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Biodegradation kinetics of trans-4-methyl-1-cyclohexane carboxylic acid

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Abstract Naphthenic acids are a complex mixture of organic compounds which naturally occur in crude oil. Low molecular weight components of the naphthenic acids are known to be toxic in aquatic environments and there is a need to better understand the factors controlling the kinetics of their biodegradation. In this study, a relatively low molecular weight naphthenic acid compound (trans-isomer of 4-methyl-1-cyclohexane carboxylic acid) and a microbial culture developed in our laboratory were used to study the biodegradation of this naphthenic acid and to evaluate the kinetics of the process in batch cultures. The initial concentration of trans-4-methyl-1-cyclohexane carboxylic acid (50–750 mg l^{-1}) did not affect the maximum specific growth rate of the bacteria at 23°C (0.52 day^{-1}) to the maximum biodegradable concentration (750 mg l^{-1}). The maximum yield observed at this temperature and at a neutral pH was 0.21 mg of biomass per milligram of substrate. Batch experiments indicated that biodegradation can be achieved at low temperatures; however, the biodegradation rate at room temperature (23°C) and neutral pH was 5 times faster than that observed at 4°C. Biodegradation at various pH conditions indicated a maximum specific growth rate of 1.69 day^{-1} and yield (0.41 mg mg^{-1}) at a pH of 10.

Keywords Biodegradation · Kinetics · Naphthenic acids

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Introduction

Canada ranks the second largest in terms of global proven crude oil reserves (15% of world reserves), after Saudi Arabia. The majority of these reserves are found in Alberta's oil sands located in the Athabasca Basin in northeastern Alberta, Canada (Department of Energy, Government of Alberta 2007). In 2004, Alberta Energy reported that the production of crude oil from these reserves had reached 1.1 million barrels per day (Department of Energy, Government of Alberta 2007).

Oil sand is comprised of bitumen, mineral content including sands and clays, and water (Department of Energy, Government of Alberta 2007). The bitumen is extracted from the oil sand ores with a hot water extraction process which produces large volumes of slurry waste. Approximately, 0.1–0.2 m^3 of tailings

pond water is accumulated for each ton of oil sands processed (Lo et al. 2003). In 2003, it was estimated that approximately 4×10^8 m^3 of tailings comprised of a slurry of sand, clay, water, and unrecovered bitumen in a stable aqueous suspension are retained in the Athabasca region and that the total volume was expected to increase to 10^9 m^3 by 2020 (Lo et al. 2003). The toxicity of the liquid wastes from the tailings pond water has been largely attributed to the salinity and presence of organic compounds which are believed to be a complex mixture of organic acid surfactants referred collectively as naphthenic acids (NAs) (Headley et al. 2002a; Lo et al. 2003; Bataineh et al. 2006; Quagraine et al. 2005; Headley et al. 2007; Hsu et al. 2000).

NAs are a complex mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids with the general chemical formula $C_nH_{2n+Z}O_2$, where n indicates the carbon number and Z specifies the number of hydrogen atoms that are lost as the structure becomes more compact when compared with those with linear hydrocarbon chains.

NAs are extracted from crude oil during the refining process due to their acidity. There are many different classes of carboxylic acids present in crude oils (Meredith et al. 2000). The commercial applications for NAs are varied and include: fuel additives, wood preservatives, paint driers, anti-wear additives in lubricants, high aluminum ceramics, enhancement of the water proofing properties in cement, and in the manufacturing of tires (Deineko et al. 1994; Clemente et al. 2003; Brient et al. 1995; Lower 1987).

Low molecular weight components in oil sands naphthenic acids, such as 4-methyl-1-cyclohexane carboxylic acid (4MCHCA) are known to be biodegradable in aquatic environments. The trans-isomer of 4MCHCA (trans-4MCHCA) was thus investigated in this study, to elucidate factors (concentration, pH, and temperature) controlling the kinetics of biodegradation using batch cultures. An earlier research study indicated that the trans-isomer would be more amenable to biodegradation than the cis-isomer (Headley et al. 2002b). The chemical formula for trans-4MCHCA is $CH_3C_6H_{10}CO_2H$ with a formula weight of 142.2 g mol^{-1}. It has a single ring structure fitting the formula of NAs where the Z series is equal to -2 (CAS Number 13064-83-0). The simple naphthenic acid compound trans-4MCHCA selected in the present study, although not representative of oil sand naphthenic acids or any commercially prepared mixture, facilitates the understanding of the principles governing the biodegradation kinetics of this compound which could then be used as a stepping stone to study the biodegradation of more complex naphthenic acid mixtures.

Materials and methods

Microbial culture and medium

The original culture utilized throughout this study was isolated using a commercially prepared NA mixture available as Fluka technical NAs (manufactured and supplied by Sigma-Aldrich, CAS No. 1338-24-5) as substrate. The culture was then used to inoculate trans-4MCHCA prepared in modified McKinney's medium at concentrations of 100 mg l^{-1}.

McKinney's modified medium containing non-growth rate limiting concentrations of all required mineral nutrients was used for the growth and maintenance of the microbial consortium and biodegradation studies (Hill and Robinson 1975; Yu et al. 2006). The medium was prepared in 2 l batches of reverse osmosis (RO) water and had the following composition: KH_2PO_4 (840 mg l^{-1}); K_2HPO_4 (750 mg l^{-1}); $(NH_4)_2SO_4$ (474 mg l^{-1}); NaCl (60 mg l^{-1}); $CaCl_2$ (60 mg l^{-1}); $MgSO_4 \cdot 7H_2O$ (60 mg l^{-1}); $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (20 mg l^{-1}). Trace mineral medium was added to the macronutrients at a concentration of 0.1% on a volumetric basis. The trace mineral medium was comprised of: H_3BO_3 (600 mg l^{-1}); $CoCl_3$ (400 mg l^{-1}); $ZnSO_4 \cdot 7H_2O$ (200 mg l^{-1}); $MnCl_2$ (60 mg l^{-1}); $NaMoO_4 \cdot 2H_2O$ (60 mg l^{-1}); $NiCl_2$ (40 mg l^{-1}); and $CuCl_2$ (20 mg l^{-1}).

The resultant solution, a buffered medium, was sterilized at 121°C for 30 min prior to use. The cultures were maintained in 250 ml flasks each containing 100 ml of medium with 100 mg l^{-1} of trans-4MCHCA. The trans-4MCHCA (substrate) was dissolved in the medium by vigorous mixing on a magnetic stirrer. This was followed by adjustment of pH to 7.0 using a 1.0 and/or 0.1 M solution of sodium hydroxide, as required. The medium was then inoculated with 10 ml of the designated culture (10% on a volumetric basis). Optical densities were monitored and recorded daily over several days with

a spectrophotometer. Upon complete biodegradation of the substrate, the culture was used as an inoculum for subculturing of fresh medium. Subculturing was initially carried out for every 10–14 days. Frequent subculturing eventually resulted in shorter lag phases of approximately 5–6 days at room temperature (23°C) and as a result the subculturing was carried out every 7 days. After several months of experimentation in shake flasks testing a variety of inoculum sources, the culture originating from the Fluka NA standard was selected as the most suitable candidate for biodegradation of the trans-4MCHCA studies.

In order to identify the dominant species of the developed microbial consortium used in this study a sample taken from a culture in the exponential phase of growth was plated on aseptic agar. Agar mixture was prepared by dissolving 3 g of Difco® Bacto agar, 3 g Difco® Bacto tryptose phosphate broth in 100 ml of RO water under sterile conditions. Trans-4MCHCA (250 mg l^{-1}) was added to this mixture to support the microbial growth. The prepared mixture was then poured into Petri dishes. Microbial identification was conducted at a commercial laboratory (EPCOR-Quality Assurance Lab, Edmonton, Canada). The MIDI® Microbial Identification System utilizing a fatty acid profile for a fingerprint technique was used.

Experimental systems and procedures for biodegradation studies

Following the establishment of a suitable microbial culture for biodegradation of trans-4MCHCA, batch experiments were conducted to study the kinetics of microbial growth and biodegradation. The effects of trans-4MCHCA initial concentration, temperature, and pH were investigated.

Batch experiments were carried out in 500 ml shake flasks each containing 150 ml of medium described previously, containing substrate (trans-4MCHCA) at various initial concentrations of 50, 100, 250, 500, 750, and 800 mg l^{-1}, with an initial pH of approximately 7.0. Each flask was inoculated by a 5-day old culture (5% on a volumetric basis). Flasks were maintained at room temperature (23°C) on a magnetic stirrer (200 rpm) to achieve sufficient mixing and oxygen transfer. pH was monitored daily and adjusted to the designated value as required with a 0.1 M solution of NaOH. In all cases, samples were taken on daily basis and tested for optical density [OD] (biomass concentration), substrate concentration, and pH. The sampling frequency was increased during the exponential phase and decreased once stationary growth was established. Progressive experiments were carried out under similar and/or modified conditions using the preceding batch culture as an inoculum to permit adaptation of the consortium to the higher substrate concentrations. For example, upon completion of batch experiments with an initial substrate concentration of 100 mg l^{-1}, the consortium capable of degrading this concentration was then used as an inoculum in the subsequent batch experiments with an initial substrate concentration of 250 mg l^{-1}.

Samples required for various analyses were collected from the reactor using a stainless steel needle and glass hypodermic syringe. Prior to determination of substrate concentration, the liquid sample was filtered through 0.22 μm nylon microfilters using a stainless steel cartridge. The samples were collected in duplicate in 2 ml amber vials and immediately stored at −17°C until the time of analyses.

To assess the effect of temperature, experiments were carried out in a temperature controlled environmental chamber where the temperature was incrementally lowered from room temperature 23°C to 16, 12, 10, 8, and 4°C and similarly raised to 30, 37, and 40°C. The initial substrate concentration used in these experiments was 500 mg l^{-1} and the pH was initially set at a value around 7.0. All other conditions and procedures were similar to those described earlier.

Batch experiments were carried out to assess the effects of initial pH (5.5, 7.0, 8.5, 10, 11, and 13) on the activity of the bacterial culture and biodegradation of trans-4MCHCA. During these experiments the pH of the culture was adjusted once a day to the designated experimental pH. The initial substrate concentrations and temperature for these experiments were 500 mg l^{-1} and 23°C, respectively. All other conditions and procedures were similar to those described earlier.

Analytical methods

The concentration of biomass was determined by direct measurement of the OD of the samples taken

from the flasks at a wavelength of 620 nm (Panikov et al. 2007). An ultraviolet (UV) spectrophotometer (Mini Shimadzu, Model 1240) was used for the determination of the OD. The OD was then related to dry-weight using a calibration curve developed in this work.

The concentration of substrate (trans-4MCHCA) was measured throughout the course of this work using an Agilent 6890 N gas chromatograph (GC) equipped with a flame-ionization detector (FID), and a split/splitless injector. The injector was operated in splitless mode with an initial column inlet temperature of 90°C. The oven was heated to a final temperature of 180°C at a rate of 30°C per minute. The column was an Agilent 19091N-113 HP_INNO-WAX Polyethylene Glycol capillary column with a length of 30 m and a nominal diameter of 320 μm. Calibration was carried out with eight standard solutions prepared in nutrient medium at concentrations covering those used in the batch reactor experiments (2–1,000 mg l^{-1} trans-4MCHCA). The upper range of calibration represents the solubility limit of trans-4MCHCA in the medium as determined experimentally. Samples of standard solutions were filtered and prepared in a manner similar to that of the actual samples taken from the experimental system. The standards were stored in amber vials at −17°C. Two working standards were tested each time, a set of samples were analyzed to confirm the GC calibration. The retention time for trans-4MCHCA was determined to be 4.20 min. Each sampling event included the collection of three samples using a clean glass syringe with a stainless steel needle. The injection volume was 0.5 μl. Each sample was analyzed in triplicate. Millipore water was injected into the column following the analyses of a maximum of five samples (15 injections) to prevent the build-up of substrate or medium components in the column. The injection syringe was cleaned with Millipore water prior to analyses. The inlet was heated to a temperature greater than the operating conditions prior to each batch of analyses (maximum of 12 samples) for a minimum of 1 h in order to clear the inlet of any residual NAs and/or medium minerals. The column inlet was cleaned or replaced as required when the Millipore blanks injected between samples were not sufficient to return to the detection limit of 10 mg l^{-1}. The effectiveness of this procedure in clearing the inlet and column of any residual mineral or substrate build-up was confirmed by a minimum of three injections of Millipore water prior to analyses to ensure a background concentration of <10 mg l^{-1}. This maintenance procedure was performed as required in order to ensure minimal interference of the medium minerals on the inlet liner.

Results and discussion

Microbial identification carried out for the developed microbial consortium capable of degrading trans-4MCHCA at room temperature indicated that the consortium was comprised of two bacterial species. The first bacterial species which was dominant on the agar plates and formed colorless (transparent) colonies was identified as *Variovorax paradoxus* (previously referred to as *Alcaligenes paradoxus*). The second species which formed larger colonies with a beige or brown color and were present in smaller numbers was identified as *Pseudomonas aeruginosa*. Microbial identification of the culture capable of biodegradation of trans-4MCHCA at 8°C indicated that the dominant bacteria were *Pseudomonas putida*. It was observed through plating of the microbial consortium at 12°C and 4°C that the predominant genera at these temperatures were represented by white, small, uniform colonies similar to those observed in the culture observed at 8°C.

Figure 1 illustrates the results of substrate biodegradation and biomass growth in batch reactors at four selected initial substrate concentrations (50 mg l^{-1}, 250 mg l^{-1}, 500 mg l^{-1}, and 750 mg l^{-1}). In all the cases a direct relationship between the microbial growth and substrate utilization were seen (i.e., the maximum substrate utilization corresponded to the exponential growth phase of the biomass). It can also be observed that the lag phase generally increased with increase in initial concentration of substrate.

Table 1 presents the biodegradation rates observed at different initial concentrations of substrate. In order to calculate the rate of substrate utilization the lag phase was excluded. Included in this table are the calculated values of specific growth rate and biomass yield. The observed specific growth rates ranged between 0.31 day^{-1} for the lowest initial substrate concentration (50 mg l^{-1}) and 0.52 day^{-1} observed at the highest initial concentration (750 mg l^{-1}). The mean value of specific growth rate was 0.45 day^{-1}

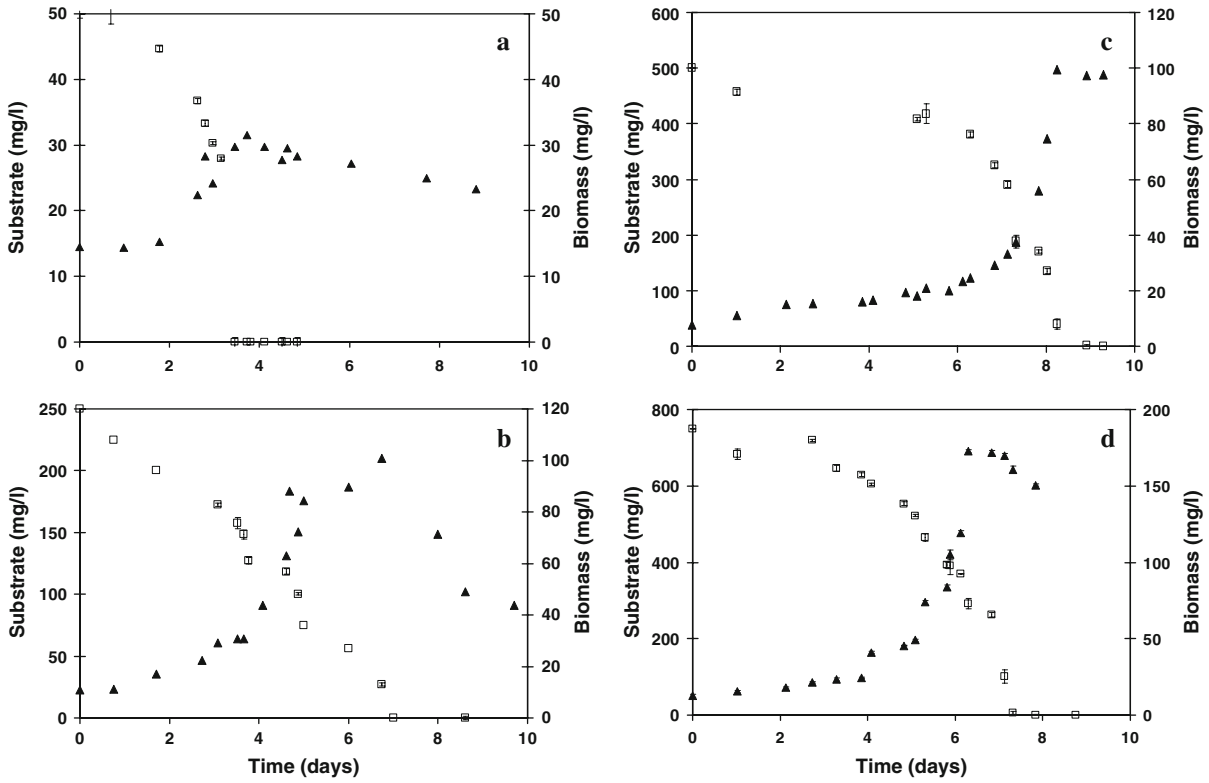

Fig. 1 Substrate biodegradation and biomass growth with time for various initial substrate concentrations and neutral pH (7). (**a**) Initial substrate concentration of 50 mg l^{-1}, (**b**) 250 mg l^{-1}, (**c**) 500 mg l^{-1}, (**d**) 750 mg l^{-1}. (▲), Biomass concentration (mg l^{-1}); (□), Substrate concentration (mg l^{-1}). *Note*: Error bars represent 95% confidence interval and may not be visible in some cases

Table 1 Summary of specific growth rate, biodegradation rate and overall yield determined at various initial substrate concentrations for trans-4MCHCA at room temperature (23°C) and neutral pH conditions (7)

Initial substrate concentration (mg l^{-1})	Average specific growth rate (day^{-1})	Biodegradation rate (mg l^{-1} day^{-1})	Overall yield (mg biomass/mg substrate)
50	0.31	11.0	0.34
100	0.45	27.8	0.28
250	0.51	45.3	0.31
500	0.45	114	0.21
750	0.52	134	0.22

with a 95% confidence interval of 0.08 day^{-1}. The overall yield ranged between 0.21 mg mg^{-1} (at 500 mg l^{-1}) and 0.34 mg mg^{-1} (at 50 mg l^{-1}) with the average value being 0.26 ± 0.07 mg mg^{-1}. The observed biodegradation rates increased proportional to the initial substrate concentration as follows:

$$r_A = 0.192 \times C_{init} \quad (1)$$

where r_A equals the biodegradation rate [mg (l day^{-1})]; C_{init} equals initial substrate concentration [mg (l^{-1})]. Similar biodegradation studies with simpler contaminants have reported maximum specific growth rates which are significantly higher than those determined in the present work (i.e., up to 6 times greater). For example, for phenol and naphthalene the maximum specific growth rate was reported to be 0.09 h^{-1} (2.1 day^{-1}) (Nikakhtari and Hill 2006) and 0.13 h^{-1} (3.1 day^{-1}) (Purwaningsih 2002), respectively. Research on biodegradation of trans-4MCHCA in Athabasca River water (both non-amended and

amended to a pH of 7) resulted in a maximum specific growth rate of 0.07 day^{-1} for an initial substrate concentration of 9 mg l^{-1} (Tanapat 2001). The average maximum specific growth rate observed in this study was 6.4 times higher than the previously reported one. However, the specific growth rates for pure naphthenic acid compounds are not as high as those for simpler organic compounds. Future studies involving environmental or cosubstrate factors may lead to further enhancements in biodegradation rates.

Similar trends in biodegradation performance were observed for various temperatures as represented in Fig. 2 and Table 2. A decreased lag phase was observed in the biomass growth and corresponding substrate utilization upon increasing the temperature from 23°C to 37°C. However, biodegradation was not achieved at 40°C and 42°C. Interestingly, the developed microbial consortium was able to grow and utilize substrate at temperatures as low as 4°C. However, the lag phase did increase considerably with each incremental decrease in temperature (23 to 16, 12, 10, 8, and 4°C). As presented in Table 2, the specific growth rates observed reached the maximum value of 0.45 day^{-1} at room temperature (23°C) which was 5 times greater than the specific growth rate observed at 4°C. Similarly, the maximum values of cell yield and biodegradation rate were observed at room temperature. Cell yield was decreased as temperature increased or decreased above room temperature. Cell yield coefficients ranged from 0.13 (at 37°C) to 0.21 mg of biomass mg^{-1} of substrate (at 23°C). However, the decrease in cell yield between 23°C and 4°C did not exceed the standard deviation ($\sigma = 0.09$) of the mean cell yield (0.20 mg mg^{-1}). The typical reported values for *Pseudomonas* grown with various carbon sources such as glucose, ethanol, and methanol ranges between 0.4 and 0.6 mg mg^{-1}. The yield for *Pseudomonas* in acetate has been reported as

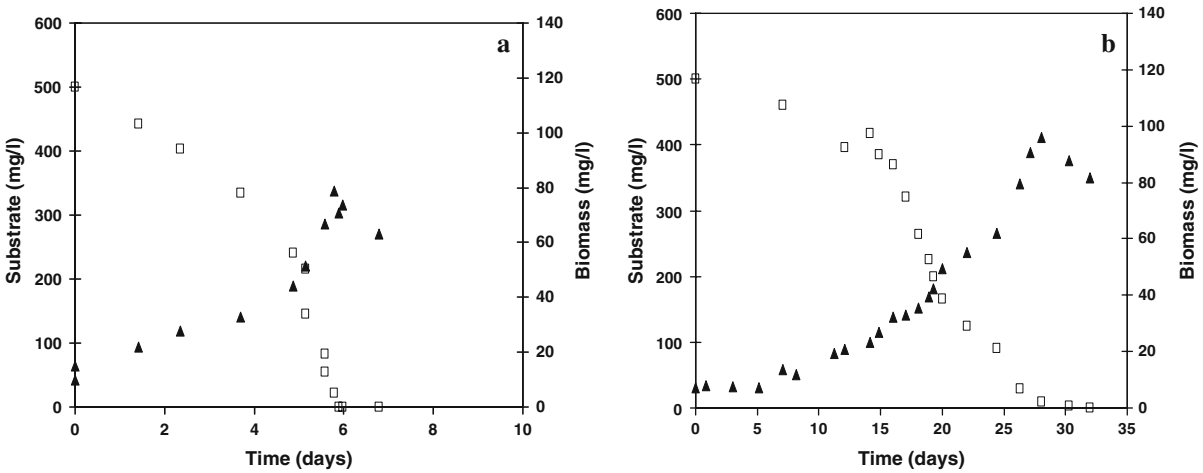

Fig. 2 Substrate biodegradation and biomass growth with time at various temperatures. (**a**) 37°C, (**b**) 4°C. (▲), Biomass concentration (mg l^{-1}); (□), Substrate concentration (mg l^{-1}).

Note: Error bars represent 95% confidence interval and may not be visible in some cases

Table 2 Summary of specific growth rate, biodegradation rate and overall yield determined at various temperatures for initial substrate concentrations of 500 mg l^{-1} and neutral pH (7)

Batch experiment temperature (°C)	Average specific growth rate (day^{-1})	Biodegradation rate (mg l^{-1} day^{-1})	Overall yield (mg biomass/mg substrate)
4	0.09	25.1	0.18
12	0.11	28.1	0.16
16	0.27	21.2	0.16
23	0.45	114	0.21
37	0.29	86.3	0.13

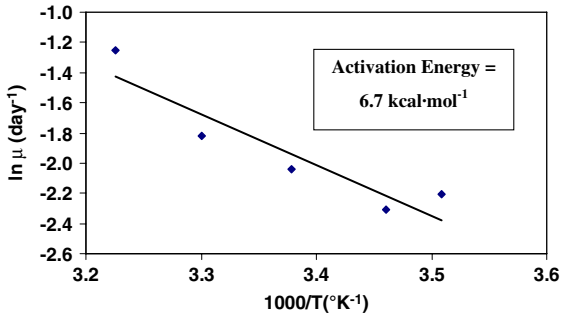

Fig. 3 Arrhenius dependency of specific growth rates (μ) on temperature

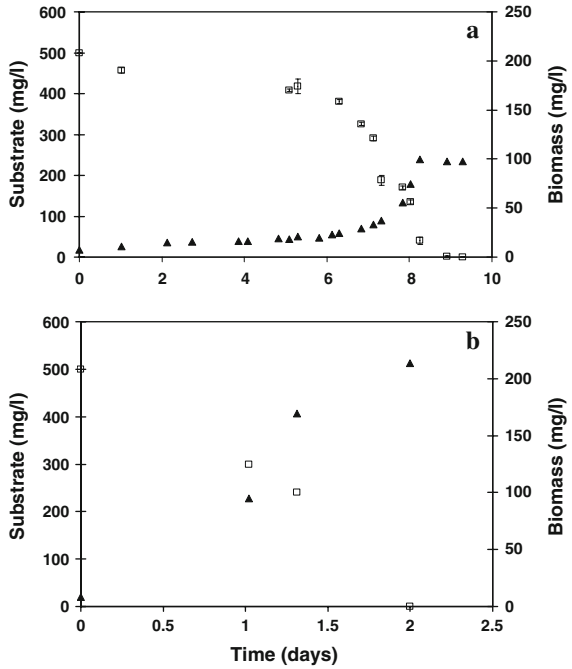

Fig. 4 Substrate biodegradation and biomass growth with time at various pH conditions (initial substrate concentration 500 mg l^{-1}; temperature 23°C). (**a**) pH 7, (**b**) pH 10. (▲), Biomass concentration (mg l^{-1}); (□), Substrate concentration (mg l^{-1}). *Note*: Error bars represent 95% confidence interval and may not be visible in some cases

0.28 mg mg^{-1} (Shuler and Kargi 1992). This value is more comparable with the cell yield coefficients observed with trans-4MCHCA at room temperature.

Figure 3 shows the Arrhenius type dependency of the specific growth rates of trans-4MCHCA on temperature. The activation energy calculated on the basis of the Arrhenius equation was 6.7 kcal - mol^{-1} (28 kJ mol^{-1}). Higher values of activation energy indicate a limitation in the biochemical reaction at low temperatures. Lower values of activation energy are associated with diffusion control. The activation energy of molecular diffusion is approximately 6 kcal mol^{-1} (Shuler and Kargi 1992). Reactions with high activation energies are very temperature-sensitive, while those with low activation energies are relatively temperature insensitive (Levenspiel 1972). Using a different culture than that used here, Tanapat (2001) reported an activation energy of 17 kcal mol^{-1} for biodegradation of trans-4MCHCA. However, the reported activation energy for the cis-isomer of 4MCHCA was 7.4 kcal mol^{-1}.

The effect of pH on the microbial growth and substrate utilization is shown in Fig. 4. The calculated values of specific growth rate, cell yield, and biodegradation rate are presented in Table 3. During these experiments the pH of the culture was adjusted to the designated experimental pH on a daily basis. For the cultures with initial pH values above 8, pH decreased to values in the range 7.7–8.0 which was then readjusted to the designated values. For the culture with an initial pH of the 7.0 the change in pH was not significant (from 7 to 6.8). Biodegradation was successfully achieved within the pH range of 7–11 with a maximum specific growth rate, biodegradation rate and cell yield at pH 10 (see Table 3). Biodegradation did not occur in the culture with a pH of 5.5 which represents the natural pH of a solution of 500 mg l^{-1} of trans-4MCHCA in the medium.

Table 3 Summary of specific growth rates, biodegradation rates and yield determined at various pH conditions for initial substrate concentrations of 500 mg l^{-1} at room temperature (23°C)

Batch experiment pH	Average specific growth rate (day^{-1})	Biodegradation rate (mg l^{-1} day^{-1})	Overall yield (mg biomass/mg substrate)
7	0.45	114	0.21
8.5	0.71	184	0.19
10	1.69	244	0.41
11	0.68	171	0.28

Conclusions

The biokinetics of a single ringed NA compound (trans-4MCHCA) was studied in a batch system in order to determine the rates of biodegradation of trans-4MCHCA and the environmental factors affecting the biodegradation. This study served to develop a viable consortium capable of biodegradation of trans-4MCHCA and to verify the factors influencing the activity of this culture.

The microbial consortium capable of biodegradation of trans-4MCHCA at moderate temperatures (23°C) was dominated by *Variovorax paradoxus*, while *Pseudomonas putida* was the dominant species when biodegradation was carried out at low temperatures (4–12°C). The microbial growth and biodegradation of the pure trans-4MCHCA was achieved at a concentrations as high as 750 mg l^{-1}, with a corresponding maximum specific growth rate of 0.52 day^{-1} and biodegradation rate of 134 mg $(l\ day)^{-1}$. Biodegradation was influenced with temperature ranging between 4°C and 37°C with the highest rate of 114 mg $(l\ day)^{-1}$ achieved at a temperature of 23°C for initial substrate concentrations of 500 mg l^{-1}. The activation energy calculated on the basis of the Arrhenius equation was 6.7 kcal mol^{-1}. Cell yield decreased slightly as temperature increased or decreased from 23°C.

Biodegradation was achieved at pH values in of the range 7–11. The maximum specific growth rate (1.69 day^{-1}), biodegradation rate [244 mg $(l\ day)^{-1}$], and cell yield (0.41 mg mg^{-1}) were obtained at pH 10.

The maximum specific growth rate observed at neutral pH and room temperature in this study (0.52 day^{-1}) was greater than that observed in a previous study of trans-4MCHCA (0.07 day^{-1}). However, this specific growth rate is lower than that reported for simpler organic compounds such as phenol and naphthalene.

The results of the present study indicate that biodegradation of naphthenic acid is influenced by the environmental conditions such as pH, temperature, and concentration of naphthenic acid and that maintaining the condition of biodegradation at the optimal level could lead to enhancement of biodegradation. The selected naphthenic acid compound trans-4MCHCA studied may not be representative of oil sand naphthenic acids as a mixture; however, this study permits the understanding of the principles governing the biodegradation kinetics of this compound which could facilitate future studies aiming at biodegradation of mixtures and/or more complex naphthenic acids.

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