectric point, which causes the lysozyme to bind strongly to the cation exchange resin used, even with the low pH condition at pH 3 used in these trials.

Figure 12 - Lysozyme Structure (Rypniewski *et al.*, 1993)

The experimental hypothesis that cell viability would not be strongly affected by a combined fermentation and recovery system was examined by investigating several key parameters thought to be of critical importance: 1) the effect of the resin itself on growth of the culture; 2) the determination of any toxic or nutrient-binding effects of the resin which would reduce capacity for protein binding; 3) the assessment of nutrient deprivation the culture would experience while passing through the EBC column; and 4) the determination of the lysozyme binding capacity under various conditions including the combined fermentation and recovery process.

Although the CM Hyper-Z resin has the ability to bind positively charged metal components in the trace metal supplement (**Figure 4**, page 23), it is apparent that during an actual fermentation induced with methanol, this binding effect will be reduced due to the presence of competing secreted proteins in the culture medium. This conclusion is

supported by the fact that during the actual combined recovery process, the cells were not limited by heavy metal uptake of the resin. The difference between the heavy metal result noted in **Figure 4** and the combined fermentation and recovery trial is that during the combined recovery trial, the resin was not exposed to the culture medium until 20 hours into the induction phase. The heavy metal trial, which showed limitation on heavy metals due to resin binding, was in effect a worst case scenario. Further, the demonstrated maceration effect of the resin on the cells indicated that more significant factors were present in the heavy metal trial which had shown adverse effects on the culture.

The effect of nutrient deprivation was evaluated by substituting a second, smaller bioreactor for the EBC column, and using residence times, or the total time a portion of fermentation broth is out of the main fermenter, to determine whether the deprivation of a carbon and oxygen source was harmful to the fermentation. The second fermenter was presumed to be ideally mixed (**Equation 2**). Since the control experiments were fed 33% more methanol over the course of the study compared to the experimental fermenters, due to both the small and large fermenters feeding methanol, the data for the control system wet weights were normalized to the volume being fed. The rate of change after the experiment was begun was lowered by 25%, due to the increased volume of methanol fed to the system. The data was not altered for the entire course of the experiment, due to that the control and experimental systems were fed the same during glycerol batch, glycerol fed-batch, and 20 hours of methanol feeding, and that it was only then that the systems began to differ. The values of CO_2 evolution were not altered, due to that it is a reading of an instantaneous flow rate, whereas wet weight is a cumulative value.

This study showed that CO_2 is being evolved at a slower rate, with increased residence times, and that the trend is inconclusive for wet weight comparison (**Table 7** and **Table 8**). There is a statistically significant difference between the experimental systems for the CO_2 measurements ($P < 0.001$), but not for the wet weights ($P > 0.3$). There was also sufficient data to produce a statistically significant correlation ($P < 0.001$). This suggests that there is something adverse occurring to the cells, but it is not a major problem, as the cells within the system were not strongly affected. Using a producing strain, the change in product titer will prove to be an important parameter to be studied in the future to determine the cause of these effects.

$$t_m = \frac{\int_0^\infty tE(t)dt}{\int_0^\infty E(t)dt} \cong \int_0^\infty tE(t)dt$$

Equation 2 - Equation for the Determination of Ideal Residence Time

	Slope (g/L·hr)	Average slope (g/L·hr)
Control	3.588	3.416
Control (2)	3.244	
7.5 minute residence time	3.216	3.302
7.5 minute residence time (2)	3.387	
60 minute residence time	3.036	3.036
130 minute residence time	3.139	3.600
130 minute residence time (2)	4.061	

Table 7 - Rate of Change in Wet Weight, Normalized for Feed Volume

	Slope (mL CO_2 per minute/hr)	Average slope (mL CO_2 per minute/hr)
Control	1.440	1.356
Control (2)	1.273	
7.5 minute residence time	0.944	1.000
7.5 minute residence time (2)	1.055	
60 minute residence time	0.940	0.940
130 minute residence time	0.852	0.870
130 minute residence time (2)	0.889	

Table 8 - Rate of Change in CO_2 Output, Normalized for Feed Volume

In order to assess factors which might potentially affect binding capacity, the effect of various BSM components on the binding capacity of lysozyme was investigated. All BSM components were used in the same concentrations as the media. As may be seen in **Table 3**, irrespective of the medium component, binding was always greater than 94% which indicates a minimal affect on binding capacity.

The binding capacity of the resin for lysozyme ascertained in the test-tube study was low, as expected, due to inefficient binding kinetics inherent in the system. This study was used to gain a basic understanding as to the approximate binding capacity of the resin. The test-tube binding capacity of the resin was determined to be just below 25 mg/mL. Using this data, a dynamic binding study in sodium citrate buffer was performed. The data showed that the 2% breakthrough for flow-through speeds of 250 and 350 cm/hr, occurred at 50 and 75 mg/mL, respectively. While it is often the case that an increased flow rate through the EBC column corresponds to decreased binding efficiency, the opposite was found here. This was likely due to poor binding kinetics in the slower feed rate due to insufficient expansion. Using the data, a dynamic binding capacity study was performed using whole broth, to model conditions inside the column, and prepare for later experiments. In this study harvested, undiluted broth was passed through the T-column containing 15 mL of resin, reaching 2% breakthrough at 300 mg of lysozyme (**Figure 8**), which corresponds to a 20 mg/mL dynamic binding capacity. As expected, the binding in whole broth was less efficient than in buffer, due to the very high ionic strength and density of the whole broth. The feed rate into the column was lowered partially into the experiment due to that the resin bed was over-expanded. In subsequent runs the feed rate was lower, in order to avoid this difficulty.

Using the experience gained in the whole broth DBC study, a recirculating binding study was performed. The goal of this study was to determine the saturation capacity of the resin, as well as determine a binding profile for a recirculating system. For this reason, there was insufficient resin provided to bind all of the lysozyme added. This study determined that the majority of the binding lysozyme was adsorbed within 12 hours, reaching a saturation capacity of 30 mg/mL (**Figure 9**).

The combined fermentation and recovery process was performed using data from previous studies in order to maximize efficiency. The binding capacities of the resin for lysozyme determined were 31-43 mg/mL, which was an improvement on traditional, one-pass, binding capacities. It was also determined that the binding capacities in the second run were lower than those in the first run, likely due to that the resin wasn't as fresh in the second run. Also, all runs performed showed approximately a 20% disappearance of the lysozyme (**Table 9**). It is not fully understood where this portion of the lysozyme went, but it is possible that the remainder is being degraded by the low pH of the fermentation, or that a byproduct of the fermentation is acting as an inhibitor. Known inhibitors of hen egg lysozyme include heparin, histidine methylester, chitotriose, and chitobiose as well as large, acidic polymers, some of which may possibly be found in *P. pastoris* cultures (Valisena *et al.*, 1996; Wang *et al*., 1991; Skarnes and Watson, 1955). It is also possible that X-33, the *P. pastoris* strain used, was releasing proteases into the culture medium, as it is unknown if X-33 is a protease deficient mutant (Cereghino and Cregg, 2000). It is likely that X-33 produces proteases, as protease-deficient mutants are known to be less vigorous, and have slower growth rates (Cereghino and Cregg, 2000). The effects of proteases on the assays performed would be similar to those of inhibitors; the proteases

would lower the apparent activity of the lysozyme. During the course of these studies, a container of lysozyme purchased was determined to contain proteases, and while the lysozyme activity was decreased, the effects were significantly more pronounced than in the experimental studies. For this reason, it is unlikely that proteases alone are the cause for the difference in the lysozyme quantities added, and the apparent loss of 20% of the lysozyme by the end of the study. Due to the mechanism of proteases, it would be possible to determine the presence of proteases by using gel electrophoresis, and determined if there are cleaved pieces of lysozyme in the culture medium. This test may be complicated by the large quantities of other proteins, excreted by the *P. pastoris*, in the culture medium. It may also be complicated by that the gel electrophoresis would only show the presence of cleaved pieces of lysozyme if the lysozyme is uniformly degraded. If the lysozyme structure was degraded at random points, then a gel would not be able to show what happened to the lysozyme. The previously encountered difficulty with the resin absorbing the PTM1 salts and causing diminished growth was not noticed over the course of the combined system.

		Lysozyme Activity (10^6 Units)	Percent of Initial Lysozyme Activity
First Run	Initially in the fermenter	10.0	
	Remaining in the fermenter	3.20	32%
	Recovered from the EBC	5.02	50%
	Not fully accounted for	1.80	18%
Second Run	Initially in the fermenter	5.50	
	Remaining in the fermenter	2.50	46%
	Recovered from EBC	1.77	32%
	Not fully accounted for	1.23	22%

Table 9 - Combined Fermentation and Recovery Results

The use of a combined fermentation and recovery process was determined to be an effective method for separations of high cell density cultures. There appeared to be no major loss of viability caused by the binding conditions. The binding of the target protein to the resin increased with recirculation, as compared to a single-pass study using whole broth (**Table 10**).

	Binding Capacity (mg/mL)
Test-tube binding	25
DBC in sodium citrate buffer	50 – 75
DBC using Whole Broth	20
Recirculating in Whole Broth	30
Combined Fermentation and Recovery Process	31 – 43

Table 10 - Comparison of Binding Capacities

Due to the significant loss of activity encountered using a lysozyme system; future studies should investigate other, more stable, protein systems which are available. A study should also be performed to determine the cause of the activity loss although choosing the right production system and protein should eliminate this degradation factor. The efficiency of the fermentation and recovery processes should be improved upon by isolating and improving the aspects deemed important. One of the more important aspects should be the scale-up of the recovery process. The 200 mg/L protein concentrations used in this experiment are lower than a typical *Pichia pastoris* titer. It would be a worthwhile study to determine if this process would work efficiently with higher titers, a larger EBC column, and more resin. It would also be important to use a strain of *P. pastoris* which produces protein, in order to gain a better understanding of the effect of carbon and oxygen deprivation. Such a strain would allow the investigator to monitor the AOX1 gene for changes during the deprivation, as well as provide the ability to analyze titer for metabolic changes.

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APPENDIX

1. Process Recipes:

1.1. BSM (Basal Salt Media):

	Concentration
H_3PO_4 (conc.)	26.7 mL/L
$CaSO_4$	0.93 g/L
K_2SO_4	18.2 g/L
$MgSO_4 \cdot 7H_2O$	18.4 g/L
KOH	4.13 g/L
Glycerol	40 g/L

1.2. Biotin Solution:

	Concentration
Sterile DI water	10 mL
2M NaOH	A few drops
D-biotin	0.4 g/L
Sterile DI water	Remaining vol.

1.3. BMGY (Buffered Minimal Glycerol complex) Medium:

	Concentration
Biotin	400 μg/L
Peptone	20 g/L
Yeast Extract	10 g/L
Glycerol	10 mL
Yeast Nitrogen Base	13.4 g/L

1.4. PTM1 Trace Salts:

	Concentration
H_2SO_4	5 mL/L
$CuSO_4 \cdot 5H_2O$	6 g/L
NaI	0.08 g/L
$MnSO_4 \cdot H_2O$	3.36 g/L
Molybdic acid	0.2 g/L
Boric acid	0.02 g/L
$CoCl_2 \cdot 6H_2O$	0.82 g/L
$ZnCl_2$ (work in hood)	20 g/L
$FeSO_4 \cdot 7H_2O$	64.6 g/L

1.5. YEP (Yeast Extract Peptone) Plates:

	Concentration
Yeast Extract	5 g/L
Peptone	10 g/L
Glucose	10 g/L
Agar	20 g/L

Autoclave, mix, and pour at 50-60°C

1.6. YNB (Yeast Nitrogen Base) media:

	Concentration
Yeast Nitrogen Base	6.7 g/L
KH_2PO_4	11.5 g/L
K_2HPO_4	2.66 g/L
Glycerol	20 g/L

1.7. Phosphate Buffer, 0.1 M, pH 7.0:

	Concentration
0.1M Na_2HPO_4	800 mL
0.1M NaH_2PO_4	200 mL
Concentrated NaOH	To pH 7.0

1.8. Tris-acetate buffer:

1.8.1 Tris-acetate buffer, 20 mM, pH 7.5

	Concentration
Tris (base)	0.484 g/L
$C_2H_4O_2$	To pH 7.5

1.8.2 Tris-acetate buffer, 20 mM, pH 9.5 + 1 M NaCl

	Concentration
Tris (base)	0.484 g/L
NaCl	58.44 g/L
$C_2H_4O_2$	To pH 9.5

1.8.3 Tris-acetate buffer, 20 mM, pH 9.5 + 2 M NaCl

	Concentration
Tris (base)	0.484 g/L
NaCl	116.88 g/L
$C_2H_4O_2$	To pH 9.5

1.8.4 Tris-acetate buffer, 40 mM, pH 8.5 + 1 M NaCl

	Concentration
Tris (base)	0.969 g/L
NaCl	58.44 g/L
$C_2H_4O_2$	To pH 8.5

1.8.5 Tris-acetate buffer,40 mM, pH 8.5 + 2 M NaCl

	Concentration
Tris (base)	0.969 g/L
NaCl	116.88 g/L
$C_2H_4O_2$	To pH 8.5

2. Pump Calibration

The Cole-Farmer Instrument Company Masterflex three-roller peristaltic pumps were calibrated using #16 Pharmed Masterflex tubing ranging in flowrates from 7.67 mL/min to 180 mL/min. The settings are arbitrary values written on the pump motor. All calibrations used fermentation broths which were both growing and aerated. (**Table 11**)

Setting	mL	Time (minutes)	mL/min	Residence Time for 1L (minutes)
0.5	23	3	7.67	130.4
0.75	35	2	17.5	57
1.00	43	2	21.5	46.5
1.50	47	1	47	21.27
2.00	70	1	70	14.28
2.50	170	2	85	11.76
3.00	212	2	106	9.43
3.50	125	1	125	8.00
3.75	135	1	135	7.40
4.00	150	1	150	6.67
4.50	165	1	165	6.06
5.00	180	1	180	5.55

Table 11 - Cole-Farmer Masterflex Pump Calibration Using #16 Tubing and Aerated Fermentation Broth

3. Growth curve of *Pichia pastoris*

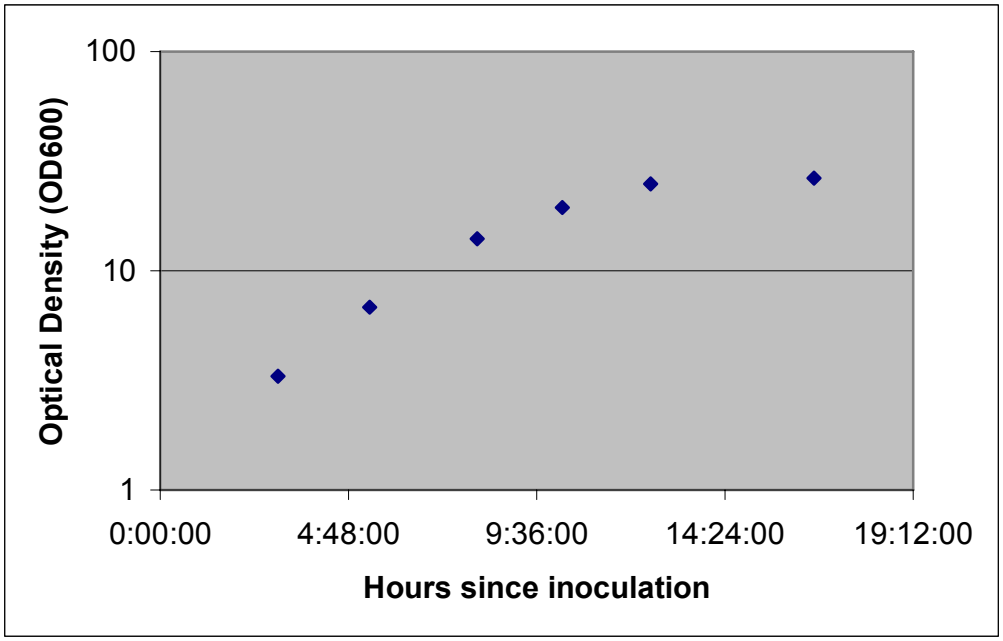


Figure 13 - Growth curve of *P. pastoris* at 27°C and 200 RPM in YNB media.

4. Maceration Effects of the CM Hyper-Z Resin on *P. pastoris* in a Bioflo 3000 Fermenter

Time Since inoculation	With Resin	Without Resin
0.566667	0.445	
1.25		0.69
2.833333	0.707	
4.75		1.396
21.15	2.252	
21.91667		4.9
22.73333	3.182	
24.16667		5.82
24.21667	4.11	
25.5		6.28
28.75		7.04
32.23333	4.82	
32.91667		9.56
51.75		65.4
56.11		77.6
57.65		86
72.4		117.5

Table 12 – Maceration Effects of CM Hyper-Z in a Bioflo 3000 on *P. pastoris*

5. Determining the Effect of Nutrient Deprivation on *P. pastoris*

Time into Methanol (hours)	Wet weight (g/L)	Adjusted Wet weight (g/L)	CO_2 (mL/min)
20:13	266	266	146.115
22:34	286	281	149.24
26:38	318	305	148.46
29:58	348	327.5	156.78
42:09	384	354.5	176
45:08	390	359	177.38
49:06	418	380	187.92

Table 13 - The Effect of Residence Time on CO_2 and Wet Weight – Control

Time into Methanol (hours)	Wet weight (g/L)	Adjusted Wet weight (g/L)	CO_2 (mL/min)
20:05	290	290	144.12
21:00	296	294.5	149.89
22:35	304	300.5	149.84
25:40	322	314	155.68
40:10	368	348.5	169.6
43:15	394	368	178.41
45:00	398	371	178.57
48:30	422	389	182.85

Table 14 - The Effect of Residence Time on CO_2 and Wet Weight – Control (2)

Time into Methanol (hours)	Wet weight (g/L)	CO_2 (mL/min)
19:29	280	144.56
22:22	302	147.68
25:28	302	152.36
29:33	328	158.6
40:58	344	163.8
44:10	372	169.78
46:34	374	171.6

Table 15 - The Effect of Residence Time on CO_2 and Wet Weight – 7.5 Minutes

Time into Methanol (hours)	Wet weight (g/L)	CO_2 (mL/min)
20:15	294	150.24
23:31	328	148.96
27:54	326	155.28
32:35	350	156.16
43:40	375.1	171.2
48:25	403.6	178.8

Table 16 - The Effect of Residence Time on CO_2 and Wet Weight - 7.5 Minutes (2)

Time into Methanol (hours)	Wet weight (g/L)	CO_2 (mL/min)
20:25	280	143.1
23:45	312	153.9
25:25	326	157.68
29:15	346	155.25
32:55	358	163.08
44:05	376	169.56
49:30	404	173.61
52:55	418	180.9
55:35	420	180.125
67:25	434	192.78
69:30	448	195.48

Table 17 - The Effect of Residence Time on CO_2 and Wet Weight - 60 Minutes

Time into Methanol (hours)	Wet weight (g/L)	CO_2 (mL/min)
19:55	316	154.71
22:40	318	156.87
25:24	326	156.87
27:34	346	159.3
29:59	358	160.38
32:12	352	162
43:48	394	170.395
47:32	398	180.63
49:45	408	178.345

Table 18 - The Effect of Residence Time on CO_2 and Wet Weight - 130 Minutes

Time into Methanol (hours)	Wet weight (g/L)	CO_2 (mL/min)
19:30	298	CO_2 detection system error
24:00	344	148.19
27:25	352	150.8
30:55	364	153.8125
33:15	360	156.6875
44:10	414	166.32
48:05	426	169.12

Table 19 - The Effect of Residence Time on CO_2 and Wet Weight - 130 Minutes

5.1. Statistical Analysis of the CO_2 Output using SPSS 14.00

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
Condition	1	Control (1)	7
	2	Control (2)	8
	3	7.5 Min (1)	7
	4	7.5 Min (2)	6
	5	60 Min	11
	6	130 Min (1)	9
	7	130 Min (2)	6

Tests of Between-Subjects Effects

Dependent Variable: CO_2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9354.776(a)	13	719.598	122.657	.000
Intercept	70838.835	1	70838.835	12074.590	.000
Condition	201.476	6	33.579	5.724	.000
Time	6270.374	1	6270.374	1068.795	.000
Condition * Time	266.492	6	44.415	7.571	.000
Error	234.671	40	5.867		
Total	1446849.585	54			
Corrected Total	9589.447	53			

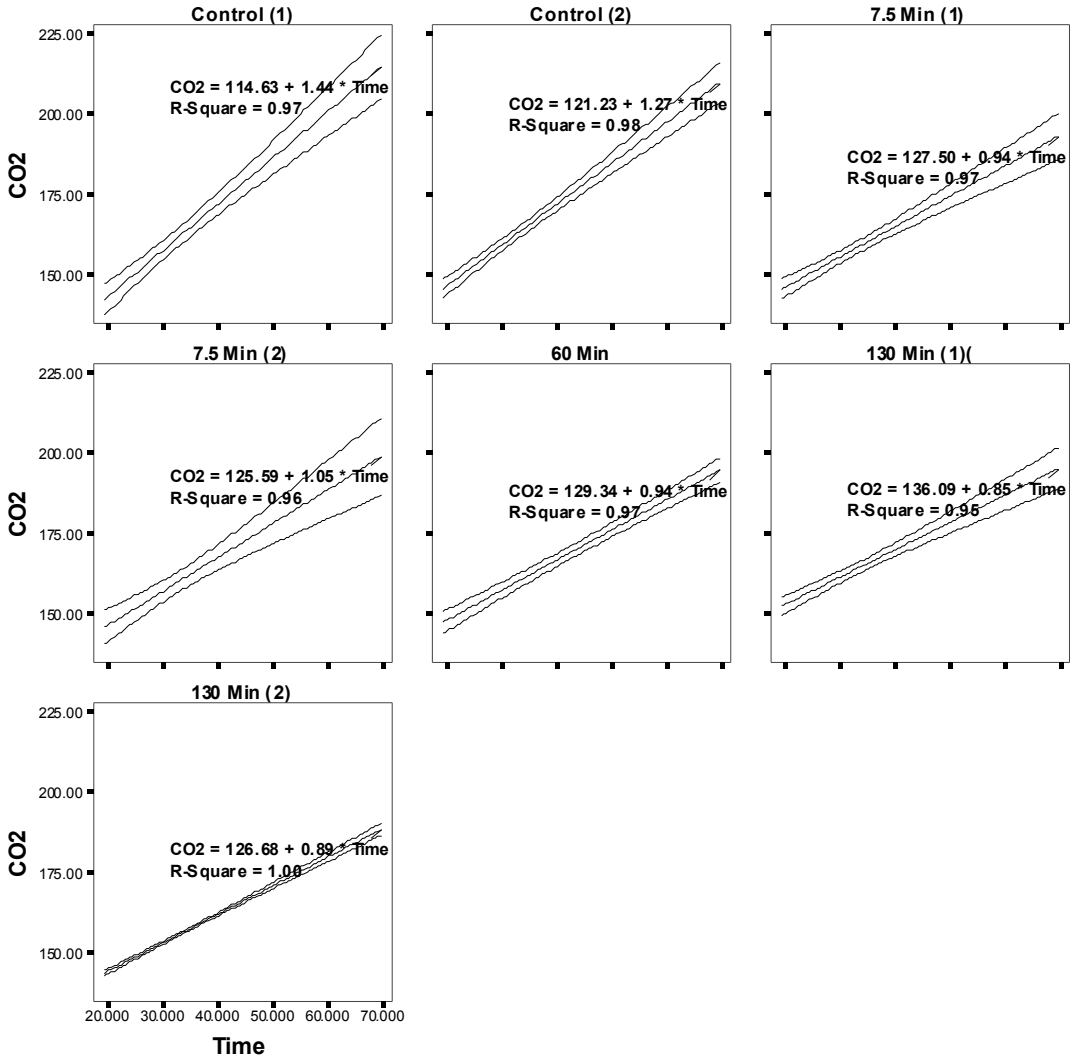
a R Squared = .976 (Adjusted R Squared = .968)

Parameter Estimates

Dependent Variable: CO2

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	126.684	4.066	31.160	.000	118.467	134.901
[Condition=1]	-12.059	5.059	-2.384	.022	-22.284	-1.834
[Condition=2]	-5.450	4.866	-1.120	.269	-15.284	4.384
[Condition=3]	.818	5.086	.161	.873	-9.461	11.097
[Condition=4]	-1.099	5.242	-.210	.835	-11.694	9.497
[Condition=5]	2.651	4.533	.585	.562	-6.511	11.813
[Condition=6]	9.408	4.874	1.930	.061	-.443	19.260
[Condition=7]	0(a)	.	.	.	.	.
Time	.889	.114	7.811	.000	.659	1.119
[Condition=1] * Time	.551	.142	3.874	.000	.263	.838
[Condition=2] * Time	.384	.137	2.806	.008	.108	.661
[Condition=3] * Time	.055	.145	.381	.705	-.237	.348
[Condition=4] * Time	.166	.149	1.110	.274	-.136	.467
[Condition=5] * Time	.051	.122	.421	.676	-.195	.298
[Condition=6] * Time	-.037	.138	-.269	.789	-.315	.241
[Condition=7] * Time	0(a)	.	.	.	.	.

a This parameter is set to zero because it is redundant.



Linear Regression with
95.00% Mean Prediction Interval

5.2. Statistical Analysis of the Wet Weight using SPSS 14.00

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
Condition	1	Control (1)	7
	2	Control (2)	8
	3	7.5 Min (1)	7
	4	7.5 Min (2)	6
	5	60 Min	11
	6	130 Min (1)	9
	7	130 Min (2)	7

Tests of Between-Subjects Effects

Dependent Variable: WetWeight

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	103158.047(a)	13	7935.234	85.345	.000
Intercept	265192.159	1	265192.159	2852.191	.000
Condition	1166.652	6	194.442	2.091	.075
Time	69563.009	1	69563.009	748.163	.000
Condition * Time	683.887	6	113.981	1.226	.313
Error	3812.114	41	92.978		
Total	6825723.220	55			
Corrected Total	106970.161	54			

a R Squared = .964 (Adjusted R Squared = .953)

Parameter Estimates

Dependent Variable: WetWeight

					95% Confidence Interval	
Parameter	B	Std. Error	t	Sig.	Lower Bound	Upper Bound
Intercept	233.557	12.820	18.218	.000	207.666	259.448
[Condition=1]	-29.690	17.551	-1.692	.098	-65.134	5.755
[Condition=2]	-7.057	16.662	-.424	.674	-40.707	26.593
[Condition=3]	-9.700	17.674	-.549	.586	-45.393	25.993
[Condition=4]	1.732	18.384	.094	.925	-35.394	38.859
[Condition=5]	11.241	15.104	.744	.461	-19.262	41.744
[Condition=6]	19.584	16.702	1.173	.248	-14.146	53.314
[Condition=7]	0(a)	.	.	.	.	.
Time	4.061	.378	10.729	.000	3.296	4.825
[Condition=1] * Time	-.473	.508	-.930	.358	-1.499	.554

[Condition=2] * Time	-.817	.485	-1.686	.099	-1.796	.162
[Condition=3] * Time	-.845	.519	-1.627	.111	-1.893	.204
[Condition=4] * Time	-.674	.539	-1.249	.219	-1.763	.416
[Condition=5] * Time	-1.025	.416	-2.460	.018	-1.866	-.183
[Condition=6] * Time	-.922	.488	-1.891	.066	-1.907	.062
[Condition=7] * Time	0(a)	.	.	.	.	.

a This parameter is set to zero because it is redundant.

6. Binding Experiments

6.1. Dynamic Binding Capacity in Buffer

time (minutes)	mg Lys	UV 280	C/C_0
10	195	0.006	0.000968
25	487.5	0.01	0.001613
35	682.5	0.011	0.001774
45	877.5	0.028	0.004516
55	1072.5	0.176	0.028387
65	1267.5	2.01	0.324194
75	1462.5	3.96	0.63871
95	1852.5	5.2	0.83871
105	2047.5	5.7	0.919355
115	2242.5	6.13	0.98871

Table 20 - Dynamic Binding Capacity in Buffer - 250 cm/hr

time (minutes)	mg Lys	UV280	C/Co
10	270	0.007	0.001129
20	540	0.014	0.002258
30	810	0.005	0.000806
40	1080	0.006	0.000968
50	1350	0.033	0.005323
60	1620	0.338	0.054516
70	1890	2.52	0.406452
80	2160	4.56	0.735484

Table 21 - Dynamic Binding Capacity in Buffer - 350 cm/hr

6.2. Dynamic Binding Capacity in Whole Fermentation Broth

	Max U/min	ml into the column	Amt gone into column (mg)
9:12			
9:27	0	90	18
9:35	0	138	27.6
9:53	0	246	49.2
10:00	0	288	57.6
10:12	0	360	72
10:29	0	462	92.4
10:40	0	528	105.6
10:56	0	624	124.8
11:09	0	702	140.4
11:24	0	792	158.4
11:43	0	906	181.2
12:05	0	1038	207.6
12:26	0	1140.9	228.18
12:53	0	1273.2	254.64
13:15	0	1381	276.2
13:29	0	1449.6	289.92
13:45	100	1528	305.6
13:58	120	1591.7	318.34
14:14	0	1670.1	334.02
14:46	220	1826.9	365.38
15:10	180	1944.5	388.9
15:43	250	2106.2	421.24
16:15	340	2263	452.6
16:56	420	2463.9	492.78
17:22	530	2591.3	518.26
17:45	620	2704	540.8
18:20	920	2875.5	575.1
18:51	1050	3027.4	605.48
19:50	1700	3316.5	663.3
Ingoing	**2500**		

Table 22 - Binding Capacity of Lysozyme in Whole *Pichia pastoris* Fermentation Broth

6.3. Recirculating Binding Capacity in Whole Fermentation Broth

Hours into Methanol	Hours from Experimental Start	Max U/min
10:12:00	0:00:00	2350
10:35:00	0:23:00	2300
11:07:00	0:55:00	2300
11:26:00	1:14:00	2200
11:43:00	1:31:00	2250
12:04:00	1:52:00	2250
12:32:00	2:20:00	2725
13:01:00	2:49:00	2275
13:33:00	3:21:00	2650
14:36:00	4:24:00	1750
14:59:00	4:47:00	1700
15:30:00	5:18:00	1750
16:04:00	5:52:00*	1550
16:45:00	6:33:00	1700
17:26:00	7:14:00	1500
18:39:00	8:27:00	1400
19:05:00	8:53:00	1525
19:33:00	9:21:00	1125
21:15:00	11:03:00	1650
22:10:00	11:58:00	1050
34:27:00	24:15:00	900
37:00:00	26:48:00	1180
37:57:00	27:45:00	1200
39:29:00	29:17:00	1000

Table 23 - Recirculating Binding Capacity in Whole *Pichia pastoris* Fermentation Broth
(* Temperature lowered from 30 to 20.5°C)

7. The Complete Experimental System

Hours into fermentation	Hours from Column Start	U/mL in fermenter
73.67	0.00	3033
76.05	2.38	3000
79.33	5.67	2130
82.93	9.27	1620
95.42	21.75	1350
98.87	25.20	1000
100.65	26.98	1000
103.40	29.73	960
120.35	46.68	795

Table 24 - Run #1 of the Complete Experimental System

Hours into fermentation	Hours from Column Start	U/mL in fermenter
74.93	0.00	1350.00
76.77	1.83	1200.00
83.50	8.57	1095.00
91.45	16.52	905.00
96.28	21.35	780.00
99.70	24.77	750.00
102.87	27.93	740.00
119.70	44.77	610.00

Table 25 - Run #2 of the Complete Experimental System