PRODUCTION OF A RECOMBINANT PROTEIN USING *Pichia pastoris* GROWN IN KRAFT EVAPORATOR CONDENSATE

by

Preston Yee Ming Hoy
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Preston Hoy
Name of Author (please print) 16/07/2004 Date (dd/mm/yyyy)

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Department of Chemical and Biological Engineering
The University of British Columbia
Vancouver, BC Canada
ABSTRACT

Increasingly, environmental regulations are guiding industries to adopt practices which minimize water use and associated effluent discharge. In the pulp and paper industry, this has resulted in a trend to recycle waste streams as process water within various mill operations. Since instituting environmentally sound practices is often a balance between satisfying regulatory requirements and plant economics, any process which may satisfy both goals should be investigated further.

In this study, the feasibility of cultivating a recombinant strain of the methylotrophic yeast, *Pichia pastoris*, on combined evaporator condensate from a Kraft mill was investigated. The use of a recombinant yeast in this application is novel and the reasoning behind this research is two-fold. Firstly, to facilitate complete methanol removal from a waste stream, rendering it suitable for re-use within the mill or discharge. Secondly, to generate a product of potential economic value in the pulp and paper industry. A literature review was conducted to generate a list of *Pichia* transformants expressing potentially useful products. Through a selection process that rated each candidate based on a criteria set, a recombinant *Pichia* strain expressing lipase from *Geotrichum candidum* was selected.

A series of shake flask experiments were performed to gauge the effect of various media compositions on yeast growth. This was followed by fed-batch cultivations in a 1.8 L reactor which was monitored and controlled via a program written in LabVIEW [National Instruments, Austin, Texas]. Feeding was automated using a feedback algorithm loosely based on the SCF technique which used DO patterns to initiate cycling. Cell densities of 8-12 g/L (dry weight) were reached within the reactor grown on a condensate/methanol feed.

Lipase activity was determined titrimetrically and was found to occur only in the presence of yeast-peptone. Maximum enzyme activities for the reactor trials were in the range of 10.8 – 13.9 μmol/min/mL. Protein concentrations for the two final runs were

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measured at 57 and 48 mg/L, yielding respective specific activity values of 220 and 140 μmol/min/mg. These values are roughly 6-7 times lower than cited in literature and is thought to have been resultant from various factors including proteolytic deactivation.

This study has demonstrated the possibility of growing a recombinant yeast in a kraft waste effluent to produce a useful product.
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<td>AOX</td>
<td>alcohol oxidase</td>
</tr>
<tr>
<td>AST</td>
<td>activated sludge treatment</td>
</tr>
<tr>
<td>BOD</td>
<td>biological oxygen demand</td>
</tr>
<tr>
<td>CH₃SH</td>
<td>methyl mercaptan</td>
</tr>
<tr>
<td>COD</td>
<td>chemical oxygen demand</td>
</tr>
<tr>
<td>DHA</td>
<td>dihydroxyacetone</td>
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<tr>
<td>DHAS</td>
<td>dihydroxyacetone synthase</td>
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<tr>
<td>DMS</td>
<td>dimethyl sulfide</td>
</tr>
<tr>
<td>DMDS</td>
<td>dimethyl disulfide</td>
</tr>
<tr>
<td>DO</td>
<td>dissolved oxygen</td>
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<tr>
<td>GAP</td>
<td>glyceraldehydes 3-phosphate</td>
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<tr>
<td>GCL</td>
<td><em>Geotrichum candidum</em> lipase</td>
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<tr>
<td>H₂S</td>
<td>hydrogen sulfide</td>
</tr>
<tr>
<td>HAP</td>
<td>hazardous air pollutants</td>
</tr>
<tr>
<td>HRT</td>
<td>hydraulic retention time</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-{N-Morpholino}popanesulfonic acid</td>
</tr>
<tr>
<td>Mut(⁺/⁻/⁻)</td>
<td>methanol utilization phenotype (plus/slow/minus)</td>
</tr>
<tr>
<td>PID</td>
<td>proportional/integral/derivative</td>
</tr>
<tr>
<td>PFR</td>
<td>packed bed reactor</td>
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<tr>
<td>SBR</td>
<td>sequencing batch reactor</td>
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<tr>
<td>SCF</td>
<td>self-cycling fermentation</td>
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<tr>
<td>SCP</td>
<td>single cell protein</td>
</tr>
<tr>
<td>TRS</td>
<td>total reduced sulfur</td>
</tr>
<tr>
<td>USAB</td>
<td>upflow anaerobic sludge blanket</td>
</tr>
<tr>
<td>VOC</td>
<td>volatile organic compound</td>
</tr>
<tr>
<td>X₅P</td>
<td>xylulose 5-monophosphate</td>
</tr>
<tr>
<td>YP</td>
<td>yeast-extract peptone</td>
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CHAPTER I - INTRODUCTION

Methanol is a ubiquitous substrate in nature, and is present in many industrial waste streams including those from Kraft pulp mills. The low concentration of methanol in combined Kraft mill effluent precludes its economic use as a dedicated substrate for microbial growth. However, internal process streams such as combined and foul condensates contain much higher concentrations of methanol which must be removed prior to re-use to meet methanol emission standards US EPA Cluster rules (Berube and Hall, 1999). Energy intensive steam stripping is currently the method of choice to achieve this end.

Biological treatment of condensate is an alternative means to remove the methanol fraction. Its advantage over steam stripping is a much reduced energy cost as well as complete removal of the compound, although it is a slower and more complex process to operate. Biological treatment processes are typically run in either aerobic or anaerobic mode with mixed bacterial cultures adapted to methanol consumption (Bérubé and Hall, 1999, Welander et al., 1999). The use of a methylotrophic yeast in this application is thus a wholly novel approach. The key aspect which sets this technology apart from the other methods is its ability to potentially generate a valuable product.

Over the past two decades, the methylotrophic yeast *Pichia pastoris* has been developed as a commercial expression system for recombinant proteins. The promoter for the alcohol oxidase gene (AOX1) is exceptionally active, allowing for the production of high concentrations of foreign proteins. Unlike bacterial expression systems, the yeast is capable of exercising post translational modifications which can be crucial to the activity of the expressed protein (Cereghino and Cregg, 2000). This active expression system provides the potential for production of valuable products from methanol-bearing streams such as Kraft mill condensates. The purpose of this study was to determine whether *Pichia pastoris* could be grown in condensates, and whether engineered products could be produced in such industrial media using engineered strains of the yeast.
CHAPTER II - LITERATURE REVIEW

2.1 Literature Review of *Pichia pastoris*

The methylotrophic yeast *Pichia pastoris* has garnered much interest and use as an expression system for recombinant proteins during the past decade. Its value in this role stems primarily from the fact that this yeast has the ability for high production levels of foreign proteins which is controlled by the presence of methanol in the substrate media. A further advantage, of no less importance, is the ability for the yeast to perform the type of post translational modifications which are characteristic in mammalian type recombinant systems. This section will provide an overview of the historical development of the *P. pastoris* expression system and review the pertinent theory concerning the biological aspects and cultivation of this yeast.

2.1.1 Historical Background and Development of the *P. pastoris* Expression System

During the late 1960's, certain yeast species were identified as having the ability to utilize methanol as a sole energy and carbon source (Cereghino and Cregg, 2000; Walker, 1998). Termed *methylotrophs*, initial interest in these yeasts centered on their potential for development as a source of single-cell protein (SCP) to be used as high protein animal feed (Wegner, 1990). In the following decade, growth protocols and media formulations were developed for the methylotrophic yeast *Pichia pastoris* by the Philips Petroleum Company. However, the oil crisis of the 1970's resulted in a sharp increase in the cost of methane, the precursor component for synthesizing methanol. This, coupled with a fall in the price of soybeans, an alternative source of animal feed, rendered the marketing of the yeast as a SCP source economically unviable (Cereghino and Cregg, 2000).
Development of *P. pastoris* into an organism for the expression of foreign proteins was initiated during the 1980’s when Philips Petroleum contracted this project to the Salk Institute Biotechnology/Industrial Associates, Inc. (SHIBA, La Jolla, CA). Researchers at the institute identified and isolated a powerful promoter responsible for the transcription of an alcohol oxidase (AOX) gene. This discovery served as a launch point for the subsequent generation of cloning vectors and protocols for the genetic manipulation of the yeast (Cereghino and Cregg, 2000). It followed quickly that new *Pichia* strains were created, some of which contained genes selected from various other organisms. Using the methanol fermentation protocols developed in the prior decade, it was found that high level expression of heterologous proteins could be achieved due to the strong regulating mechanism of the AOX promoter (Siegel and Brierley, 1988; Walker, 1998; Houard et al., 2002).

Phillips Petroleum eventually sold the patent rights for the *P. pastoris* expression system in 1993 to Research Corporation Technologies (Tucson, AZ) which currently licenses the marketing of portions of the system to Invitrogen Corporation (Carlsbad, CA) (Cereghino and Cregg, 2000). The fermentation procedures have since been refined and well established with a standard set of media formulations and growth protocols available from Invitrogen Corp (Invitrogen, 2001). Both the availability of ready-to-use expression kits and the ease of genetic manipulation techniques (due in part to similarities to the well-understood *Saccharomyces cerevisiae* yeast) have established *P. pastoris* as one of the organisms of choice for the expression of a multitude of foreign genes.

2.1.2 Alcohol Oxidase Promoter, Construction of Expression Vector and Gene Regulation

In *Pichia pastoris*, methanol metabolism requires the presence of several unique enzymes. Primary among these is alchohol oxidase which catalyzes the first step in the methanol utilization pathway. Though levels of this enzyme are virtually undetectable when a rich carbon source (ie. glucose, glycerol) is present in the substrate, it has been observed that AOX is hyper-produced when methanol is introduced in growth-limiting
concentrations. Sub-cellular vesicles known as peroxisomes are formed under these conditions with the alcohol oxidase enzyme comprising over 30% of the total soluble protein content (Duff and Murray, 1986; Cereghino and Cregg, 2000; Sreekrishna et al., 1997; Zhang and Wang, 1993; Ohashi et al., 1998).

The cornerstone rationale of using the P. pastoris system is to take advantage of the sheer strength of the AOX promoter during foreign gene expression. This is done by constructing a compatible expression vector and introducing it into the yeast cell. The Pichia pastoris expression vector consists of a plasmid, a self replicating circular ring of DNA, with several general features; marker genes for both E. coli and P. pastoris, the alcohol oxidase gene with its associated promoter, and the foreign gene of interest (Sreekrishna et al., 1997). The specific components of the vector will vary according needs of the expression, such as insertion of various secretion signals or engineered deficiencies which would allow identification of the modified yeast strain. As shown in Figure 2.1, the foreign gene is inserted between the promoter and the AOX gene. Thus when the promoter signals for the transcription of the oxidase gene, the foreign DNA sequence is likewise transcribed at a similar copy number.

Figure 2.1  P. pastoris Expression Vector (adapted from Cereghino and Cregg, 1999)
Expression of the alcohol oxidase enzyme is controlled by two genes; AOX1 and AOX2, with AOX1 contributing to the large majority of enzyme activity (Cereghino and Cregg, 2000; Chiruvolu, 1997). The regulation of these genes is, in turn, performed on the level of transcription, through a joint repression/derepression and induction mechanism. As suggested above, the presence of rich carbon sources favors a metabolic pathway which does not result in the synthesis of key enzymes required for methanol consumption; thus transcription of AOX1 (and AOX2) is repressed. Upon depletion or removal of these carbon sources, transcription of the alcohol oxidase genes may still not commence until induced through the presence of methanol. Figure 2.2 illustrates the process by which the production of alcohol oxidase, and thus heterologous proteins, is controlled.

![Figure 2.2](image)

**Figure 2.2 P. pastoris Transcription Control Mechanism (adapted from Shuler and Kargi, 1992)**

### 2.1.3 Methanol Utilization Pathway

The formation of peroxisomes during methanol induction is indicative of alcohol oxidase synthesis. Within these small organelles, the first stages of methanol catabolism occur where AOX catalyzes the initial oxidation of methanol to formaldehyde and
hydrogen peroxide (Figure 2.3) (Cereghino and Cregg, 2000; Duff and Murray, 1986; Zhang and Wang, 1993). Hydrogen peroxide is in turn degraded to oxygen and water by reaction with the enzyme catalase. The formaldehyde next faces two possible routes in the metabolic pathway. A portion exits the peroxisome to be used in energy synthesis reactions via oxidation to carbon dioxide and formate. The remainder of the formaldehyde undergoes a condensation reaction with xylulose 5-monophosphate (XU₅P) and is catalyzed by dihydroxyacetone synthase (DHAS). The resulting products, glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone (DHA), exit the peroxisome and progress through a cyclic pathway which convert them into raw material for construction of cellular components.

![Figure 2.3 Methanol Utilization Pathway (adapted from Cereghino and Cregg, 1999)](image)

It has been observed that methanol concentrations beyond 1 v/v% impair growth of the yeast cells and may even lead to cell death (Zhang et al., 2000; Guarna et al., 1997). This phenomenon is due to the build-up of the products from the initial methanol oxidation stage. In an excess methanol environment, the alcohol is rapidly degraded to peroxide and formaldehyde due to the large AOX enzyme presence. However, the two other enzymes within the peroxisome, catalase and DHAS, are present in considerably lower levels and thus cannot breakdown these chemicals fast enough to prevent toxic accumulation. In light of this, the strong activity of the AOX1 promoter can be regarded
as a potential drawback, rendering the yeast quite sensitive to methanol substrate concentrations. As an alternative, researchers have developed *Pichia* strains which do not vigorously utilize methanol. This will be discussed in the next section.

### 2.1.4 *Pichia* Strains of Differing Methanol Utilization Capacity

The wild type *Pichia* strain can metabolize methanol quickly because it can synthesize alcohol oxidase in large quantities. Most host strains have the same methanol utilization rate as the wild type; these are known as Mut\(^+\) strains (methanol utilization plus phenotype) (Cereghino and Cregg, 2000; Chiruvolu et al, 1997). Sometimes, however, it is disadvantageous for certain fermentation operations to function with such rapid substrate uptake. The high utilization rate may necessitate that large amounts of methanol feed be stored for the continuous growth and induction of the yeast, thus posing a potential health and fire safety concern. Also, the Mut\(^+\) strains are sensitive to high residual methanol concentrations due to build-up of peroxide and formaldehyde within the cell, as discussed previously. Sudden changes in high MeOH concentrations will also adversely affect the cells and may cause the AOX enzyme to lose activity.

To counter some of these issues, researchers have developed alternate *Pichia* host strains. The Mut\(^S\) strain (methanol utilization slow phenotype) is a yeast strain with the AOX1 gene partially deleted from the *P. pastoris* genome. This causes the yeast to rely solely on the much weaker AOX2 promoter to generate the alcohol oxidase enzyme (Cereghino and Cregg, 2000). The result is a much reduced methanol utilization rate and hence growth rate. This was demonstrated by Cregg and Madden (1987) who found the specific growth rates of Mut\(^S\) strains to be 0.01-0.04 h\(^{-1}\), approximately 4-14 times slower than their Mut\(^+\) counterparts (Cregg, and Madden, 1987). The benefit of the lower AOX presence is a dramatic decrease in sensitivity to high MeOH residual concentrations thereby potentially simplifying substrate feeding control systems.

Another mutant host strain, the Mut\(^-\) strain (methanol utilization minus phenotype), has both AOX1 and AOX2 genes completely deleted. This renders the yeast
completely unable to produce alcohol oxidase and therefore incapable of growth on methanol. In this case, the yeast must be fed a highly regulated and limiting concentration of a rich carbon source such that repression effects, though still present, are low enough to permit a satisfactory level of expression (Cereghino and Cregg, 2000, Chiruvolu et al, 1997). The circumstances in which the use of this yeast may be favorable include the situation where methanol is not a desirable feed source for the production of certain therapeutic or food-grade proteins.

In both Mut$^8$ and Mut$^*$ strains, the AOX1 promoter is left intact in the yeast genome such that both retain the ability for high protein expression in the presence of methanol (even though it is not utilized in the Mut$^*$ Pichia). An added advantage of these strains over the Mut$^+$ phenotype occurs when the foreign protein is expressed intracellularly. Since AOX levels are lowered and nonexistent in each of these strains respectively, there is more room in the cell that can be devoted to the accumulation of the protein, rather than accumulation of alcohol oxidase (Chiruvolu et al, 1997).

### 2.1.5 Post Translational Modifications

Eukaryotic proteins secreted from their native host organisms often undergo a degree of post processing that enables their activity. Types of post translational modifications include; protein folding, disulfide bridge formation, lipid addition, and glycosylation (Cereghino and Cregg, 2000). The *P. pastoris* expression system has the ability to emulate many of these modifications, giving it a distinct advantage over bacterial expression systems in this aspect. There are, however, differences in protein processing, particularly in glycosylation, between the yeast and higher eukaryotes that could prove to be problematic.

In the process of glycosylation, sugar chains are attached to the synthesized protein. Depending on the attachment site and location of the modification (which will not be discussed in detail here) the sugar addition is distinguished as either O-linked or N-linked glycosylation. O-linked glycosylation in mammalian cells occurs when
oligosaccharides of various sugars are attached to the hydroxyl groups of serine or threonine (Rawn, 1989). In *P. pastoris*, however, the oligosaccharide complexes are composed entirely of mannose units (Bretthauer and Castellino, 1999; Houard et al., 2002). Also, there may be a tendency for the yeast to glycosylate a protein even if this does not normally take place in its native organism (Cereghino and Cregg, 2000).

The process of N-linked glycosylation occurs in the endoplasmic reticulum of the cell (Rawn, 1989). But whereas in mammalian systems where oligosaccharide residues are added, processed, and trimmed, yeasts expression systems may continue to add large numbers of mannose units, resulting in a condition called hyperglycosylation (Cereghino and Cregg, 2000). This would consequently interfere with the correct folding pattern of the expressed protein. Although this is occasionally observed in *Pichia*, it occurs with far less frequency than for recombinant proteins secreted from *S. cerevisiae* (Cereghino and Cregg, 2000; Bretthauer and Castellino, 1999).

### 2.1.6 Established Fermentation Practices and Growth Considerations

Recombinant *Pichia* fermentations typically revolve around a procedure where there are several distinct feeding regimes. These correspond to the various phases of yeast growth and induction. The Invitrogen growth protocols form the basic template that most fermentation operations use, barring minor modifications dependent on the specific needs of the process (Invitrogen, 2001). These guidelines detail; (1) an initial batch phase where the yeast metabolizes a rich carbon source (glucose or glycerol) followed by (2) a limiting fed-batch phase of the rich substrate and concluding with (3) a methanol fed-batch phase.

The goal of the first stage is simply to promote rapid growth of the yeast culture to a high density (25 – 40 g/L dry cell density). During this time induction of the recombinant *Pichia* does not occur because of the presence of the rich carbon source and also because of the absence of methanol. When the substrate has been exhausted in batch mode, the second stage is initiated where the culture is fed glucose or glycerol at a
growth limiting rate. This allows the consumption of metabolites (ie. acetate and ethanol) that accumulate during the batch phase and further increases the culture density prior to induction. In some fermentations, such as the study performed by Zhang et al. (2000), this stage is coupled with a transition phase where methanol is introduced at low levels and slowly increased while the rich substrate is simultaneously ramped down. The strategy allows for a gradual transition to the new metabolic pathway and is reported to result in a reduced time for complete adaptation to methanol (from 4-5 hours to 2 hours). In the final phase, methanol is continuously fed into the reactor at growth limiting concentrations. It is during this stage that expression of foreign proteins is facilitated. The yeast may also experience slow but continued growth on the MeOH feed. It should be noted that in the absence of a transition phase, methanol must initially be added very slowly otherwise the sudden concentration shock will cause cell death (Invitrogen, 2001).

Conditions of *Pichia* fermentation are generally well established and require little optimization. Normally, the process is operated at 30°C with an ideal dissolved oxygen (DO) content of approximately 20% of saturation level. During methanol metabolism a considerable amount of heat is generated and cooling is required to maintain temperature below 32°C, above which protein expression is negatively affected (Invitrogen, 2001). Oxygen limiting conditions will likewise adversely affect protein production and cell growth. The desired pH may vary depending on the properties of the recombinant protein but usually lie in the range between 5 and 6. The acidic environment favors yeast growth and also discourages bacterial contamination.

In addition, *P. pastoris* has the ability to be cultured in a defined media formulation consisting of carbon sources, biotin, trace elements and water. These chemicals are fairly inexpensive and the defined nature means that the medium is free of undesirable elements (ie. toxins) which would render the production and harvesting of human pharmaceutical grade proteins unviable.
2.2 Selection of Recombinant Pichia Strain

One of the initial goals of this project was to select and obtain a recombinant *Pichia* strain which had been shown to successfully express a product. It was beyond the scope of the research to perform the necessary genetic manipulation; as such a literature search was conducted to identify potential candidate strains. Due to the popularity of the expression system, there were a large number of possible organisms from which to choose. Because of the pulp and paper perspective of this project, however, it was desired that the expressed product reflect a potential value in this industry.

Through the literature review, a number of prospective strains were identified and short-listed (Table 2.1). Through a selection procedure more fully covered in the Methods and Materials section, a recombinant *Pichia* strain expressing a lipase from *Geotrichum candidum* was selected.

<table>
<thead>
<tr>
<th>Expressed Product</th>
<th>Reference</th>
<th>Potential Application in the Pulp &amp; Paper Industry</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichoderma reesei</em></td>
<td>2000 [4]</td>
<td>cellulose degrader; potential application in pulp bleaching</td>
</tr>
<tr>
<td>Celllobiohydrolase (Cel7A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td>1998 [20]</td>
<td>degradation of fatty acids; can be used to treat extractives in paper making process streams</td>
</tr>
<tr>
<td>Lipase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Geotrichum candidum</em></td>
<td>1997 [15]</td>
<td>degradation of fatty acids; can be used to treat extractives in paper making process streams</td>
</tr>
<tr>
<td>Lipase(s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candida guilliermondii</em></td>
<td>1998 [14]</td>
<td>xylose fermentation; ethanol production during fermentation of spent sulphite liquor (SSL)</td>
</tr>
<tr>
<td>Xylose reductase (xyl1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trametes veriscolor</em></td>
<td>1997 [18]</td>
<td>potential lignin degrader, applications in pulp bleaching</td>
</tr>
<tr>
<td>Laccase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b-(1-6)-glucanase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces viridosporus</em> (T7A)</td>
<td>1998 [36]</td>
<td>depolymerization and solubilization of lignin, applications in pulp bleaching</td>
</tr>
<tr>
<td>peroxidase/endo glucanase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3 Background on *G. candidum* Recombinant *Pichia* strain

The native *Geotrichum candidum* organism is the most common species of the *Geotrichum* genus, a fungus often found in fruits, plants, soil, dairy products, and is a part of the normal microbial population of the human body (Sutton et. al, 1998). During the 1960’s, it was discovered that this fungus secretes lipases with a high substrate affinity for cis (ω-9) unsaturated fatty acid residues such as those in oleic acid (Holmquist et. al, 1997). This property is exploited in industrial processes where lipids and oils are enzymatically restructured (Holmquist et. al, 1997). Further investigation into the genome of *G. candidum* revealed that two genes were responsible for the synthesis of the isoenzymes (Shimada et al., 1989; Shimada et al., 1990). The resulting lipases, GCL I and GCL II, were subsequently identified to have slightly differing substrate specificities.

Initially, Holmquist et al. (1997) had attempted to culture a recombinant strain of *S. cerevisiae* containing the lipase genes but achieved only low levels of expression. This prompted them to switch the host organism to *P. pastoris* which favors much higher levels of foreign protein production. Ultimately, through controlled expression of GCL I/GCL II and corresponding mutant variants, the researchers sought to further study the microbiological, structural, and genetic aspects of the lipases.

Growth of the recombinant *Pichia* occurred in 250 mL shake flask cultures maintained at 30°C and agitation speed of 250-300 rpm for approximately 4-5 days. Induction of the yeast was maintained by daily additions of 0.5 mL methanol. Polyacrylamide gel electrophoresis and Western blot analysis were used to verify the presence and purity of the expressed isoenzymes. Lipase concentration was determined spectrophotometrically by the Bradford method (Bradford, 1976) and enzyme activity was measured titrimetrically with a pH stat system; both these procedures will be discussed in greater detail in the Materials and Methods section.

Holmquist et al. (1997) found GCL I and GCL II expressed to approximately 60 mg/L, surpassing levels obtained from *S. cerevisiae* by a factor of 60. Also notable was
the virtual absence of contaminating proteins produced during yeast growth. The expressed products were demonstrated to be fully active by titrimetric assay with a triglyceride (triolein). The specific activities of GCL I and GCL II on triolein was reported to be 1200 and 700 (μmol/min/mg) respectively. The substrate specificity of the two isoenzymes, conducted by reaction with oleic (C18:1) versus octanoic (C8) acid compounds, confirmed that GCL I has a considerably higher preference to oleic substrates (~3 times) than GCL II, which demonstrates a more general substrate affinity.

2.4 Literature Review of Kraft Pulp Mill Condensates

In the kraft process, vapors containing volatile organics and sulfur bearing compounds are evolved at various locations in the pulping and chemical recovery stages. The fraction of these vapors which are subsequently condensed are aptly termed condensates. Though condensate streams may constitute only 5% of an overall bleached pulp mill effluent volume, it may account for 25-40% of the total BOD load and up to 30% of the toxicity (Blackwell et al, 1979). The significant environmental impact of these waste streams necessitate that they be aggressively targeted for treatment. This section will briefly review sources of condensates within the mill, their general chemical composition, and current treatment methods.

2.4.1 Origin of Condensates in the Kraft Process

There are several primary sources of condensate within the Kraft mill process; the chip digester, the blow tank, and the multiple effect evaporators (Figure 2.4). Within the digester, wood chips are impregnated with cooking liquor, Na₂S and NaOH. The ensuing reaction at elevated temperature and pressure solubilizes roughly 80% of lignin content of the pulp (Smook, 1992). Vapors formed at this stage are rich in volatile organics compounds (VOCs) resulting from the oxidation reactions. These gases are relieved from the vessel via a pressure control valve and vented to a condenser, with the subsequent liquid flow sent to a turpentine decanter for turpentine recovery (Milet, 1997). The
fraction of the stream not recovered by the decanter constitutes one of the condensate flows.

For operations using a batch digester, the cooked chips are discharged from the reaction vessel to the blow tank at high pressure, causing the chips to disintegrate into fibers upon entry. Flash steam and organic vapors evolved during this process proceed to an accumulator heat recovery system where the condensed liquid flow comprises the second primary condensate stream. Vapors escaping from the accumulator are condensed and combined with the flow entering turpentine recovery. In continuous pulping operations, the only source of digester condensate originates from the indirect flash steam condenser, with the liquid flow sent to the turpentine decanter (Blackwell et al., 1979).

Figure 2.4 Kraft Condensate Flowchart (source: Milet 1997)

After the blow tank, the pulp mass travels to the brown stock washers where the dissolved organics and spent cooking liquor are separated from the pulp as weak black liquor. The liquor stream then proceeds to the multiple effect evaporators for the first stage in the chemical recovery cycle (Figure 2.5). Here, the solids are concentrated from a consistency of 13-17% to 60-80% through a series of reboilers operated at various
pressures (Smook, 1992). Usually, the incoming liquor is initially split between two effects (shown as evaporators 5 and 6 in Figure 2.5). The vapors produced here and perhaps in the next two reboilers contain the greatest amount of organics as compared to the remaining evaporators. Most mills divert these condensate streams and combine them with the flows originating from the digester vent gases and blow tank vapors. These streams, collectively know as ‘foul’ condensates, are so named because of the high concentration of organic, particularly reduced sulfur, compounds. Separate treatment is required for foul condensates prior to reuse or discharge into the wastewater plant. Condensates evolved from the remaining evaporators are considerably less contaminated. These streams are merged into a combined flow which is hence termed ‘combined’ or ‘clean’ condensate. Depending on the mill operation and characteristics of the combined condensate, it may be reused directly as process water or require further treatment.

![Multi-Effect Evaporator Schematic](source Smook, 1992)

**Figure 2.5  Multi-Effect Evaporator Schematic (source Smook, 1992)**

### 2.4.2 Composition of Kraft Condensates

Volatile organics evolved during the various pulping processes described above constitute the major chemical contaminants found in condensate (Table 2.2). These include alcohols, ketones, terpenes, and reduced sulfur compounds. Among the organics,
methanol, acetone, and methyl ethyl ketones are regarded as hazardous air pollutants (HAPs) by the 1990 EPA Clean Air Act Amendments (NCASI, 1995). The presence of non-volatile components such as resin acids and sodium salts are most likely due to entrainment of black liquor in the multiple-effect evaporators (Milet, 1997).

Table 2.2. Concentration Ranges for Chemicals in Kraft Mill Condensates (adapted from Blackwell et al., 1979)

<table>
<thead>
<tr>
<th>Chemical Constituents</th>
<th>Evaporator Combined Condensate (mg/L)</th>
<th>Stripper Feed (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TRS components</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2S</td>
<td>1-90</td>
<td>5-660</td>
</tr>
<tr>
<td>CH3SH</td>
<td>1-30</td>
<td>5-720</td>
</tr>
<tr>
<td>DMS</td>
<td>1-15</td>
<td>10-1000</td>
</tr>
<tr>
<td>DMDS</td>
<td>1-50</td>
<td>10-150</td>
</tr>
<tr>
<td><strong>BOD contributors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methanol</td>
<td>180-700</td>
<td>140-10000</td>
</tr>
<tr>
<td>ethanol</td>
<td>1-190</td>
<td>20-1100</td>
</tr>
<tr>
<td>acetone</td>
<td>1-15</td>
<td>15-500</td>
</tr>
<tr>
<td>methyl ethyl ketone</td>
<td>1-3</td>
<td>20-25</td>
</tr>
<tr>
<td>terpenes</td>
<td>0.1-150</td>
<td>1-9600</td>
</tr>
<tr>
<td>phenolics</td>
<td>-</td>
<td>1-82</td>
</tr>
<tr>
<td>resin acids</td>
<td>28-230</td>
<td>1-20</td>
</tr>
<tr>
<td>total BOD5</td>
<td>60-1100</td>
<td>800-13000</td>
</tr>
<tr>
<td>sodium</td>
<td>4-20</td>
<td>-</td>
</tr>
<tr>
<td>suspended solids</td>
<td>30-70</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>6-11.1</td>
<td>8.0-10.5</td>
</tr>
</tbody>
</table>

In terms of biochemical oxygen demand (BOD), methanol is the constituent of greatest concern. It is believed to be formed by the cleavage and subsequent reaction of methyl glucuronic acid residues in hemicellulose and methoxyl groups on lignin during chip digestion (Blackwell et al., 1979). Ethanol may also be present, though in considerably less and highly variable quantities. It is formed after logging of a tree when aerobic fermentation of the wood begins to occur, with chip handling and storage practices affecting the amount of ethanol volatilization (Blackwell et al., 1979). Terpenes occur naturally in wood as one of the extractives components and vary in concentration according to the specific wood species. As previously mentioned, it is the usual practice
of most mills to operate a turpentine recovery system such that it is removed as a large source of BOD.

Hydrogen sulfide (H$_2$S), methyl mercaptan (CH$_3$SH), dimethyl sulfide (DMS) and dimethyl disulfide (DMDS) are collectively known as total reduced sulfur (TRS) compounds and are the other contaminants of key interest besides methanol. TRS compounds result from reactions involving Na$_2$S and its derivatives during the cooking process. Two main concerns surrounding the presence of TRS compounds are firstly their intense mal-odorous nature and second, the degree of toxicity that they impart on the effluent. It has been reported that up to 95% of condensate toxicity is caused by TRS's (Milet, 1997).

2.4.3 Current Treatment Options

Treatment of condensates is usually reserved for the foul fraction, with the combined fraction sometimes reused directly as process water for brownstock washing, recausticizing, or in the bleach plant. However, it is often the case that contaminant concentrations, particularly methanol, warrant treatment of the combined condensate prior to reuse. According to guidelines set out in the EPA Cluster Rules, streams that exhibit methanol concentrations exceeding 500 mg/L should be treated to remove 90% of the original amount before being sent to the wastewater facility (Swan, 1995). In the most recent incarnation of the MACT I portion of the Cluster Rules, methanol emissions from pulp washing system vents must be reduced and controlled in kraft mills by an April 15, 2006 deadline (Teilbaard et al., 2003). Also of concern is the release of HAPs from sources within the mill associated with direct condensate recycling (NCASI, 1995).

Steam stripping which accomplishes the dual task of removing both TRS compounds and methanol, is the favored option of most pulp mills for treatment of foul condensates. It has been reported in an NCASI survey of 15 mills that foul condensate steam stripping units achieved high methanol removal efficiencies, ranging from 95-99% (NCASI, 1995). It is, however, an energy intensive operation that is best performed on
low volume flows with high contaminant concentration. Removal of equivalent amounts of methanol in cleaner, more dilute condensate streams require increasing levels of steam throughput and constitutes a greater cost per unit of methanol removed. It should also be noted that in the same NCASI survey, the exit methanol concentration averaged 610 mg/L, which exceeds the aforementioned Cluster Rule guidelines (Milet, 1997). Alternatively, if the goal is only to extract the TRS components from the stream, air stripping of condensate would suffice, since these compounds have been shown to be quite volatile in neutral pH conditions (Blackwell et al., 1980). There are, however, very few full scale operations of this type employed by mills (Milet, 1997).

Biological treatment of condensate is an alternative means to remove TRS and methanol. In contrast to steam stripping, the cost of biological treatment is proportional to the strength, rather than the flow, of the stream. Higher contaminant concentrations require higher biomass levels which increases costs associated with nutrient addition and sludge handling. Its advantage over steam stripping is a much reduced energy requirement although it is usually associated with longer hydraulic retention times (HRT) and a greater complexity of operation. Biological treatment processes are typically run in either aerobic or anaerobic mode with mixed bacterial cultures adapted to methanol consumption (Bérubé & Hall, 1999; Welander et al., 1999).

Laboratory-scale studies during the 1980’s found that anaerobic treatment of kraft condensates often exhibited difficulties including reactor instabilities and variable BOD removal efficiencies (Milet, 1997). However, recent pilot scale experiments demonstrated good methanol removal efficiencies with an HRT of less than 4 hours (Teilbaard et al., 2003). Their test effluent consisted of the recycled condensate stream used during brownstock washing. Inlet methanol concentrations ranged from 100 – 400 mg/L with the treated flow consistently displaying concentrations of 1-2 mg/L. They also reported the successful operation of a full-scale, high-rate anaerobic reactor in a pulp mill located in Jackson, Alabama. In this case study, treatment of a foul condensate stream yielded a 99% removal efficiency for methanol.
The viability of dedicated biological treatment systems were also examined in an NCASI pilot plant study (1999) was carried out in a southern kraft mill. Two options were examined; conventional activated sludge treatment (AST), and upflow anaerobic sludge blanket (USAB). The two treatment systems were operated in parallel and the effectiveness of each was judged for various loading conditions. It was found that both processes were effective at degrading methanol to low concentrations for moderate strength condensate feeds (COD ~ 900 ppm). The suspended solids concentration in the resulting effluent was approximately 50 mg/L, allowing it to be viable for reuse within the mill. The AST could further handle higher strength mixed condensate effluents (COD ~ 6000 ppm) though the hydraulic residence time needed for sufficient treatment may limit the economic practicality of this option. In comparison, the effectiveness of the USAB to treat the same higher strength effluent was initially good, affecting removal efficiencies of over 80% and 95% for COD and methanol, respectively. However for longer durations especially after shock periods of high organic loading, the system was found to be inconsistent, providing averaged removal rates of 65% for COD and 80% for methanol.

2.5 Overview of Self-Cycling Fermentation

The cycling logic for the reactor scale experiments were loosely based on the principles for self-cycling fermentation (SCF). This control technique was originally developed for applications in the production of commercially valuable microbial products (Sheppard and Cooper, 1990; Brown and Cooper, 1991; Zenaitis and Cooper, 1994). Basically, the SCF method entails controlling the hydraulic retention time of a semi-continuous reactor by linking the cycling time to expected patterns in dissolved oxygen levels. For a constant aeration rate, there is a distinguishing difference in DO concentration between the time when substrate is being consumed by the microbial population and when this substrate has been completely depleted. Upon substrate depletion, a positive DO spike will result, which signals the onset of endogenous metabolism (Figure 2.6). By continuously monitoring the dissolved oxygen level, this spike can be detected by the control system which then initiates reactor cycling. At this
point, a portion of the reactor broth is harvested and an equal volume of feed is introduced. In terms of wastewater treatment, this control technique ensures the complete removal of biodegradable materials from the effluent stream with the minimum required hydraulic retention time. Another advantage of SCF is the relative simplicity it affords compared to other control methods based on respirometry which may require more sophisticated apparatuses containing DO sensors, biological sludge, and wastewater (Milet, 1997).

![Figure 2.6 Typical DO Profile in a SCF](image)

In the study performed by Milet (1997), the SCF technique was applied to the treatment of kraft mill condensates. Experiments were performed on a laboratory-scale recirculating packed bed reactor (PFR) and sequencing batch reactor (SBR), with the emphasis of the study on the latter. The PFR consisted of a cyclone column bioreactor (1.5 L working volume) containing a packed bed of Pall Rings which served as the biofilm support surface for attached microbial growth. Effluent feeding and harvesting occurred on the recirculation line and was controlled via a computer program resident on a 286 PC. For the SBR, the primary components were a 1.5 L working volume stirred tank reactor and a dosing vessel (0.725 L) from which fresh feed would be added. As with the former set-up, cycling of the SBR system was controlled using the same algorithm resident on a 286 PC. The systems were seeded with biomass obtained from an activated sludge reactor treating combined effluent from a Western Pulp kraft mill. Both
systems were able to consistently reduce COD by approximately 88% from influent values of 3060 mg/L for accumulator condensate and 64% from initial values of 1740 mg/L for evaporator condensate. However, it was found that the overall performance of the SBR was superior than that of the PFR, typically achieving COD removal rates of 39 kg COD/m³/day which was seven times greater than those observed for the PFR and comparable to values seen in full scale activated sludge reactors.
CHAPTER III - RESEARCH OBJECTIVES

The overall goal of this research was to culture a recombinant strain of the yeast *Pichia pastoris* in a Kraft mill condensate and have it successfully express a desired product. To achieve this end, a stage-wise approach was taken, starting with preliminary shake flask studies and culminating in automated reactor-scale fermentations.

The specific research objectives in approximate chronological order were as follows:

1. Perform preliminary shake-flask growth trials of the wild-type *Pichia* in Kraft mill condensate
2. Select and obtain a suitable recombinant *Pichia* strain
3. Perform similar shake-flask growth trials with the recombinant *Pichia* strain
4. Develop/adapt procedures for the assay of expressed enzyme activity
5. Assemble a reactor and design an automated control system suitable for the fed-batch growth of yeast
6. Conduct reactor scale fermentation studies for the yeast and quantify the activity of expressed enzyme
CHAPTER IV - METHODS AND MATERIALS

4.1 Ranking of Candidate Organisms

Research on each of these _Pichia_ transformants had occurred in the recent past years and expression of their respective proteins had been reported as successful. The recombinant strains were evaluated according to a criteria set (Table 4.1). This set included; quality and level of expressed protein, ease of the enzyme assay, type of equipment required for the assay, ability to express extra-cellularly, and applicability to the pulp and paper industry. In terms of quality of expression, it was recognized that though the protein molecule was synthesized, it must be correctly folded and glycosylated to be active (especially important for recombinant enzymes). Extra-cellular expression was desired since intra-cellular expression would necessitate the additional steps of lysing the yeast cell and harvesting the cytoplasm prior to assay.

Table 4.1  Ranking Criteria of the Candidate Organisms

<table>
<thead>
<tr>
<th>Candidate Organism</th>
<th>Expression</th>
<th>Assay Protocol</th>
<th>Total points</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>relevance to pulp and paper</td>
<td>quality</td>
<td>intra / extra difficulty</td>
</tr>
<tr>
<td><strong>Trichoderma reeset</strong></td>
<td>Cellobiohydrolase (Cel7A)</td>
<td>very good (5)</td>
<td>fair (3.5)</td>
</tr>
<tr>
<td><strong>Rhizopus oryzae</strong></td>
<td>Lipase</td>
<td>good (4.5)</td>
<td>good (4.5)</td>
</tr>
<tr>
<td><strong>Geotrichum candidum</strong></td>
<td>Lipase(s)</td>
<td>good (4.5)</td>
<td>v. good (5)</td>
</tr>
<tr>
<td><strong>Candida guilliermondii</strong></td>
<td>Xylose reductase (xyl1)</td>
<td>good (4.5)</td>
<td>good (4.5)</td>
</tr>
<tr>
<td><strong>Trametes veriscolor</strong></td>
<td>Laccase</td>
<td>fair (3)</td>
<td>fair (3)</td>
</tr>
<tr>
<td><strong>Tricodermera</strong></td>
<td>b-(1-6)-glucanase</td>
<td>fair (3)</td>
<td>good (4)</td>
</tr>
<tr>
<td><strong>Streptomyes viridosporus</strong></td>
<td>(T7A) peroxidase/endoglucanase</td>
<td>fair (3)</td>
<td>good (4.5)</td>
</tr>
</tbody>
</table>
Ranking of the candidate transformants were done via a points system, where each recombinant was assigned a score out of a maximum value for each criteria. The *Pichia* strain with the highest total points was judged the best overall candidate for the study. Based on this procedure, the *Pichia* strain expressing a lipase from *Geotrichum candidum* was chosen.

4.2 Origin of Yeast Strains

The *Pichia pastoris* wild-type strain was obtained from the Northern Regional Research Laboratory (NRRL-1603), Peoria, Illinois. The *Geotrichum candidum* recombinant *Pichia* strain (GS115 (*his4*) harboring plasmid YpDC420) was obtained from M. Holmquist and D.C. Tessier of the Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec. This particular yeast strain was encoded with the *Geotrichum candidum* lipase (GCL) I gene only, as opposed to the strain containing both GCL I and II which was studied in the paper by Holmquist and Tessier (1997).

4.3 Culture Media

Yeast cultures were grown in rich media or defined media made up in either distilled water or combined evaporator condensate obtained from a local Kraft pulp mill (Western Pulp, Squamish, BC). Rich media consisted of 1 wt% yeast-extract and 2 wt% peptone dissolved in either distilled water (rich medium) or condensate (rich condensate medium). Defined media consisted of 3.0 g/L (NH₄)₂SO₄, 17.42 g/L K₂HPO₄, 6.95 g/L citric acid, 0.30 g/L MgSO₄·H₂O, 1.0 ml/L trace element solution, and 1.0 ml/L vitamin solution dissolved in distilled water (defined medium) or condensate (defined condensate medium). Trace element solution contained 1.0 g/L FeSO₄·7H₂O, 0.005 g/L CuSO₄·5H₂O, 0.01 g/L H₃BO₃, 0.01 g/L MnSO₄·H₂O, 0.07 g/L ZnSO₄·7H₂O, 0.01 g/L Na₂MoO₄·2N₂O. Vitamin solution contained 200 µg/L biotin, 200 µg/L folic acid, 40 mg/L calcium pantothenate, 40 mg/L niacin, 40mg/L pyridoxine-HCl, 200 mg/L inositol,
20 mg/L riboflavin, 20 mg/L p-aminobenzoic acid, 100 mg/L thiamine. The four possible media combinations are summarized in Table 4.2 below.

Table 4.2 Summary of Media Combinations

<table>
<thead>
<tr>
<th>Media</th>
<th>Yeast Peptone (rich)</th>
<th>Mineral (defined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O (clean)</td>
<td><strong>Media 1</strong></td>
<td><strong>Media 2</strong></td>
</tr>
<tr>
<td>Condensate</td>
<td><strong>Media 3</strong></td>
<td><strong>Media 4</strong></td>
</tr>
</tbody>
</table>

All solutions were either autoclaved (121°C, 10 psig, 20 min) or filter sterilized (0.22 µm pore size, Fisher Scientific) prior to use. In the case of condensate media, methanol concentrations prior to autoclaving were in the range of 500 mg/L. After autoclaving, methanol concentrations were measured at 80 - 120 mg/L.

4.4 Condensate Stripping

Excluding the initial shake flask experiments, all shake flask and reactor trials involving a condensate medium required that the condensate be air-stripped prior to use. Stripping was done at an ambient room temperature of 26°C for a duration of 1 hour. The volumetric stripping rate was 3.75 L air/L condensate/hr.

4.5 Shake-Flask Cultures

Shake flask studies were performed in 250 mL Erlenmeyer flasks corked with foam plugs to allow air permeation. Appropriate culture broth was added to each flask to a volume of 50 mL. Prior to yeast inoculation, the flasks were autoclaved (121°C, 10 psig, 20 min). Subsequently, they were placed within an orbital shaker (Innova 4230 refrigerated Incubator, New Brunswick Scientific) and incubated at 30°C and agitated at 150 rpm.

Inoculum was prepared by aseptic transfer of a single yeast colony from stored agar cultures to a 250 mL Erlenmeyer flask. The inoculum growth broth contained a 50
mL solution of 1 wt% yeast-extract and 2 wt% peptone dosed with 2 ml of 50 wt% glucose. The inoculum culture was placed in the orbital shaker and grown overnight (16 hours) to an approximate cell concentration of 6 g/L. One mL aliquots of the inoculum were then transferred to each growth flask.

4.6 Determination of Cell Density

Cell concentration (dry weight per volume) was determined by measuring the optical densities (OD) of yeast culture samples using a spectrophotometer (HACH DR/2000). Samples (0.5 mL) were centrifuged for 5 minutes at 6700 \( \times g \) and the pellet re-suspended in 10 mL of distilled water. These tubes were then inserted into the spectrophotometer and the absorbance read at 600 nm. Calibration curves of cell concentration vs. absorbance were constructed using absorbance measurements of dense cell culture serial dilutions plotted against their dry cell weights obtained by drying known volumes at 103°C for 2 hours (Figure 4.1).

![Figure 4.1 Pichia Cell Calibration Curves (Dry Cell Weight)](image)

\[
\begin{align*}
\text{lipase recombinant} & : y = 12.762x \\
\text{wild type} & : y = 12.88x
\end{align*}
\]
4.7 Determination of Methanol Concentration

Methanol concentration was measured using a Varian CP-3800 gas chromatograph with a Supelco (Supelcowax-10™ 24080-U) capillary column (fused silica, 30m × 0.32id × 0.25μm film thickness). Helium was used as the carrier gas at a flow rate of 25 mL/min with hydrogen and air as the fuel gases at flowrates of 30 mL/min and 300 mL/min, respectively. For all determinations 1-butanol was used as the internal standard. The detection method consisted of initially holding the column oven temperature at 45°C for 2 minutes then ramping at 20°C/min up to 65°C and holding for 2.75 minutes. The flame ionization detector and injector were both maintained at 250°C.

Methanol calibration curves were produced with each analysis set using seven methanol standards with concentrations ranging from 79.15 – 4749 mg/L (Figure 4.2).

![Sample Methanol Cell Calibration Curve](image)

Figure 4.2 Sample Methanol Cell Calibration Curve

4.8 Determination of Protein Concentration

Culture samples were first centrifuged at 6700 ×g for 5 minutes and 1.0 mL volumes of the supernatant were withdrawn and centrifuged again at 2000 ×g for 30 minutes in Centricon YM-30 (Millipore, Bedford, Mass) centrifugal filters. The retentate
was then re-suspended in 1.0 mL of 20mM 3-{N-Morpholino}propanesulfonic acid (MOPS) buffer, pH 7.0. Following the method of Bradford (Bradford, 1976), protein concentration was determined spectrophotometrically using the ‘Protein Assay Kit’ supplied by Bio-Rad (Hercules, CA) with bovine serum albumin as the standard. A calibration curve provided the correlation between absorbance and protein concentration (Figure 4.3).

![BSA calibration curve](image)

**Figure 4.3** BSA calibration curve

### 4.9 Measurement of Lipase Activity

The determination of lipase activity was adapted from the procedures used by Holmquist and Tessier (1997), and Minning et al. (1998). In both studies, activity was determined by the hydrolysis of triolein, which results in the release of hydride ions and a corresponding drop in pH. A pH-stat device was employed to hold the pH constant by controlled titration with a base. Activity of the lipase would be subsequently expressed as the amount of base required to maintain a constant pH for a specific time interval. However, due to equipment limitations (i.e. lack of a pH-stat) improvisation to the procedure was required. This is described below.
Lipase activity was measured titrimetrically by the enzymatic hydrolysis of triolein at 25°C. The substrate mixture consisted of 0.6 mL triolein (Sigma) emulsified in a 100 mL solution containing 1 wt% CaCl\(\cdot\)2\(\text{H}_2\text{O}\) and 2 wt% gum arabic. The freshly prepared mixture was emulsified using an electrical Braun Handblender for 2 minutes and then adjusted to \(\text{pH} = 7.0\). The titrimetric reaction was initiated when 50 \(\mu\text{L}\) of the resuspended lipase solution (in 20 mM MOPS buffer) was added to 20 mL of the substrate solution. \(\text{pH}\) was continuously monitored (Orion 710A pH/ISA meter) and logged into a computer with a sampling frequency of 30 seconds. After 30 minutes, the reaction was discontinued. The drop in \(\text{pH}\) was correlated to the theoretical moles of \(\text{NaOH}\) required to maintain \(\text{pH}\) at 7.0 by means of a standard substrate mixture/\(\text{NaOH}\) titration curve (Figure 4.4). In other words, the change in \(\text{pH}\) was correlated to the theoretical change in moles \(\text{NaOH}\) from the initial \(\text{pH}\) using a fitted polynomial curve. Natural \(\text{pH}\) drift of the substrate was compensated by the subtraction of control baseline curves formed when substrate mixtures were injected with 50 \(\mu\text{L}\) of blank MOPS buffer. Lipase activity is defined as the \(\mu\text{moles}\) of equivalent \(\text{NaOH}\) hydrolyzed per mL of culture broth per minute.

4.10 Estimation of Error

Duplicate trials were performed on selected sample sets to gauge the relative error for results of the various analyses. Relative error values for these sample sets are assumed to be representative for other results obtained by the same analyses. Standard deviations were calculated using the \text{STDEV} function in Excel and divided by the respective mean values to yield percent standard deviation values. For methanol uptake rates the average percent standard deviation was 9% for the shake flask trial shown in Figure 5.8. Similarly, the average percent standard deviation for measured lipase activity was 25% for the results displayed in Figure 5.10. The average percent standard deviation for protein concentration was 10% for the results of reactor trial F7 shown in Figure 5.19.
Figure 4.4 Inverse Standard Substrate Titration Curve

4.11 Fermenter Setup

All fermentation cultures were carried out in fed-batch mode within a 1.8 L (working volume) glass reactor, specially fabricated by Canadian Scientific Glassblowing (Richmond, BC). The vessel was jacketed and the water bath temperature was kept constant at 30°C while pH was maintained at 5.6. Aeration was supplied by filter-sterilized building air in the range of 0 – 15 L/min and regulated via a digital airflow controller (Aalborg, GFC mass flow controller). Agitation was facilitated using a stir bar and placing the vessel on a stir plate. A dissolved oxygen probe (Cole Parmer, polargraphic, autoclavable) and pH probe (Cole Parmer, sealed, double junction, autoclavable) continuously monitored conditions within the reactor. NaOH (1.0 M) was used to compensate for pH changes and a defoaming agent (Antifoam A, Sigma) was used to control excessive foaming. Feed solution (~10 %v/v methanol in distilled water or condensate) was automatically dosed into the reactor and evaporation was compensated by addition of appropriate make-up solution (defined mineral medium).
4.12 Description of Feedback Control System

The system used for the control and data acquisition during the fermentation trials was adapted from the existing system used for the self-cycling batch reactor assembled by Norman Woo. Signals were sent, received, or processed through four pieces of equipment. These consisted of a 286 PC equipped with a data acquisition and control board (Strawberry Tree Inc., Model Acjr, Sunnyvale, California), a Pentium PC containing the Instrumentation and Automation board manufactured by National Instruments (DAQ PCI-6024E), a signal data logging box, and a digital output switch box (Figure 4.6).
The data logging box was equipped with a bank of signal ports that could each be selected to receive either current or voltage signals. It is here that measurements were acquired from the pH probe, DO probe, and air flow controller. Essentially, the function of this unit was not to process the raw signals, but to render them suitable for transmission via a parallel port to the National Instruments board housed within the Pentium PC. The versatility of the data logging box was such that various measurement devices can be easily incorporated or removed from the overall apparatus as needs dictate. In addition to signal acquisition, the unit is equipped with a 5 volt analog output port which is used to send commands to the air flow controller.

The Pentium PC together with the National Instruments Automation board constituted the 'brain' of the control system. The NI-DAQ board functioned as the central interface device capable of sending and receiving both analog and digital signals. As the raw input signals were received from the data-logging box, they were converted to numerical values which were read into the Labview control program (described in greater detail in the next section). Corresponding control responses, as determined by the control algorithm, were sent to an auxiliary 286 computer via a parallel port connection. These responses were all in the form of digital on/off signals that actuated the stir plate and various pumps, the exception being the analog air controller signal which was sent to the data logging box.
The digital control signals fed into the 286 PC is processed through a Pascal program that access the Strawberry Tree control board. The Pascal program, originally developed by J.D. Sheppard (1990) and applied towards a self-cycling fermentation system, was adapted by Milet in his study of biological treatment of condensates (Milet, 1997). The current form of the program is resultant from the revisions made by Norman Woo for his investigation into the process control aspects of self-cycling condensate treatment (unpublished). Digital signals exiting the Strawberry tree control board were lastly routed to a switch box with a capacity of eight 120 V AC outlet ports. As previously mentioned, the stir plate, feed pump, make-up pump, defoamer pump, and pH control pump are controlled via the switch box in an on/off fashion only (ie. speed/intensity is not controlled).

4.13 Description of LabVIEW Program and Feed Cycling

A sophisticated reactor control system was developed based on the LabVIEW (National Instruments, Austin, Texas) instrumentation and control programming package. LabVIEW employs a graphical environment for the construction of program structures rather than traditional text based environments, allowing for a more intuitive approach in the creation of practical user interfaces. The resultant graphical interface for the control and monitoring of the reactor allows the user to input various parameters which dictate the operational behavior of the system (Figure 4.7).

Within the program, several control loops regulated the various fermentation conditions; pH, dissolved oxygen concentration, liquid level, and feed cycling. A separate program running concurrently with the primary control algorithm was responsible for the addition of defoamer. This consisted simply of adding a small amount (~3-4 mL) of the anti-foam agent at fixed time intervals, usually between 6-8 hours. Due to the constraints of the reactor set-up (10 ports used for measurement devices and inlet/outlet streams), it
Figure 4.7  Reactor Control User Interface
was only feasible to implement a uni-directional pH adjuster (ie. the pH could only be adjusted up or down). Base addition was chosen according to the general fermentation guidelines from Invitrogen (2001) since it was reported that methanol metabolism is usually accompanied by a drop in pH.

It was desired to maintain the dissolved oxygen concentration at approximately 2 mg/L so as to ensure non-limiting conditions. This was accomplished by employing a PID control algorithm to regulate the air flow rate. The PID algorithm itself is a subroutine resident in the LabVIEW program library and called upon in the main program. Different values were tested for each of the P (proportional), I (integral), and D (derivative) settings in the early stages of reactor operation. Although usually a quick response is desirable to rapidly achieve set point, it was found that the system was unusually sensitive and even slightly aggressive PID settings would eventually result in instability. This would be manifested in very large air flow rate fluctuations, cycling from perhaps 4 L/min to the maximum of 15 L/min within the span of several seconds. A contributing factor to this behavior is believed to be the variability of the DO measurements. Though the readings sent from the probe were quite stable during the initial hours of a fermentation run, as the biomass density increased the values became more erratic, sometimes exhibiting cycling fluctuations or momentary DO spikes. To compensate for this behavior, the control algorithm was required to act slowly enough such that short term fluctuations were disregarded and only the general offset was corrected. The values that resulted in acceptable control performance were P=1 and I=0.01. Derivative control was nonexistent since this type of response is not suitable for 'noisy' systems. In comparison, the studies of self-cycling biological condensate treatment conducted by N. Woo, which employed a similar DO control concept, found that the best PID control performance was provided when P was merely set to 1 (with I and D equal to 0) (unpublished).
begin feed

cycling loop

check timer

loop

NO

calculate
average flow
rate (R)

compare new air
flow rate with
current max value

NO

YES

cycle time <
cycle interval?

set R = max

set new max = 0
set cycle time = 0

Figure 4.8  Flowchart for Cycling Algorithm
Feed cycling was strongly coupled to the control of the air flow rate. As previously discussed, methanol concentrations in excess of 1 v%/v would impair yeast growth and viability. It was therefore paramount to design an algorithm which would ensure that methanol did not accumulate within the reactor. The principle underlying the cycling program was that it would monitor the progress of substrate consumption via the exerted oxygen demand. Since dissolved oxygen is maintained at a fixed set point, the inlet air flow rate would vary accordingly. As methanol metabolism occurred, the yeast culture would increase its oxygen utilization and hence cause a rise in the air flow rate, which was continuously monitored by the computer. Upon complete consumption of the substrate, the yeast would revert back to a state of endogenous respiration, causing a positive DO spike. To compensate for this, the air flow rate would then be scaled back. When the system detected the air flow rate at 30% of the maximum value observed for the current feed cycle, methanol addition was initiated. By setting the cycling cut-off value to be a variable of the maximum air flow rate instead of a fixed value, the problem of correct feed cycling in the initial stages of a fermentation run was avoided. During this period, which usually lasts 18-24 hours, biomass levels slowly increase and overall oxygen uptake is only a fraction of the observed levels during the bulk of the experimental run.

To compensate for the short term variability of the air flow rate, the feed algorithm calculated the average flow rate value at ten minute intervals. Thus at each interval, the algorithm performed a comparison check of the newly averaged value against the max flow rate recorded at that time. Figure 4.8 summarizes the decisional logic and response actions of the cycling algorithm.

The end result of this cycling technique was a series of discreet dose cycles of throughout the course of the fermentation (Figure 4.9). Methanol addition was calibrated such that each dose would result in a methanol concentration of 500-600 mg/L within the reactor. This ensured a perpetual methanol limiting environment to better facilitate protein expression.
Liquid level within the reactor was regulated at 1.6 L using a control algorithm in the LabVIEW program. The algorithm compensated for liquid evaporation due to air-sparging by activating a pump to add make-up liquid to the reactor broth. This involved using an empirical equation to estimate the evaporation rate as a function of the air flow rate. The composition of the make-up liquid was either distilled water or air-stripped condensate depending on the desired media conditions of the reactor trial.

While the fermenter allowed air sparging, the sparger system used was unable to meet the oxygen demand in the reactor once biomass levels exceeded 12 g/L. Usually within 30 hours of growth, the maximum air flow capacity (15 L/min) would have already been reached and the remainder of the run was conducted under oxygen limited conditions. Cell densities achieved during these runs are almost certainly lower than corresponding values would be if oxygen demand was fully met. Further, the effect of cycling between anoxic and aerobic conditions on protein expression and activity is not known.

![Fed-Batch Cycling Behavior](image)

**Figure 4.9  Fed-Batch Cycling Behavior**

Though the sampling frequency of the various instruments (ie. pH, DO, air flow controller) was set at 0.5 Hz (1 data sample every 2 seconds), the actual recorded data consisted of ten minute averaged values. The reasoning behind the large time discrepancy between sampling frequency and actual data logging was two fold:
1.) Real-time response actions of the various pumps and air flow controller required a relatively short sampling interval for adequate control of the reactor conditions.

2.) It was neither necessary nor practical to accumulate an excessively large amount of data which would have resulted if the data logging and sampling frequency were the same.

Considering that times for the fermentation trials averaged in the 120 hour range, the slower logging frequency still provided excellent resolution on the reactor trends. Averaging the raw sampling data also had the added advantage of ‘smoothing’ out short term fluctuations observed for DO and air flow rate measurements, thus providing for a clearer depiction for the behavior of these fermentation conditions.

### 4.14 Reactor Media Conditions

Various growth conditions were examined for five fed-batch trials. Table 4.3 summarizes the defining characteristics for each of the reactor runs:

<table>
<thead>
<tr>
<th>Run</th>
<th>Yeast Strain</th>
<th>Growth Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3</td>
<td>Wild type</td>
<td>Defined minerals/condensate</td>
</tr>
<tr>
<td>F4</td>
<td>Lipase recombinant</td>
<td>Defined minerals/condensate</td>
</tr>
<tr>
<td>F6</td>
<td>Lipase recombinant</td>
<td>Yeast peptone/condensate</td>
</tr>
<tr>
<td>F7</td>
<td>Lipase recombinant</td>
<td>Yeast peptone/condensate + initial glucose dose</td>
</tr>
<tr>
<td>F8</td>
<td>Lipase recombinant</td>
<td>Yeast peptone/distilled water + initial glucose dose</td>
</tr>
</tbody>
</table>
CHAPTER V - RESULTS AND DISCUSSION

This section will discuss the results and subsequent analysis of results. The progress of the experimentation can be distinguished as two main stages; preliminary shake flask studies followed by fed-batch reactor runs.

5.1 Preliminary Shake Flask Studies

The shake flask experiments were initially conducted to observe growth capacity of the yeast as a function of various media formulations and other variables. In short, they were sensitivity studies that allowed for a survey of different growth conditions in addition to comparison between wild-type and lipase recombinant Pichia strains. Conducting these studies and examining a general field of conditions facilitated the formation of a knowledge base useful towards the design of reactor scale trials.

5.1.1 Growth in Condensate

One of the first objectives of the preliminary trials was to attempt P. pastoris cultivation in a condensate-based medium. A great majority of the work involving this yeast had been done in ‘clean’ media supplemented with rich nutrients. This is understandably the environment of choice for maximizing protein expression. However, given the scope of this project, it was desired to observe the growth capacity of the yeast in a less ideal environment. Early experiments with the wild-type strain showed that growth in non-airstripped condensate was severely inhibited. Figure 5.1 displays the growth curves of P. pastoris cultures grown in two media types spiked to varying methanol concentrations. Cultures in the ‘clean’ rich media (yeast-peptone in distilled water) displayed growth on methanol, in that their cell densities exceeded the corresponding growth observed in the control flask (rich media blank). In contrast, cell yield in all rich condensate media (yeast-peptone in condensate) cultures were consistently below that of the control with virtually no differentiation between different
methanol concentrations. The result implied that cell generation in these flasks was due entirely to the uptake of rich nutrients in the yeast-peptone mixture while metabolism of methanol was completely suppressed.

![Figure 5.1 Pichia Growth Comparison on Rich Media and Non-Airstripped Rich Condensate Media](image)

This observation was reinforced through various other shake flask trials where *Pichia pastoris* was cultured with a defined mineral formulation (i.e. in the absence of yeast-peptone) in unmodified condensate. When methanol was used as a sole carbon source in this growth media, cell yield was practically zero (Figure 5.2). The minor growth observed in the distilled water control culture was due to the uptake of remaining nutrients in the 1 mL transfer volume from the inoculum.

It was hypothesized that suppression of growth was due to the presence of sulfurous compounds present in the condensate. Blackwell et al. (1979) found that TRS concentration was the dominant factor contributing to condensate toxicity and that, even for stripped condensates, there was a high correlation between TRS concentration and
toxicity. Among the sulfurous compounds, hydrogen sulfide has a particularly damaging
effect on aerobic organisms (Milet, 1997). H₂S toxicity stems from the binding of sulfide
to iron in cytochrome c oxidase, a key enzyme in the electron transport chain (Vismann,

![Graph](image)

**Figure 5.2  Pichia Growth in Unmodified Defined Mineral Condensate**

To counteract the problem, it was desired to air-strip the condensate prior to use. A study performed by Blackwell and Oldham (1980) estimated the vapor/liquid equilibrium behavior of common contaminants found in Kraft condensates. According to their investigation some of these compounds (including methyl mercaptan, dimethyl sulfide, dimethyl disulfide, and hydrogen sulfide) are only sparingly soluble in water. Figure 5.3 below summarizes their findings and illustrates the relative ease of stripping for the various condensate components. It is noted that for a temperature of 30°C, less methanol is removed as compared to the various TRS species, by a factor of at least 100.

Similar results were obtained in condensate stripping experiments performed by Milet (1997). It was found that the gas flow rate (0.03 L/min – 3 L/min) did not significantly influence the stripping rate of methanol or TRS. After 40 minutes of stripping only 18% of the methanol was removed, whereas 100% of the DMS and 78% of
the DMDS was stripped. H$_2$S and CH$_3$SH were completely removed within the first 10 minutes.

Figure 5.3  Vapor/Liquid Equilibrium for Various Kraft Condensate Contaminants

The *P. pastoris* wild type grew well in air-stripped condensate and demonstrated growth with methanol uptake, though final cell densities were consistently lower (20-30%) than their non-condensate counterparts in the shake flask experiments (Figure 5.4). This strongly suggests that, if present in high enough concentrations, sulfurous compounds present in the condensate completely shut down the methanol utilization pathway. Accordingly, all further experiments were conducted with air-stripped condensate (refer to Methods and Materials for specific stripping conditions).

Upon examination, the various methanol concentration curves in Figure 5.4 exhibit two distinct phases. The first phase, between time zero and 22 hours, corresponds
to the period when the yeast preferentially metabolized the rich nutrients present in the yeast-peptone mixture. When this food source was exhausted, yeast metabolism was fully switched to the secondary carbon source, methanol. This was characterized by a dramatic increase in methanol removal and a slower rise in cell density. The exact point at which the cultures exhausted the yeast-peptone nutrients and began to fully metabolize methanol is not known since data points were not taken between zero and 22 hours.

![Graph showing Pichia Growth in Air-Stripped Condensate](image)

**Figure 5.4  Pichia Growth in Air-Stripped Condensate**

### 5.1.2 Growth of Recombinant *Pichia pastoris*

The lipase bearing *P. pastoris* strain was cultured using the same methodology as the wild type. The goal of these trials were to compare the characteristics of the two strains and observe if any growth related deficiencies were apparent when cultured in the various media types.
Comparison of growth between the wild type *Pichia* and the lipase recombinant strain yielded nearly identical profiles for cultivations in rich media supplemented with glucose, indicating similar metabolic rates on a rich carbon source (Figure 5.5).

**Figure 5.5 Growth Comparison of Lipase Recombinant and Wild-Type *Pichia***

Growth of the recombinant was also compared in defined media versus defined condensate media (Figure 5.6). An initial dose of substrate, either glucose or glycerol, was used to encourage the rapid build-up in cell density during the initial phase of the culture prior to methanol feeding. It was found that the cultures grown in the condensate media were 20-30% lower in cell density than their non-condensate counterparts, which was consistent with the results observed for the wild type *Pichia* strain. It was also apparent that the cultures initially grown on glycerol achieved higher cell yield than those grown on glucose.

**Table 5.1 Specific Methanol Uptake Rates for Recombinant *Pichia* Grown in Defined Media with Initial Rich Carbon Source**

<table>
<thead>
<tr>
<th>Media Type</th>
<th>defined mineral</th>
<th>defined condensate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Substrate</td>
<td>glucose</td>
<td>glycerol</td>
</tr>
<tr>
<td><strong>Specific Methanol Uptake Rate (mg/hr/g dry cell weight)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose Period 1</td>
<td>40.6</td>
<td>31.8</td>
</tr>
<tr>
<td>Dose Period 2</td>
<td>59.4</td>
<td>46.5</td>
</tr>
</tbody>
</table>
Values of specific methanol uptake rates are summarized in Table 5.1. Methanol uptake was higher in the cultures dosed with glycerol than those dosed with initial glucose. The defined condensate *Pichia* also showed higher uptake rates than the defined mineral ones; though the difference between initial glucose cultures were minor (3-6%), the difference between the corresponding glycerol cultures were quite pronounced (70-77%). Since the specific uptake rates are lower for the cultures exhibiting a greater cell yield (defined mineral cultures), it is hypothesized that in the defined condensate cultures, a greater portion of the energy metabolized from methanol consumption was used in cell maintenance rather than cell generation, owing to the more toxic environment of the condensate.

![Graph](image)

**Figure 5.6** Initial Substrate Comparison for Lipase Recombinant *Pichia* in Defined Media

pH was also monitored during the run and was observed to decline in each of the four flasks (Figure 5.7). Although the pattern of the pH curves closely approximate each other, it was noted that cultures grown in the defined condensate media experienced a slightly greater pH decline as compared to those in defined media. The three segments of
pH decline were noted to correspond to the distinct phases of substrate metabolism, first during consumption of the yeast-peptone nutrients and later during the two periods of methanol consumption.

After successfully growing the yeast in a condensate medium, the next step was to attempt expression of the *Geotrichum* lipase through methanol induction. The induction procedure for the shake flask cultures involved first growing the recombinant yeast to a sufficient cell density (6-9 g/L) using glucose, then switching to methanol dosing at appropriately spaced intervals, similar to the procedure used in the prior growth trial.

![pH curves for Lipase Recombinant *Pichia* in Defined Media](image)

**Figure 5.7**  pH curves for Lipase Recombinant *Pichia* in Defined Media

Unlike the previous experiment however, the culture broth combinations did not consist of a condensate medium. As a first time attempt to quantitatively produce and detect the recombinant enzyme, it was decided to initially culture the *Pichia* in a clean nutrient formulation deficient of potentially harmful or inhibiting components. Figure 5.8 illustrates the growth behavior and methanol consumption curves for the recombinant strain grown in rich yeast-peptone medium and defined mineral medium. The initial fast growth phase is associated with the metabolism of a rich carbon source (glucose), with methanol induction starting at approximately 68 hours. It is evident that the yeast-peptone cultures fared better not only on the initial glucose dose, but also during methanol
consumption. Final cell densities showed that the cultures grown on the mineral solution were 40% lower in cell concentration than the rich media cultures.

![Graph showing comparison of methanol concentration and cell density between rich and defined media cultures](image)

**Figure 5.8 Rich vs. Defined Media Comparison for Lipase Recombinant *Pichia***

Analysis of the degradation curves revealed that the specific methanol uptake rates of the rich media and defined mineral cultured yeasts were within a close range (24.6 and 23.2 mg MeOH/hr/g dry cell weight respectively) at the start of methanol addition. During the progression of the run, however, the specific uptake rate of the defined mineral culture remained essentially constant whereas that of the rich media increased steadily to 34.6 mg MeOH/hr/g dry cell weight. This can be understood in light of the relative energy demand associated with synthesizing cellular components in the two media formulations. The specific uptake rates observed here are lower than those in the previous defined media cultures. These rates were also slightly lower than those observed by Ren et. al. (2003) whose values were in the range of 38.4 – 51.2 mg MeOH/hr/g dry cell weight for the *Pichia* expression of human serum albumin. It is noted that these growth trials were performed within a 30 L bioreactor rather than in shake flasks. A logical hypothesis would be that the difference in oxygen delivery between a shake flask and a bioreactor would lead to differences in oxygen consumption and hence
metabolic rates and methanol uptake. However, results in a subsequent fed-batch reactor study showed that this is not the case. Specific methanol uptake rates observed for reactor trial F7 (to be discussed in Section 5.2.4) were approximately 31 mg MeOH/hr/g dry cell weight, similar to the value observed for the rich media culture in this shake flask study.

Detection of recombinant protein for this growth trial will be discussed in the following section.

The pH behavior of the recombinant strains in the two media formulations were quite different. Whereas the defined mineral culture displayed a slight and gradual pH decline to approximately 5 (similar to the trend seen in the prior experiment), the sets grown in the rich medium experienced several pH shifts over the course of the trial (Figure 5.9). Initially, a relatively brief pH drop to 5.2 was observed followed by a long rise to 8.3 where it stabilized and was maintained for a large portion of the run. Gradually, however, the pH shifted again and gradually declined to approximately 6.

![Rich vs. Defined Media pH Curves for Recombinant Pichia](image)

**Figure 5.9 Rich vs. Defined Media pH Curves for Recombinant Pichia**

The first pH inflection of the yeast-peptone cultures occurred within the first 15 hours of glucose metabolism. However, pH stabilization near the 70 hour mark seems to correspond to the exhaustion of yeast-peptone and the introduction of methanol. Similar
behavior was observed during the growth of a cystatin recombinant *Pichia* strain when it was grown in a yeast-peptone broth (Appendix A).

5.1.3 Expression of Lipase in Shake Flask Studies

Lipase assays were performed on the latter shake flask studies containing the recombinant *Pichia* strain. Figure 5.10 depicts observed enzyme activity over the course of the recombinant shake flask experiment discussed in the previous section.

![Figure 5.10 Lipase Activity for Rich Media Recombinant Culture](image)

As shown in the plot, lipase activity of the yeast-peptone cultures were detected approximately 54 hours after initial methanol dosing. Enzyme activity reached a maximum level of 55 μmol/min/mL at 130 hours and rapidly declined thereafter. No lipase activity was observed during growth in the defined medium over the duration of the experiment. This result was reaffirmed by a similar shake flask study where lipase activity was not detected when recombinant cultures were grown in the defined mineral broth (results not shown). It is hypothesized that the defined media is deficient in key nutrients which fully enable the methanol utilization and expression pathway. This is in agreement with the results obtained by Holmquist et al. (1997) who found high levels of enzyme expression in a growth culture consisting of a yeast-peptone formula while only
trace levels of lipase was detected when an otherwise identical minimal formula without yeast-peptone was used.

5.2 Fed-Batch Reactor Studies

Whereas the shake flask cultures allowed for the examination and comparison of yeast growth and behavior among a cross section of culture conditions, the aim of the reactor studies was to more rigorously examine \textit{Pichia} growth and lipase expression in a highly regulated environment.

Using the feed cycling algorithm, several fermentation runs were conducted. It was desired to establish a control run to define the growth characteristics of the wild type \textit{Pichia} strain. Subsequent reactor trials would be compared against this control to observe operational anomalies or deviations from expected trends. It also allowed a check on system behavioral aspects, such as methanol cycling, oxygen feed stability, and pH control stability, among other factors. The four following fermentations consisted of recombinant \textit{Pichia} cultures grown in condensate supplemented media with the last conducted in a ‘clean’ distilled water environment.

For the sake of brevity, the following discussions will refer to the individual runs by their designation (F3 through F8). Media characteristics for each trial were given earlier (Methods and Materials). Run F5 encountered contamination problems and as such is excluded from discussion.

5.2.1 Comparison of Yeast Growth (Fermentation)

Figure 5.11 displays the growth curves obtained for the various fed-batch runs. The duration of the fermentations lie in the range between 140 to 220 hours, which is comparable to the time of a shake flask experiment. F4 and F6 were discontinued sooner
than the others because their cultures were well into their stationary phases and would be unlikely to display further increases in cell density.

As stated above, the wild-type run (F3) was performed to establish a baseline performance record from which to compare the subsequent fermentations. This was conducted in a condensate media supplemented with defined minerals and achieved a maximum cell density of 11.6 g dry weight/L. The following run (F4) was identical in terms of media formulation with the exception that the lipase recombinant strain was cultured. The resulting growth trend closely matched that of F3 during two-thirds of the run before biomass levels stabilized and began to slowly decline. The maximum cell density achieved here was 8.4 g/L, approximately 70% of the wild-type value, similar to the shake flask studies.

It is also apparent that within a short time span oxygen demand from the yeast outpaced the available supply, which had a maximum flow rate varying between 14 - 15 L/min. This was the usual case for the fed-batch cultures with the exceptions of runs F6 and F8, which will be discussed later.

![Figure 5.11 Growth Curve Comparison for Fed-Batch Cultures](image)

Figure 5.11  Growth Curve Comparison for Fed-Batch Cultures
When cultured in a yeast-peptone environment, the logarithmic growth phase was more rapid and shorter, owing to the presence of rich nutrients. This was apparent in the next three fermentations (F6, F7, F8). For the first 24 hours, the growth rate in all three runs was nearly identical as the yeast-peptone nutrients were preferentially metabolized. After this period however, deviations soon became noticeable as the specific characteristics of each culture medium exerted their influence.

The reactor trial involving the lipase recombinant in YP-condensate media (F6) attained steady-state biomass levels similar to those observed for run F4. In contrast cultures F7 and F8, in which 1 wt%/v glucose was initially added, displayed continual increases in cell density until near the end of their runs, similar to the trend observed for the wild type trial (F3). It may be that the recombinant *Pichia* is more resistant to inhibitors in condensate after initial growth on glucose (and possibly other rich carbon sources).

Upon closer examination of the F7 growth curve, three distinct phases are identifiable. A rapid exponential growth phase (0-30 hrs) is followed by a transition period (30-50 hrs) where the yeast adapted to the methanol substrate, and lastly a second growth phase (50-170 hrs) where methanol metabolism yielded slow but steady cell growth. The maximum cell density attained in this run slightly exceeded that of the wild-type (11.9 g dry weight/L).

Likewise, F8 exhibited three distinct phases, differing primarily in the length of the transition phase which was approximately twice as long as the corresponding period for run F7. This was contrary to expectation since the culture media for F8 consisted of a yeast-peptone solution (with initial glucose dose) in distilled water. Intuitively, it would be expected that yeast growth would be considerably enhanced, and perhaps experience a shorter transition phase, in the absence of inhibitory compounds present in condensate. It is noted, however, that overall growth rates in the second growth phase of F7 and F8 appear to be nearly identical, with the latter being displaced roughly 60 hours from the former. It is also observed that these growth rates are similar to that observed for the wild
type culture. The effect of initial glucose dosing may not only render the yeast more resistant to inhibitors, but also result in promoting biomass production rates typically observed for a \textit{Pichia} wild strain. The final cell density achieved in F8 was 10.8 g/L, roughly 10\% lower than that observed for F7.

5.2.2 Reactor Cycling Characteristics

To observe the actual behavioral aspects during a fed-batch trial, it is necessary to examine the reactor cycling characteristics. As was stated in the Methods and Materials section, several key parameters were logged continuously during each run, namely; dissolved oxygen, air flow rate, volume of feed added, and pH.

![Air Flow Rate and Dissolved Oxygen Reactor Characteristics](image)

Figure 5.12 Air Flow Rate and Dissolved Oxygen Reactor Characteristics
(a) Run F3 (b) Run F7
Figure 5.12 (a and b) illustrates the air flow rate and DO cycling behavior for runs F3 and F7, while Figure 5.13 (a and b) shows the corresponding trends in feed addition and pH (only partial segments of the entire runs are shown). Both graphs display the typical trends observed during a normally functioning reactor trial.

For the air flow/DO charts, system cycling is indicated by the valleys formed in the air flow rate plot-line or concordantly, by the spikes in the dissolved oxygen plot-line. It initially appears that the dosing cycles were of a constant length throughout the run, as indicated by the uniform 'steps' made by the feed addition (Figure 5.13). It is observed, however, that along the progression of the entire run, there was a slight and very gradual lengthening in cycling times, which was common to all fermentation trials though it is more easily seen for F3 (Figure 5.14.). This seems at first counter intuitive since logic would suggest that as biomass levels increase, time taken to consume equivalent doses of methanol would decrease. In actuality, as cell density steadily increased, the response time in the scale back of air flow rate was slowed after successive feed cycles. The reason
is rooted in the principle of the cycling algorithm, which was explained in the Materials and Methods section. In short, cycling is activated by the comparison of the oxygen (air flow rate) required during substrate metabolism and that during the endogenous state of the yeast. When biomass levels are low, the transition time from a high oxygen demand (during methanol consumption) to a low oxygen demand (after substrate exhaustion when the culture reverts to the endogenous state) is quick. In contrast, when the culture has reached high biomass levels, the cumulative oxygen demand within the reactor is relatively high even during the endogenous state. Thus to satisfy this DO demand, air flow is scaled back more slowly and signaling to initiate the following feed dose occurs at increasing lengths of time.

![Graph](image_url)

**Figure 5.14  Cycle Length vs. Cycle Number (Run F3)**

One key difference between the two plots of Figure 5.12 is apparent during the initial portion of the runs. Whereas F3 was cultured in a defined mineral broth, the F7 culture was grown in the presence of yeast-peptone. This difference is manifested by the absence or presence of a prolonged air-flow spike during which no cycling occurs (usually lasting the first 25 hours). This is indicative of the period when yeast-peptone cultures are actively metabolizing the rich nutrients and/or glucose preferentially. Cycling is then initiated upon the exhaustion of these substrates during the gradual scaling back in
air flow rate. pH behavior, as well, is slightly different between growth in the two media types.

As evidenced in the shake flask experiments, *Pichia* cultures in the presence of yeast-peptone will experience several pH shifts; initially a tendency to go acidic, followed by a brief basic phase, and finally an acidic progression lasting for the remainder of the run. Figure 5.13b reveals this in the minor pH disturbance during the 10-25 hour period, which was a consequence of the limitation of the uni-directional pH controller (set to only compensate for acidic shifting). This is in contrast to defined mineral cultures which only exhibited pH decline (Figure 5.13a).

In runs F3, F4, and F7, oxygen demand could not be satisfied with the air supply system. It is thus reasonable to assume that these cultures may have yielded higher biomass levels had oxygen not been limiting. Runs F6 and F8, however, did not exhibit the expected oxygen limitation conditions experienced by the aforementioned cultures. The air flow rate for F8 (Figure 5.15) reveals that the air flow spikes are erratic near their maximums (as compare to F7) with the average maximum air flow rates within the bounds of 8.8 – 10.4 L/min, significantly lower than the 15 L/min cut-off which is usually observed. Also evident in the graph is the consistency of the median DO measurement, which easily maintains a value of roughly 2 mg/L. This is in contrast to the F3, F4, and F8 in which dissolved oxygen concentrations routinely drop to, or below, 1 mg/L. Figure 5.16 provides an expanded view of several dose cycles in F8; once again notice the irregular shape of the air flow spike apexes (as compared to the uniform curves of Figure 4.9 in the Methods and Materials Section).
Figure 5.15  Airflow and Dissolved Oxygen Cycling (Run F8)

Figure 5.16  Expanded View Cycling Behavior for Run F8

5.2.3  Limitation to Biomass Growth

For each fed-batch trial there was an upper limit to the cell density attainable using the reactor system. Two factors contributed to this; limitations of the air supply
system and the lengthening of dosing intervals. Whereas the former affects metabolic activity via the inability to fully satisfy oxygen demand, the latter pertains to growth restrictions of a limiting substrate.

As biomass accumulated during the progression of the runs, the methanol in each feed dose would be consumed quicker. However dosing frequency did not increase but instead declined slightly throughout the trial, as was previously discussed. The combined effect of increasing cell density and decreasing dose cycling translated into an overall reduction in methanol consumed per unit mass of yeast. Increasingly, any available methanol would then only be used for cell maintenance rather than further cell growth. Depending on the specific medium conditions, this limit would vary, but a limit would inevitably be reached in each case.

5.2.4 Methanol Consumption

Although regular feed cycling indicated the consumption of methanol, it was still desired to examine in detail a sample of the methanol uptake behavior. Figure 5.17 displays methanol uptake and the corresponding airflow rate trend during two separate dose cycles during run F7. As was the case seen in the shake flask experiments, methanol consumption with respect to time appears to be practically linear. The specific methanol uptake rates for the two dose cycles corresponded closely to each other; 31.7 mg MeOH/hr/g dry cell weight for cycle 5 and 30.7 mg MeOH/hr/g dry cell weight for cycle 98. These values were within close range to the specific uptake rate observed in the shake flask study examining recombinant Pichia growth in rich media (Figure 5.8) which was found to be 34.6 mg MeOH/hr/g dry cell weight.

It is observed, that a lag time is present between the point of methanol exhaustion and the point of minimum air flow rate (which signals the onset of the following dose cycle). This was addressed earlier in the discussion concerning the response time for the scaling back of the air flow rate. The principle is simply demonstrated by comparing the two plots. In the figure depicting dose cycle 5 (near the beginning of the fermentation),
the lag time is 0.3 hrs (18 min), whereas for dose cycle 98, the delay is twice as long at 0.6 hrs (36 min).

![Graph showing methanol concentration and airflow rate curves for Reactor Trial F7.](image)

**Figure 5.17** Methanol Concentration and Air Flow Rate Curves for Reactor Trial F7 (a) Dose Cycle 5 (b) Dose Cycle 98

A possible solution to compensate for cycle lengthening involves changing the value of the signaling variable in the cycling algorithm. As explained in the Materials and Methods section, dose signaling occurs when the averaged airflow rate falls to a set fraction of the maximum value recorded for the current cycle (the default setting was 30%). To obtain a quicker signaling response, this setting can be increased to a higher value (ie. 80% of the observed maximum air flow rate). Although this may not eliminate cycle lengthening, the effects will be much less pronounced and should result in nearly constant cycle lengths (depending on the setting). This method, however is only reliable
when reactor conditions are stable and DO fluctuations (and therefore airflow rate fluctuations) are not present. To illustrate, once again examine the airflow rate patterns of F8 shown in Figure 5.16. Here, the airflow rate cycles are characterized by curves with broken peaks. An immediate decline from the maxima during a given cycle may or may not indicate methanol exhaustion. Thus if the signaling variable is set at a high value (60%-90%), cycling may be activated prematurely which will eventually lead to accumulation of methanol in the reactor.

5.2.5 Recombinant Protein Expression for Reactor Studies

For the four fermentation trials involving the recombinant Pichia strain, lipase activity was detected only in the cultures containing yeast-peptone, that is: F6, F7, and F8. This is consistent with earlier observations for the shake flask experiments which showed no expression in cultures grown in a mineral formulation.

In fermentation 6, lipase activity was detected at 92 hours from the start of the run. Enzyme activity then increased to a maximum of 13.7 μmol/min/mL within 30 hours, then appeared to rapidly decline thereafter (Figure 5.18).

Figure 5.18 Lipase Activity for Reactor Trial F6

Lipase activity in F7 was detected early in the run, within 24 hours, which is in contrast to the results of earlier shake flask trials and those seen for the previous
fermentation. Near the 100 hour mark, however, enzyme activity levels roughly doubled and appeared to remain relatively steady within the range 10 – 13 µmol/min/mL. In addition to enzyme activity, protein concentration measurements were also taken for this and the next run (F7 and F8). Figure 5.19 shows that the trend in increasing lipase activity is roughly mirrored by that of the protein concentration. Initially within the first 80 hours, protein concentration did not exceed 3 mg/L. At the corresponding time that enzyme activity is seen to abruptly increase, protein concentration experienced a similar increase. However, toward the conclusion of the run, protein concentration continued to increase to a final value of 56.7 mg/L whereas activity appeared to level off.

![Graph](image)

**Figure 5.19** Lipase Activity and Protein Concentration for Reactor Trial F7

In comparison, lipase activity was observed to be noticeably lower in F8 (Figure 5.20). Here, negligible activity was observed until 125 hours into the run, followed by a gradual increase lasting for the remainder of the culture period. The maximum activity attained, 10.7 µmol/min/mL, is within the range of values observed in the latter half of run F7, though the considerably slower rise to this reflects delayed growth rate as previously discussed. Protein concentration followed a similar pattern, a gradual increase which corresponds to the trend in enzyme activity. As with F8, protein concentration
continues to increase until the end of the fermentation, eventually reaching a value of about 48.5 mg/L, approximately 14% lower than the previous run.

![Graph showing NaOH hydrolysis rate and protein concentration](image)

**Figure 5.20 Lipase Activity and Protein Concentration for Reactor Trial F8**

The average specific activity of the lipase enzyme in F7 and F8 were determined to be approximately 220 and 140 μmol/min/mg protein, as compared to 1200 μmol/min/mg protein observed by Holmquist et. al. (1997) for the GCL I isoenzyme. Protein concentrations, however, were within proximity (-20%) to the level achieved by the previous work at maximum values of 60 mg/L. This result, together with the comparatively low specific activity, suggests that though protein production was proceeding at expected levels, incorrect expression or deactivation of a significant percentage of the enzyme may have occurred. It was observed by Holmquist et. al. (1997) that a portion (6-8 wt%) of the enzyme received N-linked high-mannose-type glycosylation, which would impair its ability to properly react with the substrate. It is possible that cultivation in a condensate media exacerbates glycosylation of the expressed protein.
Enzyme deactivation could have also occurred via the presence of proteases which are released during cell lysis. Previous work indicated that the presence of truncated lipase products which were thought to have resulted from proteolytic deactivation (Holmquist et. al., 1997). Although seen in shake flask cultures, this is a much more frequent and pronounced problem in reactor fermentations given the high cell concentrations. Even a small fraction of the culture undergoing cell lysis would result in significant protease activity (Cereghino and Cregg, 2000, Romanos, 1995). This may provide a partial explanation to the high enzyme activity observed in the prior shake flask trial (55 μmol/min/mL) compared to results in the reactor trials (~11-14 μmol/min/mL). The common remedy for this is the addition of protease inhibitors (ie. casamino acids) into the culture medium or lowering the pH to reduce proteolytic activity (Cereghino and Cregg, 2000; Sreekrishna et al., 1997; Invitrogen Corporation, 2001; Romanos, 1995).

Another explanation for the discrepancy between protein production and lipase activity would be the significant generation of non-lipase protein products (ie. contaminating proteins), although this was not previously observed with this strain (Holmquist et. al., 1997). Alternatively, it is possible that the condensate itself is deactivating the lipase enzyme after it is produced.

It should also be noted again that the procedure to assay enzyme activity was different than the method used by Holmquist et. al. (1997) due to lack of proper equipment (pH-stat device). Though care was taken in designing the assay procedure to closely match the theoretical principles of the original method, the procedure may still contain inherent flaws that could skew the results.

In comparing the three runs bearing lipase activity, it is observed that maximum values for protein concentration are within reasonable range (+/- 30%). A difference in trends however is noticed for each; a clear maximum and decline for F6, establishment of a plateau for F7, and a continual increase for F8. In reference to run F8, the comparatively sluggish rise in activity is reflective of the delayed increase in biomass.
when compared to F7. It was suggested that the growth curve was nearly identical though displaced 60 hours later from it’s counterpart in the prior run.

For run F6, though cell density levels achieved were considerably less than those attained in the latter two fermentations, lipase activity was clearly present in comparable amounts. The rapid decline in activity, however, is a trait unique to this reactor trial. The conclusion that can be drawn from this is two fold. Firstly, the presence of an initial glucose growth phase may not be needed to achieve appreciable levels of lipase activity from a culture. However, without the early metabolism of glucose (or another rich carbon substrate) the yeast may not be able to maintain consistent levels of enzyme production.
CHAPTER VI - CONCLUSIONS

This study examined the viability of culturing a lipase recombinant strain of *P. pastoris* in a kraft mill condensate effluent. A series of shake flask experiments were conducted to observe growth behavior in various media formulations. This was then followed by several fed-batch reactor trials to assess the effectiveness of growing the yeast using an automated feedback control system. For selected trials, enzyme activity and protein concentration of the expressed lipase were measured. The primary conclusions of this study are summarized below:

1. Growth of *P. pastoris* was severely inhibited when cultured in non-air stripped combined condensate. Initial shake flask studies revealed that uptake of methanol was completely suppressed in this situation. It was hypothesized that sulfurous compounds in the effluent were in sufficient concentration to shut down the methanol utilization pathway. Cultures grown in air-stripped condensate grew well, though still 20-30% lower in cell density than their non-condensate counterparts.

2. Lipase activity was only detected when the recombinant *Pichia* strain was grown in a medium supplemented with yeast extract and peptone. Cultures grown in defined mineral formulations yielded no or only trace levels of enzyme activity. This was first observed in the shake flask experimentation and later reaffirmed in the reactor scale studies. The result is consistent with previous work by Holmquist et al. (1997).

3. Addition of a rich carbon source such as glucose or glycerol was not required for the production of recombinant protein.

4. A feed-back control program based on DO monitoring was successfully implemented in the reactor system. The algorithm ensured complete depletion of methanol before initiating subsequent feed cycles.
5. Biomass growth in the fed-batch trials was constrained due to oxygen limiting conditions and increase in cycle time.

6. Maximum enzyme activity levels detected in the three reactor trials (F6, F7, and F8) were in the range of 10.8 – 13.9 μmol/min/mL. The average specific activities of the latter two runs were 220 and 140 μmol/min/mg protein respectively. These levels are approximately 6-7 times lower than reported by previous work (Holmquist, 1997). Protein concentrations however, were found to be within 20% of the value cited in literature. Biomass levels in the previous work were not reported.
CHAPTER VII - FUTURE WORK

The following recommendations are suggested for future work:

1. The integration of an online methanol sensor would allow the methanol concentration to be continuously controlled at a set point. The current control algorithm cycles between a limiting methanol concentration and its complete exhaustion. It has been reported that consistent methanol concentrations promote significantly higher levels of protein expression (Guarna et al., 1997).

2. If it is not feasible to incorporate a continuous methanol sensor, the cycling algorithm should be modified to eliminate (or reduce as much as possible) the cycle lag which develops over the course of a fed-batch trial.

3. Oxygen limitation remains a major concern for the reactor trials. The current air supply is restricted to a maximum flowrate of 15 L/min. Sparging pure oxygen into the reactor would undoubtedly improve O$_2$ limiting conditions.

4. SDS-PAGE analysis should be performed on selected supernatant samples. This would be used to check the observation by Holmquist et al. (1997) that the enzyme was expressed in relative high purity with little contaminating protein. The analysis may also be helpful to determine the extent to which over-glycosylation of the recombinant product is occurring. A third possible application is to confirm the absence of the expressed lipase in defined mineral formulations (ie. absence of yeast-peptone). If the recombinant product is detected in these cultures, then it would suggest that though expression is proceeding, incorrect synthesis and secretion renders the enzyme inactive.

5. Protease inhibitors, such as casamino acids, should be added to the culture broth to reduce the possibility of proteolytic deactivation of the expressed enzyme.
6. The effect of condensate on the activity of the lipase should be investigated. To do this, it will be necessary to acquire a standard grade of the *Geotrichum candidum* lipase I (GCL I) and assay its activity after exposure to condensate and compare results when exposed to a control media (ie. distilled water).

7. The use of other condensate cuts should be examined. This study focused exclusively on cultivation within combined condensate, however higher methanol concentrations are present in foul and digester condensate streams. These streams are also accompanied by higher levels of other contaminants (ie. TRS) and the difficulties they impose on yeast growth need to be addressed.

8. The next major phase of the project would be to modify the current reactor system into a SBR, such that large quantities of condensate could be treated semi-continuously. Alternatively, the system could be set up to operate as a chemostat, using the dilution rate as the prime variable to control the yeast growth rate.
REFERENCES


41. Yan, J. (2002) Growth of *Pichia Pastoris* on Kraft Mill Condensate, Undergraduate Thesis, Chemical and Biological Engineering, University of British Columbia, Vancouver, BC.


APPENDIX A

Shake Flask Growth Trials of Cystatin Recombinant Pichia – Summary of Results

Prior to the acquisition of the Geotrichum lipase bearing recombinant strain, it was desired to observe the growth and expression capabilities of a conveniently available recombinant strain of the yeast in condensate media. This was to serve as a learning model and test organism while efforts to acquire the lipase strain were in transit.

The test organism was a recombinant Pichia strain which expressed the cysteine protease inhibitor, cystatin C, and was generously donated by Dr. Sue Baldwin and Jason Pritchett of the UBC Department of Chemical and Biological Engineering. A growth survey of the yeast was then modeled into an undergraduate thesis project undertaken by Yan Jin (Jin, 2002). The project entailed performing growth comparison between the recombinant and wild type strain and a simple $2^2$ factorial design experiment examining the effects of media type and agitation speed on shake flask cultures. Tables A.1 and A.2 summarize the conditions used in the factorial design experiment.

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<th>Agitation Speed</th>
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<td>Low</td>
</tr>
<tr>
<td>B1</td>
<td>B2</td>
<td>Low</td>
</tr>
<tr>
<td>C1</td>
<td>C2</td>
<td>High</td>
</tr>
<tr>
<td>D1</td>
<td>D2</td>
<td>High</td>
</tr>
</tbody>
</table>

Table A.1 Operating Conditions for $2^2$ Factorial Experiment

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<th>Media</th>
<th>Agitation Speed (rpm)</th>
</tr>
</thead>
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<td>Defined Mineral in Condensate</td>
<td>175</td>
</tr>
<tr>
<td>High</td>
<td>Yeast Peptone in Condensate</td>
<td>250</td>
</tr>
</tbody>
</table>

Table A.2 Factorial Experiment Design Levels

The growth comparison between the wild type strain and the cystatin recombinant strain was carried out in defined mineral and defined mineral in condensate media, with
an initial dose of a carbon rich substrate (glycerol) and subsequent methanol addition. The resultant growth curves for the two yeast strains in their respective media formulations are shown in Figure A.1. As expected, the wild strain in defined media exhibits the greatest initial growth rate and final cell concentration at 8.3 g dry cell weight/L, followed by the wild strain cultured in condensate supplemented with defined minerals at 7.5 g dry cell weight/L. The cystatin strain in defined media yielded roughly two thirds of the growth observed by its wild-type counterpart. The recombinant strain cultured in the defined mineral condensate displayed a final cell density of approximately 5 g dry cell weight/L. All cultures were observed to experience a drop in pH though there seemed to be a minor distinction between those grown in defined media and those in the condensate based broth, with the condensate cultures becoming slightly more acidic.

Figure A.1  Growth and pH Comparison for Wild-Type and Cystatin Pichia Strains

For the results of the factorial experiment, it was observed that the strongest factor influencing cell growth was media type. Specifically, the presence of yeast-peptone was
seen to induce 30% higher biomass concentrations than cultures grown in a defined media formulation (Figure A.2). Shaker speed, however, did not produce any noticeable effect on cell growth. It was initially speculated that oxygen transfer would be a crucial growth factor and that it was directly related to the agitation rate. This did not appear to be the case and it was theorized that either the concentration of the cell culture may have been too large to facilitate adequate oxygen transfer or \( \text{O}_2 \) was in excess for both agitation rate.

Trends in pH were also monitored and two distinct patterns emerged for the media types (Figure A.2). Growth in defined mineral broth exhibited a gradual pH decline and stabilization around 5.1 whereas the yeast-peptone culture initially experienced a pH dip and subsequent rise to approximately 8, where it remained. The time when the rise reached a plateau roughly corresponds to when the rich yeast-peptone nutrients were exhausted and methanol dosing began.

Figure A.2  Growth and pH for Factorial Experiment
During the second half of the shake flask trial, after methanol dosing had been initiated, culture supernatant samples were assayed for cystatin activity, where one unit of enzyme activity was defined as 1 mg of inhibited papain per minute (Figure A.3). Though no obvious pattern was discerned from the data, the positive activity values were indicative of the presence of cystatin.

![Figure A.3 Expressed Enzyme Activity](image-url)
APPENDIX B

Copies of the fed-batch reactor control program are available through the Special Collections division of the UBC library. The program was written using the graphical programming package LabVIEW (National Instruments, Austin, Texas) and requires an installed version of this to run correctly.