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Effects of elevated temperature on bacterial community structure and function in bioreactors treating a synthetic wastewater

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The impact of elevated temperature on bacterial community structure and function during aerobic biological wastewater treatment was investigated. Continuous cultures, fed a complex growth medium containing gelatin and α-lactose as the principal carbon and energy sources, supported mixed bacterial consortia at temperatures ranging from 25–65°C. These temperature- and substrate-acclimated organisms were then used as inocula for batch growth experiments in which the kinetics of microbial growth and substrate utilization, efficiency of substrate removal, and mechanism of substrate removal were compared as functions of temperature. Bacterial community analysis by denaturing gradient gel electrophoresis (DGGE) revealed that distinct bacterial consortia were supported at each temperature. The efficiency of substrate removal declined at elevated temperatures. Maximum specific growth rates and the growth yield increased with temperature from 25–45°C, but then decreased with further elevations in temperature. Thus, maximum specific substrate utilization rates did not vary significantly over the 40°C temperature range (0.64 ± 0.04 mg COD mg^{-1} dry cell mass h^{-1}). A comparison of the degradation of the protein and carbohydrate portions of the feed medium revealed a lag in α-lactose uptake at 55°C, whereas both components were utilized simultaneously at 25°C. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 140–145.

Keywords: biodegradation; DGGE; growth kinetics; temperature; thermophilic

Introduction

Aerobic biological wastewater treatment systems utilize mixed microbial consortia to transform organic and inorganic pollutants to innocuous byproducts, allowing municipal and industrial wastewaters to be released to the environment without detrimental impact. Because the majority of these facilities are operated at ambient temperatures (5–35°C), the effect of temperature on process kinetics has been studied primarily in this range. Many industrial waste streams, however, are produced at high temperatures or are of sufficient strength to promote reactor autoheating, such that biological wastewater treatment at elevated temperatures is possible and potentially economically advantageous compared to other process alternatives. A recent survey of industrial treatment facilities revealed that fewer than five thermophilic ($T > 45$°C) aerobic processes were operating in the United States [9].

Although the impact of temperature on pure bacterial cultures is well understood, the relationship between temperature and the metabolic properties of the bacterial consortia associated with biological wastewater treatment remains unclear. Ecological principles dictate that the dominant members of these mixed microbial communities will change such that those best able to utilize the available resources will proliferate [1,21,23]. Only sparse information can be found in the technical literature comparing the biokinetic rates and pertinent catabolic abilities of mesophilic and thermophilic consortia associated with biological wastewater treatment (see [18] for a review).

Despite this lack of technical understanding and operational experience, a number of new thermophilic treatment facilities are now being designed and installed [25,26]. The assumption inherently made regarding these processes is that thermophilic treatment offers improved biokinetic rates with similar abilities to metabolize pollutants as analogous mesophilic treatment alternatives. There is little scientific evidence, however, to support these assumptions. Previous researchers have either evaluated process kinetics [7,27,28] or pollutant removal efficiency [6,8,29], with the optimum temperature for treatment ranging from as low as 30°C [29] to as high as 58°C [7,28].

The aim of the present work was to investigate the impact of elevated temperature on aerobic biological wastewater treatment of a readily biodegradable wastewater. The hypothesis was that biokinetic rates would increase with temperature, but that bacterial diversity would decline as temperature increased such that the treated wastewater quality and the overall metabolic ability of the bacterial community would decline. Chemostat cultures were operated on a synthetic waste stream containing a simple protein (gelatin) and carbohydrate (α-lactose) as its principal organic constituents at temperatures ranging from 25–65°C. Overflow from these reactors was then used as inoculum for batch reactor experiments in which microbial growth and substrate degradation were studied. This experimental design allowed for both the kinetics of microbial growth and substrate utilization and the efficiency of substrate removal to be studied concurrently. In addition, the mech-

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anism of substrate removal was compared as a function of temperature.

Materials and methods

Chemostat cultures

Continuous cultures were each inoculated with 1-ml samples collected from the aeration tanks of the West Lafayette, IN municipal wastewater treatment facility. Addition of the feed medium commenced immediately following inoculation. The feed medium contained (per liter of deionized water): 1.5 g gelatin (Aldrich Chemical Company, Milwaukee, WI, USA), 1.5 g α-lactose, 50 mg yeast extract (Difco Laboratories, Detroit, MI, USA), 830 mg $NaH_2PO_4 \cdot H_2O$, 570 mg Na_2HPO_4, 500 mg NH_4Cl, 60 mg $MgSO_4 \cdot 7H_2O$, 2 mg KCl, 600 μg $CaCl_2$, and 1 ml of SL7 trace mineral solution [4]. Chemical analysis showed that this medium had a chemical oxygen demand (COD) of 3.1 g L^{-1}.

Sterile medium was pumped into the 580-ml laboratory reactor (CYTOLIFT glass airlift bioreactor, Kontes, Vineland, NJ, USA) at the specified rate using a Masterflex variable-speed console drive pump (Cole Parmer, Vernon Hills, IL, USA). The substrate feed rate was 1.5 ml min^{-1} for all chemostats to ensure that the bacterial inocula used for all batch experiments had a similar physiological state. The exception was at 65°C where a flow rate of 1.0 ml min^{-1} was used due to unstable bacterial growth at higher flow rates. Cultures were aerated and mixed at a rate of 0.5–1.0 L min^{-1}. Reactor temperatures were maintained at 10°C (± 0.2°C) intervals from 25–65°C by circulating water through the jacket of the reactor. Bacterial cultures in the chemostats were assumed to be suitable for batch experiments after steady-state had been reached with respect to biomass level plus an additional four hydraulic residence times.

Batch reactor experiments

Inocula for batch experiments were collected from the overflow of the chemostat cultures described above. This cell suspension was centrifuged (20 min, 10 000 × *g*), and the pellet resuspended in 100 ml of 0.1 mM sodium phosphate buffer (pH 7). Resting cells were then reheated for a period of 10 min in a 4-L jacketed-reactor operated at the specified temperature, after which 3 L of fresh, preheated feed medium (described above) were added. Mixing and aeration were provided by passage of 1–2 L min^{-1} of air through the reactor. Reactor temperatures were maintained at the set value (±1°C) throughout the duration of each batch experiment. The initial ratio of substrate to biomass levels was controlled to be at least 20:1 on a COD basis to ensure that intrinsic growth and substrate utilization rates were elucidated [12,16].

Maximum specific growth rates (μ_m) were determined by regression of the cell concentration during the exponential phase of growth. The duration of the exponential growth phase was determined by visually inspecting natural logarithm plots of cell concentration over time. Growth yields were determined by calculating the ratio of the increase in dry cell mass to substrate depletion during the exponential growth phase. Maximum specific substrate utilization rates (q_m) were calculated by dividing μ_m by the cell yield. All kinetic assays were performed at least in triplicate, and results are reported as the arithmetic mean ± standard error. Statistical analysis was performed by single factor analysis of variance (ANOVA). Statistical significance was defined as $P < 0.05$.

Analytical procedures

Cell concentration was measured as optical density (OD_{600}) and/or dry cell mass. COD analysis was performed on filtered samples (0.45 μm pore size) according to the closed reflux, colorimetric method [2]. Soluble protein and soluble carbohydrate levels were also determined on this filtrate according to the Hartee [14] modification of the Lowry method [19], and the anthrone method [15], respectively. Bovine serum albumin (BSA) and glucose were used as protein and carbohydrate standards, respectively. Experimental analysis revealed that 1 g of gelatin corresponded to 0.6 g of protein (as BSA) and 1.08 g of COD; 1 g of α-lactose was equal to 0.9 g of carbohydrate (as glucose) and 1.04 g of COD. β-galactosidase activity was measured by the *o*-nitrophenyl-β-D-galactoside (ONPG) method [20], modified such that incubations were performed at the temperature from which the cells were obtained. All of these assays were performed in triplicate and values are reported as the arithmetic mean.

DNA extraction, PCR, and denaturing gradient gel electrophoresis (DGGE)

Samples were collected from each of the steady-state chemostats operated at a different temperature. Approximately 100 ml of well-mixed reactor contents from each reactor were collected and centrifuged. The pellet was resuspended in 10 ml of lysis buffer (120 mM sodium phosphate buffer, pH 8.0, 5% sodium dodecyl sulfate), split into 1.2-ml aliquots, and stored at −20°C. Cells were lysed by performing a 75-min incubation at 70°C followed by two consecutive freeze-thaw cycles. Chromosomal DNA was then purified from this solution using the FastDNA Spin Kit per manufacturer's instructions (BIO 101; Vista, CA, USA).

Partial 16S rRNA genes were amplified from the extracted genomic DNA by PCR. The hypervariable V3 region of the domain bacteria was amplified using the PRBA338F (5′-ACTCCTACGGGAGGCAGCAG-3′) [17] and PRUN518R (5′-ATTACCGCGGCTGCTGG-3′) primers with a GC clamp [22]. The 50-μl reaction mixture contained: 5 μl of 10× Promega PCR buffer (Promega, Madison, WI, USA), 4 μl of 25 mM $MgCl_2$, 0.4 μl of 10 mM deoxynucleoside triphosphates, 2.5 μl of bovine serum albumin (2% wt/vol), 1 μl of forward and reverse primers (100 μM each), 2 U *Taq* polymerase, 33.6 μl of sterile water, and 1 μl chromosomal DNA extract (~1 ng of template DNA). The PCR protocol included a 5-min denaturation at 94°C, 30 cycles of 92°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 7 min at 72°C for final extension.

DGGE was performed as described elsewhere [22]. Briefly, equivalent amounts of PCR amplicons were loaded onto 8% (wt/vol) polyacrylamide gels (37.5:1, acrylamide: bisacrylamide) in 0.5 × TAE buffer using a denaturing gradient ranging from 30 to 55% denaturant. Following

electrophoresis, the gel was stained with SYBR Green I (Molecular Probes, Eugene, OR, USA), then visualized on an UV transillumination table.

Results

Following inoculation of the continuous flow bioreactors, noticeable turbidity due to bacterial growth developed within 24 h at all temperatures. Steady-state with respect to cell density was reached after 15–20 reactor volumes of synthetic feed medium had been passed through the reactors. The presence of different bacterial populations at each temperature was verified by denaturing gradient gel electrophoresis (DGGE) of the hypervariable V3 region of the 16S rRNA gene (Figure 1). Twenty-eight unique band positions were identified, of which only five were common to multiple-temperature reactors. Three of these five bands were common to both 35°C and 45°C reactors (lanes 3 and 4); another was common to the 45°C and 55°C (lanes 4 and 5); and the other was common to the 45°C, 55°C, and 65°C chemostats (lanes 4, 5 and 6). The number of bands also varied with temperature: 25°C, 7 bands; 35°C, 10 bands; 45°C, 10 bands; 55°C, six bands; 65°C, four bands.

After initiation of individual batch assays, using steady-state chemostat overflow as inoculum, bacterial growth commenced immediately at all temperatures. Observations

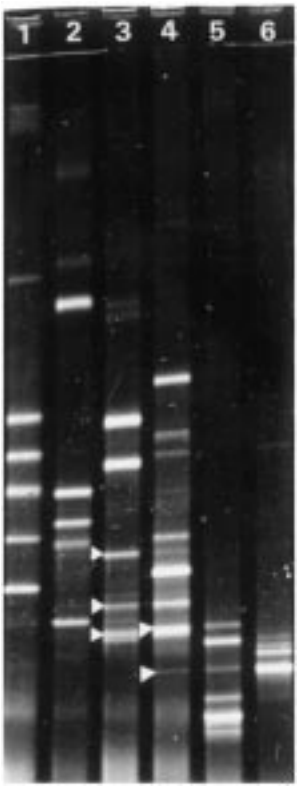

Figure 1 Denaturing gradient gel electrophoresis revealing community fingerprints of the consortia supported by chemostats operated at temperatures of 25–65°C. Individual lanes contain 16S rDNA PCR products from genomic DNA extracts. Lane 1, marker (from top to bottom: *Pseudomonas putida*, *Acinetobacter* sp ADP1, *Comamonas acidovorans* ATCC 15668, *E. coli* DH5α, *Alcaligenes* sp BR40, *C. testosteroni*); lane 2, 25°C; lane 3, 35°C; lane 4, 45°C; lane 5, 55°C; and lane 6, 65°C. White arrows identify bands common to multiple lanes.

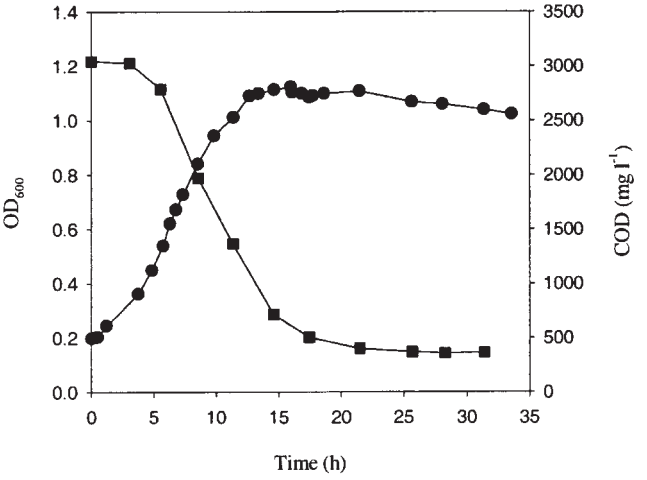

Figure 2 Bacterial growth coupled to COD removal at 55°C. ● OD_{600}; ■ COD.

over an extended time period indicated that the biomass underwent the exponential, stationary, and endogenous phases of growth; this growth occurred with a concomitant disappearance of soluble COD (Figure 2). Because the removal of COD ceased 20–25 h after the initiation of each experiment, residual soluble substrate levels (including protein and carbohydrate) were measured after 25–30 h to determine removal efficiency at each temperature.

Residual COD levels revealed a temperature effect in that removal efficiency declined (ie, concentrations increased) from 25–45°C, with a moderate increase in removal efficiency at 55°C compared to 45°C (Table 1). Residual COD levels were statistically lower at 25°C than at 45°C or 55°C. Overall, these removal efficiencies varied from 86–94% of the initial COD levels. No discernible pattern was identified for the removal efficiency of either carbohydrate or protein as a function of temperature. There was, however, a key difference in the relative fraction that carbohydrate and protein contributed to the residual COD. Assuming that the ratios of protein : COD and carbohydrate : COD remain constant throughout each experiment (initially, 1.71 and 1.16, respectively), the fraction of residual COD comprised by these two constituents was considerably higher at 25°C (95%) compared to 35–

Table 1 The effect of elevated temperature on the final concentrations of COD, protein, and carbohydrate, as produced by batch culture

Reactor temperature (°C)	Final concentrations		
	COD (mg L^{-1})	Protein (mg L^{-1})	Carbohydrate (mg L^{-1})
25	195 ± 2	52 ± 7	73 ± 8
35	243 ± 34	49 ± 1	56 ± 4
45	421 ± 10	56 ± 1	120 ± 1
55	354 ± 5	45 ± 7	83 ± 5
65	nd	nd	nd

Values are means ± standard error.
Initial concentrations were: COD = 3.1 g L^{-1}, protein = 950 mg L^{-1}, and carbohydrate = 1.35 g L^{-1}.
nd, not determined.

55°C (53–62%). For example, at 25°C the mean residual protein and carbohydrate levels were 73 and 52 mg L^{-1}, respectively, for a total of 189 mg COD L^{-1} out of the 195 mg COD L^{-1} measured. At 55°C, protein and carbohydrate levels were 83 and 45 mg L^{-1}, comprising 198 mg L^{-1} of the 354 mg L^{-1} of COD measured.

Regression of the increase in biomass density during the exponential growth phase allowed for the maximum specific growth rate (μ_m) to be determined as a function of temperature. The μ_m increased by a factor of 1.4 as temperature increased from 25–45°C, but then declined almost by half with further increases in temperature to 65°C (Figure 3). The growth yield followed a similar parabolic pattern. The maximum specific substrate utilization rate (q_m), therefore, appeared to be relatively constant over the entire 40°C temperature span studied (Figure 3).

The removal of protein and carbohydrate was compared at 25°C (Figure 4a) and 55°C (Figure 4b) as representative mesophilic and thermophilic temperatures, respectively. At 25°C, both protein and carbohydrate were immediately and simultaneously utilized. In the thermophilic system, however, a lag in carbohydrate degradation was initially observed, though protein degradation began immediately.

An additional batch growth experiment at 55°C on an α-lactose/yeast extract-only medium demonstrated initial rapid growth (~1 h), followed by a short period (~3 h) in which no growth occurred (Figure 5a). A sharp decrease in β-galactosidase activity was measured during this period. Once growth resumed, β-galactosidase activity increased and the rate and extent of carbohydrate degradation were similar to that on the complete growth medium (Figure 4b). Batch growth on a α-lactose-only medium revealed immediate and continuous growth; β-galactosidase activity remained relatively constant throughout the duration of the batch experiment (Figure 5b). Subsequent experiments verified that this growth could be sustained on α-lactose as sole carbon source at 55°C by successfully maintaining it in a chemostat for a period of more than 20 hydraulic residence times (data not shown). Both the rate and extent of carbohydrate removal were less than that measured when yeast extract was included in the growth medium.

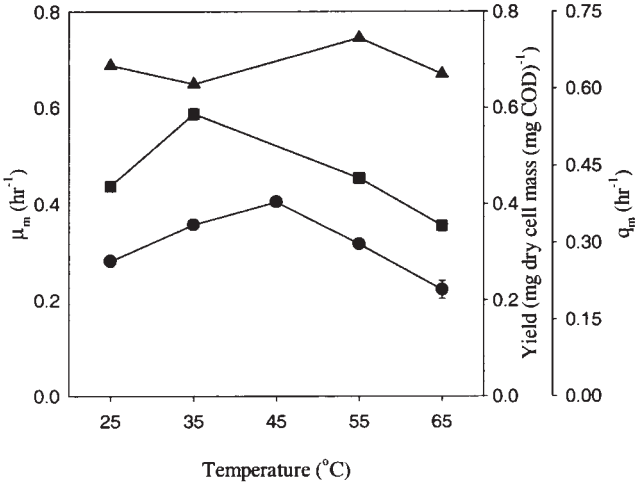

Figure 3 The effect of temperature on the maximum specific substrate utilization rate (μ_m), growth yield, and maximum specific substrate utilization rate (q_m). Values for μ_m and yield are reported as the arithmetic mean ± standard error; q_m was calculated as the mean μ_m divided by mean yield. ● μ_m; ■ yield; ▲ q_m.

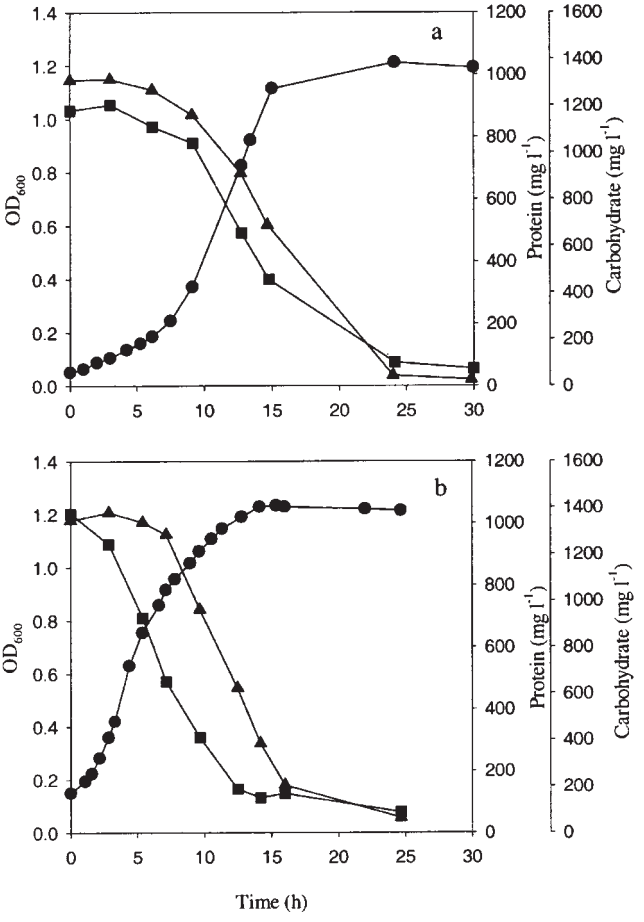

Figure 4 Bacterial growth coupled to the removal of protein and carbohydrate at: (a) 25°C, and (b) 55°C. ● OD_{600}; ■ protein; ▲ carbohydrate.

Discussion

The goal of this research was to investigate the impact of elevated temperature on bacterial community structure and function during aerobic biological wastewater treatment. This work is of practical significance because a number of thermophilic aerobic biological processes are now being installed to treat high temperature and high strength industrial wastewater assuming that these systems exhibit more rapid biokinetic rates without loss of metabolic function. Results indicated that elevated temperature failed to improve substrate utilization rates, thereby offering no biokinetic advantage to thermophilic treatment (Figure 3). Furthermore, the bacterial consortia supported by thermophilic reactors demonstrated a reduced metabolic potential, as exhibited by higher levels of residual pollutants (Table 1) and an inability to simultaneously degrade multiple substrates (Figure 4).

An implicit hypothesis of this work was that bacterial community structure would change with temperature, and that the respective mesophilic and thermophilic consortia would exhibit different metabolic abilities. Selection of

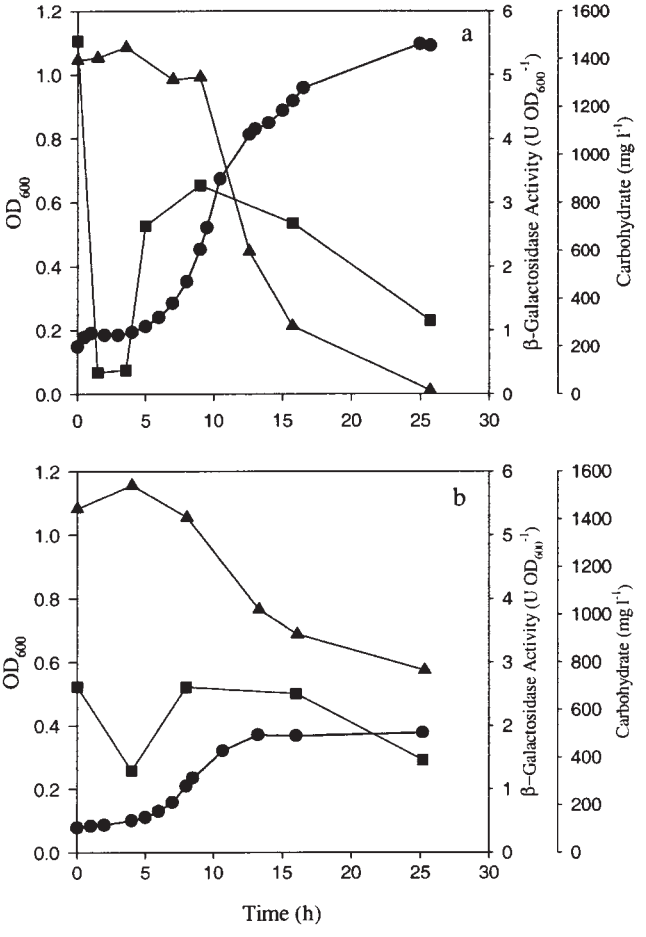

Figure 5 Bacterial growth coupled to carbohydrate removal at 55°C in media containing the following carbon and energy sources: (a) yeast extract (50 mg L^{-1}) and α-lactose (1.5 g L^{-1}), and (b) α-lactose (1.5 g L^{-1}). ● OD_{600}; ■ β-galactosidase activity; ▲ carbohydrate.

unique bacterial communities at each temperature studied was verified by DGGE. The small number of DGGE bands common to multiple chemostat reactor samples indicated that these bacterial communities were almost completely distinct. Although a number of PCR artifacts are known to occur during the amplification of mixed genomic DNA extracts, such as preferential amplification [13] and heteroduplex formation [11], DGGE is generally accepted as a technique that provides a fingerprint of the dominant phylotypes in the original mixed culture [11,22,30]. Because these communities were therefore determined to be temperature-specific, the subsequent experiments provided a suitable basis to confirm or refute our hypothesis on the functional impact of elevated temperature on aerobic biological wastewater treatment.

The increase in the number of DGGE bands from 25°C (seven bands) to 35–45°C (10 bands) seems to contradict the hypothesis that bacterial diversity would decrease as temperature increased, but the decrease in the number of DGGE bands with further increases to 55°C (six bands) and 65°C (four bands) provides some evidence to support this hypothesis. DGGE, however, fails to provide conclusive evidence on this matter for several reasons. First, DGGE measures phylogenetic diversity (ie, the number of distinct 16S rRNA genes), not the functional diversity of the respective phylotypes. For example, two phylogenetically identical *Comamonas* spp differed in their abilities to auto-aggregate during growth on phenol [30]. Second, two distinct phylotypes could migrate the same distance on the denaturing gel, such that diversity is underestimated. And finally, the sensitivity of the DGGE technique allows for single base differences of the hypervariable V3 region of the rRNA gene to be detected. Strains of very similar phylogeny could therefore produce multiple bands.

Although different bacterial communities were supported at different temperatures, μ_m was only somewhat impacted, increasing by a factor of 1.4 over the 20°C span from 25–45°C and then decreasing as temperature continued to increase. Measurement of the highest μ_m at 45°C is similar to that previously reported [27], though others [7,28] have measured the highest μ_m at 58°C. A more important kinetic parameter for biological wastewater treatment is q_m, which was relatively constant over the entire temperature range studied. This occurred because temperature had a similar effect on both μ_m and cell yield, such that the ratio of these parameters (q_m) remained relatively constant. Elevated temperature, therefore, has only minimal impact on the rate of pollutant removal during aerobic biological wastewater treatment, and the assumption that thermophilic aerobic treatment offers biokinetic advantages is likely invalid.

The different bacterial consortia supported by conventional mesophilic reactors (eg, 25°C) also produced a better quality effluent than at thermophilic temperatures (eg, 55°C). This result corresponds well with that of previous researchers [6,8,29]. This deterioration in effluent quality at elevated temperature indicates a decline in overall metabolic ability at elevated temperature. The ratios of residual protein : COD and carbohydrate : COD also seem to indicate a difference in the chemical composition of residual COD.

In addition to producing a higher quality effluent, the mechanism of substrate removal was characteristically different at 25°C and 55°C (Figure 4). Both protein and carbohydrate levels simultaneously declined without an initial lag period at 25°C. Although protein degradation began immediately at 55°C, a lag was apparent in carbohydrate degradation. This provided a further indication of reduced metabolic ability of thermophilic consortia relative to analogous mesophilic communities in that multiple substrates were not utilized simultaneously.

A complicating factor in reaching this conclusion, however, was the presence of yeast extract in the feed medium. Yeast extract was originally included in the feed medium at a low concentration (50 mg L^{-1}) to provide organic micronutrients to support growth. While prototrophic growth is common at mesophilic temperatures, pure strains of thermophilic cultures are rarely able to grow on a single carbon source and inorganic nutrients [3,5,24]. Yeast extract, however, is also a complex substrate containing readily degradable organic carbon sources in addition to micronutrients, and its presence perhaps initially inhibited α-lactose degradation at 55°C, even though it also promoted considerably more rapid and complete degradation of α-lactose when gelatin was not included in the feed medium. The slow, somewhat weak growth of the thermo-

philic culture on α-lactose as a sole carbon source was somewhat surprising, as the authors are aware of only two previous reports of prototrophic growth at thermophilic temperatures [10,31].

In conclusion, long-term operation of biological wastewater treatment systems at elevated temperature has significant impacts on bacterial community structure and function. Unique consortia develop as a function of temperature, and measurable differences exist in the abilities of these communities to achieve the goals of biological wastewater treatment. In addition to a lack of biokinetic advantage to operating aerobic biological wastewater treatment systems at elevated temperatures, there appears to be a reduced ability to simultaneously degrade multiple substrates. This work demonstrates that biological wastewater treatment is feasible at elevated temperature. Thus, it may be advantageous to pretreat high temperature waste streams without first reducing their temperature. Further work is necessary to determine if these conclusions are broadly applicable to other waste streams and the associated bacterial consortia supported by those biological treatment processes.

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