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Date August 25, 1986
ABSTRACT

Waste fibre (cellulose) in the form of primary clarifier sludge (PCS) from pulp mills is an abundant and renewable resource which is currently used merely as a dewatering agent for activated sludge, and as a fuel source in hog fuel boilers. One company which follows this practice of disposal is Tembec Inc., a low yield sulphite pulping operation located in Témiscaming, Québec. Tembec produces 40 tonnes/day of PCS on a dry basis, primarily as a by-product of sulphite pulping, but also from chemithermomechanical pulp (CTMP) production. The operation also produces azeotropic (97%) ethanol from the hexose fraction of its spent sulphite liquor (SSL) in a fermentation process that is currently underutilized. While the market for pulp is historically volatile, ethanol has always been a highly-valued product with a stable market and selling price. Bioconversion of PCS to ethanol through enzymatic hydrolysis and fermentation is a well understood process which could be implemented at a modest cost by using existing equipment and personnel. Therefore a unique opportunity exists at Tembec to increase ethanol productivity, and at the same time reduce the amount of PCS of which it must dispose. In this thesis, the suitability of PCS as a substrate for ethanol production through a variety of technologies has been explored.

Efforts to characterize the amenability of PCS to enzymatic hydrolysis have shown that initial (1 hour) hydrolysis rates in acetate buffer as high as 7.9 g·L⁻¹·h⁻¹ are possible at an initial enzyme loading of 10 filter paper units (FPU)/g PCS, using cellulases derived from Trichoderma reesei. The hydrolysis rate was found to be proportional to enzyme loading. A 66.4% conversion of PCS to reducing sugars was possible, coinciding with a 56% increase in residual solids dewaterability.
Wood sugars derived from PCS were readily fermented to ethanol by *Saccharomyces cerevisiae* yeast, with yields as high as 66% of the theoretical obtained.

To reduce the deleterious effects of end product inhibition, simultaneous hydrolysis and fermentation (SSF) experiments were carried out. In batch trials, sugar accumulations (in amounts proportional to the enzyme loading) were observed over the first 10 hours of SSF. After this time, yeast populations multiplied and the sugar was converted to ethanol. In SSF reactions using 100% SSL (20% total solids) as the reaction matrix ethanol production could be increased by 25% over a 24 hour period (the residence time in the fermenters at Tembec). This result could possibly be improved upon if the SSL were pre-fermented prior to the addition of PCS and enzymes, hence simulating the conditions of the industrial process and reducing the effect of hexose inhibition of cellulase activity. It should be stressed however that pre-fermented SSL was found to significantly inhibit hydrolysis and fermentation; hence, a kinetic penalty is associated with fortifying SSL with sugars via PCS hydrolysis *in situ*.

A novel system of simultaneous saccharification and extractive fermentation (SSEF) was developed using PCS as a substrate. It was found that ethanol production (relative to aqueous volume) from Tembec PCS could be increased by 50% through simultaneous *in-situ* liquid extraction of ethanol by oleyl alcohol. The benefit of ethanol extraction increased in proportion to the concentration of ethanol produced.

A model for SSF of PCS in Tembec's existing continuous fermentation was presented, and selected kinetic and yield parameters for use in with the model were obtained. The model remains in the early stages of development.
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1. INTRODUCTION

Considerable improvements have been made in technologies for the production of ethanol from lignocellulosic materials; however, several factors have hindered economic exploitation, including: low prices for conventional energy resources, high costs of dedicated biomass crops, and costly, multiple step biomass processing schemes which result in significant capital and operating expenditures (Coughlan, 1992; Ladisch and Svarczkopf, 1991; Lynd et al., 1991; Vallander and Eriksson, 1990). For this reason, the possibility of utilizing waste lignocellulosic material for ethanol production rather than dedicated biomass crops has been the subject of much research (Duff et al., 1995; Duff et al., 1994; Wayman, 1990).

Biomass is an abundant and renewable resource. However, conventional growth and harvesting techniques are responsible for a minimum cost of $40/t and $33/t for biomass from either conventional forests or from woody crops, respectively (Foody and Foody, 1991). While wood costs alone do not necessarily render biomass-to-ethanol schemes uneconomic, it is important to consider that fibre supply is also an issue. It is unlikely that two tonnes of wood which are suitable for the production of 1 tonne of Kraft pulp worth $700.00 will ever be directed instead to make $200-400 worth of fuel ethanol. As such, with the present (and possible future) fibre shortage in North America, any cellulose-to-ethanol industry must identify and use fibre which is not suitable for alternative uses. In the work described in this thesis, waste sludge from the primary clarifier of a low-yield sulphite pulping operation was used in the production of ethanol. This substrate has many advantages which will be described in greater detail in later sections of this report.

Regardless of the method chosen for ethanol production from biomass, the cellulose and hemicellulose fractions must first be hydrolyzed to produce fermentable sugars, which are
subsequently fermented by ethanol-producing yeast strains. Enzymatic hydrolysis using cellulases has the advantage over chemical methods of degradation (e.g. dilute acid hydrolysis) of being highly specific, and compatible with the fermentation process. High bioprocessing costs can be directly related to the recalcitrant problem of cellulase inhibition by cellobiose, and to a lesser extent glucose (a glucan), which reduces the rate of sugar production. One of the most promising technologies for addressing this problem is the simultaneous saccharification and fermentation (SSF) process. In SSF reactors, the conversion of liberated sugars to ethanol results in a significant kinetic advantage over separate hydrolysis and fermentation (SHF) reactions, due to reduced glucan inhibition. The advantages of the technology include the potential for use of low enzyme loading, single vessel processing, and reduced potential for microbial contamination. The most obvious disadvantage of SSF technology is the necessity of operating at temperatures well below the optimum for cellulases in order to meet the cultural requirements for growth of the fermentative organism. Another negative effect is the ethanol inhibition of cellulase activity, although this is not as significant as the effect of operating at a reduced temperature.

This thesis describes the efforts made to define optimum conditions to produce ethanol from pulp mill primary clarifier sludges using SSF technology. This includes the development of a kinetic model to predict the results of the implementation of the SSF process at the Tembec sulphite mill located in Témiscaming, Quebec. Throughout the work, the focus was on both the advantages afforded by, and limitations imposed through, implementation of the technology at Tembec's pulp and paper operation.
1.1. Pulp mill primary clarifier sludge (PCS) as a feedstock for ethanol production

1.1.1. Waste cellulosic material from the pulp and paper industry

The pulp and paper industry in Canada employs 70,000-80,000 people and its products account for approximately 3% of the total gross national product of the country (Sinclair, 1990). Balanced against this very positive contribution to the Canadian economy is the fact that the pulp and paper industry has been identified as a major contributor of pollutants to air and water (Murray and Richardson, 1993). New federal regulations were introduced in 1991 which set more stringent limits on effluent biochemical oxygen demand (BOD), total suspended solids (TSS), and toxicity. In response to these regulations, the Canadian pulp and paper industry has made dramatic strides in decreasing both the quantity and environmental impact of liquid effluents which it discharges to the receiving environment. These gains have been made through a combination of technologies which allow for reduced water consumption and improvements in primary and secondary treatment processes for plant effluents. As a by-product of the latter treatment processes, primary and secondary sludges are generated. The scale of many pulping operations means that significant quantities of these materials are produced; in the U.S., an estimated 2.1-12.2 million tonnes of combined primary and secondary sludge are produced annually (Anon, 1992; Anon 1988; Linderoth, 1989; Dowell, 1989). Handling and disposal of such quantities of sludge can represent upwards of 40% of the cost of operating a wastewater treatment system (Anon, 1988).

Approximately 60 kg of primary and 20 kg of secondary sludge are produced per tonne of pulp produced (Anon, 1992). The way in which excess sludge is handled and disposed of varies from mill to mill; however, dewatering followed by incineration or landfilling remain the preferred options. Landfilling costs have risen dramatically over the past decade. In 1986 disposal by landfilling was estimated to cost US$50-US$80/t exclusive of tipping fees. 
More recent estimates for landfilling costs place the figure for sludge disposal by this means at Can$100/t (McCubbin et al., 1992). In addition to high costs, increasingly limited space for landfilling and concerns about the environmental impact of sludge landfilling act to limit the future for this disposal method.

**Sludge combustion:** While usually not an option for mechanical pulp mills, when on-site combustion facilities exist, the 95% reduction in solid matter which can be achieved through incineration of combined waste primary and secondary sludges in hog fuel or power boilers makes this method of disposal an attractive alternative to landfilling. For a primary sludge, recovered at 3-5% solids, the cost of mechanically dewatering the sludge to a moisture content of 35-50%, conveying and combustion has been estimated to be in the range of US$29-$60/t (Anon, 1988). This cost does not include loss of boiler efficiency nor does it account for operational problems which can result from sludge burning. At some sites, the requirement for cofiring with conventional fuels can be reduced or eliminated by pre-drying the sludge with waste heat in the boiler flue gases, thereby improving the economic picture (Linderoth, 1989). However, the cost associated with such conventional sludge handling technologies, combined with concerns over emissions from mill boilers, continue to provide incentive for the development of improved solid waste handling and upgrading processes. Particularly attractive are those technologies which show a potential for a return in the form of salable products.

1.1.2. **Advantages of pulp mill primary clarifier sludge (PCS) as an ethanol feedstock**

Niche opportunities, such as those which exist in the pulp and paper industry, have a number of advantages over bioconversion schemes which use dedicated cellulosic energy crops. Firstly, lignocellulosics such as PCS have a very low or negative cost, as compared to biomass which is being produced specifically as an energy crop (Lonther, 1990). Secondly, solids captured in the primary clarifier have been through extensive mechanical and/or
chemical pretreatment. The result is that, for chemical pulping processes in particular, primary clarifier sludge has been pretreated to remove lignin and hemicellulose, yielding a sludge which has a higher proportion of cellulose and is much more amenable to bioconversion than the wood from which it was derived. Thirdly, hydrolysis is carried out in an aqueous slurry, removing the need for expensive, polymer-enhanced dewatering operations. Finally, association with a pulp mill can reduce operating costs through the provision of materials handling equipment, waste heat, and maintenance personnel. All of these factors combine to significantly reduce both the capital and operating costs as compared to a process for the conversion of wood to ethanol. In addition to these processing advantages, the environmental benefits of using PCS instead of wood include a reduced amount of waste material to be landfilled and the production of a proven, clean-burning motor fuel additive.

1.2. Biomass-to-ethanol production technologies

It is beyond the scope of this thesis to give a detailed review of the state of the art of biochemical technologies for the conversion of cellulose to ethanol. Instead, a very general overview will be provided, along with pertinent references to which the reader can refer for more detailed information.

1.2.1. The structure of biomass

The majority of biomass is comprised of lignocellulosic material. Although variation exists between species, wood is approximately 50% cellulose, 25% lignin, and 25% hemicellulose on a mass basis. The structures of cellulose and lignin are presented in Figure 1 (Smook, 1992). Chemical pulping processes remove most of the lignin and hemicellulose, and a small portion of the cellulose, from the wood chip furnish. The cellulose and hemicellulose fractions are hydrolyzed by cellulase enzymes to yield oligosaccharides and simple sugars.
Figure 1. Structures of cellulose and lignin (Smook, 1992)
Cellulose is a linear polymer of glucose units joined by β-1,4 glycosidic bonds. Due to the large number of hydroxyl groups situated on the superstructure of the molecule, hydrogen bonding occurs between adjacent strands. Consequently, individual strands group to form fibrils which are insoluble in water due to their large collective molecular weights. The fibrils themselves are enclosed in a hemicellulose-lignin matrix (Lehninger, 1978). A portion of cellulose, however, is amorphous, the result of the formation of side chains from the main linear structure.

Whereas glucose is the sole building block of cellulose, hemicelluloses are shorter-chain polymers built from five different sugars. Glucose, mannose and galactose are hexoses (contain six carbon atoms), while xylose and arabinose are pentoses (contain five carbon atoms). Lignin is a polymer of aromatic alcohols (Smook, 1992). In sulphite pulping, the lignin is removed from the cellulose fibres through sulphonation. The lignosulphonates are soluble in the cooking liquor; therefore, the solid cellulose is separated from the liquor via a process of washing and screening. It is common practice to burn the lignosulphonates in a recovery boiler to produce process steam and recover sulphur (as $\text{SO}_2$), as is done at the Tembec sulphite mill (Témiscaming, Quebec). This is accomplished by first increasing the total solids concentration of the red liquor from 10 to 50% (w/w) through evaporation and then combusting the (heavy) liquor.

**Primary clarifier sludge production:** PCS is primarily waste cellulose (derived from fibres which are discarded during pulp screening and cleaning processes), as well as undigested wood chips, sand and grit. The fibres are present in PCS due to screen inefficiencies (i.e. they are acceptable as pulp but are discarded nonetheless), and are nearly 100% carbohydrate. Tembec PCS also contains CTMP fibres which have retained most of the original hemicellulose and lignin. Sand and other foreign matter enter the mill with the
wood chips and must be removed from the raw pulp, and (in addition to bark) may enter from the effluent stream of the mill's debarking process if water is used for this purpose.

1.2.2. Enzymatic hydrolysis of cellulose

The first stage in the production of ethanol from biomass is the conversion of the cellulose and hemicellulose fractions to soluble sugars. It is advantageous to use enzymes to accomplish hydrolysis, since enzymes are highly specific catalysts, and the reaction matrix is amenable to yeast fermentation.

However, enzymes are expensive, and high bioprocessing costs can be directly related to the recalcitrant problem of cellulase inhibition by cellobiose, and to a lesser extent glucose, which reduces the rate of sugar production. One of the most promising technologies for addressing this problem is simultaneous saccharification and fermentation (SSF).

The conversion of cellulose to glucose is a two-step, enzyme-catalyzed reaction. Enzymes are proteins, which are large polymers of amino acids. They are highly specific biological catalysts, owing to their extensive tertiary structures which restrict the nature of their interactions with substrates. Each enzyme has both a binding region which enables adsorption to a substrate, and a region responsible for performing a specific function, for example, bond cleavage. The two active regions have structures which restrict activity to a specific task on a specific type of substrate. The lock and key analogy applies to substrates and enzymes. Cellulases, enzymes which degrade cellulose to glucose, are comprised of three distinct types of enzymes (Wald and Wilke 1984): (1) β-1,4-glucan glucanohydrolase (an endoglucanase), (2) β-1,4-glucan cellobiohydrolase (an exoglucanase) and (3) β-glucosidase. Wood and MacRae (1972) have proposed a model which describes the interdependence of the system of enzymes in the hydrolysis of cellulose. The following relation is a summary of the reaction:
The reaction occurs at the interface between the solid substrate and the aqueous bulk liquid. According to Wood and MacRae, both endo- and exoglucanases work in concert to degrade the cellulose strands to cellobiose, a glucose dimer, in the first stage of hydrolysis. Endoglucanases randomly attack the β-1,4-glycosidic bonds of the water-insoluble cellulose, yielding water-soluble oligosaccharides of varying molecular weights. Exoglucanases attack both the reducing and nonreducing ends of these intermediates, in the process yielding cellobiose. The conversion of cellobiose to glucose is achieved by the activity of β-glucosidase.

The rates at which the series of reactions progress are limited by the inhibition of enzyme activities as reaction products build up in the bulk liquid. Exo- and endoglucanases are inhibited by cellobiose accumulations. The activity of β-glucosidase is inhibited to a lesser extent by glucose (Grohmann, 1993).

The effect of enzyme loading, or concentration of enzyme activity relative to the mass of substrate, is shown quite clearly by the different sugar production rate curves in Figure 3 (Duff et al., 1995). The rate of hydrolysis and final conversion of the substrate (deinking sludge (DIS)) both increase with an increase in enzyme loading (the initial DIS concentration was 50 g/L in each case). This is a result of the high reactivity of cellulose to enzyme attack - increasing the surface concentration of enzymes (relative to the substrate) increases the number of interactions between enzymes and active sites. Currently the cost of enzymes (US$ 2665/Mg of cellulase (Lynd et al., 1991)) are such that economically viable
**Figure 3.** Effect of enzyme loading on hydrolysis of 50 g/L deinking sludge

FPU Cellulase/gram DIS: • 10; ○ 20; ▲ 30; ■ 40.
biomass conversion processes would be run at rates well below those which the reactivity of the cellulosic substrate would allow (Walker and Wilson, 1991).

**Effect of surfactant addition on hydrolysis:** It has been shown that the presence of a surfactant can significantly increase the rate of hydrolysis and final conversion of highly crystalline cellulose (Helle et al., 1993). It is possible that the reduction in interfacial energy caused by the adsorption of a surfactant increases the rate of production of new surface during hydrolysis, since the rate of hydrolysis is proportional to the number of active sites available on the cellulose surface. Another explanation is that the surfactant competes with cellobiose and glucose for enzymes on which to adsorb. By virtue of the surfactant's large size, inhibitory glucans are displaced from the immediate vicinity of the enzymes. This could also occur if surfactant adsorbed to the cellulose, thereby displacing sugars (but not enzymes) from the surface of the substrate where hydrolysis takes place.

The possibility of increasing the rate of PCS hydrolysis through the addition of a nonionic surfactant (Tween 80) will be explored in this thesis.

1.2.2.1. Hydrolysis kinetics

The rate equation for the enzymatic hydrolysis of cellulose can be determined from the hydrolysis mechanism itself. The following relation (Nakasaki, Murai and Akiyama, 1988), which can be fit to experimental data, is a simplified version of the actual mechanism:

\[
E + S \rightleftharpoons E \cdot S \rightleftharpoons E + G
\]

\[
E + G \rightleftharpoons E \cdot G
\]

The enzyme (E) binds to an active site (S) on the substrate, whereby the enzyme-substrate complex E·S is formed. S and E represent cellobiose and β-glucosidase, respectively.
Hydrolysis occurs and the complex breaks apart to yield the end product, glucose (G). This process involves (1) the diffusion of the enzyme from the bulk solution to the solid/liquid interface, (2) adsorption of the enzyme onto the active site, (3) chemical reaction (hydrolysis) and (4) diffusion of product and enzyme from the interface back to the bulk solution.

Also included in the mechanism (line 2) is the competitive inhibition of enzyme activity by glucose. The E·G complex forms when a glucose molecule adsorbs to a hydrolytically active site on an enzyme.

The mechanism is a greatly simplified representation of what actually occurs during hydrolysis. It considers only in the final stage of the process - the production of glucose from cellobiose. However, the accumulation of cellobiose also greatly inhibits further cellobiose production in a more realistic model of cellulose hydrolysis which includes feedback inhibition of glucanase. For our purposes, the more simplified model of hydrolysis serves as a convenience for applying experimental data to a rate law derived from the mechanism proposed by Nakasaki, Murai and Akiyama (1988).

The rate equation corresponding to the mechanism is:

\[
\frac{-dS}{dt} = a \frac{dG}{dt} = \frac{V_{\text{max}} S_n}{K_m + \frac{K_m^m}{K_{ig}} G_n + S_n}
\] 

Where \( t \) represents time and \( V_{\text{max}}, K_m \) and \( K_{ig} \) are constants; \( a = 0.909 \). The coefficient \( a \) accounts for the fact that one water molecule is consumed in the production of each molecule of glucose during hydrolysis. Equation (1) is the Monod equation, which is
applicable to substrate-limited systems, with an additional term in the denominator, \((K_m/K_{ig})\cdot G\), to account for competitive inhibition by glucose. \(V_{max}\) and \(K_m\) are determined from a Lineweaver-Burk plot (Figure 4, Aiba et al., 1973) of initial hydrolysis rate data (G=0):

To determine \(K_{ig}\), equation (1) is integrated:

\[
\frac{G}{t} = \frac{K_m(1 + K_{ig}S_o)}{1 - K_m/(K_{ig}\cdot a)} \cdot \frac{1}{t} \ln \left( \frac{S}{S_o} \right) + \frac{V_{max}}{1 - K_m/(K_{ig}\cdot a)} \tag{2}
\]

where \(S_o\) is the initial substrate (PCS) concentration. Hence plotting \(G/t\) versus \((1/t)\ln(S/S_o)\) (Foster-Niemann coordinates (Nakasaki et al., 1988)) will yield a linear relationship, whence \(K_{ig}\) is normally obtained from the slope.

Applying rate equations obtained from batch data in models of continuous systems:

Equation (1) is a general expression which can be used to determine the rate of cellulose hydrolysis, when the cellulose and reducing sugar concentrations are known. The constants are determined by analyzing hydrolysis data obtained from parallel runs having different initial substrate concentrations. The constants are valid for a specific initial level of enzyme loading. When derived from batch data, the results are directly applicable to continuous, plug flow reactors (PFR's) if ideal plug flow is assumed. As in batch reactors, all particles in plug flow have equivalent residence times; the same is true of enzyme molecules.

However, in continuous, stirred tank reactors (CSTR's), such as those employed in Tembec's fermentation system, both substrate and enzymes have a residence time distribution. Also, the substrate concentration and enzyme loading remain constant in a CSTR, whereas these parameters vary in batch reactors and PFR's. While it is beyond the scope of this thesis to completely account for these differences in developing a SSF model
Figure 4. Lineeweaver Burk plot of initial hydrolysis rate data (Aiba et al., 1973)
for a sequence of CSTR's, we will obtain a reasonable solution by calculating the average level of enzyme loading over the course of a batch reaction. This will be done in a later section on model development, which covers the results of experiments done using SSL as a reaction matrix. To obtain a more accurate expression for enzymatic hydrolysis, a continuous system must be used to extract model data.

1.2.2.2. Pretreatment of Lignocellulosic Substrates

The rate of hydrolysis of biomass is proportional to the extent to which the cellulose fraction of the material is accessible to enzymes. An enzyme molecule must be in physical contact with an active site on the cellulose structure for the cleavage of the glycosidic bond to occur. Normally the liquid/solid mixture is maintained as a homogeneous slurry through continuous agitation. Mass transfer effects such as diffusion rates of enzymes to the active sites on the substrate, and products from the substrate/bulk liquid interface to the bulk liquid are not considered to have as great an effect on the rate of hydrolysis as does the availability of substrate (Duff, personal communication). The rate of hydrolysis would not increase with an increase in available substrate (enzyme dosage constant) in a diffusion-limited system.

Since the cellulose in untreated biomass is encased in a recalcitrant hemicellulose/lignin matrix, it must undergo an initial processing step prior to its enzymatic conversion to hydrolysis sugars. The most effective pretreatments make use of chemicals; dilute-acid prehydrolysis is a commonly used process by which all of the lignin and hemicellulose are dissolved and separated from the insoluble cellulose fraction.

The primary clarifier sludge obtained from the Tembec pulp mill in Témiscaming, Quebec had already undergone a chemical pretreatment (acidic \((\text{NH}_4)_2\text{SO}_3/\text{H}_2\text{SO}_3\) digestion) whereby most of the hemicellulose and lignin was degraded and removed with the liquor.
This feature, in addition to those already mentioned, makes PCS an ideal candidate for a feedstock in the production of ethanol. The substrate can be fed to the ethanol production system without additional pretreatment steps.

1.2.3. Ethanol production via fermentation

Yeasts are the most commonly used microorganisms for the production of ethanol from saccharides (Atkinson and Mavituna, 1983). The high selectivity of all *Saccharomyces* strains has led to their widespread use in industrial fermentations. The anaerobic fermentation of ethanol from hexose (e.g. glucose) is represented by the following equation:

\[
C_6H_{12}O_6 \xrightarrow{\text{yeast cells, nutrients}} 2 \cdot CH_3CH_2OH + 2 \cdot CO_2 \uparrow + \text{yeast cells}
\]

The theoretical (stoichiometric) yield is 0.51 g ethanol and 0.49 g CO\(_2\)/g glucose fermented (neglecting the substrate consumed in the production of yeast cells). Due to the large number of metabolic pathways in the production of ethanol and CO\(_2\) from hexoses (e.g. glucose), other products such as glycerol, acetic and formic acids are also produced. However, ethanol conversion efficiencies as high as 89% of the theoretical have been obtained using *Saccharomyces cerevisiae* (Atkinson and Mavituna, 1983).

1.2.3.1. Cell growth

It is commonly understood that, under many conditions, the transport of fermentable sugars is the rate determining step in the consumption of sugars by yeast cells in the production of additional yeast cells and ethanol (Sols, 1961).

During the initial stages of a batch fermentation, fermentable sugars are consumed in the production of yeast cells as a viable population is propagated. During this stage the dissolved oxygen level in the aqueous broth is high enough to favour yeast production,
which occurs in an oxygen-rich environment (Duff, personal communication). The production of cells via oxidative pathways requires large amounts of oxygen - it has been found that an equal weight of oxygen is required to produce a given weight of yeast on a dry basis (Maxon and Johnson, 1953). As long as the substrate (sugar) is in excess, the cell growth rate is proportional to the number of cells present, i.e. an exponential growth rate ensues. This is often referred to as the logarithmic growth phase. The cell growth rate reaches a limiting value as the enzymes which enable the oxidative pathways (e.g. carboxylase) become saturated with sugars (Harrison, 1963). The specific cell growth rate, \( \mu \), is defined as:

\[
\mu = \frac{1}{X} \frac{dX}{dt} \quad \text{(h}^{-1}\text{)}
\]

where \( X \) is the concentration of yeast cells (g/L). There are many empirical expressions for the specific logarithmic growth rate which have been developed. One such relationship combines the \textit{Monod equation}, which applies to substrate-limited systems, with an additional term to account for the reduction of yeast activity by ethanol (Erickson and Fung, 1988):

\[
\mu = \mu_{\text{max}} \left( \frac{G}{K_s + G} \right) \left( 1 - \frac{P}{P_{\text{max}}} \right)^m \quad \text{(h}^{-1}\text{)}
\]

where \( \mu_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\)), \( P \) is the ethanol concentration (g/L), \( P_{\text{max}} \) is the ethanol concentration at which yeast metabolism ceases (g/L); \( m \) (unitless) and \( K_s \) (g/L) are constants.

Following the initial logarithmic growth phase, the cell population reaches a maximum level, beyond which the rate of cell multiplication equals the rate of endogenous cell decay. This
is known as the *stationary phase* of cell growth. No significant change in cell mass occurs over this interval. The time of the onset of stationary growth, as well as the maximum cell mass concentration, are functions of substrate availability as well as oxygen and ethanol levels in the reaction matrix.

As the original substrate becomes depleted, the rate of endogenous cell decay eventually surpasses the rate of cell multiplication, whereupon the cell population enters the final stage of growth known as the *death phase*.

**Yeast inhibition:** *S. cerevisiae* is inhibited by the presence of alcohols (e.g. ethanol and methanol) and fatty acids (e.g. acetic and formic acids). Both acids and alcohols interfere with membrane functions. Alcohols also inhibit cytoplasmic enzymes, and interact with the yeast cell at the genetic level. Since the undissociated forms of organic acids are particularly toxic to cell growth and cell metabolism, the fermentation matrix is maintained (above pH 5) where dissociation occurs to a lesser degree (Erickson and Fung, 1988).

Spent sulphite liquor also exerts an inhibitory effect on cell growth and metabolism (Yu and Wayman, 1985). This is due in part to the acetic acid present. Also, the presence of sulphite greatly increases the yield of glycerol (Wilkinson and Rose, 1963), the formation of which is represented by the formula:

\[
\text{Glucose} + \text{SO}_2^- \rightarrow \text{Glycerol} + \text{Acetaldehyde} \cdot \text{SO}_3^- + \text{CO}_2
\]

**Cell recycle:** Most continuous industrial fermentation processes utilize a system of cell recycle. This allows for a portion of the cells which would otherwise be removed with the product stream to be concentrated and added to the feed stream. Figure 5 is a schematic of Tembec's continuous fermentation system which makes use of cell recycle technology. The
Figure 5. Tembec's continuous fermentation system
use of cell recycle allows for higher cell concentrations and hence greater fermentation rates than would otherwise be possible. There should be an upper limit to the fraction of cells exiting the system which may be recycled, which would be determined by the maximum number of younger (more active) cells which could be maintained in the system (or more specifically, the maximum average activity of the cell population, which has an age distribution).

1.2.3.2. Ethanol fermentation

As the yeast cell count reaches a certain level, the production of ethanol rather than cells is favoured as CO₂, a by-product of cell metabolism, displaces much of the oxygen originally present in the broth. The fraction of sugar consumed in the production of ethanol, rather than in the production of yeast, can be increased by maintaining a low level of dissolved oxygen after the required cell population has been produced. Low substrate concentrations also favour the production of ethanol. At glucose concentrations between 3 and 100 g/L, the oxidative pathways that enable yeast growth are repressed to a degree, which permits the production of ethanol in the presence of oxygen (Atkinson and Mavituna, 1983). However, yeast growth will occur as long as oxygen is present.

In modeling the behaviour of a continuous fermentation system such as the one employed by Tembec, the rate of ethanol production is related to the rate of cell production by means of a yield coefficient, \( Y_{P/X} \) (g ethanol produced/g cells produced). This relationship does not apply to the overall yield in batch systems where the coefficient varies with cell concentration, as well as with sugar and ethanol concentration. However, over short intervals, \( Y_{P/X} \), can be determined from batch fermentations. Care must be taken to note the concentrations of reactants and products, and the phase of cell growth.
The rate of ethanol production in a continuous system can be expressed as:

\[
\left( \frac{dP}{dt} \right)_{\text{production}} = \mu XY_{P/X} \quad \text{(gL}^{-1}\text{h}^{-1})
\]

The modeling of Tembec's fermentation system will be discussed in a following section.

1.2.4. Simultaneous hydrolysis and fermentation (SSF)

Simultaneous hydrolysis (saccharification) and fermentation is, as the name implies, a single-step process in which the enzymatic hydrolysis and alcoholic fermentation are carried out simultaneously in the same vessel (Philippidis et al., 1993). Depending on the concentration of reactants used, typical SSF reactions are run for 7 days at 35 - 37°C with crude cellulase, pretreated lignocellulose, and an ethanologenic microorganism, such as Saccharomyces cerevisiae, or Zymomonas mobilis. As sugars are released, that fraction which is fermentable using the particular organism involved is immediately taken up by the fermentative microbe, and ethanol is produced. In batch reactions, fermentation is the rate limiting step initially, as the rate of sugar production is high and the yeast cell population is low. As the cell population attains a maximum, the opposite conditions prevail and the rate of ethanol production is typically determined by the rate of hydrolysis. Since ethanol is a much less potent inhibitor of cellulase than is cellobiose or glucose, the reaction rate is increased or, alternatively, lower enzyme loading can be used to accomplish the same conversion in the same time as would be required were hydrolysis and fermentation accomplished separately.

SSF reaction systems depend on an adequate β-glucosidase activity to ensure that cellobiose is hydrolyzed to the less inhibitory (and more fermentable) glucose. Many fungal cellulases are deficient in β-glucosidase, and if such an unsupplemented crude cellulase preparation is used in a hydrolysis reactor, it is possible for cellobiose to accumulate. In experimental
work, β-glucosidase is generally added in excess to ensure cellobiose conversion. Rather than augment the cellulase with additional β-glucosidase, one group of researchers has conducted SSF using two yeasts, *S. cerevisiae* and the cellobiose-fermenting yeast *Brettanomyces clausenii*. Using this approach, they found that ethanol yield was improved over that attained in a monoculture. Using a coculture they achieved an 88% sugar yield from a 10% cellulose substrate, which in turn was fermented to give 4.5% ethanol (Wyman *et al.*, 1986). Higher ethanol concentrations in batch systems can be attained by SSF if substrate is fed sequentially.

Although promising, a number of problems remain to be addressed before SSF technology progresses to the stage of industrial application. The major disadvantage of SSF is that it is conducted at a temperature which is a compromise between the optima for fermentation (20 - 33°C, depending on the microbe) and hydrolysis (45 - 50°C). As a result, neither fermentation nor hydrolysis occurs at an optimum rate, necessitating incubation periods of up to 7 days, and concomitantly large reaction vessels. On the positive side, reduced thermal denaturation of cellulases should occur at the lower temperature, thereby allowing more efficient use of cellulase enzymes. It would be useful to carry out a kinetic study to determine the relative benefits of high versus low temperature operation.

Few fermentative yeasts survive well at temperatures in excess of 35°C, and it is unlikely that stable thermotolerant yeast strains will be developed. The optimum temperature for *Z. mobilis* wild type is 30°C; however, there are reports of strains capable of fermentation at 45°C (Lee *et al.*, 1981). Also, the genes encoding *Z. mobilis* ethanol production have been expressed in *Escherichia coli* (Ohta *et al.*, 1991). Accordingly, integration and expression of these ethanologenic genes may be possible in an acid-tolerant thermophilic microbe, and this microbe applied to SSF.
Another potential disadvantage of SSF is the high potential for microbial contamination. Steam explosion may sterilize the cellulosic substrate; however, microbes are reintroduced during the subsequent, and necessary, water wash. When crude cellulase is added to the hydrolysis mixture, it brings with it nutrients and metabolites from the *T. reesei* growth medium, in addition to fungal spores and pieces of mycelium. A readily available sugar substrate, plus long-term incubation at 35 - 37°C is conducive to the competitive proliferation of contaminants. Selective inhibitors could be added to the SSF process, but this would entail additional costs. It may be possible to discourage contamination and conduct a successful long-term SSF at 50°C, pH 4.8, under an anaerobic headspace, if an acid-tolerant, thermophilic, high-yield ethanologenic anaerobe can be either isolated or genetically developed. It should be noted that the potential contamination hazard is no more acute for SSF than for any other fermentation process, nor is it any more acute that it would be for separated hydrolysis and fermentation operations.

Another possible disadvantage of SSF is the mild inhibitory or denaturing effect that ethanol has on cellulases. While this ethanol effect is not nearly as pronounced as cellobiose end-product inhibition, the rate of cellulose hydrolysis does slow as ethanol accumulates (Holtzapple *et al.*, 1990; Abe and Takagi, 1991). While the effect of accumulation of ethanol on further production of the metabolite by yeast may be more important than the inhibitory effect of ethanol on cellulases, it is clear that the development of a technology which would allow for continuous ethanol removal would speed the commercialization of SSF technologies for the production of ethanol from biomass.

In SSF reactors, the conversion of liberated sugars to ethanol results in a significant kinetic advantage due to reduced end-product inhibition. The advantages of the technology include the potential for use of low enzyme loading, single vessel processing, and reduced potential for microbial contamination. The most obvious disadvantage of SSF technology is the
necessity of operating at temperatures well below the optimum for cellulases in order to meet the cultural requirements for growth of the fermentative organism. Simultaneous hydrolysis and fermentation has been the subject of intense research and development, with most recent work focusing on the use of recombinant bacteria for the metabolism of oligomeric and five carbon sugars (Ingram and Doran, 1995; Katzen and Fowler, 1994), agricultural residues (Grohmann, 1993), and on modeling and reactor development (Philippidis et al., 1994; South and Lynd, 1994).

1.2.5. Integrated fermentation and product recovery
Technologies for the conversion of lignocellulose to ethanol generally result in the production of fermentation broths that contain moderate to low ethanol concentrations. One of the major deterrents to the large-scale production of fuel ethanol is the high cost of dilute product recovery. The separation and concentration of ethanol to a fuel-grade product is an energy-intensive operation. The traditional recovery process consists of separation of the ethanol from the fermentation broth and concentration to 95% by distillation. This is followed by an azeotropic distillation step, with the addition of benzene, to dehydrate the ethanol to 99%. The total energy input for these two distillation steps is 7.63 MJ/L of fuel-grade ethanol (Dzenis and McNab, 1984). For a fermentation broth containing approximately 9% ethanol, distillation cost can represent 40 to 60% of total plant energy consumption, and it becomes progressively less economical to recover ethanol at concentrations below 6% (Dzenis and McNab, 1984; Cysewski and Wilke, 1978).

A number of studies have focused on the development of novel ethanol extraction systems for recovery of dilute ethanol streams at low cost. In addition, techniques have been developed for the continuous removal of ethanol from fermentation systems. In these studies the ethanol concentration in the broth was kept at a low level to prevent end-product inhibition. In this manner high glucose to ethanol productivities could be maintained in a
continuous culture. Generally, these latter techniques also can be applied to low-cost dilute ethanol recovery.

Cysewski and Wilke (1977) showed that fermenter ethanol productivities could be increased 12-fold by conducting a continuous fermentation under a vacuum (50 mmHg). The vacuum fermenter eliminated ethanol inhibition by continuously vaporizing ethanol as it was formed at a fermentation temperature of 35°C. Similarly, the application of a vacuum to a broth containing a low ethanol concentration would allow ethanol recovery at a reduced cost. Vacuum fermentation does, however, have the disadvantage of concentrating nonvolatile compounds to inhibitory levels. Also, the trace oxygen requirement of the fermenting yeast must be supplied by sparging the broth with oxygen, as the solubility of oxygen in the broth under a continuous vacuum is somewhat lower than the level under atmospheric pressure.

A variety of membrane technologies have been developed for the selective removal of ethanol from fermentation broths. These include pervaporation (Groot et al., 1984), perstraction (Matsumura and Markl, 1986), and membrane distillation (Udriot et al., 1989). Pervaporation can be used when the components of a liquid mixture exhibit different diffusivities in a membrane. In an ethanol-water mixture, ethanol selectively diffuses through the membrane and is removed by a gas stream or vacuum on the other side. The ethanol is then condensed on a chilled surface and collected (Groot et al., 1984).

Perstraction is very similar in principle, except the membrane separates the fermentation broth from an organic solvent. The ethanol is preferentially soluble in the organic solvent and crosses the membrane. The membrane barrier ensures the separation of the aqueous and organic solvent phases. This separation allows the selection of an organic solvent which has a high ethanol extraction coefficient, but which might be quite toxic to the fermenting yeast. In these systems the ethanol is usually recovered from the organic solvent in a flash.
vaporization unit or by passing the solvent through a selective packed bed column for ethanol adsorption (Matsumura and Markl, 1986).

In membrane distillation, a hydrophobic porous membrane separates two aqueous solutions. The fermentation broth is kept at a higher temperature than the extraction water on the other side. This causes a vapour pressure gradient and the selective mass transfer of ethanol across the membrane as a vapour. Since the receiving water is cooler, ethanol can accumulate to a higher concentration than present in the broth. This is a form of distillation at temperatures below the boiling point of the components to be separated, with the membrane pores forming the gaseous phase (Udriot et al., 1989).

Supercritical CO₂ extraction of ethanol also has been accomplished. Fermenter pressurization and addition of supercritical CO₂ to the reactor resulted in inhibition of the fermentation (Thibault et al., 1987). However, it was shown that a broth stream could be pressurized and passed countercurrent to the CO₂ stream, through a supercritical extraction column, and then recycled to the fermenter. Ethanol could then be removed from the supercritical fluid by passage through an activated carbon bed, and recovered with a second CO₂ stream. This process has a number of advantages over distillation: (1) solvent loss is not a critical concern as CO₂ is a by-product of the fermentation process, (2) ethanol inhibition of the fermentation is reduced, and (3) it requires 50% less energy than conventional distillation.

A biological system has been developed for the upgrading of dilute ethanol streams to acetaldehyde for low-temperature recovery (Murray et al., 1990). This system uses the methylotrophic yeast Pichia pastoris. When grown on methanol, the highly active alcohol oxidase enzyme pathway is synthesized. Alcohol oxidase is relatively nonspecific and can oxidize a variety of short chain primary alcohols, while the second enzyme in the pathway,
formaldehyde dehydrogenase, is specific for formaldehyde. Accordingly, P. pastoris grown on methanol can be used to oxidize ethanol to acetaldehyde, which accumulates, since it can not be oxidized further (to acetic acid).

Methanol-grown P. pastoris cells were successfully used for the conversion of quite dilute ethanol concentrations to acetaldehyde. A 1.8% ethanol solution was oxidized to acetaldehyde at 95% efficiency, and at 100% of theoretical yield. The alcohol oxidase enzyme system was psychrotolerant, and only showed a 32.5% decrease in activity when the bioconversion temperature was lowered from 30 to 3°C. This ability to carry out the bioconversion over a wide temperature range is very important, since heating and cooling costs are of critical importance. In an industrial setting, ambient temperature can vary widely, and flexibility in operating temperatures can result in substantial savings.

As an alternative fermentation product, acetaldehyde is more volatile, with a boiling point of 20.8°C, as compared to 78.5°C for ethanol. With a heat of vaporization of 30.41 kJ/mol, acetaldehyde requires less energy to distill than ethanol, which has a heat of vaporization of 40.48 kJ/mol (Weast, 1987). Unlike ethanol, acetaldehyde does not form an azeotrope with water. Therefore, acetaldehyde is easily separated from mesophilic fermentation broths. Murray et al. (1990) found that acetaldehyde readily evaporated from fermentation broths at 22°C, and offered an attractive alternative to the expense of ethanol distillation. An additional advantage to producing acetaldehyde is its much greater market value as a commodity chemical (Anon, 1989). However, if the desired end product is ethanol, then the acetaldehyde can be readily reduced to ethanol using either nickel or copper oxide catalysts (Hagemayer, 1978).
1.2.5.1. Extractive fermentation:

Extractive fermentations, perhaps, have been the most studied means of dilute ethanol recovery. These fermentations are conducted as two-phase systems, and consist of the aqueous fermentation broth and an immiscible organic solvent. Solvent extraction followed by separation of solute from solvent via distillation is generally the most inexpensive method of separation in dilute aqueous systems, due to the high heat of vaporization of water relative to other solvents (Treybal, 1987). It is important that the correct organic solvent be chosen. The organic solvent must be insoluble in water and non-toxic to the yeast biocatalyst. In addition, the extraction coefficient of the organic solvent must be sufficiently high, such that ethanol selectively partitions into it (Park et al., 1988; Ruiz et al., 1987; Roddy, 1981).

Daugulis et al., (1987) conducted an extractive ethanol fermentation in a 7 litre fermenter where commercially available oleyl alcohol was used as the extractive solvent. The extractive solvent was introduced as the discontinuous phase through the bottom of the reactor via a diffuser, and individual droplets floated to the top to form a solvent layer. The fermentation broth was mixed at 150 rpm, a speed that gave adequate mixing while the quiescent upper solvent layer was maintained. The organic solvent was withdrawn from the top for product recovery and recycle. Extracted ethanol was recovered from the organic solvent in a flash vaporization unit (80 kPa, 75°C), thereby regenerating the solvent for recycle. In this continuous system, 300 g L⁻¹ of glucose were converted to ethanol at a 96% efficiency. Ethanol recovery from the flash vaporization unit operated at 90% efficiency, and contained between 65 - 85% (by weight) ethanol, with the remainder being water. To date, there have been no reports of the successful combination of extractive fermentation and cellulose hydrolysis.
The simultaneous saccharification and extractive fermentation (SSEF) process in this thesis involves the application of extractive fermentation technology to the SSF process. Extractive fermentation, as the name implies, is a conventional fermentation which in addition uses an immiscible solvent (extractant) to continuously remove the solute (ethanol) from the fermentation matrix (broth) as it is produced. To achieve this, interfacial contact between the broth and the extractant must occur - the duration of phase contact in continuous systems is determined by (1) the extractant flowrate, (2) broth flowrate and (3) the residence time of each phase in the contactor. In conventional industrial processes the broth flowrate is set to meet a required production rate; hence, if ethanol extraction were implemented, the extractant flowrate would be set according to (1) the broth flowrate, (2) the desired ethanol concentration in the broth, i.e. the percent recovery of ethanol, (3) the partitioning of ethanol between phases, (4) the rate of interfacial ethanol diffusion and (5) the interfacial area within the phase contactor. A schematic of the SSEF process is presented in Figure 6.

It is desirable, but not necessary, for ethanol to have a greater affinity for the extractant than for the broth. In fact, it is highly unlikely that ethanol would be preferentially absorbed by an extractant (which is by definition immiscible with water), since ethanol is completely miscible with water (ethanol and water are very similar compounds - both have the structure \( R-OH \), where \( R = H \) for water and \( CH_3CH_2 \) for ethanol). However, ethanol is soluble in a number of extractants which are also biocompatible with fermentation systems. These extractants (e.g. oleyl alcohol) have been used successfully in laboratory-scale extractive fermentations (Daugulis, 1987).

**Partition coefficient:** The parameter which indicates the degree to which a solute distributes itself between phases, when both phases are in physical contact and at equilibrium, is known as the partition coefficient. It is one of the parameters used in
Figure 6. Schematic of the SSEF system
determining the minimum ratio of extractant to feed solvent flowrate used in a
countercurrent extraction process, such as a packed column. Mass ratio (Bancroft)
coordinates are commonly used to plot equilibrium data when determining the partition
coefficient of a particular two-phase, three-component system. The coordinate system
ensures a linear equilibrium curve over a range of low solute concentrations, as expressed by
the following equation (Perry and Green, 1984):

\[ K' = \frac{\gamma}{\chi} \]  

(6)

where \( K' \) = the partition coefficient of the solute
\( \gamma \) = the weight ratio of solute (e.g. ethanol) to solute-free extractant (oleyl
alcohol) in the extractant phase
\( \chi \) = the weight ratio of solute to solute-free raffinate (e.g. broth) in the raffinate
phase

For a given system, at least \( 1/K' \) kg of extractant would be required to remove all the
ethanol from 1 kg of raffinate (Perry and Green, 1984).

1.2.6. Model development of SSF technology applied to Tembec's fermentation
system

In order to facilitate the development of a rational process design for implementation of SSF
technology at Tembec, a standard model for 3 continuous stirred tank reactors (CSTR's) in
series was employed. Kinetic parameters can be used to characterize the rates of production
and consumption of cells, reducing sugars, PCS and ethanol in Tembec's fermentation
system. Modeling of sugar uptake, ethanol production, and yeast cell yield in Tembec's
continuous fermenters was carried out using the method of Aiba et al. (1973). The model is
of the form:
1. Yeast cells: 
\[ \frac{dX_n}{dt} = D(X_{n-1} - X_n) + \mu_n X_n \]  

\[ (7) \]

2. Reducing sugars:
\[ \frac{dG_n}{dt} = D(G_{n-1} - G_n) - \left( \frac{dG_n}{dt} \right)_{\text{consumption}} + \left( \frac{dG_n}{dt} \right)_{\text{production}} \]  

\[ (8) \]

3. Ethanol:
\[ \frac{dP_n}{dt} = D(P_{n-1} - P_n) + Y_{p/X} \mu_n X_n \]  

\[ (9) \]

4. PCS:
\[ \frac{dS_n}{dt} = D(S_{n-1} - S_n) - \left( \frac{dS_n}{dt} \right)_{\text{consumption}} \]  

\[ (10) \]

In these equations,

\[ X_n = \text{the yeast cell concentration in reactor } n \text{ (g/L)} \]

\[ G_n = \text{the reducing sugar concentration in reactor } n \text{ (g/L)} \]

\[ P_n = \text{the ethanol concentration in reactor } n \text{ (g/L)} \]

\[ S_n = \text{the PCS concentration in reactor } n \text{ (g/L)} \]

\[ D = \text{the dilution rate (h}^{-1}) \]

\[ \mu_n = \text{the specific cell growth rate (h}^{-1}) \]

\[ Y_{p/X} = \text{the ethanol yield coefficient relative to cell yield (unitless)} \]

The sign convention in the above equations is that the rates subscripted \textit{production} and \textit{consumption} are positive. As previously mentioned,

\[ \mu_n = \mu_{\text{max}} \left( \frac{G_n}{K_s + G_n} \right) \left( 1 - \frac{P_n}{P_{\text{max}}} \right)^n \]  

\[ (4) \]
The term \( Y_{P/X} \) is the ethanol yield coefficient for cell growth \( \frac{\text{ethanol produced (g)}}{\text{yeast cells produced (g)}} \).

In equation (8),

\[
\frac{dG_n}{dt}_{\text{consumption}} = \frac{Y_{P/X}}{Y_{P/G}} \frac{\mu_n}{\mu_p} X_n
\]

In addition, it can be seen that

\[
\frac{dG_n}{dt}_{\text{production}} = \frac{dG_n}{dt}_{\text{consumption}}
\]

where

\[
d\left( \frac{dG_n}{dt} \right)_{\text{production}} = \frac{V_{max}S_n}{K_m + \frac{K_m}{K_i} G_n + S_n}
\]

Ideal mixing is assumed \( (D = \text{constant} = \nu/V) \), where \( V = \text{tank volume and } \nu = \text{volumetric flowrate} \).

Equations (4) and (7-13) can be solved simultaneously for each of the three CSTR's in Tembec's continuous process.

**Limitations of the model:** The purpose of the model is to provide a preliminary indication of the amount of ethanol which can be produced via the SSF process, in excess of the amount which is currently produced by SSL fermentation alone. Based on this information, one should be able to determine whether further study at the pilot plant level is warranted. The results should not be used in place of those obtained from pilot plant trials, since certain physical phenomena, which can only be studied using a larger scale continuous process, have not been considered. For example, ideal mixing has been assumed \( (D = \text{constant}) \), when in fact deviations from ideal mixing normally occur (Fogler, 1991). Also, the yeast cell balance will certainly be affected by the deposition of cells onto the PCS; this may also reduce the access of enzymes to the substrate. Finally, the effects (on hydrolysis) of thermal denaturing of enzymes, and irreversible enzyme adsorption to PCS, have not been separated from the effects of PCS depletion and enzyme inhibition by reducing sugars. However, the
results obtained are more than sufficient to determine the merits of carrying the SSF process to the pilot plant stage.

2. RESEARCH OBJECTIVES

The fundamental objective of the research related to this thesis is to optimize the conditions for the production of ethanol from sulphite primary clarifier sludge. Our efforts in this regard can be subdivided into three streams. The first considers the characteristics of the enzymatic hydrolysis of PCS and the fermentation of hydrolysis sugars. This includes the study of each reaction separately as well as the an examination of the SSF process.

The second body of work carries the SSF process further to include a solvent extraction step. The promise of improved ethanol production rates and simplified product recovery led to our examination of the SSEF process. Application of extractive fermentation technology to the simultaneous hydrolysis and fermentation of PCS has not been attempted before, and one of our goals was to determine whether it was possible to do so.

The third stream is dedicated to the development of a mathematical model of Tembec's existing continuous SSL fermentation system, adapted to run in SSF mode. Experiments using SSL as a reaction matrix have been designed to obtain the necessary kinetic and stoichiometric parameters for use in the model. The model is meant to predict the increase in ethanol production that is possible by introducing simultaneous PCS hydrolysis to the current fermentation system. The results can be used to determine whether work should progress to the pilot plant level.
3. EXPERIMENTAL

3.1. MATERIALS

3.1.1. Substrates and enzymes

Primary clarifier sludge (PCS) was obtained from Tembec (Témiscaming, Quebec) and was supplied as approximately 10 mass % solids from the mill. Solka floc (James River Corporation, Berlin, NH), was used as the substrate (pure, amorphous cellulose) in experiments designed to determine the efficacy of carrying out SSEF at very low aqueous phase volumes, and as a standard in some PCS hydrolysis reactions. Sigma Cell 50 (Sigma, St. Louis, MO) was used in some hydrolysis experiments as a source of pure, crystalline cellulose. Cellulase enzymes derived from *Trichoderma reesei* were obtained from Novo-Nordisk (Denmark) as Celluclast CCN #1.5L (78 FPU/mL glucanase activity) and Novozym TN #188 (780 IU/mL β-glucosidase activity).

3.1.2. Yeast culture

The yeast strain *Saccharomyces cerevisiae* was used in all experiments. This was obtained directly from Tembec's SSL fermentation system.

3.1.3. Spent sulphite pulping liquor

Spent sulphite liquor (SSL) was obtained from Tembec (Témiscaming, Quebec) and was supplied as 20 mass % solids. This is the concentration of SSL which is currently fermented by the mill, and referred to in this thesis as "100%" SSL. The pH of the SSL was raised from 1-1.5 as received to 5.3 using 30% ammonium hydroxide (Fisher Scientific, Nepean, ON) before hydrolysis or fermentation.
3.1.4. Organic solvents for SSEF experiments

Oleyl alcohol was provided by Henley Chemicals Ltd. (Concord, ON) under the trade name ADOL® 85 NF. Oleic acid was provided by Fisher Scientific (Fair Lawn, NJ).

3.2. METHODS

3.2.1. Hydrolysis experiments

3.2.1.1. Hydrolyses in buffered solution:

Reactions were performed in 125 mL Erlenmeyer flasks (50 mL liquid volume), in a 0.2 M sodium acetate buffer solution (pH 4.8). The solution also contained 0.1% sodium azide (Fisher Scientific, Fair Lawn, NJ) as a biocide. The flasks were placed in a shaker (New Brunswick Scientific Co., NJ), in which the hydrolyses were performed at 50°C, at a rate of agitation of 150 rpm. In each flask, 0.2% of a nonionic surfactant was used (Tween 80, Sigma) to ensure that an adequate amount of surface active agent was present in each system. The sludge, buffer solution, and surfactant were combined and allowed to reach an isothermal equilibrium under reaction conditions for 1 h. Hydrolyses were initiated by the addition of enzymes (β-glucosidase followed by glucanase). Unless otherwise specified, the substrate level in all flasks was 50 g PCS/L. Also, the enzyme loading was 10 FPU of glucanase and 100 IU of β-glucosidase activity per gram of PCS in all hydrolyses and SSF runs, except where noted. PCS, SSL and enzymes were stored at 4°C until use. Duplicate flasks were run in hydrolysis experiments (and in all other experiments, except where noted).

Effect of hydrolysis on PCS dewaterability: To determine the effect of enzymatic hydrolysis on dewaterability of the residual sludge, samples of sludge were removed after various hydrolysis times and centrifuged at a fixed speed (3500 rpm) and duration (5 min) in a centrifuge (Model CU-5000, IEC Ltd., MA).
Effect of xylose on hydrolysis of Sigma Cell 50: Each flask contained 50 g Sigma Cell 50/L at an enzyme loading of 5 FPU glucanase and 50 IU β-glucosidase. Xylose levels of 0 g/L (as a control) and 46 g/L were present in parallel runs.

3.2.1.2. Hydrolysis experiments in spent sulphite liquor
Reactions were performed in 250 mL Erlenmeyer flasks (100 mL liquid volume) in media containing SSL of various concentrations up to full strength. To mimic the continuous fermentation system at Tembec in which steady state sugar concentrations are low, the SSL was prefermented to remove hexoses (and the resulting ethanol was removed by flash vaporization). Unless otherwise specified, 50 g PCS/L and 10 FPU/g enzymes were used. The flasks were incubated in a rotary shaker (New Brunswick Scientific Co., New Jersey) at 33 and 37°C, and at 150 rpm.

3.2.2. Fermentation, SSF and SSEF experiments
The conditions for fermentation and SSF experiments were nearly identical to those under which hydrolyses in SSL were performed. As was the case in hydrolysis experiments, all runs were carried out in attrition bioreactors. Whereas shake flasks were used for all hydrolyses, some fermentation, SSF and SSEF experiments were also conducted using a bench-scale fermenter with a working volume of 10 L.

3.2.2.1. Fermentation and SSF experiments in non-SSL medium
The growth medium was prepared using distilled water. Experiments were carried out at temperatures of 33 and 37°C, in a growth medium comprised of 0.6% ammonium sulphate (Anachemia Chemicals, Toronto, ON), 1.742% potassium phosphate dibasic (Fisher Scientific, Fair Lawn, NJ)), 0.695% citric acid (Fisher Scientific, Fair Lawn, NJ), 0.1% yeast extract (Difco Laboratories, Detroit, MI) and 0.2% Tween 80 in distilled water.
In addition, 1 mL/L of trace element solution and 2 mL/L of vitamin solution (Duff et al., 1994) were added to the growth medium. The final solution was pH 5.0-5.1.

Saccharomyces cerevisiae inoculum was grown for 24 h prior to its use, in growth medium containing 1% glucose (Fisher Scientific, Fair Lawn, NJ) as a carbon source. SSF and fermentation runs were initiated via the addition of 20 vol-% inoculum solution to the flasks containing various amounts of PCS and enzymes.

Aseptic conditions were maintained during all fermentation and SSF experiments; therefore, the reaction media and sludge were autoclaved separately at 121°C for 30 min prior to the addition of medium to the flask containing sludge. The filter-sterilized vitamin solution was then added to the sterile reaction medium. Sterilized pipettes were used to withdraw samples, and the flasks were flame-sterilized before and after sampling.

3.2.2.2. Fermentation and SSF experiments in SSL, shake flask scale
To initiate a fermentation, 37 vol-% inoculum solution was added to flasks containing unfermented SSL in various concentrations including full strength (the solvent for the inoculum solution was distilled water). Prior to its use, Saccharomyces cerevisiae inoculum for fermentation and SSF experiments was grown for 24 hours in an enriched media consisting of 5% glucose, 2.5% peptone (ICN Biomedicals, Aurora OH) and 0.3% yeast extract, in addition to trace elements and vitamins (Duff et al., 1994). The growth medium was 2.5% peptone and 0.3% yeast extract in SSL. For SSF reactions, the SSL was fortified with various concentrations of PCS, and cellulase enzymes were added at the same time as the yeast inoculum. Unless otherwise noted, all runs were performed at 33°C. All runs were carried out at pH 5.3.
3.2.2.3. SSF experiments, fermenter scale

The inability to add fresh substrate limited SSF reactions at the shake flask scale. To overcome this limitation, a series of SSF reactions were carried out at 37°C in a BioFlo IV 20-L fermenter (New Brunswick Scientific Co., NJ), which was operated in batch and fed-batch modes. All reactions were performed using a working volume of 10 L of non-SSL medium. The initial mixing rate was 300 rpm, which was decreased to 150 rpm as the viscosity of the slurry decreased as a result of PCS hydrolysis. Two clusters of shear-type blades provided the mixing required. An intrinsic steam sterilization system ensured aseptic conditions in the reactor during both normal operation and sampling. Both pH and dissolved oxygen were monitored during runs; the latter variable was controlled through the sparging of sterilized air.

3.2.2.4. SSEF experiments, shake flask scale

Batch experiments with the use of an immiscible extracting solvent (oleyl alcohol, or oleic acid, where noted) in situ were carried out in 500 mL Erlenmeyer shake flasks in the same manner as SSF runs in non-SSL medium. Both phases were sampled.

The preparation was identical to that used for simultaneous hydrolysis and fermentation experiments without extraction, except for the addition of the organic solvent. The reaction temperature was 37°C. The organic phases were autoclaved separately and added to the flasks after the addition of enzymes. As with the aqueous phase, the organic phase was centrifuged to separate the three main components: sludge, aqueous broth and solvent. To maintain the degree of agitation in the aqueous fermentation broth at the intensity that was applied to the SSF experiments, the shaker speed was set at 225 rpm (compared with 150 rpm for the fermentation runs).
3.2.2.5. **SSEF experiments, fermenter-scale**

To improve upon the results obtained by performing a fed-batch SSF run in the BioFlo IV fermenter, the experiment was repeated, with the introduction of an ethanol extracting solvent (oleyl alcohol) *in situ*. The operating conditions that existed during the SSF run were duplicated for the SSEF run. The total working volume was 10 L of non-SSL medium. This included both phases; the aqueous phase volume was 1.67 L, while the volume of oleyl alcohol was 8.33 L (1:5 fermentation medium:extractant ratio).

3.2.3. **Water-Ethanol-Oleyl Alcohol partition coefficient**

Solutions of various concentrations of ethanol in distilled water were prepared in 20 mL vials. Equal volumes of oleyl alcohol were added to the aqueous solutions, and the vials were immediately capped. The total volume in each vial was 20 mL. This minimized the amount of head space in the vials, which would impact on the results. The vials were then heated to 37°C in a shaker (New Brunswick Scientific Co.) under quiescent conditions for 30 min. The vials were then vigorously shaken by hand for 15 s and allowed to settle to reform two distinct phases. This procedure was repeated a total of 5 times over a period of 1 h. Samples of each phase were then analyzed.

3.3. **ANALYSES**

3.3.1. **Sampling protocol, all experiments**

Representative 1.4 mL samples were withdrawn and centrifuged (13000 g, 5 min) using a Fisher micro-centrifuge (Model 235C). The supernatant liquids were then placed in capped 2 mL vials and frozen for later analysis.
3.3.2. Reducing sugars assay

The concentration of total reducing sugars in each sample was determined colorimetrically using a dinitrosalicylic acid (DNS) reagent (Miller, 1959). Three mL of an appropriately diluted sample was combined with an equal amount of DNS reagent in a sealed 10 mL HACH test tube and placed in boiling water for 15 min.

An HACH single-beam spectrophotometer (Model DR/2000) was used to read the absorbances of the coloured solutions. The calibration curve (using glucose as a standard) for use with the method was found to have a linear dynamic range 0.1-0.3 g/L at a wavelength of 575 nm. The error in using the method was due primarily to the method itself rather than the result of error in sample preparation, and was estimated to be ±15%.

3.3.3. Ethanol in aqueous solution

A Hewlett-Packard Gas Chromatograph (5880A Series) equipped with a flame ionization detector (FID) and a Carbowax 20M packed column (Supelco Inc., Bellefonte, PA) was used to determine the ethanol concentrations of the aqueous samples. The injected volume was 1 μL. External standards ranging from 0.1-1.0 g/L ethanol and containing 1.5 g/L isopropanol as an internal standard were used. The oven temperature was operated isothermally at 70°C; the injection port and detector temperatures were 160 and 200°C, respectively.

3.3.4. Ethanol in oleyl alcohol and oleic acid

For analysis of ethanol in oleyl alcohol and oleic acid, a SPB™-5 column (Supelco, Inc.) was used. The oven temperature was increased from an initial value of 70°C to a final value of 310°C at a rate of 45°C/min. Samples were diluted 1:1 with n-octanol to lower their viscosity; the internal standard was 8 g n-butanol/L. The standards ranged from 1-25 g ethanol/L. The identical method was used in a following experiment to determine the
ethanol content of oleic acid, where external standards were prepared in the same manner. In both cases, the error associated with the assay was estimated as being ±7%, due primarily to the error involved in the preparation of the samples for analysis.

4. RESULTS AND DISCUSSION

4.1. Experiments in non-SSL medium

4.1.1. Hydrolyses
Before conducting hydrolyses in SSL, a number of experiments were performed using distilled water rather than SSL as a solvent. These experiments were designed to study the nature of the substrate (PCS), and how variables such as PCS moisture content, enzyme loading and surfactant concentration would affect hydrolysis kinetics and conversion of PCS to reducing sugars. Besides providing a basis for further work using an SSL medium, the experiments provided some answers regarding the enzymatic hydrolysis of PCS in general. Experiments which were used to determine maximum PCS conversion, the effect of hydrolysis on PCS dewaterability, and the effect of initial moisture content (of PCS added to the system) on hydrolysis were useful in determining the suitability of the substrate for SSF in SSL.

4.1.1.1. Determination of maximum conversion of PCS to total reducing sugars (TRS)
To determine the fractions of cellulose and hemicellulose in PCS accessible to hydrolytic enzymes, extreme conditions were applied to the substrate. An enzyme loading of 100 FPU/g PCS was used. In addition, a low (10 g/L) initial PCS concentration served to minimize the effect of end product inhibition of cellulase activity. As shown in Figure 7,
Figure 7. Maximum conversion of PCS to reducing sugars.

- Initial enzyme loading: 100 FPU/g PCS
- Initial substrate concentration: 10 g PCS/L
after 24 h the hydrolysis had reached the point of completion. The maximum possible conversion of PCS to reducing sugars was 66.4%. The remaining 33.6% was likely comprised of cellulose and hemicellulose inaccessible to enzymes (e.g. in undigested wood chips, CTMP fibre and bark), lignin, extractives (e.g. turpenes), sand and other inorganic matter. The value for the degradable fraction of PCS obtained in this experiment was used in developing the SSF model, where the concentration of PCS is based on that which can be converted to reducing sugars via hydrolysis.

4.1.1.2. Hydrolysis of PCS of different moisture contents

It is advantageous to remove as much water from the sludge prior to hydrolysis and fermentation as is possible, since this would lower the liquid load on the downstream ethanol distillation system. However, both the surface and internal hydrogen bonding characteristics of the substrate, which influence enzymatic hydrolysis, are altered by the removal of water. It is possible that the removal of water results in more intimate contact between hydroxyl groups on adjacent cellulose fibrils, thereby reducing the number of available hydrolytic sites. The extent to which the PCS could be dehydrated without significantly decreasing its ability to be hydrolyzed was determined in this experiment.

PCS was air-dried to various moisture contents, rehydrated in acetate buffer (to 50 g PCS/L), and subjected to cellulase hydrolysis. The final (44 h) conversions are presented in Table 1. The initial PCS concentration and enzyme loading was 10 g/L and 100 FPU/g PCS, respectively.
Table 1  Effect of initial moisture content on the 44 h conversion of PCS to TRS

<table>
<thead>
<tr>
<th>Initial moisture content (% solids)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>42.3</td>
</tr>
<tr>
<td>28</td>
<td>42.7</td>
</tr>
<tr>
<td>40</td>
<td>48.6</td>
</tr>
<tr>
<td>46</td>
<td>48.5</td>
</tr>
<tr>
<td>61</td>
<td>43.8</td>
</tr>
<tr>
<td>93</td>
<td>34.6</td>
</tr>
</tbody>
</table>

No relationship between moisture content and extent of hydrolysis existed between PCS having moisture levels within the range of 23 and 61% solids; however, beyond this range, moisture content exerted a significant effect. A 21% reduction in conversion was obtained by drying sludge from 61 to 93% solids. As the PCS loses water, it possibly becomes more difficult for enzymes to obtain access to the surfaces of the fibrils, as the hydrogen bonds must be broken for this to occur. Since all PCS samples were rehydrated to equal solids concentrations, it is evident that the increased level of hydrogen bonding is permanent.

The results have shown that PCS can be dried to solids contents as high as 61% and used in a SSF process, thereby minimizing the amount of additional water added to the process with the substrate. Since the effect of drying is noticeable only between solids levels of 61 and 93%, additional work should be done to determine at what point (between 61 and 93% solids) drying has a significant effect on substrate conversion.
4.1.1.3. Effect of PCS extract on hydrolysis of Sigma Cell 50

As received from the mill, the PCS had a sharp odour characteristic of free SO₂. It was necessary to determine if the SO₂ or other species present in the aqueous fraction of the PCS slurry inhibited the hydrolytic enzymes. The results would indicate whether a PCS washing stage prior to hydrolysis would be warranted.

Parallel hydrolyses were performed using 100 g Sigma Cell 50/L, and 5 FPU/g enzymes, both in 0.2 mols/L acetate buffer, and in a 0.2 mols/L acetate buffer solution prepared using the aqueous fraction of the PCS rather than distilled water. Enzymes were added to a PCS extract solution, in which no cellulose was added, as a control.

The type of medium had no effect on the hydrolysis of Sigma Cell 50 (Figure 8), hence it was concluded that a washing step would not be required prior to the enzymatic hydrolysis of Tembec PCS.

4.1.1.4. Effect of surfactant addition on PCS hydrolysis

A series of hydrolyses of PCS were conducted in which the concentration of a nonionic surfactant, Tween 80 (a polyoxyethylene molecule), was varied. Figure 9 shows the sugar production rate over 24 h for each level of surfactant.

Apart from variations caused by experimental error, no significant modification of hydrolysis kinetics or final conversion of PCS resulted from the presence of Tween 80 (polyoxyethylene (20) sorbitan mono-oleate). It is possible that PCS, which contains products of incomplete delignification and lignin redeposition, may also contain natural surfactants (e.g. lignosulphonates, extractives) such that additional surfactants are unnecessary (Duff, personal communication). It is also possible that the cellulose fraction of PCS is predominantly amorphous and therefore easily hydrolyzed, unlike the more
Figure 8. Effect of medium on hydrolysis of Sigma Cell 50

Figure 9. Effect of surfactant (Tween 80) addition on hydrolysis of PCS
crystalline form of cellulose (Sigma Cell 50) used by Helle et al (1993). In this study, the presence of a surfactant significantly increased the rate and extent of hydrolysis.

4.1.1.5. Effect of enzyme loading on hydrolysis of PCS

Parallel hydrolyses of PCS were conducted at different levels of enzyme loading. The sugar production curves are shown in Figure 10; their main features are presented in Table 2.

It is evident that an increase in enzyme loading results in an increase in the rate of sugar production as well as in an increase in final sugar concentration. However, the specific initial (and overall) hydrolysis rate, or hydrolysis rate per FPU, decreases as the level of enzyme loading increases. This is the result of the limited number of active sites on the PCS surface which are accessible to enzymes; as the enzyme dosage is increased, the surface approaches the point of saturation. The upper bound to the final sugar concentration indicates that there is a limit, based on the accessible cellulose fraction, to which the substrate can be converted to sugars.

The results indicate that, as is the case with many industrial processes involving catalysts, there must be a compromise between production rate and turnover (number of catalytic reactions per catalytic site expended). Optimization is not a task that can be avoided as research is carried to a larger scale.

4.1.1.6. Effect of hydrolysis on dewaterability of PCS

Whether disposing of excess sludge by incineration or landfilling, it is desirable to dewater it to the greatest extent possible (Metcalf and Eddy, 1991). Many forms of sludge reduction such as biological digestion are able to reduce the mass of solids requiring disposal by up to 50%; however, the residual solids are more difficult to dewater, particularly aerobically digested sludges (Metcalf and Eddy, 1991). Dewaterability of the PCS was markedly
Figure 10. Effect of enzyme loading on hydrolysis of PCS

Table 2. Effect of enzyme loading on hydrolysis of Tembec PCS

<table>
<thead>
<tr>
<th>Enzyme loading (FPU/g PCS)</th>
<th>Final (48) h sugar concentration (g/L)</th>
<th>Initial rate of hydrolysis (g/(L·h))</th>
<th>Specific initial hydrolysis rate (g/(L·h·FPU))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>18.6</td>
<td>2.1</td>
<td>1.07</td>
</tr>
<tr>
<td>5</td>
<td>27.7</td>
<td>4.8</td>
<td>0.95</td>
</tr>
<tr>
<td>7</td>
<td>30.3</td>
<td>6.6</td>
<td>0.94</td>
</tr>
<tr>
<td>10</td>
<td>31.5</td>
<td>7.9</td>
<td>0.79</td>
</tr>
</tbody>
</table>
enhanced through hydrolysis (Figure 11), notably during the first 2 h of the reaction. Over 27 h, the dewaterability increased by 56%. This improvement in water removal would result in a reduction in the use of dewatering chemicals and/or sludge of a higher heating value if it is to be incinerated in the power boiler. For sludge destined for the landfill, transportation costs would decrease, since the weight of the sludge would be reduced.

4.1.1.7. Effect of xylose on hydrolysis of Sigma Cell 50

An accumulation of nonfermentable sugars was observed during the hydrolysis of PCS, possibly pentose sugars. To determine whether these products inhibit enzyme activity, crystalline cellulose (Sigma Cell 50) was hydrolyzed in the presence of xylose (a pentose) and in the absence of xylose (as a control). The initial cellulose concentration was 50 g/L. The results are shown in Figure 12.

Xylose was found to inhibit hydrolysis to a degree, although in this experiment its level was much higher than that which would occur during PCS hydrolysis, due to the low amount of hemicellulose present in the substrate, from which pentose sugars are derived. However, higher levels of pentoses are present in SSL, especially when a hardwood furnish is used (since hardwoods contain a greater fraction of hemicellulose than do softwoods). Therefore SSL may significantly inhibit hydrolysis in this instance.

4.1.1.8. Hydrolysis kinetics: hydrolysis of different concentrations of PCS

Hydrolyses of various concentrations of PCS in (non-SSL) acetate buffer were performed (Figure 13). An expression for the hydrolysis rate equation was obtained by fitting the data to equations (1) and (2). As a means by which to overcome scatter in the data, the first 4 h of hydrolysis were considered in determining the initial hydrolysis rates corresponding to different substrate concentrations - i.e. the average rate of hydrolysis over the first 4 h of the
Figure 11. Effect of hydrolysis on dewaterability of Tembec PCS

Figure 12. Effect of xylose on the hydrolysis of Sigma Cell 50
reaction was taken as the initial hydrolysis rate. The initial rate data are presented in Table 3.

The initial hydrolysis data were plotted using Lineweaver-Burk coordinates to yield values for $V_{max}$ and $K_m$ of 3.4 g L$^{-1}$ h$^{-1}$ and 16.8 g/L, respectively. A linear relationship was obtained ($R^2 = 0.999$). The third and final constant, $K_{ig}$, was found by plotting the data on a Foster-Niemann coordinate system. For $S_0 = 50$ g/L, $K_{ig}$ was found to be 12.6 g L$^{-1}$ h$^{-1}$ ($R^2 = 0.836$). Hence the hydrolysis rate law (equation (1)) becomes:

$$\frac{dS}{dt} = \frac{3.4S}{16.8 + 13G + S} \text{ (g·L}$^{-1}$·h$^{-1}$)$$

(14)

It is evident that glucose inhibition assumes a significant role in determining the rate of hydrolysis. For example, with an initial PCS concentration of 50 g/L, a 28% reduction in the initial rate of hydrolysis (from 2.5 to 1.8 g·L$^{-1}$·h$^{-1}$) would result from increasing the initial glucose concentration from 0 to 20 g/L. Equation (14) is valid for batch and continuous plug flow systems, but must be modified for continuous systems where stirred tank reactors are employed.

4.1.2. Fermentation experiments

Preliminary experiments were undertaken to characterize the effects of variables (temperature, ethanol concentration) that exist in a fermentation process such as the system employed by Tembec. Once these effects were determined, work was allowed to proceed on fermentations in the much harsher environment of Tembec SSL. Experiments using PCS-derived wood sugars were useful in determining the yields which could be obtained using this substrate, in the absence of SSL-based wood sugars which would interfere with the results.
Figure 13. Hydrolysis of PCS of different concentrations

Table 3. Initial hydrolysis rates at different initial PCS concentrations in acetate buffer

<table>
<thead>
<tr>
<th>Initial PCS concentration (g/L)</th>
<th>Initial hydrolysis rate (g L⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.61</td>
</tr>
<tr>
<td>20</td>
<td>1.07</td>
</tr>
<tr>
<td>35</td>
<td>1.66</td>
</tr>
<tr>
<td>50</td>
<td>2.05</td>
</tr>
</tbody>
</table>
4.1.2.1. Effect of temperature on the fermentation of wood sugars

The operation of an industrial process is rarely isothermal; rather, a temperature range based on daily and yearly ambient temperature patterns exists. Since pulp mills are limited in their cooling capacities, they invariably operate their liquid effluent treatment systems at higher than optimal temperatures during the summer months. For this reason, it was necessary to determine the results of conducting a fermentation at a temperature higher than the optimum for *Saccharomyces cerevisiae* yeast (33°C (Erickson et al., 1988)). Two parallel trials were performed using PCS-derived wood sugars, one at 33°C, and the other at 37°C.

Temperature was shown not to have a significant effect on either ethanol production or yeast growth within the temperature range tested (Figure 14). Hence an industrial fermentation process, for example Tembec's continuous SSL fermentation system, would have a certain level of flexibility in its control of temperature.

4.1.2.2. Effect of initial ethanol concentration on the fermentation of wood sugars

Solutions containing initial wood sugar concentrations of 100 g/L and varying initial ethanol concentrations were fermented at 33°C. The aim was to determine the level at which ethanol has a significant inhibitory effect on the fermentative ability of the yeast. Over a range of initial ethanol concentrations of 0 to 28 g/L, no significant relationship between initial ethanol concentration and the conversion of sugars to ethanol and CO₂ after 70 h was evident (Table 4).

Despite the absence of a distinct correlation between initial ethanol concentration and conversion, perhaps due to the limited number of levels (3) of initial ethanol concentration, one would expect conversion to increase to a maximum, followed by a decline. The maximum would correspond to the highest initial ethanol concentration at which all the sugars are consumed. Since ethanol represses the oxidative pathways responsible for yeast
Figure 14. Effect of temperature on the fermentation of PCS-derived wood sugars
growth, the least amount of yeast should be produced at the highest ethanol concentration; therefore, the greatest fraction of sugar would be available for the production of ethanol. In this experiment, the highest conversion of sugar to ethanol was obtained when 28 g/L, rather than 0 g/L, of ethanol was present initially, which is in agreement with this (accepted) theory (Atkinson and Mavituna, 1983). This range of initial ethanol concentrations (0 - 28 g/L) is similar to that normally achieved in the series of CSTR's in Tembec's SSL fermentation process, which produces 12 g ethanol/L as a final product during hardwood runs, compared with 20 g ethanol/L from softwood-derived SSL. Therefore our experimental range should encompass that which could be obtained by fortifying Tembec's present system via PCS hydrolysis.

The rates of ethanol fermentation, at initial ethanol concentrations greater than 28 g/L decreased dramatically, to the point where negligible amounts of ethanol were produced over a period of 70 h (Figure 15). Beyond an initial ethanol concentration of 53 g/L, the yeast exhibited no significant fermentative capacity. The results obtained agree with those of others who have worked with \textit{S. cerevisiae} (Erickson \textit{et al.}, 1988).

### 4.1.3. Simultaneous saccharification and fermentation (SSF)

Tembec PCS proved to be highly amenable to hydrolysis, and in separate experiments as much as 66\% of the wood sugars produced were converted to ethanol and CO\textsubscript{2}. The low yield is most likely due to the high levels of nonfermentible sugars present in the TRS (e.g. xylose and complex sugars) and the production of other byproducts such as glycerol. However, the result was reasonable given the feedstock, and it was concluded that PCS would be a good a feedstock for ethanol production. Since the accumulation of reducing sugars in the batch reactors was found to inhibit hydrolytic activity, SSF experiments were conducted to reduce the levels of these products during reactions. From the results it would
Table 4. Effect of initial ethanol concentration on conversion of wood sugars to ethanol

<table>
<thead>
<tr>
<th>Initial ethanol concentration (g/L)</th>
<th>Conversion after 70 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>11</td>
<td>55</td>
</tr>
<tr>
<td>28</td>
<td>66</td>
</tr>
</tbody>
</table>

Figure 15. Effect of initial ethanol concentration on fermentation of wood sugars
be possible to determine the kinetic benefit the SSF process would have, if any, over the SHF process.

4.1.3.1. Effect of substrate concentration and enzyme loading on SSF of PCS

PCS from Tembec's sulphite pulping process proved highly amenable to SSF to produce ethanol (Figure 16). In all SSF runs, a transient peak of reducing sugars was observed at the onset of the SSF reaction. The size of the initial sugar peak was a function of substrate concentration and enzyme loading. Peak reducing sugar concentrations varied from a low of approximately 3 g/L in flasks with an enzyme loading of 5 FPU/g and 50 g/L PCS, to 18 g/L in flasks with an enzyme loading of 10 FPU/g and 100 g/L PCS. The appearance of this peak of sugar indicated that, at the enzyme loadings tested, an imbalance existed between the rate of production of sugars by the enzymes and the rate of conversion of these sugars to ethanol by the yeast. Because end-product sugars are well known inhibitors of cellulase activity, the presence of substantial quantities of hydrolysis sugars in the reactor would serve to reduce the effectiveness of the enzymes present. Since the cellulase enzymes represent most of the cost of biomass-to-ethanol processes, it would be highly desirable to eliminate such losses in enzyme efficiency.

Efforts to reduce the size of the initial sugar peak by doubling the inoculum of yeast produced little effect. However, there are two observations which should be made concerning the sugar peak produced in the reactor system employed. First, these initial experiments were carried out in batch reactors and a lag in yeast growth was experienced which would not normally be observed in continuous cultures (particularly those employing cell recycle). Second, in continuous or semi-continuous cultures, substrate could be fed at such a rate as to ensure that the level of reducing sugar would not be inhibitory to cellulase (Duff et al., 1994).
Figure 16. Effect of enzyme loading and PCS concentration on SSF of PCS
4.1.3.2. Effect of enzyme loading on ethanol production in the SSF of PCS

Parallel SSF runs were performed at different levels of enzyme loading. The initial PCS concentration in all runs was 100 g/L. The reducing sugar and ethanol production curves are presented in Figure 17. The conversions of PCS corresponding to the different levels of enzyme loading are listed in Table 5.

The conversion is based on the fraction of substrate converted to both ethanol and carbon dioxide. By the stoichiometry of the conversion of glucose to ethanol and CO$_2$, 51% of the substrate by weight is theoretically converted to ethanol (neglecting substrate consumed in cell growth and in the production of other fermentation products such as glycerol). In reality it is not possible to obtain as high a conversion from hydrolysis (wood) sugars, as a portion of the glucose is consumed in the production of new yeast cells, and pentose sugars (e.g. xylose) are not fermented by *S. cerevisiae*.

It is evident that beyond a level of enzyme loading of 5 FPU/g PCS, substantial increases in ethanol concentration and PCS conversion were not obtained. It can also be seen that the ethanol conversion per FPU/g PCS was inversely related to enzyme loading (Table 5). This trend with respect to TRS also exists in hydrolysis experiments (Figure 10). Since TRS inhibition of enzyme activity should be insignificant during SSF experiments, the decreased benefits to increases in enzyme dosage is most likely due to the depletion of active enzyme sites over time. This should be true for both hydrolysis and SSF reactions, judging from the similar production curves.

Recall that the maximum conversion of wood sugars to ethanol and carbon dioxide observed was 66% (Table 4). A possible explanation for the low yield, compared to that which is theoretically possible with a glucose substrate, is the presence of nonfermentible sugars (pentoses and complex sugars). It was determined in an earlier experiment (Figure 7) that
Figure 17. Effect of enzyme loading on SSF of PCS

Table 5. Effect of enzyme loading on conversion of PCS to ethanol

<table>
<thead>
<tr>
<th>Enzyme loading (FPU/g PCS)</th>
<th>Final (74 h) ethanol concentration (g/L)</th>
<th>Conversion of PCS to ethanol (%)</th>
<th>PCS conversion per unit enzyme loading (%/(FPU/g PCS))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.1</td>
<td>15.9</td>
<td>15.9</td>
</tr>
<tr>
<td>3</td>
<td>11.8</td>
<td>23.1</td>
<td>7.70</td>
</tr>
<tr>
<td>5</td>
<td>17.6</td>
<td>34.5</td>
<td>6.90</td>
</tr>
<tr>
<td>10</td>
<td>18.8</td>
<td>36.9</td>
<td>3.69</td>
</tr>
</tbody>
</table>
the maximum conversion of PCS to reducing sugars was 66.4%. Therefore the conversion of PCS to ethanol should be no greater than 44% (66% of 66.4%). At an enzyme loading of 10 FPU/g PCS, the conversion (36.9%, Table 5) approaches this upper value. However, using half the enzymes a conversion only marginally lower (than that corresponding to the higher enzyme loading) was obtained. The optimal use of enzymes in a complete PCS-to-fuel ethanol process is dependent upon the cost of enzymes and the cost of dilute product recovery. Advancements in enzyme production technology may result in the more liberal use of enzymes in the future; at present the cost of enzymes for biomass-derived ethanol has been estimated as being US$0.10/L (Chahal and Chahal, 1995).

4.1.3.3. SSF of PCS in the BioFlo IV bench-scale fermenter
The SSF runs were scaled up to the 20 litre fermenter scale to address two objectives. First, we wished to ascertain whether fed-batch addition of PCS could be used to increase the concentration of ethanol beyond what was observed in shake flasks. Second, by not adding fresh enzymes throughout the run, we hoped to be able to determine the upper time limit of enzyme activity. The initial PCS concentration was 50 g/L, with subsequent 25 g/L additions of substrate.

A number of observations can be made from the results of the fermenter run (Figures 18a and 18b). First, note that when ethanol production is expressed in concentration terms (Figure 18a), the impact of addition of water along with sludge is visible.

Second, despite the fact that no new enzyme was added throughout the run, hydrolysis continued to occur through 4 substrate feeding cycles. This is tantamount to having a reactor in which cellulase enzymes are recycled until exhaustion, although it is possible that some cellulase components remained active throughout the experiment. The determination of the activities of the different types of enzymes was beyond the scope of the study.
Figure 18. SSF of Tembec PCS in the BioFlo IV bench scale fermenter. Ethanol expressed in (a) concentration (g/L) and (b) mass (g) units.
Third, it appears as though addition of fresh substrate will cause an increase in the rate of ethanol production, despite the fact that, for example at the end of cycle 3 (100 h), unhydrolyzed PCS was still present. This seems to indicate that cellulase enzymes will preferentially attack the fresh substrate rather than the residual material from the previous run. It is possible that the enzymes, which continuously adsorb and desorb from active hydrolytic sites on the substrate, naturally concentrate on the surface of the fresh substrate, which has a much greater concentration of active sites than has the older substrate.

Fourth, the incremental increase in sugar production decreased with each subsequent addition of sludge, which indicated that there was a time dependent loss of enzyme activity occurring. Our data are not capable of distinguishing the mechanism responsible for this loss of activity; however, it is likely due to a combination of interfacial and shear denaturation, and irreversible binding of cellulase to inactive sites on residual solids.

Finally, it is interesting to note from these data that, despite the fact the substrate specific ethanol yield \( Y_{P/S} \) is decreasing with each incremental cycle, the amount of ethanol produced per unit of enzyme added \( Y_{P/E} \) increased over the course of the SSF run (Table 6). Since cellulase enzymes are a major component of the cost of such a process, it may be advantageous to operate the SSF reactor in such a way that PCS is added until enzyme activity is exhausted. This is particularly true when the substrate is a low-cost waste material such as PCS. In such a case, the reduction in enzyme cost must be balanced against the increased capital and operating costs associated with the larger reactor required to achieve the same ethanol production rate (as that achieved using a higher level of enzyme loading).
4.1.4. Simultaneous saccharification and extractive fermentation (SSEF)
The results from the SSF runs showed that PCS hydrolysis and ethanol fermentation could be sustained in a common reactor. The advantages of this configuration over a system of separate hydrolysis and fermentation are (1) a reduction in enzyme inhibition by hydrolysis sugars and (2) the elimination of a second reactor. However, the result of SSF is a dilute product which must undergo an energy-intensive (and therefore costly) distillation step.

Extractive fermentation is a technology which holds much promise for product separation from dilute mixtures. It is currently the most economical way to achieve separation in dilute systems (Treybal, 1987), although this has not been proven industrially in the case of dilute ethanol-water mixtures, such as those resulting from SSF. However Daugulis (1987) has had much success in using oleyl alcohol as an ethanol extractant in ethanol fermentations on a pilot-plant scale, in which a much more concentrated product (>80%) was produced. Our goal was to further research in this area by applying the technology to SSF.

4.1.4.1. Partition coefficient of ethanol in Water-Ethanol-Oleyl alcohol (WEO) systems

The most important quality of an ethanol extractant is its ability to remove ethanol from the fermentation medium. The partition coefficient $K'$ is a measure of this quality. An experiment was performed to determine the partition coefficient of ethanol in the WEO system for a range of concentrations which could possibly arise in an extractive fermentation system using PCS as a substrate. The results could be used to determine the minimum extractant (oleyl alcohol) flowrate required to achieve a desired separation of ethanol from the aqueous phase.
The equilibrium ethanol concentrations in the water and oleyl alcohol phases which were brought into physical contact with each other are presented in Table 7. Included are the corresponding values of $\gamma$ and $\chi$ (in Bancroft coordinates (Perry and Green, 1984)), where:

\[
\gamma = \text{the weight ratio of ethanol to oleyl alcohol in the oleyl alcohol phase}
\]
\[
\chi = \text{the weight ratio of ethanol to water in the water phase}
\]

The relationship between $\gamma$ and $\chi$ has been plotted (Figure 19). It is well understood that over a range of low solute concentrations, the equilibrium curve will be linear when expressed in terms of Bancroft coordinates (Perry and Green, 1984). This is in agreement with the results obtained.

The slope of the equilibrium curve, $K' = \gamma/\chi$ (partition coefficient), was found to be $0.275 \pm 0.041$ (95%). It is one of the parameters used in determining the minimum flow ratio of extractant (oleyl alcohol) to feed solvent (fermentation broth) used in a continuous, countercurrent extraction process, such as a packed column. Since $K'$ for this system (@ 35°C) was found to be 0.275, at least $1/0.275$ or 3.6 kg of oleyl alcohol would be required to remove all the ethanol from 1 kg of water (on an ethanol-free basis).

It would appear that oleyl alcohol is a poor extractor of ethanol, by conventional liquid-liquid extraction standards. However, ease of ethanol recovery and low extractant volatility (and therefore extractant longevity), in addition to extractant biocompatibility with the SSF process, are qualities oleyl alcohol has which offset the penalty of the low partition coefficient. It has been shown (Daugulis, 1987) that ethanol can be recovered by passing the extracting solvent stream through a single flash drum operated under relatively mild conditions (80 kPa, 75°C). Under these conditions, 80% ethanol was recovered as the top product from a flash separator. When recovering ethanol from dilute streams, the larger amount of the liquid load is borne by the bottom section of the distillation column, since the
Table 6. Ethanol production during fed-batch SSF of PCS (fermenter-scale)

<table>
<thead>
<tr>
<th>Cycle</th>
<th>PCS* (g/L)</th>
<th>Enzyme Loading (FPU/g)</th>
<th>Incremental [Ethanol] (g/L)</th>
<th>(Y_{P/S}) (g/g)</th>
<th>(Y_{P/E}) (g/(FPU/g))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>10</td>
<td>10.6</td>
<td>0.21</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>6.7</td>
<td>6.1</td>
<td>0.24</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>5.0</td>
<td>3.9</td>
<td>0.16</td>
<td>0.04</td>
</tr>
<tr>
<td>4</td>
<td>125</td>
<td>4.0</td>
<td>2.6</td>
<td>0.10</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Total amount of PCS added since start of experiment, based on initial reactor volume

Table 7. Water-Oleyl Alcohol-Ethanol equilibrium data

<table>
<thead>
<tr>
<th>Ethanol concentrations (g/L)</th>
<th>(Y)</th>
<th>(\chi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous phase</td>
<td>Organic phase</td>
<td></td>
</tr>
<tr>
<td>8.2</td>
<td>2.1</td>
<td>0.0025</td>
</tr>
<tr>
<td>10.7</td>
<td>2.4</td>
<td>0.0028</td>
</tr>
<tr>
<td>11.2</td>
<td>2.9</td>
<td>0.0035</td>
</tr>
<tr>
<td>12.7</td>
<td>2.9</td>
<td>0.0034</td>
</tr>
<tr>
<td>14.6</td>
<td>2.9</td>
<td>0.0034</td>
</tr>
<tr>
<td>14.8</td>
<td>3.4</td>
<td>0.0040</td>
</tr>
<tr>
<td>19.6</td>
<td>4.7</td>
<td>0.0055</td>
</tr>
<tr>
<td>22.8</td>
<td>6.4</td>
<td>0.0076</td>
</tr>
<tr>
<td>41.7</td>
<td>8.8</td>
<td>0.0104</td>
</tr>
<tr>
<td>60.1</td>
<td>14.5</td>
<td>0.0171</td>
</tr>
</tbody>
</table>
Figure 19. Water-Ethanol-Oleyl alcohol (WEO) equilibrium curve
bottom product is primarily water containing very little ethanol - progressively less water reaches higher stages. Consequently, a column known as a *rectifier* is often placed upstream of the main distillation column to remove the bulk of the water, with the concentrated ethanol top product going to the distillation column of a smaller diameter. If extractive fermentation were employed, a rectifier may not be required, since the 80% ethanol stream could be processed by the distillation column alone.

Daugulis (1987) also observed negligible extractant losses, which suggests that high extractant recirculation rates are possible without the need to supply large amounts of make-up solvent. The upper limit to the circulation rate would be dictated by the size of phase contactor which in turn would depend upon process economics.

### 4.1.4.2. Effect of presence of an extractant (oleyl alcohol) on the hydrolysis of PCS

An extracting solvent (extractant) must satisfy certain criteria to be eligible for use in a SSEF process utilizing PCS. As was previously mentioned, it must (1) be able to extract a sufficient amount of ethanol from the aqueous phase, (2) it must be nontoxic to yeast, and (3) it must not interfere with the access of enzymes to the substrate. Oleyl alcohol has been found to satisfy the first two conditions (Daugulis, 1987); however, the last condition is unique to the system in which hydrolysis of solid substrate is required to generate fermentable sugars, and had not been verified by experiment prior to the execution of this thesis.

The ability of oleyl alcohol to satisfy the last condition was determined by experiment, in which PCS was hydrolyzed in the presence of different amounts of the extractant. A series of oleyl alcohol:fermentation broth ratios were used. The enzyme loading in each case was 5 FPU/g, applied to 50 g/L PCS.
A slight inverse relationship between the oleyl alcohol/broth phase ratio and the final conversion of PCS to reducing sugars was observed (Figure 20). However, this can be attributed to lower levels of mixing in the flasks containing the higher phase ratios, rather than to a reduction in enzyme access to the substrate, or to enzyme inhibition. Since it has also been shown that oleyl alcohol is a suitable extractant in processes utilizing liquid substrates such as glucose (Daugulis, 1987), oleyl alcohol would appear to hold much promise as an extractant for fermentations utilizing solid substrates like PCS (Moritz and Duff, 1996).

4.1.4.3. Comparison of SSF and SSEF ethanol productivities

Since oleyl alcohol did not interfere with the hydrolysis of PCS, it was reasonable to expect that SSEF runs using PCS as a substrate could be performed. Since the primary motivation for developing SSEF technology is the kinetic advantage which could be gained over the SSF process through the reduction of ethanol inhibition, parallel SSF and SSEF runs were performed to test this hypothesis. An oleyl alcohol:fermentation broth ratio of 0.6:1 was employed in the SSEF experiment.

Figure 21a shows 3 ethanol production curves; the upper curve represents the SSF trial, while the middle and bottom curves represent the ethanol production in the respective aqueous and extractant phases of the SSEF trial. These productivities were achieved using 10 FPU/g enzymes and 50 g/L PCS at a temperature of 37°C. The ethanol concentration in the fermentation broth of the SSEF run was lower than the corresponding SSF concentration, due in part to the migration of ethanol from the broth to extractant. Marginally less ethanol was produced in the SSEF run than was produced in the SSF run (Figure 21b). However, this result was encouraging, since it was in accordance with Daugulis' results which proved the compatibility of oleyl alcohol with \textit{S. cerevisiae}. In
Figure 20. Effect of extractant/broth ratio on hydrolysis of Tembec PCS
Figure 21. Comparison of SSF and SSEF ethanol productivities: (a) ethanol concentrations (g/L) and (b) total ethanol (g) produced in each system.
addition, it is evident that PCS hydrolysis can be successfully incorporated into an extractive fermentation process.

4.1.4.4. Comparison of SSF and SSEF processes under fed-batch conditions

The results from the previous experiment indicated that SSEF experiments using PCS as a substrate are possible; however, it remained to be determined whether the SSEF process could have an advantage over the SSF process in terms of the rate of ethanol production.

It was thought that the SSEF process could have an advantage over the SSF process by (1) increasing the substrate concentration from the values of the previous runs, and (2) also increasing the extractant/fermentation broth ratio. These changes would have the effect of increasing the ethanol concentrations in the aqueous phases of both the SSF and SSEF reactors (over values from the previous experiment), perhaps to levels where ethanol would have a significant inhibitory effect on enzyme activity and yeast fermentation. In addition, the difference in ethanol concentration between the aqueous phases of the SSF and SSEF runs would be greater than it was in the previous experiment. Since the aqueous-phase ethanol concentration would always be lower in the SSEF reactor than in the SSF reactor, it is possible that a significant reduction in end product inhibition could be obtained through the use of an extractant.

The production of TRS and ethanol in the aqueous phases of the SSF and SSEF runs over the experiment (0-57.3 h) are plotted in Figure 22a. The notable features of the sugar and ethanol curves are:

1. The TRS concentrations in both the SSF and SSEF flasks rise sharply from the start of the experiment to maximum values at a time of about 5 h, whereupon nearly as sharp a decline in sugar concentration is seen over the next 6-7 h. This was a result of the low yeast cell count at the start of the experiment. After 5 h, the cell count was sufficiently
Figure 22a. Comparison of SSF and SSEF under fed-batch conditions, production of TRS and ethanol in the aqueous phase

(The oleyl alcohol:fermentation broth ratio was increased from the value of 0.6:1, used in the previous experiment, to 1:1. Since initial PCS concentrations greater than 50 g/L were found not to form fluid slurries, the PCS was fed to the reactors in increments of 50 g/L (based on initial reactor volume) after 0 and 24.2 h. The initial enzyme loading was 10 FPU/g; no enzymes were added after the start of the experiment).
high for noticeable amounts of ethanol to be fermented by the yeast. The CO₂ produced as a by-product of fermentation effectively maintained the dissolved oxygen concentration at a level low enough to favour the production of ethanol from the hydrolysis sugars rather than cells. After the addition of sludge at 24.2 h, such that the total PCS concentration was increased to approximately 100 g/L (effectively less than this amount since some PCS had already been converted to TRS and ethanol, and water was added with the wet sludge); another local maximum in sugar concentration occurred in the aqueous phase in each reactor. However, the sugar peaks were less dramatic than those previous for a number of reasons: First, the water added with the wet sludge diluted the aqueous phase in each reactor. Second, the level of enzyme loading in each case was less than it was at the beginning of the experiment. Finally, the yeast cell count was higher than that present initially; hence, the population was more dynamic in its consumption of the hydrolysis sugars.

2. The reducing sugar concentration reaches a minimum value at a time of 24.2 h and gradually increases over the balance of the experiment. This was likely due to the accumulation of non-fermentable sugars (e.g. pentoses such as xylose).

3. The initial rate of reducing sugars production was greater for the nonextractive fermentation run than it was for the run in which oleyl alcohol was present. This is most likely due to the disparity between the level of agitation in the two flasks (as was witnessed in the experiment in which PCS was hydrolyzed in the presence of oleyl alcohol). Both were placed in the same shaker at 225 rpm. The stirring speed was sufficiently high to disperse the oleyl alcohol phase into the aqueous slurry, thus ensuring good interfacial contact beneficial to ethanol extraction. However, due to the lower amount of liquid in the SSF flask, and the dimensions of the flasks, less obstruction to the movement of sludge and enzymes was encountered in this flask, and
hence better mixing was achieved in the SSF flasks. This disparity in mixing diminished as the experiment progressed and the PCS particle size decreased as a result of hydrolysis.

4. The rate of accumulation of ethanol in the aqueous phase was found to be nearly identical for both the SSF and SSEF runs up to an elapsed time of 20 h. After 20 h, the ethanol concentration in the SSEF flask had increased to the point where the amount of ethanol extracted by the oleyl alcohol became significant. This transition is marked by a noticeably lower aqueous-phase ethanol concentration in the aqueous phase of the SSEF flask than that in the SSF flask. Both the SSF and SSEF runs were done in duplicate (as were all experiments presented in this thesis), with little variation between duplicates (approximately ±5%), hence it is possible to have confidence in the results obtained.

Figure 22b shows the concentrations of TRS, aqueous-phase ethanol, and ethanol in oleyl alcohol. A steady increase in the concentration of ethanol in the oleyl alcohol phase occurs, which mirrors the increase in the ethanol concentration in the aqueous phase. As can be seen from the relatively low concentration of ethanol in the extractant phase, the amount of ethanol which can be removed from the broth is minimal in batch systems such as these. Extracting solvent must be continuously fed to the system to significantly reduce the ethanol concentration in the reactor and increase the rate of ethanol extraction, although higher extractant:broth ratios may also be used. It is possible that if the fermentation step remained a batch operation, but with an increase in solids concentration in the broth, it would be possible to demonstrate the advantage of the extractive fermentation process over one employing no extractive step. It has been shown in previous work (Table 1) that sludge having a consistency as high as 61% solids is readily hydrolyzed; accordingly, it was concluded that it would be possible to substantially increase the PCS concentration in the
broth in future fed/batch SSEF experiments done in the BioFlo IV bench-scale fermenter, where improved mixing conditions are possible.

The total production of ethanol as a function of time is presented in Figure 22c, on a mass basis. For the SSEF run, both the oleyl alcohol and aqueous phases contribute to the total ethanol produced. The SSEF run resulted in a greater conversion of substrate to ethanol than was obtained in the SSF run. A total of 4.84 g of ethanol were produced in the SSEF run, compared with 4.23 g for the SSF run. A total of 20 g of PCS (on a dry basis) were fed to each flask. Therefore the total conversion of substrate (to both ethanol and CO₂) was found to be 47.5% for the SSEF run and 41.5% for the SSF run.

The results show that the presence of oleyl alcohol as an extracting solvent does not decrease the conversion of the substrate, but rather the opposite is true in this experiment. It should be noted that the SSEF process did not have a clear advantage over the SSF process until the addition of additional PCS after 24.2 h, i.e. when the PCS concentration was substantially increased. Therefore the application of SSEF technology in fed-batch systems could allow for the use of more concentrated substrates than are possible using the SSF process. Ease of product recovery is another advantage of the SSEF process, although one not explored here.

4.1.4.5. Comparison of oleyl alcohol with oleic acid as candidates for SSEF processes

This experiment was similar to the previous one; however, several differences exist between them: (1) an additional extractive fermentation run was performed using oleic acid as an extracting solvent, (2) although the initial concentration of PCS was 50 g/L as in the previous experiment, two additional doses of 50 g PCS/L each were added to the flasks, compared with only one dose in the previous experiment, and (3) the enzyme loading was maintained at a constant level of 10 FPU/g PCS through the addition of enzymes along with
Figure 22b. Production of TRS, aqueous-phase ethanol and ethanol in oleyl alcohol

Figure 22c. Total ethanol produced (g) in parallel SSF and SSEF fed-batch runs
fresh PCS (not including the enzymes which desorbed from the original PCS as it was consumed), whereas no additional enzymes were added after the start of the previous experiment. A SSF trial was run as a control.

The TRS and ethanol production curves are shown in Figure 23a. Doses of substrate and enzymes were added to the flasks at times of 23 and 48 h. There are a number of conclusions that can be drawn from the results, namely:

1. The initial rate of reducing sugar production was greater for the runs done in flasks containing oleic acid than for other runs. The cause is unknown, although the first addition of sludge and enzymes (after the initial dose) did not result in a great increase in the concentration of TRS in the flasks; no noticeable increase was seen for the flask containing no extractant. It is possible that the extractant interfered with the ability of the yeast to gain an initial foothold in the broth, resulting in less sugar uptake and therefore an accumulation of sugars. The second addition of sludge and enzymes at 48 h resulted in a greater increase in the TRS concentrations in all of the flasks. An insight to why this occurs can be gained by examining the nature by which both enzymes and yeast complete their respective tasks. Enzymes diffuse quickly to substrate and adsorb to the surface. The enzymatic depolymerization of cellulose occurs at the interface between the substrate and the bulk liquid (aqueous phase). Hence the change in the volume of the bulk fluid that accompanies the addition of wet sludge does not appreciably affect the fraction of the enzymes (recall that enzymes are added with the sludge) which hydrolyze the substrate. However, yeast cells ferment soluble sugars in the bulk liquid. Adding sludge immediately lowers the concentration of yeast cells, and hence the rate at which the yeast population consumes hydrolysis sugars, the level of which has increased due to a sudden burst in the rate of hydrolysis. The net result of the disturbance is the local
Figure 23a. TRS and ethanol production, SSF and SSEF (both extractants) runs
maximum in TRS concentration that occurs immediately after the addition of sludge at 48 h.

2. The ethanol remaining in the aqueous phase (rather than migrating to the extractant phase) is greatest in the SSF run, and lowest for the SSEF flask containing oleyl alcohol. The median value was obtained for the SSEF flask in which oleic acid was used as the extractant. Much of the difference can be attributed to the fact that oleyl alcohol is a more effective extractor of ethanol, as can be seen from Figure 23b. The final concentration of ethanol in oleic acid was found to be 2.7 g/L, somewhat lower than that in oleyl alcohol (3.9 g/L). Hence the oleyl alcohol removed more ethanol from the aqueous phase than did the oleic acid.

3. The differences in the final ethanol concentrations in the aqueous phases of the different runs are also due to the fact that less ethanol was produced in the SSEF flasks containing oleyl alcohol than were produced in the other flasks. As can be seen from Figure 23c, the most ethanol was produced from the flasks in which no extractant was used. Slightly less ethanol was produced in the SSEF flasks in which the extractant was oleic acid. The least amount of ethanol was produced in the SSEF flasks containing oleyl alcohol. The results are summarized in Table 8.

The amount of ethanol produced by nonextractive fermentation is approximately equal to the amount produced through extractive fermentation using oleic acid, within the limits of experimental uncertainty. It is worthy of note that the rate of ethanol production was greater in the SSEF runs with oleic acid than in other runs up to a time of 48 h. It is possible that the yeast used the oleic acid as a nutrient during this period. If this is true, it would be worthwhile to use a mixture of oleic acid and oleyl alcohol for an extracting solvent. In this way the superior extracting ability of the oleyl alcohol could be combined
Figure 23b,c. SSF and SSEF (oleyl alcohol/oleic acid extractants): (b) ethanol in aqueous and extractant phases, (c) total ethanol produced.
Table 8. Effect of extracting solvent on ethanol yield

<table>
<thead>
<tr>
<th>Type of extractant</th>
<th>Ethanol, aqueous phase (g/L)</th>
<th>Ethanol, solvent phase (g/L)</th>
<th>Total ethanol produced (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>19.7</td>
<td>----</td>
<td>3.3</td>
</tr>
<tr>
<td>oleyl alcohol</td>
<td>15.3</td>
<td>3.9</td>
<td>2.8</td>
</tr>
<tr>
<td>oleic acid</td>
<td>17.8</td>
<td>2.7</td>
<td>3.2</td>
</tr>
</tbody>
</table>

with the superior biocompatibility of oleic acid.

It is possible that both SSEF runs failed to outperform the SSF run due to better mixing in the SSF reactors. For this reason, it was necessary to scale the experiments up to the point where they could utilize the superior mixing environment of the BioFlo IV fermenter.

4.1.4.6. Multiple Stage Batch SSEF (Fermenter-scale)

Using multiple batch feeding of PCS into the laboratory-scale fermenter, the kinetic benefits of extraction became apparent (Figures 24a and 24b). By comparing these figures to the equivalent experiment carried out without the extractant (Figure 18a) we can see that the average ethanol productivity (based on aqueous phase volume) over 200 h in the extractive case was 50% higher than in the conventional SSF reactor (0.15 g/L reactor volume/h vs 0.10 g/L/h). The conditions of both the extractive and non extractive experiments were identical, including the amount of substrate and enzymes added, although the first cycle of PCS addition occurred 24 h sooner in the SSEF run. However, reactions in both SSF and SSEF runs were allowed to go to completion in calculating ethanol productivities, to mitigate the effect of this disparity. The results indicate that under equal mixing conditions
Figure 24. Fermenter-scale, fed-batch SSEF: (a) ethanol production, both phases; (b) total ethanol production, mass basis

(Fermentation medium: extractant ratio: 1:5.)
and high extractant:broth ratios, substantially higher ethanol productivities are possible in the SSEF process than are possible in the SSF process. As detailed above, it is expected that process configurations which allow for continuous solvent throughput would derive an even greater benefit from in situ ethanol extraction (Moritz and Duff, in press).

4.2. Experiments in SSL medium for development of SSF model of continuous system at Tembec

The model equations for the continuous SSL fermentation system at Tembec, modified to operate in SSF mode, have been described in Section 1.2.6. The constants of the equations were evaluated using data acquired from experiments using SSL as the reaction medium. The SSL was of the same concentration used by Tembec in its fermentation system, defined here as 100% SSL. All data to be applied towards the simulation were taken from experiments running at a temperature of 33°C (the normal operating temperature of Tembec's system).

In addition to experiments, the results of which were used directly in the development of the model, other experiments using SSL were performed to determine the effects of operating variables (SSL concentration, temperature) on enzymatic hydrolysis and yeast fermentation. The results, which indicate the sensitivity of the system to changes in these variables, are also presented in this section.

4.2.1. Effect of temperature and SSL concentration on PCS hydrolysis

To determine the interactive effect of SSL concentration and temperature on hydrolysis rate, hydrolyses were carried out at two SSL concentrations (50% and 100%) and two temperatures (33 and 37°C) (Figure 25). Higher hydrolysis rates were observed at the higher reaction temperature (37°C), although this effect was more pronounced for the runs performed in the more dilute (50%) SSL. For example, the hydrolysis rate in 50% SSL was
Figure 25. Effects of temperature and SSL concentration on PCS hydrolysis
43% higher at 37°C (0.60 g/(L·h)) than at 33°C (0.36 g/(L·h)). By contrast, the hydrolysis rate in 100% SSL was only 9% higher at 37°C (0.36 g/(L·h)) than at 33°C (0.33 g/(L·h)).

Hydrolysis of PCS was inhibited by SSL, an observation which contrasts our earlier findings with more dilute SSL concentrations at a higher temperature (50°C). However, the fact that inhibition was less severe at the higher temperature also supports these earlier findings (Duff et al., 1994). It is possible that the difference in degree of inhibition is partly due to batch to batch variation in the SSL. This contention is supported by the wide variations observed in the fermentability of the SSL as delivered from the mill. In a previous experiment, it was found that xylose inhibited hydrolysis to a degree (Figure 12), although not to the extent where SSL inhibition of enzyme activity in this experiment could be attributed solely to the presence of xylose. However, because the xylose content of SSL varies from batch to batch (more xylose is produced from hardwood than from softwood pulping), it is possible that some of the variation in SSL inhibition experienced between batches can be attributed to varying xylose levels.

If SSL inhibition of hydrolysis continues to be a concern, it is possible that sugar production could be increased by hydrolyzing PCS in SSL prior to its evaporation (from 50% to 100% SSL). However, this advantage might be offset by a hydrolysis rate reduction due to the competitive inhibition of the enzymes by the higher levels of reducing sugars which would result from choosing this method over a SSF process (where the sugars produced via hydrolysis would be continuously removed via fermentation).

4.2.2. Fermentation of SSL: effect of initial sugar concentration

In one set of runs, 33% SSL was fortified with glucose to achieve a series of initial total reducing sugar concentrations. The purpose of fermenting the SSL samples was to observe the effect substrate availability would have on yeast cell growth and on the rate of ethanol
production. Thirty three percent rather than 100% SSL was used, due to difficulties initially encountered in fermenting the more concentrated SSL. These difficulties were resolved in subsequent experiments using 100% SSL, where a larger yeast inoculum was used (approximately 2:1 inoculum:concentrated SSL (>100%)). The more concentrated SSL was produced through the rotovaporization of 100% SSL. By contrast, a ratio of inoculum:SSL of 1:2 was employed in the runs using dilute (33%) SSL.

The reducing sugar and ethanol production curves are shown in Figure 26a; the yeast cell production curve is shown in Figure 26b. It is seen immediately that the ethanol production rate was directly related to the concentration of reducing sugars, after cell growth had passed the logarithmic phase (after about 5 h) and (relatively) stable cell populations had been established. It can also be seen by inspection that Monod kinetics prevailed, since the system was substrate-limited up to an initial sugar concentration of 66 g/L (the cell growth rate increased with an increase in initial sugar concentration). No detectable increase in cell growth rate resulted when the initial sugar concentration was increased from 66 to 85 g/L. This result indicates that the cells were saturated with substrate at this point, i.e., the active sites on enzymes responsible for cell growth and metabolism were saturated with adsorbed sugars.

4.2.3. Model data

The preliminary work using dilute SSL was followed by experiments in full strength (100%) SSL dedicated to model development. The results are presented in this section.

4.2.3.1. Hydrolysis of PCS in 100% SSL

The results from a series of hydrolyses of PCS in full strength (100%) SSL (the feed concentration to Tembec's fermentation system) are given in Figure 27a. This was the first of a number of experiments done to obtain kinetic and yield parameters for the SSF process.
Figure 26. Fermentation of 33% SSL: effect of initial reducing sugar concentration: (a) Reducing sugars consumption and ethanol production; (b) yeast cell growth
An analysis of the initial rate data using Lineweaver-Burk coordinates ($1/v_0$ versus $1/S_o$, where $v_0$ and $S_o$ are the initial sugar production rate and initial substrate (PCS) concentration, respectively) produced a linear relationship between $1/v_0$ and $1/S_o$ ($R^2=0.995$). The average rates of hydrolysis over the first hour (Table 9) were used to determine the initial rate law (where the competitive inhibition by glucose is negligible since very little glucose exists):

$$v_0 = \left( \frac{dG}{dt} \right)_o = \frac{1.5S}{4.9 + S} \text{ (g/(L-h))} \tag{15}$$

Where $G$ and $S$ are the reducing sugar and PCS concentrations (g/L), respectively.

Table 9. Initial hydrolysis rate data, hydrolysis of PCS in 100% SSL

<table>
<thead>
<tr>
<th>PCS concentration (g/L)†</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial hydrolysis rate (g/(L-h))</td>
<td>0.84</td>
<td>1.05</td>
<td>1.15</td>
<td>---</td>
<td>1.29</td>
</tr>
</tbody>
</table>

† based on total sludge, although the rate equation was obtained by considering only the fraction of PCS which could be converted to reducing sugars.

Further analysis using Foster-Niemann coordinates quantified the effect of competitive inhibition by glucose on the hydrolysis rate, $v$. A linear relationship between $G/t$ and $1/t \cdot \ln\left(\frac{S}{S_o}\right)$ was obtained (Figure 27b, $R^2=0.999$), whence $K_{ig}$, the glucose inhibition constant, was found to be 0.89. When equation (15) is modified to take glucose inhibition into account, the result is equation (16):

$$v = \frac{dG}{dt} = \frac{1.5S}{4.9 + 5.5G + S} \tag{16}$$

Equation (16) is valid over the range of sugar concentrations (0-11.2 g/L) encountered in experiment. The effect of SSL inhibition of cellulase activity is apparent in comparing...
Figure 27. Hydrolysis of PCS in 100% SSL: (a) Reducing sugar production; (b) Foster-Nieman plot of hydrolysis data.
equation (14) with equation (16) - when $S_a = 50$ g/L, the initial rate of hydrolysis is 46% lower in 100% SSL (1.37 g/(L·h)) than in initial rate acetate buffer (2.54 g/(L·h)).

4.2.3.2. Fermentation of 100% SSL
Batch experiments were performed to quantify the kinetic parameters and yield coefficients of cell growth and ethanol production. SSL of the concentration currently fed to Tembec's continuous fermentation system was fermented, and the concentrations of yeast cells, reducing sugars, and ethanol were determined as functions of time.

In an effort to simulate the large number of operating conditions - i.e. the many combinations of reducing sugars, yeast cell and ethanol concentrations - that may prevail in a continuous system, fermentations were performed under a series of initial conditions. Specifically, the initial yeast cell and ethanol concentrations were varied and the results analyzed.

4.2.3.2.1. Fermentation of 100% SSL: effect of initial ethanol concentration
Ethanol accumulation in the bulk liquid (or more significantly within the yeast cells) is known to inhibit cell activity and hence lower the ethanol production rate. A set of experiments performed in 100% SSL at 33°C were designed to observe this effect (Figures 28a-d).

It can be seen immediately that ethanol inhibits yeast growth (Figure 28a), as predicted. Notably, increasing the initial ethanol concentration from 17.9 to 22.9 g/L produced the greater reduction in cell growth rate (and maximum cell concentration) than that which occurred from 12.5 - 17.9 g/L. To illustrate this, the specific cell growth rates $\mu$ were plotted against substrate (reducing sugar) concentration (Figure 28d).
Figure 28a,b. Fermentation of 100% SSL: effect of initial ethanol concentration: (a) Yeast cell growth; (b) reducing sugars uptake.
Figures 28c,d. Fermentation of 100% SSL: effect of initial ethanol concentration: (c) Ethanol production; (d) specific cell growth rate versus substrate concentration
It can be seen from Figures 28a and 28c that ethanol accumulation in the fermentation matrix inhibits ethanol production to a lesser degree than cell growth. This may be the result of the oxidative pathways which enable cell growth and multiplication being repressed to a greater degree than those pathways which enable ethanol fermentation. It should be possible to increase ethanol yield at the expense of cell growth in a process utilizing cell recycle, by maintaining a high ethanol concentration and a low concentration of substrate. In this process, the reduction in the rate of ethanol fermentation caused by the high level of ethanol could be offset by the higher concentration of viable cells.

The effects of substrate availability \(G\) and ethanol accumulation \(P\) on the specific cell growth rate \(\mu\) are conveniently summarized by equation (4) (Aiba et al., 1973):

\[
\mu = \mu_{\text{max}} \left( \frac{G}{K_s + G} \right) \left( 1 - \frac{P}{P_{\text{max}}} \right)^m
\]

Equation (4) is valid for the logarithmic phase of cell growth, and is not valid for the model (which describes SSF under the condition of stationary-phase cell growth). The constants \(\mu_{\text{max}}, K_s, P_{\text{max}}\) and \(m\) were obtained by fitting equation (4) to experimental data. Corresponding values of \(\mu, P\) and \(G\) were read from Figures 28a-c, thus enabling \(\mu\) to be represented as a function of both \(P\) and \(G\). Using an Excel™ spreadsheet, values of \(\mu\) as determined by equation (4) were placed alongside their corresponding experimental values. Using the equation solver feature, values for the four unknown parameters were obtained by setting the sum of the residuals (differences between experimental and fitted values) to zero. The values were then substituted into equation (4):

\[
\mu = 1.3 \left( \frac{G}{54 + G} \right) \left( 1 - \frac{P}{28} \right)^{0.95} \quad \text{(h}^{-1})
\]
Equation (17) is valid over the range of reducing sugars and ethanol concentrations encountered over the logarithmic cell growth phase of the experiment (50.9-36.2 and 16.4 - 26.6 g/L, respectively).

To determine the goodness of fit, specific cell growth rates predicted by equation (17) were plotted against their respective experimental values (Figure 28e). There is a considerable amount of scatter in the data points ($R^2 = 0.54$); however, the fact that the slope of the graph is $0.98\pm0.30$ (95% confidence) attests to the suitability of the model - a slope of 1 would indicate that the experimental values for the specific growth rate are equal to the fitted values. The validity of the equation is confirmed by the residual plot (Figure 28f), in which the points are randomly distributed over the zero-residual line.

It should be noted that equation (17) is not a valid expression for the specific cell growth rate for Tembec's continuous system, which does not operate within the logarithmic phase of cell growth - another expression must be determined for the model. However, the expression does provide a convenient means by which to observe how the presence of substrate and ethanol affects yeast growth. For the practical purposes of a model, values for $\mu_n$ should be determined for each of Tembec's 3 fermenters, along with corresponding ethanol and cell yield coefficients. It is simply not practical to obtain these parameters from small-scale batch fermentations.

4.2.3.2.2. Fermentation of SSL: effect of initial yeast cell concentration

In previous experiments it was difficult to achieve sufficiently high cell mass concentrations, due to the tendency for SSL to suppress cell growth. This effect is apparent when comparing the cell mass concentrations obtained in 33% SSL (Figure 26b) with those obtained in 100% SSL (Figure 28a). This limited the amount of data available at cell
Figures 28e,f. Fermentation of 100% SSL, effect of initial ethanol concentration: (e) Predicted (equation (17)) versus experimental specific cell growth rates; (f) plot of specific cell growth rate residuals
concentrations similar to those which are achieved industrially (Tembec maintains 4 - 5 g cells/L in its continuous, cell recycle fermentation process).

By varying the initial dose of yeast inoculum, and by concentrating the inoculum via gravity settling, it was possible to vary the initial cell concentration up to a maximum level of 5.3 g/L (Figure 29a).

From the cell production curves, it can be seen that logarithmic cell growth did not occur in any of the three runs. This was due in part to the fact that the cells, which were grown in conventional (non SSL) medium, were unacclimatized to the SSL. In addition, the initial sugar concentration of the SSL was much lower (46 g/L) than that of the SSL Tembec currently ferments (≈80 g/L). These factors, in addition to high initial cell mass concentrations, induced substrate-limited conditions from the onset of the fermentations. Due to the large amount of cells relative to fermentable substrate, much of the energy produced by the production of ethanol from glucans was used for the maintenance of cell functions, rather than for the production of cell mass. Hence cell production was minimal (after an initial 5 h lag due to cell acclimation to SSL). This is beneficial from the standpoint of maximizing ethanol yield. However, new cells are needed to replace dead and moribund ones; the rate of cell replacement can be manipulated in continuous systems by controlling the cell recycle ratio (recycled cells:wasted cells) thereby controlling the cell age and concentration in the reactor. It can be expected that an optimal cell recycle ratio exists, which minimizes the rate of cell replacement (thereby maximizing yield) while at the same time maintaining a sufficiently high ethanol production rate (due to a sufficient number of viable cells). This ideal recycle ratio would be dependent upon the specific needs of the mill. For example, it may be necessary to sacrifice a high production rate in favour of a high yield (i.e. use a high recycle ratio) if a process is substrate limited.
Figures 29a. Fermentation of 100% SSL: effect of initial cell concentration on yeast cell production
Much of the task of cell replacement is accomplished by the cells themselves, as dead cells are lysed and consumed by living cells (endogenous respiration). In all experiments, fermentations were accomplished using cells with an initial age distribution over an interval of 0-24 h (that of the inoculum); therefore, the greater portion of ethanol was produced by cells which were propagated during the initial stage (0-12 h) of fermentation. In contrast, the cells in Tembec's system would have a wider age distribution due to cell recycle.

Low fermentation rates were obtained (Figure 29c), due to the limited amount of fermentable substrate and the use of unacclimatized yeast. For example, the maximum rate of ethanol production with an initial cell concentration of 5.3 g/L was 1.0 g/(L-h) (obtained after 2.5 h when the concentration of reducing sugars was 42 g/L), which is lower than Tembec's overall production rate of 1.3 g/(L-h). In the batch experiment, the rate of ethanol production decreased significantly as the fermentable substrate was depleted - 0.4 g/(L-h) were produced when 29 g/L reducing sugars remained (Figure 29b), 9 g/L of which was fermentable. Therefore it was not possible to use the data obtained to produce a realistic model of Tembec's process using the data obtained.

It is evident that the ethanol production rates observed were substantially lower than those of the previous experiment, where, for example, the maximum ethanol production rate in the run in which the initial ethanol concentration was 12.5 g/L was found to be 1.9 g/(L-h). This occurred after 4 h, despite the presence of a significant amount of inhibitory ethanol. Since only 41 g/L of reducing sugars remained after 4 h - which is similar to the substrate level at which the maximum ethanol production rate was observed in this more recent experiment - the greater ethanol production rate can be attributed to the presence of acclimatized cells. Although the cells were grown in the same medium in all SSL fermentations prior to their addition to the reactors, in this case the initial cell inoculum was
Figures 29b,c. Fermentation of 100% SSL: effect of initial cell concentration:
(b) reducing sugars consumption; (c) ethanol production
comparatively low, and as a result new cells were propagated in the SSL matrix and thereby were acclimatized to the medium.

4.2.3.3. Simultaneous hydrolysis and fermentation of PCS in 100% SSL

The hydrolysis and simultaneous fermentation of PCS was conducted in unfermented, 100% SSL as a means of determining the increase in ethanol productivity possible via this method. The ethanol concentrations after 24 h corresponding to initial PCS concentrations of 20, 35, 50 and 65 g/L as well as a control (0 g PCS/L) are presented in Table 10. The ethanol production curves are presented in Figure 30.

Preliminary SSF results indicate that Tembec could significantly increase its ethanol production rate while at the same time reduce the amount of PCS of which it must dispose. For example, over a period of 24 h (comparable to the residence time of Tembec's process) a 25% increase in the ethanol production rate over the control (no PCS addition) was forthcoming when 65 g PCS/L was simultaneously hydrolyzed in the fermentation medium. The fact that there was marginal benefit to increasing PCS loading into the vessels indicated a limited degree of hydrolysis occurring in the SSF reactions. It is likely that this inhibition was mainly due to the presence of hexoses in the unfermented SSL (although inadequate mixing at higher PCS levels is also a possibility), and that significant hydrolysis may not have occurred until after these sugars were consumed. Consumption of approximately 65 g/L of sugar initially present in 100% SSL requires nearly 24 hours (result not shown). As such, by using unfermented SSL we effectively minimized the benefit to PCS fortification over the 24 hour period of observation.

As was previously noted, our batch reactions have two major differences from the full scale system at Tembec. First, the concentration of hexoses in our system (initially 65 g/L) was significantly higher than the steady state concentration in Tembec's full-scale system.
Table 10. SSF of PCS in 100% SSL: 24 h ethanol concentrations

<table>
<thead>
<tr>
<th>Initial PCS concentration (g/L)</th>
<th>Ethanol concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>18.7</td>
</tr>
<tr>
<td>20</td>
<td>21.4</td>
</tr>
<tr>
<td>35</td>
<td>21.5</td>
</tr>
<tr>
<td>50</td>
<td>22.4</td>
</tr>
<tr>
<td>65</td>
<td>23.4</td>
</tr>
</tbody>
</table>

Figure 30. SSF of PCS in 100% SSL: ethanol production curves
Secondly, in our batch reactors, yeast concentrations range from 1-3 g/L, increasing over the time course of the SSF reaction. These values are initially much lower and rise to a yeast concentration only 60% of that observed in the full scale system. Since both of these factors have exerted strongly negative impacts on the rates of ethanol fermentation observed in the batch experimental system, it is likely that much greater improvements in ethanol production can be achieved through SSF of PCS than those already observed.

It is likely that the disadvantage of the low hydrolysis rates observed during the SSF runs could be overcome to some extent in a full-scale system, by reducing the TRS inhibition of hydrolysis, and by improving the mixing conditions. In a system such as Tembec's, PCS and enzymes could be added gradually during startup, thereby increasing the level of partially hydrolyzed PCS (which is considerably less viscous than unhydrolyzed PCS) in the reactor until the desired PCS concentration is attained. In this way favorable mixing conditions would be possible in addition to a lower level of enzyme inhibition.

4.2.3.4. Interactive effects of SSL and ethanol on cell growth

The interactive effects of SSL and ethanol concentration on yeast cell production were determined at one initial ethanol concentration (23 g/L) in both 33 and 100% SSL (Figure 31). At an initial ethanol concentration of 23 g/L, the maximum specific cell growth rates were 0.07 and 0.38 h\(^{-1}\) in 100 and 33% SSL, respectively. In runs performed in 100% SSL, the maximum specific cell growth rates at initial ethanol concentrations of 12.5 and 23 g/L were 0.12 and 0.07 h\(^{-1}\) respectively. These observations are qualified by the fact that different sizes of yeast inoculums were used.
Figure 31. Interactive effects of SSL and ethanol on cell growth
5. CONCLUSIONS

Primary clarifier sludge from Tembec's low yield sulphite pulping operation was readily hydrolyzed by cellulase enzymes. A maximum conversion of 66.4% was obtained when an initial enzyme loading of 100 FPU/ g PCS was applied to 10 g/L PCS.

Tembec PCS can be dehydrated to a solids level of 61% (w/w) without significantly decreasing its amenability to enzymatic hydrolysis. Therefore it would be possible to augment the sugar supply of a fermentation process, via the addition of PCS and cellulase enzymes, without significantly increasing the hydraulic load.

The addition of a nonionic surfactant (Tween 80) did not have a significant effect on the enzymatic hydrolysis of Tembec PCS. It is possible that sufficient natural surfactants were present with the substrate, or that the fraction of cellulose available for hydrolysis was amorphous and hence readily accessible to enzymes.

The dewaterability of PCS was enhanced through enzymatic hydrolysis. A 56% increase in dewaterability was observed over the course of 27 h. Most of this increase occurred over the first 2 h of hydrolysis.

Higher rates of hydrolysis were associated with higher levels of enzyme loading; however, the specific hydrolysis rate (relative to enzyme loading) was found to decrease with an increase in enzyme loading. Average specific rates of hydrolysis of 50 g/L PCS (in acetate buffer) were 1.07 and 0.79 g/(L·h·FPU) at enzyme loadings of 2 and 10 FPU/g PCS, respectively. This was due primarily to the effects of substrate exhaustion and reducing sugar inhibition of cellulase activity.
PCS hydrolysis in 0.2 mols/L acetate buffer was found to obey the following rate law:

\[
\frac{dS}{dt} = \frac{3.4S}{16.8 + 1.3G + S} \quad (\text{g} \cdot \text{L}^{-1} \cdot \text{h}^{-1})
\]  

(14)

This expression is valid at 33°C for an initial enzyme loading of 10 FPU/g PCS. The effect of hexose inhibition of cellulase activity is apparent upon inspection of the rate law.

Hydrolysis of PCS can be carried out in spent sulphite pulping liquor (SSL). Inhibition of the hydrolysis reaction by sugars present in the SSL can be reduced by pre-fermentation of the SSL. Other inhibitors remain in the SSL after fermentation, and their effects on hydrolysis must be considered a kinetic penalty associated with the use of SSL as a reaction matrix. Higher hydrolysis rates were observed at higher temperatures; however, this effect was greater in less-concentrated SSL. It is possible therefore that increasing the temperature not only increases the activity coefficient of hydrolysis, but also decreases the effect of cellulase inhibition (caused by the presence of hexose, pentose and possibly other chemical species which inhibit by adsorbing onto cellulase) by decreasing the fraction of species which adsorb onto the active sites of the cellulase. The rate of PCS hydrolysis at 33°C in 100% SSL was found to obey the law:

\[
v = \frac{dG}{dt} = \frac{1.5S}{4.9 + 5.5G + S} \quad (\text{g} \cdot \text{L}^{-1} \cdot \text{h}^{-1})
\]  

(16)

which is valid at an initial enzyme loading of 10 FPU/g PCS. For an initial PCS concentration of 50 g/L, the initial hydrolysis rate was found to be 44% lower in 100% SSL than the corresponding value in acetate buffer (1.4 versus 2.5 g\cdot\text{L}^{-1}\cdot\text{h}^{-1} respectively).
Temperature (over a range of 33 to 37°C) affected neither the rate of fermentation of PCS-derived wood sugars, nor their conversion to ethanol, which was as high as 66% of the theoretical yield. Hence an industrial process such as the one employed by Tembec would have a degree of freedom in its control of temperature. The maximum conversion was obtained with an initial ethanol concentration of 28 g/L. It is possible that higher yields correspond to higher ethanol levels during fermentations, as cell growth is suppressed to a greater extent than ethanol production. It would be necessary to balance the higher yield with a lower ethanol production rate when optimizing a fermentation process. Another benefit to operating a system at a high ethanol concentration would be a reduction in the cost of product recovery compared with a system operating at a lower ethanol concentration.

Ethanol inhibited the fermentation rate of wood sugars; beyond an initial ethanol concentration of 53 g/L, the yeast cells were inactive. In experiments utilizing 100% SSL as a reaction matrix, ethanol was found to inhibit cell growth to a lesser extent than ethanol fermentation. This would support the previous explanation for the higher ethanol yields experienced at higher ethanol concentrations.

An expression for logarithmic cell growth in 100% SSL was derived:

$$\mu = 1.3 \left( \frac{G}{54 + G} \right) \left( 1 - \frac{P}{28} \right)^{0.95} \quad (h^{-1}) \quad (17)$$

Equation (17) is valid over the range of reducing sugars and ethanol concentrations encountered over the logarithmic cell growth phase of the experiment (50.9-36.2 and 16.4 - 26.6 g/L, respectively). The equation quantifies the effect of ethanol ($P$) inhibition on cell growth, as well as the extent to which the system is substrate-limited. It can be seen that the
maximum specific cell growth rate possible is 1.3 h\(^{-1}\), and that the rate declines to half this value when the substrate concentration is 54 g/L. It is not possible to apply this expression to continuous industrial fermentation processes, such as the one employed at Tembec, since logarithmic cell growth does not occur, and cell concentrations are higher than those attainable in batch reactors without the option of cell recycle. It is more practical to use observed yields from these processes (which are wood species dependent) as a basis for developing a model, than yields observed from small-scale, batch reactions.

The yield of ethanol from simultaneous saccharification and fermentation reactors was not strongly influenced by the enzyme loading used, allowing the possibility of efficient conversion of PCS at low (<5 FPU/g) enzyme loading. At this level of enzyme loading, 6.9% conversion/(FPU/g PCS) was obtained over 74 h, with a final ethanol concentration of 17.6 g/L.

SSF of PCS can increase ethanol yield from 100% SSL by up to 25% in 24 hours (the residence time in Tembec's fermentation system). It is expected that this result could be improved upon in a continuous system in which the yeast concentration is high and fermentable sugar concentration is low, and where better mixing is possible.

In bench-scale SSF reactions using 10 L working volumes and the improved mixing conditions of the BioFlo IV fermenter, the rate of ethanol production relative to enzyme loading was as high as 0.76 g ethanol/(FPU/g PCS) under fed-batch conditions.

The presence of a second immiscible solvent (oleyl alcohol) did not have a deleterious effect on cellulose hydrolysis. Based on this finding, a novel simultaneous hydrolysis and extractive fermentation process has been developed and demonstrated to have a 50%
higher ethanol productivity (0.15 g·L⁻¹·h⁻¹) than the equivalent SSF process without extractant (0.10 g·L⁻¹·h⁻¹).
6. **FUTURE WORK**

Further work is required to demonstrate the kinetic benefit of SSEF technology. We were limited in our batch experiments by the amount of extracting solvent relative to aqueous phase volume we could employ. A semibatch system incorporating product separation and solvent recycle could serve to determine the extent to which the SSEF process could outperform the SSF process.

It would be useful to carry out a kinetic study to determine the relative benefits of high versus low temperature operation in SSF experiments. Low temperature benefits ethanol fermentation at the expense of a lower hydrolysis rate. An upper limit to the temperature at which *Saccharomyces cerevisiae* is capable of consuming hydrolysis sugars produced (at the same temperature) should be determined.

To fully develop the model of SSF in SSL medium in a continuous system, a pilot plant should be constructed to duplicate the conditions under which the full scale system would operate. Realistic values for such parameters such as cell mass and TRS concentration could be obtained in such a setup, in addition to better mixing. Also, the effect of SSL type (e.g. hardwood or softwood derived) should be determined.
7. NOMENCLATURE

Acronyms

CSTR  Continuous stirred tank reactor
FPU   Filter paper unit
IU    International unit
PCS   Primary clarifier sludge
PFR   Plug flow reactor
SHF   Separate hydrolysis and fermentation
SSEF  Simultaneous saccharification and extractive fermentation
SSF   Simultaneous saccharification and fermentation
SSL   Spent sulphite liquor
TRS   Total reducing sugars
WEO   Water-Ethanol-Oleyl alcohol system

Symbols

\( a \)  Hydrolysis coefficient (=0.909)
\( D \)  Tank dilution factor (h\(^{-1}\))
\( E \)  Enzyme loading (FPU/g)
\( G_n \) Hexose concentration in tank \( n \) (g/L)
\( K_{ig} \) Constant, enzyme rate equation (g/L)
\( K_m \) Constant, enzymatic hydrolysis rate equation (g/L)
\( K_s \) Constant, ethanol fermentation rate equation (g/L)
\( K' \) Partition coefficient of solute in liquid-liquid extraction systems
\( m \) Constant, ethanol inhibition of yeast activity
\( P_{max} \) Ethanol concentration at which yeast metabolism ceases (g/L)
\( P_n \) Ethanol concentration in tank \( n \) (g/L)
\( S_n \)  
PCS concentration in tank \( n \) (g/L)

\( t \)  
time (h)

\( V \)  
CSTR volume (L)

\( V_{\text{max}} \)  
Constant, enzymatic hydrolysis rate equation (h\(^{-1}\))

\( X_n \)  
Yeast cell concentration in tank \( n \) (g/L)

\( Y_{P/S} \)  
Yield of ethanol per unit substrate consumed (g/g)

\( Y_{P/E} \)  
Yield of ethanol per unit enzyme (g/FPU)

\( Y_{P/G} \)  
Yield of ethanol per unit glucose consumed (g/g)

\( \gamma \)  
the weight ratio of solute (e.g. ethanol) to solute-free extractant (oleyl alcohol) in the extractant phase

\( \chi \)  
the weight ratio of solute to solute-free raffinate (e.g. broth) in the raffinate phase

\( \mu \)  
Specific cell growth rate (h\(^{-1}\))

\( \mu_{\text{max}} \)  
Maximum specific cell growth rate (h\(^{-1}\))

\( K' \)  
Partition coefficient for WEO system

\( \nu \)  
Volumetric flowrate (L/h)
8. REFERENCES


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