Comparison of PCR-DGGE and Selective Plating Methods for Monitoring the Dynamics of a Mixed Culture Population in Synthetic Brewery Wastewater

Kawai Tam,*,† Ching-Hong Yang,‡ Mark R. Matsumoto,† David E. Crowley,§ and John D. Sheppard||

Department of Chemical and Environmental Engineering and Department of Environmental Sciences, University of California, Riverside, California 92521, Department of Biological Sciences, University of Wisconsin, Milwaukee, Wisconsin 53201, and Department of Bioresource Engineering, McGill University, Ste-Anne-de-Bellevue, QC, H9X 3V9, Canada

Enrichment of an activated sludge inoculum in synthetic brewery wastewater, which included glucose, maltose, and ethanol, was conducted in batch experiments to identify the dominant microbes present, to determine methodologies capable of monitoring the mixed culture population dynamics, and to determine the consortium's substrate degradation behavior. These results and methodologies were subsequently used in the determination of the population dynamics of suspended and attached microorganisms in a sequencing batch system in the second part of this research work. The three-membered microbial community comprised two bacterial and one fungal species that were identified as *Acinetobacter* sp., *Enterobacter* sp., and *Candida* sp. PCR-DGGE and plating on selective media were used to track the population dynamics of the consortium during the degradation of different substrates in synthetic wastewater containing glucose, maltose, and ethanol. *Enterobacter* sp. could degrade glucose and maltose but not ethanol, whereas *Acinetobacter* and *Candida* could degrade all three carbon sources. In buffered batch mixed culture experiments, *Enterobacter* was the predominant bacterium until the sugar concentrations decreased to levels that enabled *Acinetobacter* and *Candida* to degrade ethanol. PCR-DGGE was effective for detecting the dominant species, but culture-based methods were more accurate for monitoring the population dynamics of these microorganisms during growth in the wastewater medium.

Introduction

This paper is the first part in a two-part series in which the species comprising a mixed culture capable of degrading a synthetic brewery wastewater are identified and their population dynamics are characterized in batch growth. Part 2 extends this study to a sequencing batch system in which the synthetic wastewater is degraded by both suspended and attached microbial populations.

A wastewater from the brewery industry was chosen as the representative biodegradable waste. Biological treatment systems are capable of treating brewery wastewater; however, control of such systems can be difficult. Brewery effluents are highly biodegradable and have a broad range of chemical characteristics (Table 1). It is this variation in composition and flow that makes it a difficult waste to treat since biological systems prefer stable environments.

A number of aerobic biological pretreatment processes have been investigated for brewery wastewater. These include the deep shaft bioreactor (*1*, *2*), the jet loop aerator (*6*), and a fixed film sequencing batch reactor (*7*). All three involve the use of an activated sludge inoculum derived from wastewater treatment systems that have been acclimated to the brewery wastewater. Adequate treatment and sufficient reduction of TSS and BOD was achieved, but control problems occurred as a result of wastewater variability even after installation of an equalization basin in the first two systems. The fixed film sequencing batch reactor used by Nguyen et al. (*7*) provided repeatable and consistent treatment of actual brewery wastewater regardless of the concentration introduced. This was the impetus for investigating the population dynamics of the sequencing batch reactor system similar to the one used in Part 2 of this series of papers.

Only a few studies have been done to characterize the microbial communities from activated sludge that are

Table 1. Typical Industrial Brewery Wastewater Characteristics

characteristic	range	reference
BOD, g L^{-1}	0.6−4.0	2
TSS, g L^{-1}	0.2−2.6	1
ethanol, g L^{-1}	0.4−1.69	3
pH	4.5−11	2
total Kjeldahl nitrogen, g L^{-1}	0.0192−0.0692	4
orthophosphate (PO_4^{3-}), g L^{-1}	0.002−0.06	5

* To whom correspondence should be addressed. Ph: 951-827-2498. Fax: 951-827-5696. E-mail: ktam@engr.ucr.edu.

† Department of Chemical and Environmental Engineering, University of California.

‡ Department of Environmental Sciences, University of California.

§ University of Wisconsin.

|| McGill University.

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used for aerobic brewery wastewater treatment. Using microscopic inspection, LeClair (*1*) described the presence of a diverse microflora containing flocculated bacterial colonies and protozoa, including ciliates, both free swimming *Aspidiscus* sp., *Vorticella* sp., amoeba, and paramecia in the deep shaft reactor treatment system. In the aerobic jet loop activated sludge reactor treating brewery wastewater used by Dilek et al. (*8*), colonies obtained from acclimated activated sludge were examined for colony morphology and pigmentation using Gram staining, Neisser staining, and Sudan Black staining (for distinguishing polyhydroxybutyrate). In the same study, 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, K. pneumonia* ssp. pneumo., *Vibrio fluvialis* and *Aeromonas hydrophila*. Enumeration methods were not used during the experiment, but once the isolates were obtained, respirometric activity tests were conducted on the isolates for determination of their growth rates on glucose and brewery wastewater. *B. cepacia* was dominant among the active aerobic bacteria in the mixed culture treating brewery wastewater in the jet loop reactor system.

In terms of characterization of mixed culture systems, molecular based techniques are increasingly being used for analysis of wastewater systems to identify culturable and nonculturable species. Curtis and Craine (*9*) used polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S rDNA to compare microbial communities present in different activated sludge plants. A benefit of DGGE is the possibility of identifying individual members of the microbial community by sequencing bands excised from the gel. However, 16S rDNA profiles generated by DGGE have been used to estimate diversity and relative abundances of different bacterial species (*10*). Most recently, research has been done on the relationship between 16S rDNA band intensities and abundance of the bacterial species that are present (*11*, *12*), showing that PCR-DGGE is capable of studying mixed culture systems with some limitations.

Hence in Part 1 of the series, a fixed mixed culture consortium was identified, and for further understanding of the characteristics of the reactor system, in Part 2 both culture based and molecular methods were used to examine the sub-population changes that occur during the treatment of a synthetic brewery wastewater in a batch system. Information from these experiments will help to get an understanding of the consortia and their behavior in diverse environmental conditions of synthetic brewery wastewater in the semicontinuous experiments described in Part 2. Therefore, the objective of this study was to identify key microorganisms responsible for treatment of the brewery wastewater, to determine the characteristics of these dominant microbes in mixed culture batch experiments with a synthetic wastewater containing glucose, maltose, and ethanol, and to standardize methods for characterizing the mixed consortium.

Materials and Methods

Growth Medium. Simulated brewery wastewater medium was prepared by dissolving the following materials in tap water: 1 g L^{-1} malt extract, 0.5 g L^{-1} yeast extract, 0.15 g L^{-1} peptone, 0.86 g L^{-1} maltose, 2.2 g L^{-1} $(NH_4)_2SO_4$, and 2.8 mL L^{-1} ethanol. Buffering salts of 0.08 g L^{-1} NaH_2PO_4 and 0.14 g L^{-1} of Na_2HPO_4 were added to maintain the pH at 6.7. The medium was filter sterilized. This synthetic brewery wastewater had an initial BOD of approximately 2500 mg L^{-1} and was based on wastewater samples obtained from a microbrewery in Montreal, Quebec, Canada.

Inoculum. An enrichment culture capable of degrading brewery wastewater was produced from an activated sludge from a municipal wastewater treatment facility in Orange County, California. A sample of the sludge was subjected to 20 transfers in synthetic brewery wastewater. Each growth cycle spanned approximately 17 h. After enrichment, aliquots of the resulting mixed culture were frozen in glycerol at −70 °C. Microorganisms were isolated from the enrichment culture by spread plating serial dilutions on solid medium consisting of the synthetic brewery wastewater and 15 g L^{-1} of agar. Single colonies were streaked on agar plates until pure cultures were obtained. Two bacterial isolates and one fungal isolate obtained by this method were identified by sequencing of their rDNA sequences.

DNA Extraction. DNA was extracted using a FastDNA SPIN Kit (BIO101,Vista, CA) following the manufacturer's protocol.

Bacterial 16S rDNA sequences were obtained by PCR amplification using the universal primers PRBA338f (5′-ACTCCTACGGGAGGCAGCAG-3′) that targets a conserved region that flanks the V3 region of 16S rDNA from the domain bacteria**,** and PRUN518r (5′ATTACCGCGGCTGCTGG-3′), which is based on a universally conserved region of the small subunit rRNA gene (*13*). A nested PCR strategy was used to amplify the 18S rDNA genes for the fungal species (*14*). In the first PCR step, primers NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS8 (5′-TCCGCAGGTTCACCTACGGA-3′) were used to amplify a 1.7 kb fragment. The PCR mixture contained 1 Ready-To-Go PCR bead with 1 μL of each primer (10 pmol), 1 μL of the fungal DNA template, and deionized water for a total volume of 25 μL. The PCR program was 94 °C for 2 min; 30 cycles of 94 °C for 0.5 min, 50 °C for 0.75 min, and 72 °C for 1.5 min; and one final cycle at 72 °C for 5 min. The PCR products were verified on a 1.2% (w/v) agarose in 1X TAE buffer at 110V for 30 min. 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 was diluted 500-fold and was used as the rDNA template in the nested PCR amplification step with forward primer NS1-GC (5′-CCAGTAGTCATATGCTTGTC-3′) with a GC clamp attached to the 5′ end (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGG-3′) and reverse primer NS2+10 (5′-GAATTACCGCGGCTGCTG GC-3′). The PCR program for the second step was 94 °C for 2 min; 30 cycles of 94 °C for 0.5 min, 55 °C for 1 min, and 72 °C for 1.25 min; and a final cycle of 72 °C for 5 min.

Multiple bands were detected for both mixed culture and pure culture bacterial and fungal rDNA samples and were excised from the DGGE gels. Each gel slice was placed in a vial containing 20 μL of deionized water and kept at 4 °C overnight to allow the DNA to passively diffuse out from the gel slice. A 10-μL sample from the diffusion step was used as DNA templates for a subsequent PCR amplification. The rDNA fragments were cloned into *E. coli* JM109 using a commercially available kit (Promega pGEM-T Easy Vector Systems, Madison, WI). Purification of the plasmids was performed using standard procedures with a QIAprep Spin Miniprep Kit (Promega, Madison, WI). The plasmids were sequenced and the species were determined using the

Table 2. Sequence Affiliations of Plasmids Obtained from DGGE Gel Slices of Dominant Band Locations Corresponding to Figure 1

sample ID	location in Figure 1	accession no.[a]	sequence homology matches [accession no.] (sequence ID %)
dominant bands of lane 3	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 lane 1	B1	AF242485	*Acinetobacter haemolyticus* 16S rRNA gene, strain ATCC 17922 [Z93436] (100%)
dominant bands of MC (lane 2)	MC1	AF242486	*Acinetobacter haemolyticus* 16S rRNA gene, strain ATCC 17922 [Z93436] (97%)
	MC4	AF242487	*Enterobacter aerogenes* 16S rRNA, partial 5′ end, strain NCTC 10006 T [AJ001237] (100%)
dominant bands of fungal isolate		AF527824	*Candida sojae* 18S rRNA gene, [Z93436] (99%)

[a] Accession numbers of DNA sequences submitted to GenBank.

BLAST software program and database (http://www.ncbi.nlm.nih.gov) and are submitted to GenBank (accession numbers provided in Table 2).

Community Analysis (PCR-DGGE). The presence of the predominant bacterial species in the mixed culture was analyzed by PCR-DGGE. A GC clamp (5′-CGCCCGCCGCGCG CGGCGGGCGGGGCGGGGGCACGGGGGG-3′) was attached to the 5′ end of PRBA338f. PCR was performed using Ready-To-Go PCR beads (Amersham-Pharmacia Biotech, Piscataway, NJ). The PCR mixture consisted of 5 pmol of each primer, 1 μL of the DNA template, and deionized water for a total volume of 25 μL. PCR reactions were run at 92 °C for 5 min; 30 cycles of 92 °C for 1 min, 55 °C for 0.5 min, and 72 °C for 1 min; and then one final cycle at 72 °C for 6 min. 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 gels were run using a DCode TM Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA) at 60 °C at 200 V. The gel was stained in a solution of ethidium bromide (35 μg L^{-1}) and photographed on an UV transilluminator. To transform the intensity of these 16S rDNA bands into numerical data, the gel image was imported into Scion Image (Scion Corp., Frederick, MD), which was used to convert band staining intensities into x/y plot profiles. In each replicate experiment, bands of the same species 16S rDNA were normalized by taking a ratio of each 16S rDNA band divided by the brightest band of the same species 16S rDNA during the experiment as a basis for comparison.

Culture Based Enumeration of Microorganisms. Plate counts for the three microbial species were performed using selective media. Carbon substrates that were used for preparation of the selective media were based on a preliminary survey of differences in organic substrate utilization using BIOLOG GN MicroPlates, which contain an array of 95 substrates. The substrates chosen 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 that had been adjusted to pH 7. Selectivity for the growth of the yeast was achieved using synthetic brewery wastewater/antibiotic agar plates with 0.03 g L^{-1} streptomycin sulfate and 0.03 g L^{-1} chlortetracycline (*15*). For enumeration of the colonies formed on these selective carbon and antibiotic plates, samples were serial diluted by mixing 100 μL of the sample with 900 μL of 0.01 M phosphate buffer and serial diluting 10-fold to obtain a dilution that would result in growth of between 30 and 300 colonies when plated.

Wastewater Analyses. Ethanol concentrations were analyzed using a Hewlett-Packard 6890 series gas chromatograph (GC) system (Wilmington, DE) with a flame ionization detector (FID) set to 200 °C and injector and oven temperatures set to 80 °C. The temperature program was 80 °C for 2.5 min followed by a temperature ramp of 50 °C/min to 120 °C. The GC method had a detection limit of 0.01 g L^{-1} of ethanol. Maltose was detected through a reaction between maltose and a colored reagent (containing 96 mM 3,5-dinitrosalicylic acid, 5.3 M sodium potassium tartrate solution, and 2 M NaOH) at 100 °C (Sigma, St. Louis, MI). Two-milliliter samples were mixed with 1 mL of the colored reagent, placed in a boiling water bath for 15 min, cooled on ice and subsequently diluted with 9.0 mL of deionized water, after which the products were analyzed spectrophotometrically (Beckman DU 640 spectrophotometer, Fullerton, CA) at a wavelength of 540 nm. Glucose was detected through the reaction of 6% (v/v) *o*-toluidine in glacial acetic acid (Sigma, St. Louis, MO) and glucose at 100 °C, producing a colored product that was quantified at a wavelength of 635 nm. Total organic carbon (TOC) of the supernatant samples was analyzed using a Shimadzu TOC-5050 total organic carbon analyzer (Japan).

Results and Discussion

Community Analysis by PCR-DGGE of 16S rDNA. PCR-DGGE was used for the purposes of microbial identification in addition to testing the suitability of the PCR-DGGE method as a relative quantitative analysis for community analysis of the samples obtained from both pure and mixed culture. A fingerprint of the community was obtained containing multiple bands for the mixed culture and pure cultures (Figure 1). Multiple bands for pure cultures are known to exist. The presence of minor faint bands unidentified in mixed culture (MC) can be attributed to two possibilities, the first being the occurrence of unspecific amplification. Unspecific amplification occurs when the PCR primer set erroneously binds to nucleotide sequences on the rDNA template in a region other than the targeted one. The second possibility is attributable to the 16S rDNA region of bacteria having different numbers of operons. It is possible that during PCR amplification of such a bacteria the primers bind to different operons, which may result in faint bands appearing in nearby locations to that of the main bright band for that bacteria on a DGGE gel. This occurrence

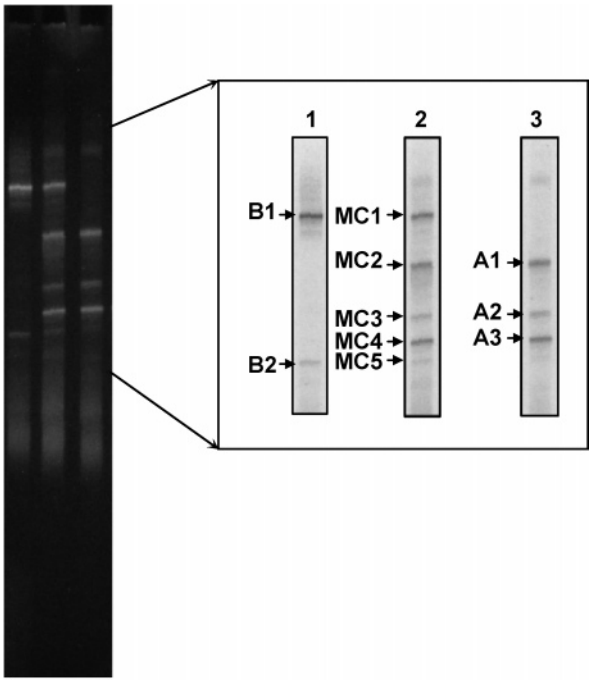

Figure 1. Profile of PCR-DGGE fingerprints of bands containing 16S rDNA for the bacterial pure and mixed cultures. The most prominent bands for *Acinetobacter* (lane 1) and *Enterobacter* (lane 3) are labeled B1 and B2 and A1 to A3, respectively. The mixed culture (MC) bands are labeled MC1 to MC5 (lane 2).

of multiple bands found for the 16S rRNA of a pure culture bacteria has been depicted in previous studies using temperature gradient gel electrophoresis (TGGE) by Nübel et al. (*16*). In these experiments, the mixed culture community contained five distinct bands (MC1 to MC5). For pure culture samples, three bands (A1 to A3) were detected for *Enterobacter* and two bands (B1 and B2) for *Acinetobacter* (Figure 1).

Comparison of the band profiles showed that all of the bands from the single isolates matched to bands visible in the mixed culture. To verify that the multiple bands were from the same microorganisms, various bands were cut from the DGGE gel and sequenced. Sequencing results of the sample 16S rDNA confirmed that the similarly located bands had the same sequence affiliation (Table 2). The three dominant bands A1, A2 ,and A3 (lane 3) were affiliated to a partial 16S rRNA sequence of *Enterobacter aerogenes* with sequence similarities of 98%, 99%, and 100%, respectively. One of the three corresponding bands in the mixed culture consortium, MC4 was also affiliated to the gene of *E. aerogenes* at a sequence similarity of 100%. Based on sequencing information where locations of A1 to A3 matched with MC2, MC3, and MC4 of Figure 1, the location on the DGGE fingerprint band profile could be used to identify *Enterobacter* sp. in a DGGE gel. Because of this finding and cost constraints, sequencing of only the brightest band excised from the DGGE gel for pure *Acinetobacter* (lane 1, B1) and a similarly located band in the mixed culture (lane 2, MC1) was done and resulted in sequence affiliations of 100% and 97%, respectively, to *A. haemolyticus*.

The identities also confirmed wet mount microscopy information where two distinct colony types for the bacterial species were detected. One consisted of motile, straight Gram-negative rods, which formed slightly iridescent, flat, and irregular edged colonies with diameters ranging from 2 to 3 mm, which we now know to be characteristic of *Enterobacter* sp. Cells from the second morphotype were short Gram-negative rods or cocci, with cream-colored colonies that were smooth edged and 1−2 mm in diameter, characteristic of *Acinetobacter* sp.

From the PCR-DGGE of the 18S rDNA samples, the fungal isolate had a 99% affiliation to *Candida sojae*, a yeast from the Ascomycota division. The results confirm wet mount microscopy data where budding ovoids and budding elongated mycelia were detected and are characteristic of vegetative cells and pseudomycelium of *Candida sojae* (*17*). The DGGE analysis of the pure culture *Candida* resulted in a band profile identical to that of the mixed consortium, 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 again only *Candida* sp. was detected.

Substrate Use Patterns. The isolation of these microbial species from the enrichment culture is consistent with the known substrate characteristics of these microorganisms. *E. aerogenes* is commonly found in sewage or activated sludge, and is capable of using various carbohydrates and amino acids, such as glucose, maltose, glycerol, L-serine, and L-alanine (*18*). 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, and sugars, as well as recalcitrant aromatic compounds (*18*). *Candida* sp. is capable of assimilating various carbon sources including glucose, maltose, and ethanol (*17*), the key carbon sources comprising this synthetic brewery wastewater. These substrate usage patterns were verified to determine the best method to characterize the mixed culture.

The degradation capabilities of the pure isolates were tested in buffered pure culture experiments to verify their carbon substrate use patterns before testing the behaviors in mixed culture experiments. Based on literature, experiments were buffered at pH values of 5, 6, and 7 for *Candida*, *Acinetobacter*, and *Enterobacter*, respectively, for optimum growth (*18*). As expected, *E. aerogenes* could grow on glucose and maltose but not ethanol. Glucose was consumed preferentially, while maltose degradation commenced after a 4-h lag. The degradation of maltose by *E. aerogenes* involved hydrolysis to glucose, which was detected from a slight increase in the glucose concentration at 15 h into the experiment (Figure 2).

Acinetobacter sp. are reported to degrade both alcohols and sugars (*18*). The preferentiability of the substrates used was glucose followed by ethanol and maltose. After a 4-h lag period, ethanol was reduced by 24%, whereas maltose had no substantial decrease in concentration, indicating that a lengthy period of adjustment is required by *Acinetobacter* to produce the enzymes necessary for maltose degradation. Once this occurs, the substrate of choice is switched from ethanol to maltose and a significant reduction of 83% is achieved within 11 h (Figure 2).

The ability of the fungal isolate *Candida sojae* to degrade glucose, maltose, and ethanol was similarly confirmed where sugar and ethanol substrates were degraded simultaneously, but at higher rates for the sugar substrates. *Candida* sp. also hydrolyzed the maltose to glucose such that the residual glucose concentration increased between 12 and 15 h as the maltose concentration declined. As seen in Figure 2, the ethanol concentration increased at the 4-h period. 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 for *Saccharomyces cerevisiae* and has been referred to as the

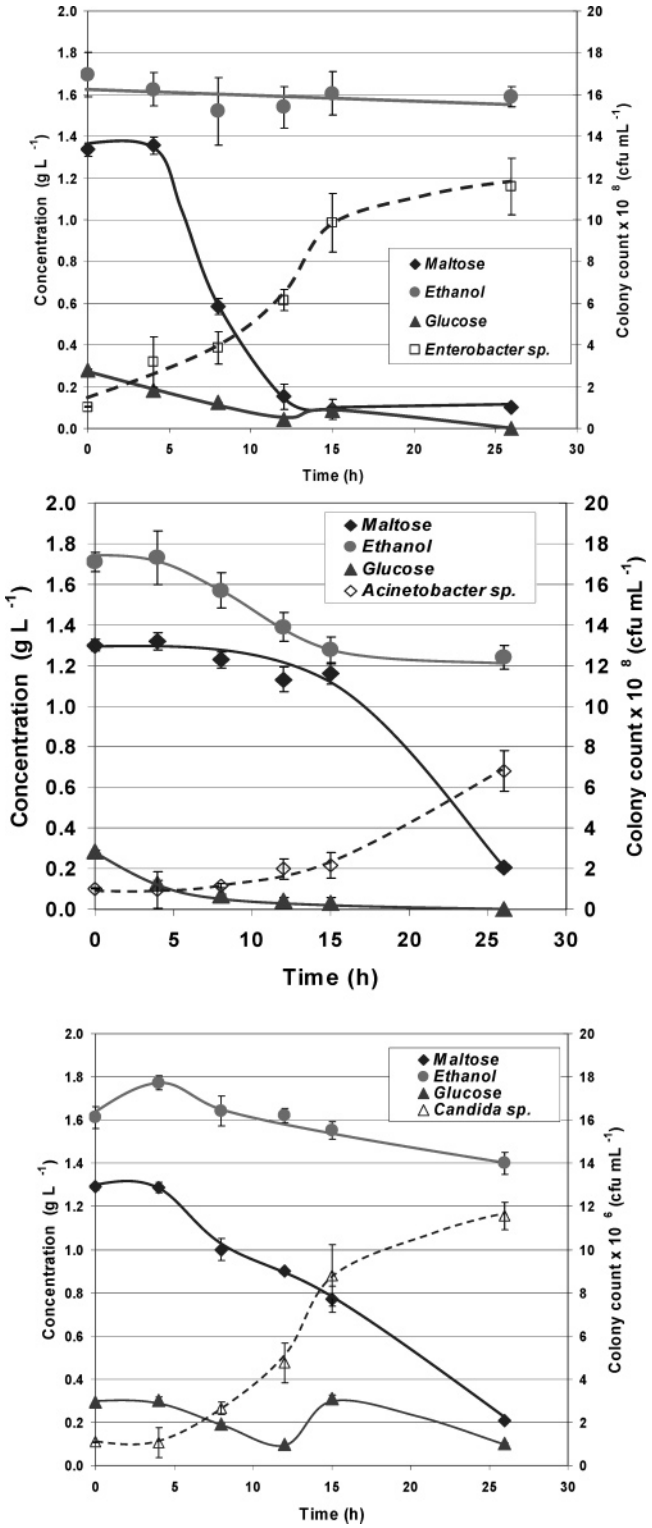

Figure 2. Residual concentrations of maltose, glucose, and ethanol in buffered pure culture *Acinetobacter* sp. (pH 6), *Enterobacter* sp. (pH 7), and *Candida* sp. (pH 5) experiments. Error bars represent standard deviations of triplicate experiments.

"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 (*19*). This trend was also seen with *Candida sojae*, which has not been previously reported.

Mixed Culture Growth Dynamics. To investigate the interactions of the microbial consortium when grown on synthetic wastewater medium, two sets of experiments were performed in triplicate. In the first set, growth and substrate degradation rates were monitored for 24 h in a nonbuffered medium, which provides the behavior when no control of pH occurs. In the second set of experiments, samples were taken during mixed culture flask experiments buffered at a pH of 7 over a 30 h period. The population changes in the mixed consortium were monitored by two methods including PCR-DGGE and selective carbon source plate counts. As we tested both methods with the first set of experiments with nonbuffered medium, it was found that PCR-DGGE provided information between sampling points and was useful in the relative changes in populations but lacked viability information. It has been reported that limitations for relative quantitation can be affected by unspecific amplification when done for environmental samples (*20*). However, it was shown that for a simple three species consortium, the amplification of 16S rDNA can be unaffected (*12*). PCR-DGGE was hypothesized to be capable of providing relative quantitation, and we evaluated this through a comparison with the selective media plate counts. If viability was decreased, 16S rDNA quantities would stabilize and exhibit no further increase in 16S rDNA.

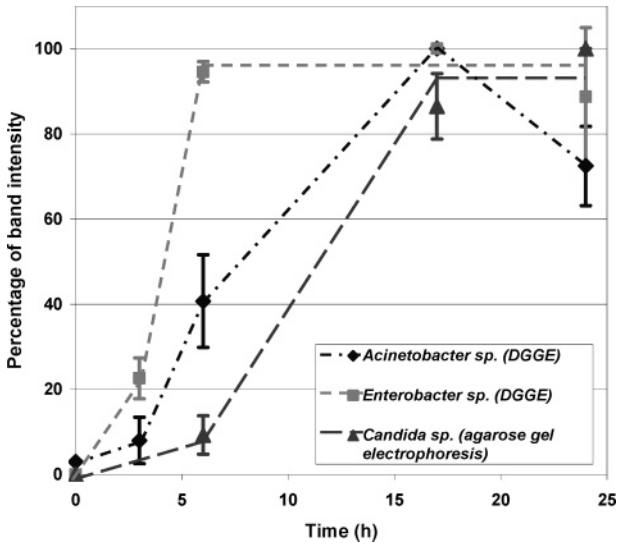

Figure 3. Changes in band intensity of the individual bacteria (*Acinetobacter* and *Enterobacter* sp.) from DGGE and changes in band intensity of *Candida* species from a 1.8% agarose gel in a nonbuffered mixed culture experiment. Error bars represent standard deviations of triplicate experiments.

In Figure 3, results of PCR-DGGE analysis of a nonbuffered mixed culture experiment are presented and proved to be a useful technique for monitoring the population dynamics of the two bacterial species in the consortium. Using the standard *t*-test to compare the average band intensity results at each time sample and of each species, the dotted trend lines were plotted in Figure 3, indicating whether the intensities were the same or changing ($P < 0.05$). The standard deviations among the triplicate experiments were within 10% and within 15% for *Acinetobacter* and *Enterobacter* species, respectively (Figure 3). It is assumed that 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. Thus, the band intensities were used as a measure of semiquantitative abundance of the individual population of microorganisms present at each time interval, relative to their initial values. Since there is only one fungal component of the

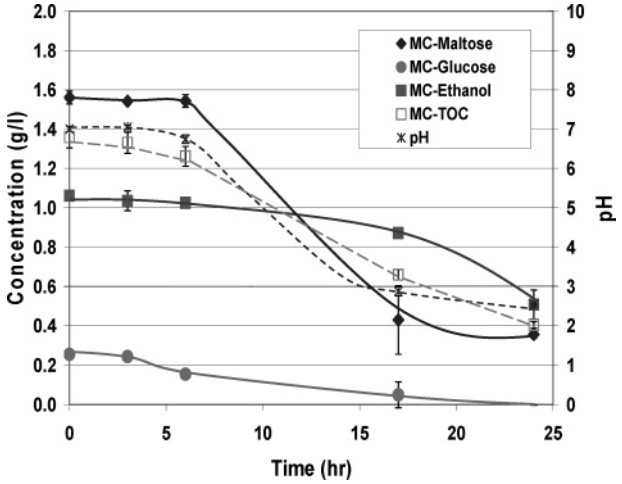

Figure 4. Residual concentrations of maltose, glucose, and ethanol in nonbuffered mixed culture experiments containing *Acinetobacter* sp., *Enterobacter* sp., and *Candida* sp. Error bars represent standard deviations of triplicate experiments.

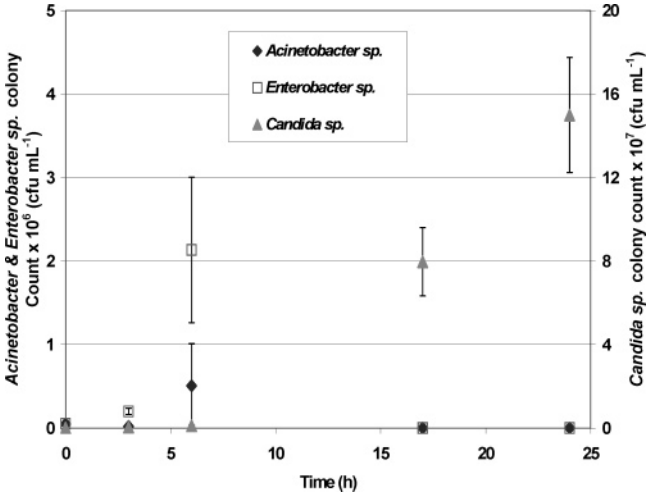

Figure 5. Viable colony counts obtained during nonbuffered mixed culture experiments using *Acinetobacter*, *Enterobacter*, and *Candida* species. Error bars represent standard deviations of triplicate experiments.

consortium, the band intensity analysis of the 18S rDNA on agarose gels alone was sufficient to track the changes in relative abundance of the yeast, avoiding potential problems in nested PCR amplifications. 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 changes in rDNA of each species. Trend lines are drawn based on a 95% confidence interval of a *t*-test on consecutive time samples.

In the nonbuffered mixed culture experiment, results for residual concentrations of glucose, maltose, ethanol, and TOC are depicted in Figure 4, PCR-DGGE results are found in Figure 3, and colony counts are presented in Figure 5. TOC was reduced by 71% during the 24-h experiment. In the first 3 h of the experiment a lag phase was evident before glucose was significantly consumed by the mixed culture. Following removal of glucose, the next substrate consumed was maltose, and finally ethanol was degraded after the sugar levels had been reduced (Figure 4). PCR-DGGE and viability data help to elucidate the dynamics of the 3-species consortium.

During the initial 3-h period, some growth occurred for *Enterobacter* as seen with increased colony counts of Figure 5. This is not surprising since the environment is optimal for *Enterobacter*. PCR-DGGE results confirm this growth and also indicate that some replication of 16S rDNA also occurred for both *Acinetobacter* and *Candida* although less significantly, keeping in mind that the increases are relative to the largest amount of 16S rDNA recovered during the experiment. The *Enterobacter* colony count increased on the order of 10^5 cfu mL^{-1} during the initial 3 h.

During the period between 3 and 6 h, when glucose was the only substrate used, *Enterobacter* out-competed *Acinetobacter* and *Candida* and increased in 16S rDNA (Figure 3) substantially during the period. From pure culture experiments, all three species can utilize glucose, but at a pH of 7, favorable to *Enterobacter*, viability data in Figure 5 confirms the rapid growth of *Enterobacter*.

During the period between 6 and 17 h, the pH dropped, the remaining glucose was used, maltose was reduced by 71%, ethanol was reduced by 14%, and only *Acinetobacter* and *Candida* showed an increase in 16S or 18S rDNA. From pure culture experiments, *Acinetobacter* and *Candida* are capable of using maltose and ethanol as substrates. *Enterobacter* had no further increase in 16S rDNA but substrate degradation is possible for maintenance of these cells without replicating rDNA. Nonbuffered pure culture experiments with *Enterobacter* sp. indicated that substrate degradation occurred even when viable population colony counts decreased (data not shown). After the 17-h time sample, when pH was less than 3, only *Candida* was expected to grow and both PCR-DGGE and viability data (Figures 3−5) reflect this.

Culture based methods show the viability of the species but are very labor and time intensive for a mixed culture consortium, even for a simple system of 3 species. Samples collected from the same time intervals analyzed by DGGE were plated onto media plates, selective for *E. aerogenes*, *A. haemolyticus*, and *Candida sojae,* in triplicate, at a number of dilutions and then counted after a 16-h incubation. Although time samples were not retrieved in the period between 6 and 17 h, PCR-DGGE provided some information. The advantage to 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 cellular rDNA activity that has occurred by monitoring changes in the total 16S rDNA.

The mixed culture dynamics were also investigated in another set of experiments where the medium was buffered to pH 7. This pH is optimal for *E. aerogenes* and as seen in Figure 6, *Enterobacter* out-competed *Acinetobacter* and *Candida* for the substrates of glucose and maltose. From pure culture experiments, it was shown that *E. aerogenes* was not capable of degrading ethanol. Consistent with this idea, there was no significant change in colony counts of *E. aerogenes* during the period when the only remaining substrate was ethanol (Figure 6 and 7). In contrast to the results obtained in nonbuffered medium where *Candida* was more competitive due to the progressively acidic environment, the colony count data in the buffered system indicated that *Acinetobacter* was the ethanol degrader which resulted in increased colony counts. Although *Candida* demonstrated no increase in colony counts, it is possible that *Candida* was still metabolically active in the degradation of ethanol due to the slow growth rate of *Acinetobacter*. Knowledge of pure culture dynamics in this multiple substrate wastewater has aided in the understanding of the dynamics of the mixed culture-multiple substrate system.

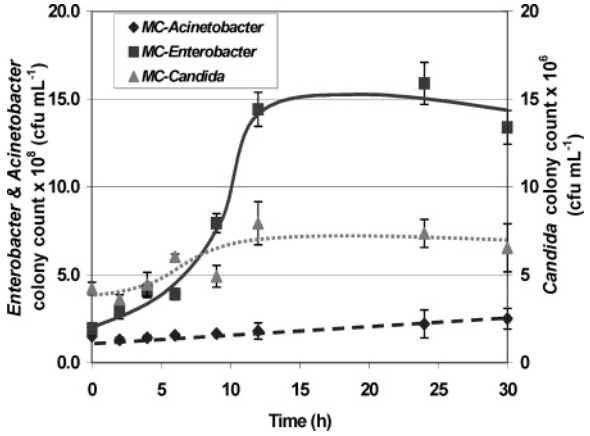

Figure 6. Viable colony counts obtained during buffered mixed culture experiments using *Acinetobacter*, *Enterobacter*, and *Candida* species. Error bars represent standard deviations of triplicate experiments.

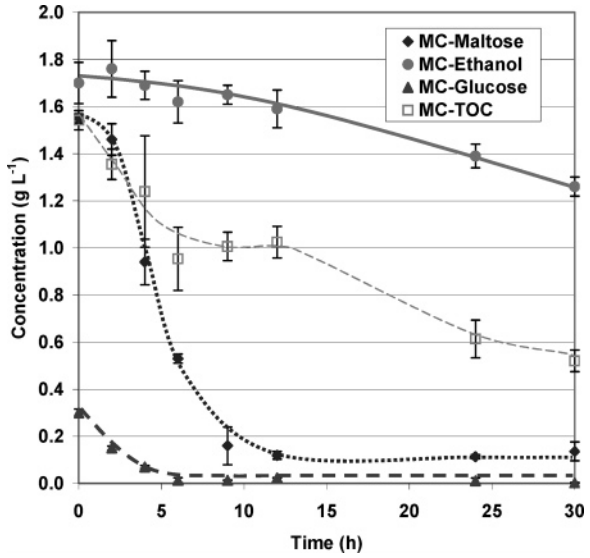

Figure 7. Residual concentrations of maltose, glucose, and ethanol in buffered mixed culture experiments containing *Acinetobacter* sp., *Enterobacter* sp., and *Candida* sp. Error bars represent standard deviations of triplicate experiments.

In comparing the two methods for characterizing the dynamics of the mixed consortium, it was determined that the better method was the selective plating procedure. Although PCR-DGGE offers a simplicity of monitoring additional microbes in a consortium with little extra labor, it does have limitations that may affect the final results. The disadvantage occurs when large differences in 16S rDNA exist between the initial and final time samples; the dilution required to achieve the exponential phase of PCR would not be possible for all of the time samples, and hence the resulting relative intensities of the of the PCR-DGGE analysis would not be correct. It is possible to overcome this limitation by using internal standards during PCR amplification (*12*); however, due to time constraints, the selective plating method was used to advance the semicontinuous experiments in Part 2 of this series.

Conclusions

The objective of this research was to identify the populations in the mixed culture, to determine which of the substrates could be degraded by each population, and to develop methods for studying the population dynamics of a defined mixed culture in a lab batch system to treat multiple substrates in brewery wastewater. Using the enrichment process, two bacterial species and one yeast species were isolated. The three microorganisms were identified as *Enterobacter aerogenes*, *Acinetobacter haemolyticus*, and *Candida sojae*. During isolation and identification of these microorganisms, PCR-DGGE of rDNA proved to be a valuable tool for confirming the species composition of the consortium and purifying them by isolation on agar media. The performance of the consortium was consistent with the growth characteristics of the individual members in pure culture. In mixed culture at pH 7, *Enterobacter* was the predominant bacterium and functioned for removal of glucose and maltose, which occurred prior to removal of ethanol by *Acinetobacter* and *Candida*. The yeast *Candida sojae* grew on all three substrates and was most competitive under nonbuffered conditions when microbial activity had resulted in acidification of the wastewater medium. The combination of culture based and molecular approaches provided complementary information on the growth dynamics of the consortium members but for relative quantitation, the culturing method was found to be a better method because cell viability is also taken into account and shall be used for subsequent characterization in Part 2.

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