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Gene Expression Analysis of Immobilized *Saccharomyces Cerevisiae*

Ryan Michael Summers

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GENE EXPRESSION ANALYSIS OF IMMOBILIZED *SACCHAROMYCES*
CEREVISIAE

by

Ryan M. Summers

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biological Engineering

Approved:

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Logan, Utah

2008

ABSTRACT

Gene Expression Analysis of Immobilized *Saccharomyces cerevisiae*

by

Ryan M. Summers, Master of Science

Utah State University, 2008

Major Professor: Dr. Timothy A. Taylor
Department: Biological and Irrigation Engineering

Immobilization is an effective method to increase ethanol production, as proven by previous research. Results almost exclusively demonstrate an increase in ethanol production by and decrease in reproduction rate of immobilized *Saccharomyces cerevisiae* cells. Recently, research has been conducted to determine the cause of this change. The extreme variance in results due to lack of technology makes it difficult to determine the cellular changes induced by immobilization. With the advent of new technology, specifically gene expression analysis, the RNA content of cells can be easily and rapidly analyzed.

S. cerevisiae cells were immobilized in 3% (w/v) calcium alginate beads and grown inside of a packed bed reactor for comparison to planktonic cells growing in batch and chemostat cultures. Temperature inside of the reactor was maintained at 33°C with a pH of 5.5. Cell concentration inside of the beads was monitored periodically in order to create growth curves. Bud scar numbers of immobilized cells were also counted and

compared to suspended cells. Scanning electron microscopy images of the alginate beads were taken to determine cell growth inside of the beads. Affymetrix Yeast 2.0 gene chips were used, and the data retrieved was analyzed with GeneSpring software using the Bioconductor packages.

Results indicated changes in expression of 3,559 genes with significant difference among treatments by a factor of 2-fold or greater. One-way ANOVA of the filtered data yielded 380 highly significantly different genes between immobilized and suspended cells. Many of the genes pertaining to glycolysis exhibited increased expression levels. Several genes necessary for reproduction were expressed at lower levels in the immobilized cells than in their planktonic counterparts. Many different gene ontologies are discussed, and the expressed genes are mapped onto biochemical pathways.

(86 pages)

ACKNOWLEDGMENTS

I would like to thank Dr. Timothy Taylor for his outstanding mentorship, friendship, and patience throughout the course of this work. Without the funding he secured, as well as his expertise and support, I would not have been able to complete this project. Also, I am grateful to Bryce Myers and Steve Broby for their advice, questions, time, and physical labor in assisting me in this study.

Many thanks are indeed owed to Dr. Frank Rosenzweig and Dr. Arle Kruckeberg at the University of Montana, and to Kate McInnerney at Montana State University. Dr. Rosenzweig significantly contributed on the initial research planning and experimental design phase of the project. Dr. Rosenzweig and Dr. Kruckeberg cultured all of the planktonic cells used in this study and analyzed the bud scar patterns of all the treatments. They also extracted RNA from all samples for gene expression analysis. Dr. Rosenzweig and Ms. McInnerney performed the sample hybridization and ran the gene chips, providing data without which this project would have been impossible. Their initial analysis of gene expression also provided great assistance when I commenced the gene expression analysis at USU. Funding for the project was provided by the University of Montana Office of Sponsored Programs and the USU Advance Program Grant for “Response of *Saccharomyces cerevisiae* proteome to growth in an immobilized cell bioreactor.”

I would also like to thank Drs. Ron Sims and Anne Anderson for their willingness to be part of my committee, as well as their suggestions and time while reviewing and editing this work.

Finally, many thanks to my parents, friends, and most especially Rachel, my wife, for their patience and encouragement while I have completed every step of the process. I certainly couldn't have finished without anyone listed above.

Ryan M. Summers

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CHAPTER 1

LITERATURE REVIEW

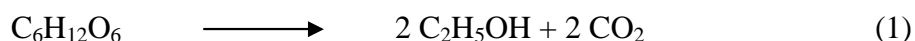
1.1 Introduction

In recent years, immobilization of cells has been researched in order to develop systems capable of producing higher yields of product than what is produced in traditional suspended cell systems (Verbelen et al. 2006). Some immobilized cell industrial processes have already been developed for vinegar production, organic and amino acid production, and wastewater treatment. This technology is also being considered to produce ethanol by both the fuel and brewing industries (Norton and D'Amore et al. 1994).

While there is great promise in this emergent technology, there are still many parameters to consider. A complete knowledge of the influence of immobilization on these parameters is necessary to improve these processes and reach an acceptable product quality (Verbelen et al. 2006). Research has indicated differing growth parameters, cellular content, and ethanol production parameters for immobilized yeast cells (Norton and D'Amore 1994). Because of the differing results, little is understood about the cellular changes induced by immobilization. However, the knowledge about both physiology and metabolic activity of immobilized cells could increase significantly with the application of recent technology in proteomics and the measurement of genome-wide gene expression (Verbelen et al. 2006).

1.2 Production of Ethanol

Due to the diminishing fossil fuel reserves, alternative energy sources need to be renewable, sustainable, efficient, cost-effective, convenient, and safe (Chum and Overend 2001). One of the many possible energy sources that fit such a description is ethanol. Production of microbial ethanol has been considered over the past decades as an alternative fuel, as depletion of fossil fuels continues. There are many microorganisms that are capable of producing ethanol from agricultural biomass, including *Clostridium sp.*, and the very well-known yeast ethanol producers *Zymomonas mobilis* and *Saccharomyces cerevisiae* (Najafpour et al. 2004). Because yeasts are chemoorganotrophic microorganisms, they obtain energy through the breakdown of organic compounds (Walker 1998). Under anaerobic growth conditions, these microorganisms have been known to produce two moles of ethanol and two moles carbon dioxide for every one mole of glucose consumed, as presented in Equation 1, below.



Because of the ease in culturing, industrial applicability, ready availability of the annotated genomic sequence, and microarray chips, the yeast *Saccharomyces cerevisiae* is an ideal microorganism to study for ethanol production (Najafpour et al. 2004). These microarray chips provide an economical and practical method for studying gene expression on an extremely large scale as they can code for thousands of genes (DeRisi et al. 1997). *S. cerevisiae* also has the ability to withstand the antimicrobial effects of ethanol, thus maintaining higher viability (Najafpour et al. 2004). Many different

fermentation techniques have been used to grow yeasts and use them in the biotechnology field.

1.3 Immobilized Cell Studies

Classical suspended cell fermentations have received the most attention in ethanol production research. This method, however, suffers from many different factors such as low cell density, nutritional limitations, and batch-mode operations with high down times. Free-cell continuous fermentations have been used in order to attempt to enhance the cell population, but they cannot operate under chemo static mode that decouples the specific growth rate and dilution rates (Ramakrishna and Prakasham 1999). Continuous fermentation does, however, yield higher conversion rates, faster fermentation rates, improved product consistency, and reduced product losses, as well as environmental advantages (Verbelen et al. 2006).

A relatively new way of growing *Saccharomyces cerevisiae* is through immobilization in calcium-alginate beads. The actual procedure of cell immobilization can markedly modify the metabolism and growth of the yeast cells (Melzoch et al. 1994). Immobilization of *Saccharomyces cerevisiae* is currently being researched to produce various products including beer, wine, ethanol, L-malate, and B-Glucanase (Walker 1998).

There are several different methods that can be used to immobilize cells. The four basic methods of cell immobilization are self-aggregation by flocculation or cross-linking agents, entrapment within preformed or polymeric matrices, surface attachment to preformed matrices, and containment behind a barrier (Masschelein et al. 1994).

Entrapment is deemed the better choice because it protects the cell without reducing activity (Gervais et al. 2000). There are two methods that can be used in cell entrapment. During the first, cells are allowed to diffuse into a preformed matrix. Cell mobility is obstructed by other cells once they begin to grow, thus entrapping the cells in the matrix. The matrix is commonly composed of sponge, ceramics, polyurethane foam, sintered glass, and stainless steel fibers (Verbelen et al. 2006). The second, and more common, form of cell entrapment is performed by adding a culture of cells to a support matrix and subsequently solidifying the matrix into a porous substance. This protects cells from shear forces and allows for higher cell concentrations. This technique also results in a considerably higher biomass loading than does immobilization in or on preformed supports (Masschelein et al. 1994).

Alginate gel, widely used in food, pharmaceutical, textile, and paper products, is a very common gel used in the entrapment of the cells. Sodium alginate is used primarily because of its stability, ease of preparation, high diffusion characteristics, and low cost. Composed primarily of D-mannuronic acid (M) and L-glucuronic acid (G), the gel forms solid calcium alginate beads when dropped into a solution of calcium chloride. The sodium and calcium ions are interchanged, thus hardening the gel without affecting the cells (Najafpour et al. 2004). Because the maximum pore size of a calcium alginate gel, 15 nm, is much smaller than the size of the yeast cells, the cells are efficiently trapped in the matrix without leakage into the media (Masschelein et al. 1994).

Despite all of the advantages of cell immobilization, there are some challenges that must be addressed. Entrapment can cause greater diffusion limitations, with the

outer cells in the beads absorbing all of the nutrients, metabolites, and oxygen in the media. It is for this reason that studies have shown the maximum mean diameter of the alginate beads to be no larger than 2-3 mm (Gervais et al. 2000; Myers 2005). This will allow the essential nutrients to reach the innermost cells in the beads, while allowing products to efficiently diffuse out. Other drawbacks include chemical and physical instability of the gel and non-regenerability of the beads (Verbelen et al. 2006).

Many advantages to immobilization of cells exist. First and foremost, the specific rate of ethanol production has been proven to be 40-50% greater by the immobilized cells than for suspended cells (Galazzo and Bailey 1990). Yields of biomass have been one-third lower than the biomass yield in suspended cells (Myers 2005). Cellular composition is also affected by immobilization (Doran and Bailey 1986), which will be discussed shortly. While cellular yield is decreased, immobilization can also provide high cell densities, which lead to short residence times when coupled with higher flow rates (Verbelen et al. 2006).

Little is known about the changes that immobilization has upon yeast physiology (Junter et al. 2002). The growth rate of immobilized cells has been reported in divergent results. One group reported that the immobilized yeast cells have been observed to exhibit four characteristic growth phases: logarithmic, linear (rapid), linear (slower), and pseudostationary (Walker 1998). Galazzo and Bailey reported no lag phase in the growth of immobilized cells, but that they do grow slower than suspended cells (Galazzo and Bailey 1988). Other groups have reported either no change or an increase in growth rate of immobilized cells (Norton and D'Amore 1994). This reported difference in behavior

between immobilized and free cells, which follow classical growth models, is often attributed to several factors, such as nutrient availability, microenvironment of cells, and physical contact of the cells with the surrounding material (Gervais et al. 2000).

When budding, haploid MAT α cells use an axial budding pattern in which mother cells bud adjacent to the previous bud site while daughter cells form buds next to the birth site (Madden and Snyder 1992). A scar is left on the wall of the mother cell at the site of budding, however the daughter cell retains no scar to indicate the bud site. The number of bud scars thus indicates the number of replications a mother cell has undergone (Hartwell and Unger 1977). It has been postulated that an increased number of bud scars may limit the surface area available for budding and nutrient transfer (Mortimer and Johnston 1959). During chronological aging of cells, the metabolism of yeast cells shifts from glycolysis to gluconeogenesis. An accumulation of iron is also found in chronologically aged cells, which is potentially toxic in high concentrations. This is all done in preparation for cell death (Reverter-Branchat et al. 2004).

Contact with the other cells in the alginate also causes changes in cell morphology and reproduction (Doran and Bailey 1986). These cellular changes have been hypothesized to cause the cells to use less energy for reproduction, thus lowering nutrient need in order to make the intercellular organelles. This, in turn, increases the ethanol production (Myers 2005).

Growth of cells immobilized in alginate beads has shown a different response at the genetic level, giving rise to changes in relative levels of enzymes and transport proteins in the cell (Galazzo and Bailey 1990). Some data have shown the average DNA

content of immobilized cells to be almost four times that of suspended cells (Doran and Bailey 1986). Immobilized cells have also been shown to have an increased concentration of both storage and structural polysaccharides, including glycogen, trehalose, glucan, and mannan (Verbelen et al. 2006). The cell wall of immobilized cells contains a higher concentration of saturated fatty acids, thus increasing the excretion of ethanol into the surroundings (Junter et al. 2002).

Yeast metabolism is also affected by immobilization. Glucose uptake and production of ethanol and glycerol has been observed to be approximately two times greater in immobilized cells. The phosphofructo kinase (PFK) activity and ATPase activity have been shown to be double that of suspended cells as well (Galazzo and Bailey 1990). Invertase, an enzyme in the cell wall that can be catabolically repressed, is also found at higher levels in immobilized cells. Measurements of intracellular pH has given discordant results, as immobilized cells have been measured as having a cytoplasmic pH both higher and lower than that of suspended cells (Norton and D'Amore 1994).

Immobilized cells have also proven to be even more resistant than free cells to ethanol and solvents such as phenol (Norton and D'Amore 1994). One possible cause for this is the increase in saturation of the fatty acids in the plasma membrane of the cells. Some groups have attributed this tolerance to the protective environment provided by the alginate (Verbelen et al. 2006). Another explanation involves the high cell densities in immobilized systems. The cell-cell contact has been thought to be the main factor in creating the protective micro-environment (Norton and D'Amore 1994).

Finally, the water activity in the cell environment is lower inside the beads, as the alginate can organize water, leaving a smaller amount for the cells (Galazzo and Bailey 1990). The yeast cells overcome this by excreting osmoprotective proteins, such as glycerol and proline (Verbelen et al. 2006). Clearly, the immobilized environment causes various changes to physiology, reproduction, and metabolism of yeast cells.

1.4 RNA Analysis

Many experiments have been performed with immobilized cells, and yet there are still many more things to be learned. Approximately 40-60% of yeast biomass is comprised of proteins (Walker 1998), which are produced following mRNA synthesis. Therefore, if ethanol production by immobilized cells is to be maximized, a better understanding of the genomic expression of the yeast must be had. A number of experiments have proven that there is a difference in both ethanol production and cellular composition of immobilized cells when compared to suspended cells.

Application of recent technologies in gene expression analysis can significantly increase understanding of yeast physiology and metabolic activity (Verbelen et al. 2006). An understanding of the differences in the genome will allow also for better analysis of immobilization technology. This can eventually lead to research of other bioconversions with immobilized cells, such as citric acid and butyric acid, antibiotic production, and applications in the brewing industry. Global transcription machinery engineering (gTME) is a tool which alters key proteins regulating the global transcriptome. This approach has already been used with *S. cerevisiae* to improve ethanol and glucose tolerance. This, in turn, led to an increase in ethanol yield (Alper et al. 2006).

One useful analytical tool for examining the metabolic state of a yeast cell is use of RNA microarray chips. The gene sequences are printed in a highly dense array on a glass slide. The pattern of expressed genes can provide detailed information of the state of the cell. Microarrays are of great importance in observing the differences in cell state, as all differences can be correlated with changes in the mRNA levels of many genes. Similarly, the changes of large groups of functionally related genes provide insight in to the systematic way that a yeast cell adapts to changes in its environment. Because the entire genome of *Saccharomyces cerevisiae* has been mapped, it is a very favorable organism on which to conduct a systematic investigation of gene expression (DeRisi et al. 1997).

1.5 Objectives

Recent research has shown that cell immobilization can be used to improve ethanol production by yeast (Myers 2005). Little is known, however, about the growth rate kinetics and the changes to the proteome of the cells. A better understanding of these is necessary in order to provide a more efficient means of ethanol production. The objectives of this project are 2-fold:

1. Determine growth kinetics for both suspended and immobilized yeast.
2. Compare the transcriptome of chemostat-grown cells and batch-grown cells in log and stationary phase to immobilized cells in order to better understand what is happening at the cellular level.

When these objectives are complete, a greater knowledge of the cellular differences due to immobilization of *Saccharomyces cerevisiae* may be achieved.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Immobilization Studies

Saccharomyces cerevisiae S288C were first obtained from the American Type Culture Collection. Frozen stock was maintained at -80°C until needed. Upon removal from the freezer, cells were first cultured in a shake flask for a period of 24 hours under aerobic conditions in order to ensure good growth. Initial growth medium used was 100 ml of a modified yeast potato dextrose (YPD) medium containing 10 g/l peptone, 5 g/l yeast extract, and 20 g/l glucose. After the culture period, cells were removed and centrifuged, and the supernatant was removed from above the cell pellet. The cells were washed with sterile water and centrifuged two more times in order to rid them of any residual phosphate from the medium.

Once suspended again in water, the cells were added to a 3% w/v high-viscosity sodium alginate solution to yield a concentration of 1×10^8 cells/ml (giving an initial number of 1×10^{10} cells in the system), while total volume of cells and alginate was 100 mL. The mixture was then hardened by adding it drop wise through a 19 gauge needle with syringe into 500 mL of 0.2 M CaCl₂ and allowed to sit with occasionally stirring for 15 minutes before being transferred into another 500 mL CaCl₂ and allowed to sit for an additional 15 minutes, again with occasionally stirring. This was done to wash excess cells from the surface of the beads, and also ensure that there was excess calcium chloride to harden the beads. Upon hardening, the beads were transferred to the sterile column (Myers 2005).

After immobilization, cells were cultured in a defined Delft medium described by Verduyn et al. (1992) containing, per liter, 100 g glucose, 5 g $(\text{NH}_4)_2\text{SO}_4$, 2 g KH_2PO_4 (Fisher, Hampton, NH), 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g CaCl_2 (Fisher, Hampton, NH), 1.25 ml Tween, 0.05 ml Antifoam B, 30 mg EDTA, 9 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.06 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.6 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.6 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.8 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 9 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 6 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg H_3BO_3 , 0.2 mg KI, 0.1 mg biotin, 2 mg calcium pantothenate, 2 mg nicotinic acid, 50 mg myo-inositol, 2 mg thiamine HCl, 2 mg pyridoxal HCl, and 0.4 mg 4-aminobenzoic acid (all reagents from Sigma, St. Louis, MO, unless otherwise specified). This medium has a lower chelating effect upon the calcium in the alginate beads, leading to greater chemical stability. It has also been shown to be an effective medium to allow for both cell growth and ethanol production (Verduyn et al. 1992).

A schematic of the immobilized reactor system is shown in Figure 1. The medium was maintained at a temperature of 33°C and pH of 5.5 by passing through a 2 L New Brunswick Celligen bioreactor.

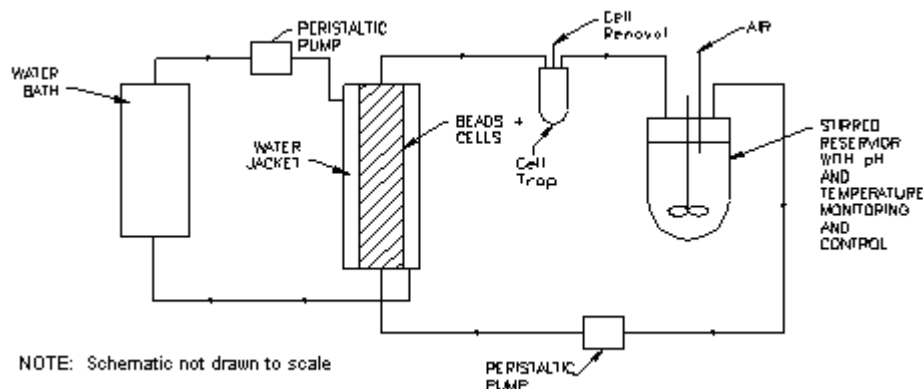


Figure 1. Schematic of the immobilized cell reactor and media control system.

2 M NaOH was used to offset the acidifying effects of yeast metabolism, and flow of the medium was controlled at 0.8 ml/s by use of a peristaltic pump. In order for the system to be truly immobilized, a cell trap also was utilized in the return line from the column containing beads to the Celligen. This was done in order to collect any cells that washed off of the surface of the beads, as well as any alginate which broke down. Glucose and ethanol levels were periodically monitored with a YSI 2700 SELECT biochemistry analyzer. These levels can be seen in Appendix A.

25 ml samples of beads were removed from the reactor on Days 0, 1, 3, 5, 10, and 17. Ten beads were subsequently dissolved in 10 ml of a 10 M sodium metaphosphate solution in order to perform cell and viability counts with use of a hemacytometer and methylene blue. This was performed by adding a mixture of 0.8 ml Phosphate Buffer Solution (PBS), 0.1 ml methylene blue, and 0.1 ml cell solution to a hemacytometer and averaging the number of cells on each grid of the hemacytometer. Five ml of beads were also dissolved in 50 ml of the sodium metaphosphate solution and then centrifuged. The pellet was then resuspended in 10 ml of a phosphate buffer with 8% paraformaldehyde, incubated for 1 hour, and stored in the refrigerator for further analysis of bud scarring. The remaining 20 ml of beads were frozen with liquid nitrogen and stored in a -80°C freezer for future RNA analysis.

Two ml flash-frozen beads were used for scanning electron microscopy (SEM) imaging. Beads were first fixed with 2% buffered glutaraldehyde for two hours, followed by three 5-minute washes of 0.1 M HEPES buffer. After washing, a post-fixing solution of 2% osmium tetroxide was added for 1 hour, followed by another 0.1 M

HEPES solution wash in three 5- minute increments. Upon fixation of the samples, a series of alcohol dehydrations were performed. Beads were soaked in a 50% ethanol solution with agitation twice for 10 minutes each. Seventy and 90% ethanol solutions were then used in replicate, also for 10 minutes each. Finally, a 100% ethanol solution was used three times for 15 minutes each.

Samples were agitated throughout the dehydration process. Upon dehydration, the beads were chemically dried in four steps, using varying concentrations of 100% ethanol and HMDS. The beads were first soaked in a 2:1 ethanol-to-HMDS solution for 15 minutes, followed by solutions of 1:1 and 1:2 ethanol-to-HMDS for 15 minutes each. Finally, beads were dried using only HMDS three times for 15 minutes each. The final HMDS solution was allowed to evaporate overnight in a fume hood. A control sample of 3% alginate beads with no cells added was prepared using the same protocol described previously. These were also frozen in liquid nitrogen and held in the -80°C freezer until preparation for SEM imaging.

2.2 Suspended Cell Studies

Suspended cell studies were performed by Dr. Frank Rosenzweig and Dr. Arle Kruckeburg at the University of Montana in ATR Sixfors, identical water-jacketed bioreactors with a 200 ml working volume. The cells were grown and harvested in three distinct phases: chemostat, batch mode log phase, and batch mode stationary phase. The chemostat operated under continuous conditions in two replicates. Two replicates were also performed on the batch mode cultures, harvesting the cells growing first in log phase and later in stationary phase from the same vessels.

The same Delft medium with 8 g/L glucose was used to culture the cells. Temperature was maintained at a constant 33°C and pH of 5.5. The chemostat cultures operated at a dilution rate of $D = 0.15 \text{ h}^{-1}$. 10 ml samples were drawn from the SixFors daily, with half of the sample fixed in the 8% formaldehyde solution. The remaining 5 ml were frozen in liquid nitrogen and stored at -80°C until further gene analysis could be performed. Cell concentration from all four Sixfor reactors was determined daily by an absorbance assay at $\lambda = 600 \text{ nm}$.

The paraformaldehyde-fixed cells from all treatments were stained with Calcofluor in order to count bud scars. Budding status was assayed at the University of Montana by observation at 1,600X magnification using Nomarski optics, while transmission and epifluorescent microscopy were performed with a Zeiss AxioSkop.

2.3 Transcriptomic Testing

RNA analysis was performed with *Affymetrix* Yeast GeneChip Genome 2.0 arrays. For suspended cell analysis, 50-100 ml of culture were filtered onto sterile 0.45 mm nylon filters, after which the filters were flash frozen in liquid nitrogen and stored at -80°C until processed. For the immobilized cell analysis, the cell-containing alginate beads were flash frozen in liquid N₂ upon removal from the reactor and stored at -80°C until processing. Physical preparation of both immobilized and suspended cells was performed at the University of Montana and Montana State University by Drs. Kruckeberg and Rosenzweig and Ms. McInnerney from the MSU Functional Genomics Core Facility.

Preparation of immobilized cells for analysis involves more steps than that of suspended cells, as the cells need to be separated from the alginate. This was done by modifying the protocol described by Kohrer and Domdey (1991). Approximately 1 ml of packed beads was ground with a pestle and mortar in the presence of liquid nitrogen until a powder was obtained. The powder was transferred to a 50 ml conical tube and mixed with 5 ml cold 0.2 M ethylenediaminetetraacetic acid (EDTA). 5 ml of RNALater was also added in order to inhibit RNase activity, and volume was brought to 50 ml with ice-cold sterile water. After 30 minutes of incubation with mixing, the mixture was passed through coarse stainless steel mesh (1 mm) into a fresh conical tube, and centrifuged for 5 minutes at 1118 g at 4°C. The supernatant was removed, and the resulting cell pellet resuspended in 5 ml EDTA, 5 ml RNALater, and 40 ml water, mixed well, and centrifuged again. The pellet was then resuspended in 50 ml sterile water and centrifuged one final time before being resuspended in 0.4 ml AE buffer (50 mM Na-acetate and 10 mM EDTA) and transferred to a screw-cap microcentrifuge tube. The frozen suspended cells were also suspended in 0.4 ml AE buffer, after which the same protocol was used for both samples.

Forty µl of 10% SDS was added with mixing to the suspension, followed by 0.5 ml acid phenol-chloroform, preheated to 65°C. After incubation at 65°C for 15 minutes with periodic mixing and brief centrifugation, the aqueous phase was transferred to a fresh centrifuge tube and extracted again with hot acid phenol-chloroform. After two more chloroform-isoamyl alcohol extraction of the aqueous phase, the solution was then brought to 0.4 ml with AE buffer. Forty µl of 3 M Na-acetate and 1 ml 95% ethanol was

added and mixed, followed by storage at -20°C . Following precipitation by centrifugation for 20 min at maximum speed, the pellet was washed with 70% ethanol and dried. After suspension of the pellet in 200 μl RNase-free water, RNA content was then analyzed by both *uv*-spectroscopy and denaturing gel electrophoresis to determine RNA yields.

The One-Cycle Eukaryotic Target Labeling Assay as described in the user's manual (Affymetrix GeneChip Expression Analysis Technical Manual 2004) was used to prepare samples for microarray hybridization. Total RNA was reverse-transcribed to cDNA with a T7-oligo(dT) primer. After second strand synthesis, the purified double stranded cDNA served as a template for the in-vitro transcription reaction (IVT). Biotin-labeled cRNA was purified, fragmented, and hybridized to the arrays at 45°C for 16 hours with constant rotational mixing at 60 rpm. Arrays were washed and stained with the Affymetrix GeneChip Fluidics Station 450 and scanned with use of an Affymetrix GeneChip Scanner 3000 and GCOS software at the Functional Genomics Core Facility at Montana State University.

The data set was then analyzed with GeneSpring software using the Bioconductor packages to determine statistically significant differences in gene expression. Definitions of gene function were obtained from the *Saccharomyces* Genome Database, <http://www.yeastgenome.org/>. Two different comparative analyses were performed. The immobilized cells harvested up through Day 5 were compared to the suspended cells, followed by a comparison of long-term immobilization by comparing samples from Days 10 and 17 to the other immobilized cells only. An understanding of these differences will

allow for determination of metabolic processes taking place in the cell, and should explain, in part, why ethanol yields have been proven to increase upon immobilization while cell reproduction remains low.

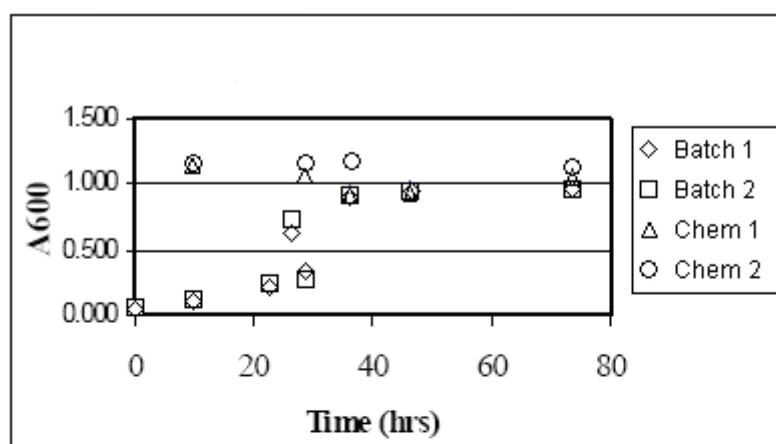
CHAPTER 3

RESULTS AND DISCUSSION

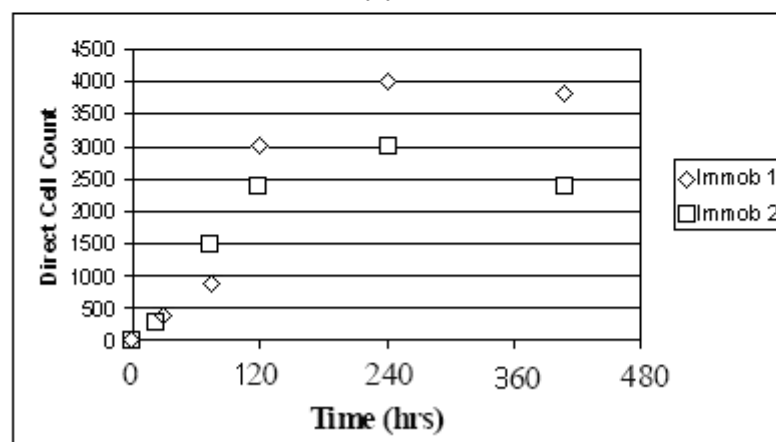
3.1 Demographic parameters

3.1.1 Cell concentration and growth curves

Cell proliferation for the different treatments can be seen in Figure 2.



(a)



(b)

Figure 2. Cell concentration of different treatments: (a) Batch- and Chemostat-grown cells and (b) Immobilized cells.

The suspended cell culture growth patterns were consistent with classical batch-log, batch-stationary, and chemostat cultures. Of interest to note is the growth pattern of the immobilized cell cultures. The concentration of cells rises sharply over the first few days before reaching a plateau at Day 10. On Day 17 in one replicate there were less cells counted than on Day 10. This is most likely attributed to the fact that the alginate beads were softening and beginning to dissolve.

3.1.2 Scanning Electron Microscopy Studies

SEM images of the beads are shown in Figures 3-6, beginning with cross-section images in Figure 3, below.

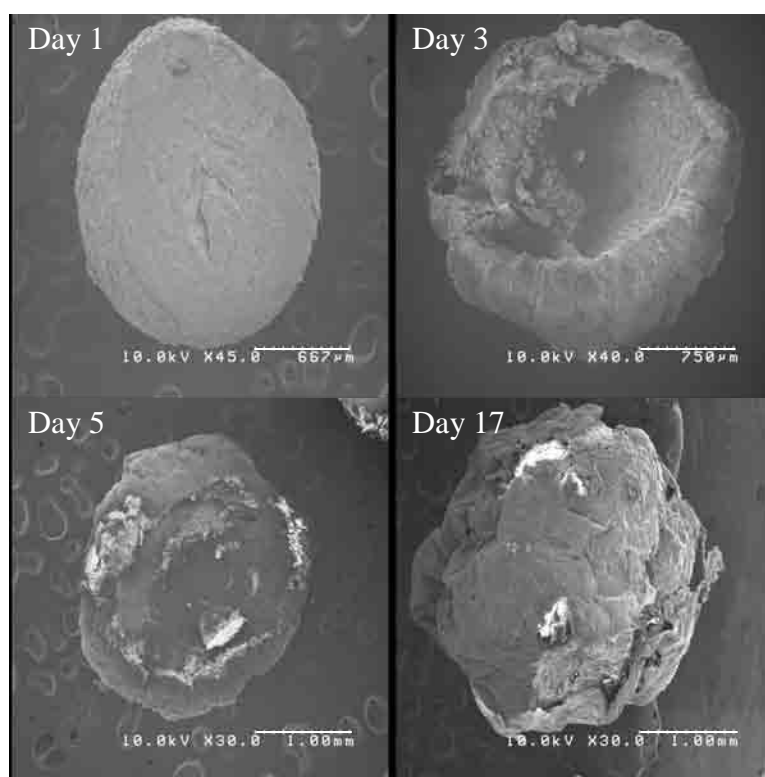


Figure 3. SEM images of bead cross sections for Days 1, 3, and 5, as well as a whole bead on Day 17. The size bar on the figures denotes 667, 750, 1.00, and 1.00 μm, respectively.

After one day in culture, the beads still appear to be solid. After three days however, the inside of the beads has become hollow, while a sheet of cells has begun to cover the inner surface. On Day 5, the center appears to have lost a little more alginate than in Day 3, while cells continue to proliferate on the inner surface as well as throughout the bead. The image of the Day 17 bead reveals that the once smooth surface has become very rough and pitted, appearing to slough off in some areas, indicating that loss of cells is most likely due to disintegration of alginate.

Images taken of the outside of the beads can be found in Figure 4. The image of the control bead (with no cells) shows a smooth surface. Upon seeding the cells into the alginate, there are very few cells that can be seen, contrasted with the number of cells beginning to grow on the outside of the beads by Day 1. Also of interest are the small bumps beginning to form on the surface of the alginate where the cells are growing under the surface, giving a chicken skin-like appearance to the beads. This appearance continues to be exhibited on the surface of the beads throughout the 17 day fermentation run, increasing with each sample.

More cells appear on the surface of the beads up until Day 10, when there are numerous colonies found on the surface of the bead. While the number of cells observed on the exterior of the beads decreases between Days 10 and 17, there are still more found on Day 17 than any prior to Day 10. Another noteworthy attribute is the appearance of small pores beginning to open up on the surface starting on Day 3, thus furthering the bead dissolution assumption.

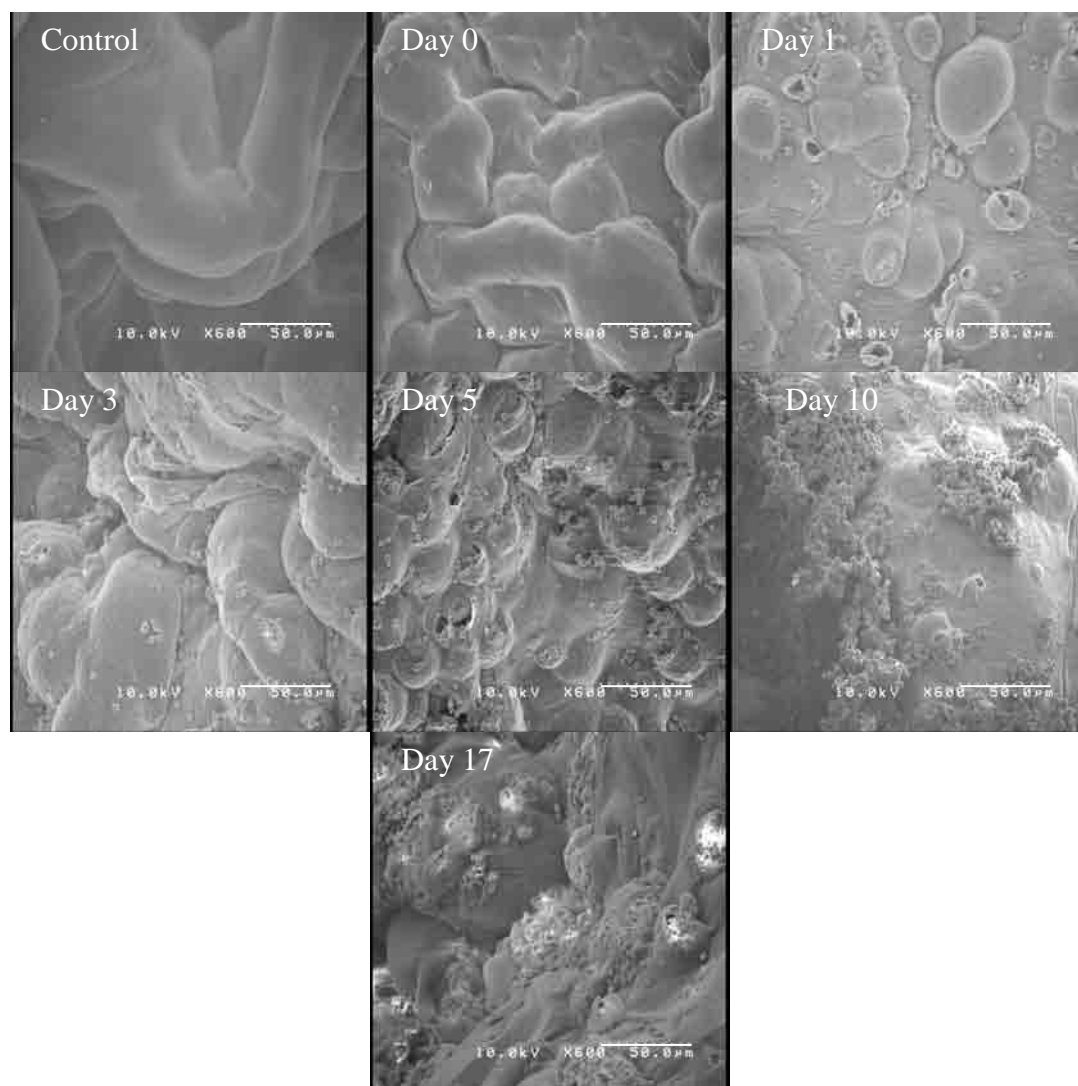


Figure 4. SEM images taken of the outside of the beads for the control and Day 0, 1, 3, 5, 10, and 17 samples. The size bar on the figures denotes 50.0 μm .

As the inside of the beads began to deteriorate, cells began to populate the inner surface. Figure 5 shows cross section images of the beads, focusing on the inner surface. Beads from Days 0 and 1 were solid throughout, whereas the remaining beads were hollow. The difference in the alginate after the first day is easily noted. The Day 0 sample had smooth surfaces with few cells, while the surface in the Day 1 sample appears

to be more brittle. Also, there are a number of cells found inside the cracks on the surface of the alginate.

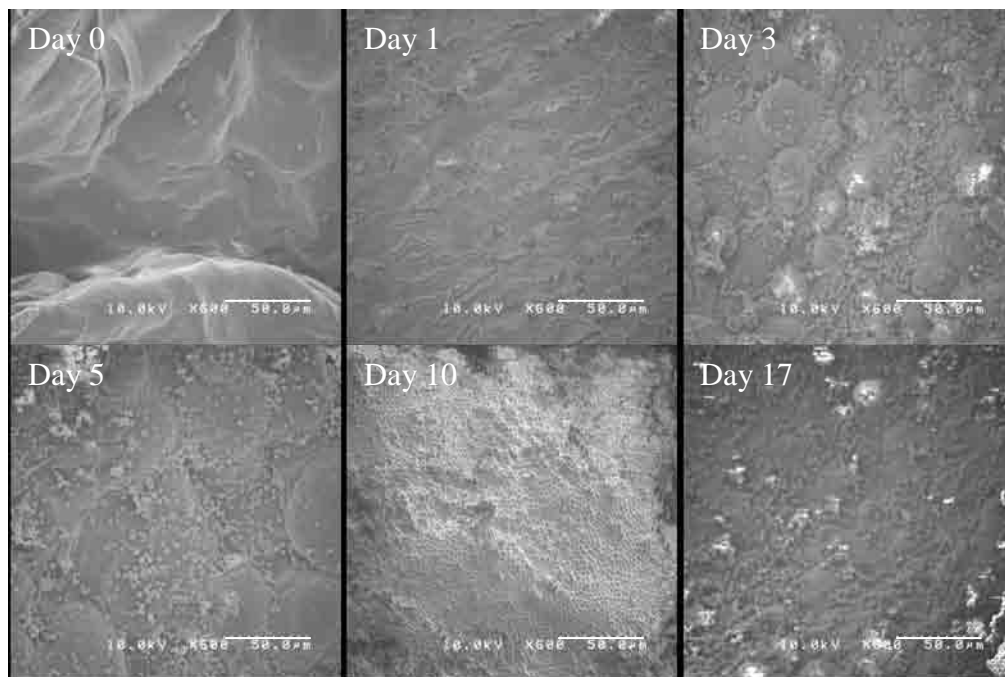


Figure 5. SEM images taken of the inside of the beads on Days 0, 1, 3, 5, 10, and 17. The size bar on the figures denotes 50.0 μm .

After and including Day 3 the inner surface of the hollow bead begins to resemble the outer surface, albeit with more cells growing on the inner surface. The surface is much smoother than the outer surface. This stands to reason, as the inside is less susceptible to shear forces of flowing media. The cells continue to populate the inner surface of the bead and begin to form confluent sheets by Day 10, as observed in Figure 5. Day 17 beads also exhibit large numbers of cells on the inner surface, with confluent sheets existing, but in much fewer numbers than in Day 10, most likely caused by alginate dissolution.

The wall of the bead, designated as the alginate between the outer and inner surfaces of the bead was also imaged, and can be seen in Figure 6. Although very few cells are observed on Day 0, by the first day many cells are already visible growing inside of the wall. These cells appear to be quickly approaching capacity. The number of cells observed inside the wall remains relatively steady throughout the duration of the study, increasing only slightly.

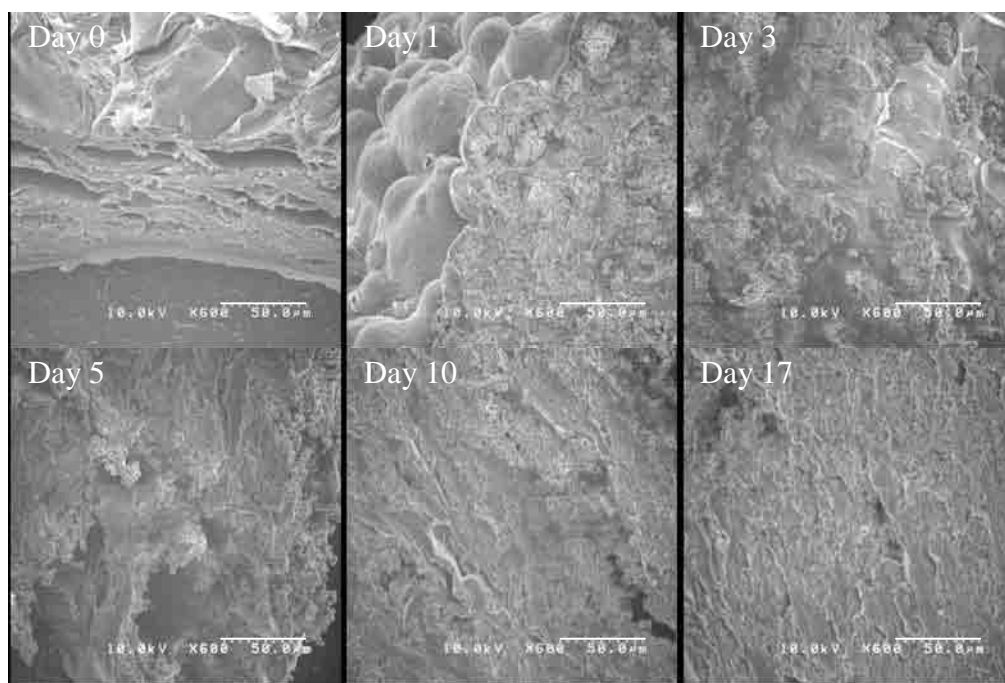


Figure 6. SEM images taken of the bead wall (between the outer and inner surfaces) for Days 0, 1, 3, 5, 10, and 17. The size bar on the figures denotes 50.0 μm.

3.1.3 Cell Bud Scar Counts

Bud scars on cells were also counted for all four treatments. A table was created to determine the distribution of scar number per cell (Table 1). It can be seen that the batch-log and chemostat cultures had higher number of cells with more bud scars than the

batch-stationary and immobilized cultures. The stationary phase and immobilized culture at Day 1 exhibited very similar patterns of budding. The majority of cells were unbudded, with a few showing one bud and an extremely low number with more than one bud scar. Even more interesting to note is the extremely high frequency of virgin, unbudded cells in the immobilized culture after Day 5. This indicates that the immobilized cells reproduce slowly over the first days before gradually exhibiting stationary phase-like cell division arrest.

Table 1. Percentage of bud scars counted for each treatment

	0	1	2	3	4	5
Chemostat	38%	31%	13%	11%	3%	5%
Batch-Log	16%	38%	13%	13%	13%	7%
Batch-Stat	46%	36%	4%	8%	4%	2%
Immob 1	45%	37%	4%	8%	4%	2%
Immob 5	69%	13%	8%	4%	3%	3%

3.2 Transcription Profiling

Of the more than 5700 genes assayed by the *Affymetrix Yeast Genechip 2.0*, 3559 genes significantly differed between treatments with a factor of 2-fold or greater. One-way ANOVA of the significantly expressed genes yielded 380 highly significant genes between immobilized and suspended cells. A heat map of the genes can be seen in Figure 7. Both replicates from each treatment are shown, including Days 1 and 5 immobilized cells. Each line represents a specific gene, where red lines indicate higher

expression and blue lines indicate down-regulation of genes. Yellow genes are neither up- nor down-regulated.

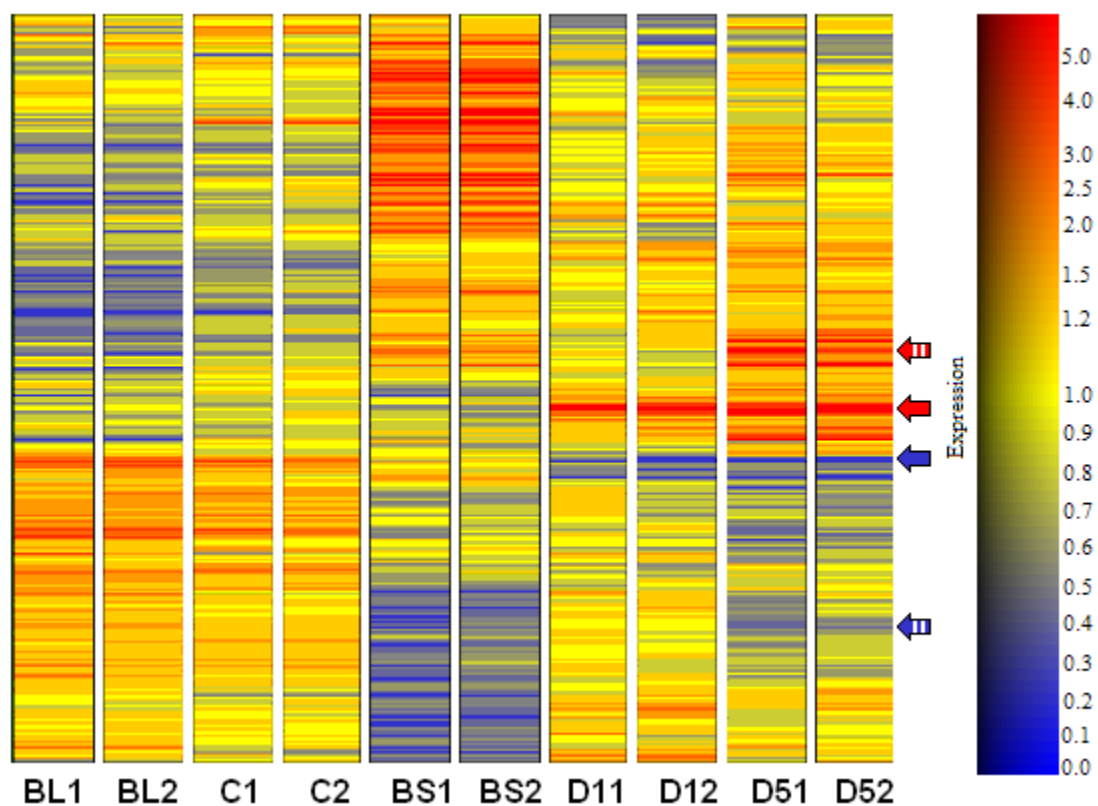


Figure 7. Heat map of significant genes expressed between treatments. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (D1), and Day 5 immobilized (D5). The expression bar describes the log-fold change represented by the colors.

Brief analysis of the map indicates bands of genes in immobilized cells with both higher and lower expressions than their planktonic cell counterparts (indicated by solid arrows). There are also bands of genes that are either up- or down-regulated on Day 5 only (shown by the striped arrows), indicating continued changes in gene expression of immobilized cells over time.

3.2.1 Gene Ontology

The ontology of each gene was downloaded from the Saccharomyces Gene Database (SGD), found at <http://www.yeastgenome.org/GOContents.shtml>. The gene names were passed through a filter to be separated into three categories: cellular component, biological process, and molecular function. The frequency of genes found within sub-categories was calculated for all significantly expressed genes.

The cellular component category includes subcellular structures, locations, and macromolecular complexes. A list of frequencies can be found in Table 2.

Table 2. Gene ontology - cellular component

Gene Ontology Term	Frequency of Significant Genes
Cytoplasm	69.5%
Nucleus	32.2%
Membrane	21.0%
Cellular Component Unknown	20.3%
Mitochondrion	18.1%
Endoplasmic Reticulum	8.1%
Endomembrane System	5.8%
Plasma Membrane	5.0%
Bud	3.6%
Site of Polarized Growth	3.6%
Cell Wall	2.2%

The highest frequency was found in genes coding for cytoplasmic components, such as transcription factors, ribosomes, mitochondrial parts, endoplasmic reticulum, Golgi bodies, microtubules, and vacuoles. 69.5% of all expressed genes were found to code for these components. 32.2% of these same genes were found to relate to the nucleus of the cell. These included genes coding for the nuclear envelope, membrane,

and lumen, as well as nucleolus, nucleoplasm, spliceosome, and signalosome complex genes.

The membrane is another cellular component of high frequency gene expression (21.0% of all significant genes). These genes encode for nuclear, Golgi, mitochondrial, and vesicular membranes, as well as the plasma membrane surrounding the cell. It is important to note that there is overlap between the gene ontology terms. This is illustrated with nuclear membrane genes, which fall under both membrane and nucleus sub-categories. Overall, 20.3% of all highly expressed genes were of unknown cellular component gene ontology.

The second category, biological process, involves such processes as metabolism, cell growth, and reproduction, and can be found in Table 3. 28.7% of the significantly expressed genes were grouped under the unknown biological process sub-category. The highest frequency of known biological processes was transport (22.2% of all significant genes). This involves all transport in the cell, including that of carbohydrates, ions, vitamins, proteins, vesicles, lipids, and peptides. The secretory pathway is also included in the transport category. The RNA metabolic process also had a relatively high frequency of genes, with 8.8% of all significant genes. Some of these processes involve RNA biosynthesis, processing, elongation, modification, and catabolism. Response to stress is another category of higher frequency (8.0% in all significant genes), including response to osmotic stress, water deprivation, and oxidative stress.

Table 3. Gene ontology - biological process

Gene Ontology Term	Frequency of Significant Genes
Biological Process Unknown	28.7%
Transport	22.2%
Organelle Organization and Biogenesis	21.6%
Protein Modification	10.4%
DNA Metabolic Process	9.7%
Transcription	9.4%
RNA Metabolic Process	8.8%
Cell Cycle	8.2%
Response to Stress	8.0%
Cell Wall Organization and Biosynthesis	3.8%

The final category, molecular function, includes tasks performed by individual gene products, such as transcription and translation regulation, chaperone and enzyme regulation, catalytic activity, and motor activity. These can be seen in Table 4.

Table 4. Gene ontology - molecular function

Gene Ontology Term	Frequency of All Significant Genes
Molecular Function Unknown	37.1%
Hydrolase Activity	19.5%
Transferase Activity	18.9%
Transporter Activity	11.7%
Protein Binding	9.9%
Structural Molecule Activity	9.5%
Transcription Regulator Activity	7.3%
Oxidoreductase Activity	7.1%
RNA Binding	5.6%
DNA Binding	4.7%
Peptidase Activity	3.7%
Protein Kinase Activity	3.5%
Ion Transporter Activity	3.4%

Slightly more than one-third of significant genes are grouped under the molecular function unknown (FUN) category. Of the remaining genes, 19.5% of all significant genes are related to hydrolase activity. Transporter activity genes, involving many similar processes to that of the transport genes in the biological process category, were expressed at frequencies of 11.7%. Expressed gene frequencies of 18.9% and 7.1% were found for transferase activity and oxidoreductase activity, respectively. Other molecular function sub-categories to show higher frequencies were transcription regulation activity, RNA binding, DNA binding, protein binding, and structural molecule activity.

3.2.2 *Glycolytic Genes*

Of the 3,559 significantly expressed genes, 14 pertain to the glycolysis ontology, while 16 are related to gluconeogenesis, shown in Figure 8. These two ontologies share eight common significant genes, including GPM1, GPM2, and GPM3. This family of genes codes for tetrameric phosphoglycerate mutase, which converts 3-phosphoglycerate to 2-phosphoglycerate. Also found in both glycolysis and gluconeogenesis are FBA1, TDH1, PGK1, ENO2, and TPI1.

FBA1 transcribes for fructose 1,6-bisphosphate aldolase, while TDH1 codes for glyceraldehyde-3-phosphate dehydrogenase 1. TPI1 is a gene induced under stress conditions which codes for triosephosphate isomerase. Expression of these three genes is very mild throughout the treatments. PGK1 forms 3-phosphoglycerate kinase, which catalyzes transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP, and is a key enzyme in both glycolysis and gluconeogenesis. Finally, ENO2, which codes for Enolase II, is the catalyst for the first

common step of glycolysis and gluconeogenesis. Expression of ENO2 is induced in response to glucose. Expression of these latter two genes, however, is lower for the Day 5 immobilized cells.

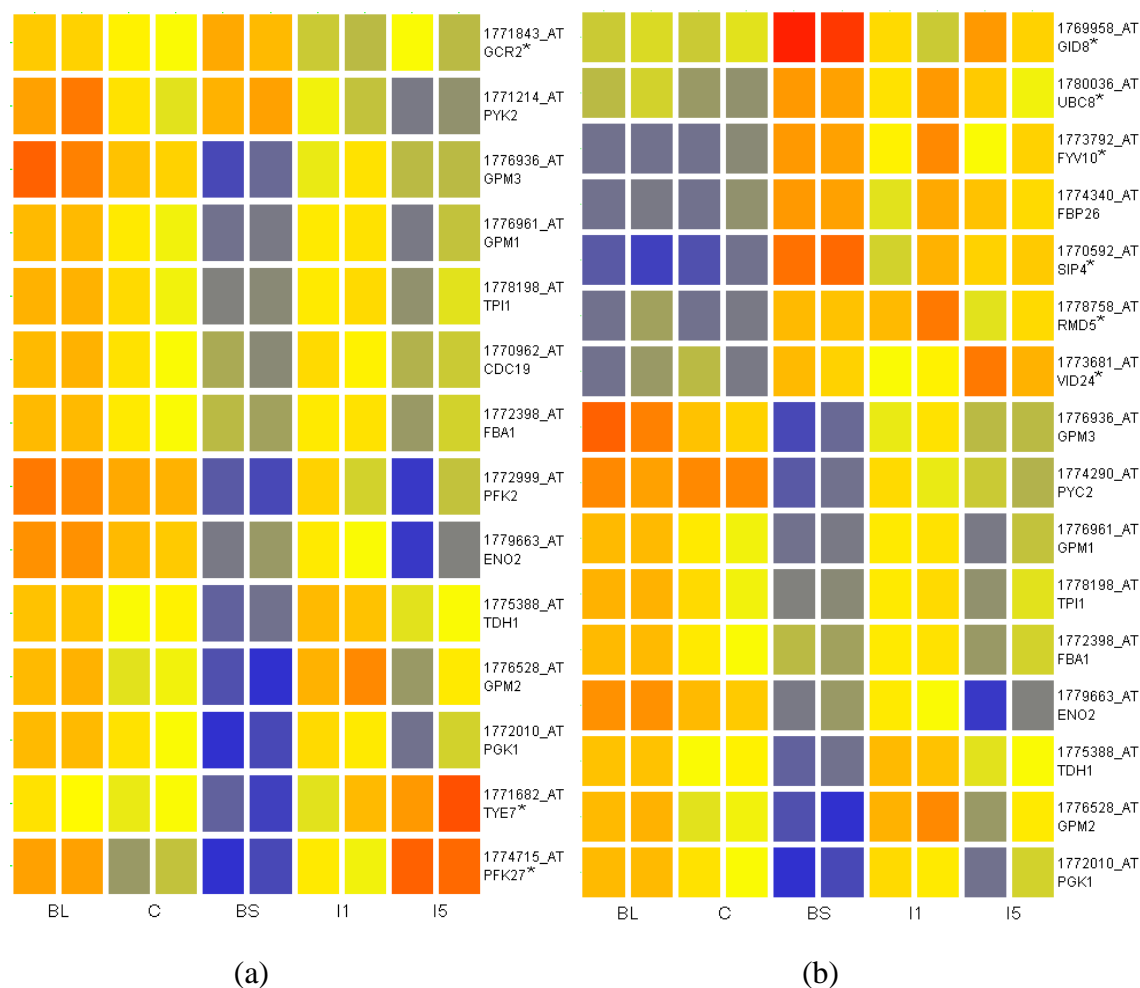


Figure 8. Expression of glycolysis (a) and gluconeogenesis (b) genes. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (I1), and Day 5 immobilized (I5). Gene names with an asterisk indicate regulatory genes.

Genes pertaining exclusively to glycolysis are CDC19, PFK2, PYK2, and glycolysis regulator genes TYE7, GCR2, and PFK27. CDC19 is a pyruvate kinase that functions as a homotetramer in glycolysis to convert phosphoenolpyruvate to pyruvate,

the input anaerobic (glucose fermentation) respiration. Although it performs such an important role in fermentation, this gene is only mildly expressed throughout all treatments. PFK2 is the beta subunit of heterooctameric phosphofructokinase involved in glycolysis, indispensable for anaerobic growth. In both the stationary culture and Day 5 immobilized cells this gene is down regulated, indicating a decrease or even lack of cell growth. PYK2 codes for pyruvate kinase, which catalyzes the final step in glycolysis. Expression of this gene is also decreased in Day 5 immobilized cells. This decrease is to be expected, however, as PYK2 transcription is repressed by glucose.

TYE7 codes for a serine-rich protein that binds E-boxes of glycolytic genes and contributes to their activation. High expression levels of this gene in immobilized cells indicate that there should be a higher rate of glycolysis. Increased expression is mildly evident in Day 1 immobilized cells, but can readily be discerned in the Day 5 immobilized cell samples.

GCR2, another transcriptional activator of genes involved in glycolysis, is only slightly expressed in the system. PFK27 codes for 6-phosphofructo-2-kinase and is induced by glucose. In both replicates of the Day 5 immobilized samples this gene is significantly up-regulated compared to the other treatments. Transcription regulation of this gene involves protein kinase A. Figure 9 depicts a pathway map with glycolysis genes overlain.

The gluconeogenesis genes that are not shared with the glycolytic pathway include PYC2 and FBP26. PYC2 levels are slightly higher in suspended cell cultures compared to immobilized cells. This gene codes for pyruvate carboxylase, a cytoplasmic

enzyme that converts pyruvate to oxaloacetate. FBP26, which codes for fructose-2,6-bisphosphatase and is required for glucose metabolism, is up-regulated in immobilized cells. Expression is higher in Day 5 cells than in Day 1 cells.

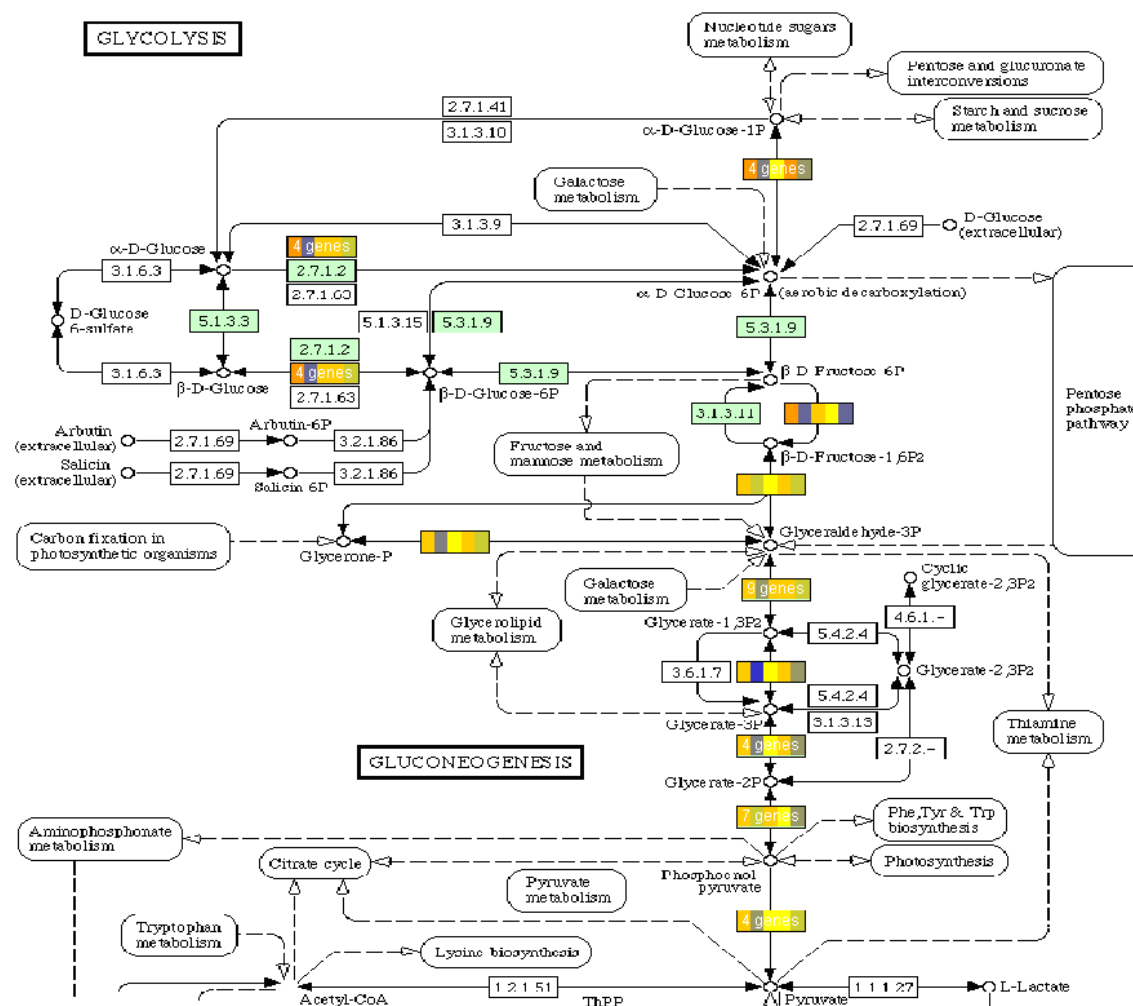


Figure 9. The glycolysis pathway with significant genes overlain. Individual color bars represent significant genes with varying colors representing (from left to right) log-phase, stationary-phase, chemostat, Day 1 immobilized, and Day 5 immobilized cultures.

Gluconeogenesis regulators GID8, UBC8, FYV10, SIP4, VID24, and RMD5 are also significantly expressed by Day 5, if only mildly. The expression levels of these genes, however, are elevated in immobilized cells compared to chemostat and batch-log cultures. All of these genes but SIP4 contribute to the inactivation and/or ubiquitination of fructose-1,6-bisphosphatase, which is essential to gluconeogenesis. SIP4 is involved in the positive regulation of gluconeogenesis.

3.2.3 Genes Pertinent to Ethanol

There are nine significant genes which directly pertain to production and utilization of ethanol. These can be found in Figure 10.

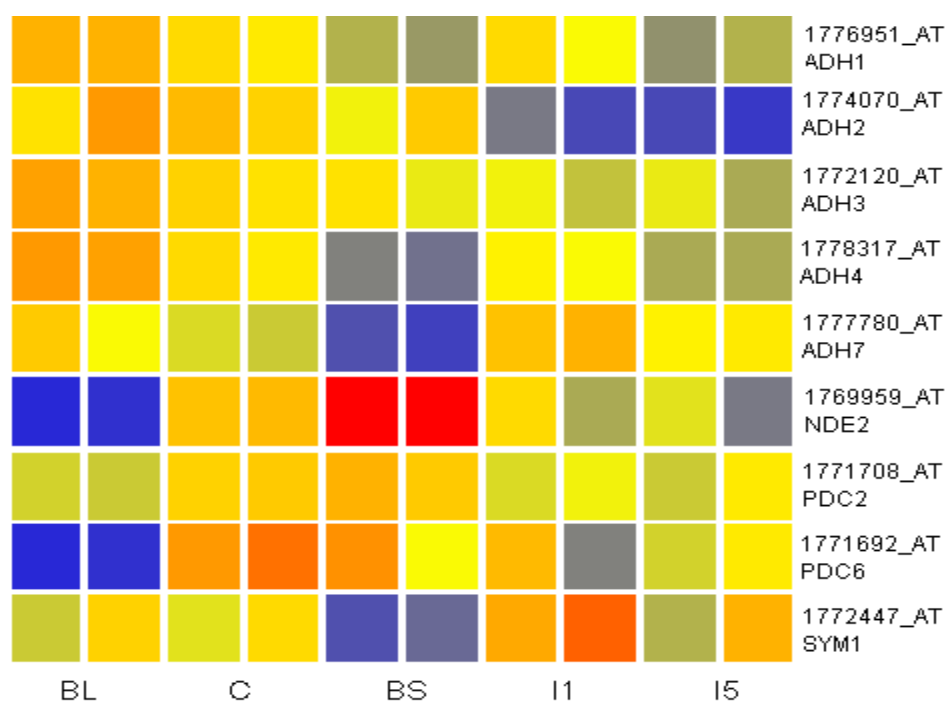


Figure 10. The genes pertaining to ethanol production and utilization.

Among these nine genes, only two exhibit a significant change of expression in immobilized cells. ADH2, an alcohol dehydrogenase gene which catalyzes the conversion of ethanol to acetaldehyde, is extremely reduced in both Day 1 and Day 5 immobilized cells. The extreme decrease in ADH2 provides additional insight into the increase in ethanol production. That the ethanol is not being converted into acetaldehyde would suggest that more is being excreted from the cell. SYM1, a FUN gene required for ethanol metabolism, however is up-regulated in the immobilized cells for both replicates on Day 1 and the second replicate only on Day 5.

Other alcohol dehydrogenase genes, ADH1, ADH3, ADH4, and ADH7 are also significant among the varying treatments. Among these, only ADH7 exhibits any change in immobilized cells. This gene is believed to be involved in alcohol synthesis. ADH1, ADH3, and ADH4 are all involved in the production of ethanol. PDC2 and PDC6, both pyruvate decarboxylases, convert pyruvate to acetaldehyde, which is then converted to ethanol. Finally, NDE2 is a mitochondrion external NADH dehydrogenase. These latter three genes, all included in the glucose conversion to ethanol ontology, are not significantly expressed (either up or down) in the immobilized cells. Significant expression is limited only to the batch reactor and chemostat samples. The seemingly sporadic expression of these ethanol-related genes is in concordance with the glycolysis genes in that the expression changes of the majority in immobilized cells is very small. Only ADH2 gives any clue as to why ethanol production is increased.

A pathway map of the continuing fermentation of pyruvate to ethanol, shown in Figure 11, reveals only that the genes leading toward ethanol production are slightly

increased in immobilized cells while those leading toward other compounds are decreased. This is most notable in the formation of acetyl-coA. The decrease observed in Day 5 immobilized cells indicates that the tricarboxylic acid (TCA) cycle is being repressed in immobilized cells. This result is logical, as the TCA cycle is not as necessary due to the extremely low growth rate observed.

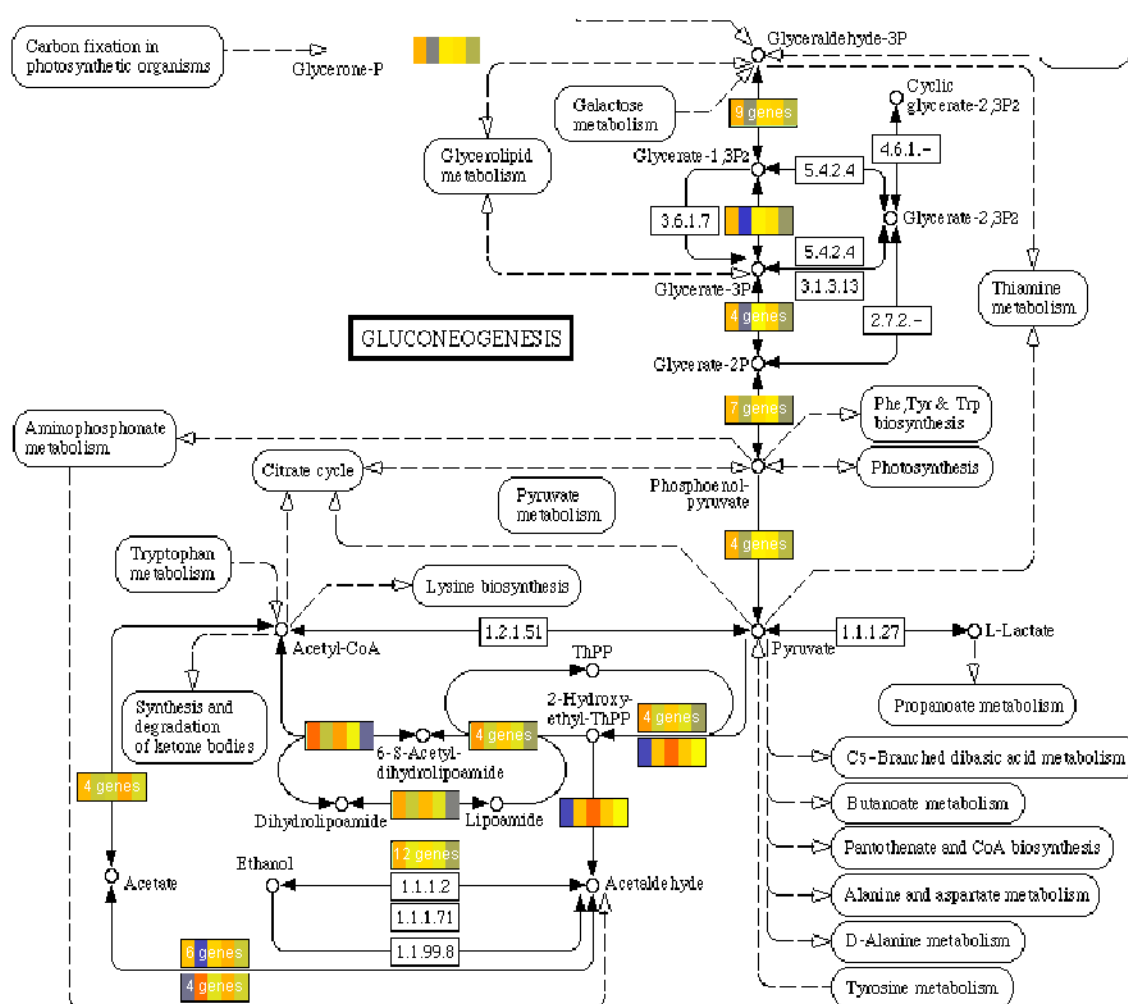


Figure 11. Continuation of the glycolysis pathway with significant genes overlain. Individual color bars represent significant genes with varying colors representing (from left to right) log-phase, stationary-phase, chemostat, Day 1 immobilized, and Day 5 immobilized cultures.

3.2.4 Other Interesting Fermentation Gene Families

The largest family of genes to be highly expressed in immobilized cells is the PAU family (Figure12).

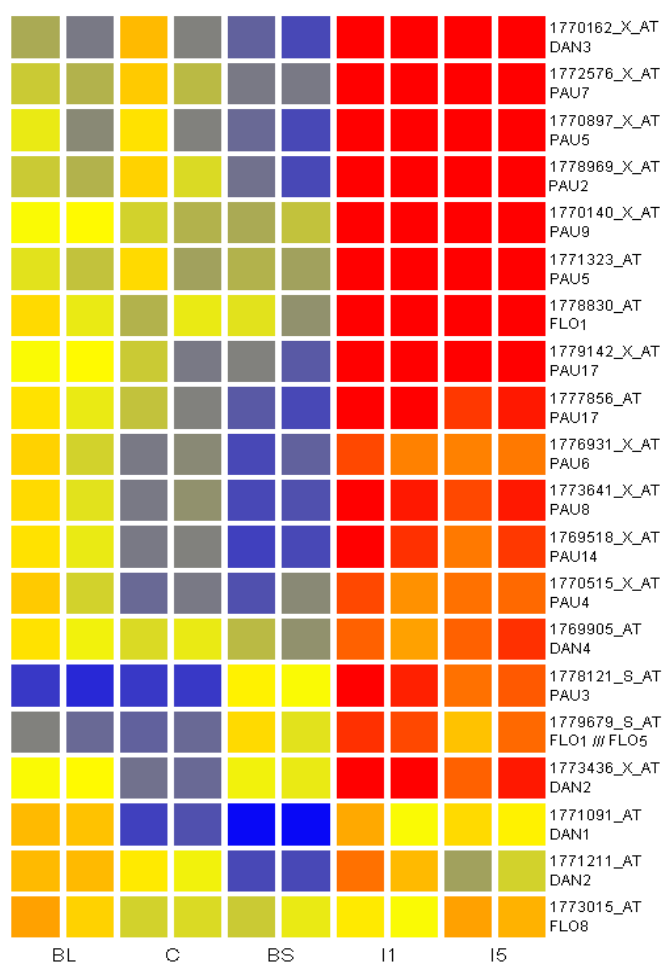


Figure 12. PAU, Dan and FLO genes highly expressed by immobilized cells. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (I1), and Day 5 immobilized (I5).

The PAU family is described as part of the 23-member seripauperin multigene family encoded mainly in subtelomeric regions, active during alcoholic fermentation, regulated by anaerobiosis, negatively regulated by oxygen, repressed by heme. While the

exact function and biological processes are unknown, these genes are activated during alcoholic fermentation. PAU5 has the largest log fold change of all genes expressed in immobilized cells. Other genes belonging to this family that are expressed are PAU2, PAU3, PAU4, PAU7, PAU8, PAU9, PAU14, PAU17, and PAU18. It can be seen that these genes are highly expressed after 24 hours of immobilization.

Another family of genes expressed is the DAN family. DAN2, DAN3, and DAN4 are also highly expressed in immobilized cells after just one day. These genes are described as cell wall mannoproteins expressed under anaerobic conditions, completely repressed during anaerobic growth. While it stands to reason that all PAU and DAN genes should be expressed due to anaerobic conditions, it is also important to note that all treatments were carried out anaerobically. The exact cause of higher expression in immobilized cells compared to suspended cells for these genes is not known, however, it does indicate that there is some factor other than lack of oxygen that contributes to their activation.

Three FLO genes, which are involved in flocculation, or natural immobilization, are also expressed by immobilized cells. FLO1 and FLO5 code for lectin-like proteins involved in flocculation. These are cell wall protein that binds to mannose chains on the surface of other cells, conferring flocculation-forming. FLO8, a transcription factor required for flocculation, is only mildly expressed. Interesting to note is that both DAN and FLO gene expression is increased in immobilized cells. While FLO genes code for adhesions that bind to mannose chains on the surface of other cells, DAN genes code for mannose chains on the cell wall. No specific interaction has been observed between

these two genes, however that they are both expressed so highly in immobilized cells is perhaps more than just coincidence.

3.2.5 Cell Cycle Genes

8.9 % of all expressed genes are related to the cell cycle. A heat map of these genes is shown in Figure 13.

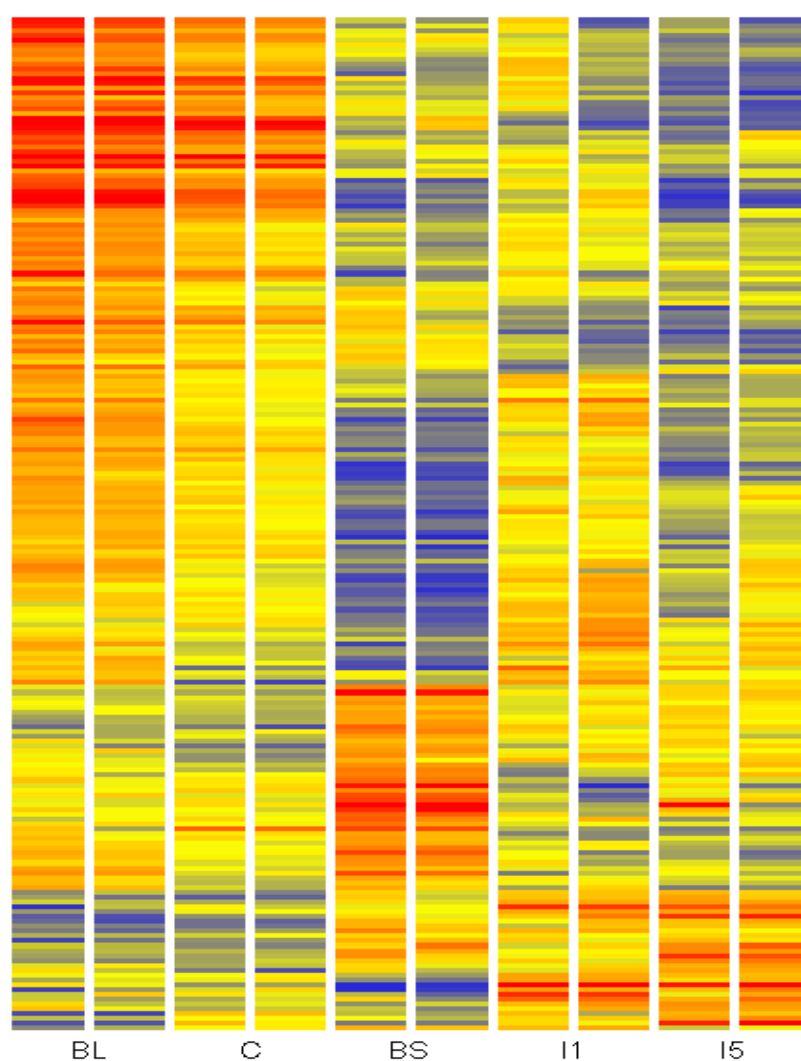


Figure 13. The significant genes pertaining to the cell cycle are shown. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (I1), and Day 5 immobilized (I5).

As expected from the decrease in immobilized cell reproduction, the majority of genes pertaining to the cell cycle are down-regulated compared to their planktonic cell counterparts. There are some genes with increased expression levels, though not nearly the same number. Most gene expression changes occur over the 5-day immobilized period, however, there are some which have already changed in the first 24 hours.

Figure 14 shows a portion of the down-regulated genes pertaining to the cell cycle.

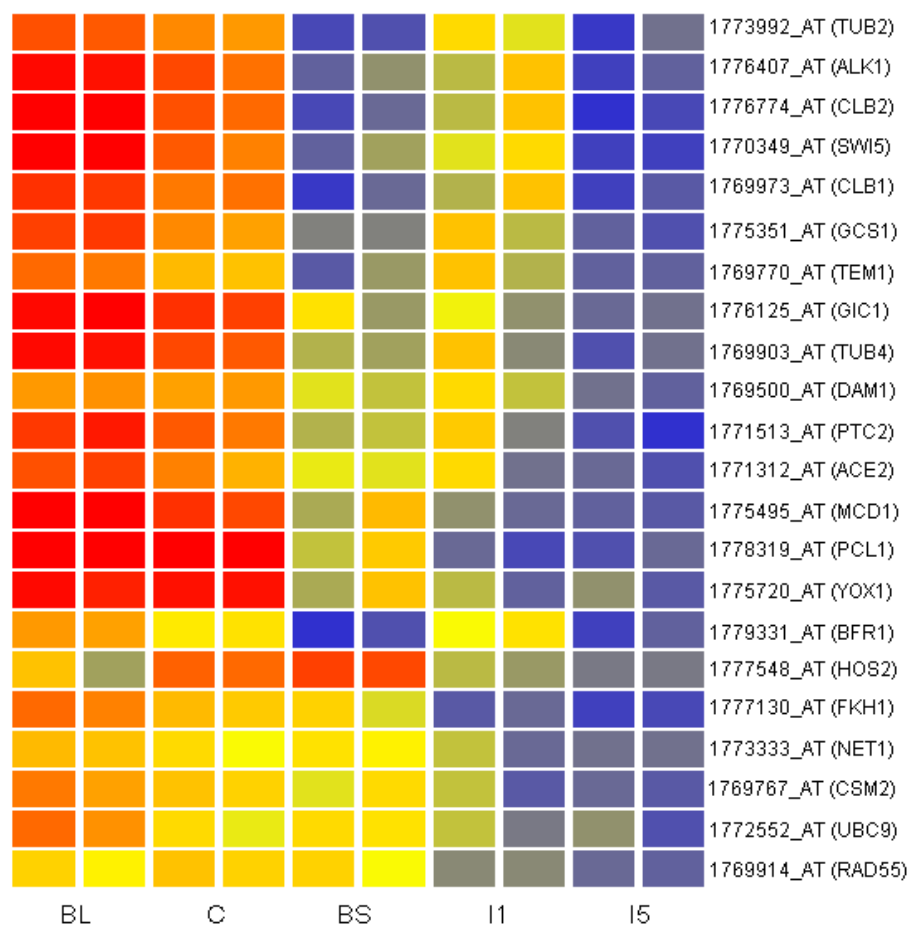


Figure 14. Genes pertaining to the cell cycle which are down-regulated in immobilized cells. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (I1), and Day 5 immobilized (I5).

Seven of the genes with lower expression in immobilized cells are expressed with a fold change factor of 2.0 or greater. Of these, only PCL1 and FKH1 have lower expression after only one day. PCL1 is involved in entry into the mitotic cell cycle and regulation of morphogenesis. As the immobilized cells reproduce much slower than the suspended cells from the initiation of the culture period, down-regulation of this gene was expected. FKH1 codes for a forkhead family transcription factor with a minor role in the expression of G2/M phase genes.

Other highly down-regulated genes are CLB1, CLB2, PTC2, SWI5, and YOX1. PTC2 plays a role in DNA checkpoint inactivation, and assists in down-regulating the unfolded protein response. PTC2 also dephosphorylates Cdc28p. CLB1 and CLB2 are B-type cyclins involved in cell cycle progression. These genes activate Cdc28p to promote the transition from G2 to M phase. SWI5 is a transcription factor that activates transcription of genes expressed at the M/G1 phase boundary and in G1 phase. This gene is regulated by phosphorylation by Cdc28p kinase. YOX1, a Cdc28p substrate, binds to Mcm1p and to early cell cycle boxes (ECBs) in the promoters of cell cycle-regulated genes expressed in M/G1 phase.

These genes all are involved in the mitotic cell cycle, shown in Figure 15. The relationship of CDC28 can be observed through M, G1, and S phases. Moreover, CLB1 and CLB2 can be seen to be extremely down-regulated in immobilized cells, thus halting passage through M phase. Lower expression of POL1, required for the initiation of DNA replication during mitotic DNA synthesis and premeiotic DNA synthesis also suggests a lower frequency of cells transitioning between G1 and S phase. The similarity between

Day 5 immobilized cells and stationary-phase batch culture cells should also be noted. In both cases, the cells have been observed to show little growth. That the genes can be proven to be down-regulated in these treatments is an especially encouraging discovery.

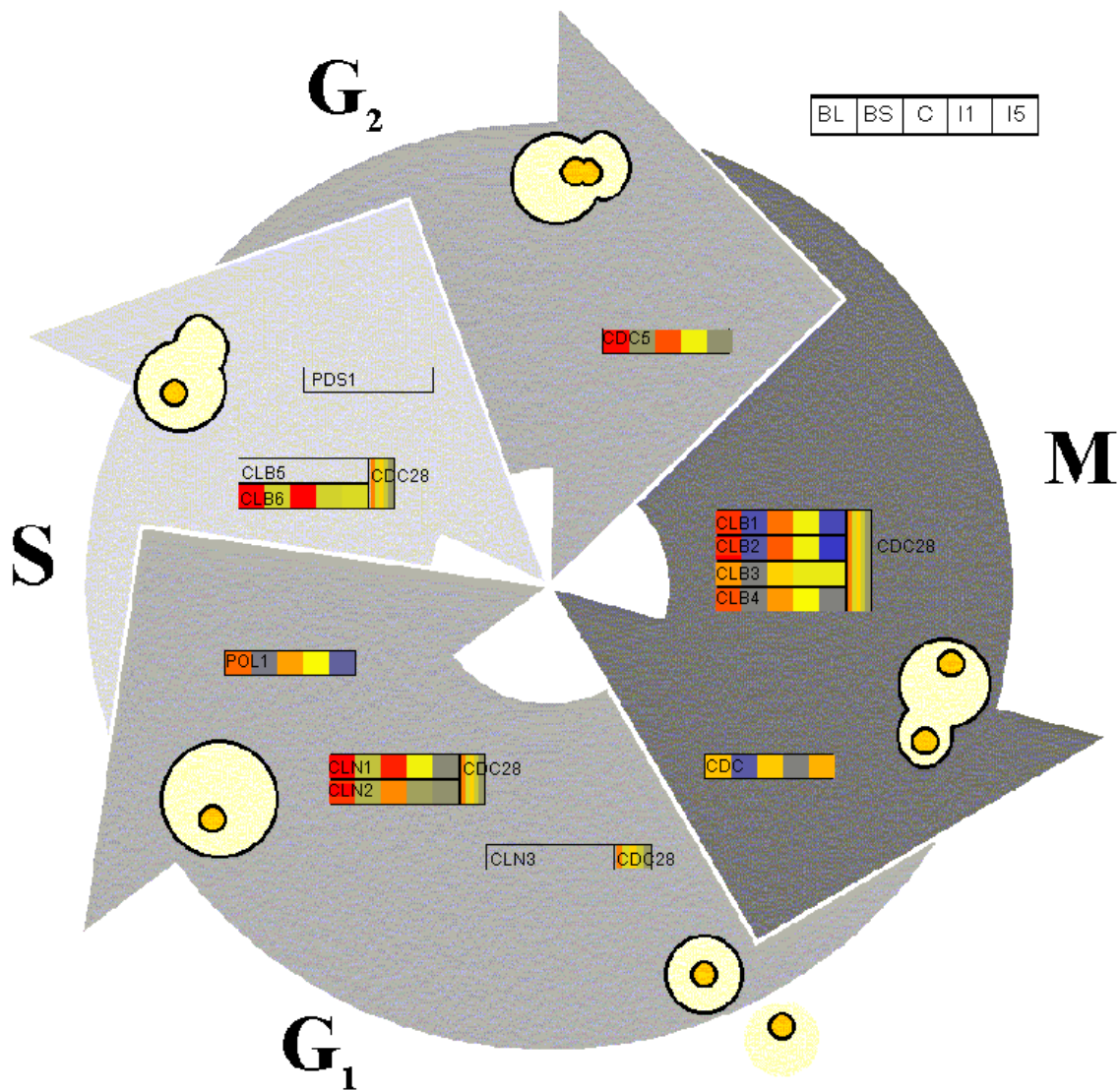


Figure 15. The mitotic cell cycle with expressed genes overlain. Individual color bars represent significant genes with varying colors representing (from left to right) log-phase, stationary-phase, chemostat, Day 1 immobilized, and Day 5 immobilized cultures.

TEM1, GIC1, ACE2, MCD1, UBC9 and NET1, which are also related to mitosis, all exhibit decreased expression in immobilized cells on Day 5. ACE2 is a transcription factor that activates expression of early G1-specific genes, while MCD1 expression is cell cycle regulated and peaks in S phase. GIC1 is involved in initiation of budding and cellular polarization. NET1 is involved in telophase exit, and TEM1 is a GTP-binding protein of the RAS superfamily involved in termination of M-phase. UBC9 is required for S- and M-phase cyclin degradation and mitotic control.

Other genes are also down-regulated in immobilized cells on Day 5. BFR1, CSM2, and DAM1 are all involved in chromosome segregation, and RAD55 codes for a protein that stimulates strand exchange. ALK1 is a protein kinase phosphorylated during the cell cycle in response to DNA damage, while GCS1 is involved in ER-Golgi transport. Finally, TUB4 and TUB2 code for gamma- and beta-tubulin, respectively. These are involved in formation of tubulin and nucleating microtubules from both the cytoplasmic and nuclear faces of the spindle pole body.

Among the genes with increased expression (shown in Figure 16), six are among the highly expressed genes with a fold change greater than 2.0. TYE7 has multiple ontologies, as it fits under both G1/S-specific transcription in the mitotic cell cycle and positive regulation of glycolysis ontologies. The HUG1 gene codes for a protein involved in the Mec1p-mediated checkpoint pathway that responds to DNA damage or replication arrest; transcription is induced by DNA damage. This DNA damage is not known, only that this gene is highly expressed after one day of immobilization. SPO19 and MND1 involve recombination and nuclear divisions. GAC1 codes for a heat shock

protein, and RCK1 is a protein kinase involved in the response to oxidative stress. All of these genes are highly expressed by the fifth day, while only HUG1 and GAC1 are expressed at elevated levels on Day 1.

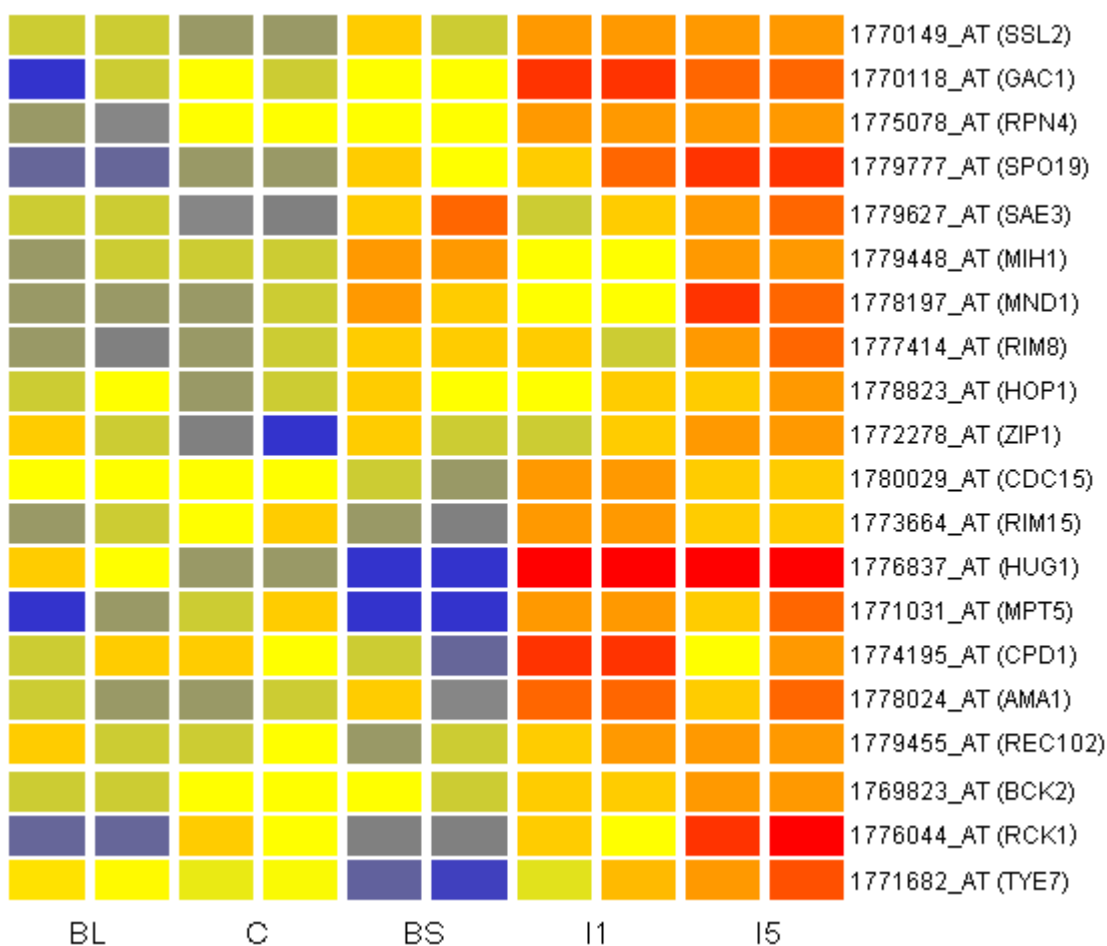


Figure 16. Cell cycle genes with increased expression in immobilized cells. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (I1), and Day 5 immobilized (I5).

Many of the genes involved in the cell cycle are also involved in chromosome synapsis and attachment, including HOP1, REC102, ZPI1, SAE3, and MND1. Other genes, such as AMA1 and SPO19 are sporulation genes. SSL2, which is only moderately

expressed in immobilized cells, is involved in DNA repair, similar to HUG1. RIM8, of unknown function, is involved in response to alkaline pH, while BCK2 is involved in the protein kinase C signaling pathway, which controls cell integrity. RPN4 stimulates the expression of proteasome genes, and is regulated by various stress responses. Finally, MPT5 is involved in longevity, maintenance of cell wall integrity, and sensitivity to and recovery from pheromone arrest.

AMA1 is a gene coding for activation of meiotic anaphase promoting complex, which would indicate an increase in cell reproduction. This gene, however, also codes for initiation of spore wall assembly, and fits under a broad category of sporulation genes discussed later on. Other genes involved in cell cycle regulation are RIM15, CDC15, and MIH1. MIH1 is simply listed as a protein tyrosine phosphatase involved in cell cycle control. Expression of RIM15, a protein kinase involved in signal transduction during cell proliferation, specifically the establishment of stationary phase, indicates a decrease in reproduction, as the cells cease to divide but maintain viability. CDC15, another protein kinase, is involved in the Mitotic Exit Network, promoting exit from mitosis. Figure 17 is a pathway map for the mitotic cell cycle in *S. cerevisiae*. The expression levels of significant genes have been overlain to provide a better understanding of the effect of immobilization on the cell cycle.

3.2.6 Sporulation Genes

Another 53 genes expressed significantly among treatments code for sporulation. A few sporulation genes are shown in Figure 18. Of these, the gene with highest expression is OSW2, which codes for a protein of unknown function proposed to be involved in the cell wall. This gene is highly expressed by Day 1, and expression continues throughout the five day study. AMA1 is also expressed by the first 24 hours, although lower than OSW2. OSW1, SPO74, and SPO 75, which are all involved in spore wall formation, are highly expressed by Day 5 in both immobilized replicates. SMA1 and SPS1, however, show very high expression in the second replicate, but only moderate expression elevation in the first replicate. These two genes are involved in prospore membrane assembly, and assist in correct localization of enzymes involved in spore wall synthesis.

SPO77 and DTR1, which are also required for spore wall formation, are only mildly expressed in Day 5 immobilized cells. Three other genes are immobilized at moderate levels on both Days 1 and 5. UBI4, which codes for ubiquitin, marks proteins for selective degradation, and is essential for the cellular stress response. NEM1 regulates nuclear growth and is required for sporulation. Finally, SPR1 contributes to ascospore thermoresistance.

Down-regulated genes in immobilized cells show lower expression in only one replicate in all but one gene. SUR7, an integral membrane protein down-regulated in both replicates, is a component of eisosomes, and associated with endocytosis. All other

down-regulated genes are significantly lower in batch-stationary cultures, with only a mild decrease of expression in immobilized cells.

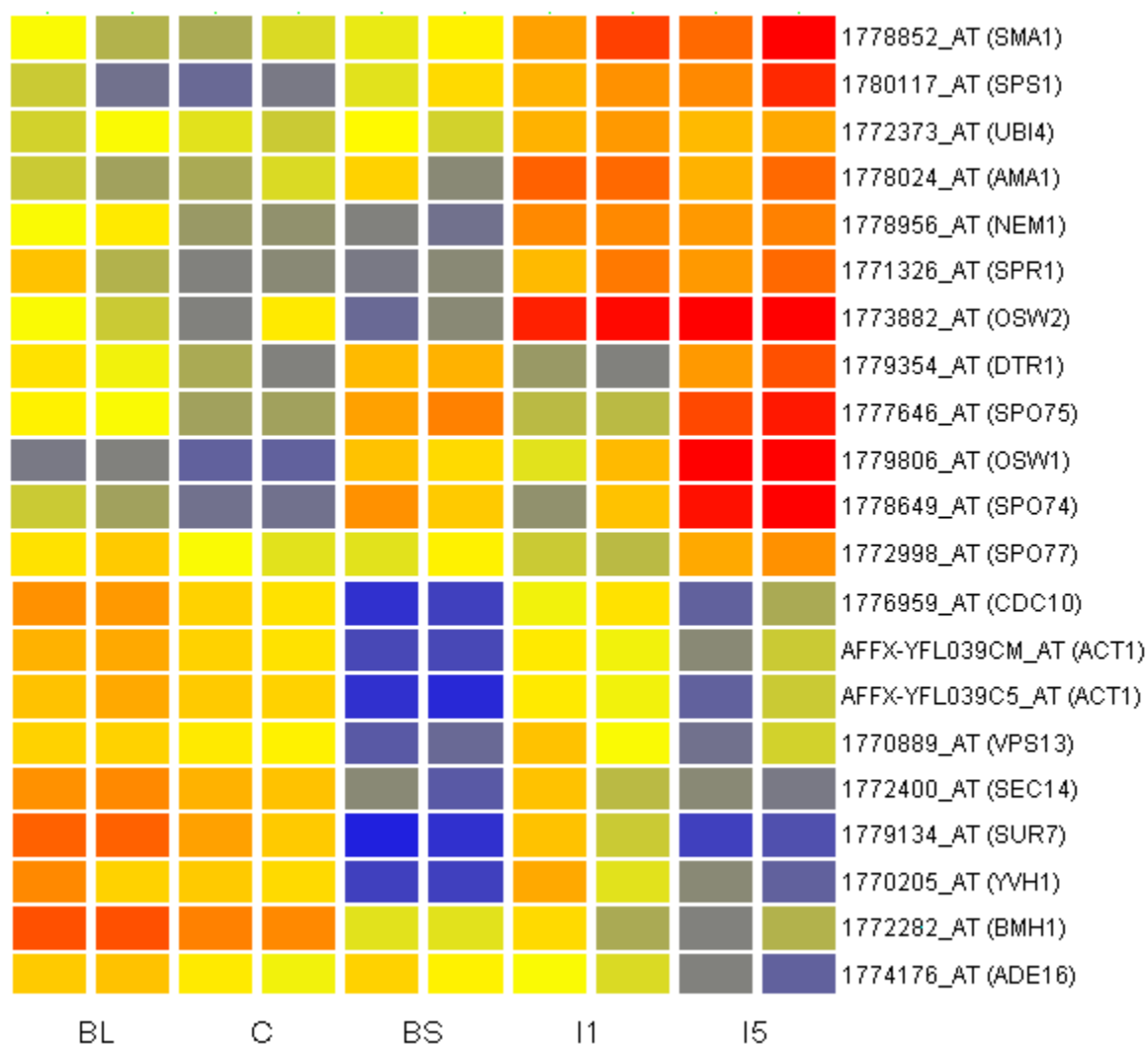


Figure 18. A sample of sporulation genes. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (I1), and Day 5 immobilized (I5).

3.2.7 Lipid Metabolism and Remodeling Genes

Ninety-four significantly expressed genes are involved in lipid metabolism and remodeling among the treatments. Of these 94, only three exhibit a highly significant expression increase in both immobilized cell replicates (see Figure 19). HES1, which regulates ergosterol biosynthesis, and YSR3, involved in sphingolipid metabolism, are both highly expressed after one day. GPI18, which is involved in glycosylphosphatidylinositol (GPI) biosynthesis, is expressed only mildly after 24 hours, but exhibits a much greater expression by Day 5. There are, in fact, nine different GPI genes that are expressed in immobilized cells during the study, if only mildly. These genes are all involved in GPI anchor synthesis, assembly, and attachment to proteins.

Two significant genes are down-regulated in both immobilized cell replicates on Day 5. DPM1 is required for GPI membrane anchoring and protein glycosylation. FEN1, a fatty acid elongase, is involved in sphingolipid biosynthesis, acting on fatty acids of up to 24 carbons in length.

Another family of genes with expression in many members is the ERG family, with 20 significant genes. These genes exhibit slightly increased expression in Day 1 immobilized cells, but mildly decreased expression in Day 5 immobilized cells. These genes are involved in sterol, ergosterol, and mevalonate biosynthesis.

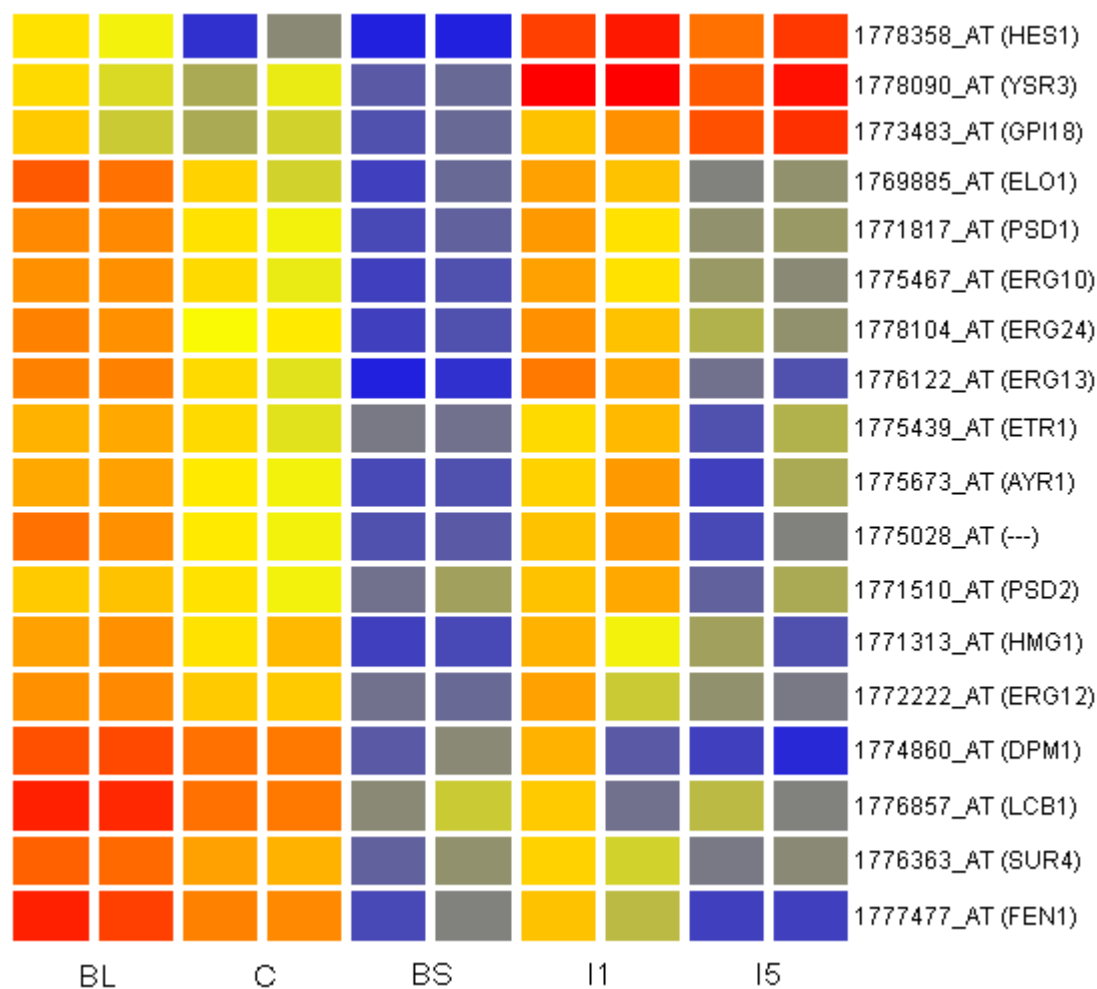


Figure 19. A sample of significant genes involved in lipid metabolism and remodeling. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (I1), and Day 5 immobilized (I5).

3.2.8 Stress Response Genes

Along with GAC1 and HUG1, MSN4 is the only other highly expressed gene in both Day 1 and Day 5 immobilized cells of the 204 significant genes associated with response to stress (Figure 20). MSN4 is a transcriptional activator activated in stress conditions, which results in translocation from the cytoplasm to the nucleus, binding DNA at stress response elements of responsive genes, and inducing gene expression.

Again, it is unknown what damage occurs to DNA upon immobilization, however, there are two highly expressed genes, MSN4 and HUG1, which are induced in response to DNA damage. RAD54 and SSL2, which are mildly expressed in immobilized cells, are also induced by DNA damage.

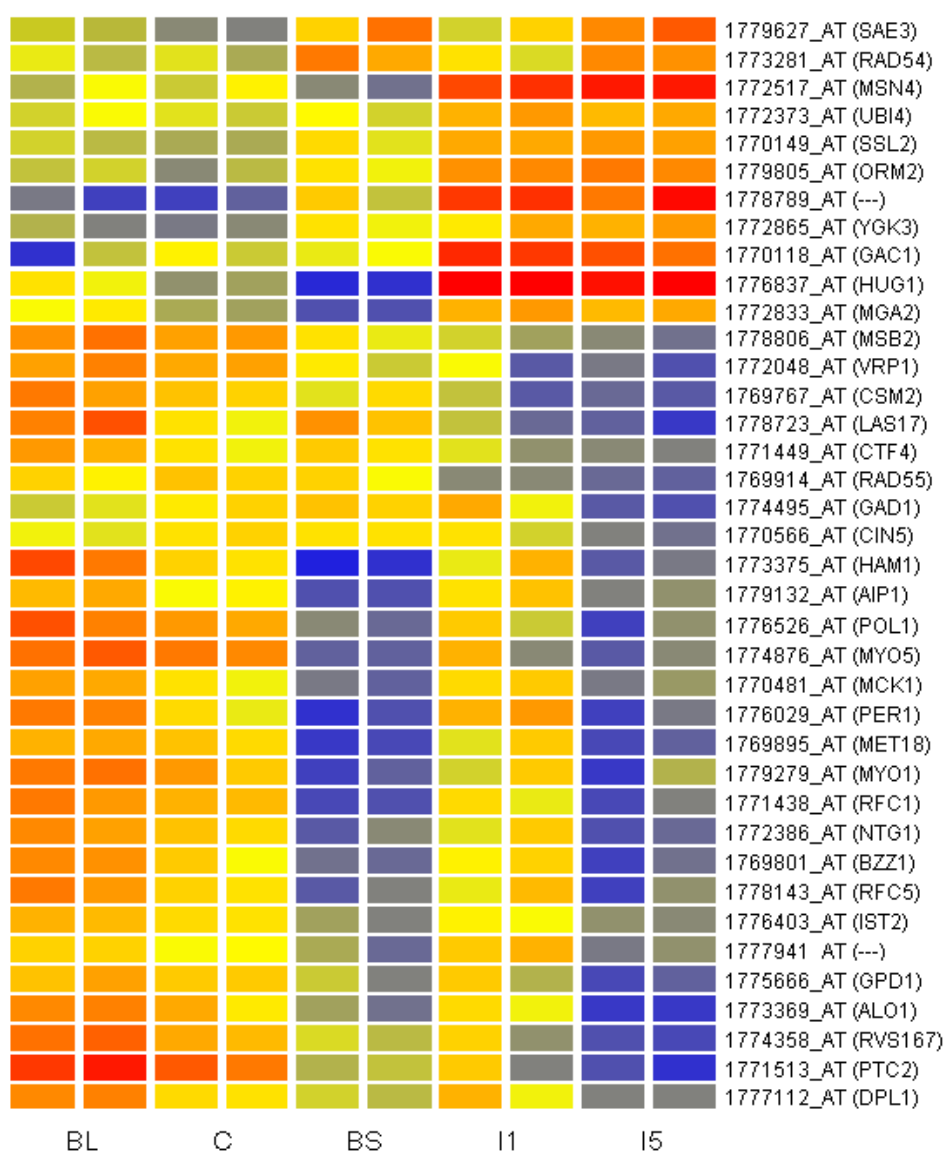


Figure 20. A selection of genes coding for stress response. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (I1), and Day 5 immobilized (I5).

The majority of genes related to stress response are mildly down-regulated in immobilized cells. VPR1, involved in cytoskeletal organization and cytokinesis, and LAS17, an actin assembly factor, are both moderately expressed lower in immobilized cells of one replicate on Day 1 and both replicates on Day 5. GAD1, a glutamate decarboxylase involved in response to oxidative stress, is also moderately down-regulated in Day 5 immobilized cells. ALO1 catalyzes the final step in biosynthesis of D-erythroascorbic acid, which is protective against oxidative stress, and is highly down-regulated in immobilized cells by Day 5. GPD1, RSV167, and PTC2 are all expressed under osmotic stress. That they are all down-regulated by Day 5 indicates a lack of osmotic stress on the cells immobilized in alginate beads.

There are also eight significant genes pertaining to pH response which all exhibit increased expression. RIM8, RIM20, and NRG1 are all expressed in response to alkaline pH. Another gene, TIR1, is down-regulated in response to acidic pH. That the gene is up-regulated is continuous with other pH-related genes. PMA1 and PMA2 are both regulators of cytoplasmic pH. These genes function to pump protons out of the cell to establish equilibrium between the cell and its surroundings. Although the pH of the media was maintained constant at 5.5, the immobilized cells appear to be reacting to an alkaline, rather than acidic solution.

3.2.9 Transport Genes

Of the 3,559 significant genes, 563 involve cellular transport. The similarity of the replicates is especially striking (see Figure 21).

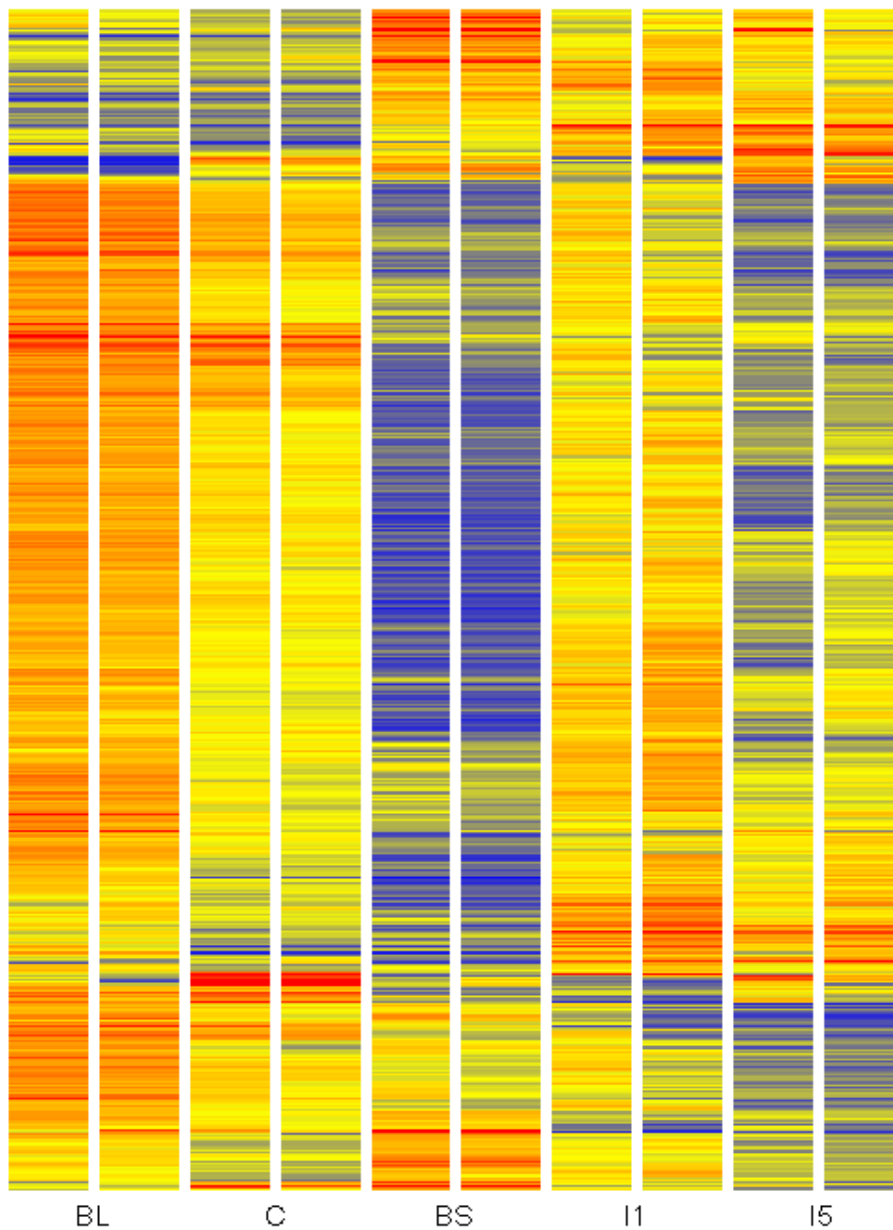


Figure 21. The expressed genes associated with transport in the cell. Replicates are shown from treatments log-phase (BL), chemostat (C), stationary-phase (BS), Day 1 immobilized (I1), and Day 5 immobilized (I5).

There are seven genes which exhibit highly increased expression in immobilized cells in both Day1 and Day 5 cells. HXT2 is a high-affinity glucose transporter, while CTR1 is a high-affinity copper transporter which mediates nearly all copper uptake under

low copper levels. These genes are both induced by low concentrations of the compound they transport. TPO4 is a polyamine transport protein which recognizes spermine, putrescine, and spermidine, which are all growth factors. AUS1 is involved in uptake of sterols and anaerobic growth, and, similar to the PAU genes, is up-regulated in immobilized cells *even though all treatments were performed anaerobically*. MCH5 is a plasma membrane riboflavin transporter which facilitates the uptake of vitamin B2. Finally, RSB1 is described as an integral membrane transporter or flippase that may transport sphingoid long chain bases from the cytoplasmic side toward the extracytoplasmic side of the membrane. This is yet another lipid/membrane gene that is up-regulated in immobilized cells.

GIT1, a plasma membrane permease which mediates uptake of glycerophosphoinositol and glycerophosphocholine, and PDR11, which mediates sterol uptake when sterol biosynthesis is compromised, are also expressed on Days 1 and 5, although more moderately. PMA2, expressed only in Day 1 cells, is involved in pumping protons out of the cell as a regulator of cytoplasmic pH and plasma membrane potential. As this gene is only up-regulated in Day 1 and not Day 5 immobilized cells, it is possible that cytoplasmic pH of the cells is under flux after immobilization, but eventually stabilizes by Day 5.

A majority of significant transport-related genes are actually down-regulated in immobilized cells. Most notably, FET3, required for high-affinity iron uptake and involved in mediating resistance to copper ion toxicity, has expression levels in immobilized cells much lower than its planktonic counterparts on both Days 1 and 5.

That this copper ion toxicity gene is down-regulated is in accordance with the increased expression level of the CTR1 copper transporter induced at low copper levels. Also highly down-regulated in immobilized cells on Days 1 and 5 are MEP1, which transports only ammonium, and PUT4, a high-affinity proline transporter.

Some genes are down-regulated only by Day 5 in immobilized cells. PAN1, which promotes protein-protein interactions essential for endocytosis, is one such gene. This correlates with the decreased expression of SUR7, another endocytosis gene. SOL2, a tRNA exporter, AVT6, a vacuolar transporter which exports aspartate and glutamate from the vacuole, and LAS17, an actin assembly factor, are also expressed exclusively in Day 5 immobilized cells.

There are three families of genes with many significant genes in immobilized cells. In this case, these genes are all mildly down-regulated by Day 5, some as early as Day 1. The SEC family, a group of genes coding for the secretory pathway, contains 25 significant genes. These genes are involved in SNARE docking and fusion, vesicle budding, and other secretory functions. The VPS family contains 20 significant vacuolar protein sorting genes. Finally, the NUP family, with 11 nuclear pore-related genes, is required to create the nuclear pore complex.

Pathway maps of less significantly changed pathways, including pyruvate metabolism, the pentose phosphate pathway, the secretory pathway, and protein export, can be found in Appendix B. The conversion of pyruvate to acetyl-coA and oxaloacetate is extremely repressed in immobilized cells, which allows for increased glucose conversion to ethanol. Less glucose is also utilized by immobilized cells in the down-

regulated pentose phosphate pathway. Finally, genes pertaining to the secretory pathway and protein export are also down-regulated in immobilized cells.

3.2.10 Other Considerations

There are a few considerations to address when analyzing the differences between immobilized and suspended cells. First of all, calcium serves various functions in the cell. It can act as a signaler for intracellular events and also serves to aid in flocculation. The media used in all treatments contained the same concentration of calcium. This was done in order to ensure that there would be no cellular changes observed due to calcium concentrations.

Another consideration is that while the cells in the differing treatments were grown under different conditions, the growth rates of the cells were also different for the four treatments. This could be one factor as to why certain changes are observed in immobilized cells. That the gluconeogenesis and lipid synthesis genes are down-regulated in immobilized cells is to be expected, as the cells are in a quasi-stationary state. These processes would not be necessary for cells with arrested growth, as is evident in both Day 5 immobilized cells and the stationary cells collected from the batch reactors.

Finally, while this study focuses primarily on the genes required for both replication and ethanol production, the strain used is not optimized for production of ethanol. It was chosen because the gene chip microarrays are specific to that strain. Given a strain optimized for ethanol production, such as those used in industry for fuel, chemical, and beverage purposes, the results might be somewhat different. An increase

in ethanol production would still be expected among the immobilized cells, as would a decrease in cell cycle promoting transcripts.

3.3 Long-Term Transcription Profiling

Analysis of only the immobilized cells from Days 0 through 17 revealed 794 significant genes. A heat map showing the significant genes of the average of the replicates is found in Figure 22.

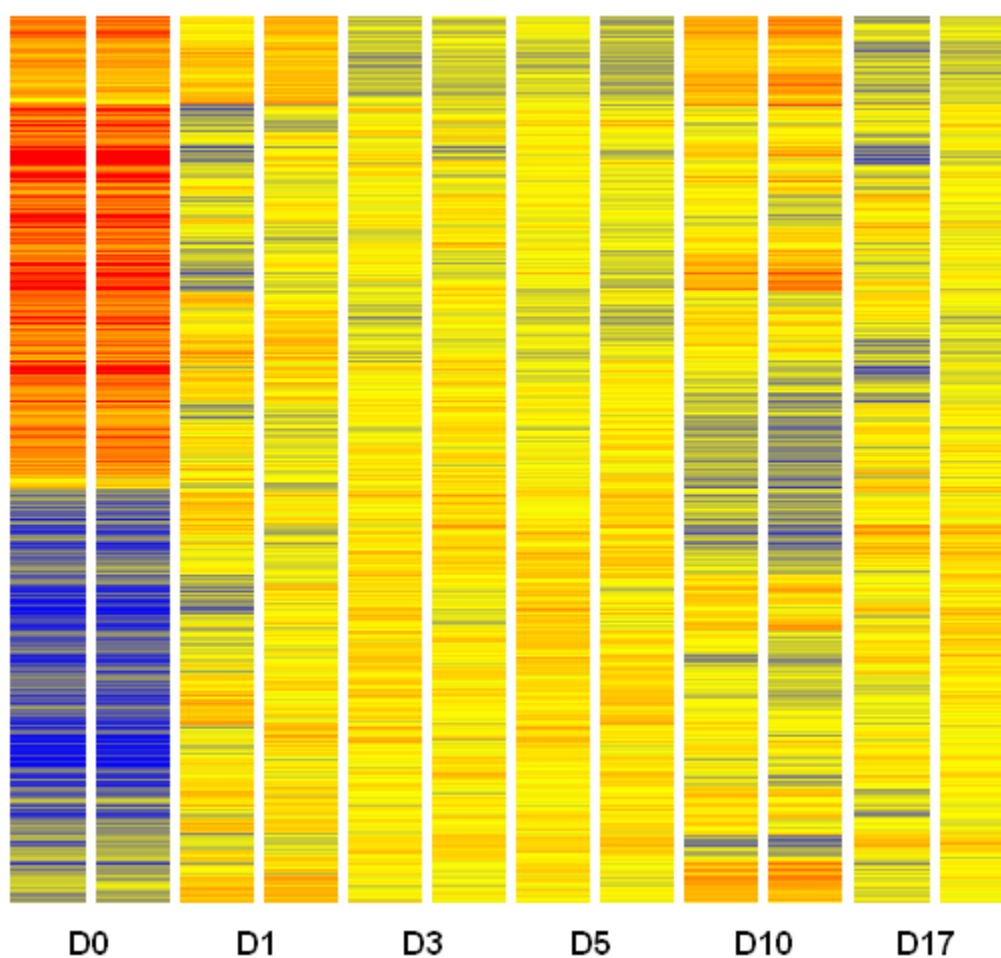


Figure 22. Immobilized cell expression for Days 0-17 (two replicates shown for each day).

There is a greater amount of different genes expressed on Day 10 than on Day 17. It can be seen that between Days 10 and 17 the expression tends to stabilize and is more similar to Day 5 on Day 17 than on Day 10. This is possibly due to a temporary change in environment during one of the two runs performed, most likely an unknown change in growth medium. Once the environment stabilized, the gene expression returned to normal immobilized expression.

Another explanation for the difference in gene expression for Day 10 is the higher presence of biofilms observed by the SEM imaging. Yeast cells in biofilms have been observed to not act as individuals in response to changing environmental conditions. These cells, however, are able to interact with other cells, change their behavior according to environmental conditions, and create colonies that allow yeast cells to differentiate for the benefit of the whole population (Kuthan et al. 2003).

Excluding the findings of Day 10, very few genes change in expression after the first day of immobilization. This is evidenced by the 48 and 21 genes expressed with a log fold change greater than 2.0 between Day 17 and Days 1 and 3, respectively.

Some of the same genes expressed in the immobilized cells after five days are also found expressed mildly on Day 17 (see figure 23). PAU5, HUG1, FLO1, FLO5, DAN1 and DAN2 are all mildly expressed on Day 17. Also mildly expressed are TIR1, TIR3, and TIR4, which are cell wall mannoproteins expressed under anaerobic conditions and required for anaerobic growth. ANB1 is a transcription factor expressed under anaerobic conditions. The glycolytic gene activator TYE7 is also expressed higher in Day 17 cells than in previous samples, indicating an increase in glycolytic rate. An increase

in glycolysis would also indicate that the cell is not preparing for death due to chronological aging, but that it is continuing to function normally.

DSE2 and DSE4 code for daughter cell-specific secreted proteins with similarity to glucanases which degrade the cell wall from the daughter side, causing the daughter to separate from the mother cell. ERG11 and ERG25, involved in ergosterol biosynthesis are both mildly expressed on Day 17, as is EXG1, another cell wall glucanase. PST1, a gene involved in maintenance of cell wall integrity is also expressed greater with time. That the cells are continuing to repair and regenerate the cell wall is an indication of continued longevity.

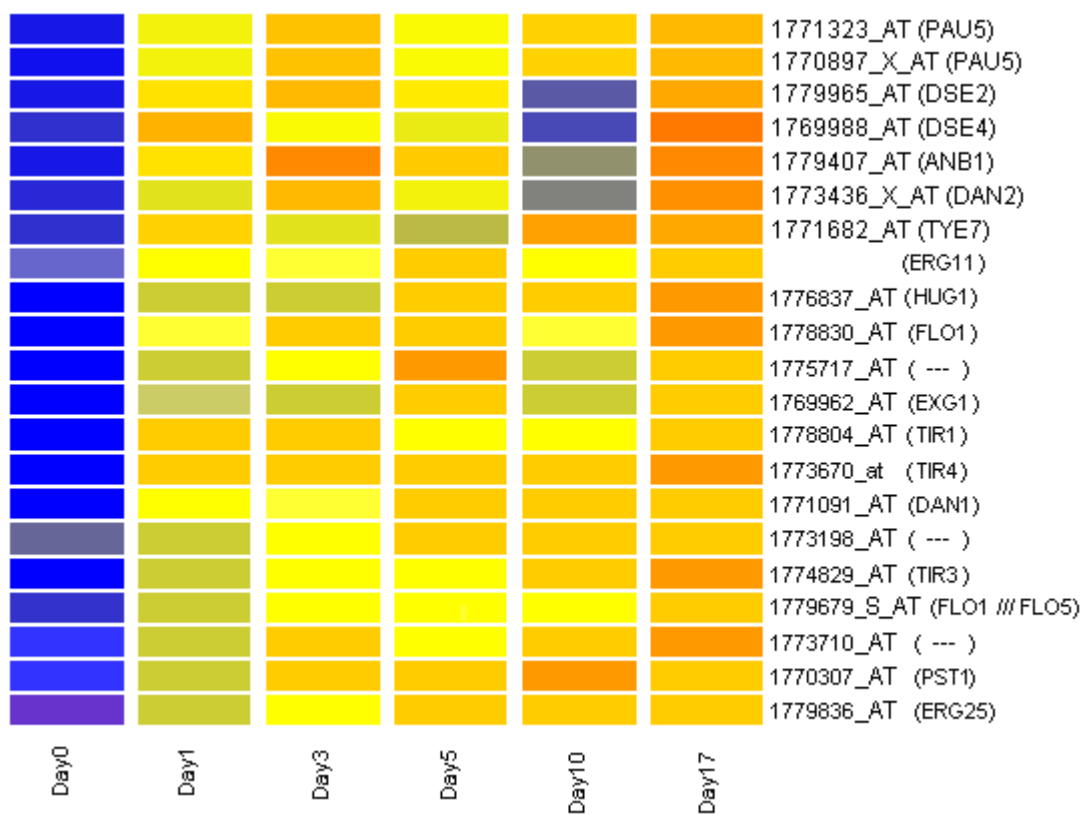


Figure 23. Mildly expressed genes after 17 days of immobilization.

Other genes continue to increase in expression after Day 5 (Figure 24). RLM1, a transcription factor involved in the maintenance cell integrity via the MAP-kinase pathway exhibits similar expression to PST1. Another gene with similar expression, SKG1, codes for a transmembrane protein with a role in cell wall polymer composition, localizing on the inner surface of the plasma membrane at the bud and in the daughter cell.

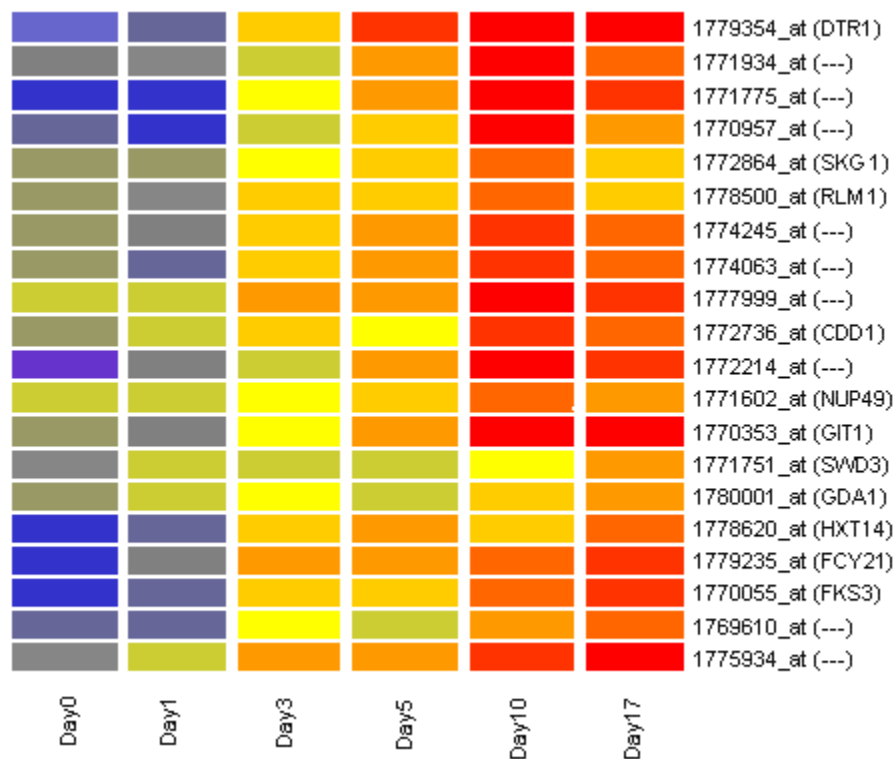


Figure 24. Highly expressed genes after 17 days.

Transporter genes DTR1, GIT1, and FCY21 are all expressed at much higher levels by Day 17 than Day 5. These genes code for transporters of dityrosine, glycerophosphoinositol and glycerophosphocholine, and purine and cytosine,

respectively. DTR1 and FKS3 are also involved in sporulation. Similarly expressed is CDD1, a cytidine deaminase which catalyzes the modification of cytidine to uridine. NUP49, which is involved in nuclear export of ribosomes is also expressed, although at a slightly lower level.

Finally there are a few moderately down-regulated genes on Day 17, as shown in Figure 25. SIP4, involved in positive regulation of gluconeogenesis, is one of these genes. This result coincides with the down-regulation of gluconeogenesis genes during the 5 day study. PET130, a protein required for respiratory growth, also exhibits a mild decreased expression. Also mildly down-regulated by Day 17 is APC9, which degrades anaphase inhibitors. Reduction in this gene indicates a reduction in mitosis of the immobilized cells. MDG1, a gene involved in pheromone signaling is also reduced with time.

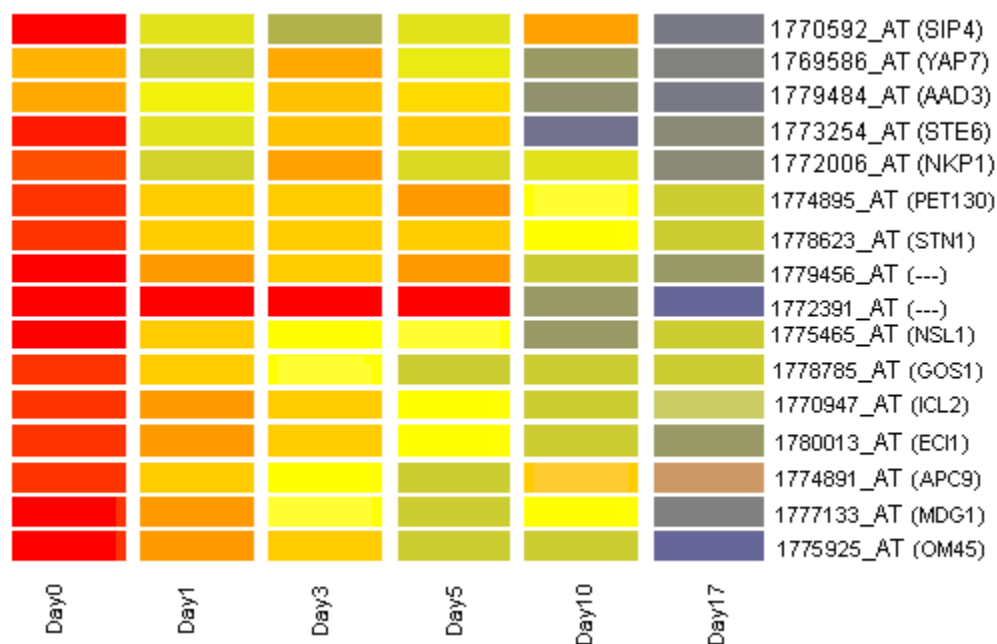


Figure 25. Down-regulated genes after 17 days.

Few significant genes among the immobilized cells are down-regulated by Day

17. Among those that are, most expression is decreased only slightly and many genes code for putative proteins of unknown function.

CHAPTER 4

CONCLUSIONS

As new technologies emerge in order to provide greater production of alternate fuels, cosmetics, chemicals, and pharmaceuticals, knowledge of what is happening at the genomic level of cells can be a great asset. Also of importance is what is happening physically to the immobilized cells. A greater understanding of these things may provide tools to improve efficiency and rates of production in various bioprocess applications.

Demographically, immobilized cells appear to reach a carrying capacity inside of the alginate beads. As the alginate began to dissolve, the number of cells inside of the beads also decreased. There were much fewer bud scars to be found in immobilized cells than in the suspended cell cultures. The growth kinetics of immobilized cells were also observed to be lower than suspended cells grown in both chemostats and batch reactors.

Increased production of ethanol with decreased production of biomass is the most visible characteristic of immobilized *S. cerevisiae*. Use of gene expression analysis has shown that this is not exactly a direct result of simply “switching on” one or two glycolytic genes. There are many genes contributing to the increase in ethanol production, and many others which decrease reproduction. Most notably, glycolysis regulators TYE7 and PFK27 are extremely expressed in immobilized cells by Day 5, causing an increase in ethanol production. Also, there are five genes inhibitory to gluconeogenesis that are expressed in immobilized cells, driving more pyruvate toward ethanol. The majority of genes expressed pertaining to the cell cycle, however, exhibit decreased expression in immobilized cells by Day 5, particularly those involved in

progression through mitosis. Elevated levels of cell cycle-related BCK2, MPT5, and RIM15 indicate that the immobilized cells exist primarily in a stationary phase with increased longevity.

Many other genes which code for a variety of biological functions are also significantly different between immobilized and suspended cells. Many sporulation genes were found to be expressed higher in the immobilized cells than their planktonic counterparts. A great number of genes pertaining to the secretory pathway have lower expression levels, especially those involved in endocytosis and vacuolar sorting and transport. There are other transporters, however, such as glucose, copper, and vitamin transporters, which exhibit increased expression. Lipid metabolism is also affected by immobilization. Many genes coding for synthesis of sterols, GPI, and sphingolipids are expressed at varying levels, both higher and lower, in immobilized cells.

There appears to be some damage that occurs to the DNA of immobilized cells, as is evidenced by the up-regulation of GAC1, HUG1, MSN4, RAD54, and SSL2 which are all induced by damage to DNA. Overall, however, the calcium alginate matrix provides a protective environment for the cells. The majority of stress response genes indicate that the oxidative and osmotic stresses are lower in immobilized cells, while the immediate surroundings are slightly alkaline.

It can also be seen that the gene expression observed after five days is very stable, as indicated by only 21 genes changing transcription abundance by 2.0 or greater over the next 12 days. Indeed, the limiting factor appears to be the life of the alginate beads, which had greatly dissolved over the 17 day test runs.

Because gene expression analysis has emerged only recently as a tool that can be used to analyze the state of a cell, there is no precedent with which to compare the results of this project. These results serve to confirm physical results already obtained in the laboratory setting, and also provide greater insight into the transcriptomic state of immobilized yeast cells over time.

CHAPTER 5

FUTURE RESEARCH

Future research opportunities include:

1. Identification and annotation of genes with unknown function. Many genes expressed significantly, especially during the 17 day fermentation runs, are either so little understood as to not be assigned a name or code for putative proteins of unknown function. This is also true for genes such as the PAU genes which are known to be up-regulated during anaerobic conditions, but have displayed other unknown characteristics during this study.
2. Determine the gene expression for yeast cells growing in a biofilm. This would enable further investigation into the deviating results of Day 10 immobilized cells during the long term immobilization study. Effects on glycolysis, cell cycle, transporter, and other ontologies could be determined.
3. Comparison of the gene expression analysis results with 2-dimensional gel electrophoresis. This treatment separates proteins inside of the cells by both size and pI. This will provide a second method to collaborate with the results found using the gene chips.
4. Determine the effects of immobilization on other organisms. Immobilization of other organisms can be performed to produce ethanol, protein, and other cellular products of interest in the pharmaceutical and chemical industries. It would be interesting to observe transcriptional changes induced by immobilization in other organisms and compare with the results of this study.

5. Genetically modify the *S. cerevisiae* strain used here in order to produce more ethanol. The findings from gene expression analysis could be useful to determine which genes to alter in order to improve ethanol production. This could also be used to determine if cells could be engineered to grow as they do when immobilized in planktonic form through gTME.
6. Develop a system to operate the immobilized column reactor continuously. Use of multiple immobilized columns placed in series could reduce the need for media recirculation. Parameters for such a system are as yet unknown and would need to be solved.

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APPENDICES

Appendix A. Glucose and Ethanol Concentrations

5 DAY RUNS

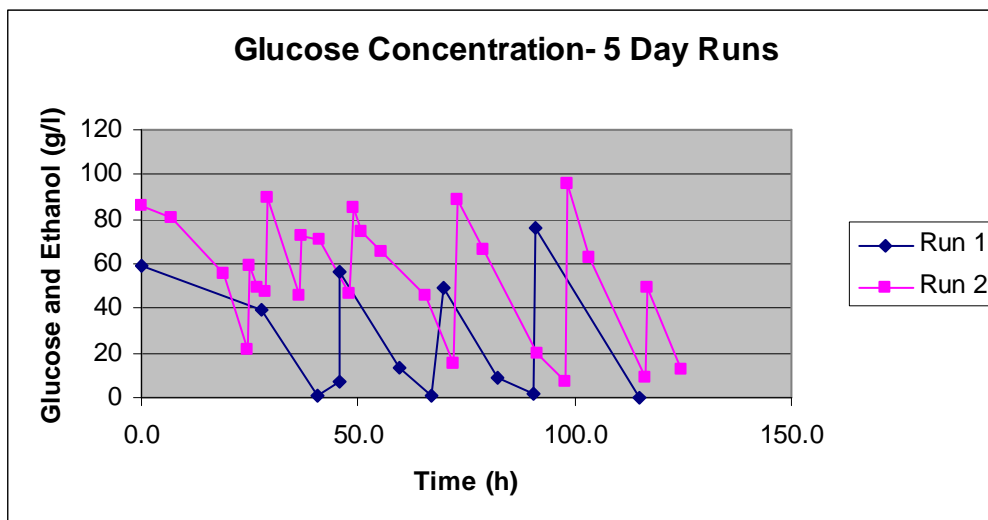


Figure 26. Concentration of glucose for the two 5 day runs.

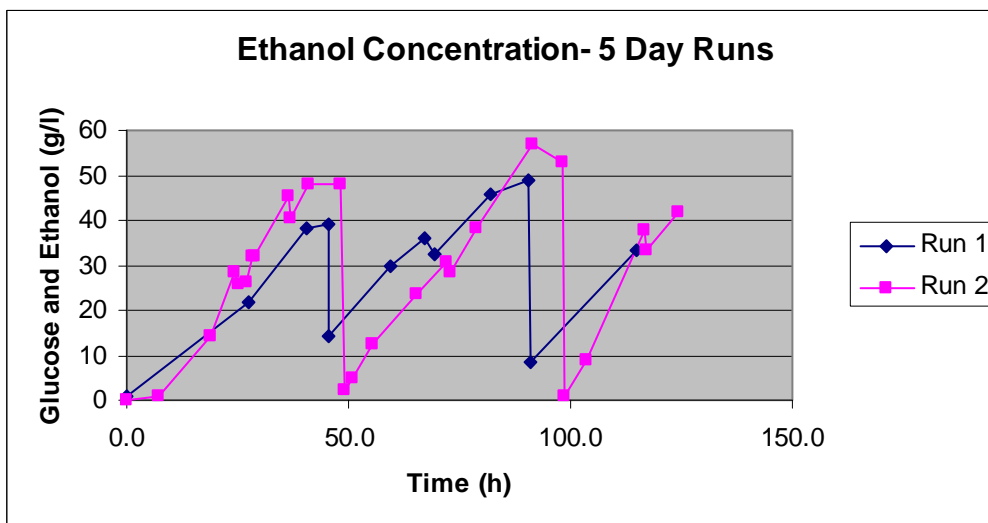


Figure 27. Concentration of ethanol for the two 5 day runs.

17 DAY RUNS

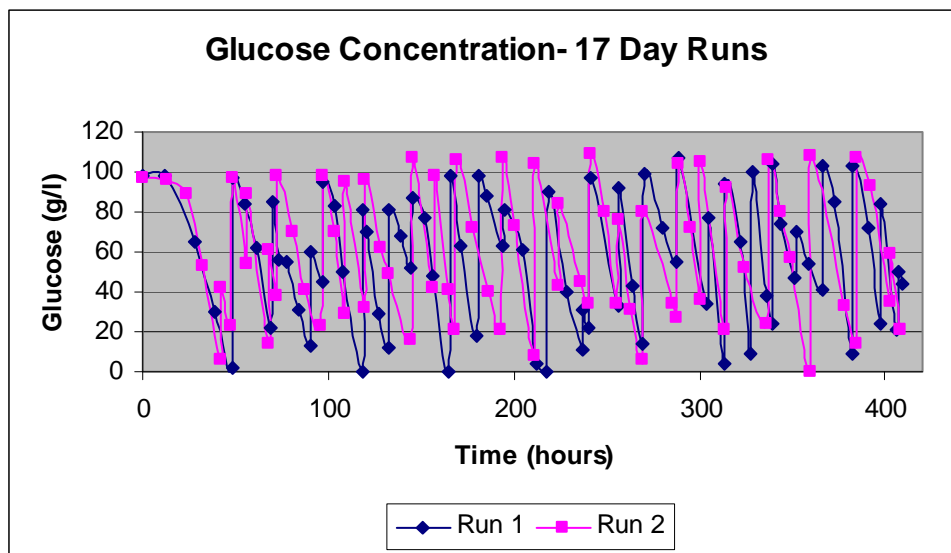


Figure 28. Concentration of glucose for the two 17 day runs.

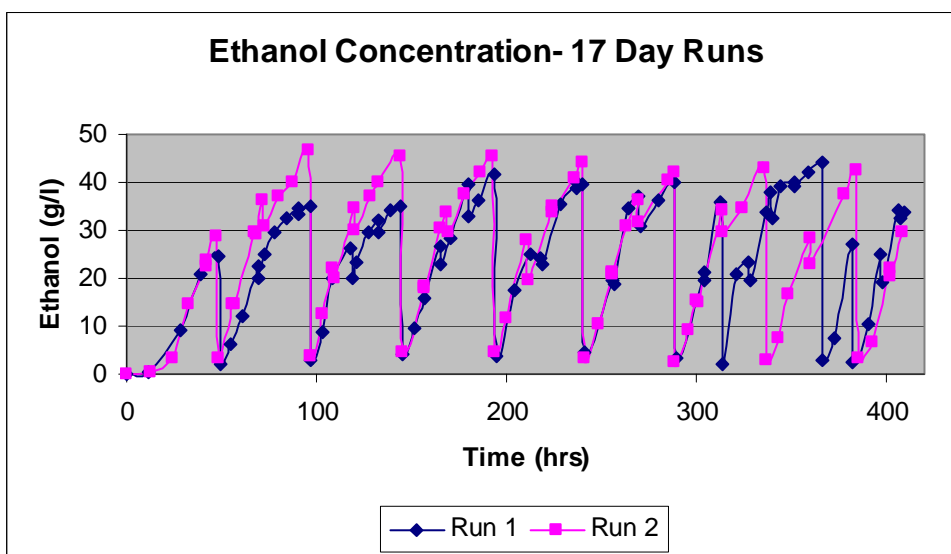


Figure 29. Concentration of ethanol for the two 17 day runs.

Appendix B. Pathway Maps

PYRUVATE METABOLISM

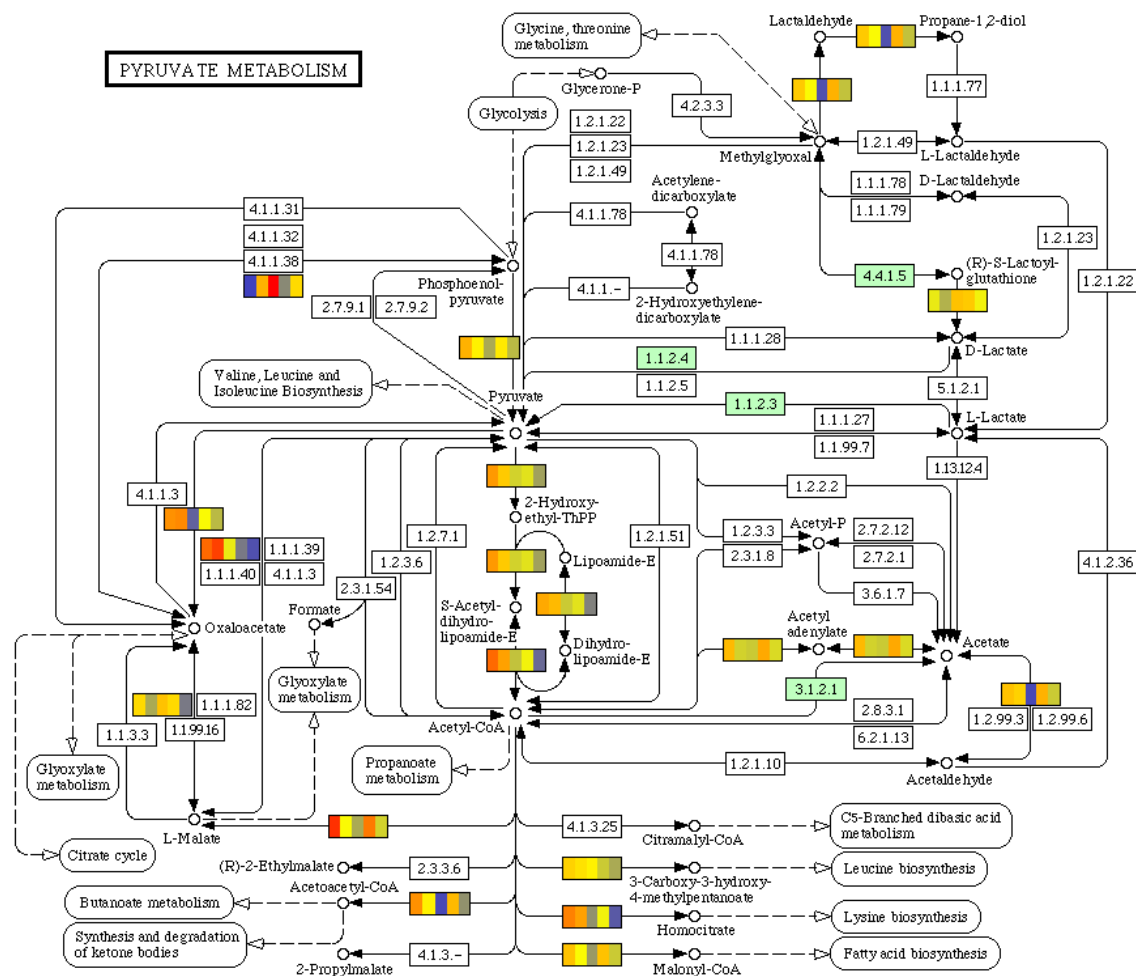


Figure 30. The fates of pyruvate with significant genes overlain. Individual color bars represent significant genes with varying colors representing (from left to right) log-phase, chemostat, stationary-phase, Day 1 immobilized, and Day 5 immobilized cultures. Conversion of pyruvate in to acetyl-coA and oxaloacetate is extremely repressed in immobilized cells, thus reducing the amount of glucose used for the TCA cycle.

PENTOSE PHOSPHATE PATHWAY

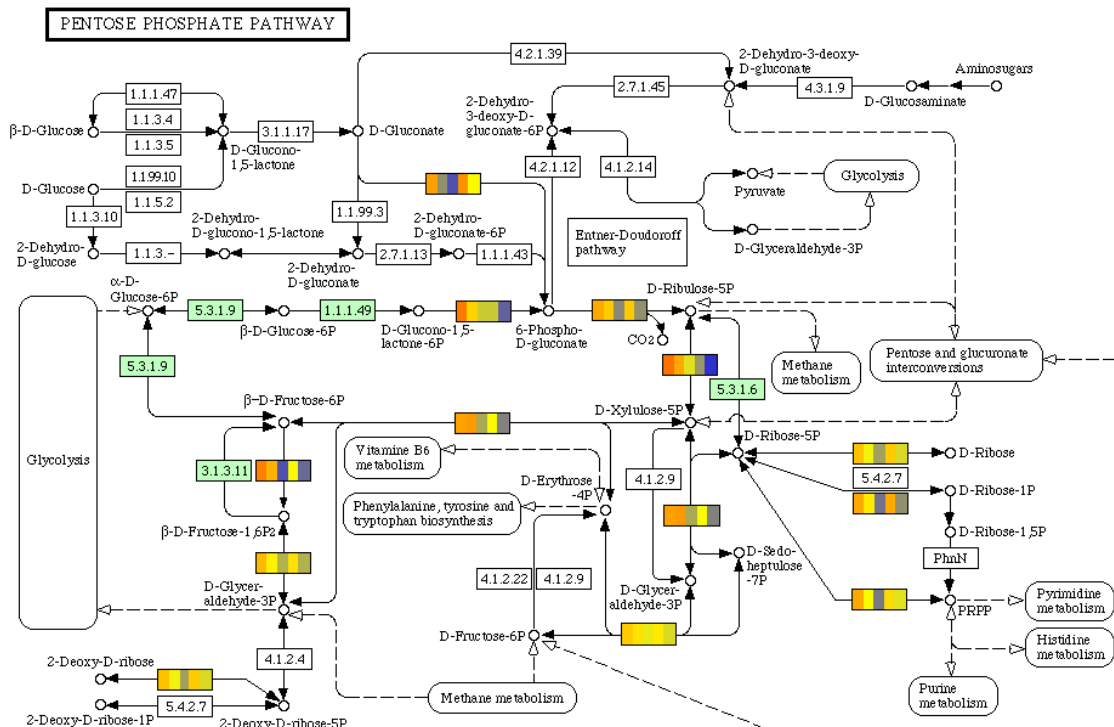


Figure 31. The pentose phosphate pathway with significant genes overlain. Individual color bars represent significant genes with varying colors representing (from left to right) log-phase, chemostat, stationary-phase, Day 1 immobilized, and Day 5 immobilized cultures. Entry into the pentose phosphate pathway is restricted greatly in immobilized cells by Day 5, especially between D-Ribulose-5P and D-Xylulose-5P.

SNARE INTERACTIONS

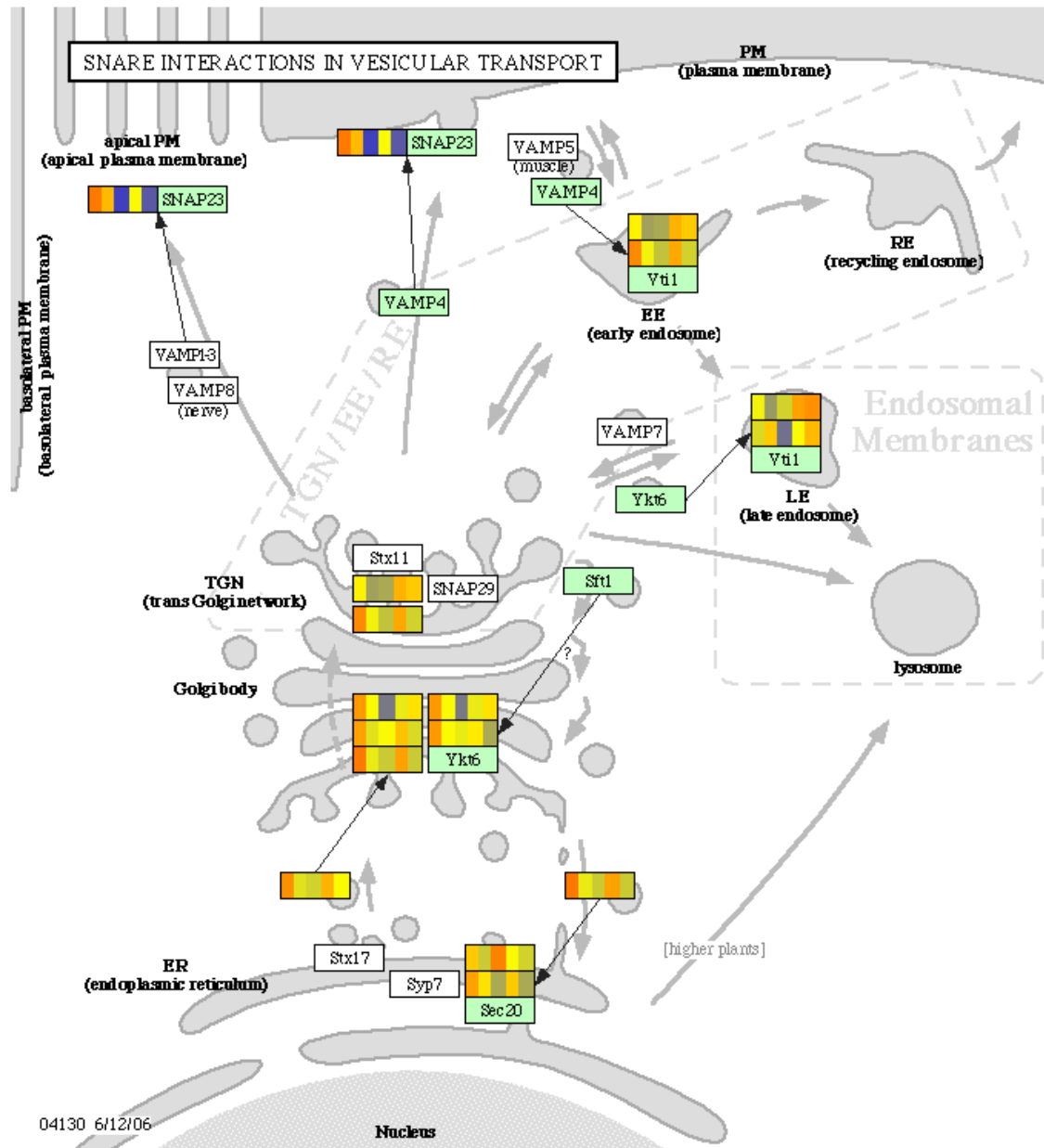


Figure 32. The secretory pathway with SNARE genes overlain. Individual color bars represent significant genes with varying colors representing (from left to right) log-phase, chemostat, stationary-phase, Day 1 immobilized, and Day 5 immobilized cultures. Genes pertaining to SNARE activity at the plasma membrane are down-regulated in Day 5 immobilized cells.

PROTEIN EXPORT

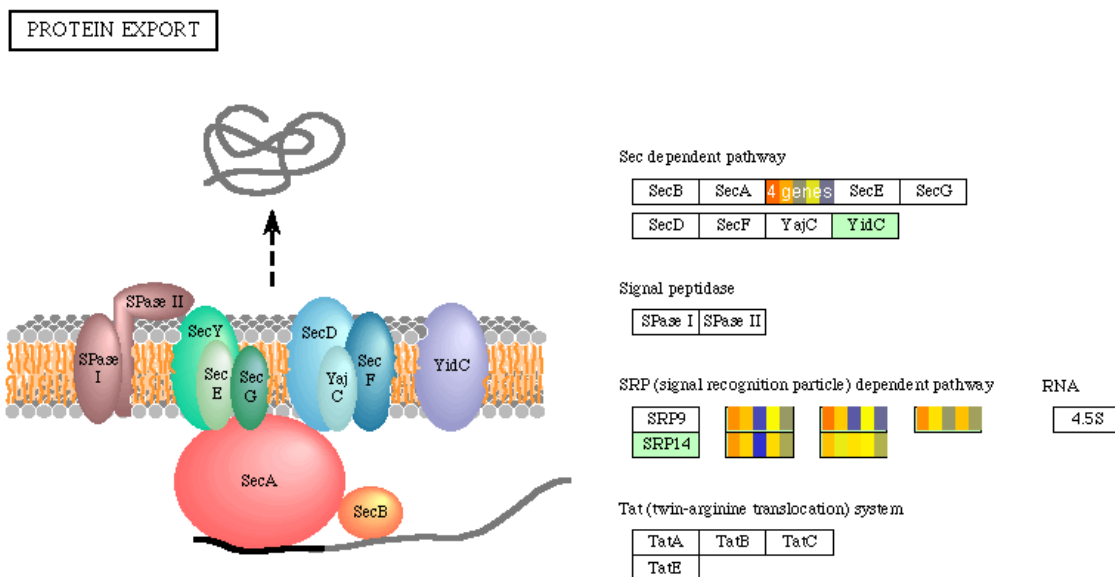


Figure 33. A diagram of the plasma membrane and genes responsible for protein export. Individual color bars represent significant genes with varying colors representing (from left to right) log-phase, chemostat, stationary-phase, Day 1 immobilized, and Day 5 immobilized cultures. Significant genes in immobilized cells are down regulated by Day 5.