REMOVAL OF MULTIPLE SUBSTRATES IN A MIXED CULTURE PROCESS FOR THE TREATMENT OF BREWERY WASTEWATER

by

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ABSTRACT

The removal of multiple substrates in a defined mixed culture process was investigated in the treatment of brewery wastewater. The study was conducted using both batch and a semi-continuous reactor system called self-cycling fermentation. Batch experiments were conducted using a synthetic brewery wastewater containing glucose, ethanol and maltose. Activated sludge from a municipal wastewater treatment plant was acclimatized in the synthetic brewery wastewater. The microbes capable of degrading this wastewater were analyzed by a combination of microscopy, spread plating, and Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and identified as *Acinetobacter sp.*, *Enterobacter sp.* and *Candida sp.* From the pure culture batch experiments, it was determined that *Enterobacter* could degrade glucose and maltose but no ethanol, while *Acinetobacter* and *Candida* could degrade all three carbon sources. In mixed culture batch experiments, *Enterobacter* was dominant in degrading the sugar concentrations to levels permissible for *Acinetobacter* to degrade ethanol. PCR-DGGE was found to be effective in identifying the dominant species but selective carbon source plating was required to determine viability and track the population dynamics. Kinetic experiments were carried out in a semi-continuous, self-cycling fermentation process using the defined mixed culture in media containing glucose and various initial concentrations of ethanol and maltose. The overall rate of substrate removal was attributable to both the suspended culture and the biofilm formed during the process. A rate expression was developed for this system for the range of substrate concentrations tested. The data indicated that substrate removal by the suspended culture

was a function of only the biomass concentration. However, substrate removal by the biofilm was found to be limited to the surface cells and determined to be a function of substrate concentration only.

RÉSUMÉ

L'enlèvement de multiples substrats par une culture mixte définie a été étudié lors d'un procédé de traitement des eaux usées de brassage. Cette étude fut menée en utilisant un système réacté en discontinu ou en semi-continu de type fermentation auto-circulatoire (self-cycling fermentation SCF). Les opérations en discontinu ont été menées en utilisant un effluent synthétique de brasserie composé de glucose, d'éthanol, et de maltose à titre de source de carbone. De la boue activée provenant d'une usine de traitement municipal des eaux usées a été introduite à l'eau usée synthétique. Les microbes pouvant dégrader cette eau usée ont été analysés en combinaison par microscopie, par mise en culture, et par électrophorèse en gel de gradient dénaturant en réaction en chaîne de polymérase (PCR-DGGE) et identifiés comme étant *Acinetobacter sp.*, *Enterobacter sp.*, et *Candida sp.* À partir des expériences en discontinu de cultures pures, il a été déterminé que l'*Enterobacter* était dominant pour dans la dégradation des sucres à des niveaux acceptables pour que l'*Acinetobacter* puisse dégrader l'éthanol. Le procédé PCR-DGGE s'est avéré être efficace pour l'identification des espèces dominantes, alors que la mise en culture spécifique des source de carbone s'est avérée nécessaire à la détermination de la viabilité et pour la localisation de la dynamique des populations. Les expériences cinétiques ont été menées en semi-continu en système auto-circulatoire de fermentation en utilisant une culture mixte en solution de glucose à des concentrations initiales variées d'éthanol et de maltose. Le taux d'enlèvement du substrat était attribuable à la culture en suspension et au mucilage se formant durant le procédé. Une relation des taux du système à été développée pour le type de substrat étudié. Les données indiquent que le taux d'enlèvement des substrats par une culture en suspension

était une fonction de la concentration de la bio-masse. Cependant le taux d'enlèvement des substrats par le mucilage était causé en grande partie par les cellules de surface, fonction seule de la concentration du substrat.

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NOMENCLATURE

ABS_{Sample} = absorbance reading at 635 nm of the sample

$ABS_{Standard}$ = absorbance reading at 635 nm of the standard

$CONC_{Standard}$ = glucose concentration of standard, g L^{-1}

$CONC_{Sample}$ = glucose concentration of the sample, g L^{-1}

BOD_5 = Biochemical oxygen demand, g L^{-1}

D_1 = Dissolved oxygen concentration of diluted sample immediately after preparation, g L^{-1}

D_2 = Dissolved oxygen concentration of diluted sample after 5 day incubation at 20°C, g L^{-1}

P = decimal volumetric fraction of sample used

B_1 = Dissolved oxygen concentration of seed control before incubation, g L^{-1}

B_2 = Dissolved oxygen concentration of seed control after 5 day incubation, g L^{-1}

f = ratio of seed in diluted sample to seed in seed control

$r_{overall}$ = overall rate of substrate removal, g L^{-1} h^{-1}

r_{susp} = rate of substrate removal due to the suspended culture component, g L^{-1} h^{-1}

$r_{biofilm}$ = rate of substrate removal due to the biofilm component, g L^{-1} h^{-1}

k_f = rate constant due to the biofilm contribution, h^{-1}

T_m = dimensionless factor representing the normalized biofilm thickness

S = substrate TOC concentration in reactor samples, g L^{-1}

r_g = rate of bacterial growth, g L^{-1} h^{-1}

μ = specific growth rate, h^{-1}

X = concentration of microorganism, g L^{-1}

Y = maximum yield coefficient, g g^{-1}

r_{su} = substrate utilization rate, g L^{-1} h^{-1}

K' = growth rate constant, h^{-1}

k_s = growth rate constant, h^{-1}

S_{in} = initial TOC concentration, g L^{-1}

S_f = final TOC concentration, g L^{-1}

t = cycle time, h

CHAPTER 1

INTRODUCTION

The production of beer has a long history dating back to 4300 B.C. by the Babylonians with around 20 varieties of beer (Raley, 1998). Throughout this period to modern day times, the production of beer has spanned most of the world in different cultures including Africa, North America, South America, Japan, China, Russia, Egypt, and the European communities. Changing economics and the availability of improved technologies have resulted in the reduction of the number of breweries in the European Economic Community (EEC) by 50% during the period from 1957 to 1978, while the quantity of beer being produced increased by 87% to 23 billion liters (Barnes *et al.*, 1984). In fact, the year 1997 saw the production of beer exceed 100 million barrels (1.6 billion liters) worldwide per year by a single company, Anheuser-Busch (Anheuser-Busch, 2002). Clearly, the production of beer is a growing industry.

With this large production of beer also comes a large generation of wastewater. It has been reported that the volume of brewery wastewater generated can be as high as seven times the volume of beer produced (Asahi Breweries, 2001). Therefore, a large brewery with an annual output of 100 million liters or more of beer can generate more than 700 million liters of wastewater per year requiring treatment. In this chapter, typical brewery wastewater characteristics, the environmental impact this waste contributes, the environmental discharge criteria and the consequences of non-compliance are discussed.

Also included in this introductory chapter is an overview of the content and organization of this thesis.

1.1 Brewery Wastewater Characteristics

Brewery wastewaters contain highly biodegradable, but complex organic mixtures. They are characterized by high biochemical oxygen demand (BOD) and total suspended solids (TSS) concentrations, as well as wide variations in flow and composition. Variations are highly dependent on whether the effluents are generated from the brewing or the packaging stages, where production rates vary independently of one another. Brewing stages produce wastes low in flow, neutral in pH and high in alcohol, carbohydrate and protein. Packaging sections produce high flow, high pH, and low concentration wastes composed primarily of residual beer and caustic bottle cleaning solutions (LeClair, 1984). Residual beer is generated as a result of spilled beer, beer rejected during improper filling and packaging, returned beer that has either expired or deemed unacceptable, exploded bottles from the pasteurization line as well as from flushing of pipe lines, all of which contribute to the combined discharge. In addition to these flow and concentration variations that occur during the five days of production, there are also two days of the week (the weekend) where, in many breweries, there are negligible amounts of wastewater produced.

This high variability in the BOD and flow make it a difficult wastewater to treat biologically. Table 1.1 contains a summary of typical wastewater characteristics from a

Table 1.1: Typical industrial brewery wastewater characteristics

Characteristic	Range	(Reference)
BOD, g L^{-1}	0.6 – 4.0	(Sheppard *et al.*, 1980)
TSS, g L^{-1}	0.2 – 2.6	(LeClair, 1984)
Ethanol, g L^{-1}	0.4 – 1.69	(Stadbauer *et al.*, 1994)
pH	4.5 – 11	(Sheppard *et al.*, 1980)
Total Kjeldahl Nitrogen, g L^{-1}	0.0192 – 0.0692	(Barnes *et al.*,1984)
Orthophosphate (PO_4^{3-}), g L^{-1}	0.002 – 0.06	(Ling and Lo, 2001)

commercial brewery. BOD levels can range from 0.6 to 4.0 g L^{-1} (Sheppard *et al.*, 1980). These large variations can cause sudden shocks to a biological treatment process and can affect the treatment and solids separation efficiency. Nutrients such as nitrogen and phosphorus, although present from cleaning agents and from some ingredients used in beer production, are low in concentration ranging from 0.0192 to 0.0692 g L^{-1} and 0.002 to 0.06 g L^{-1} for total Kjeldahl nitrogen and phosphorus respectively. These are essential nutrients in a biological treatment process and therefore, must be supplemented to the wastewater prior to its treatment. Usual sources of nitrogen and phosphorus include fertilizer grade urea and di-ammonium phosphate respectively. These nutrients are usually added to the wastewater following the approximate ratio of BOD:N:P concentrations of 100:5:1 (Department of Environmental Protection, 1999).

1.2 Environmental Impact

Untreated, direct discharge of brewery wastewater into the aquatic environment can lead to eutrophication of bodies of water into which these wastes are discharged. The high concentrations of organic substances deplete the water of oxygen as they decompose, also giving rise to noxious odours. Excess nutrients of nitrogen and phosphates in the wastewater stimulate plant growth, and cause an explosive growth of algae. These algal blooms often produce toxins in addition to unpleasant odours. Ultimately, the death of the algae results in further release of nutrients for other microorganisms to consume. The result of which is that the net respiration rate exceeds

the net photosynthesis rate and the concentration of oxygen in the water is depleted leading to death of aquatic life (Bailey and Ollis, 1986).

1.3 Environmental Legislation

A treatment process, whether aerobic or anaerobic, must be capable of reducing brewery wastewater pollution to permissible levels for discharge. The levels to which BOD must be reduced depend on whether the wastewater is to be further treated or whether it is to be discharged directly into a body of water. According to pretreatment bylaws in the city of Barrie, Ontario, a reduction of brewery wastewater BOD to below 0.3 g L^{-1} is required for discharge to municipal treatment plants for further treatment without penalty (Sheppard *et al.*, 1980). For direct discharge into the environment, effluents from breweries must have a BOD below 0.05 g L^{-1} (World Bank, 1998).

In addition to BOD reduction, it is necessary that the wastewater treatment plant also be capable of producing a clarified effluent from an efficient solid-liquid separation process. Biological material consists of organic components and if carried over into the final discharge effluent due to poor separation, the effluent total BOD concentration will, in effect, be increased to an unacceptable level. Consequently, the TSS concentration should be limited to 0.35 g L^{-1} for a pretreatment standard (Sheppard *et al.*, 1980) and 0.05 g L^{-1} for direct discharge limit (World Bank, 1998).

In the United States, minimum national standards after secondary wastewater treatment in the Clean Water Act are stipulated by the US Environmental Protection Agency, and state that BOD, TSS, and pH should be no greater than 0.03 g L^{-1}, 0.03 g L^{-1}, and in the range of 6.0 to 9.0 respectively (Metcalf & Eddy, 1991).

1.4 Enforcement and Fines

More stringent inspection and enforcement by the government would provide incentives for developing better pretreatment processes for brewery wastewater, as well as wastewaters from other industries and improve compliance. Compliance with the guidelines for discharge limits is not strongly enforced in the province of Quebec and violators have rarely been fined (Nantel, 1995).

In the United States, although the annual discharge permit fees for industries are capped at $10,000 administrative fines for industries that are in non-compliance can be substantial and detrimental to the company from a public relations viewpoint. Success of compliance to environmental limits is dependent on city level enforcement because violations directly impact the municipal treatment plants. For example, in the city of Oxnard, California, a company can be charged up to:

- $2000/day for failure to furnish a self-monitoring report,
- $3000/day for failure to comply in a timely manner to any compliance schedule ordered by the city manager,

- $5000/day for discharges in violation of the standards or limits or permit conditions,
- $10/gallon for discharges in violation of any suspension, or cease-and-desist orders.

In essence, the city has the authority to shut down a non-complying plant (City Council of the city of Oxnard, 1999).

1.5 Summary

The annual production of beer is increasing, while restrictions on discharge limits of wastewater are likely to become increasingly stringent. Therefore, further research into improving pretreatment processes would be of benefit to industry.

The general objective of this research is to obtain an improved understanding of the treatment processes in order to provide information to improve control of current treatment processes making their operations more predictable, less costly to operate and less subject to violation.

The approach taken in this research was to review the current status of brewery wastewater treatment technologies and identifying areas where additional study could provide valuable information to industry. As part of this research the study of the microbiology was conducted including the identification and the behaviour characteristics of each of the dominant microbes under various conditions. Finally, a

kinetic model was developed for substrate removal in a Self-Cycling Fermentation process using synthetic brewery wastewater.

This thesis consists of eight chapters. This introductory chapter contained an overview of the brewery wastewater characteristics, associated environmental problems, the environmental criteria for treated wastewater and consequences of failure to comply. Chapter 2 includes a literature review of the systems currently studied for treatment of brewery wastewater, the methods currently used for identification and enumeration of mixed culture consortium, and the research studies on Self-Cycling Fermentation. Chapter 3 provides a list of detailed objectives of this research work. Materials and methods of the experimental part of the project are described in Chapter 4. The identification of the dominant microbes in the mixed culture used in this research is presented in Chapter 5. Chapter 6 explores methods in characterizing the dominant microbes with respect to time to monitor population dynamics of both pure and mixed culture batch experiments. The results and discussion of semi-continuous experiments and the development of a kinetic model for substrate degradation in semi-continuous experiments are presented in Chapter 7. Finally, Chapter 8 contains the conclusions, original contributions, and recommendations for future work.

CHAPTER 2

LITERATURE SURVEY

The pertinent literature describing the currently studied anaerobic and aerobic processes for biological pretreatment of brewery wastewater is presented. Methods for the identification and enumeration of the dominant microorganisms are reviewed and the significance and scope of this Ph.D. research study are discussed.

2.1 Anaerobic and Aerobic Treatment Processes for Brewery Wastewater

Both anaerobic and aerobic processes for brewery wastewater treatment have been researched since the late 1970's. Anaerobic processes function by biologically converting organic material into methane and carbon dioxide by three reaction steps: hydrolysis, acidogenesis, and methanogenesis. Aerobic processes accomplish biological waste treatment by converting organic wastes into carbon dioxide and biomass material (Metcalf & Eddy, 1991). Each process has both advantages and disadvantages and these are presented in addition to a review of the treatment systems described in the literature. The anaerobic processes include the anaerobic fluidized bed (AFB) reactor process, the upflow anaerobic sludge blanket system (UASB), and the anaerobic digestion-ultrafiltration (ADUF^{R}) system. The Deep-shaft reactor, jet-loop reactor, and a fixed film sequencing batch reactor are alternative aerobic processes to the standard activated sludge system. Since the use of BOD and COD vary in the literature, an average

BOD:COD ratio of 0.6 was used to normalize the values for comparison when no correlation was provided.

2.1.1 Anaerobic Treatment Processes

2.1.1.1 Anaerobic Fluidized Bed Reactor (AFBR)

Several researchers have investigated the use of AFBR for the treatment of brewery wastewater. Using a laboratory-scaled AFBR, Toldrá *et al.* (1987) treated brewery wastewater with a total chemical oxygen demand (TCOD) ranging from 0.86 – 2.5 g L^{-1} and an organic loading rate (OLR) of 0.21 – 0.50 g COD L^{-1} h^{-1} at 35°C. At hydraulic retention times (HRT) of 8 h, only 50% TCOD removal efficiency was achieved. In addition, a temperature reduction to 20°C was investigated which resulted in a reduction in the COD removal efficiency by 10 – 25 %. Anaerobic reactors work optimally at temperature ranges of 30 to 38°C for mesophilic microorganisms as demonstrated by (Metcalf & Eddy, 1991) and Toldrá *et al.* (1987).

Better TCOD removal efficiencies were achieved by Tanemura *et al.* (1992) in their laboratory-scale AFBR. Influent brewery wastewater of 4.7 g TCOD L^{-1} and an OLR of approximately 0.1 g COD L^{-1} h^{-1} was treated at 37°C (310 K) during a 25-h HRT. At this optimal temperature, a higher TCOD removal efficiency of 96% was obtained.

AFBR systems require lengthy periods of time for the microbial population to become established on the support material and higher temperatures are required for this process. The reactor system used by Tanemura *et al.* (1992) required three months for the stabilization of sludge on a support material used in the fluidized bed reactor at 37°C (310 K). One advantage of the slow growth rate of anaerobic systems and the low growth yield is that very little organic waste is utilized for the synthesis of new cells. Hence sludge generation is low at approximately 0.07 to 0.13 g suspended solids g^{-1} BOD removed (UNEP, 1994). After dewatering, this sludge would be suitable for landfill, composting or application on land (Metcalf & Eddy, 1991).

Yongming *et al.* (1993) was able to reduce the startup period to 35 days at a reaction temperature of 25°C. An initial effluent range of TCOD of 2.0 –3.0 g L^{-1} was treated at an OLR of 1.12 – 1.22 g COD L^{-1} h^{-1} with a 2.5-h HRT and achieved 85% removal efficiency. However, the regiment of startup in which the system had to adhere was rigorous to achieve these results. The regiment started with initial flushing of the support material with tap water, followed by addition of 1.7 g volatile suspended solids per L^{-1}, then addition of wastewater had to be low in COD loading and have a long HRT profile. The initial bed expansion required control to 40% while implementing continuous feeding and intermittent recycle during the initial 15 days. At the first observance of biofilm growth, the height of the bed had to be increased gradually, and the recycle quantity had to be reduced accordingly. This would be impractical in a large-scale reactor system.

Anaerobic systems are highly susceptible to fluctuations in temperature, pH and loading. pH of the reactor system must be maintained between 6.6 to 7.6 in order for operational stability of the reactor to be maintained (Metcalf & Eddy, 1991). Methanogens are unable to function below a pH of 6.2, and consequently the concentration of volatile fatty acids (VFA) is increased in the effluent stream (Yongming *et al.*, 1993). The alkalinity should be in the range of 1.0 – 5.0 g L^{-1} and the VFA should be below 0.25 g L^{-1} during proper operation (Metcalf & Eddy, 1991). Variations in these conditions can cause destabilization of the reactor system, causing potential odour problems and often lengthy restabilization times (UNEP, 1994).

2.1.1.2 Upflow Anaerobic Sludge Blanket (UASB)

The upflow anaerobic sludge blanket (UASB) is also referred to as the upflow sludge blanket reactor (USBR) in the literature. The difference between this system and the AFBR reactor is that biologically formed granules and particles are used in the USAB instead of a biofilm-covered support medium as in the case of AFBR. With the use of the UASB reactor, Yan and Tay (1996) treated brewery effluent with TCOD levels of 2.03 g L^{-1}, an OLR of 0.51g COD L^{-1} h^{-1}, a HRT of 4 hours, temperature of 21.8°C and a pH of 7.1, effectively to obtain a removal efficiency of 89%. However, the formation of full-grown granules with high activity and good settleability in wastewater treatment required 6 months for start-up. Similar to the AFBR process, start-up times are quite lengthy.

Craveiro *et al.* (1986) had achieved a similar removal efficiency of 84% for a brewery wastewater with an initial TCOD 2.55 g L^{-1} value, an OLR of 0.02 g COD L^{-1} h^{-1}, within a HRT of 6 h at 35 °C (308 K) using a pilot-scale UASB reactor. Problems with temperature control equipment caused the temperature to rise from 35°C to 47°C. Return of the temperature to 35°C (308 K) required 22 h, and the system was not able to resume effluent treatment until two days later. Early detection of the temperature offset, circumvented the complete destabilization of the reactor system. However, the stability of the granules in the event of periods of downtime was not investigated.

Activated sludge seed was investigated as an alternative to digested sewage sludge seed in a lab-scale USAB reactor because of its considerable amounts of methanogenic bacteria and little sand or soil presence (Cronin and Lo, 1998). After an acclimatization/incubation period of three weeks in an airtight container, the reactor was seeded with both acclimatized and unacclimatized activated sludge equivalent to 5.93 g VSS L^{-1} for startup in the USAB reactor. The system was considered completely stabilized after 101 days, and with a HRT of 1.5 days, the reactor system treating an initial TCOD concentration of 2.51 g L^{-1} and an OLR of 0.068 g COD L^{-1} h^{-1}, was capable of COD removal of 82 ±9%. Several sludge loading rates (SLR) were tested and it was determined that SLR less than 0.02 g COD g^{-1} VSS h^{-1} was acceptable but SLR greater than 0.065 g COD g^{-1} VSS h^{-1} was too high resulting in low COD removals. Use of an activated sludge as seed worked but results were not significantly improved in terms of startup times required, and SLR appeared to be stringent.

Aside from the low cellular growth rate, anaerobic processes can produce methane gas that can then be recovered and used at a plant. Cronin and Lo (1998) achieved methane yields of 0.013 L CH_4 L^{-1} h^{-1} in the USAB reactor while Yan and Tay (1996) expressed the methanogenic activity as 0.038 g CH_4-COD g^{-1}VSS h^{-1}. Methane produced can have a purity of 85% and can be used by the plant for fuel (UNEP, 1994).

2.1.1.3 Anaerobic Digestion – Ultrafiltration ($ADUF^R$)

Another anaerobic process incorporates a modification of the conventional anaerobic digester by the addition of an ultrafiltration membrane to achieve retention of the biomass (Strohwald *et al.*, 1992; Fakhru'l-Razi, 1994). This concept resulted in treatment at enhanced loading rates and improved process efficiency. Strohwald *et al.* (1992) applied the high rate $ADUF^R$ (Anaerobic Digestion - Ultrafiltration) process to treating brewery wastewater on a pilot-scale for 80 days. After 50 days for sludge acclimatization, the system was able to accept increased loading rates. Operating at 35°C, with an average COD concentration of 6.7 g L^{-1} and an OLR of 0.63 g COD L^{-1} h^{-1}, the COD concentration was reduced by between 96% to 99% at HRTs of 12 to 19.2 h. It was also found that sludge that had been dormant for two months could be reactivated within seven days to achieve COD reductions of 92.7% to 98.5%, provided that the microbes had not been stressed before the dormant stage. This required a gradual decrease in flowrate levels to zero over a two-day period. This may

not always be possible in the case of an upset in the system unless the shut-down is planned.

2.1.1.4 Summary of Anaerobic Systems

In summary, anaerobic systems were found to be efficient at treating high strength COD wastewater of concentrations greater than 2 g L^{-1} and achieved removal efficiencies greater than 82%. Although anaerobic systems during proper operation have lower energy consumption, low sludge generation, and a fuel product for use, high COD concentrations must be used for treatment (UNEP, 1994). Deviation from the range of optimal conditions for an anaerobic system results in destabilization of the system. Although the possibility of system recovery was reported in the literature, the criterion for its success was based on a gradual decrease of the brewery wastewater for the duration of several days. In practice, unless a shutdown is planned, upsets in the system that are highly possible would crash the system, leading to high expenses or even shutdown of the plant where compliance enforcement is strict. The lengthy startup periods and the possibilities of destabilization coupled with the potential lengthy restart periods make anaerobic processes less attractive for the brewery wastewater treatment application.

2.1.2 Aerobic Treatment Processes

2.1.2.1 Deep Shaft Reactor (DSR) and Lab-Scale Jet Loop Reactor

Molson's brewery in Barrie, Ontario installed a full-scale deep shaft effluent treatment system after conducting successful pilot-scale studies in the late 1970's (Sheppard *et al.*, 1980, LeClair, 1984). The reactor system was both space and energy efficient where the DSR consisted of two concentric tubes. Injection of compressed air into the shafts caused a density gradient promoting a high velocity circulation of the mixed liquor suspension down through the inner shaft and up through the concentric region created by the two shafts promoting mixing. With an average OLR of 6.34×10^{-4} g COD L^{-1} h^{-1}, average influent TCOD concentration of 2.9 g L^{-1}, a HRT of 5 h, 94% TCOD removal efficiency was achieved. The treated effluent COD was consistently less than 0.175 g L^{-1}. Response of the DSR system after a plant shut down was also good with 97% removal efficiencies achieved after a short period (Sheppard *et al.*, 1980).

Similar removal efficiencies were also achieved by Bloor *et al.* (1995) using a laboratory-scale jet loop reactor that has a similar configuration to that of the deep shaft reactor. Treating an initial TCOD concentration of 6.0 – 8.0 g L^{-1} at 35-39°C, an OLR of 49.7 g COD L^{-1} h^{-1} and HRTs between 3.7h – 4 h, 97% TCOD removal efficiencies were obtained.

2.1.2.2 Membrane-Aeration Bioreactor (MABR)

Brindle *et al.* (1999) investigated the use of hydrophobic dead-end hollow fibers for the aeration of a biofilm that was attached to the shell side of the fibers for use in treating brewery wastewater. With a TCOD range of 1.72 – 3.07 g L^{-1} both pilot-scaled completely mixed MABR and plug flow MABR configurations were investigated. The OLRs ranged from 0.18 – 1.26 g L^{-1} h^{-1}, with HRTs ranging from 1.4 – 10.7 h for a completely mixed MABR setup. For the plug flow MABR setup, OLRs ranged from 0.38 – 1.41 g L^{-1} h^{-1}, with HRTs ranging from 1.8 – 3.6 h. The reactor temperature was maintained at 25.7 ± 0.5°C. TCOD removal efficiencies of 69 ± 13% and 88 ± 15% for the completely mixed and plug flow MABR configurations respectively. Slough-off of the biofilm in the mixed liquor contributed to the final effluent TCOD, which reduced the TCOD removal efficiency values. Better cleaning practices of the filters improved the overall TCOD removal to above 82% for the completely mixed MABR.

Startup conditions were investigated for the development of the biofilm for both configurations (Brindle *et al.*, 1999). After 36 h of recirculating wastewater to the MABR, visible biofilm growth was detected. Using the plug flow configuration, biofilm development on the fibers by the third day achieved 74% removal efficiency with a HRT of 3.6 h and by the ninth day with a three hour HRT, 93% removal efficiency of TCOD was achieved. Development of the biofilm through the completely mixed flow configuration was found to be less successful and stabilization of the biofilm did not occur until the tenth day.

Shock loading in terms of influent concentration and OLR were also investigated in the MABR. With a three-fold increase in influent concentration over two HRTs, the dissolved COD removal efficiency settled to a low of 64%, but the system completely recovered within three HRTs of the shock load. A two-fold increase in organic loading rate (OLR) caused deterioration in the dissolved COD removal efficiency and after four HRTs after the increased OLR, the removal efficiency increased to only 70%. The robustness of the system was more affected by OLRs but this could be circumvented by the use of an equalization tank prior to treatment.

2.1.2.3 Fixed Film and Suspended Growth Sequencing Batch Reactors (SBR)

Ling and Lo (1999) investigated both suspended-growth and attached-growth SBR systems. Wastewater collected from a local brewery where the COD concentrations ranged between 1.04 – 4.52 g L^{-1}, was used as the initial feed to the SBR systems. At 20 ± 2°C, the OLRs varied between 0.08 – 0.79 g L^{-1} h^{-1}, with HRTs ranging between 13.4 – 145 h. TCOD removal efficiencies were on average 97.2% and 89.6% for the suspended–growth and attached-growth SBR systems respectively. By the better TCOD removal efficiencies observed, Ling and Lo determined that suspended-growth SBR systems were better than the attached-growth SBR systems.

In another fixed film SBR, brewery wastewater collected from Molson Brewery Ltd., in Vancouver, B.C. was treated employing a feedback control strategy (Nguyen *et al.*, 2000). The strategy was based on the correlation between dissolved oxygen

concentrations and respiration rates in the biomaterial. Computer detection of declining respiration rate, as indicated by an increasing dissolved oxygen concentration, was correlated to the depletion of a limiting substrate. This automatically triggered the removal of a portion of the treated wastewater and supplementation with fresh brewery wastewater for treatment in the next cycle.

Temperature effects were studied and removal efficiencies of TCOD were 81% after three hours at 25°C with an OLR of 0.40 g L^{-1} h^{-1} and 74% after 1.5 h at 35°C with an OLR of 1.11 g L^{-1} h^{-1}. An increase in organic loading with respect to increased total operating volume from 0.8 L to 1.2 L of the reactor resulted in TCOD removal efficiencies being maintained between 83 to 87%. This highlighted the benefit of the feedback control system and the adaptability of the system whereby the control system easily adjusted the residence time to compensate for changes in loading rate. Nguyen *et al.* (2000) also found that operation of the reactor system under excess ammonia-nitrogen conditions promoted the increase in sludge content in the packed bed of the reactor, eventually plugging and flooding the bed. Therefore, for a packed bed configuration, nitrogen-limiting conditions were recommended.

While this process was found to be adaptable to changes in parameters and the system was treated as a black box, no study was made with respect to population dynamics or kinetic modelling for substrate removal.

2.1.2.4 Summary of Aerobic Systems

In summary, aerobic systems were found to have the advantage of being more robust than anaerobic systems. Recovery from shock loads and upsets took less time in comparison to anaerobic systems. In the worst case scenario, restart would be in the order of hours or days, not months. The operation of aerobic systems is optimal in the temperature range of 25 – 35°C (UNEP, 1994). Therefore, energy requirements for heating would be substantially reduced for aerobic treatments when compared with anaerobic systems that must operate above 30°C for optimal treatment. Although sludge generation is greater than that of anaerobic systems, recycle of a portion of the settled cells back into the reactor maintains the desired treatment efficiency in the reactor, and the remainder of the settled cells are either sent to a digester or dewatered (Metcalf & Eddy, 1991).

While aerobic system were found to be capable of greater than 82% TCOD removal efficiencies at steady state conditions, these efficiencies are somewhat lower than the over 90% efficiencies found in the anaerobic systems.

Since brewery wastewater is known to be variable, the present research focused on an aerobic treatment process because of the robustness and adaptability of aerobic systems. The feedback control strategy on a semi-continuous reactor was shown to provide repeatable and adaptable treatment on a variable wastewater concentration (Nguyen *et al.*, 2000). Therefore, this strategy was applied to a semi-continuous reactor

system using suspended-growth biomass for the investigation of the kinetics of a defined mixed culture on a synthetic brewery wastewater. Identification of the dominant microorganisms that are present in the treatment process may also provide some insight into the process kinetics of the system for the purpose of enhanced control and improved substrate removal efficiencies.

2.2 Enumeration and Identification of Microorganisms in Mixed Cultures

2.2.1 Brewery Wastewater Treatment Processes

Few studies have characterized microbial communities of activated sludge used for aerobic brewery wastewater treatment. Using microscopic inspection, LeClair (1984) described the presence of a diverse microflora that contained flocculated bacterial colonies and protozoa, including ciliates, both free swimming *Aspidiscus sp.*, *Vorticella sp.*, amoeba, and paramecium in the Deep Shaft reactor treatment system.

In the aerobic jet loop activated sludge reactor treating brewery wastewater used by Dilek *et al.* (1996), colonies grown on CGY agar (Pike *et al.*, 1972) inoculated with acclimatized activated sludge were examined for colony morphology and pigmentation. Gram staining as well as two other staining techniques, including Neisser stain (for distinguishing poly-P) and Sudan Black (for distinguishing poly-B-hydroxybutyrate) were also used to characterize the different species present. Identification of the bacterial species was achieved using the Analytical Profile Index (API) method and associated

APILAB computer software. This method characterized the activated sludge as containing mainly aerobic bacteria, all belonging to *Pseudomonas* sp. and facultative anaerobes in small numbers, including *Klebsiella oxytoca, Klebsiella pneumonia ssp.*, *Vibrio fluvialis* and *Aeromonas hydrophila.* No enumeration methods were used during the experiment but once the isolates were obtained, respirometric activity tests were conducted on these isolates for determination of their individual activity on glucose and brewery wastewater. *P. cepacia* was found to be the most dominant and active aerobic bacteria in the mixed culture treating brewery wastewater in the jet loop reactor system.

Methods of enumeration and identification that have not been used to examine brewery wastewater treatment are numerous and will be limited to those studies that have been used for characterizing activated sludge in other aerobic wastewater treatments. These methods include a range of biological analyses to molecular analyses where the genetic material including deoxyribonucleic acids (DNA) are used to identify the microrganisms.

2.2.2 Activated Sludge Treatment Processes

In the characterization of phenol-degrading bacteria in activated sludge, Okada *et al.* (1991) used the plate count methods by using the same medium as the wastewater and solidifying this with agar. Total colony counts were obtained from dilutions of the activated sludge flocs. This method proved to be inadequate since this was a mixed culture. In performing a total colony count analysis for mixed cultures, use of dilution to

obtain the proper concentration for spread plate purposes may dilute out slower growing microorganisms. Not unexpectedly, no clear relationship could be identified between colony counts and phenol degrading activity.

Watanabe *et al.* (Apr. 1998) performed bioaugmentation experiments whereby *Pseudomonas putida* BH and *Comamonas sp.* strain E6 were individually added to an activated sludge treating phenolic wastewater in separate experiments. Using a set of strain-specific primers particular for a *gyrB* gene fragment of the introduced bacteria, Polymerase Chain Reaction (PCR) amplification was performed and quantification of the electrophoresced PCR product was done by densitometry. Total colony count data was obtained from plating of the samples on Luria-Bertani (LB) agar plates. In this case, because phenol was a difficult substrate to degrade, the results from total plate counts on LB agar were not significantly different from the relative quantitation obtained from the densitometric results of the PCR products of each of the introduced bacteria.

In a separate study, Watanabe *et al.* (Nov.1998) isolated DNA from phenol-digesting activated sludge with no bioaugmentation, for detecting, isolating and determining the functionally dominant phenol-degrading bacteria in activated sludge. A specific gene encoding the largest subunit of multicomponent phenol hydroxylase was amplified by using PCR. The PCR products were subjected to Temperature Gradient Gel Electrophoresis (TGGE) to illustrate the appearance of major bands detected after phenol acclimatization. These bands were excised from TGGE and sequenced and identified the principal phenol degrader in activated sludge as *Valivorax paradoxus*.

A combination of molecular analyses and cell counts were used in analyzing activated sludge samples taken from a municipal sewage plant and a sequencing batch reactor (SBR) treating dairy-sewage wastewater (Manz *et al.*, 1994). Specific oligonucleotide probes targeting the 16S and 23S ribosomal ribonucleic acid (rRNA) of defined phylogenetic groups of bacteria were used. Cell counts determined by a combination of in situ hybridization with fluorescently-labelled oligonucleotide probes and epifluorescence microscopy allowed for detection of physiologically active cells. Bacteria from the proteobacteria belonging to the beta-subclass were dominant in the municipal sewage plant whereas cells hybridizing with a cytophaga-flavobacterium-specific probe were most abundant in the SBR. Unfortunately, samples were not taken at various intervals of time to determine how useful this method could be for population dynamics.

Muyzer and Ramsing (1995) reviewed the various molecular methods to study the organization of microbial communities. These methods included fluorescent in situ hybridization, and TGGE amongst them. A similar analysis to TGGE is denaturing gradient gel electrophoresis (DGGE). Muyzer and Ramsing found that it was promising molecular approach because the method is based on the concept that slight differences in rDNA sequences can be used as the means for distinguishing the "ribotypes" of individual microorganisms using 16S rDNA oligonucleotide primers to amplify bacterial rDNA. Instead of cloning the PCR amplified products into *E. Coli* and laboriously sequencing numerous clones, DGGE separated out the rDNA of the PCR products into bands specific to different microorganisms that could then be individually cloned.

Application of DGGE to a consortium such as activated sludge resulted in a fingerprint of DNA bands unique to the consortium. Curtis and Craine (1998) were able to use DGGE to compare the diversity of total microbial communities present in different activated sludge plants.

More recently, Muyzer conducted a review of the advancement of DGGE/TGGE systems in 1999, and reiterated that DGGE/TGGE allowed the simultaneous analysis of multiple samples allowing community changes over time to be followed. An additional benefit to DGGE is the possibility of identifying members of the microbial community by sequencing bands excised from the gel. Other fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) do not have this option. Muyzer (1999) also commented that little research had been done on quantification of band intensity from DGGE except for one research study of DGGE analysis on hypersaline microbial mats where the number of bands was an indicator of diversity and the band intensities were used for a measure of proportional abundance (Nübel *et al.*, 1999). It would appear that DGGE has the potential for being a useful tool for differentiating between the consortium of degraders present, and tracking individual degraders by monitoring the abundance of ribosomal DNA with respect to time in relation to environmental variables. This DGGE method was investigated in this research work in combination with plate count methods.

2.3 Self-Cycling Fermentation (SCF)

When disturbances occur in practice, most solutions have to be developed in a time consuming and difficult process of trial and error or through the experience of the on-site operator. However, unlike commonly used activated sludge processes, SCF incorporates a feedback control system that adjusts the residence time of the mixed liquor in the treatment process according to the treatment requirements (Nguyen *et al.*, 2000). Even with changes in the nutrient environment or influent composition, SCF results in repeatable behaviour of the microbial population, facilitating the development of an optimal treatment strategy.

SCF was developed by Sheppard *et al.* (1990) as a modification to the continuous phasing process (Dawson, 1972) used for synchronizing pure cultures of microorganisms. Continuous phasing is actually a semi-continuous process that consists of periodically diluting a microbial culture with an equal volume of fresh nutrients at a frequency equal to the cell doubling time. Before dilution, half of the reactor contents are removed so that the working volume of the reactor remains constant and the environmental conditions are consistent for each nutrient "cycle". SCF is based on the addition of a feedback control system that monitors changes in cellular respiration as reflected by the level of dissolved oxygen in the system, thus automatically adjusting the time period (or cycle time) between nutrient addition. SCF consists of three main steps (Figure 2.1). The first step is a reaction step where batch growth occurs until nutrient limitation is reached. As microbial growth occurs in the batch reactor, the dissolved oxygen level decreases as a

result of an increasing rate of microbial respiration. Once the limiting substrate is exhausted, cellular respiration slows down and the dissolved oxygen level increases abruptly. This abrupt change is the trigger point for the computerized feedback control system to initiate the second step of SCF. In the second step, one half of the cells and end products are harvested from the reactor and in the last step, a volume equivalent to the harvested volume is supplemented back into the reactor with a nutrient solution. The three-step SCF process can be repeated indefinitely.

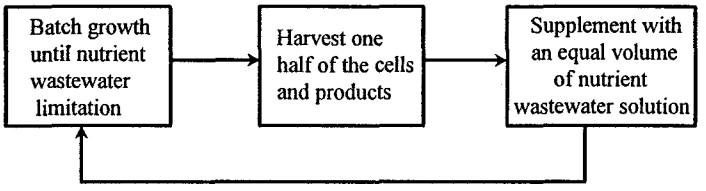

Figure 2.1 : The self-cycling fermentation process

The SCF procedure of harvesting and dosing after the depletion of a limiting nutrient in the growth medium, results in a population whose cells are for the most part, at the same stage of cell development and are behaving in the same manner at the same time. In pure microbial populations, this occurrence is referred to as growth synchrony. Since the starting microbial population in each cycle is similar and the nutrient environment is the same in each cycle, the growth of a pure microbial population will also be the same from cycle to cycle. This phenomenon is useful in determining the dynamic behaviour of a pure microbial population resulting from changes in the nutrient environment as seen from the recent research using SCF.

The process of harvesting and dosing must be accomplished in a quick manner. The use of a cyclone column reactor suits this purpose well. Liquid can be easily removed and added and the thin film of mixed liquor that swirls down the column reactor enhances aeration of the liquid. The following researchers have successfully used this type of reactor and strategy.

Sheppard *et al.* (1990) tested the metabolic response of *Bacillus subtilis* ATCC 21332 to the availability of iron and manganese and the utilization of nitrogen. It was determined that a proportionality of 920/7.7/1.0 on a molar basis of nitrogen to iron to manganese was required for an optimal production of surfactin, a lipopeptide surfactant. In other research, it was shown that SCF was a better fermentation process in terms of inoculum effects and startup times than both the batch and the chemostat fermentations for emulsan production using *Acinetobacter calcoaceticus* RAG-1 (Brown *et al.*, 1991). *Acinetobacter calcoaceticus* RAG-1 was also used to degrade limiting concentrations of hexadecane, 1-hexadecene, or 1-chlorohexadecane, in order to determine which carbon substrate was best utilized to produce higher emulsan concentrations (Brown *et al.*, 1992). The SCF technique provided stable and repeatable results, which concluded that hexadecane, was the carbon source that provided the highest specific emulsan yields.

Hughes *et al.* (1996) investigated SCF as a control strategy for the degradation of phenol by *Pseudomonas putida* ATCC 12633. They were successful in achieving complete substrate consumption as well as stable and repeatable treatment. Hughes *et al.* (1996) treated one pollutant with a pure culture.

Nguyen *et al.* (2000) was successful in using the SCF control strategy for treating brewery wastewater with a mixed culture in a fixed-film bioreactor. This provided the impetus in this research to characterize the population dynamics of a suspended and attached growth system using the SCF control strategy for treating a synthetic brewery wastewater of variable composition.

2.4 Significance and Scope of this Study

The process kinetics and behaviour of a mixed culture consortium in treating brewery wastewater has normally been viewed as a "black box" because of a lack of understanding of the interactions of the microbes in the consortium. Studies to predict biological treatment have been limited to chemical analyses of input and output from this black box and have not provided information on the population dynamics of a mixed culture in treating brewery wastewater. A better understanding of the relationships between the dominant microbes in the mixed culture and how they relate to the degradation of key components, such as sugars and ethanol in the brewery wastewater could help in designing better control procedures to overcome and troubleshoot problems that may arise during operation of a treatment system.

As described in the literature, brewery wastewater has been treated by a variety of reactor systems including both suspended and attached growth formats. With a focus on aerobic systems, the self-cycling fermentation process with feedback control strategy showed promise as an experimental system to study the contribution to treatment by

suspended and attached growth because of its repeatability. Understanding the contributions from suspended and attached growth could shed light on the development of a more effective treatment system.

CHAPTER 3

OBJECTIVES

As government regulations regarding wastewater emissions become more and more strict, the development of more effective, manageable and economical treatment processes is desirable. A better understanding of how treatment processes respond at the level of the microbial populations will provide information that can be used to improve control of current treatment plants, making their operations more predictable and less likely to violate local effluent discharge criteria.

Aerobic treatment processes were found to be more robust and adaptable than anaerobic processes in handling the variability in concentration that is characteristic of brewery wastewater. In particular, one study by Nguyen *et al.* (2000) used a feedback control strategy applied to a fixed-film bioreactor to produce repeatable cycles of treatment regardless of the influent brewery wastewater concentration. This provided the impetus to further investigate the application of the self-cycling fermentation process to a mixed culture system.

The primary objective of this research work is to obtain an understanding of the process kinetics in a self-cycling fermentation system using a mixed culture growing on a mixed substrate. The focus of the research is in identifying the dominant microbes in the mixed culture treatment system, monitoring their growth and capabilities for degradation of maltose and ethanol. In addition, the effects on treatment will be characterized as a

result of the formation of a biofilm, and the dominant microbe's shift from suspended to attached growth during the progress of treatment.

3.1 Statement of objectives

1. To identify the key microorganisms responsible for treatment of the synthetic brewery wastewater (using DGGE-sequencing, and BIOLOG).

2. To determine pure culture characteristics of the dominant microbes of the mixed culture in batch experiments treating synthetic brewery wastewater.

3. To evaluate PCR-DGGE for tracking changes in the population of a mixed culture during the semi-continuous treatment of brewery wastewater.

4. To establish a plate count method for tracking changes in the population of a mixed culture during the semi-continuous treatment of brewery wastewater.

5. To determine mixed culture interactions and degradation capacities at various ethanol and maltose concentrations during semi-continuous operation.

6. To develop a model to describe the kinetics of substrate degradation of wastewater containing ethanol and maltose and the relative contributions by microbes in both suspended and attached growth.

CHAPTER 4

MATERIALS AND METHODS

The following chapter describes the apparatus and procedures that were used throughout the research project for collection of the experimental data. Many procedures were utilized in the analysis of the wastewater and for the detection of the dominant microorganisms present. The method of preparation of the synthetic brewery wastewater as well as variations of this wastewater for the study of substrate degradability is included. Identification and enumeration of the dominant mixed culture microorganisms are identified through DNA analyses and microbiological techniques, and an enumeration method was devised for tracking these microorganisms. The batch and semi-continuous experimental set-up and a description of a typical semi-continuous experiment along with the biofilm contribution experiments are outlined. Finally, the method for determining the oxygen mass transfer coefficient of the cyclone column is described.

4.1 Synthetic Brewery Wastewater Media

In reviewing the characteristics of actual brewery wastewater as reported in the literature, as well as from analysis of samples obtained from McAuslan brewery in Montreal, Quebec, typical values of BOD, pH, ethanol, and nutrients were determined. The analyses characterized the brewery wastewater from the McAuslan Brewery as having a BOD concentration of 2.4 g L^{-1}, a pH of 6.20, a total Kjeldahl nitrogen (TKN) content of 0.0614 g L^{-1}, an ammonia-nitrogen (NH_3-N) concentration of 0.004 g L^{-1}, a phosphate content of 0.007 g L^{-1} and an ethanol content of 0.0012 L L^{-1}. Since actual

brewery wastewater can fluctuate from day to day, brewery wastewater was simulated to produce a consistent composition in which process kinetics could be tested. The synthetic wastewater was based on the following ingredients added in various concentrations to tap water: peptone, malt extract, maltose, ethanol, yeast extract as a source of vitamins to improve performance of the mixed culture, buffering salts of sodium phosphate (monobasic) (NaH_2PO_4), and sodium phosphate (dibasic) (Na_2HPO_4) for the batch experiments to maintain the pH at approximately 6.7 and as a trace phosphorus source in the semi-continuous experiments, and ammonium sulphate $(NH_4)_2SO_4$ was added as an inorganic nitrogen source.

The yeast extract, peptone, and ammonium sulphate components were constituted in a ten liter capacity carboy and then autoclaved at 121°C for three hours. It was determined in previous thermal tests for a ten liter carboy filled with eight liters of water that three hours was required to ensure that 121°C was reached at the center. After sterilization, the solution was allowed to cool overnight before addition of the remaining components that had been filter sterilized. This two step process was necessary because components such as peptones can form complexes with sugars and excess phosphates can also form complexes with sugars at high temperatures rendering these metabolically inactive (Gerhardt *et al.*, 1994). When the synthetic wastewater is completed, it is connected to the reactor set-up as the substrate source for the mixed culture. Sterilization of the synthetic wastewater was necessary in order to prevent contamination and to maintain a consistent composition of the wastewater throughout the period of experimentation. Eight liters of solution were produced each time.

Five variations of the wastewater were used in this research work and a summary of each substrate concentration is listed in Table 4.1. They are denoted as Low Maltose & Mid Ethanol, High Maltose & Mid Ethanol, Mid Maltose & Low Ethanol, Mid Maltose & High Ethanol and Mid Maltose & Mid Ethanol. All tested synthetic wastewaters had an initial pH value of approximately 6.7 and their BOD values are also listed.

4.2 Inoculum Preparation

An activated sludge sample obtained from a local wastewater treatment facility in Orange County, California was subjected to 20 cycles of complete biodegradation and re-inoculation into synthetic Mid Maltose / High Ethanol brewery wastewater for selection of the dominant microorganisms capable of treating this wastewater. Each cycle of complete biodegradation of the synthetic wastewater spanned approximately 17 h. After this acclimatization period, aliquots of this mixed culture consortium from the final cycle were frozen in glycerol at -70°C to preserve the starting culture. Cultures from this acclimatized mixed culture consortium were isolated by spread plating serial dilutions of the mixed culture on solid medium consisting of the synthetic brewery wastewater and 15 g L^{-1} of agar. Three morphologically different colonies from these mixed culture plates were then streaked out on similar solid media four consecutive times, each time from a single colony to isolate and obtain three pure cultures to be used as inocula in subsequent brewery wastewater treatment experiments. Each of the three pure cultures was also frozen in glycerol at –70°C.

Table 4.1: Synthetic Brewery Wastewater Contents

Substrate	Low Maltose & Mid Ethanol	High Maltose & Mid Ethanol	Mid Maltose & Low Ethanol	Mid Maltose & High Ethanol	Mid Maltose & Mid Ethanol
Yeast Extract (g L^{-1})	0.5	0.5	0.5	0.5	0.5
Peptone (g L^{-1})	0.15	0.15	0.15	0.15	0.15
Ammonium Sulfate (g L^{-1})	1.42	1.71	1.22	2.28	1.52
Malt Extract (g L^{-1})	1	1	1	1	1
Sodium Phosphate, mono-hydrate (monobasic) (g L^{-1})	0.05	0.06	0.04	0.08	0.05
Sodium Phosphate, Anhydrous (dibasic) (g L^{-1})	0.08	0.1	0.07	0.14	0.09
Maltose (g L^{-1})	0.43	1.72	0.86	0.86	0.86
95% Ethanol (L L^{-1})	0.00122	0.00122	0.00061	0.0028	0.00122
BOD (g L^{-1})	1.822	2.181	1.954	2.496	2.045

To ensure consistency, the final preparation of the mixed culture inoculum for use in semi-continuous experiments required growth of the pure cultures separately. Based on the optical density of the culture in each of the flasks, the pure cultures were added together to produce a final inoculum consistent in concentrations for each experiment.

4.3 Methods for Identification of the Dominant Microorganisms in the Mixed Culture

The mixed culture inoculum that was defrosted from the –70°C starter stock, was cultured in a series of enrichment steps. Batch flask experiments were conducted whereby 1 ml of the inoculum (either mixed culture or pure culture) was added to 99 ml of Mid Maltose & High Ethanol synthetic brewery wastewater. The flasks were shaken at 200 rpm on a New Brunswick Scientific Model C24 incubator shaker at 25°C. After 24 hours of growth, 1-ml samples were centrifuged at 14 000 x g and pellets of these cells were used in the Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) method and steps of ligation, transformation and sequencing of the resulting ribosomal DNA obtained from PCR-DGGE. Previously isolated pure cultures that were frozen at –70°C were also cultured in a series of enrichment steps for use in BIOLOG microplate analyses. The procedures are outlined in the following sections.

4.3.1 Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

4.3.1.1 Extraction of DNA from the mixed culture and pure cultures

One-ml samples from the 24-h mixed culture and the pure culture batch flask brewery wastewater experiments were centrifuged at 14,000 x g for three min. and the resultant pellet was re-suspended in 9.78×10^{-4} L of a 200 mM sodium phosphate buffer (pH 8.0) provided in the FastDNA® SPIN Kit (BIO101,Vista, CA). DNA was extracted by following the manufacturer's protocol for the FastDNA® SPIN Kit. The re-suspended cell mixture was transferred into tubes containing a lysing matrix, such as glass or ceramic beads, and vigorously shaken in a FastPrep® Cell Disruptor Instrument (Model FP120, Golbrook, New York) to physically break the walls of the cells. This mixture was once again centrifuged and the supernatant that contained the cell contents was added to tubes containing a protein precipitating solution. The proteins were then removed by centrifugation. A DNA binding matrix suspension was then added to the protein-free supernatant for isolation of the DNA. The DNA attached to the matrix and was then filtered and washed with a salt and ethanol wash solution. The final step of the protocol consisted of adding ultra pure water (free of DNase and pyrogen) to the matrix to elute the DNA. After centrifugation, the filtrate that contained the DNA was ready for further applications such as PCR.

4.3.1.2 PCR

The process of PCR is a series of steps that produces replicates of the template DNA. The template DNA (double stranded) is denatured to form two separate individual strands. The second step involves attachment of the selected primers (short strands of DNA) to the targeted sites on the single stranded DNA. The third step allows for primer extension whereby the primers extend to form the complementary strand of the DNA template. These three steps are repeated for usually 30 cycles to create a higher concentration of the initial DNA strand (Figure 4.1). Ready-To-Go PCR beads (Amersham-Pharmacia Biotech, Piscataway, NJ) are commercially available for the application of PCR. As specified by the manufacturer, the beads contain approximately 1.5 Units of *Taq* DNA polymerase, 1 x 10^{-2} M Tris-HCl, (pH 9.0 at room temperature), 5 x 10^{-2} M $MgCl_2$, 2 x 10^{-4} M of each dNTP and stabilizers. These were used in all PCR applications in this research.

4.3.1.2.1 PCR - Bacterial Species

For the bacterial species universal primers PRBA338f and PRUN518r which correspond to E. Coli rRNA positions 338 and 518 respectively were used to amplify the conserved 16S rDNA regions of the mixed culture DNA resulting in a product of approximately 200 bp in length. Forward primer PRBA338f targets a conserved region that flanks the V3 region of 16S rDNA from the domain bacteria, while PRUN518r is based on a universally conserved region of the small subunit rRNA gene (Øvreås *et al.*,

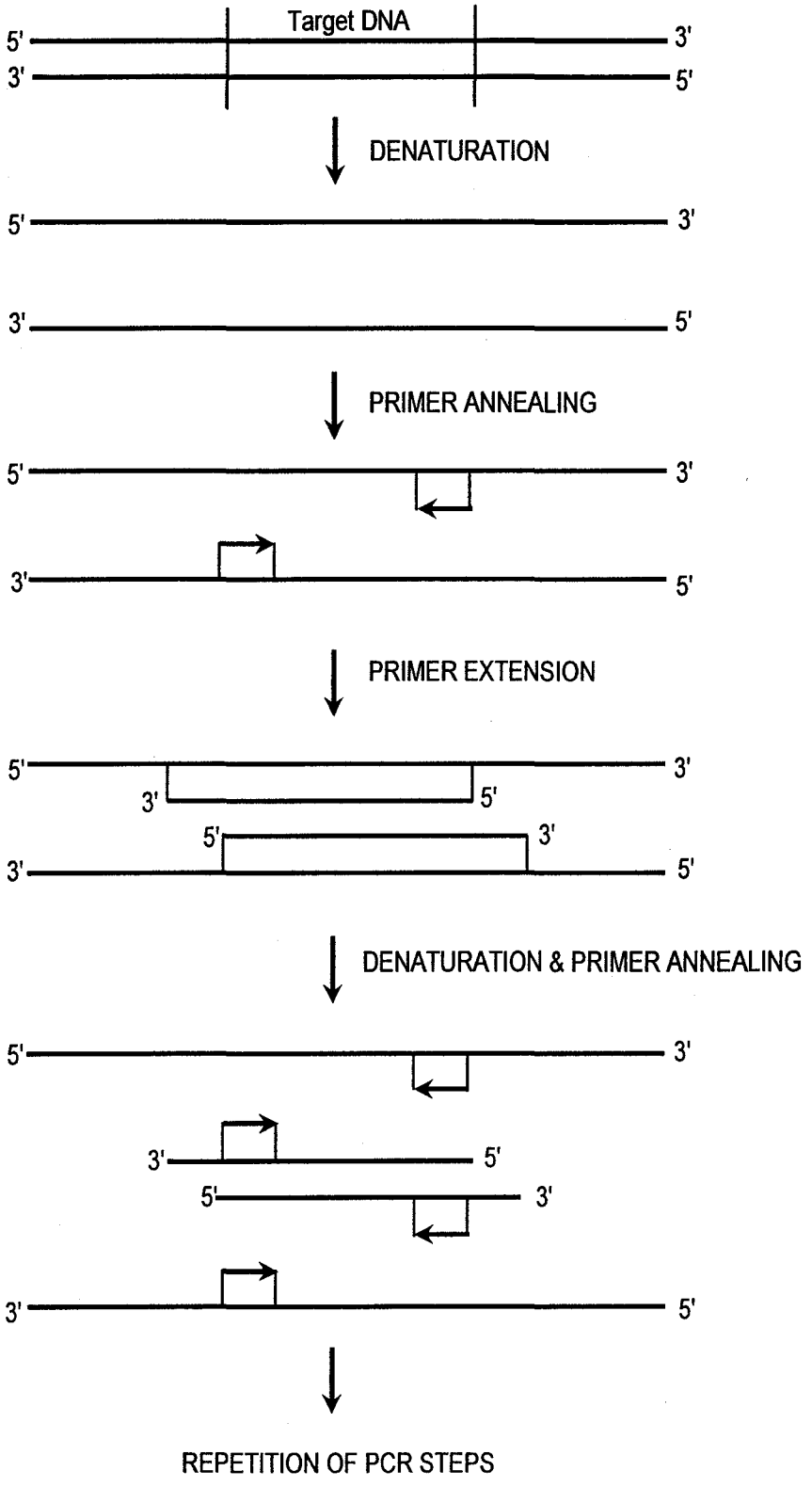

Figure 4.1: Polymerse Chain Reaction (PCR) process

1997). The sequences of base pairs for the two bacterial primers are listed in Table 4.2. A GC clamp was attached to the 5' end of PRBA338f.

This GC clamp is necessary for DGGE because it provides a sequence domain that is resistant to denaturation preventing the complete denaturation of melted DNA fragments. Ready-To-Go PCR beads with 1 x 10^{-6} L of each primer (5 x 10^{-6} M), 1 x 10^{-6} L of the DNA template (approximately 5 x 10^{-4} g L^{-1}) and deionized water for a total volume of 2.5 x 10^{-5} L was used for PCR amplification. PCR amplification was performed using a Peltier Thermal Cycler PTC-200 (MJ Research, Watertown, MA) with the following program:

92°C for 0.083 h for the initial denaturation;

30 cycles of:

92°C for 0.016 h for denaturation,

55°C for 0.0083 h for primer annealing, and

72°C for 0.016 h for primer extension;

and then one final cycle at 72°C for 0.1 h.

The amplified products were analyzed by electrophoresis in a 1.8% (w/v) agarose gel in 1 X TAE buffer at 114 V for 0.33 h for detection of 200 bp products as well as any unspecific products with the use of DNA ladders. DNA ladders contain standardized segments of base pairs of DNA that are compared to sample DNA to determine the sample DNA fragment size. Table 4.3 provides the chemical compositions of all buffers and solutions used in the electrophoresis analyses.

Table 4.2: Primers for amplification of bacterial and fungal ribosomal DNA genes

Type	Primer	Sequence	Reference
Bacteria	PRBA338f [a]	5'-ACTCCTACGGGAGGCAGCAG-3'	Øvreås *et al.*, 1997
Bacteria	PRUN518r	5'-ATTACCGCGGCTGCTGG-3'	Øvreås *et al.*, 1997
Fungal	NS1	5'-GTAGTCATATGCTTGTCTC-3'	White *et al.*, 1990
Fungal	NS8	5'-TCCGCAGGTTCACCTACGGA-3'	White *et al.*, 1990
Fungal	NS1-GC [b]	5'-CCAGTAGTCATATGCTTGTC-3'	Kowalchuk *et al.*, 1997
Fungal	NS2+10	5'-GAATTACCGCGGCTGCTGGC-3'	Kowalchuk et al., 1997

[a] GC clamp 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3' attached to 5' end of PRBA338f.

[b] GC clamp 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGG-3' attached to 5' end of NS1-GC.

Table 4.3: Chemical Compositions of Solutions used in Agarose Electrophoresis and DGGE

Solution	**Chemicals Used**	**Quantities**
50 X TAE buffer	Tris base	242 g L^{-1}
	Acetic acid, glacial	0.0571 L L^{-1}
	0.5 M EDTA* (pH 8.0)	0.100 L L^{-1}
1 X TAE buffer	50 X TAE buffer	0.020 L L^{-1}
0% Denaturing solution	40% Acrylamide / Bis	0.0002 L L^{-1}
(for 8% (w/v)gel)	50 X TAE buffer	0.00002 L L^{-1}
100% Denaturing solution	40% Acrylamide / Bis	0.0002 L L^{-1}
(for 8% (w/v) gel)	50 X TAE buffer	0.00002 L L^{-1}
	Formamide (deionized)	0.0004 L L^{-1}
	Urea	0.42 g L^{-1}

*EDTA = Ethylene diamine tetraacetic acid

4.3.1.2.2 PCR - Fungal Species

A nested PCR strategy was necessary for amplifying the 18S rDNA gene of fungal species to an appropriate size for denaturing gradient gel electrophoresis (Kowalchuk *et al.*,1997). The two primer sets used for this nested PCR technique for fungal species are found in Table 4.2. In the first PCR loop, primers NS1 and NS8 amplify nearly the entire 18S gene of fungi (White *et al.*, 1990) and yield a fragment approximately 1.7 kb. Ready-To-Go PCR beads with 1 x 10^{-6} L of each primer (1 x 10^{-5} M), 1 x 10^{-6} L of the fungal DNA template and deionized water for a total volume of 2.5 x 10^{-5} L was used for the first PCR amplification with the following thermocycling program: 94°C for 0.033 h; 30 cycles of 94°C for 0.0083 h, 50°C for 0.0125 h, and 72°C for 0.025 h; and then one final cycle at 72°C for 0.083 h. The PCR products were verified on a 1.2% (w/v) agarose in 1 X TAE buffer run with a DNA ladder at 110V for 0.5 h. The 1.7 kb bands were excised from the agarose gel and the DNA was then extracted from these gel slices using a QIAEX II Agarose Gel Extraction kit (Qiagen, Hilden, Germany). The rDNA extracted from the agarose gel and diluted 500-fold, was used as the rDNA template in the nested PCR amplification loop with primers NS1-GC (forward primer with GC clamp attached) and NS2+10 (reverse primer). The proportions of volume used for the nested PCR amplification using Ready-To-Go PCR beads is as described above for the first fungal rDNA PCR loop. The thermocycling program for the nested PCR loop includes: 94°C for 0.033 h; 30 cycles of 94°C for 0.0083 h, 55°C for 0.0167 h, and 72°C for 0.021 h; and a final cycle of 72°C for 0.083 h. The PCR products were again analyzed by agarose gel electrophoresis and found to be approximately

570 bp for the nested PCR products which is an adequate length for proper migration in DGGE.

4.3.1.3 Analysis of PCR products by DGGE

The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE), using 8% (w/v) acrylamide gels with linear denaturant gradients ranging from 20% to 60% for the bacterial species 16S rDNA and from 15% to 35% for the fungal species 18S rDNA. The gradient gels were prepared from two stock solutions including a 0% denaturing solution (40% Acrylamide/Bis, 50 X TAE buffer, and nanopure water), and a 100% denaturing solution (40% Acrylamide/Bis, 50 X TAE buffer, 40% (v/v) formamide, 7M urea and nanopure water). The concentrations of the solutions used for manufacturing the DGGE gels are listed in Table 4.3. The gels were polymerized with 0.09% (v/v) Temed and 0.9% (v/v) 10% ammonium persulfate solution. The polymerized denaturing gradient gels were run using a DCode TM Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA), containing 7 l of 1 X TAE buffer at 60°C.

A volume of 2×10^{-5} L PCR amplified rDNA samples, each mixed with 5×10^{-6} L of loading dye were added to the wells of the acrylamide gel and subjected to electrophoresis at 200 V for 3 hours for the bacterial species and at 200 V for 4 hours for the fungal species. The gel was then stained in a solution of ethidium bromide

(3.5×10^{-5} g L^{-1}) for 0.167 h before being photographed on an UV transilluminator (Fisher Biotech, Model FBT1V-816).

4.3.1.4 Sequencing of selected rDNA PCR products

Multiple bands that were detected in DGGE for both bacterial and fungal rDNA samples were excised from the DGGE gels. Each gel slice was placed in a vial containing 2×10^{-5} L of deionized water and kept at 4°C overnight to allow the DNA to passively diffuse out from the gel slice. A volume of 1×10^{-5} L of deionized water containing rDNA fragments were used as DNA templates for another PCR amplification. Bacterial primers PRBA338f and PRUN518r were used for the bacterial 16S rDNA samples and nested loop fungal primers NS1-GC and NS2+10 were used for 18S rDNA samples for re-amplification. Bacterial and fungal samples were verified on a 1.8% (w/v) and 1.2% (w/v) agarose gels respectively. 200 bp fragments of bacterial samples and 570 bp fragments of fungal samples were excised and gel extracted using a QIAEX II Agarose Gel Extraction kit. Using a commercially available kit (Promega pGEM®-T Easy Vector Systems, Madison, WI) for ligation and transformation, these amplified 16S and 18S rDNA fragments were cloned into pGEM®-T Easy vectors by ligation and transformed into *E. coli* JM109 competent cells where the fragments of inserted DNA are replicated naturally by the competent cells. The cells are then spread plated onto appropriate agar plates for growth and clones that have had successful insertion of the fragment of sample DNA, appear as white coloured colonies.

Purification of the plasmids of all clones obtained above were performed using standard procedures available with a QIAprep Spin Miniprep Kit (Promega, Madison, WI). Purified plasmids from these clones were sent out for sequencing. Sequence analysis was achieved through the use of the program BLAST (Basic Local Alignment Search Tool) which is available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) to search the database GenBank.

4.3.2 BIOLOG Microplate Analysis

The BIOLOG GN MicroPlate (BIOLOG, Hayward, CA) consists of a test panel of 95 different carbon sources used for testing the capability of inoculum microorganisms to utilize these carbon sources. The BIOLOG GN MicroPlate is specific for gram negative bacteria. Oxidation of the carbon sources by the bacteria produces a pattern of color changes in the wells, which constitutes the metabolic fingerprint of the capabilities of the inoculated microorganisms. The pure culture sample is first streaked onto Tryptic Soy Agar (TSA) (Fisher Scientific, Tustin, CA) plates and allowed to incubate at 28°C for 16 hours. Preparation of a uniform suspension of the inoculum solution for use on each microplate was accomplished by dispersing a swab of cells grown on the TSA plate into a sterile saline solution and mixing with a sterile pipet. Both the sample preparation and the inoculum preparation were done for each pure culture. Using an 8-Channel Repeating Pipetter (Brinkmann Transferette™ –8) and sterile pipet tips, the BIOLOG GN MicroPlates were inoculated 8 wells at a time with 1.50×10^{-4} L of the inoculum solution per well. The BIOLOG GN MicroPlates were then covered and incubated at 28°C for

4 to 16 hours. The microplate inoculated with the suspected *Enterobacter sp.* was incubated for 4 hours while the other pure culture was incubated at 28°C for 16 hours. Incubation of *Enterobacter sp.* for greater than 4 hours results in false positive readings potentially due to utilization of extracellular polysaccharides, lysed cell material or even stored endogenous substrates. Positive reactions produce a purple color while negative reactions produce no visible purple color. This fingerprint of coloured wells is then matched with a database provided by BIOLOG software of metabolic fingerprints to identify the pure culture inoculum.

4.4 Methods for Enumeration of Microorganisms

Two methods were used for measuring the quantities of microorganisms present in samples taken from either batch or semi-continuous experiments. Optical density was used as a quick measure of the cell density in the mixed culture solution and was correlated to biomass dry weight concentration. Selective carbon source plate counts provided further information on the number of viable cells from each species in the mixed culture samples.

4.4.1 Optical Density

Measurements of biomass concentration were achieved by measuring the absorbance of a ten-fold diluted sample solution at a wavelength of 600 nm using a Beckman DU® 640 Spectrophotometer (Fullerton, CA). A volume of 2×10^{-4} L of the

cell sample solution was mixed in 1.8 x 10^{-3} L of 0.01M phosphate buffered solution. The phosphate buffered solution (pH 7.6) consisted of 12.36 g L^{-1} sodium phosphate, anhydrous (dibasic), 1.8 g L^{-1} sodium phosphate, monohydrate (monobasic), and 85 g L^{-1} sodium chloride, that was then diluted ten-fold with distilled water and sterilized before being used for dilution of samples. This 0.01M phosphate buffered solution was also used as the blank solution in the absorbance readings. Ten-fold dilutions of the samples ensured absorbance readings of less than 1.0, above which Beer's Law deviates substantially for correlating absorbance with bacterial cell concentrations (Gerhardt *et al.*, 1994).

A correlation between optical density and biomass dry weight concentration was established. Solutions were made where 10%, 25%, 50%, 75%, and 100% of the solution was comprised of mixed culture cells. The optical densities from these samples were measured at a wavelength of 600 nm. The procedure for obtaining biomass dry weight concentrations of the diluted samples, consisted of centrifuging 0.01 L samples for 0.25 h at 4000 x g using a Beckman J2-HS centrifuge (Fullerton, CA). After removal of the supernatant, the resultant pellet of cells was washed and re-suspended with 0.01 L of 0.01M phosphate buffered solution, and centrifuged again at 4000 x g for 0.25 h. After removal of the supernatant, the resulting pellet of washed cells was re-suspended with 0.01 L of 0.01M phosphate buffered solution once more and transferred to a pre-weighed aluminum dish and dried overnight at 105°C. Ten milliliter solutions of the 0.01M phosphate buffered solution were also dried overnight at the same conditions to offset the weight added by the phosphate buffered solution when the samples were dried. A plot of

the biomass dry weight concentrations of cells and the corresponding optical density is illustrated in Figure 4.2. Linearity between biomass dry weight and optical density is observed up to an absorbance of 1.5.

4.4.2 Selective Carbon Source Plate Count Analysis

Results from the BIOLOG GN MicroPlates for both bacterial pure culture samples that were used for identification of the microorganisms were used to determine the carbon source that would be most selective for either of the pure cultures. The degree to which the inoculum can utilize the carbon sources is directly related to the color intensity of the reaction products, as detected by reading the absorbance of the reaction products with a BioRad Microplate Reader (Model 450). Although there was not a single carbon source that was completely selective for each of the pure cultures, there were some choices whereby the reactivity of the carbon source was higher for one culture in comparison to the other. These were L-pyroglutamic acid for *Acinetobacter sp.* and D-galacturonic acid for *Enterobacter sp.* Selective agar plates were made by addition of 15 g L^{-1} of agar to solutions of 2 g $L^{-1}$of L-pyroglutamic acid and 2 g L^{-1} D-galacturonic acid which had been adjusted to approximately neutral pH by addition of 0.2 N sodium hydroxide (NaOH). Selectivity for the growth of the yeast or fungi was achieved using synthetic brewery wastewater/antibiotic agar plates with 0.03 g L^{-1} streptomycin sulfate and 0.03 g L^{-1} chlortetracycline (Page *et al.*, 1982).

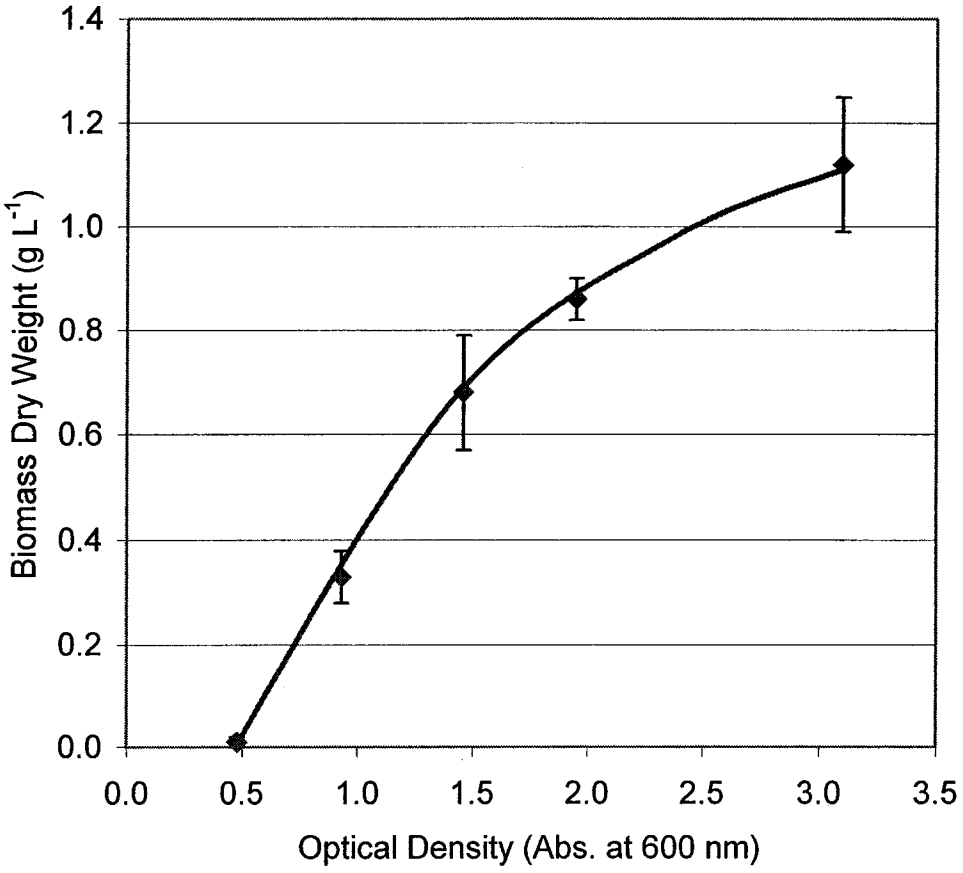

Figure 4.2: Correlation of biomass dry weight concentration and optical density measured at 600 nm.

For enumeration of the colonies formed on these selective carbon and antibiotic plates, a revised plate count method was used so that reading of plates for multiple samples and dilutions would be facilitated. Samples were serial diluted by mixing 1×10^{-6} L of the sample with 9.0×10^{-4} L of 0.01M phosphate buffered solution and serial diluted ten-fold each time to obtain the correct dilution that would result in growth of between 30 to 300 colonies when plated. Instead of spread plating 1×10^{-4} L of the diluted sample onto an agar plate as with the traditional spread plate method, 1×10^{-5} L of the well-mixed diluted sample was dropped onto the agar plate. Three replicate drops were placed onto the plate as well as replicate drops at several dilutions. The drops were allowed to air dry for 1 hour in a laminar fume hood, then incubated at 25°C for 16 hours and subsequently read by using a colony microscope at a magnification of 25 times (Leitz Wetzlar, Germany). Comparisons between the traditional spread plate method and revised droplet method were analyzed by the statistical comparison of means t-test. The results were statistically the same within a 95% confidence interval. The comparative data are shown in Appendix A.

4.5 Analyses for Effluent Characterization

4.5.1 Ethanol Analysis

Ethanol concentration was analyzed using a Hewlett Packard 6890 Series Gas Chromatograph (Wilmington, DE), fitted with a FID detector set to 200°C, and injector and oven temperatures set to 80°C. A temperature program of 80°C for 0.042 h followed

by a temperature ramp of 50°C per 0.0167 h to 120°C was used. The GC method was calibrated to a detection level of 0.01 g L^{-1} of ethanol. The calibration curve is shown in Figure 4.3.

4.5.2 Maltose Analysis

Maltose was detected through a reaction between maltose and a coloured reagent (containing 9.6 x 10^{-2} M 3,5-dinitrosalicylic acid, 5.3M sodium potassium tartrate solution and 2M NaOH) at 100°C (Sigma, St. Louis, MI). Cell-free samples of 3.33 x 10^{-4} L were mixed with 1.67 x 10^{-4} L of the coloured reagent in 0.002-L capacity microfuge tubes. The 0.002-L tubes of the mixture are then mounted on a floating rack where the volume of the mixture was completely submerged in a boiling water bath for exactly 0.25 h. To stop the reaction, the tubes were quenched on ice to room temperature. The contents were then diluted with 1.5 x 10^{-3} L of deionized water before being analyzed spectrophotometrically (Beckman DU® 640 Spectrophotometer, Fullerton, CA) at a wavelength of 540 nm. Calibration curves were generated each time this analysis was performed. A sample calibration curve is shown in Figure 4.4.

4.5.3 Glucose Analysis

Glucose was detected through the reaction of 6% (v/v) o-toluidine in glacial acetic acid (Sigma, St. Louis, MI) and glucose at 100°C, producing a coloured byproduct that was then quantified at a wavelength of 635 nm. The manufacturer's protocol consisted of

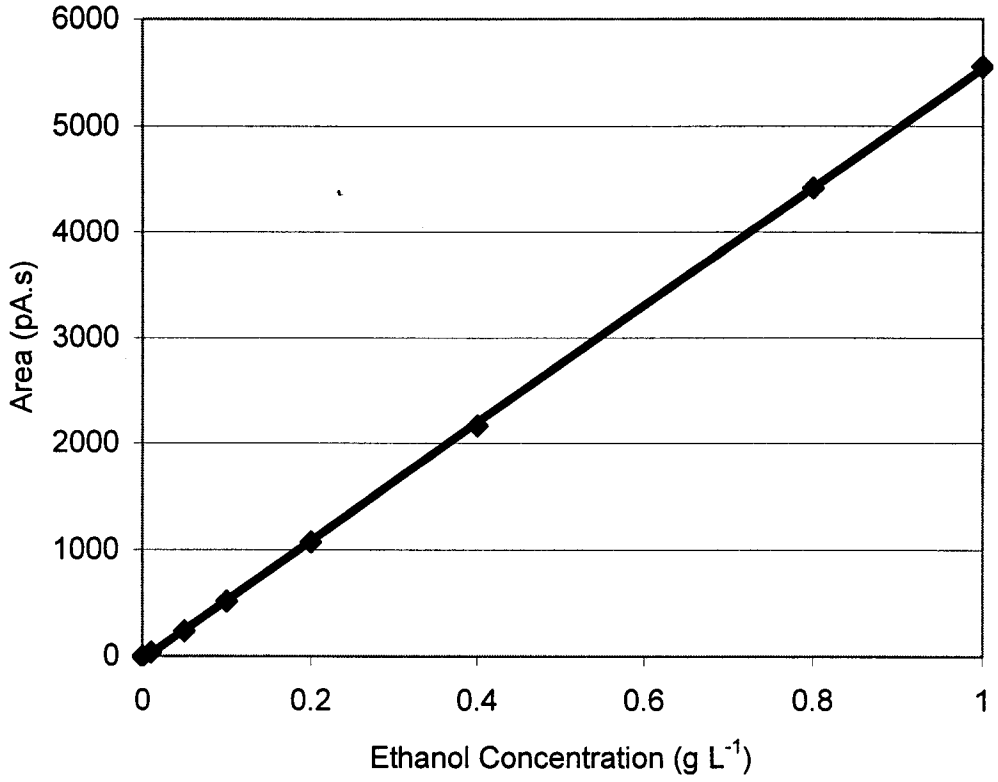

Figure 4.3: Ethanol calibration curve.

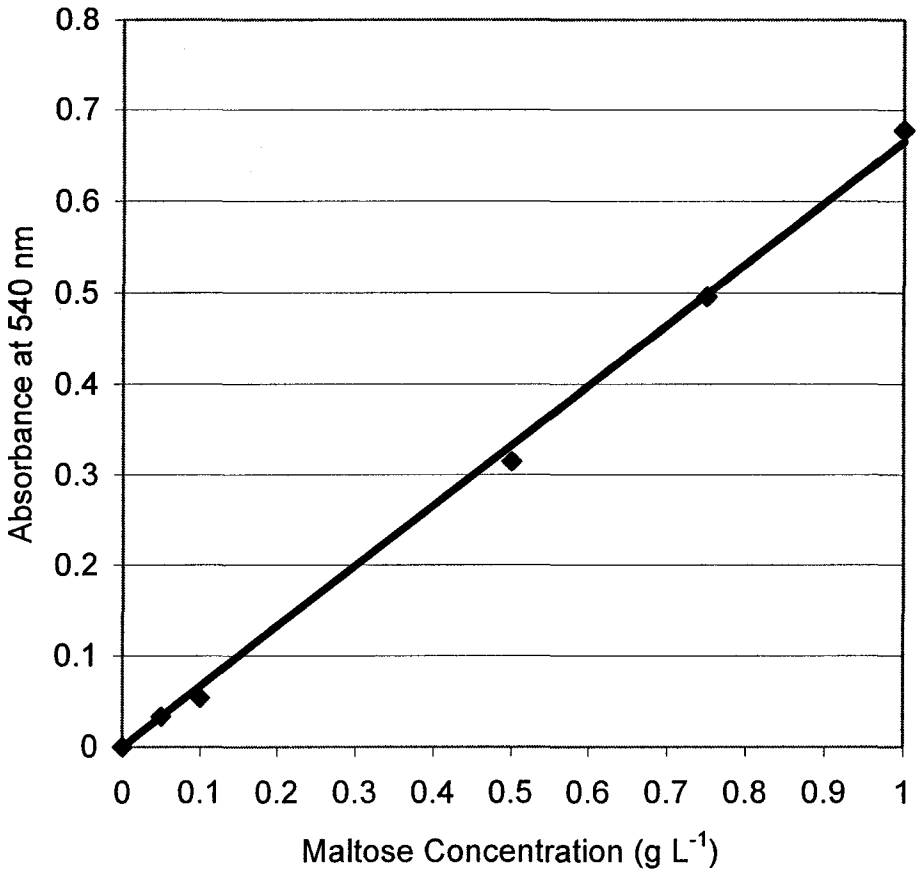

Figure 4.4: Maltose calibration curve.

mixing 5.0 x 10^{-3} L o-toluidine reagent with 1 x 10^{-4} L of the cell-free sample in test tubes, heating the solution in a vigorous boiling water bath for 10 minutes and cooling the solution to room temperature with tap water. Contents of the test tubes were then analyzed colourimetrically using the Beckman spectrophotometer. A standard glucose solution of 0.1g L^{-1} (Sigma, St. Louis, MO) was run with each set of samples in order to obtain the absorbance for the standard for calculating the resulting glucose concentration of the sample according to equation 4.1:

$$\frac{ABS_{Sample}}{ABS_{Standard}} \times CONC_{Standard} = CONC_{Sample} \qquad (4.1)$$

where ABS_{Sample} and $ABS_{Standard}$ are the absorbance readings of the sample and the standard respectively and $CONC_{Standard}$ and $CONC_{Sample}$ are the concentrations of the standard and sample respectively.

4.5.4 Biochemical Oxygen Demand (BOD)

The standard 5 day BOD analysis, sometimes denoted BOD_5, was performed on the initial synthetic brewery wastewater solutions. Samples of the solutions in the proper dilution, along with aerated and seeded dilution water are poured into 0.3 L airtight bottles (Yellow Springs Instrument, YSI). Dilution water is made up of a phosphate buffer solution, a magnesium sulphate solution, a calcium chloride solution and a ferric chloride solution according to the standard procedure found in Standard Methods for the Examination of Water and Wastewater (APHA *et al.*, 1989). The seed used was a mixed culture sample taken from the reactor and mixed into the dilution water at a ratio of 5 x

10^{-4} L of a 1.5 g L^{-1} mixed culture sample to 1 L of dilution water. The dissolved oxygen readings are taken initially and after the fifth day using the Orion O_2 electrode (Model 97-08-00). BOD readings are calculated according to the following equation (APHA *et al.*,1989):

$$BOD_5 = \frac{(D_1 - D_2) - (B_1 - B_2)f}{P} \tag{4.2}$$

where BOD_5 = Biochemical oxygen demand, g L^{-1}

D_1 = D.O. of diluted sample immediately after preparation, g L^{-1}

D_2 = D.O. of diluted sample after 5 day incubation at 20°C, g L^{-1}

P = decimal volumetric fraction of sample used,

B_1 = D.O. of seed control before incubation, g L^{-1}

B_2 = D.O. of seed control after 5 day incubation, g L^{-1} and

f = ratio of seed in diluted sample to seed in seed control = (% seed in diluted sample)/(% seed in seed control)

4.5.5 Total Organic Carbon (TOC)

TOC of the initial synthetic brewery wastewater and cell-free samples was analyzed using a Shimadzu TOC-5050 Total Organic Carbon Analyzer (Japan). 8-microliter injections were used and samples were measured in triplicate. The standard solution was made with 4.25 g L^{-1} anhydrous potassium hydrogen phthalate which is equivalent to 2 g L^{-1} TC (total carbon). The calibration curve for the TOC analyzer is shown in Figure 4.5.

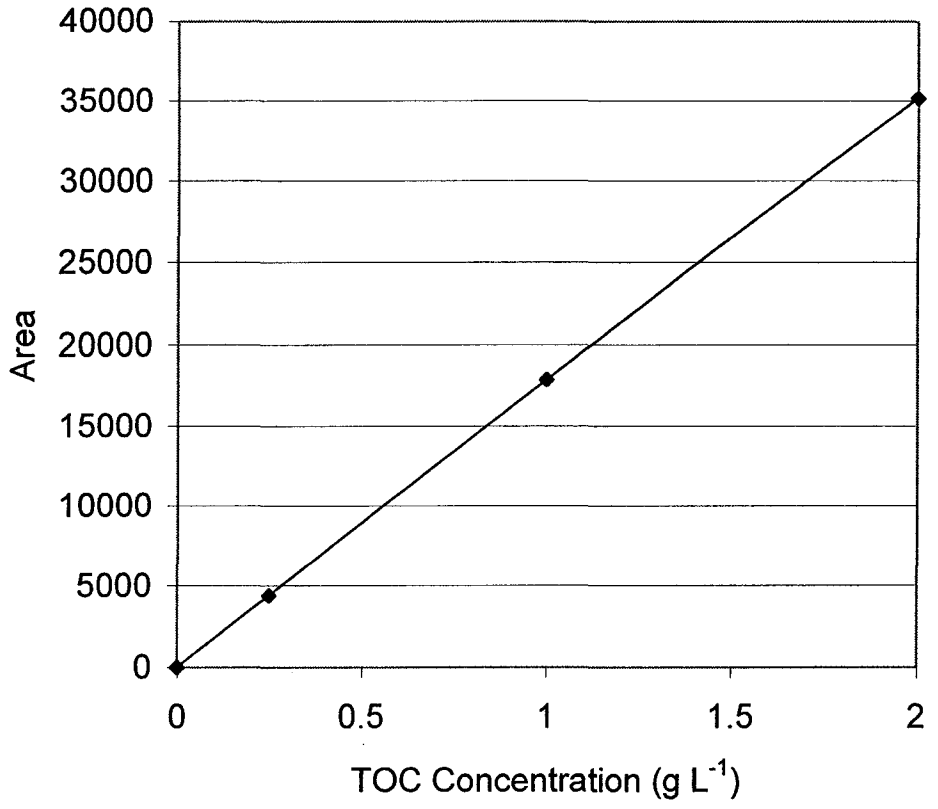

Figure 4.5: Total organic carbon (TOC) calibration curve.

4.6 Experimental Apparatus

The semi-continuous reactor system used in treating the synthetic brewery wastewater is shown in Figure 4.6. A one liter working volume of mixed liquor was pumped out the bottom of the cyclone column bioreactor with a 0.018 hp (MagneTek Model MDX) centrifugal pump and re-circulated past a Signet 2714 pH sensor (El Monte, CA) that provided a voltage that was proportional to pH values to the computer acquisition/controller. The mixed liquor was then
passed through a water jacket that maintained the mixed liquor temperature at 25°C using an Isotemp 1013S circulator bath (Pittsburgh, PA), past an Ingold dissolved oxygen (D.O.) probe and finally returned to the top of the cyclone bioreactor. The mixed liquor would enter the side arm of the cyclone reactor tangentially, causing it to swirl down the sides of the reactor in a thin liquid film.

To provide oxygen to support microbial growth during the treatment process, compressed air that was both filtered and humidified was delivered at a flowrate of 180 L h^{-1} through a Dwyer rotameter (Michigan City, Indiana), to the bottom of the cyclone bioreactor. The air then flowed upward countercurrent to the falling liquid film and exited through the top of the reactor after passing through a foam trap and air filter. Hence, the reactor was continuous with respect to the airflow and semi-continuous with respect to the treatment of the waste which was added on a cyclical basis as described in Section 2.3. All connections between pieces of equipment were made with flexible and sterilizable latex tubing.

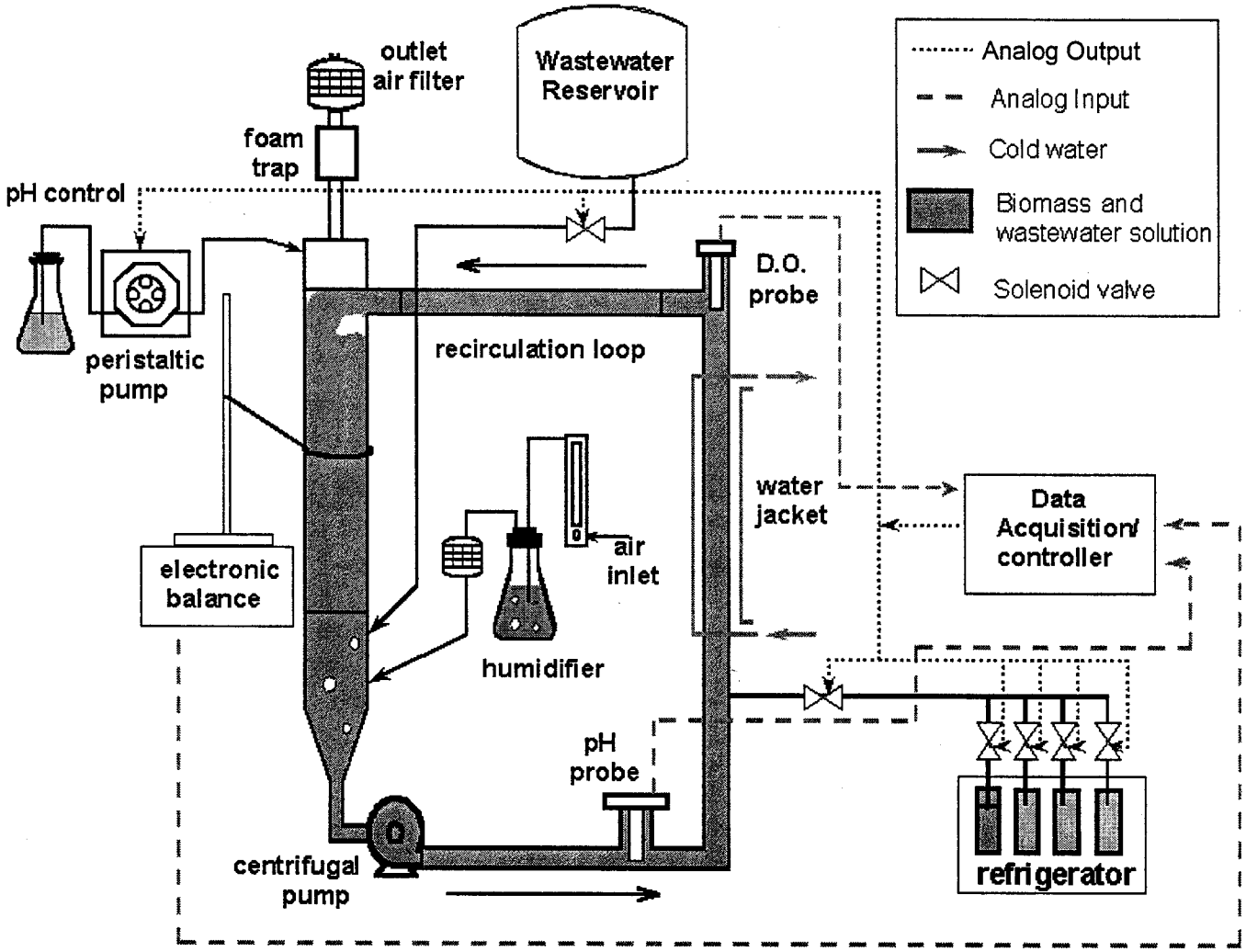

Figure 4.6: Experimental Apparatus.

Other components of the reactor set-up included a wastewater reservoir, sterilizable solenoid valves (Skinner Model V52LB2052) and a refrigerated storage area. The system was controlled by a National Instruments Data Acquisition/ Control system using Labview software (Austin, TX) which monitored the output from the on-line dissolved oxygen meter, pH probe and an electronic balance (Pennsylvania Scale Model 7300, Leola, PA) which measured the weight of the cyclone reactor and its contents. The computer and data acquisition / control system also controledl the solenoid valves, the centrifugal pump for recirculation, and a Masterflex peristaltic pump for addition of 0.2 M sodium hydroxide. The solenoid valves were opened and closed to accomplish both the harvesting of approximately 0.5 L of mixed liquor and addition of an equal volume of fresh synthetic wastewater each treatment cycle, adjusted for any volume added by the pH control system.

4.7 Description of a Typical Semi-continuous Experiment

Preparation of the mixed culture inoculum involved enrichment of each of the three pure culture species from *Acinetobacter*, *Enterobacter*, and *Candida*. A volume of 1×10^{-4} L of each pure culture defrosted from –70°C was enriched overnight in test tubes containing 8×10^{-3} L of the pH buffered synthetic wastewater to be treated. Samples of 1×10^{-3} L from these test tubes were subsequently inoculated into individual flasks containing 9.9×10^{-2} L of the same buffered synthetic wastewater. This was also incubated on a shaker overnight at 25°C. Optical densities were then obtained for each of

these pure cultures from the flasks for the purpose of reconstituting the initial mixed culture consortium proportions to be similar in for all experiments.

Startup of the computer program used for data acquisition and control, initiated the beginning of the semi-continuous experiment. A volume of 0.9 L of synthetic wastewater was automatically added to the cyclone reactor followed by manual addition of a 0.1 L volume of mixed culture inoculum. After inoculation, the first batch cycle is commenced. A cycle is defined as the period from when fresh synthetic wastewater is added, to when the Dissolved Oxygen (D.O.) reaches a maximum after a minimum in D.O. has been reached. The cycle time of the first batch cycle usually required more time since only a 10% inoculum was used for degradation of the synthetic wastewater. In subsequent cycles, 50% inoculum was used since half the working volume was removed and supplemented with fresh synthetic wastewater to remake the 1 L working volume.

The control strategy for detecting the completion of the cycle is shown in Figure 4.7 and was based on either dissolved oxygen and a critical cycle time criteria. Although oxygen was never limited during the reaction period because the D.O. value never reached below 30% saturation, fouling of the D.O. probe would result in a sluggish increase of the D.O. readings; hence a critical cycle time criterion was used.

After initialization of variables including resetting the variable "Comparison" for dissolved oxygen back to 100% Saturation, and logging of the previous cycle time as the critical cycle time, the reactor was started. Both D.O. and pH values were read by the

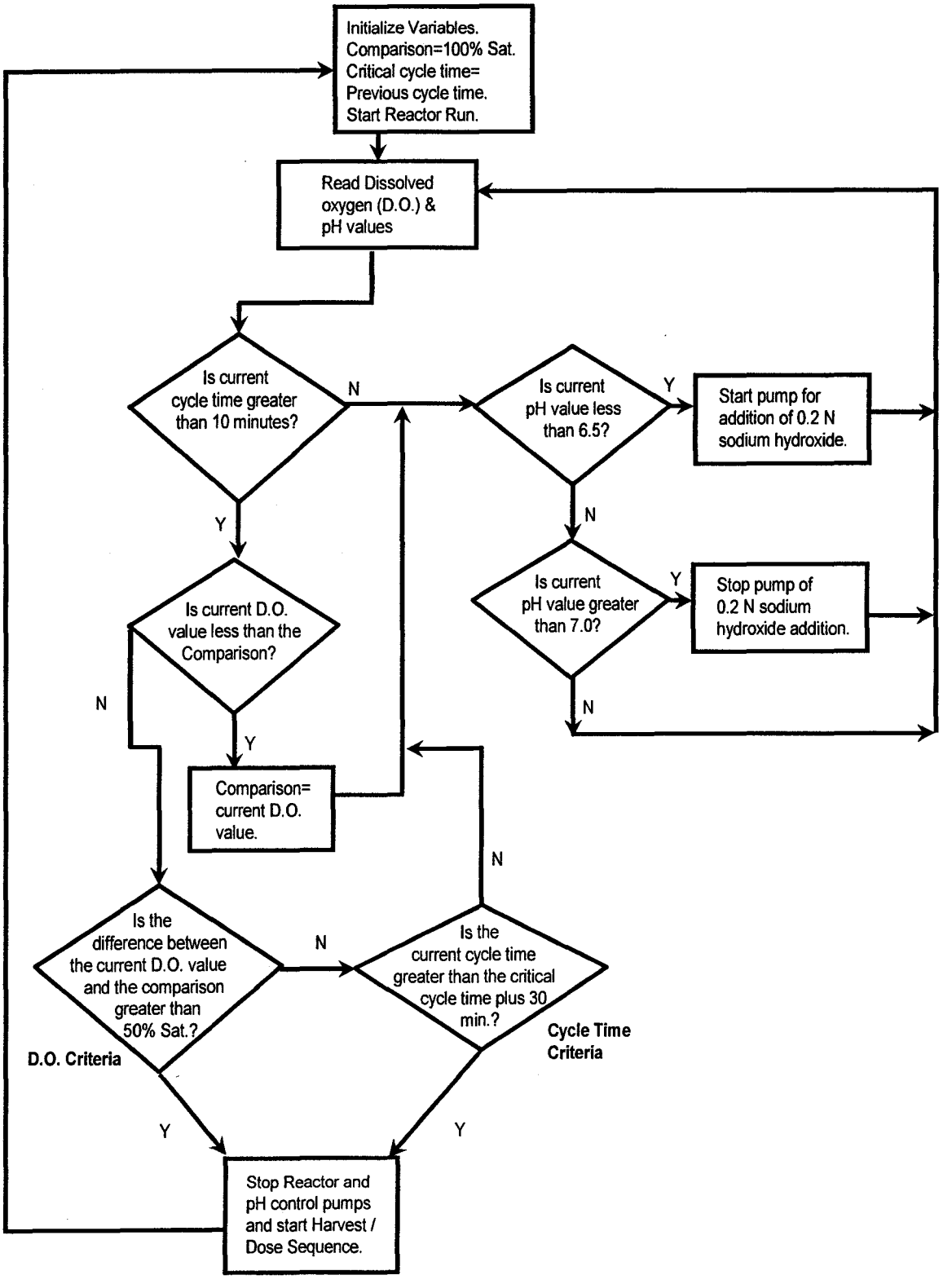

Figure 4.7: Control strategies of the semi-continuous reactor system

data acquisition. The initial criterion in the control strategy was that of making the "Comparison" variable equal to the current D.O. value as long as it was decreasing with time. This process ensured that the minimum D.O. value would be determined. The second criterion was that of a delay of 10 minutes whereby initial fluctuations in the D.O. values would not falsely trigger the D.O. Criteria control strategy. Although not depicted in Figure 4.7, after the 10 minute period, any spikes or dips in the electrical output from the D.O. meter were filtered by a criteria whereby any highs or lows must be read three consecutive times in order to be registered as true readings.

Completion of the cycle was triggered by either the increase in the D.O. reading from the "Comparison" by 50% Saturation or the exceeding of the cycle time plus 30 minutes. The half an hour inclusion enabled the D.O. criteria to trigger the cycle's end even if fouling of the Ingold D.O. probe had occurred. If either the D.O. criterion or the cycle time criterion were true, the pumps were stopped and the harvesting and dosing sequences were begun. At the completion of these sequences, the next reactor run was started and the control process continued for the next treatment cycle.

Each experiment was continued for up to 23 to 30 cycles depending on when the biofilm accumulation inside the reactor system had stabilized. These semi-continuous experiments were repeated for each of the five synthetic brewery wastewaters tested. To determine when stabilization of the biofilm in the reactor occurred, biofilm contribution experiments were conducted throughout each of the semi-continuous experiments.

4.8 Biofilm Contribution Experiments

Biofilm contribution experiments were conducted at every fifth to ninth cycle of the semi-continuous experiment. The process in chronological order, consisted of:

a. stopping the re-circulation pump and control system,
b. emptying out the mixed liquor contents of the reactor,
c. rinsing the reactor system with supernatant of previous cycles and draining the rinse solution out,
d. removing a section of latex tubing from the re-circulation loop, draining for approximately 0.083 h and subsequent weighing,
e. reattachment of the latex tubing into the re-circulation loop,
f. addition of 0.5 L of cell-free supernatant solution from previous cycles,
g. addition of fresh synthetic brewery wastewater to complete the 1 L working volume,
h. restarting the computer acquisition / controller and pump.

The purpose of the above procedure was to duplicate the previous cycle in the semi-continuous experiment but without any contribution by the suspended culture in the reactor. Thus, samples from the previous cycles were centrifuged, and only the supernatant and fresh synthetic wastewater was used in the biofilm contribution runs. The difference in cycle time between the previous cycle and the cycle without any suspended culture in the mixed liquor provided a relative measure of the contribution of the biofilm to the treatment of the wastewater. The weight of the latex tube was used as

the basis for determining when biofilm accumulation had stabilized and to estimate the total quantity of biofilm in the system.

CHAPTER 5

CONSORTIUM CHARACTERIZATION AND IDENTIFICATION

Three methods contributed to the identification of the dominant species in the mixed culture consortium. These methods included the traditional method of culturing and isolation on solid agar media, microscopic inspection and gram staining. In addition, molecular techniques involving PCR-DGGE and sequencing described in Sections 4.3.1.2 to 4.3.1.4, resulted in fingerprints of bands of the 16S rDNA of the bacterial species and 18S rDNA of the fungal species. Finally, BIOLOG microplates that were used for the purpose of determining their ability to use selective carbon sources confirmed the identifications of the bacteria.

Preliminary characterization of the mixed culture (MC) consortium was done using synthetic brewery wastewater/agar plates. Three dominant colony types (PC_A, PC_B and PC_C) were detected from this culture-based method. Microscopic analysis of cells from PC_A colonies were observed to be motile, straight Gram-negative rods, which formed slightly iridescent, flat, and irregular edged colonies with diameters ranging from 2 to 3 mm. Cells from colony PC_B were characterized as being short, plump, Gram-negative rods or cocci, and the colonies were cream-coloured, smooth edged and were 1 - 2 mm in diameter. PC_C colonies that were formed on brewery wastewater/agar plates at pH 5.5 were round, yellowish white in colour, smooth edged and had a dull-shine to the surface of the colony and were approximately 1mm in diameter. The cells from these

colonies were budding ovoids, in singles and in pairs, and budding elongated mycelia were also present.

Species characterization using DGGE has recently been documented for the molecular analysis of the eukaryal community in local activated sludge treatment plants (Marsh *et al.*, 1998), as well as a tool for comparing the diversity of various activated sludge plants (Curtis and Craine, 1998). In this research, DGGE analysis provided a fingerprint of the community that was present in treating synthetic brewery wastewater. The pure and mixed cultures were subjected to PCR-DGGE and the fingerprints of the bands containing 16S rDNA are shown in Plate 5.1.

With two dominant bacterial microorganisms detected from culture-based methods, it was expected that results from the PCR-DGGE characterization of the bacterial mixed culture consortium would display at least two bands in its fingerprint band profile. Multiple bands were seen for the mixed culture and pure culture band profiles seen in Plate 5.1. The mixed culture consortium had 6 distinct bands (MC1 to MC6), pure culture PC_A had 3 bands (A1 to A3) and PC_B had two bands (B1 and B2).

For pure cultures, the presence of multiple bands may be due to potentially nonspecific PCR amplification or multiple 16S rDNA operons. Nonspecific amplification can occur when the PCR primer set erroneously binds to nucleotide sequences on the rDNA template in a region other than the targeted one. In the case of multiple operons in the 16S rDNA, during PCR amplification of such bacteria, the

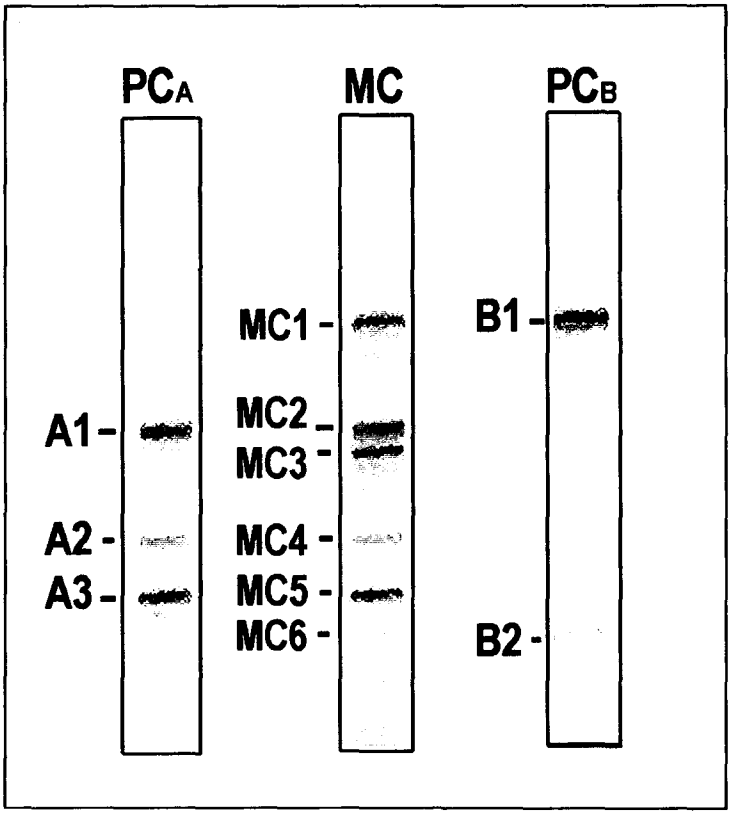

Plate 5.1: Profile of PCR-DGGE fingerprints of bands containing 16S rDNA for the bacterial pure and mixed cultures. The most prominent bands for pure cultures PC_A and PC_B are labeled A1 to A3 and B1 and B2 respectively. The mixed culture (MC) bands are labeled MC1 to MC6.

primers bind to different operons and may result in faint bands appearing in nearby locations to that of the main bright band for the bacteria on a DGGE gel. Multiple bands have been found for 16S rRNA sequences of bacteria in previous studies using Temperature Gradient Gel Electrophoresis (TGGE) by Nübel *et al.* (1996).

Comparison of the band profiles from PC_A, PC_B and MC showed that all bands in the PC_A and PC_B profiles matched to bands visible in the MC profile at similar locations in the denaturing gradient gel. Bands A1, A2 and A3 matched in location to that of MC2, MC4 and MC5. Bands B1 and B2 matched to those of MC1 and MC6. However, based solely on migration patterns in a DGGE gel it is not possible to positively conclude that similarly located bands are the same species because 16S rDNA fragments that have quite divergent sequences can comigrate on DGGE gels (Vallaeys *et al.*, 1997). To verify that the similarly located bands were the same microorganism, various bands were cut from the DGGE gel and sequenced. Similarly for the fungal PCR-DGGE analyses, the most prominent bands of the pure culture PC_C from the fungal DGGE were excised and sequenced and the homology match of the bands were identical and the identification is presented in Table 5.1.

Sequencing results of the sample 16S rDNA verified that the similarly located bands had the same sequence homology match. The three dominant bands A1, A2 and A3 of PC_A were homologous to a partial 16S rRNA sequence of *Enterobacter aerogenes* with sequence homology matches of 98%, 99% and 100% respectively. Therefore, three operons exist for *Enterobacter aerogenes*. One of the three corresponding bands in the

Table 5.1: Sequence homologies of plasmids obtained from DGGE gel slices of dominant band locations corresponding to Plate 5.1

Sample ID	Location in Plate 5.1	Accession No.*	Sequence Homology Matches [Accession No.] (Sequence homology ID %)
Dominant bands of PC_A	A1	AF242482	*Enterobacter aerogenes* 16S rRNA, partial 5'end, strain NCTC 10006 T [AJ001237] (98%)
	A2	AF242483	*Enterobacter aerogenes* 16S rRNA, partial 5'end, strain NCTC 10006 T [AJ001237] (99%)
	A3	AF242484	*Enterobacter aerogenes* 16S rRNA, partial 5'end, strain NCTC 10006 T [AJ001237] (100%)
Dominant bands of PC_B	B1	AF242485	*A. haemolyticus* 16S rRNA gene, strain ATCC 17922 [Z93436] (100%)
Dominant bands of MC	MC1	AF242486	*A. haemolyticus* 16S rRNA gene, strain ATCC 17922 [Z93436] (97%)
	MC5	AF242488	*Enterobacter aerogenes* 16S rRNA, partial 5'end, strain NCTC 10006 T [AJ001237] (100%)
	MC3	AF242487	Uncultured bacterium SP44-7 gene for 16S rRNA [AB028114.1] (100%)
Dominant bands of PC_C	C1	-	*Candida sojae* 18S rRNA gene, [Z93436] (100%)

* Accession Numbers of DNA sequences submitted to GenBank

mixed culture consortium, MC5 was also homologous to *Enterobacter aerogenes* at a sequence homology of 100%. With this information and the matching locations of A1 to A3 with MC2, MC4 and MC5 of Plate 5.1, this verified the identity of *Enterobacter sp.* in the mixed consortium and that the fingerprint band profile could be used to identify this bacteria in a DGGE gel.

Identification of the second bacterial species was obtained by sequencing band B1 from the pure culture PC_B. The sequence of PC_B was homologous to the *Acinetobacter haemolyticus* gene at a sequence homology of 100%. The faint DGGE band B2 of the pure culture sample PC_B was unsuccessfully identified. The band intensity of B2 was not as intense as that for B1 and thus the band for B1 was used as the main identifier of the *Acinetobacter haemolyticus* gene present in the mixed consortium used in treatment experiments. Similarly located MC1 of the mixed culture consortium had a sequence homology of 97% and was homologous to *Acinetobacter haemolyticus* similar to results of B1 for pure culture PC_B.

Although the database of species in BIOLOG is limited, results from GN BIOLOG microplates for both culturable bacterial species were also identified as being *Enterobacter sp.* and *Acinetobacter sp.* The results of the GN BIOLOG microplate analyses are presented in Appendix B.

Results obtained for the pure cultures made it simple to determine the extent to which PCR-DGGE was able to detect uncultured bacteria in this mixed culture

consortium for treating synthetic brewery wastewater. The remaining DGGE band MC3 of the consortium was unaccounted for by either PC_A or PC_B pure cultures obtained through culturing methods. Sequencing results showed that band MC3 was homologous to an uncultured bacterium SP44-7 gene for 16S rRNA with a sequence homology identification of 100%. PCR-DGGE technique was able to detect one unculturable bacterium in addition to the culturable ones, which were also detectable by standard agar plating methods.

For the fungal species, the DGGE gel slice from the DGGE specific for fungal species was excised and sequenced. The fungal nucleotide sequence obtained from this slice matched with a sequence found in GenBank. A 100% homology to *Candida sojae* 18S rRNA gene, a yeast from the *Ascomycota* division matched to the 18S rDNA sample. The results confirm wet mount microscopy data where budding ovoids, and budding elongated mycelia that had been detected are characteristic of vegetative cells and pseudomycelium of *Candida sojae* (Nakase *et al.*, 1994).

CHAPTER 6

BATCH EXPERIMENTS

Having matched PC_A, PC_B and PC_C with the species *Enterobacter aerogenes* and *Acinetobacter* sp. and *Candida sp.* respectively, a review of the known characteristics from literature can provide information on the capability for preferential substrate degradation of these microorganisms. Both bacterial species are from the *Proteobacteria* sub class. The occurrence of these two degraders was not unexpected since *Acinetobacter* sp. usually grow well in enriched media, which is the case of brewery wastewater as well as this synthetic brewery wastewater, and *Enterobacter aerogenes*, being gut bacteria, are commonly found in sewage or activated sludge. *Enterobacter aerogenes* is capable of using various carbohydrates and amino acids, such as glucose, maltose, glycerol, L-serine and L-alanine. Although it is relatively uncommon for *Acinetobacter* to degrade carbohydrates, some strains are able to metabolize a range of compounds including some amino acids, fatty acids, aliphatic alcohols, sugars, as well as recalcitrant aromatic compounds (Balows *et al.*, 1992). *Candida sp.* is capable of assimilating various carbon sources including glucose, maltose and ethanol (Nakase *et al.*, 1994), the key carbon sources comprising this synthetic brewery wastewater. Hence, it was not unexpected to find its presence in the mixed consortium. Both pure and mixed batch culture experiments were performed using these microorganisms to verify the individual contributions to degradation of the synthetic brewery wastewater and the interactions between them as they coexist in a mixed culture. The results of these experiments are presented and discussed in this chapter.

6.1 Pure Culture Experiments

6.1.1 Non-buffered experiments - O.D., pH, and plate counts

In 26-h batch flask experiments using a high ethanol/mid maltose synthetic brewery wastewater and no added buffering capacity, *Acinetobacter sp, Enterobacter sp.*, and *Candida sp.* were inoculated into individual flasks containing 0.099 L of the synthetic brewery wastewater. These experiments were performed in triplicate. Samples were obtained at time intervals of 0, 4, 8, 12, 15, and 26 h. Five-milliliter samples were retrieved from the flasks aseptically and subjected to analyses for optical density (O.D.), viable plate counts, and concentrations of the various carbon sources. Figure 6.1 illustrates the average changes in optical density as the experiments progressed. All three pure cultures were capable of growth in the synthetic brewery wastewater. The O.D. readings of the *Acinetobacter sp.* and *Enterobacter sp.* leveled off after 8 h and 12 h respectively. The growth of *Candida sp.* included an extended lag phase, until approximately 14 h, thereafter the growth rate increased significantly.

The pH of the culture broths throughout the 26-h batch experiment is depicted in Figure 6.2. The non-buffered nature of these batch experiments demonstrated that the assimilation of the carbon sources by all three pure cultures produced products that were acidic. It is known that many *acinetobacters* are able to acidify media containing sugars (Hauge, 1960). *Enterobacters* also are capable of producing acidic products in the presence of sugars including glucose and maltose (Balows *et al.*, 1992). *Candida sp.* also

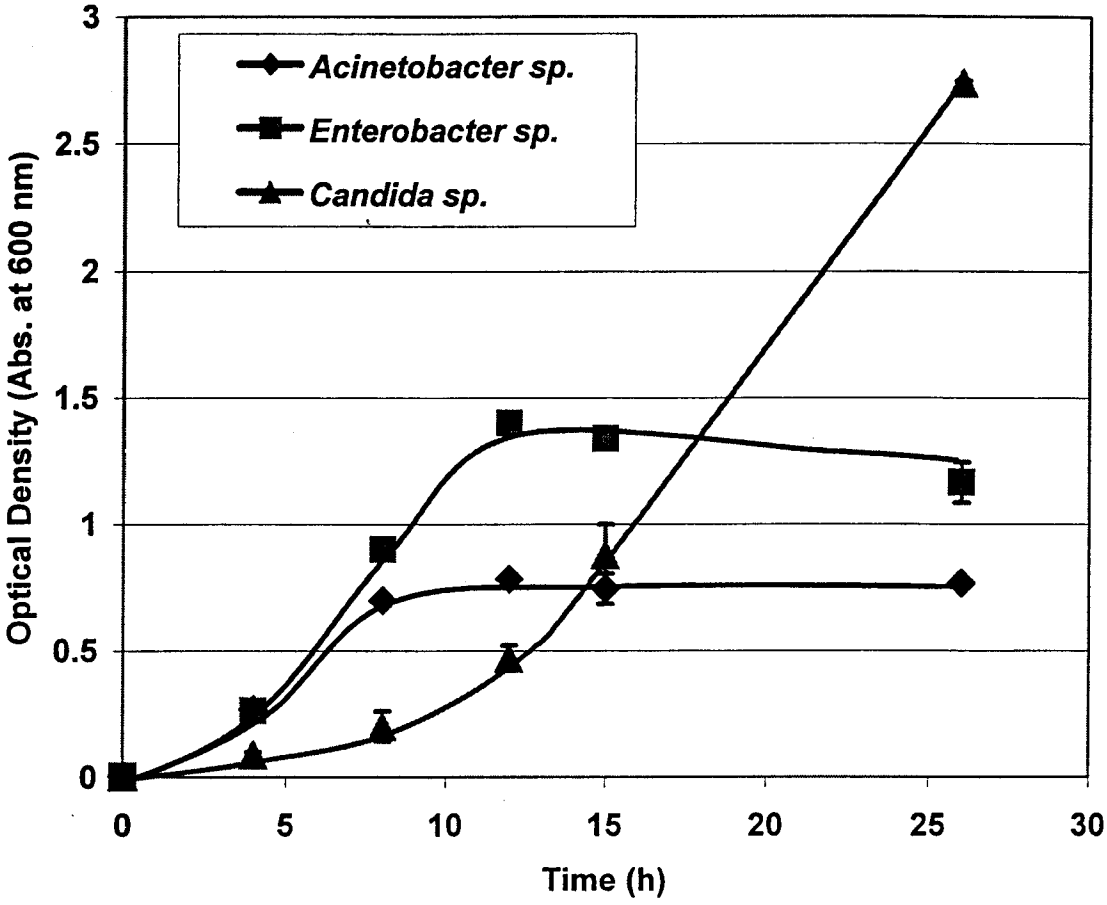

Figure 6.1: Optical densities monitored during non-buffered pure culture experiments using *Acinetobacter*, *Enterobacter* and *Candida* species.

(Error bars represent standard deviations of triplicate experiments)

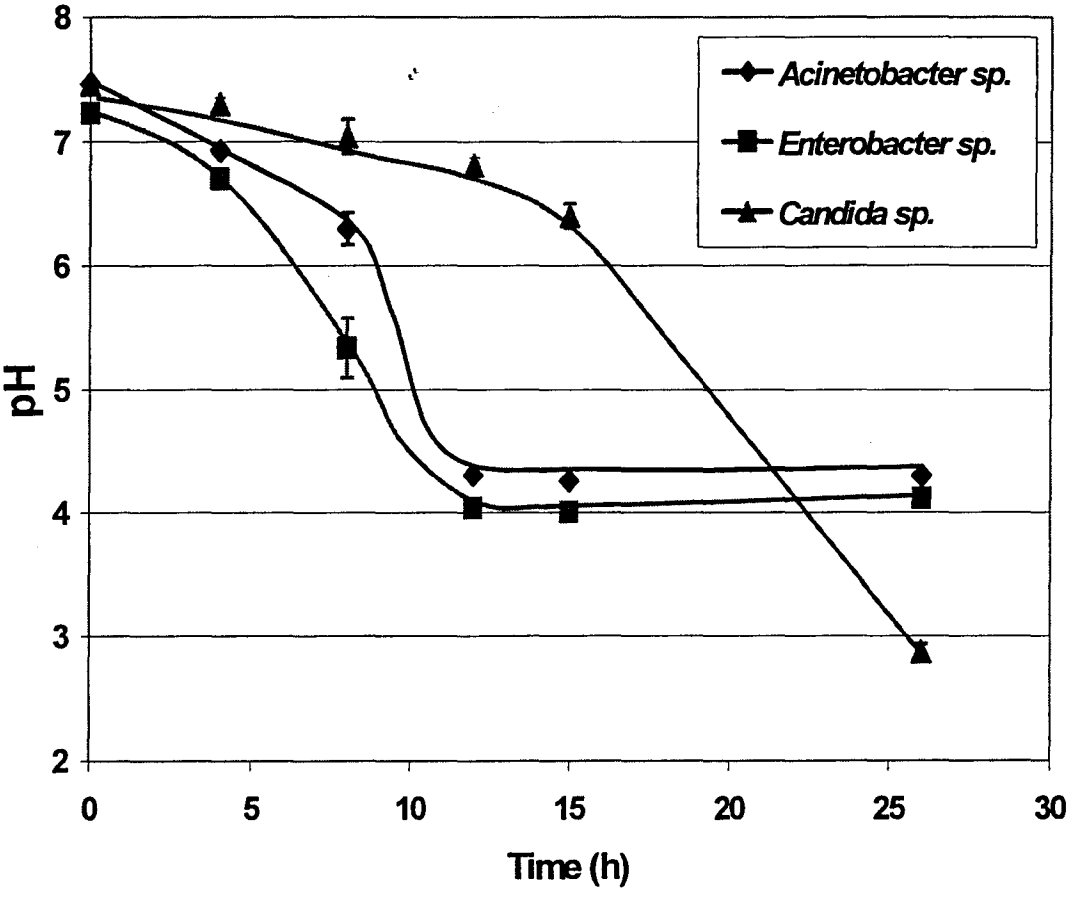

Figure 6.2: pH values measured during non-buffered pure culture experiments using *Acinetobacter*, *Enterobacter* and *Candida* species.

(Error bars represent standard deviations of triplicate experiments)

produced acids which is a self beneficial mechanism because yeast grow optimally at pH values between 5.1 to 6.4 but are capable of growth between pH values of 2.5 to 9.5 (Saltarelli, 1989). The initial pH of all flasks was approximately 7.3 ±0.1. After eight hours of growth, experiments using *Enterobacter sp., Acinetobacter sp.* and *Candida* species produced pH values of 5.3, 6.3 and 7.0 respectively. The deviation of the former from neutrality was expected to deter growth of the *Enterobacter sp.* but the O.D. continued to increase between 8 to 12 hours (Figure 6.1). The literature reports that *Acinetobacter sp.* grows at an optimum pH of between 5.5 to 6.0 during vigorous aeration (Balows *et al.*, 1992). However, contrary to what was expected, the results from O.D. measurements showed that the growth of *Acinetobacter sp.* increased no further after the 8-h sample, even as its optimal pH for growth was approached. After the optimum pH of 6.5 was achieved after 15 hours of growth of *Candida sp.,* the O.D. continued to increase up to 26 hours, corresponding to a final pH of 2.8.

However, optical density is a gross parameter that provides information on the turbidity of the solution but provides no information on the viability of the cells present. Results from selective carbon source plate counts performed on each sample are presented in Figure 6.3. Both *Acinetobacter* and *Enterobacter sp.* showed increases in viable cell counts during the first eight hours. After this period, the viability dropped considerably for both *Enterobacter sp.* and *Acinetobacter sp.* at the 12- h sample. This illustrated that O.D. was an insufficient measure of growth when microbes were exposed to adverse growth conditions. Potential lysis of cells would increase the turbidity of the solution providing higher O.D. readings. These high O.D. values would be a false

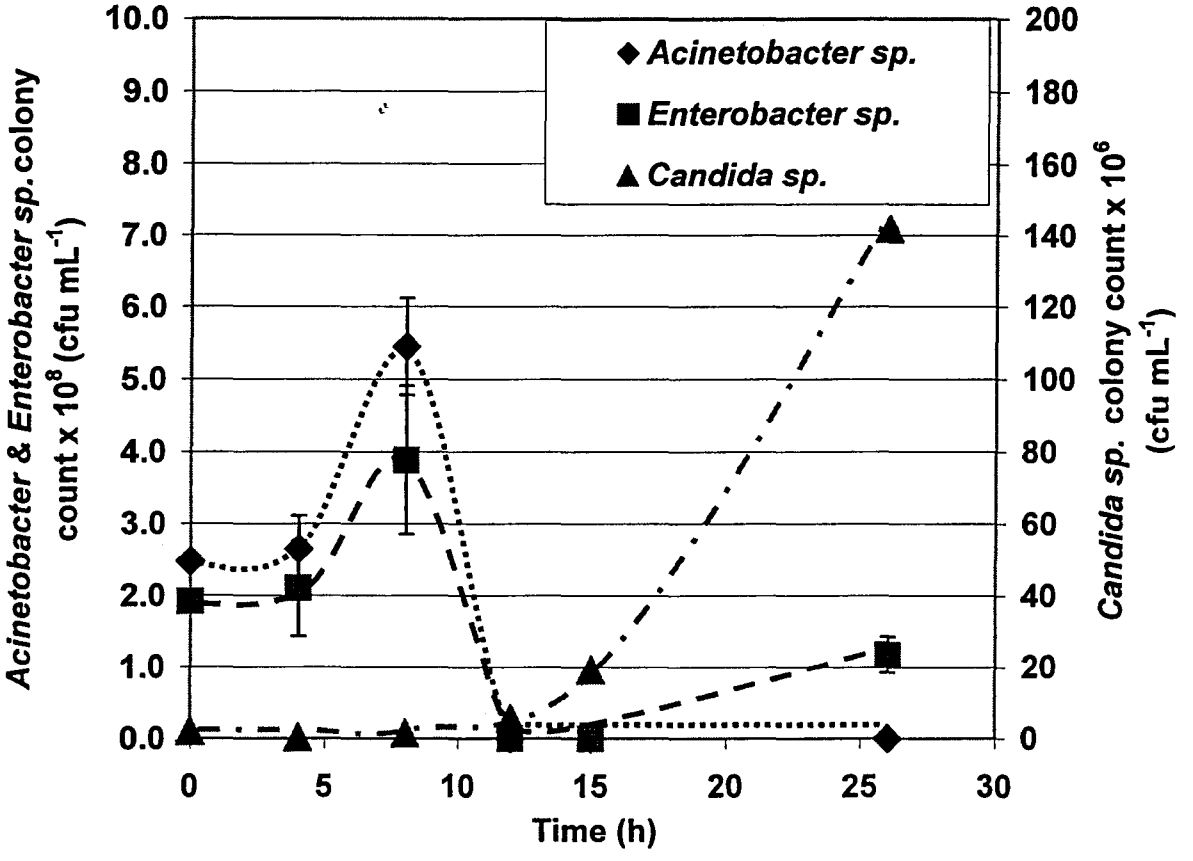

Figure 6.3: Viable colony counts obtained during non-buffered pure culture experiments using *Acinetobacter*, *Enterobacter* and *Candida* species.

(Error bars represent standard deviations of triplicate experiments)

indicator of growth and as seen from Figure 6.3, the viability had, in fact, been significantly decreased. However, it should be noted that in situations where optimal growth conditions are present, O.D. has been shown to be a good measure of growth (see Section 6.1.3).

From Figure 6.2, the pH of the bulk liquid during this period for both the bacterial experiments had decreased to between 4 to 4.3 for *Enterobacter sp.* and *Acinetobacter sp.* respectively. Figure 6.3 illustrated the substantial decrease in colony count at these pH values because these two bacterial species do not prefer extreme acidic conditions. Even though the optical density readings for *Acinetobacter* and *Enterobacter* species remained constant and increased respectively during the period between 8 h to 12 h, the colony counts of the 12-h samples were 3.98×10^4 cfu mL^{-1} and 1 cfu mL^{-1} for *Acinetobacter* and *Enterobacter* species respectively.

The decrease in viability did not correspond to a cessation in growth, but rather a decrease due to an "adaptation" period because growth continued despite a loss in viability. This "adaptation" period was seen from results of plating the 26-h samples on selective carbon source plates which produced colony counts of 3.93×10^4 and 1.17×10^8 cfu mL^{-1} for *Acinetobacter* and *Enterobacter sp.* respectively.

Finally, as expected *Candida* sp. flourished as the pH levels dropped. The initial drop in the colony count corresponded to the lag period in Fig. 6.1, and was perhaps due to adaptation to the initial pH of approximately 7.4 in the batch flask experiment.

6.1.2 Non-buffered experiments - substrate degradation

The capacity of *Enterobacter sp., Acinetobacter sp.* and *Candida* species for degradation of maltose, ethanol, and glucose was investigated. Figures 6.4 to 6.6 depict the residual concentrations of maltose, glucose, and ethanol from cell-free samples obtained at each time interval. As expected, *Enterobacter sp.* was capable of degrading glucose and maltose but not ethanol. Glucose was consumed preferentially, while maltose was significantly reduced during the period between 8 to 12 hours. The degradation of maltose by the *Enterobacter sp.* appeared to be through an initial hydrolysis step. This was apparent from the increase in the glucose concentration as maltose concentration reached a minimum in the 15-h sample. Degradation of the mixed substrates during the period between 8 to 12 h confirmed that the *Enterobacter sp.* were still metabolically active even though viability had decreased.

As previously mentioned, *Candida* species are capable of degrading glucose, maltose, and ethanol. This was confirmed by the results shown in Figure 6.5. The sugar substrates were degraded first until the concentration of sugar decreased to a critical level and then metabolism of ethanol occurred. Similar to the trend seen with *Enterobacter* sp., the sequence of maltose degradation was through hydrolysis. The residual glucose concentration increased during the period between 4 and 15 hours as the maltose concentration declined. Note that the ethanol concentration increased between 4 and 8 hours. Under aerobic conditions when sugars are present in excess, yeasts are capable of producing ethanol due to a respiratory "bottleneck". This phenomenon has been reported

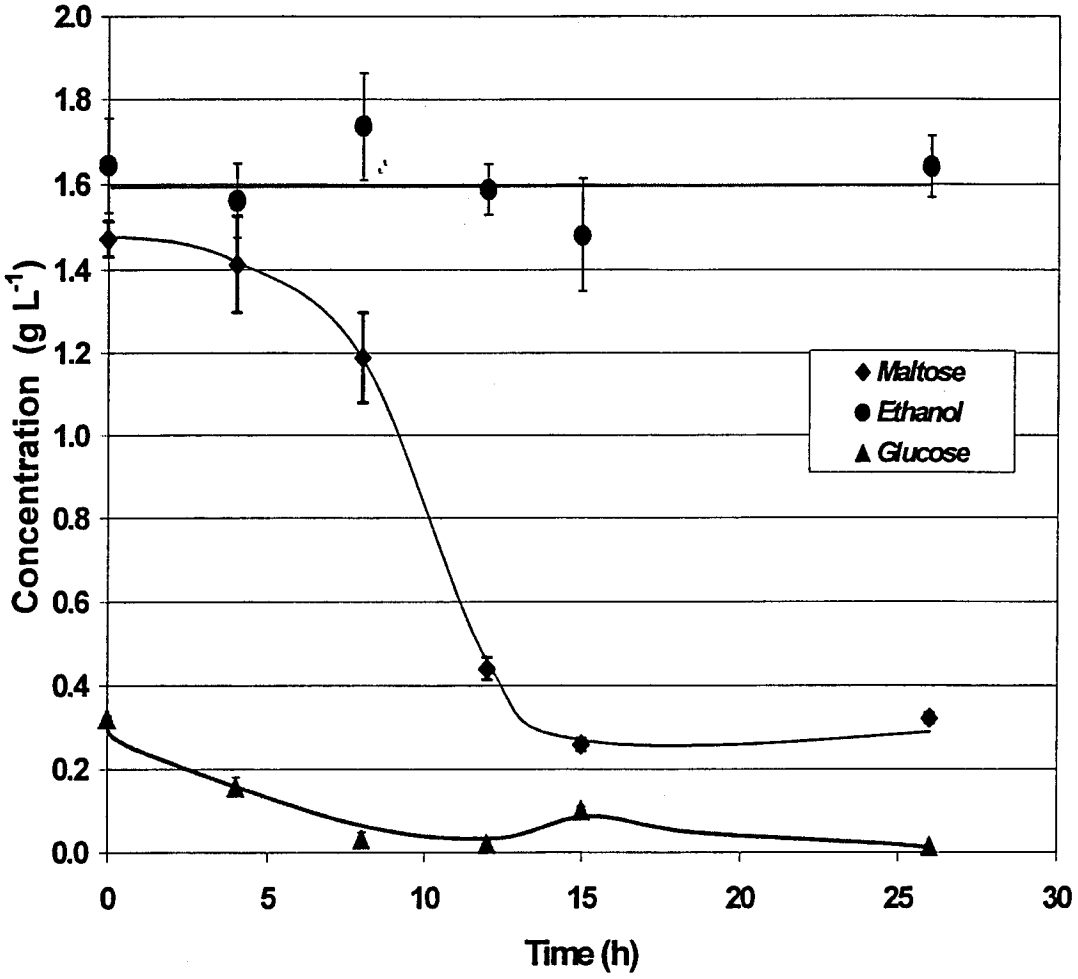

Figure 6.4: Residual concentrations of maltose, glucose, and ethanol in non-buffered *Enterobacter sp.* experiments.

(Error bars represent standard deviations of triplicate experiments)

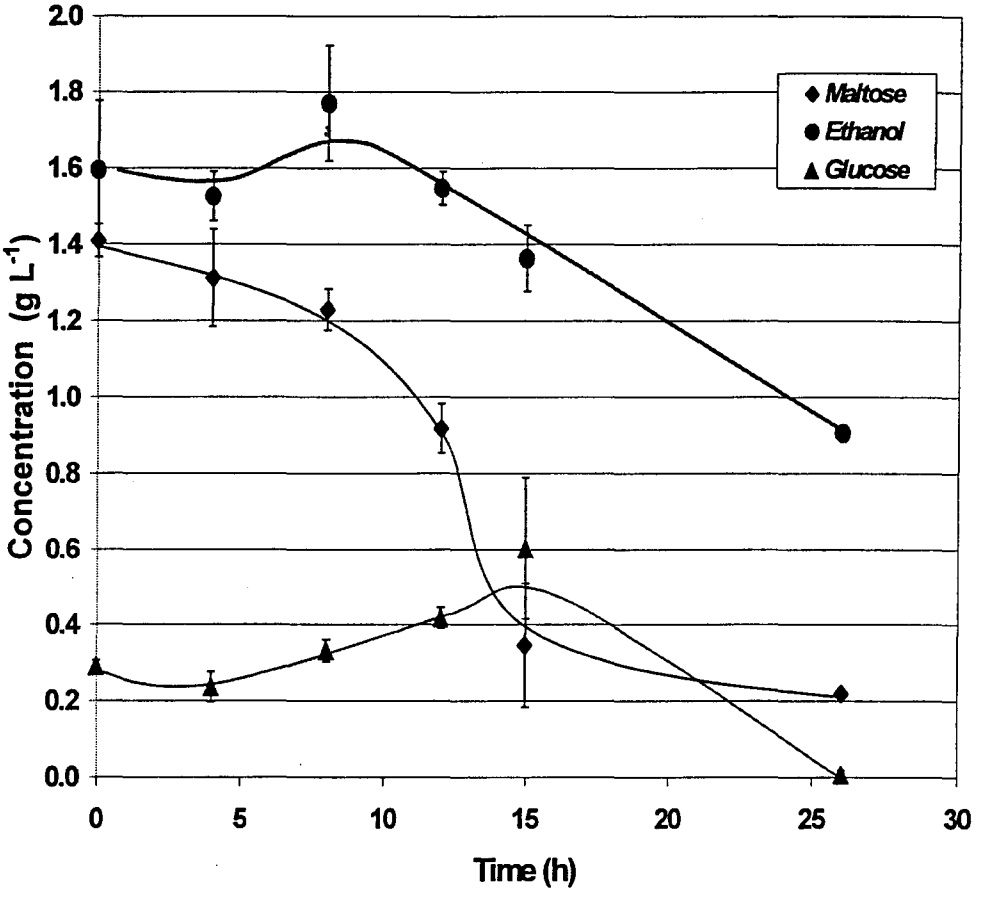

Figure 6.5: Residual concentrations of maltose, glucose, and ethanol in non-buffered *Candida sp.* experiments.

(Error bars represent standard deviations of triplicate experiments)

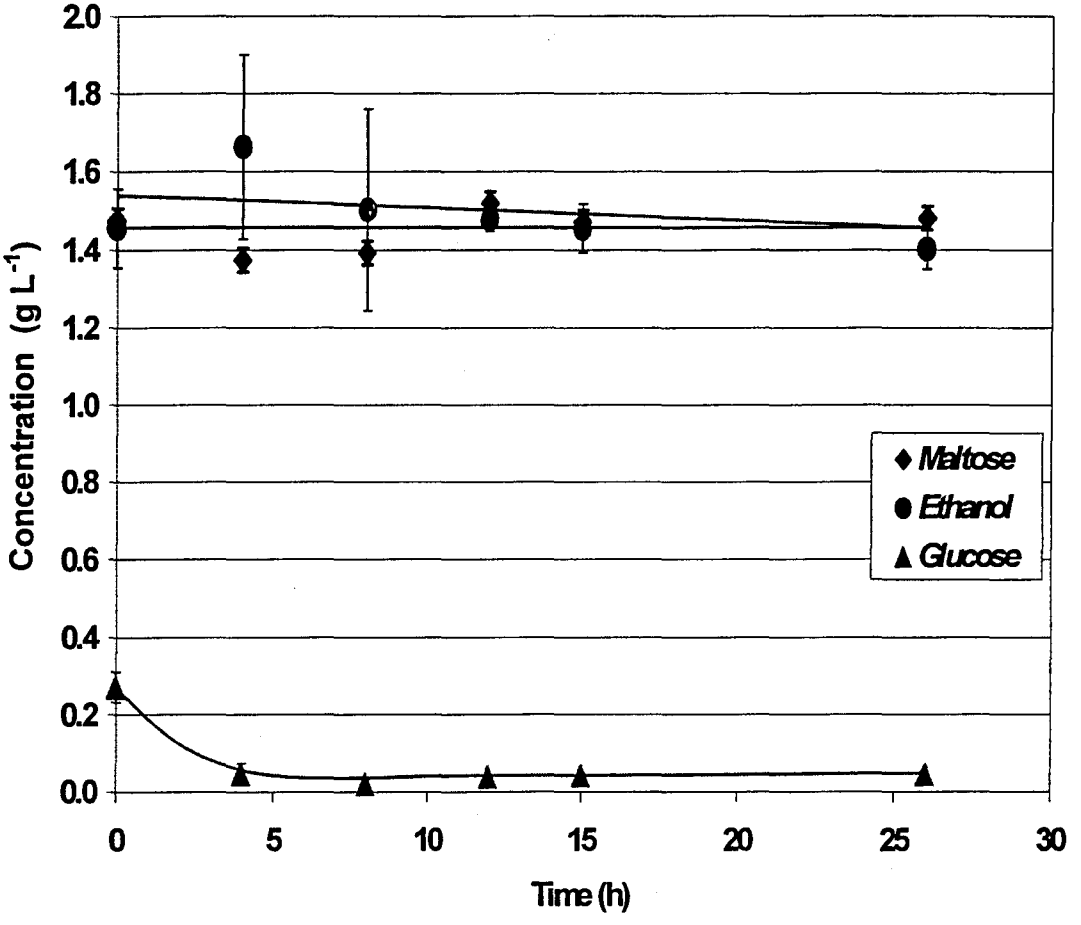

Figure 6.6: Residual concentrations of maltose, glucose, and ethanol in non-buffered *Acinetobacter sp.* experiments.

(Error bars represent standard deviations of triplicate experiments)

for *Saccharomyces cerevisiae* and has been referred to as the 'Crabtree effect' and can be affected by a variety of factors including the mode of sugar transport and the regulation of enzyme activities involved in respiration and alcoholic fermentation (Van Dijken *et al.*, 1993). As the sugar concentration fell and the pH became more favorable, *Candida* sp. was able to degrade all three substrates simultaneously.

From the results shown in Figure 6.6, *Acinetobacter* sp. was only capable of degrading glucose. The concentration of maltose remained unchanged and the concentration of ethanol decreased by less than 5% throughout the 26-h experiment. It was expected that *Acinetobacter* sp. would be capable of degrading sugars and alcohols as mentioned above, however, the concentrations of maltose and/or ethanol present may have been inhibitory to the *Acinetobacter* sp. This is postulated because in mixed culture experiments, *Acinetobacter* sp. was capable of growth (see Section 7.2).

6.1.3 Comparison of non-buffered and buffered experimental results

In other batch flask experiments run in triplicate, the High Ethanol & Mid Maltose solutions were buffered to approximately pH 6 for *Acinetobacter* sp, pH 7 for *Enterobacter* sp. and pH 5 for the *Candida* sp. using buffering agents of citric acid and either monobasic or dibasic sodium phosphate. Figure 6.7 illustrates that during these experiments the pH remained within 5% of the initial pH value.

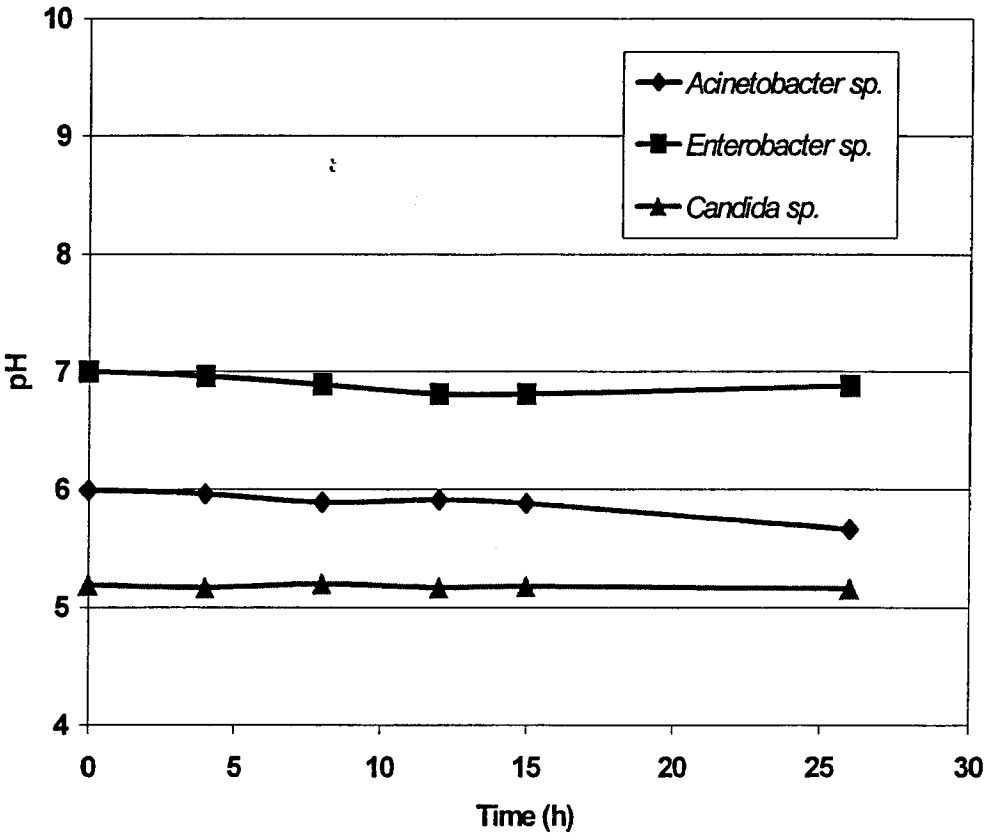

Figure 6.7: pH values obtained during buffered pure culture experiments using *Acinetobacter*, *Enterobacter* and *Candida* species.

(Error bars represent standard deviations of triplicate experiments)

6.1.3.1 *Enterobacter* species

For the *Enterobacter sp.*, the experiment began at the same initial pH of 7 as the non-buffered experiments. Similar trends in optical density were observed with the non-buffered solutions and the buffered solutions (Figure 6.1 and 6.8). In both experiments the maximum O.D. was reached at the 12-h sample. However, with respect to viable cell count (Figure 6.9), the cells in the buffered solutions showed increases in number throughout the rest of the 26-h period similar to the increases in O.D. values (Figure 6.8). This showed that O.D. measurement was a better indicator of viable colony count at optimum environmental conditions.

However, the increased microbial growth in the buffered experiments did not improve degradation of ethanol, confirming that *Enterobacter sp* is incapable of ethanol degradation. As seen in Figure 6.10, it was observed that upon significant reduction of the maltose substrate after the 12-h period, only the remaining glucose was degraded further, while the ethanol concentration remained unchanged. At a more optimal pH of 7, it was observed that the onset of maltose reduction occurred earlier than in the non-buffered experiment. Lag phases occurred for both non-buffered and buffered experiments during the initial 4 hours.

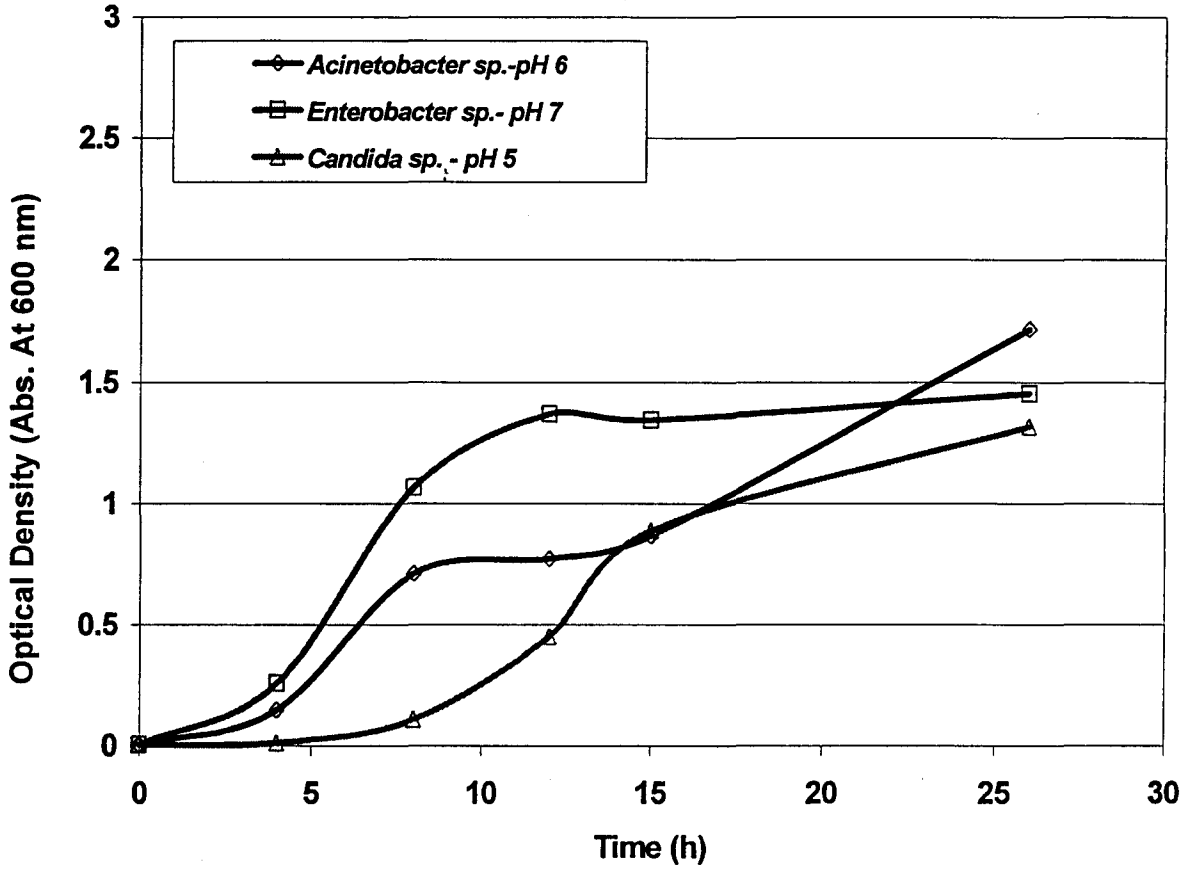

Figure 6.8: Optical densities measured during buffered pure culture experiments using *Acinetobacter*, *Enterobacter* and *Candida* species.

(Error bars represent standard deviations of triplicate experiments)

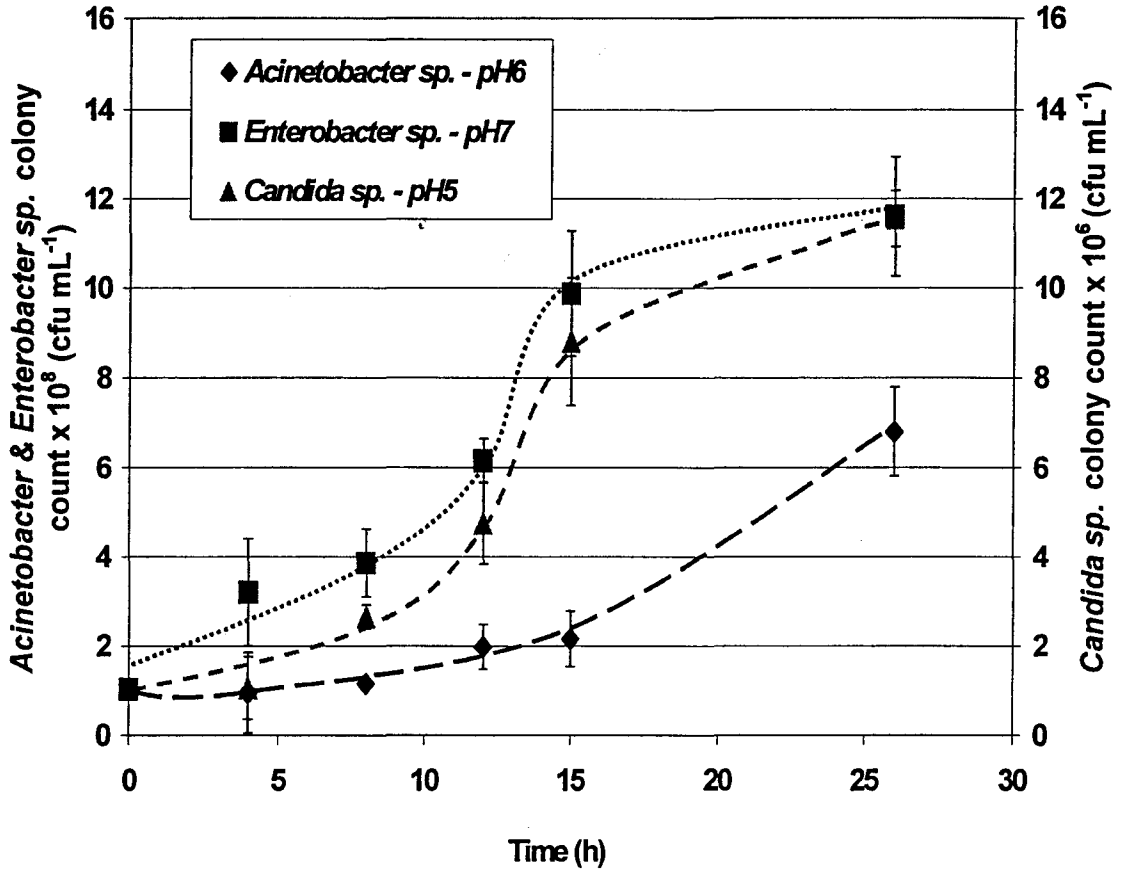

Figure 6.9: Viable colony counts obtained during buffered pure culture experiments using *Acinetobacter*, *Enterobacter* and *Candida* species.

(Error bars represent standard deviations of triplicate experiments)

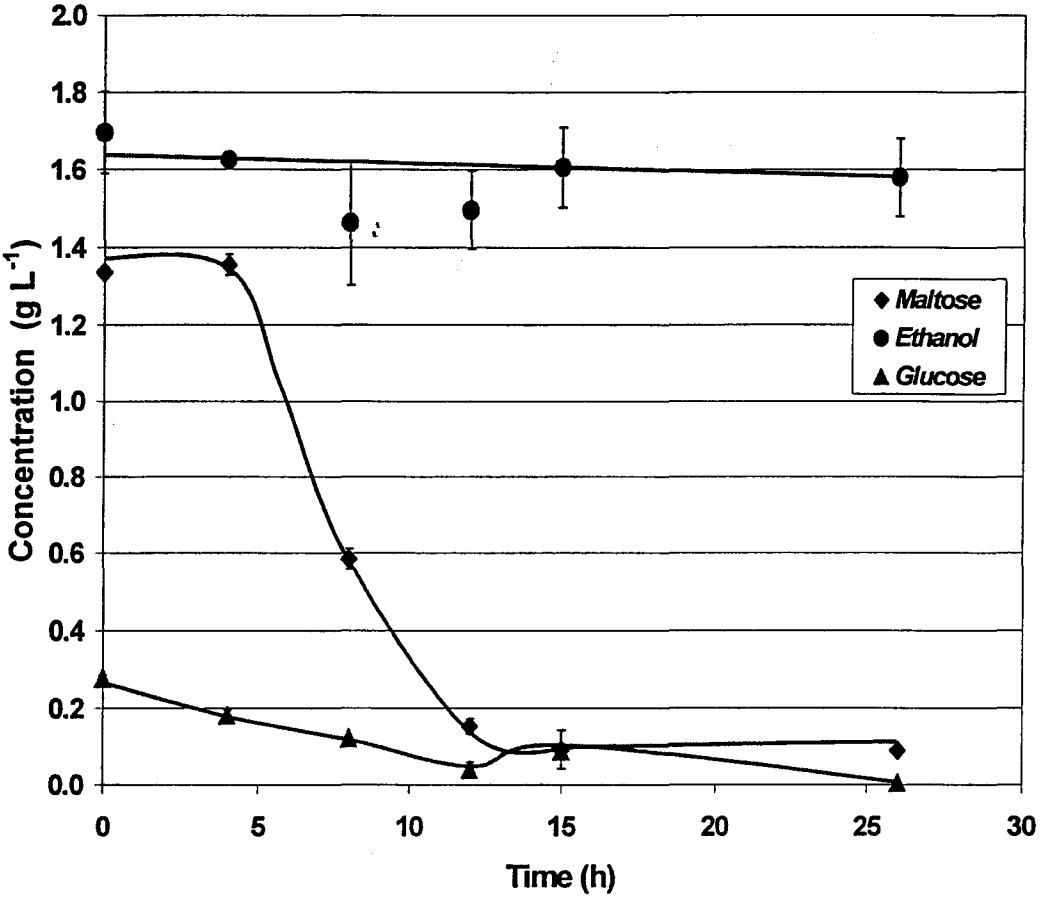

Figure 6.10: Residual concentrations of maltose, glucose, and ethanol in buffered *Enterobacter sp.* experiments.

(Error bars represent standard deviations of triplicate experiments)

6.1.3.2 *Candida* species

Results of experiments buffered at pH 5 with *Candida* species contrasted with the results of the other buffered experiments. Comparison of the optical densities showed that both required lag phases for the initial 12 hours (Figure 6.8). However, in the buffered medium, the maximum optical density and colony counts achieved were reduced, at approximately 1.32 and 1.16 x 10^7 cfu mL^{-1} respectively in comparison to an O.D. of 2.73 and colony count of 1.42 x 10^8 cfu mL^{-1} for the non-buffered case.

Comparison of the plots for carbon degradation of the buffered system and non-buffered systems (Figure 6.11 and Figure 6.5) demonstrated that the degradation capacity was lower at a pH of 5. With respect to maltose degradation, by the 15-h sample maltose had been reduced to approximately 0.35 g L^{-1} in the non-buffered case (approximately pH 6.4) but only to 0.77 g L^{-1} at a pH of 5. As expected, the previously discussed 'Crabtree effect', also referred to as a respiratory bottleneck, occurred in the buffered system also. Increased glucose concentrations in either case illustrated that the degradation of maltose involved hydrolysis. Although capable of synthetic brewery wastewater degradation at a pH of 5, the growth of *Candida* species appeared to be better when the pH was allowed to decrease further.

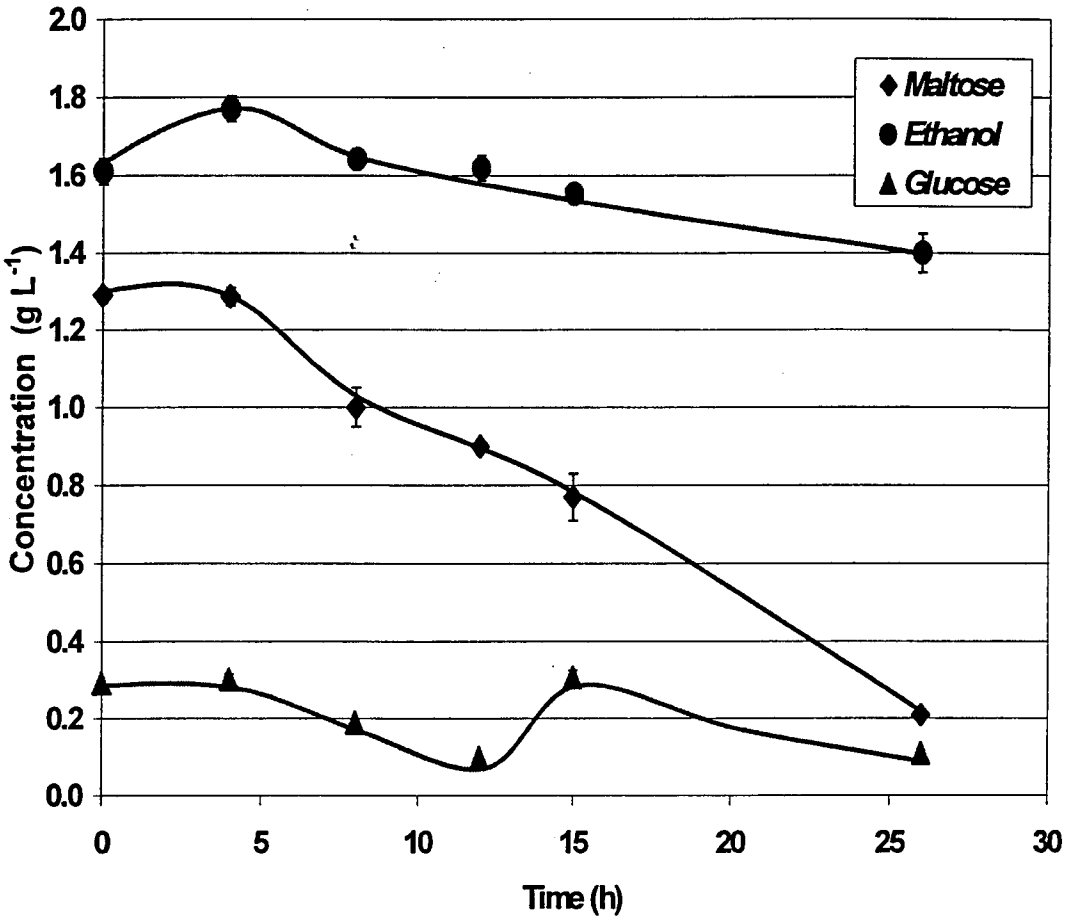

Figure 6.11: Residual concentrations of maltose, glucose, and ethanol in buffered *Candida sp.* experiments.

(Error bars represent standard deviations of triplicate experiments)

6.1.3.3 *Acinetobacter* species

Performance of the *Acinetobacter sp.* was poor in the non-buffered system with an ability to substantially degrade only glucose, with minimal ethanol degradation and no maltose degradation during the 26-h experiments. Performance of the same microorganism in a more optimal pH environment of 6 yielded substantially better results. After the 15-h time sample, *Acinetobacter sp* continued to grow as detected by the increase in O.D. (Figure 6.8). The colony count continued to increase during the period between 15 h and 26 h (Figure 6.9) and Figure 6.12 verified that *Acinetobacter sp* was capable of degrading all carbon sources in the synthetic brewery wastewater. Following a lag period and an initial increase in the O.D. between 4 h and 8 h, a plateau in O.D. was observed spanning the period between 8 h and 15 h. Ethanol degradation was apparent during this period of time. It is postulated that during this period of time *Acinetobacter sp.* produced the enzymes and metabolites necessary for maltose assimilation. As the *Acinetobacter sp* became capable of degrading maltose, this sugar became the preferential substrate in comparison to ethanol. These buffered experiments verified information from the literature in which *Acinetobacter* species were reported to be capable of degrading both alcohols and sugars (Balows *et al.*, 1992).

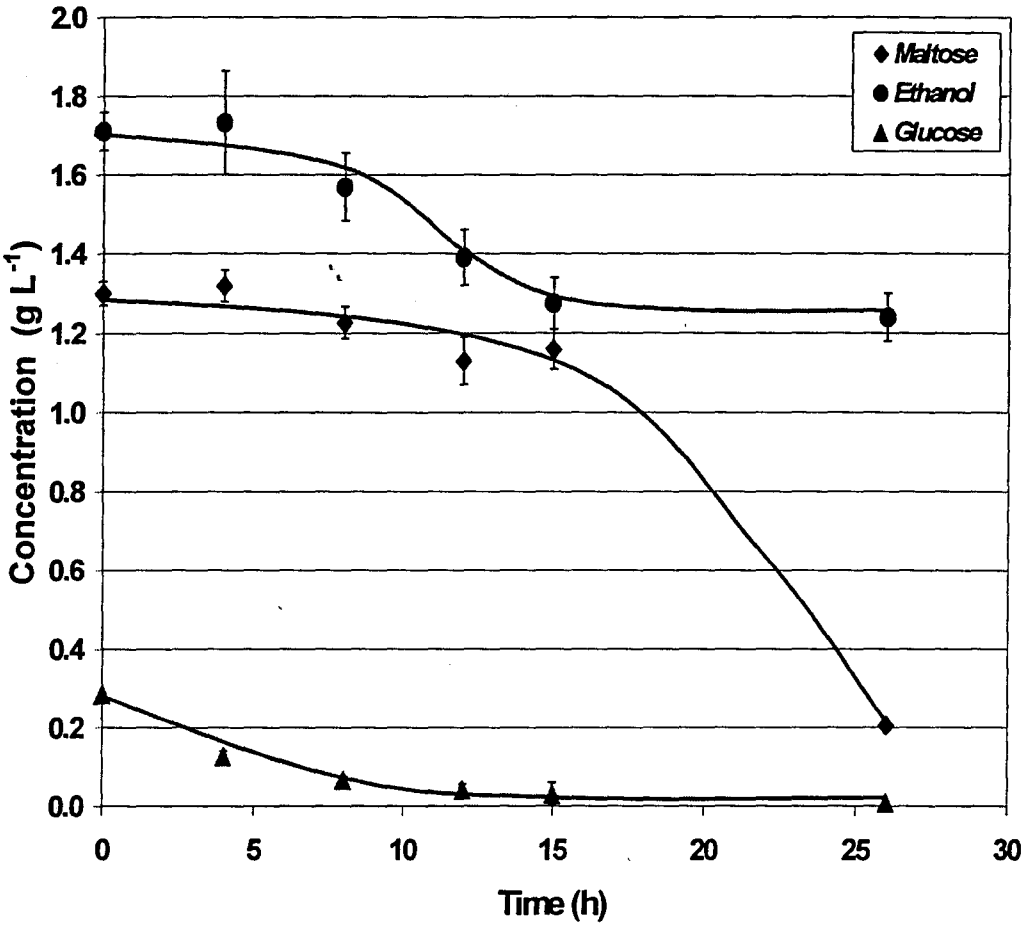

Figure 6.12: Residual concentrations of maltose, glucose, and ethanol in buffered *Acinetobacter sp.* experiments.

(Error bars represent standard deviations of triplicate experiments)

6.2 Mixed Culture Experiments

From the pure culture experiments, it was determined that *Enterobacter sp* could degrade glucose and maltose but not ethanol, *Acinetobacter sp.* could degrade glucose, ethanol and maltose at optimal conditions, and *Candida sp.* could degrade all three carbon sources. The question then arises as to how these behaviours change in a mixed culture. To investigate this, two sets of experiments were run in triplicate with all three pure cultures mixed together. In the first set, growth and substrate degradation was monitored for 24 hours in a non-buffered medium with samples taken at 0, 3, 6, 17 and 24 h. Two approaches, including PCR-DGGE and selective carbon source plate counts, were used to ascertain the population changes of each species in the mixed consortium. These population results, in addition to data on the substrate degradation are presented. In the second set of experiments, samples were taken during mixed culture flask experiments buffered at a pH of 7 in 30-h experiments. The viable colony counts and substrate degradation capacity are also presented in the following sections.

6.2.1 Non-buffered mixed culture experiments

6.2.1.1 Substrate degradation

The mixed consortium containing *Acinetobacter sp*, *Enterobacter sp*, and *Candida sp.* was able to degrade the synthetic brewery wastewater as shown by the residual concentrations of glucose, maltose and ethanol as presented in Figure 6.13, in

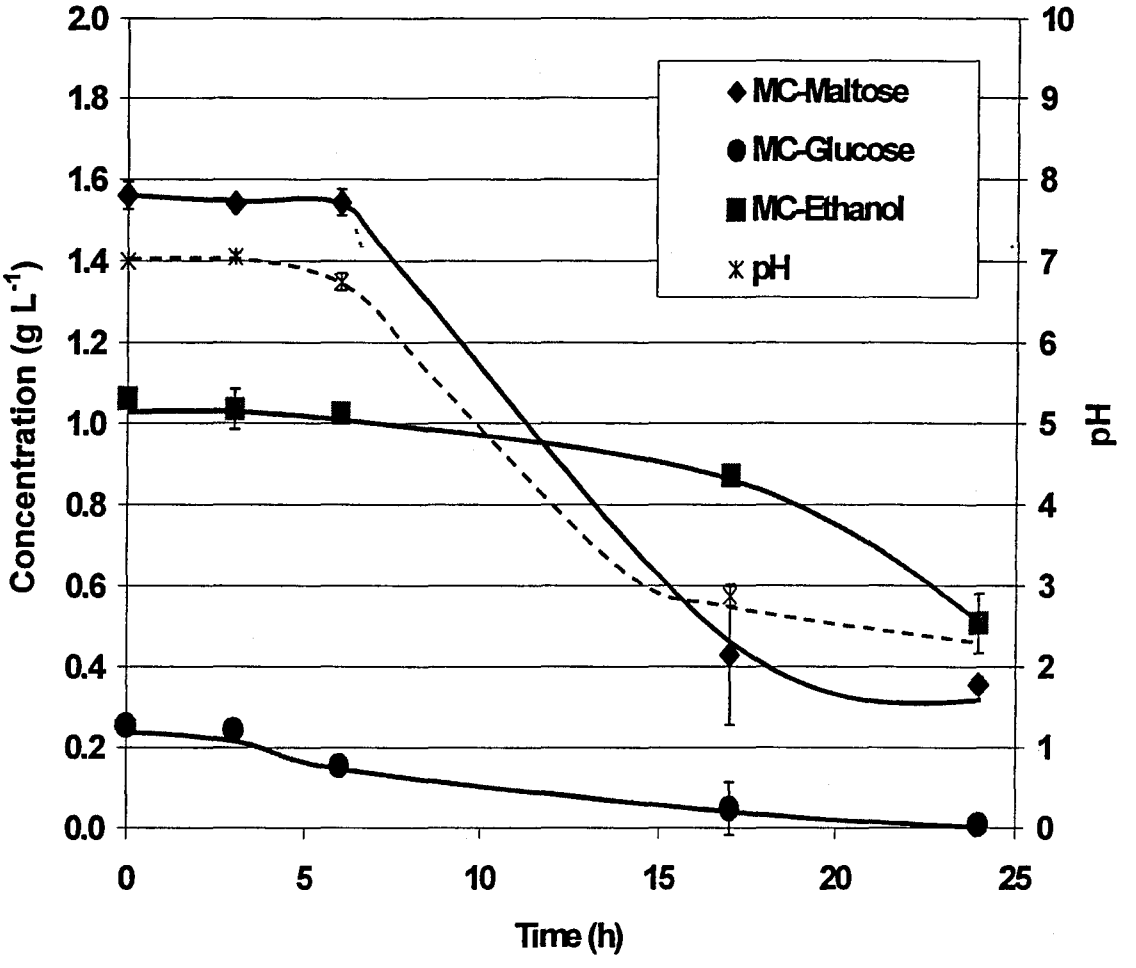

Figure 6.13: Residual concentrations of maltose, glucose, and ethanol in non-buffered mixed culture experiments containing *Acinetobacter sp.*,*Enterobacter sp.* and *Candida sp.*

(Error bars represent standard deviations of triplicate experiments)

addition to the pH measurements obtained throughout the experiment. A lag phase was evident during the first 3 h of the experiment before glucose was consumed by the mixed culture. The next substrate consumed was maltose, and finally ethanol was degraded after the sugar levels had been reduced. However, to determine the contributions to this degradation by each of the key microorganisms, characterization of the relative populations within the mixed culture had to be achieved.

6.2.1.2 Mixed culture characterization

In the first method, DNA was extracted from the sample, PCR amplified and electrophoresced on a polyacrylamide DGGE gel for the bacterial and fungal species. One benefit of DGGE is that a mixed culture sample subjected to DGGE electophoresis can result in the separation of the mixed culture 16S rDNA into individual bands corresponding to the different pure cultures present in the consortium as seen in Chapter 5. In a simplistic consortium containing the three microorganisms presented in this research, PCR-DGGE worked with minimal interference from problems that have been demonstrated with complex mixed consortiums in the past. Hansen *et al.* (1998) found PCR amplification of *Pseudomonas putida, Microbacterium arborescens, Nocardioides simplex*, and a β-subgroup proteobacterium to be highly biased where one out of four was preferentially amplified. The observed bias was attributed to these species having segments of genomic DNA outside of the amplified region that inhibited the initial PCR steps. Potential problems associated with congruence of location of bands on the DGGE gel for different bacterial species in a rhizosphere community were demonstrated by

Yang and Crowley (2000) whereby different taxonomic bacterial species were identified from a single band from DGGE. Cloning of the bands from DGGE of the mixed culture in comparison to results from cloning of the isolated pure cultures from this mixed consortium were reconciled to eliminate the potential problem of multiple species in one band. With respect to PCR bias, results of DGGE from triplicate batch experiments presented in Plate 6.1 and densitometry results of the DGGE bands in Figure 6.14 indicated that the band intensities of each time sample had standard deviation values within 10% and within 15% for *Enterobacter* species. The intensity of the bands at each time interval is directly proportional to the abundance of 16S rDNA present in each bacterial species represented by the sample. Trends of the change in abundance of 16S rDNA present were observable. Thus the band intensities were used as a measure of abundance of the individual population of microorganisms present at each time interval, relative to their initial values.

In Plate 6.1, the fingerprint of bands from DGGE for each mixed culture sample taken at the specified time intervals are presented in addition to standards in lanes A, I, and Q. The standard that had been frozen at -70°C consisted of previously investigated mixed culture 16S rDNA used in the identification of the key microorganisms as described in Chapter 5. Matching the locations of the standard bands with those of the sample helped to determine the previously identified bacterial species present. The top band highlighted by the upper arrow represents *Acinetobacter* species. *Enterobacter* species' fingerprint profile consisted of 3 bands and the brightest of these three bands,

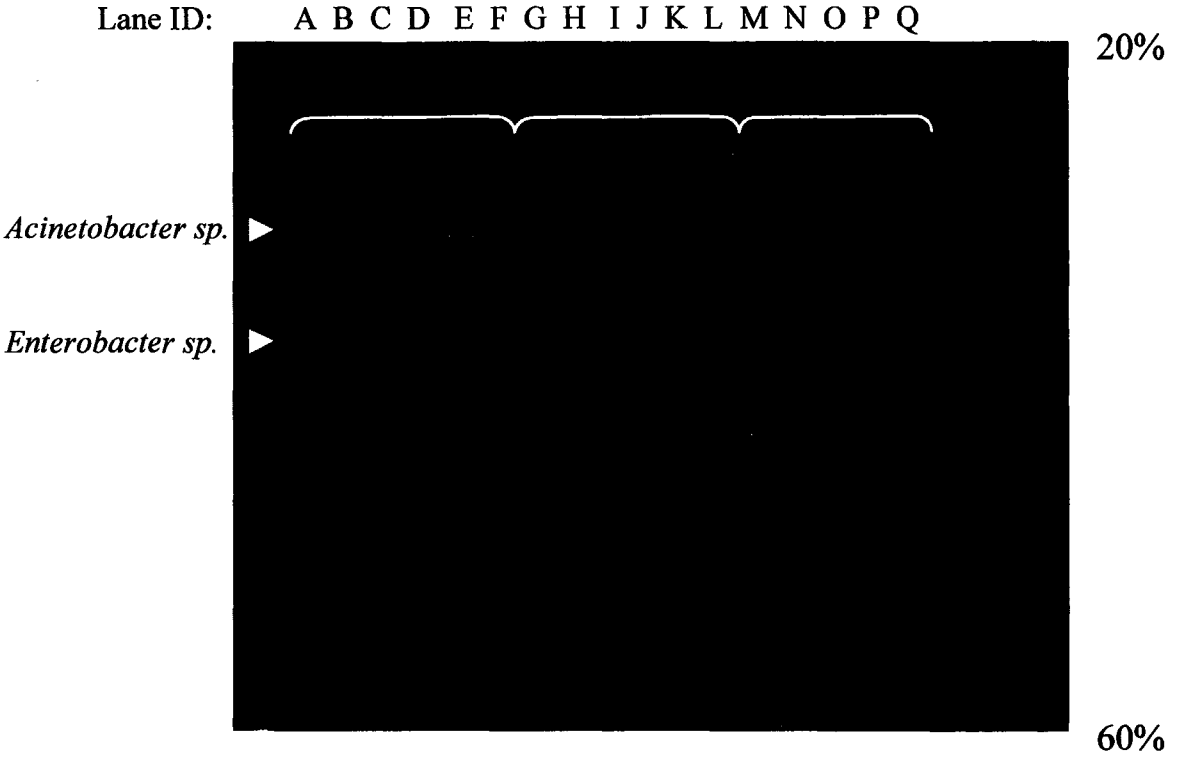

Plate 6.1: DGGE gel with denaturant concentrations from 20 to 60% of bacterial PCR products from the mixed culture.

First set of Batch Flask Experiments:(Letters denote Lane IDs)
A) Standard; B) t=0; C) t=3 h; D) t=6 h;
E) t=17 h; F) t=24 h;

Replicate: G) t=3 h; H) t=6 h; I) Standard; J) t=17 h; K) Blank;
L) t=24 h;

Triplicate: M) t=3 h; N) t=6 h; O) t=17 h; P) t=24 h; Q) Standard.

which is indicated by the lower arrow, was used for its representation due to its higher intensity.

In order to transform the intensity of these representative bands into numerical data, the gel image was imported into Scion Image (Scion Corp., Frederick, MD), an image analysis program for conversion of the band intensities of the lanes into x/y plot profiles. This Cartesian data was then transferred into EXCEL files (Microsoft Inc., Seattle, WA) for compilation of the data obtained for the lanes of band profiles before being analyzed and integrated using peak analysis software (Peakfit vs.4, SPSS Inc., Chicago, IL). The result of the peak analysis software provided a plot of each lane correlating each band with a peak and each band's intensity to the area underneath the peak. Normalizing all the bands to the brightest band on the gel, a plot depicting the relative intensity and hence abundance of 16S rDNA present for each species is presented in Figure 6.14.

Similarly for the fungal species, DGGE analysis resulted in a profile of bands for the *Candida sp.* depicted in lane A of an inverted image of the gel in Plate 6.2. An inverted image was depicted for easier detection of the bands present since very bright lanes were obtained. In this inverted image, the darkened areas corresponded to the bright areas on an actual photo of the gel. Inspection of the profile showed that little separation between the bands occurred, rendering the peak analysis software used for the bacterial species inadequate. Distinct peaks in this band profile would be difficult to obtain with the added difficulty of a very bright background in the lanes.

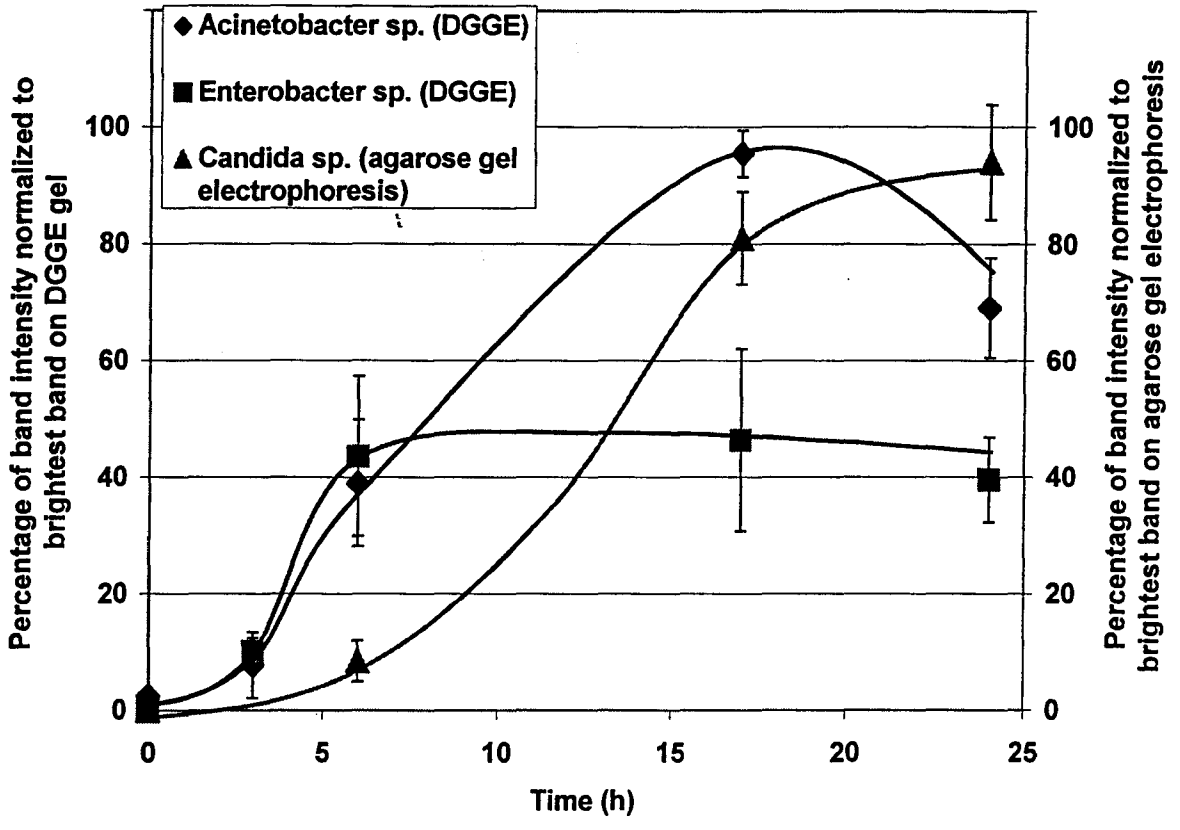

Figure 6.14: Changes in band intensity of the individual bacteria (*Acinetobacter*, and *Enterobacter sp.*) from denaturing gradient gel electrophoresis (DGGE), and changes in band intensity of *Candida* species from an 1.8% agarose gel.

(Error bars represent standard deviations of triplicate experiments)

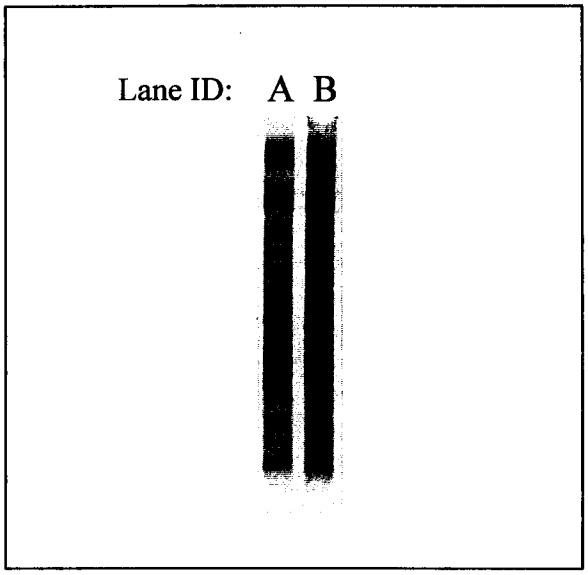

Plate 6.2: Inverted DGGE photo of fungal species
(Letter denotes Lane ID).
A) *Candida sp.*
B) Mixed Culture

The fungal band profile of the mixed consortium is illustrated in Lane B. It was noted that this band profile was identical to that of the pure culture *Candida sp.*, indicating that *Candida sp.* was the only yeast or fungal species present in the consortium. This was verified by plating the mixed culture onto antibacterial agar plates where only *Candida sp.* was detected. Therefore, band intensity analysis of the 16S rDNA present on a 1.8% agarose gel would provide the information required to track the change in rDNA present for the yeast. Plate 6.3 is a photo of the 1.8% agarose gel used for *Candida sp.* where lanes C, M and R contained DNA ladders where the bands are correlated to standard fragment sizes of DNA. With the use of these DNA ladders, the band location of the samples on the agarose gel was confirmed to be yeast because the DNA fragments obtained were approximately 1.7 kb (kilobases). These band intensities are also shown in Figure 6.14 where the percentages are obtained by normalizing the bands to the brightest band on the agarose gel. These percentages should not be compared directly to the percentages obtained for *Acinetobacter* and *Enterobacter sp.* since they are not normalized in the same way in comparison to the *Candida sp.* However, inspection of the trend of the percentages provided information on the increase in 16S rDNA of each species.

In the second approach, samples from the same time intervals were plated onto carbon source plates selective for *Enterobacter sp.*, and *Acinetobacter sp.,* and antibacterial synthetic brewery wastewater agar plates for fungal species present in the mixed culture. This provided the viable population of the microorganisms as seen in Figure 6.15. Viable plate counts showed that during the first 6 hours of growth, while the

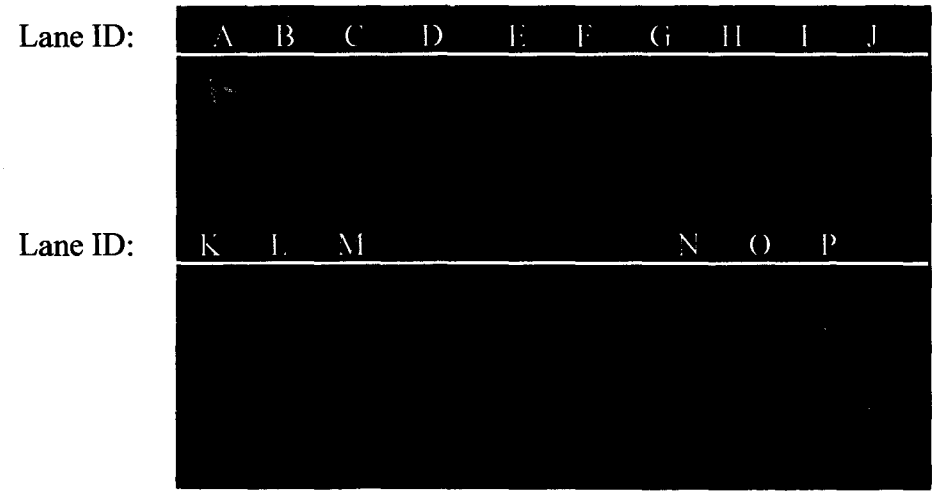

Plate 6.3: 1.8% agarose gel for fungal PCR products obtained from the Mixed Culture (MC).

First set of Batch Flask Experiments: (Letters denote Lane IDs)

A) DNA ladder;
B) t=0;
C) t=3 h;
D) t=6 h;
E) t=17 h;
F) t=24 h;

Replicate: G) t=3 h;
H) t=6 h;
I) t=17 h;
J) t=24 h;

Triplicate: K) DNA ladder;
L) t=3 h;
M) t=6 h;
N) t=17 h;
O) t=24 h;
P) DNA ladder.

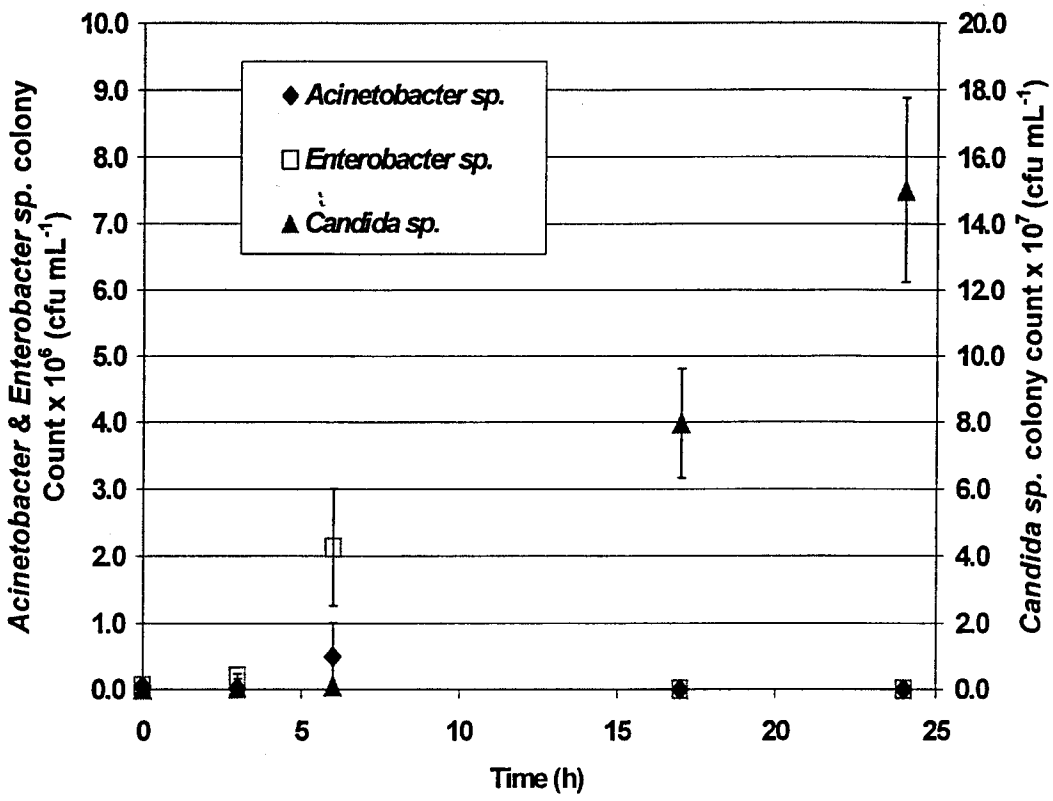

Figure 6.15: Viable colony counts obtained during non-buffered mixed culture experiments using *Acinetobacter*, *Enterobacter* and *Candida* species.

(Error bars represent standard deviations of triplicate experiments)

pH was stable at a pH of approximately 7, the viable colony forming population increased for both *Enterobacter sp.*, and *Candida sp.*, while *Acinetobacter sp.* increased after the 3-h time interval. After this period, as the pH dropped to 3 (Figure 6.13) due to lack of buffering capacity, the viability of both *Enterobacter sp.*, and *Acinetobacter sp.*, dropped substantially as the yeast proliferated in the more acidic environment.

The rDNA abundance data from Figure 6.14 was in agreement with the plate counts during the first 6 hours for an overall increasing trend in colony forming units and increased rDNA. It has been stated that the average RNA concentration within a cell increases directly with the population growth rate and is usually observed only for growth–rate variations caused by differences in nutrient medium (Bailey and Ollis, 1986). Therefore qualitative information can be obtained from Figure. 6.15. During the period between the 6 h and 17 h samples, the abundance of 16S rDNA increased. Hence, the observed increase in 16S rDNA values provided information that cells were growing during this period and consuming substrates. This coincided with results of substrate degradation in Figure 6.13, where reduction of maltose, glucose and ethanol were observed at a low pH condition. In addition to the detection of microbial activity, monitoring of the 16S rDNA also illustrated that during the period between 6 h and 17 h, only *Acinetobacter* and *Candida* species replicated in rDNA, indicating some growth of the cells had occurred. *Enterobacter* species had no further increase in 16S rDNA but substrate degradation is possible for maintenance of these cells without replicating rDNA. The advantage to the method monitoring the abundance of 16S rDNA of the samples is that in the absence of viability data, DGGE can still provide some information pertaining

to the activity that has occurred by monitoring changes in the total 16S rDNA. This is possible because genetic material remains even if the cells are no longer viable.

Although the DGGE method has the advantage of providing data on the abundance of rDNA even during adverse conditions for growth, and can provide information for multiple species of bacteria in one DGGE gel, it does not provide the information of whether the cells present are viable or not. Furthermore, the PCR amplification process limits the sensitivity of this method for tracking population changes with dramatic changes in population. In one PCR analysis where the range of 16S rDNA present at each time interval was large, difficulties were encountered in choosing a correct dilution of the 16S rDNA template for optimal PCR amplification for samples at all time intervals. Thus, the use of the PCR-DGGE method alone for tracking population dynamics is insufficient.

However, new developments in molecular technology have enabled the isolation of cells with newly synthesized DNA. Borneman (1999) used a thymidine nucleotide analog, bromodeoxyuridine (BrdU), in the examination of microorganisms in soil with various nutrient supplement additions. After a 3-day incubation period, the DNA was extracted from the soil samples, and the newly synthesized DNA was isolated by immunocapture of the BrdU-labeled DNA. Results from the total DNA analysis in comparison to the BrdU-labeled DNA showed significantly different banding patterns. BrdU-labeled DNA provided information on the active cells. Similar studies were also

performed by Urbach *et al.* (1999) with cultured bacteria and natural populations of aquatic bacterioplankton.

Since PCR-DGGE provided information on the total DNA present indiscriminately between dead or viable cells, the culturing method on agar plates will continue to be used for tracking the changes in the populations of *Enterobacter sp.*, *Acinetobacter sp.* and *Candida sp,* the three dominant genera present.

6.2.2 Buffered mixed culture experiments

In an experiment where the pH was buffered at 7, samples were taken at time intervals of 0, 2, 4, 6, 9, 12, 24, and 30 h for mixed culture experiments. These samples were plated onto selective carbon source plates for tracking the population changes of *Enterobacter, Acinetobacter*, and *Candida* in the mixed culture and the results are presented in Figure 6.16. It was observed that the dominant microorganism in these experiments was the *Enterobacter* species. At a pH optimal for its growth, *Enterobacter* species out-competed *Acinetobacter* and *Candida* for the substrates present in the Mid Maltose & High Ethanol synthetic wastewater.

Buffered pure culture experiments with *Enterobacter* species demonstrated that at pH 7, *Enterobacter* degraded glucose, and then maltose but was unable to degrade ethanol (Figure 6.4). *Candida* species also demonstrated a capacity for glucose and maltose degradation at a pH of approximately 7 from Figures 6.2 and 6.11, in addition to

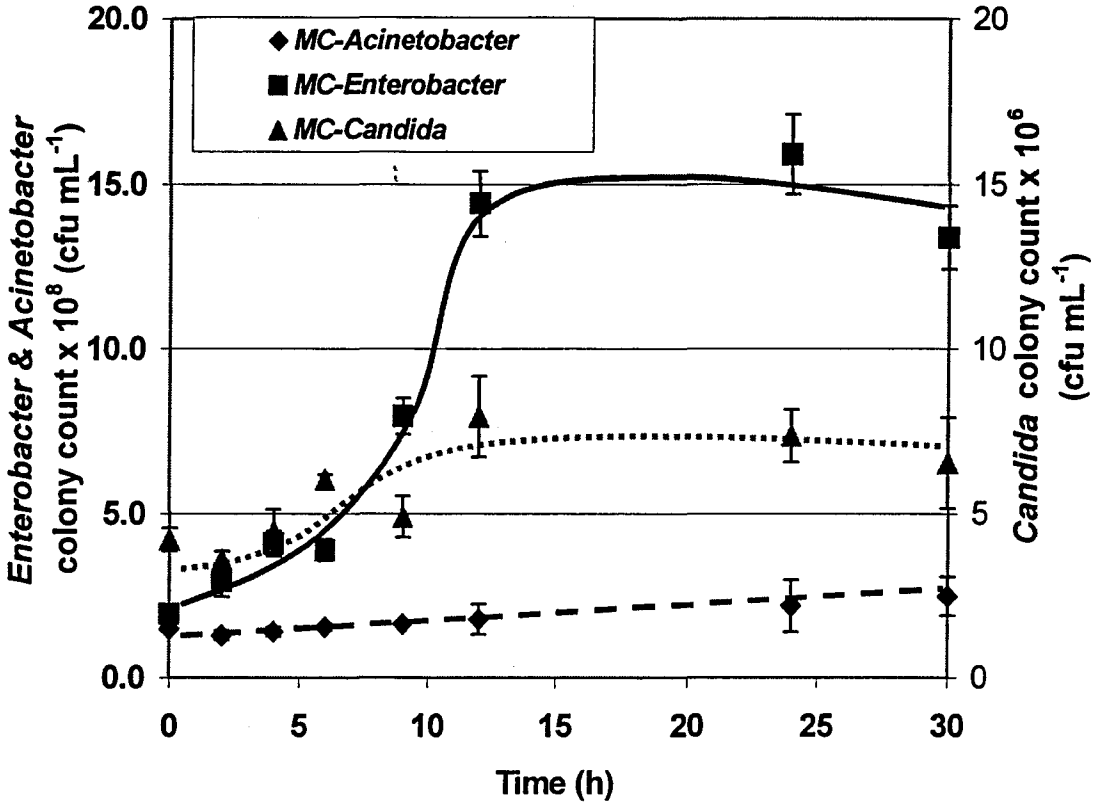

Figure 6.16: Viable colony counts obtained during buffered mixed culture experiments using *Acinetobacter*, *Enterobacter* and *Candida* species.

(Error bars represent standard deviations of triplicate experiments)

the ability to produce ethanol through a respiratory bottleneck when high concentrations of sugar are present. These behaviours from the pure culture experiments were detected in the buffered mixed culture experiments.

During the initial 12-h period, glucose and maltose were easily degraded (Figure 6.17) and this coincided with the increasing colony counts by both *Enterobacter* and *Candida* species. A slight increase in the ethanol concentration was also detected at the 2-h sample. This could be explained by the respiratory bottleneck effect demonstrated by *Candida* species in pure culture experiments when the maltose concentration was still high at the 2-h time interval.

Acinetobacter species were found to be capable of degrading only glucose at a pH of approximately 7 from Figures 6.2 and 6.6 of the non-buffered *Acinetobacter* experiments. Therefore, it was expected that little change in colony counts would be detected as seen in Figure 6.16 during this period. After the 12-h period, little maltose remained and no residual glucose was detected (Figure 6.17). Both *Acinetobacter* and *Candida* species had demonstrated ethanol degradation in pure culture experiments at pH values of 6 and 5 respectively (Figures 6.12 and 6.11). The slight reduction in residual ethanol concentration would be attributed to either *Candida* or *Acinetobacter*.

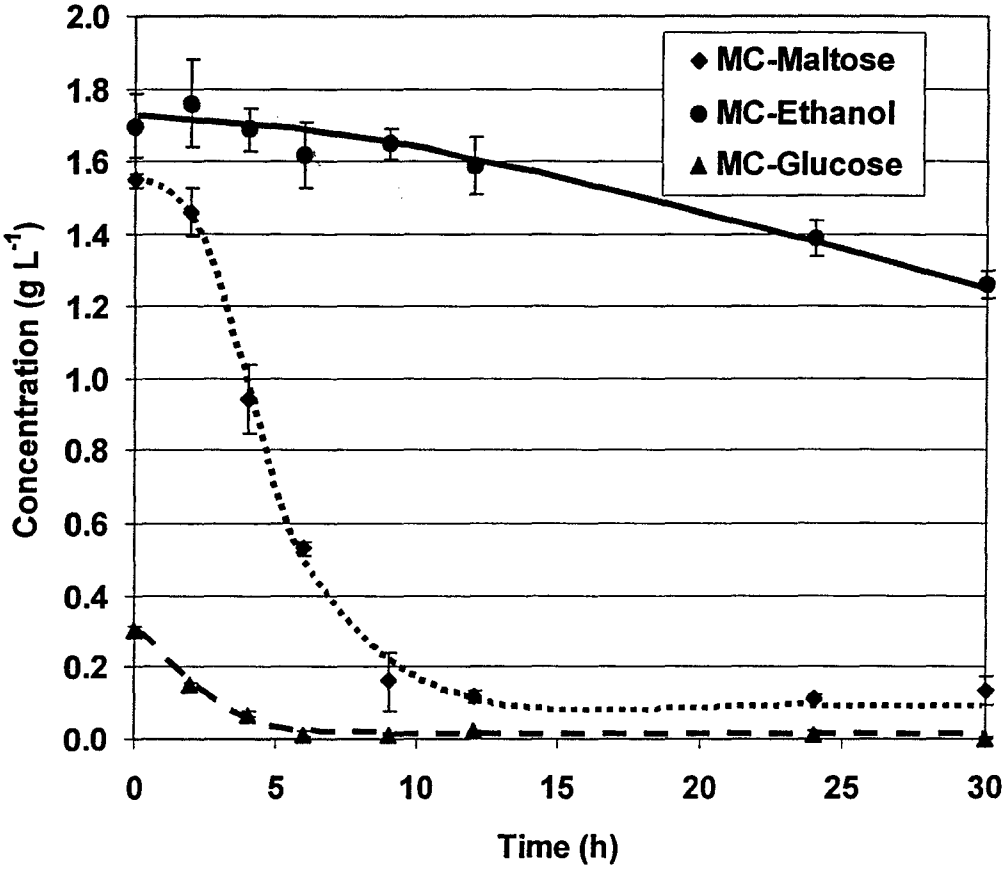

Figure 6.17: Residual concentrations of maltose, glucose, and ethanol in buffered mixed culture experiments containing *Acinetobacter sp.*, *Enterobacter sp.* and *Candida sp.*

(Error bars represent standard deviations of triplicate experiments)

6.3 Summary

In this chapter individual behaviour in buffered and non-buffered experiments were discussed. The substrate degradation behaviours were established for each of the species considered in this research, *Acinetobacter, Enterobacter* and *Candida* species. In addition, correlation between optical density and viable plate counts was found to be valid in the buffered case only. Mixed consortium behaviour was also studied in buffered and non-buffered media. The analytical techniques of PCR-DGGE and selective carbon source plate counts were used to evaluate population dynamics in a mixed culture. Although PCR-DGGE was found to be effective in identifying the dominant microorganisms in the consortium, this technique was found to be limited in that viability of cells could not be determined. Finally, the pure culture behaviours of the individual species in the defined mixed culture consortium aided the understanding of the degradation results in the mixed culture experiments.

CHAPTER 7

KINETICS OF SUBSTRATE DEGRADATION IN SEMI-CONTINUOUS EXPERIMENTS

The self-cycling fermentation process, a semi-continuous system, has been well studied for pure culture cases both experimentally and with models (Wincure *et al.*, 1995; Hughes and Cooper, 1996). In the case of mixed cultures, an initial study was conducted by Nguyen *et al.* (2000) where a mixed culture consortium was used for studying the degradation performance of a semi-continuous packed bed reactor for the treatment of brewery wastewater from Molson's brewery in Vancouver. It was determined that treatment using a mixed consortium produced repeatable cycles similar to experiments using pure cultures. No kinetic models were developed nor was the population identified. The objective of this chapter is to examine the population dynamics and to determine the kinetics of a defined mixed culture in a similar semi-continuous reactor system controlled by feedback control based on the concentration of dissolved oxygen.

7.1 Kinetic experiments for substrate degradation

Five kinetic experiments were conducted in replicate to investigate the behaviour of the semi-continuous reactor system. All experiments were performed at a constant temperature of 25°C and pH controlled within a range of 6.5 to 7.0. Different concentrations of ethanol and maltose were used in the synthetic brewery wastewaters and were tabulated in Table 4.1. These were denoted as Low Maltose & Mid Ethanol,

High Maltose & Mid Ethanol, Mid Maltose & Low Ethanol, Mid Maltose & High Ethanol, and Mid Maltose & Mid Ethanol concentrations.

Biofilm contribution runs that were performed throughout these semi-continuous experiments were designed to provide kinetic data of substrate degradation due solely to the biofilm as it developed throughout the experiment. Repeated weighing of a piece of tubing in the reactor system provided a semi-quantitative measure of the biofilm accumulation and showed that biofilm increased in mass through most of the experiment but would eventually stabilize at a constant level. For each of these kinetic experiments, the biofilm accumulation varied in its final thickness, which was measured at the end of between 22 to 30 cycles.

For the purpose of comparing the biofilm contribution kinetics for the different experimental conditions, the weight of biofilm was normalized by dividing the weight of biofilm to the constant biofilm weight obtained at the end of the experiment. Biofilms have been reported in literature to have a water content ranging from 87% to 99% (Characklis and Marshall, 1990). Analysis of the biofilm from the semi-continuous reactor system showed that the mixed culture biofilm consisted of approximately 92.7% (w/w) water; hence the biofilm density could be assumed constant. In addition, the change in the surface area of the biofilm on the reactor was considered to be negligible in comparison to the surface area of the reactor system. With these assumptions, the dimensionless weight would be equivalent to the dimensionless thickness of the biofilm.

Degradation of the synthetic brewery wastewaters was attributable to mixed culture microorganisms growing in the mixed liquor suspension as well as in the accumulated biofilm in the reactor system. Overall substrate degradation rates and substrate degradation rates due to biofilm were plotted as functions of the dimensionless thickness of the biofilm for each of the experimental conditions. Substrate degradation rates due to suspended culture were calculated by the difference between the overall degradation rate and the rate due to the biofilm. These plots are also shown for their respective experimental conditions in Figures 7.1 to 7.5.

Similar trends in the rates were observed between each of these experiments. The overall degradation rates of the first few cycles of all experiments ranged between 0.1 to 0.185 g L^{-1} h^{-1} based on the intermediate TOC concentration and decreased as the normalized biofilm thickness increased. This drop in degradation rate coincided with the decrease in the suspended culture degradation rates and may be attributed to the following factors. Increased competition for substrates between the biofilm and the suspended culture in combination with harvesting and dosing sequences of the semi-continuous reactor system where the volume in the reactor is halved each cycle would lead to increased dilution and eventual washout of the suspended cells with time. This possibility is supported with population dynamics data showing decreasing suspended solids population in Figures 7.6 to 7.10. Another possibility for the decline in the suspended culture's degradation rates would be the adsorption of the cells from the suspended medium to the biofilm strata itself, leaving less biomass in the suspended

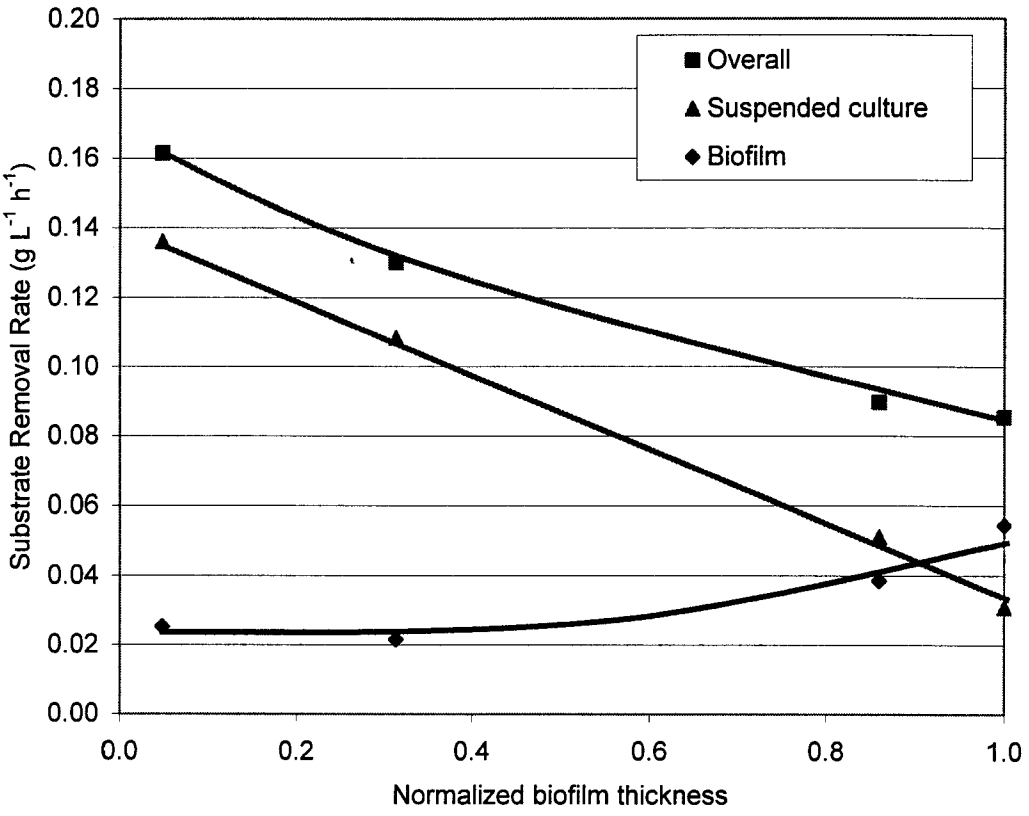

Figure 7.1: Substrate removal rates for synthetic brewery wastewater Low Maltose & Mid Ethanol

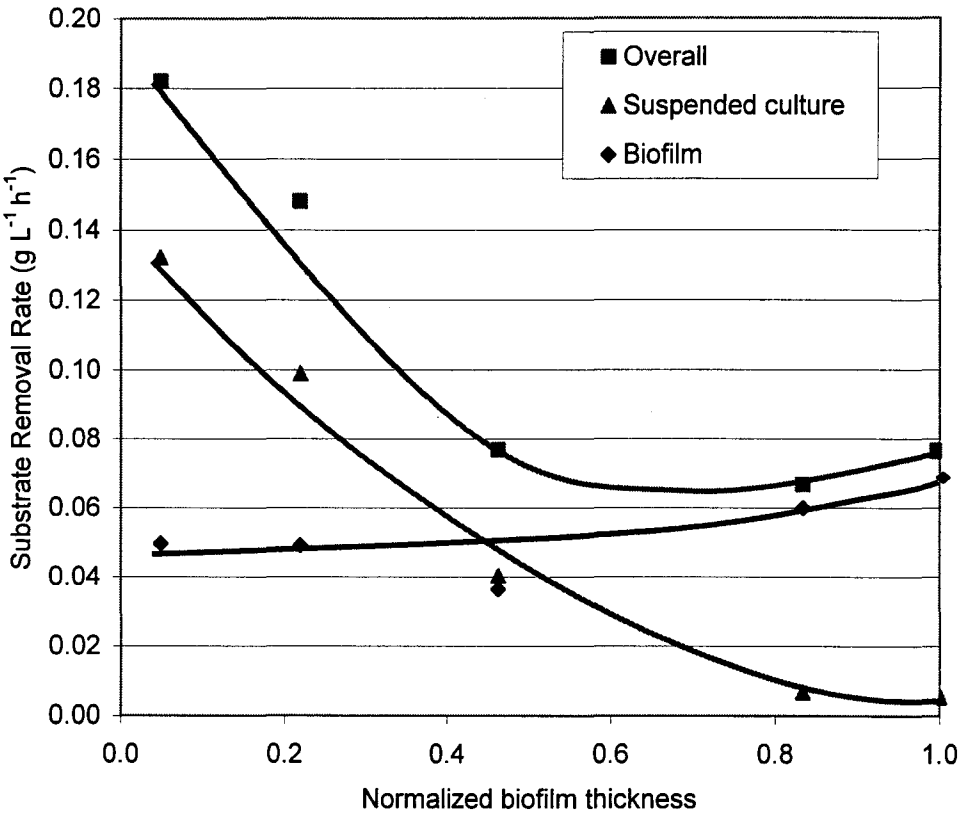

Figure 7.2: Substrate removal rates for synthetic brewery wastewater High Maltose & Mid Ethanol.

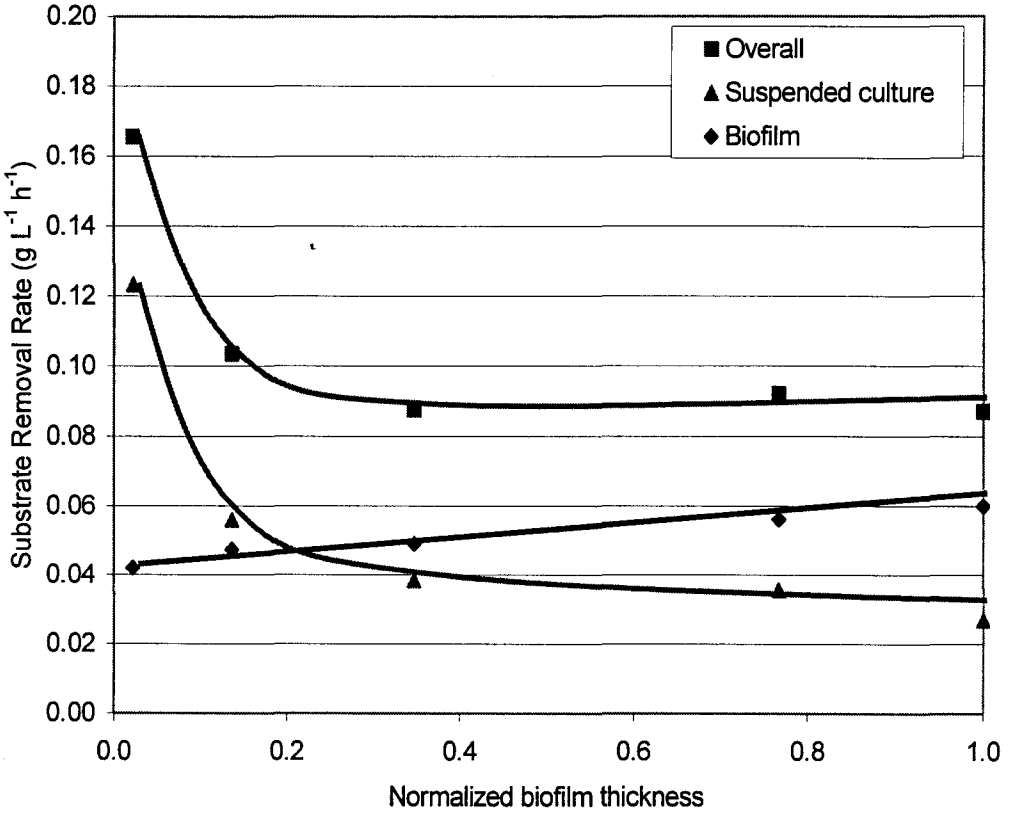

Figure 7.3: Substrate removal rates for synthetic brewery wastewater Mid Maltose & Mid Ethanol.

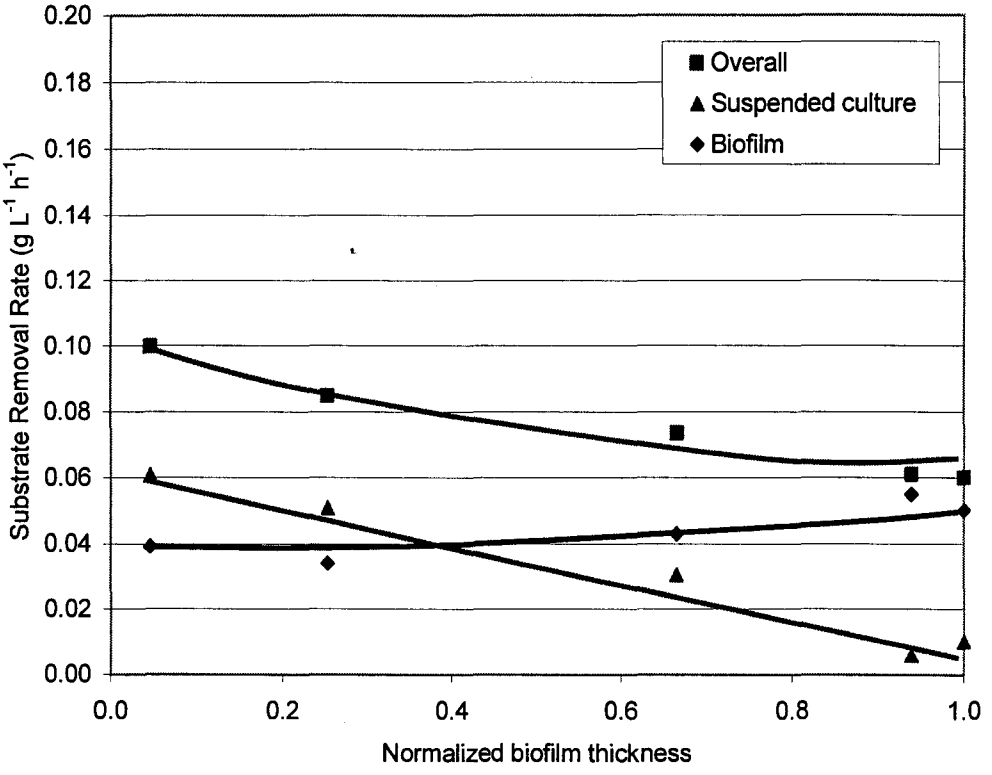

Figure 7.4: Substrate removal rates for synthetic brewery wastewater Mid Maltose & Low Ethanol.

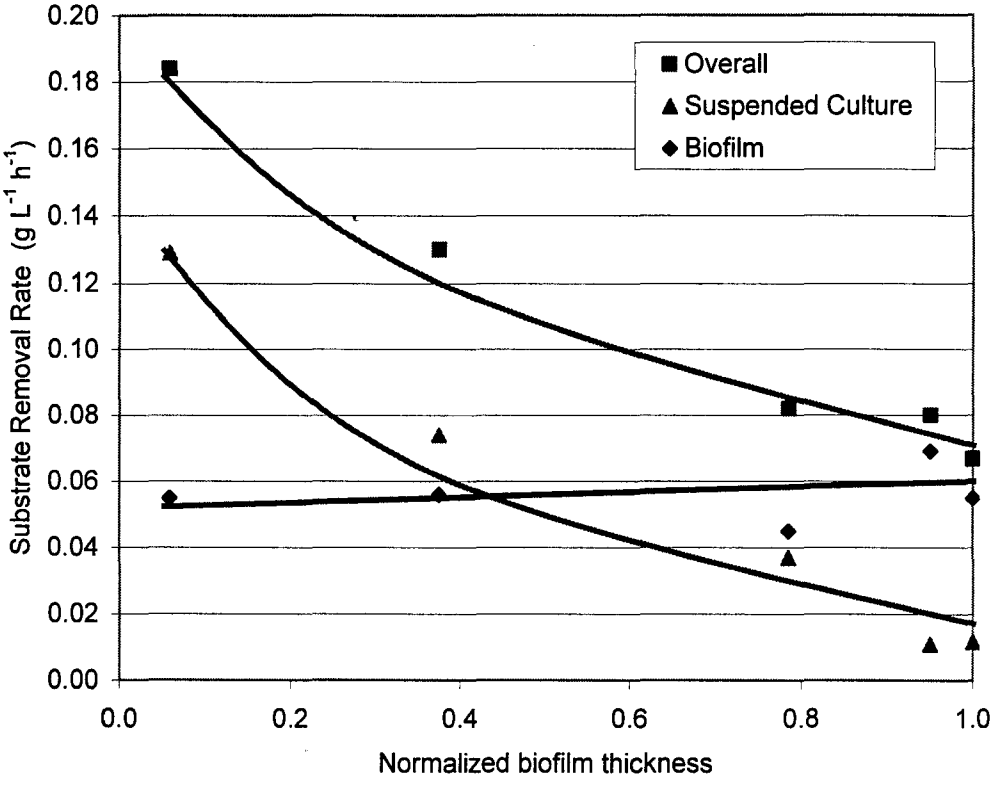

Figure 7.5: Substrate removal rates of synthetic brewery wastewater Mid Maltose & High Ethanol.

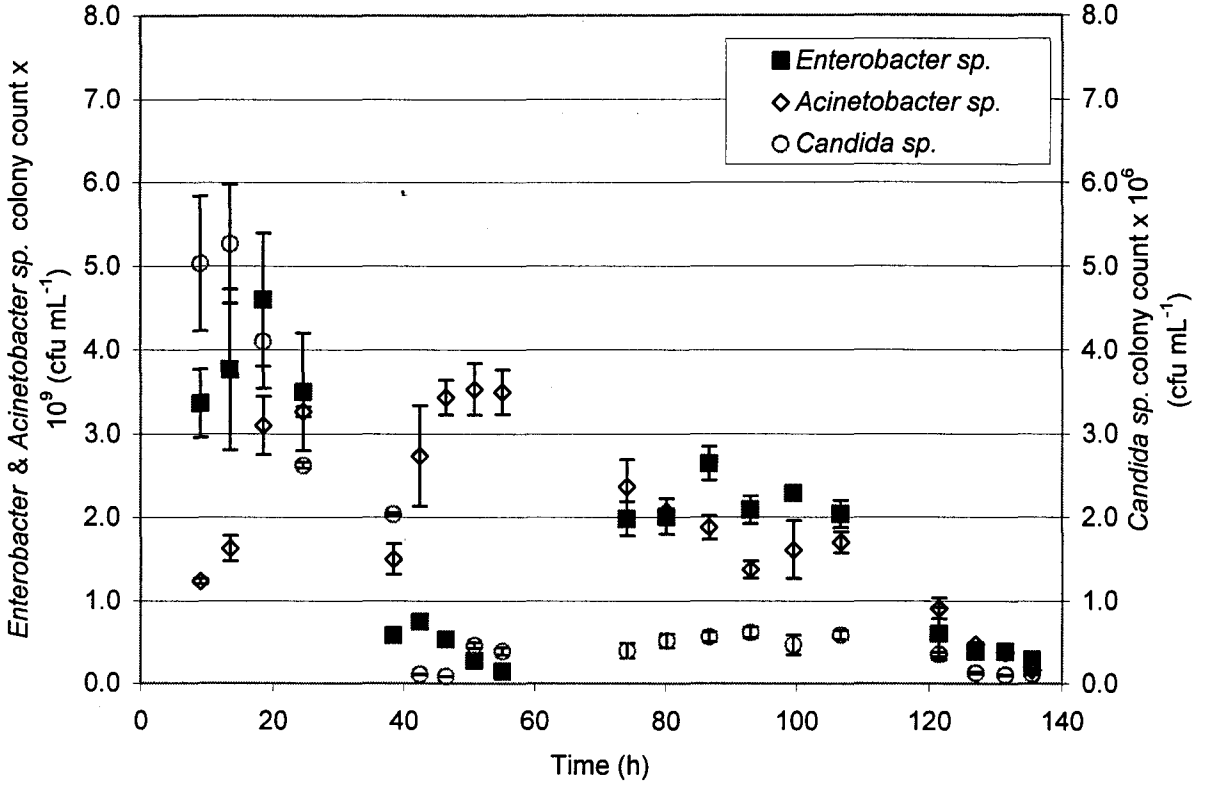

Figure 7.6: Population dynamics of suspended culture for synthetic brewery wastewater Low Maltose & Mid Ethanol experiments.

(Error bars represent standard deviations of six measurements)

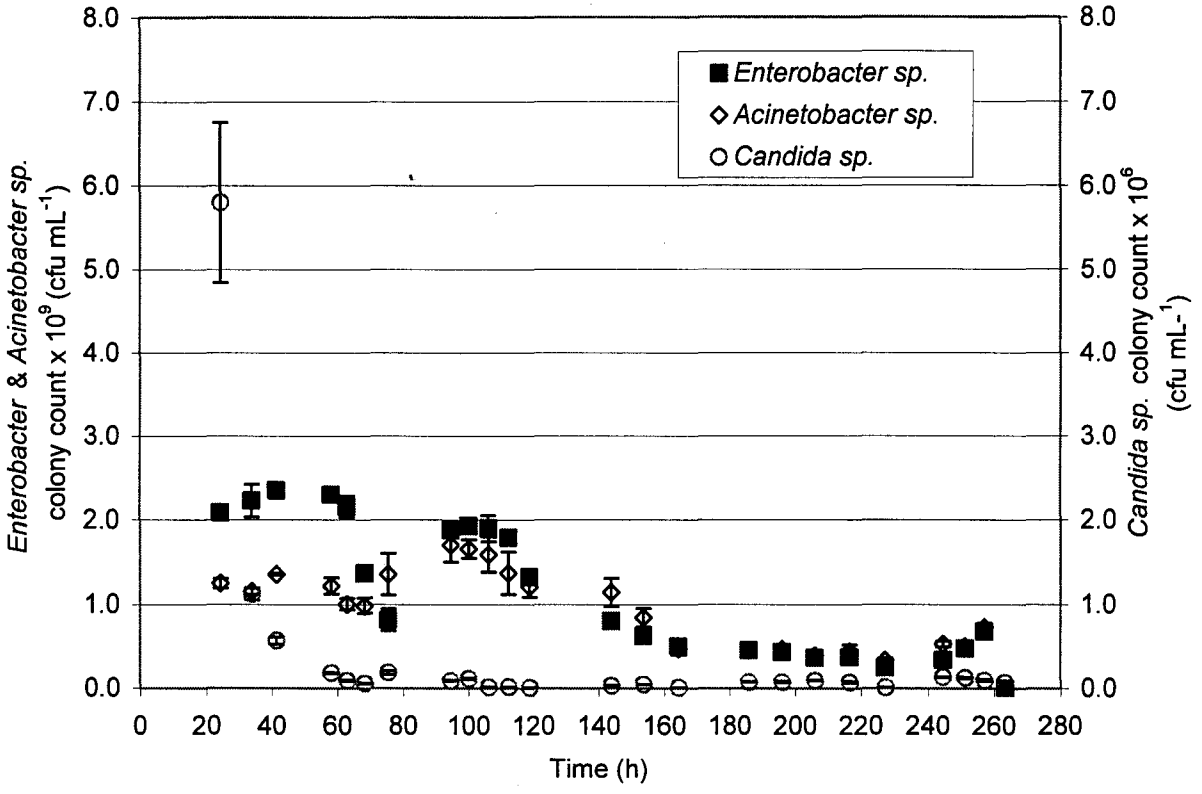

Figure 7.7: Population dynamics of suspended culture for synthetic brewery wastewater High Maltose & Mid Ethanol experiments

(Error bars represent standard deviations of six measurements)

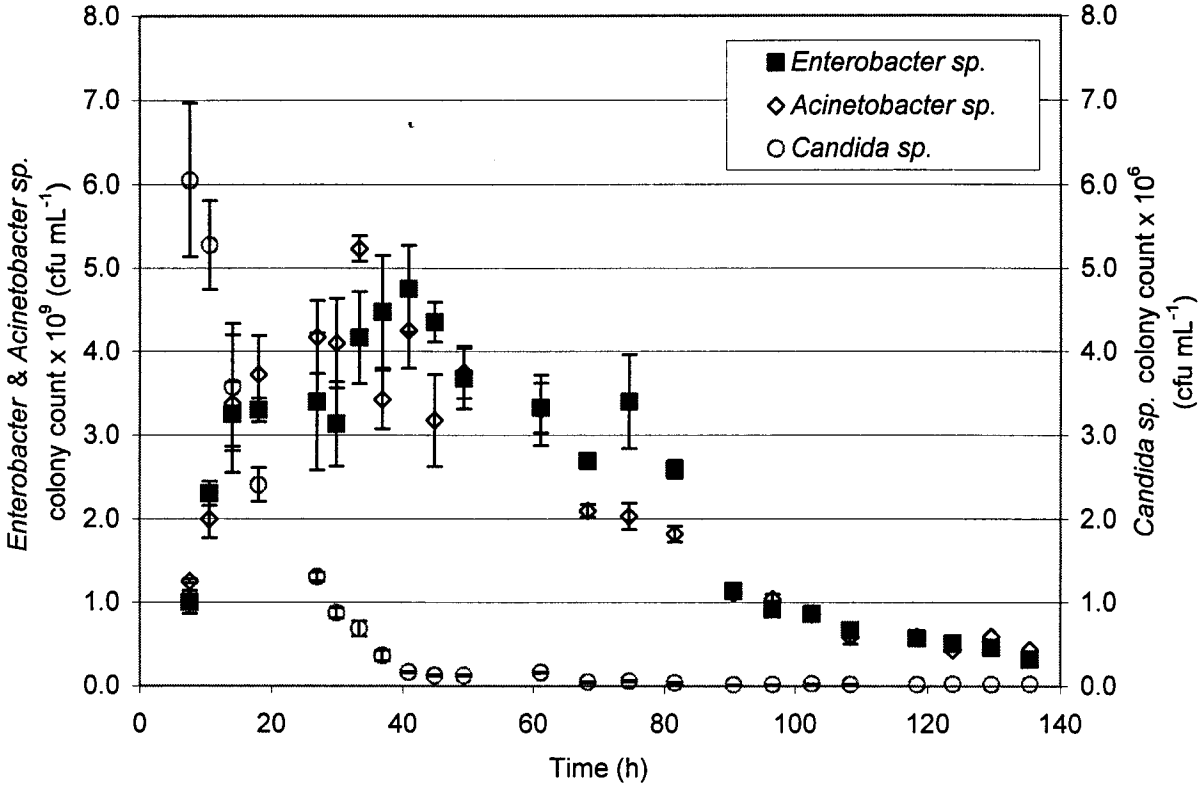

Figure 7.8: Population dynamics of suspended culture for synthetic brewery wastewater Mid Maltose & Mid Ethanol experiments.

(Error bars represent standard deviations of six measurements)

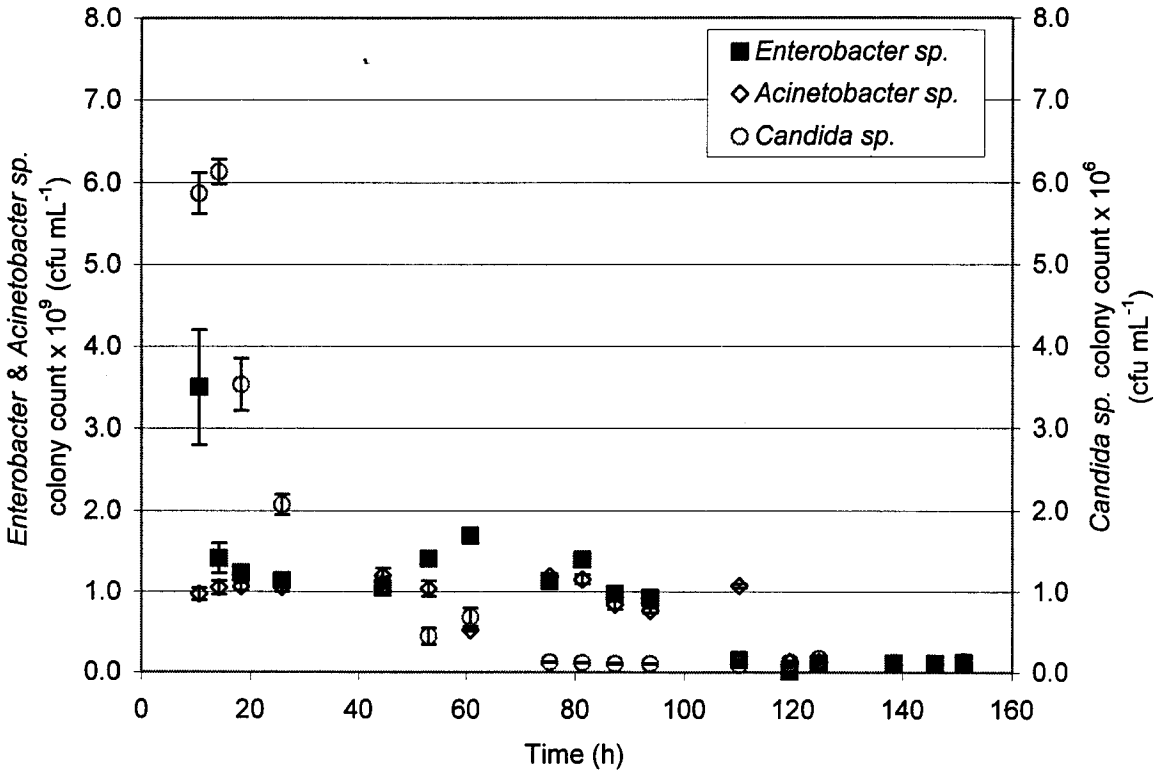

Figure 7.9: Population dynamics of suspended culture for synthetic brewery wastewater Mid Maltose & Low Ethanol experiments.

(Error bars represent standard deviations of six measurements)

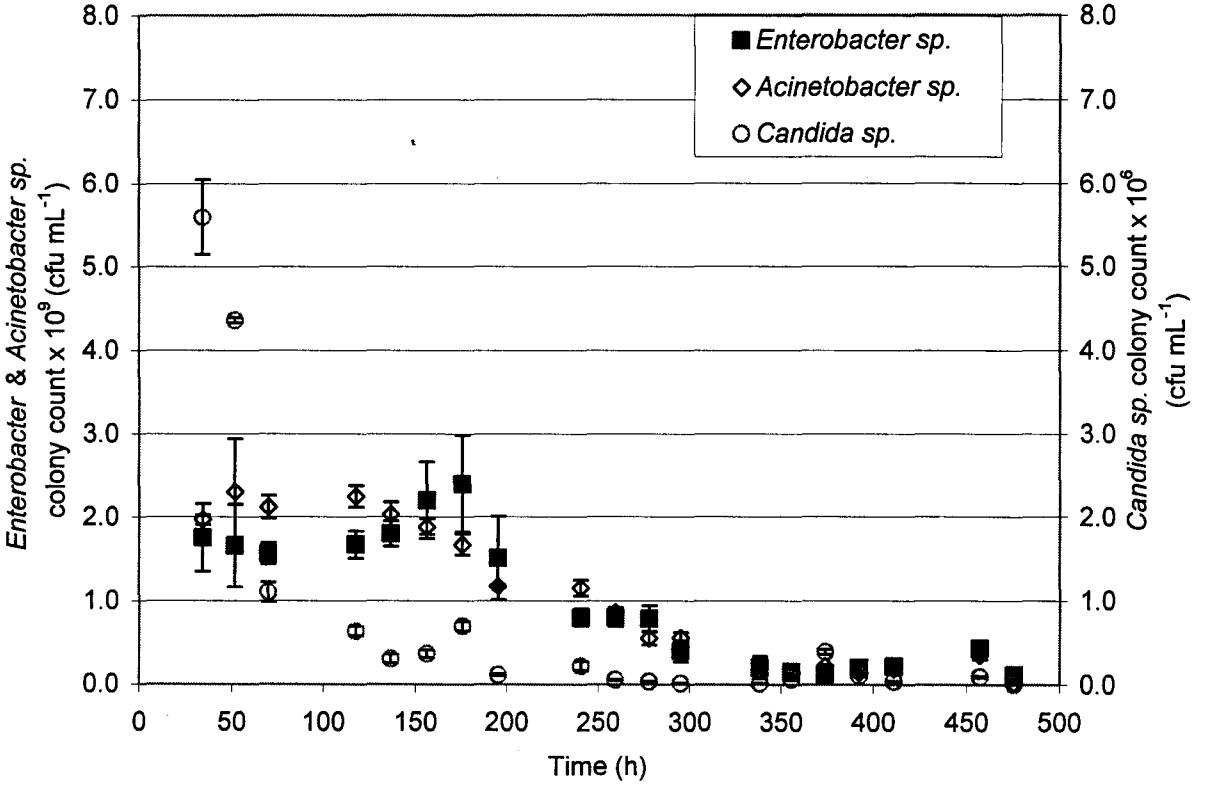

Figure 7.10: Population dynamics of suspended culture for synthetic brewery wastewater Mid Maltose & High Ethanol experiments.

(Error bars represent standard deviations of six measurements)

culture for its contribution to degradation of the wastewater. Microscopic inspection, plating and PCR-DGGE analysis of the biofilm confirmed that microorganisms belonging to the genera *Acinetobacter, Enterobacter* and *Candida sp.* that were present in the suspended solids were also present in the biofilm. The mechanisms in which these attachments occurred were not closely studied in this research however, it has been found in other research that *Acinetobacter* species were highly capable of attachment (Characklis and Marshall, 1990) and *Candida* species with their production of hyphae in their vegetative state contribute to the establishment of the biofilm in producing a dense matrix capable of trapping unattached cells. McCourtie and Douglas (1985) demonstrated that *Candida albicans* utilized sugar substrates to produce extracellular polymers, promoting adhesion to surfaces.

Figures 7.1 to 7.5 also illustrate degradation rate increases ranging from 0.01 to 0.03 g L^{-1} h^{-1} for the biofilm contribution as the normalized thickness of the biofilm increased. This indicated that most of the degradation due to the biofilm was on the surface of the biofilm and that mass transfer of substrate into the biofilm was limited. In the case where the biofilm thickness was small, no measurable increase in substrate degradation with increase in biofilm thickness was observed. As the biofilm accumulation increased, slight increases in the degradation rates were observed as the end of the experiment was approached, indicating that either mass transfer resistance, while substantial was not complete, mass transfer was limited by oxygen availability or that only the surface cells were viable. Lastly, the overall degradation rate followed the rate

of degradation by the suspended culture since little increase in the biofilm degradation rate was detected.

7.2 Population dynamics of the suspended culture

Colony counts of the suspended culture obtained from end of cycle samples for each of the kinetic experiments are illustrated in Figures 7.6 to 7.10. Insight with respect to population interactions, washout or potential adsorption of the suspended cells onto the biofilm strata could be detected from these plots. In the experiment treating Low Maltose & Mid Ethanol, Figure 7.6 illustrated that competition between the suspended bacteria and yeast occurred during the initial few cycles. As the experiment continued, the *Candida sp.* was outcompeted for substrates and since its growth was slower than that of bacteria, these yeast cells were slowly diluted out of the reactor system. Competition between *Enterobacter* and *Acinetobacter* species continued with *Acinetobacter* thriving during cycles between 39 to 58 hours into the experiment. This was not unexpected because *Acinetobacter* species are capable of degrading ethanol more readily than *Enterobacter* species. However, from the pure culture experiments, it was noted that the presence of sugar somewhat inhibited the degradation of ethanol until maltose had been reduced to a critical concentration. With the lower initial maltose concentration, *Acinetobacter* species were able to compete with the *Enterobacter* species. This competition and degradation by the suspended culture contribution explained the gradual decrease in the degradation rate by the suspended culture in Figure 7.1.

Low degradation rates by the suspended culture were detected in Figure 7.4 for the Mid Maltose & Low Ethanol concentration. Examination of the colony counts from the suspended culture in Figure 7.9 showed that the number of colonies that formed on the selective agar plates was also low. However, the colony count of *Acinetobacter* and *Enterobacter* species were very close in number, indicating that both bacteria grew equally well in their competition for the available substrates. *Candida* species were quickly diluted out of the reactor system with less substrate available in this wastewater. The bacterial species also dropped in the number of colony counts towards the end of the experiment and this can be due to either the dilution effect or the adsorption of the cells onto the reactor surface forming the biofilm. It should be noted that the feedback control strategy would normally not allow cell washout because the cycle time would automatically adjust to growth rate. However, since the biofilm is not being replaced each cycle, it will be given a competitive advantage over the cells in suspension and it is probably inevitable that a condition will be created in which the suspended cells will be growing too slowly to reproduce each cycle.

Synthetic wastewaters of High Maltose & Mid Ethanol and Mid Maltose & High Ethanol had the highest TOC concentrations of the wastewaters tested. Both exhibited similar trends in degradation rate as seen in Figures 7.2 and 7.5, and both had the onset of biofilm formation early in the experiment. This occurred at approximately 58 h and 99 h for High Maltose & Mid Ethanol and Mid Maltose & High Ethanol respectively. Examination of the suspended culture colony count in Figures 7.7 and 7.10, illustrated that the bacterial species were fairly similar in colony count in both high TOC

experiments. High Maltose & Mid Ethanol experiments had started out with lower colony counts of *Acinetobacter* species in the experiment but after approximately 78 hours into the experiment, the two bacterial colony counts became similar. Similar to the other wastewater concentrations tested, the bacterial species and yeast decreased as the experiment progressed.

In the experiments treating Mid Maltose & Mid Ethanol synthetic brewery wastewater, the colony counts for the bacterial species increased in cycles during the first 30 hours as seen in Figure 7.8. After this period, the higher colony count alternated between *Acinetobacter* and *Enterobacter* species as the experiment continued, indicative of competition between the two bacteria. At approximately 90 h, the colony counts were equal and decreased towards the end of the experiment.

7.3 Development of a Model for the Overall Rate of Substrate Degradation

The overall rate of substrate degradation in the self-cycling fermentation system consisted of two components including substrate degradation by the suspended culture and degradation due to biofilm, and can be represented by the following overall rate model.

$$r_{overall} = r_{susp} + r_{biofilm} \tag{7.1}$$

where $r_{overall}$ is the overall rate of substrate removal in units of g L^{-1} h^{-1}, r_{susp} is the rate of substrate removal due to the suspended solids component in units of g L^{-1} h^{-1}, and $r_{biofilm}$ is the rate of substrate removal due to the biofilm component in units of g L^{-1} h^{-1}.

7.3.1 Development of Rate Model of Biofilm Contribution

As the self-cycling fermentation experiments progressed, it was demonstrated that the rate of substrate degradation was a weak function of time, and hence thickness of biofilm. However, the rate of substrate degradation by the biofilm was found to be a function of substrate concentration. From this information, it was determined that the form of a rate model for substrate removal due to only the biofilm can be the following.

$$r_{biofilm} = k_f T_m S \tag{7.2}$$

In equation 7.2 k_f was defined as the rate constant due to the biofilm contribution in units of h^{-1}, T_m was a dimensionless factor representing the normalized biofilm thickness, and S represented the substrate TOC concentration in g L^{-1} in the reactor samples.

7.3.2 Development of Rate Model of Suspended Culture Contribution

These semi-continuous experiments were considered as a series of batch processes that were reinoculated at the beginning of each cycle with 50% v/v of a mixed consortium. The rate of growth of cells can be defined by the following equation 7.3.

$$r_g = \mu X \tag{7.3}$$

Where r_g is the rate of bacterial growth in units of g L^{-1} h^{-1}, μ is the specific growth rate (h^{-1}) and X is the concentration of microorganism in g L^{-1}. The rate of substrate degradation is related to the rate of cell growth where a portion of the substrate is converted to new cells and another portion is converted to organic and inorganic end products and can be defined by equation 7.4.

$$r_g = Yr_{su} \tag{7.4}$$

where r_g has been previously defined, Y is called the maximum yield coefficient, mg/mg and is defined as the ratio between the mass of cells formed to the mass of substrate consumed during a finite period during the logarithmic phase, and r_{su} is defined as the substrate utilization rate in units of g L^{-1} h^{-1} (Metcalf & Eddy, 1991).

Combining equations 7.3 and 7.4, the following equation can be written,

$$r_{su} = (\mu/Y)X \tag{7.5}$$

where μ is a function of substrate concentration, S, during exponential growth and can be represented by the following equation with K' being a constant.

$$\mu = K'S \tag{7.6}$$

Substitution of equation 7.6 into 7.5 yields equation 7.7

$$r_{su} = (K'/Y)XS \tag{7.7}$$

and finally by replacing the constant (K'/Y) with the constant k_s, an equation of the form

$$r_{su} = k_sXS \tag{7.8}$$

was obtained. Therefore, combining equations 7.2 and 7.8, the overall rate of substrate removal can be represented by equation 7.9.

$$r_{overall} = k_s XS + k_f T_m S \quad (7.9)$$

7.4 Determination of rate constants of the overall rate model

As previously mentioned, each cycle in the self-cycling fermentation system can be considered as a batch process. In each cycle, both the initial and final TOC concentrations in addition to the cycle time were obtained. This was used to calculate the overall rate of substrate removal where

$$r_{overall} = (S_{in} - S_f)/ t \quad (7.10)$$

S_{in} and S_f are the initial and final TOC concentrations respectively in units of g L^{-1}, and t is equal to the cycle time in h. From the result of this equation, determination of the rate constants in equation 7.8 of k_s and k_f was possible and are further discussed in the following sections.

7.4.1 Determination of the rate constant for biofilm rate of substrate removal

From biofilm contribution runs performed throughout each kinetic experiment in which the suspended culture had been removed from the reactor, the calculated overall rate of substrate removal is equal to the rate of substrate removal due to biofilm and can be expressed by combining equations 7.2 and 7.10.

$$(S_{in} - S_f)/ t = k_f T_m S \quad (7.11)$$

Rearrangement of equation 7.11 provides an equation for the rate constant for the biofilm and can be represented by equation 7.12.

$$k_f = (S_{in} - S_f)/ t T_m S \quad (7.12)$$

The rate constants for the biofilm contribution runs were calculated for each kinetic experiment and are included in Table 7.1. These rate constant values were similar and had an average value of 0.19 ± 0.03 h^{-1}. However, examination of the rate constants indicated that the values were fairly consistent throughout each experiment only until the final one or two measurements of biofilm thickness. This demonstrated that all substrate removal due to the biofilm probably occurred mainly on the biofilm surface during the initial cycles of the self-cycling fermentation experiments as the biofilm was being established. As the biofilm approached its constant biofilm thickness, slightly increased degradation rates became apparent, indicating sub-surface activity or increased cell viablity. Since insufficient data was available to determine the mass transfer effects or the cell viability in the biofilm, the average value of the rate constant k_f from equation 7.2 was obtained from the data points where the plots of the rate of substrate degradation was linear with respect to normalized biofilm thickness. This rate constant was calculated to be 0.149 h^{-1} and will be used in the evaluation of the rate model.

Table 7.1: Rate constants obtained from Biofilm Contributions Tests

Wastewater Type	T_m	TOC Conc. ($g\ L^{-1}$)	k_f (h^{-1})
Low Maltose & Mid Ethanol	0.05	0.20	0.13
	0.31	0.20	0.11
	0.86	0.23	0.17
	1.00	0.23	0.24
Average k_f:			**0.17**
Standard Deviation			**0.06**
High Maltose & Mid Ethanol	0.05	0.36	0.14
	0.22	0.39	0.13
	0.46	0.33	0.11
	0.83	0.26	0.23
	1.00	0.36	0.20
Average k_f:			**0.17**
Standard Deviation			**0.05**
Mid Maltose & Mid Ethanol	0.02	0.30	0.14
	0.14	0.32	0.15
	0.35	0.28	0.18
	0.77	0.30	0.19
	1.00	0.32	0.19
Average k_f:			**0.18**
Standard Deviation			**0.02**
Low Ethanol & Mid Maltose	0.05	0.26	0.15
	0.25	0.20	0.17
	0.66	0.19	0.22
	0.94	0.24	0.25
	1.00	0.18	0.28
Average k_f:			**0.23**
Standard Deviation			**0.05**
High Ethanol & Mid Maltose	0.06	0.36	0.18
	0.38	0.38	0.18
	0.79	0.37	0.15
	1.00	0.35	0.18
	0.95	0.36	0.21
Average k_f:			**0.18**
Standard Deviation			**0.02**

T_m: normalized biofilm thickness

7.4.2 Determination of the rate constant for substrate removal by the suspended culture

From equation 7.1, the rate of substrate removal by the suspended culture can be obtained from the difference between the overall rate of substrate removal and the rate of substrate removal by the biofilm. Overall rates of substrate removal were calculated at cycles immediately after biofilm contribution cycles and just prior to the last biofilm contribution run. The initial rates of removal by the suspended culture were similar for 4 of the 5 conditions with an average of 0.16 ± 0.03 g L^{-1} h^{-1} for the different wastewater concentrations tested. As the biofilm accumulated on the inside surface of the reactor, the rate of removal of substrate by the suspended culture decreased. This was attributed to the dilution and eventual washout of the cells as the experiment progressed. Because the rates were constant with varying TOC and rates dropped as washout occurred it can be concluded that the rate of removal by the suspended culture was primarily a function of the biomass concentration and not of the substrate concentration. Therefore, determination of the rate constant k_s from equation 7.8 for the suspended culture contribution was obtained from plots of the rates of removal by the suspended culture as a function of the biomass concentration of the suspended solids (Figure 7.11). The slopes of these plots are equal to the rate constant k_s of equation 7.8 and are listed in Table 7.2. The values of the rate constant k_s were very similar for the different substrate concentrations tested with an average value 0.095 ± 0.006 h^{-1}.

Therefore, the rate equation for the overall rate of substrate removal is modeled by the following equation, which is valid only while the biofilm is accumulating.

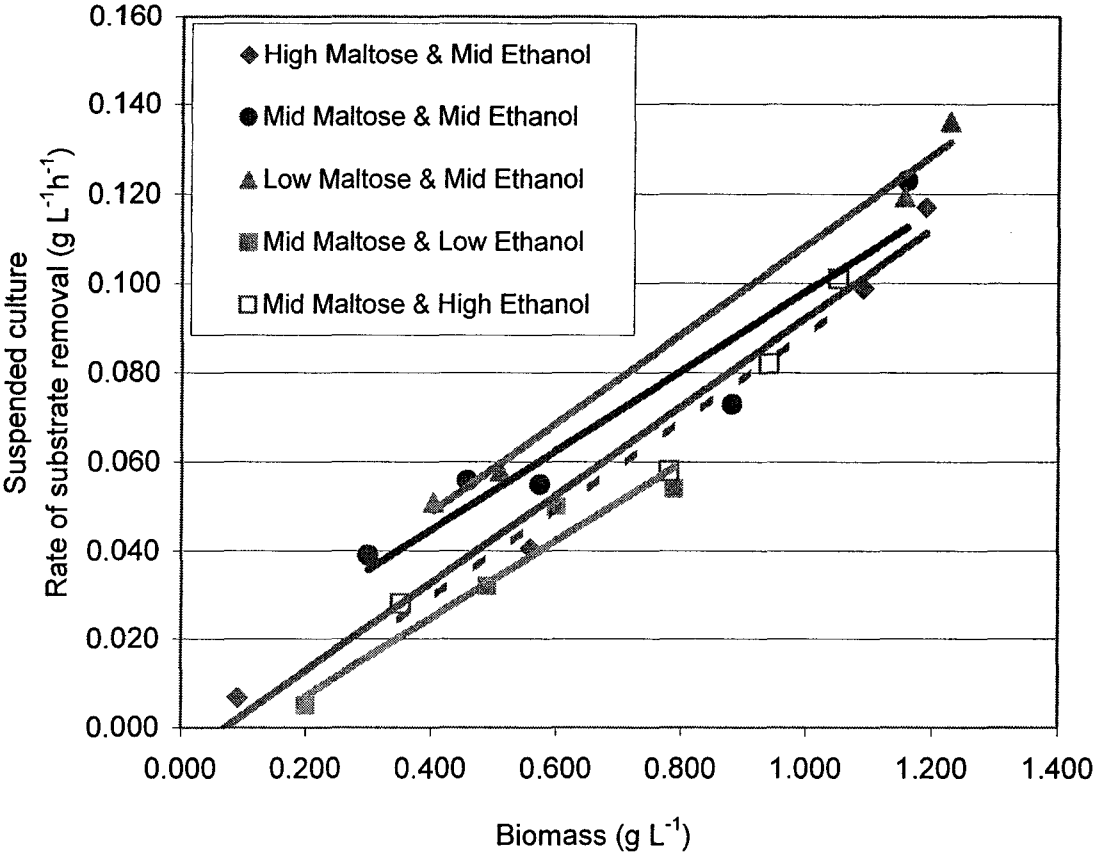

Figure 7.11: Determination of suspended culture rate constant k_s.

Table 7.2: Rate constants obtained for suspended culture contribution

Wastewater Type	k_s (hr^{-1})
Low Maltose & Mid Ethanol	0.100
High Maltose & Mid Ethanol	0.099
Mid Maltose & Mid Ethanol	0.089
Mid Maltose & Low Ethanol	0.088
Mid Maltose & High Ethanol	0.100
Average:	0.095
Standard Deviation:	0.006

$$r_{overall} = 0.095(X) + 0.149(S) \quad (7.13)$$

(numerical values in equation 7.13 have units of h^{-1}).

7.5 Model Evaluation

The above model has been developed based on the assumption that the overall rate of substrate removal is comprised of two components, the combined contributions of the suspended culture and the biofilm. The biofilm contribution was assumed to be a surface phenomenon and, therefore, not influenced by mass transfer through the biofilm layer. The cells on the surface of the biofilm were assumed to be those primarily responsible for the degradation of the substrate. In order to determine the validity of these assumptions, the experimental data were compared with rates predicted by the model. The results are presented in Table 7.3 for the various intermediate substrate concentrations. The deviations from the predicted values were determined at each of the experimental data points. Analysis of these deviations as functions of substrate concentration and of normalized biofilm thickness shows that they are randomly distributed and yield no trend with respect to either of these variables. Therefore, the model does represent the experimental data indicating that the origin of the TOC concentration whether from ethanol or maltose does not affect the performance of this mixed culture system. Furthermore, the assumptions of biofilm activity being based predominantly on surface activity and the rate of substrate removal by the suspended culture being influenced solely by biomass concentration were valid.

Table 7.3: Comparison of Predicted & Experimental Rates of Overall Substrate Removal (g L^{-1} h^{-1})

Wastewater Type	Biomass (g L^{-1})	TOC Conc. (g L^{-1})	Experimental Rate	Predicted Rate	Residuals
Low Maltose & Mid Ethanol	1.128	0.280	0.161	0.149	0.012
	0.982	0.288	0.130	0.136	-0.006
	0.545	0.285	0.090	0.094	-0.004
	0.309	0.281	0.085	0.071	0.014
High Maltose & Mid Ethanol	1.089	0.432	0.182	0.168	0.014
	0.896	0.430	0.148	0.149	-0.001
	0.337	0.403	0.077	0.092	-0.015
	0.134	0.403	0.067	0.073	-0.006
	0.059	0.400	0.078	0.065	0.013
Mid Maltose & Mid Ethanol	1.152	0.391	0.165	0.168	-0.003
	0.881	0.337	0.103	0.134	-0.031
	0.458	0.339	0.088	0.094	-0.006
	0.574	0.343	0.092	0.106	-0.014
	0.506	0.343	0.087	0.099	-0.012
Mid Maltose & Low Ethanol	1.000	0.273	0.100	0.136	-0.036
	0.918	0.262	0.085	0.126	-0.041
	0.177	0.262	0.073	0.056	0.017
	0.071	0.261	0.061	0.046	0.015
	0.187	0.264	0.060	0.057	0.003
Mid Maltose & High Ethanol	1.150	0.430	0.184	0.173	0.011
	0.890	0.420	0.130	0.147	-0.017
	0.545	0.430	0.082	0.116	-0.034
	0.320	0.410	0.067	0.091	-0.024
	0.190	0.420	0.080	0.081	-0.001

CHAPTER 8

CONCLUSIONS, ORIGINAL CONTRIBUTIONS AND RECOMMENDATIONS

The primary objective of this thesis was to obtain an understanding of the process kinetics in a self-cycling fermentation system using a defined mixed culture to treat multiple substrates. Batch and semi-continuous reactor systems were used for identification of the dominant microbes and investigation of their growth and kinetic capabilities for degradation of various substrates. Pure culture batch experiments were conducted in order to determine the behaviours of the individual species, *Acinetobacter, Enterobacter* and *Candida* in both buffered and non-buffered media. A study was also included for investigating population dynamics during mixed culture batch experiments. Finally, a semi-continuous self-cycling fermentation system was used to evaluate the population dynamics, substrate removal kinetics, and to develop a rate model to predict substrate consumption.

8.1 Conclusions from the Study of Consortium Characterization and Identification Methods

The main conclusions that can be drawn from the study of characterization and identification methods include the following.

(i) Traditional method of culturing and isolation on solid agar media followed by microscopic inspection and gram staining of the mixed culture provided rapid general classification of the types of microorganisms present. Microscopic analysis also provided information regarding shape, size and motility of these microorganisms. Traditional plating techniques determined that there were three dominant colony types in the acclimatized activated sludge. Using these methods alone, the genus or species cannot be determined.

(ii) The molecular technique of Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was capable of identifying microbes present at the genus level. The three dominant microbes present in the acclimatized activated sludge were identified as *Acinetobacter sp.*, *Enterobacter sp.*, and *Candida sp.*

(iii) The PCR-DGGE method was found to be advantageous because isolation of the individual microbes was not required for identification.

(iv) When used for population dynamics characterization, PCR-DGGE cannot provide data with respect to the microbial quantitation or viability. However, changes in microbial population can be detected using this method.

(v) The identification of the bacteria from the mixed culture was verified using BIOLOG GN Microplates. These microplates can be considered as an alternative for PCR-DGGE for the identification of individual microbes, however, additional steps are required to isolate the microbes from the consortium before application of BIOLOG GN Microplates.

(vi) BIOLOG GN Microplates can be used for identifying the selective carbon source required for isolating microbes in a mixed consortium on solid agar media.

(vii) The revised plate count methodology using well-mixed diluted sample drops "drop plating" instead of spread plating was found to provide colony count information equivalent to the standard spread plating method at the 95% confidence interval.

8.2 Conclusions from the Batch Reactor Studies

The main conclusions that can be drawn from the batch reactor studies include the following.

(i) *Enterobacter sp.* can consume glucose and maltose but no ethanol in both non-buffered and buffered media at pH 7.

(ii) *Acinetobacter sp.* can consume glucose, maltose and ethanol only in a buffered media of pH 6. In non-buffered media, only glucose was consumed and acidic byproducts were produced lowering the pH of the media to 4.3 inhibiting further growth.

(iii) *Candida sp.* can consume glucose, maltose and ethanol and consumption of substrates is enhanced at pH values lower than 5.

(iv) Favourable media conditions must exist in order to have a positive correlation between optical density and viable plate count. In this study, correlation between optical density and viable plate counts was found to be valid in the buffered case only.

(v) Behaviour in mixed culture experiments containing *Acinetobacter, Enterobacter* and *Candida sp.* can be related to their individual behaviours as pure cultures. In this study, it was shown that *Enterobacter sp.* reduced glucose and maltose concentrations before *Acinetobacter sp.* could degrade ethanol. *Candida* behaviour in the mixed culture was similar to its behaviour as a pure culture.

8.3 Conclusions from the Semi-Continuous Reactor Studies

The main conclusions that can be drawn from the semi-continuous self-cycling fermentation reactor studies include the following.

(i) The overall rate of substrate removal can be attributed to rates of substrate removal by both suspended culture and biofilm.

(ii) The rate of substrate removal due to biofilm is predominantly due to the activity of surface cells as evidenced by the constant rate of substrate removal with increasing biofilm thickness. The rate of substrate removal due to the biofilm is a first order function of substrate concentration only.

(iii) The rate of substrate removal due to the suspended culture is a function of biomass concentration only.

(iv) The rate of substrate removal associated with the biofilm is sufficient to reduce the growth of suspended culture. Over time, the suspended culture population declines due to washout.

8.4 Original Contributions

Biological treatment processes for brewery wastewater treatment have been extensively studied in the past for both anaerobic and aerobic systems. However, the published work is far from complete.

Much of the work that has been performed in the past with the objective of optimization of the treatment of brewery wastewater systems has focused on reactor design and configuration, chemical analyses of reactor input and output, and not on the process kinetics and behaviour of the mixed culture consortium. Brewery wastewater treatment has been viewed as a "black box" because of a lack of data associated with the behaviour of the microbes.

The present work contributes a body of knowledge to the field of brewery wastewater treatment by focusing on improving the understanding of the relationships in the consortium including the interactions between the dominant microbes and the how each contributes to the degradation of sugars and ethanol. Several methods for identification of microbes in a mixed consortium were examined and it was shown how factors such as

pH, substrate concentration, and biomass concentration affect the wastewater treatment system.

Other information claimed to be new findings are

(i) The identification and characterization of the dominant microbes in a mixed culture for brewery wastewater treatment using a combination of identification methods including microscopy, spread plating and "drop plating", PCR-DGGE, and BIOLOG GN Microplates.

(ii) A comparison of spread plating with "drop plating" methods demonstrated equivalent colony count information at the 95% confidence interval.

(iii) Development of a biofilm in the self-cycling fermentation (SCF) reactor system can make the SCF system an ineffective tool for the study of suspended culture. In this study it was shown that the rate of substrate removal due to the biofilm was sufficient to reduce the growth of the suspended culture; thereby creating washout conditions.

(iv) A rate equation was developed for the overall removal of substrate from simulated brewery wastewater using a semi-continuous self-cycling fermentation reactor system. The overall rate was found to be due to suspended culture and biofilm.

8.5 Recommendations

The following is a list of recommendations for future research to be taken into consideration in the field of brewery wastewater treatment.

(i) Additional parameters in the self-cycling fermentation system could be investigated including different temperatures and pH values to expand the rate model.

(ii) Since it was determined that biofilm played a substantial role in the removal of substrate from the wastewater, an additional investigation using packing material with known surface area to further study the biofilm contribution in relation to mass transfer effects on substrate removal rates would be beneficial.

(iii) Although a basic understanding of the processes involved in the treatment of a synthetic wastewater has been obtained in this study, it may be beneficial to study the more general case of treating actual brewery wastewater to test the overall rate of substrate removal.

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APPENDIX A

STATISTICAL COMPARISON OF MEANS (T-TEST)

Hypothesis: $\mu_A \neq \mu_B$

Knowledge of the standard deviation of A and B: σ_A and σ_B both known

Test to be made is (if the inequality is satisfied the hypothesis is accepted.):

$$\left|\bar{X}_A - \bar{X}_B\right| > U_{1-\alpha/2}\left(\frac{\sigma_A^2}{n_A} + \frac{\sigma_B^2}{n_B}\right)^{1/2}$$

Results of the comparison of means t-test for this hypothesis are shown in Table A.1

Hypothesis: $\mu_A > \mu_B$

Knowledge of the standard deviation of A and B: σ_A and σ_B both known

Test to be made is (if the inequality is satisfied the hypothesis is accepted.):

$$\left(\bar{X}_A - \bar{X}_B\right) > U_{1-\alpha}\left(\frac{\sigma_A^2}{n_A} + \frac{\sigma_B^2}{n_B}\right)^{1/2}$$

Results of the comparison of means t-test for this hypothesis are shown in Table A.2.

TABLE A.1: T-test Comparison between the Spread Plate Method and the "Drop Plate" Method

(A NOT EQUAL to B) (Variances not equal)

	Drop Plate Method	Spread Plate Method	Mean Diff.	t(1-α/2) 95% C.I.	Degrees of freedom	Standard Deviation Calculation	Criteria 95% C.I.	Mean > Criteria? 95% C.I.
	Candida sp. (cfu mL^{-1})							
Mean	4.63×10^{6}	4.80×10^{6}	*1.75×10^{5}*	2.571	5.0	5.81×10^{5}	1.49×10^{6}	**FALSE**
St. Deviation	3.71×10^{5}	9.54×10^{5}						
Variance	1.38×10^{11}	9.10×10^{11}						
	Acinetobacter sp. (cfu mL^{-1})							
Mean	3.01×10^{9}	1.92×10^{9}	*1.09×10^{9}*	2.776	4.0	1.19×10^{9}	3.30×10^{9}	**FALSE**
St. Deviation	2.75×10^{8}	1.67×10^{9}						
Variance	7.56×10^{16}	2.79×10^{18}						
	Enterobacter sp. (cfu mL^{-1})							
Mean	3.63×10^{9}	1.59×10^{9}	*2.04×10^{9}*	2.776	4.0	1.01×10^{9}	2.81×10^{9}	**FALSE**
St. Deviation	5.42×10^{8}	1.38×10^{9}						
Variance	2.94×10^{17}	1.90×10^{18}						

TABLE A.2: T-test Comparison between the Spread Plate Method and the "Drop Plate" Method

(A GREATER THAN B) (Variances not equal)

	Drop Plate Method	Spread Plate Method	Mean Diff.	t(1-α) 95% C.I.	Degrees of freedom	St.Dev. Calc.	Criteria 95% C.I.	Mean> Criteria? 95% C.I.
	Candida sp. (cfu mL^{-1})							
Mean	4.63×10^{6}	4.80×10^{6}	-1.75×10^{5}	2.015	5	5.81×10^{5}	1.17×10^{6}	**FALSE**
St. Deviation	3.71×10^{5}	9.54×10^{5}						
Variance	1.38×10^{11}	9.10×10^{11}						
	Acinetobacter sp. (cfu mL^{-1})							
Mean	3.01×10^{9}	1.92×10^{9}	1.09×10^{9}	2.132	4	1.19×10^{9}	2.54×10^{9}	**FALSE**
St. Deviation	2.75×10^{8}	1.67×10^{9}						
Variance	7.56×10^{16}	2.79×10^{18}						
	Enterobacter sp. (cfu mL^{-1})							
Mean	3.63×10^{9}	1.59×10^{9}	2.04×10^{9}	2.132	4	1.01×10^{9}	2.16×10^{9}	**FALSE**
St. Deviation	5.42×10^{8}	1.38×10^{9}						
Variance	2.94×10^{17}	1.90×10^{18}						

APPENDIX B

GN BIOLOG Microplate Results

The GN BIOLOG Microplate consisted of 96 wells of different carbon sources. The carbon sources of the microwells are presented in Tables B.1 and B.2. Positive assimilation of the carbon source is indicated by a "+" sign, partial assimilation by a "/" sign, and finally, no assimilation by a "-" sign. Keying in of these assimilation results enables Biolog's Microlog computer program to cross-reference the pattern of assimilation to an extensive library of species. The results of the analyes of the GN BIOLOG Microplates are presented in Tables B.3 and B.4 for pure cultures *Acinetobacter sp.* and *Enterobacter sp.* respectively.

TABLE B.1: List of carbon sources in microwells A1 to D12

Microwell ID	Carbon source	Microwell ID	Carbon source
A1	water	B1	i-erythritol
A2	α-cyclodextrin	B2	D-fructose
A3	dextrin	B3	L-fucose
A4	glycogen	B4	D-galactose
A5	tween 40	B5	gentiobiose
A6	tween 80	B6	α-D-glucose
A7	N-acetyl-D-galactosamine	B7	m-inositol
A8	N-acetyl-D-glucosamine	B8	α-lactose
A9	adonitol	B9	α-D-lactose lactulose
A10	L-arabinose	B10	maltose
A11	D-arabitol	B11	D-mannitol
A12	cellobiose	B12	D-mannose
Microwell ID	Carbon source	Microwell ID	Carbon source
C1	D-melibiose	D1	acetic acid
C2	β-methyl D-glucoside	D2	cis-aconitic acid
C3	psicose	D3	citric acid
C4	D-raffinose	D4	formic acid
C5	L-rhamnose	D5	D-galactonic acid lactone
C6	D-sorbitol	D6	D-galacturonic acid
C7	sucrose	D7	D-gluconic acid
C8	D-trehalose	D8	D-glucosaminic acid
C9	turanose	D9	D-glucuronic acid
C10	xylitol	D10	α-hydroxy butyric acid
C11	methyl pyruvate	D11	β-hydroxy butyric acid
C12	mono-methyl succinate	D12	γ-hydroxy butyric acid

TABLE B.2: List of carbon sources in microwells E1 to H12

Microwell ID	Carbon source	Microwell ID	Carbon source
E1	p-hydroxy phenylacetic acid	F1	Bromo succinic acid
E2	itaconic acid	F2	succinamic acid
E3	α-keto butyric acid	F3	glucuronamide
E4	α-keto glutaric acid	F4	alaninamide
E5	α-keto valeric acid	F5	D-alanine
E6	D,L-lactic acid	F6	L-alanine
E7	malonic acid	F7	L-alanyl-glycine
E8	propionic acid	F8	L-asparagine
E9	quinic acid	F9	L-aspartic acid
E10	D-saccharic acid	F10	L-glutamic acid
E11	sebacic acid	F11	glycyl-L-aspartic acid
E12	succinic acid	F12	glycyl-L-glutamic acid
Microwell ID	Carbon source	Microwell ID	Carbon source
G1	L-histidine	H1	urocanic acid
G2	hydroxy L-proline	H2	inosine
G3	L-leucine	H3	uridine
G4	L-ornithine	H4	thymidine
G5	L-phenyl alanine	H5	phenyl ethylamine
G6	L-proline	H6	putrescine
G7	L-pyro glutamic acid	H7	2-amino ethanol
G8	D-serine	H8	2,3-butanediol
G9	L-serine	H9	glycerol
G10	L-threonine	H10	D,L-α -glycerol phosphate
G11	D,L-carnitine	H11	glucose-1-phosphate
G12	γ-amino butyric acid	H12	glucose-6-phosphate

TABLE B.3: Results of carbon assimilation by the pure culture *Acinetobacter sp.* and the identification matched by Biolog Microlog's computer program.

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	<+>-	(/)	<+>	<+>	<+>	-	-	-	<+>	-	-
B	-	-	-	<+>	(/)	<+>	-	-	-	-	-	<+>
C	-	-	-	-	-	-	-	-	-	-	<+>	<+>
D	<+>	<+>	<+>	<+>	-	-	-	-	-	<+>	<+>	-
E	(/)	(/)	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>
F	<+>	<+>	<+>	<+>	<+>	<+>	<+>-	<+>	<+>	<+>	-	-
G	<+>	-	<+>	<+>	<+>	<+>	<+>	-	<+>	<+>	<+>	<+>
H	<+>	-	-	-	-	<+>	<+>	<+>	-	-	-	-

=> Species ID : ACINETOBACTER CALCOACETICUS/GENOSPECIES 3 <=

ML4.0	SPECIES	PROB	SIM	DIST	TYPE
=>1)	ACINETOBACTER CALCOACETICUS/GENOSPECIES	100	0.904	1.42	GN-NENT OXI-
2)	ACINETOBACTER BAUMANII/GENOSPECIES 2	0	0.001	3.82	GN-NENT OXI-
3)	ACINETOBACTER CALCOACETICUS/GENOSPECIES	0	0.000	7.44	GN-NENT OXI-
4)	ACINETOBACTER GENOSPECIES 13	0	0.000	8.78	GN-NENT OXI-
5)	ACINETOBACTER GENOSPECIES 14	0	0.000	10.32	GN-NENT OXI-
6)	PSEUDOMONAS CITRONELLOLIS	0	0.000	10.56	GN-NENT OXI+
7)	AQUASPIRILLUM DISPAR	0	0.000	11.21	GN-NENT OXI+
8)	PSEUDOMONAS VIRIDILIVIDA	0	0.000	12.42	GN-NENT OXI-
9)	PSEUDOMONAS PUTIDA BIOTYPE A	0	0.000	12.61	GN-NENT OXI+
10)	PSEUDOMONAS MACULICOLA	0	0.000	13.53	GN-NENT OXI+
Oth)					

TABLE B.4: Results of carbon assimilation by the pure culture *Enterobacter sp.* and the identification matched by Biolog Micrologʼs computer program.

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	(/)	<+>	<+>	(/)	(/)	(/)	<+>	<+>	(/)	<+>	<+>
B	-	<+>	<+>	<+>	(/)	<+>	<+>	<+>	-	<+>	<+>	(/)
C	<+>	<+>	<+>	<+>	(/)	<+>	<+>	<+>	(/)	-	<+>	<+>
D	<+>	(/)	- +	<+>	(/)	<+>	<+>	- +	(/)	-	(/)	-
E	<+>	-	-	-	-	<+>	<+>-	-	<+>	<+>-	-	<+>
F	<+>	(/)	<+>	(/)	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>
G	<+>	<+>	-	(/)	(/)	<+>	-	<+>	<+>	(/)	-	-
H	(/)	<+>	<+>	<+>	-	(/)	-	-	<+>	(/)	<+>	<+>

=> Species ID : ENTEROBACTER AEROGENES (KLB. MOBILIS) <=

ML4.0	SPECIES	PROB	SIM	DIST	TYPE
=>1)	ENTEROBACTER AEROGENES (KLB. MOBILIS)	99	0.730	4.01	GN-ENT
2)	KLEBSIELLA PNEUMONIAE SS PNEUMONIAE	1	0.005	5.68	GN-ENT
3)	KLEBSIELLA PLANTICOLA	0	0.000	6.77	GN-ENT
4)	KLEBSIELLA PNEUMONIAE SS OZAENAE	0	0.000	7.54	GN-ENT
5)	KLEBSIELLA TERRIGENA	0	0.000	7.54	GN-ENT
6)	PHOTOBACTERIUM LOGEI	0	0.000	7.63	GN-NENT OXI+
7)	SERRATIA RUBIDAEA	0	0.000	8.70	GN-ENT
8)	KLEBSIELLA PLANTICOLA/ORNITHINOLYTICA	0	0.000	9.31	GN-ENT
9)	ENTEROBACTER CLOACAE	0	0.000	9.38	GN-ENT
10)	KLEBSIELLA OXYTOCA	0	0.000	10.05	GN-ENT
Oth)					

APPENDIX C

OXYGEN TRANSFER COEFFICIENT DETERMINATION

The rate of transfer of the air bubble to the liquid phase can be described by equation C.1.

$$dC_L/dt = K_L a(C^* - C_L) \qquad \text{(C.1)}$$

where: C_L is the concentration of dissolved oxygen in the liquid (% saturation)

t is time (h),

dC_L/dt is the change in the oxygen concentration in the liquid over a time period (% saturation h^{-1})

K_L is the mass transfer coefficient (cm h^{-1})

a is the gas / liquid interface area per liquid volume (cm^2 cm^{-3})

C^* is the saturated dissolved oxygen concentration (% saturation)

It is difficult to measure K_L or a independently, hence these are usually combined to obtain the volumetric mass-transfer coefficient, $K_L a$. This value was determined to establish that the aeration efficiency of the reactor was sufficient. Oxygen was not limited during the reaction period because the D.O. value never reached below 30% saturation. The static method of gassing in / gassing out was used (Wise, 1951) to determine $K_L a$. This method was performed three times using 1 L of tap water in the reactor set-up and an air flowrate of 180 L h^{-1}. A typical plot of $ln(C^*-C_L)$ versus time for these tests is depicted in Figure C.1. The slope is equal to - $K_L a$, or 124 h^{-1} in this example. Similarly, the gassing in / gassing out method was performed on the synthetic brewery wastewater

three times. A typical $ln(C^*-C_L)$ versus time plot for the synthetic brewery wastewater is included in Figure C.2 and resulted in an average K_La of 70 h^{-1}.

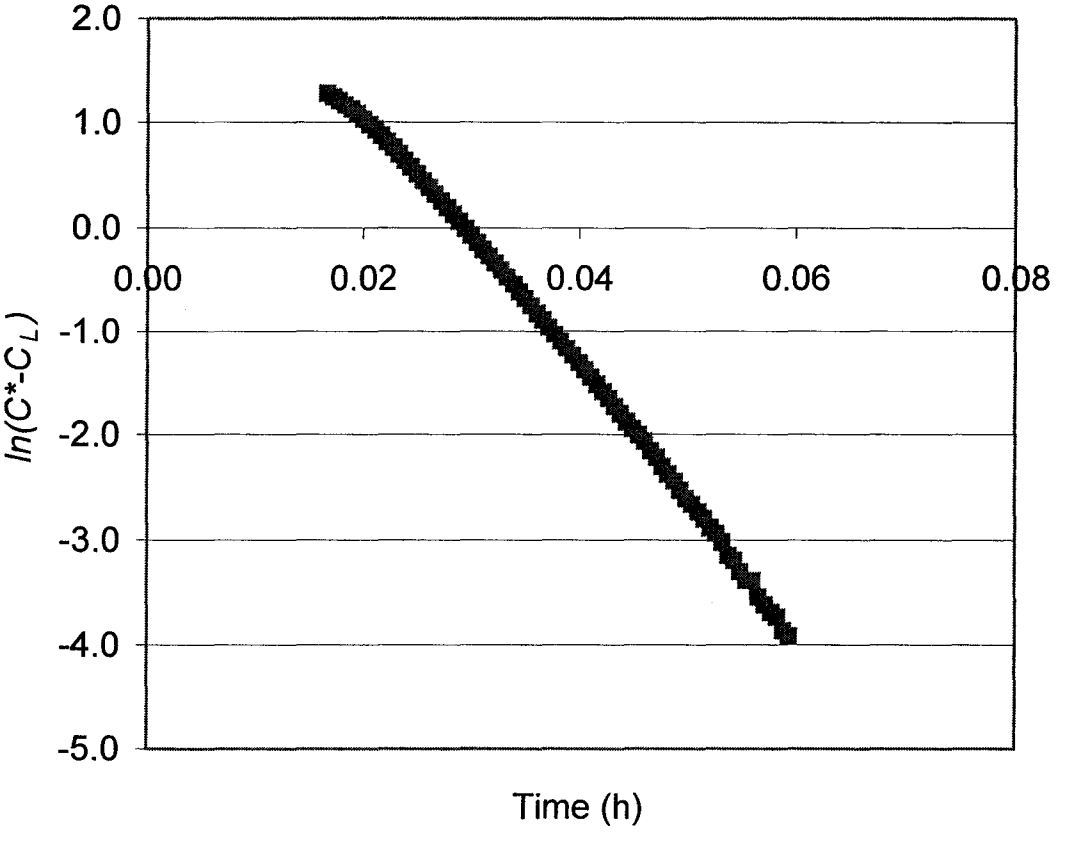

Figure C.1: Volumetric mass transfer coefficient for tap water determined to be 124 h^{-1}.

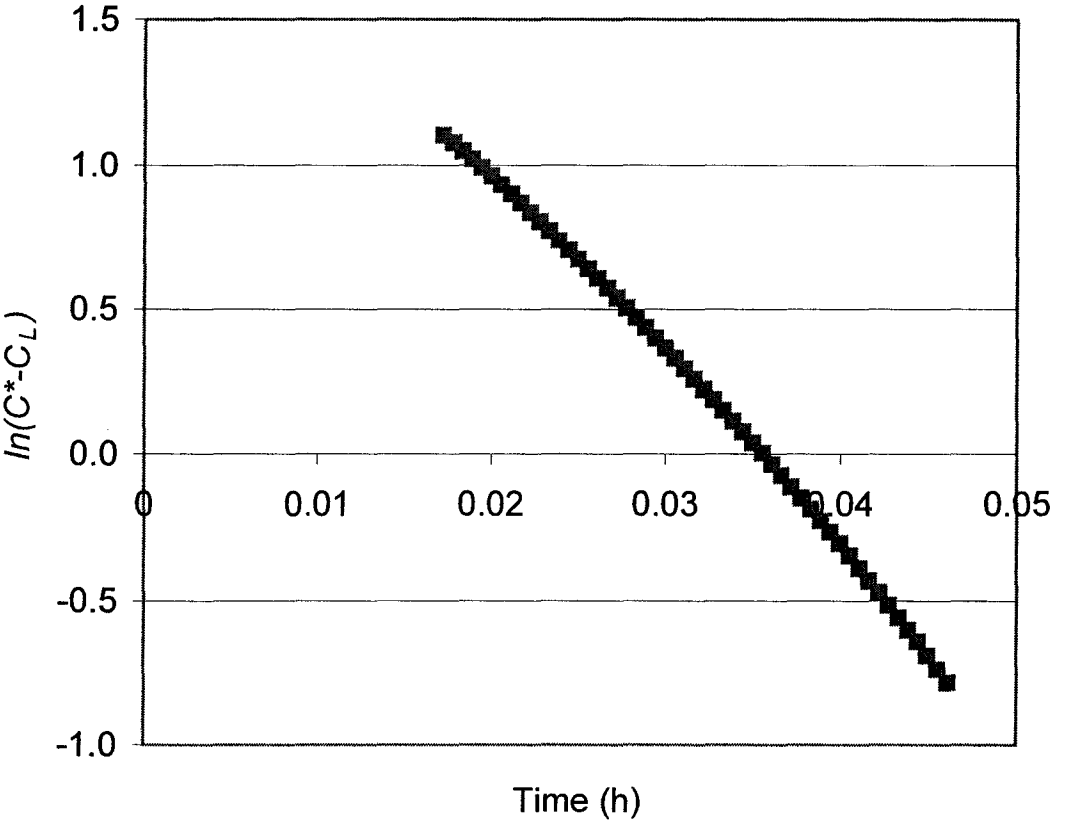

Figure C.2: Volumetric mass transfer coefficient for sterile feed determined to be 70 h^{-1}.