BIOREMEDIATION OF NAPHTHENIC ACIDS IN A CIRCULATING PACKED BED BIOREACTOR

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Chemical Engineering

University of Saskatchewan

Saskatoon

By

Li Yang Huang

Permission to Use

In presenting this thesis in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Chemical Engineering

University of Saskatchewan

Saskatoon, Saskatchewan S7N 5A9

Canada

ABSTRACT

Naphthenic acids (NAs) comprise a complex mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids. NAs are present in wastewaters at petroleum refineries and in the process waters of oil sands extraction plants where they are primarily retained in large tailing ponds in the Athabasca region of Northern Alberta. The toxicity of these waters, primarily caused by NAs, dictates the need for their treatment.

Bioremediation is considered as one of the most cost-effective approaches for the treatment of these wastewaters. *Ex*-situ bioremediation conducted in a bioreactor optimizes the microbial growth and activity by controlling environmental conditions resulting in efficient conversion of the contaminants to less harmful compounds. In this work, a circulating packed bed bioreactor (CPBB), with improved mixing, mass transfer and biomass hold-up has been used to study biodegradation of several model NA compounds: namely *trans*-4-methyl-1-cyclohexane carboxylic acid (*trans*-4MCHCA), a mixture of *cis*- and *trans*- 4-methyl-cyclohexane acetic acid (4MCHAA), and octanoic acid as well co-biodegradation of these naphthenic acids with octanoic acid, using a mixed culture developed in our laboratory. The biodegradation rates achieved for *trans*-4MCHCA in the CPBB are far greater than those reported previously in the literatures. The maximum biodegradation rate of *trans*-4MCHCA observed during batch operation was 43.5 mg/L-h, while a rate of 209 mg/L-h was achieved during continuous operation. Although *cis*-4MCHAA is more resistant to biodegradation when compared with *trans*-4MCHCA, the experimental results obtained from this study indicated both isomers were effectively biodegraded in the CPBB, with the maximum biodegradation rates being as high as 2.25 mg/L-h (*cis*-4MCHAA) and 4.17 mg/L-h (*trans*-4MCHAA) during batch operations and 4.17 mg/L-h

(*cis*-4MCHAA) and 7.80 mg/L-h (*trans*-4MCHAA) during the continuous operation. Optimum temperature for biodegradation of 4MCHAA was determined as 25 °C. Furthermore, the biodegradation rate of single ring NAs (*trans*-4MCHCA and 4MCHAA) were found to be significantly improved through utilization of octanoic acid as a co-substrate. For example, the maximum biodegradation rate of *trans*-4MCHCA obtained during batch operation with the presence of octanoic acid was 112 mg/L-h, which was 2.6 times faster than the maximum value of 43.5 mg/L-h when *trans*-4MCHCA was used as a sole substrate. Similarly, the highest biodegradation rates of *cis*-4MCHAA and *trans*-4MCHAA were 16.7 and 28.4 mg/L-h in the presence of octanoic acid, which were 7.4 and 6.8 times higher than the maximum rates of 2.25 and 4.17 mg/L-h in the absence of octanoic acid.

ACKNOWLEDGMENTS

I would like to give special thanks to Dr. Mehdi Nemati and Dr. Gordon A. Hill for their guidance, expertise, and endless encouragement throughout my graduate program. I am sincerely grateful to my committee members, Dr. Jafar Soltan and Dr. Dae Kun Hwang for their valuable opinions and comments through my research. I am also appreciative of Richard Blondin and Heli Eunike for their invaluable technical assistance and advice.

I would like to express my gratitude to the University of Saskatchewan, Natural Sciences and Engineering Research Council of Canada (NSERC), and National Water Research Institute, Environment Canada, Saskatoon for their finical support for this research project.

Most importantly, thank you to my Mother, Huaguo Cheng, for your support and patience.

DEDICATION

This thesis is dedicated to the loving memory of my father Caihai Huang.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE AND ABBREVIATIONS

Nomenclature

D_d - down comer diameter (cm)

D_r - riser Diameter (cm)

h_d - down comer height (cm)

h_r - riser height (cm)

$r_{trans\text{-}4MCHAA}$ – biodegradation rate of *trans*-4MCHAA (mg/L-h)

$r_{trans\text{-}4MCHAA}$ – biodegradation rate of *trans*-4MCHAA (mg/L-h)

$r_{trans\text{-}4MCHCA}$ – biodegradation rate of *trans*-4MCHCA (mg/L-h)

S_i – initial substrate concentration (mg/L)

V_R - reactor volume (ml)

V_w - volume of free liquid at completion /working volume (ml)

W_b - dry weight of biofilm (g)

Abbreviation

4MCHAA - 4-methylcyclohexane acetic acid

cis-4MCHAA - *cis*-isomer of 4-methylcyclohexane acetic acid

CPBB - circulating packed bed bioreactor

CSTR - continuous stirred tank reactor

ESI - electrospray ionization

FID - flame ionization detection

FTIR - fourier transform infrared

GC - gas chromatography

HDPE - high density polyethylene

HPLC - high-performance liquid chromatography

LSI - liquid secondary ion

MS - mass spectrometry

NA - naphthenic acid

NAs - Naphthenic acids

OD - optical density

QTOF - quantitative quadrupole time of flight

RO - reverse osmosis

trans-4MCHCA - 4 methyl-1-cyclohexane carbpxylic acid

trans-4MCHAA - *trans*-isomer of 4-methylcyclohexane acetic acid

Greek Symbols

η - porosity (unitless)

ρ_{ss} - density of stainless steel (g/cm^3)

1 INTRODUCTION

Canada has a highly sophisticated energy industry and is both an importer and exporter of oil and refined products. In 2008 the average petroleum production was about 438,000 m^3/d (2,750,000 bbl/d), of which 45% was conventional crude oil, 49.5% was bitumen from oil sands, and 5.5% was condensate from natural gas wells (NEB, 2009).Currently, Over 99% of Canadian oil exports are sent to the United States, and it is consistently the top source of U.S. oil imports (NEB, 2009; USEIA, 2011).

Most of Canadian oil sands are located in three major deposits in northern Alberta, namely Athabasca-Wabiskaw , the Cold Lake, and the Peace River oil sands deposits. Alberta has the second largest petroleum reserves in the world, second only to Saudi Arabia. It is estimated that about 27.6 billion m^3 (175 billion barrels) of bitumen exist in the northern oil sands. Currently, industry in Alberta extracts around 236,700 m^3 (1.49 million barrels) of bitumen each day, representing about 76% of the province's total crude oil production, this rate expected to rise to 429,000 m^3 (2.7 million barrels) by 2015 (ERCB, 2011).

The rapid expansion of Canada's oil sands industry requires a sustainable supply of fresh water to meet the water consumption of surface mining operations. Presently, each barrel of oil produced from surface mining consumes 3 barrel of fresh water (Syncrude Canada, 2004; Suncor Energy, 2005; Shell Canada 2005). The produced waste slurry, comprised of sand, clay, water, residual bitumen, organic acids, and polycyclic aromatic hydrocarbon (PAH), is retained in the large tailing ponds in Northern Alberta. In 2003, the estimated volume of tailings in the Athabasca region was approximated 4 x 10^8 m^3 and the total volume is expected to increase to over 1 billion m^3 by 2020 (Paslawski, 2008).

The primary source of toxicity of the liquid wastes in the tailing ponds has been traced to a complex mixture of organic acids, collectively referred to as naphthenic acids (NAs) (Headley et al., 2002a, Quagraine et al., 2005, Paslawski, 2008). NAs occur naturally in oil sands bitumen, and are a complex mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids with general chemical formula $C_nH_{2n+Z}O_2$, where n indicates the carbon number and Z specifies the hydrogen deficiency (Brient et al., 1995; Clemente et al., 2005). During the processing of oil sands produced by surface mining, NAs are released from bitumen through the alkaline hot water extraction process. Since oil sands companies intend to improve the water use efficiency, NAs are concentrated through process water recycling and repeated extraction cycles. Currently, the NAs concentrations in the tailing ponds range from 40 to 120 mg/L (Schramm et al., 2000; Holowenko et al., 2002; Mishra, 2009). Due to Canadian environmental regulations, these waters must be retained on-site to prevent the release of the toxic NAs into the environment (Quagraine et al., 2005, Paslawski 2008). To preserve the fragile Northern ecosystem, development of efficient water treatment technologies aiming to remove NAs has appeared as a major challenge.

Over the past few decades, various methods have been developed to remediate NAs in tailings water such as microfiltration and ultrafiltration, biological treatment, and advanced oxidation (Allen, 2008). Among these methods, bioremediation is considered as one of the most economical methods. However, natural biodegradation is generally slow and can not cope with increasing wastewater production. Therefore, enhancement of natural biodegradation has become the focus of engineers and researchers aiming to address this problem.

On the basis of existing literatures, information regarding the impact of NA structure on the biodegradation rate is limited. Only a few studies have included the information about the

dependency of biodegradation kinetics on the molecular structure of individual NA (Tanapat, 2001; Paslawski, 2008). Therefore, further research on biodegradation of single NA with various structures is needed. Additionally, biodegradation of NAs can be enhanced in an improved bioreactor deign. Further investigations on the *ex-situ* process were necessary in order to evaluate the performance of these novel bioreactors and the influential factors influencing the degradation process.

In this work, using a mixed culture developed in our laboratory and a novel circulating packed bed bioreactor (CPBB), biodegradation of several model NA compounds including *trans*-4-methyl-1-cyclohexane carboxylic acid (*trans*-4MCHCA), *cis*- and *trans*- 4-methyl-cyclohexane acetic acid (4MCHAA), and octanoic acid have been investigated under batch and continuous modes of operation. Effects of NA molecular structure, concentration, and temperature on the performance of the bioreactor and extent of bioremediation have been quantified. Possibility of enhancing the biodegradation rate through co-metabolism of NAs and octanoic acid also has been conducted.

2 LITERATURE REVIEW

2.1 Naphthenic Acids

In the petroleum industry, naphthenic acids (NAs) are commonly referred to as a complex mixture of carboxylic acids present in crude oil. The term NA is derived from the early discovery of monobasic carboxylic acids in petroleum, where these acids are based on a saturated single-ring structure (Brient et al., 1995; Tanapat, 2001). NAs are mainly composed of alkyl-substituted cycloaliphatic carboxylic acids and a small amount of acyclic aliphatic (paraffinic or fatty) acids, with a general chemical formula of $C_nH_{2n+z}O_2$, where n represent the number of carbon atoms, and *Z* specifies the hydrogen deficiency and usually has a zero or negative value (Brient et al., 1995; Clemente et al., 2005). Generally, the structure of NAs becomes more compact due to the loss of hydrogen atoms (Holowenko et al., 2001; Clemente et al., 2005). For example, NAs can be saturated, acyclic acids with Z equals to 0, monocyclic acids (Z= -2), bicyclic acids (Z= -4), or tricyclic acids (Z= -6) etc. NAs mixture consists mainly of monocyclic acids as the carbon number in the range of 7 to 12 (n =7 to 12), and multicyclic acids become predominate as carbon number increased above 12 (n >12) (Brient et al., 1995). Based on the current reports it seems that NAs with Z= -4 predominate in Athabasca oil sands tailings ponds waste waters of Suncor and Syncrude (McMartin, 2003; Headley et al., 2004; Mishra, 2009). Additionally, as shown in Figure 2.1, the carboxyl group (-COOH) is attached to aliphatic side chain ($-CH_2$) and alkyl group (-R) is directly attached to the cycloaliphatic ring. The molecular weight of NAs at various Z series and carbon number (n) is also shown in Table 2.1.

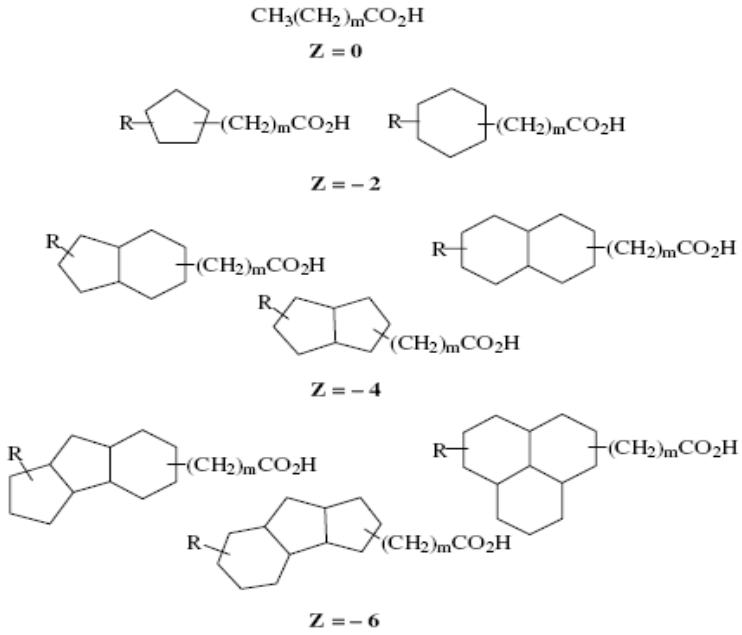


Figure 2.1: Structures of the homologues of naphthenic acids, where *Z* represents hydrogen deficiency, R is an alkyl chain, and m indicates the number of CH_2 units (Clemente et al., 2005)

Table 2.1: Molecular weight (M.W.) of naphthenic acids at various Z series and carbon number (n) (CEATAG, 1998; McMartin, 2003)

Number of Carbon Atoms	M.W. Z=0 (liner chain)	M.W. Z=-2(1 ring)	M.W. Z=-4(2 rings)	M.W. Z=-6 (3 rings)
10	172	170	168	166
11	186	184	182	180
12	200	198	196	194
13	214	212	210	208
14	228	226	224	222
15	242	240	238	236
16	256	254	252	250
17	270	268	266	264
18	284	282	280	278
19	298	296	294	292
20	312	310	308	306

Commercial NA preparations are extracted from petroleum distillates, categorized by acid number, impurity level, and color, and commonly used to produce metal salts such as copper naphthenate, which are largely consumed in the wood preservation industry. Other uses

of NAs include oil lubricants, fuel additives, paint dryers, and manufacturing of tires (Deineko et. al., 1994; Brient et al. 1995; Paslawski 2008).

2.2 Physical and Chemical Properties of NAs

Naphthenic acids (NAs) are viscous liquids and generally are non-volatile, chemically stable, and toxic compounds. Their colors range from pale yellow to dark amber. Their characteristic odour develops upon storage of refined acids and is mainly attributed by the presence of phenolic and sulphur impurities in the mixture. NAs mixture is slightly soluble in water, but soluble in certain organic solvents. Additionally, the boiling points of NAs range from 250 to 350 ºC (Brient et al. 1995).

Chemically, NAs behave as carboxylic acids such as long chain fatty acids (Brient et al. 1995) and the associated pH correlates with their solubility (Headley et al., 2002a; McMartin, 2003). Their acidity are slightly weaker than some lower molecular weight carboxylic acids like acetic acid (pH=4.76), but stronger than phenol (pH = 9.95). NA dissociation constants are in the range 10^{-5} to 10^{-6}. Additionally, due to their acidic nature, naphthenic acid corrosion has been a problem in the petroleum refining operations since the early 1900s. On the other hand, NA derivatives have been used as corrosion inhibitors for the purpose of protection of refining units (Brient et al., 1995). A summary of general physical and chemical characteristics of NAs that can describe the overall mixture is shown in Table 2.2.

Table 2.2: Summary of physical and chemical properties of naphthenic acid (NAs) mixture (Brient et al. 1995; Herman et al., 1993; Headly et al., 2002a; McMartin, 2003).

Parameter	General Characteristics
Colour	Pale yellow, dark amber, yellowish brown , black
Odour	Primarily imparted by the presence of phenol and sulphur impurities, musty hydrocarbon odour
State	Viscous liquid
Molecular Weight	Generally between 140 and 450 amu
Solubility	<50 mg/L at pH7 in water, Completely soluble in organic solvents
pKa	Between 5 and 6
Boiling Point	Range between 250 to 350 ºC

2.3 Occurrence and Toxicity of Naphthenic Acids

NAs are oxidative products of petroleum hydrocarbons, which occur naturally in crude oil and/or bituminous oil sands. In Athabasca oil sands, the carboxylic acids (particularly NAs) content is approximately 2% (Strausz 1988; CEATAG, 1998; Quagraine et al., 2005). The corrosive nature of NAs, especially at high temperature conditions (>230 °C), can potentially affect the safety and reliability of oil refining processes. Additionally, their presence in crude oil normally increases the oil acidity (total acid number), which reduces the commercial value of the petroleum product. Therefore, Canadian oil-processing companies tend to separate NAs from the crude bitumen during the extraction process before the upgrading and refining stages (Brient et. al., 1995; Clemente et al., 2005; Quagraine et al., 2005).

Currently, the Clarke caustic hot water extraction (79-93 °C) process has been widely adopted in processing of oil sands to separate bitumen from oil sands ore. Through this procedure, the viscosity of bitumen is reduced which favors the subsequent refining and upgrading processes. However, hot water extraction causes the transfer of NAs into the water fraction as naphthenates (Quagraine et al, 2005).Currently, both Syncrude Canada Ltd and

Suncor Energy Inc, involved with surface mining activities in Northern Alberta, use this procedure to extract bitumen from oil sands ore (Rogers et.al., 2002; Quagraine et al, 2005).

A large amount of water is consumed by this process which results in the generation of NAs contaminated wastewaters. It is reported that each cubic meter of mined oil sands requires up to 3 m^3 of water and produces about 4 m^3 of slurry wastes (Holowenko et al., 2002; Quagraine et al., 2005). At present, 117 million tonnes of oil sands ore are mined annually by Syncrude Canada Ltd at the Aurora North Site (Syncrude Canada, 2011). Typically, 200 mg of NAs are produced per kg of processed oil sands (Clemente et. al., 2005; Mishra, 2009). Thus, there is a potential to release 23,400 tonnes of NAs from the ore into oil sands process water (OSPW) every year.

The produced waste slurry from the oil sands extraction process is mainly comprised of sands, clay, water, unrecovered bitumen, and dissolved inorganic and organic compounds (MacKinnon 1989; Schramm et al., 2000). Due to a "zero discharge" policy, these wastes have been contained on-site, primarily in lager settling ponds. In 2003, it was estimated that about 4×10^8 m^3 of process-affected water was retained in the Athabasca region, and the total volume is expected to increase to over 1 billion m^3 by 2020 (Lo et al.,2003; Quagraine et al., 2005). Through the recycling and reuse of tailing waters, the NAs concentrations in the tailings ponds are typically in the range 40 -120 mg/L (Schramm et al., 2000; Holowenko et al., 2002; Mishra, 2009).

Naphthenic acids are acutely and chronically toxic to aquatic biota, and their toxic nature is associated with their surfactant characteristics (Dokholyan et al., 1983; Rogers et al., 2002[a]; Quagraine et al, 2005). The source of toxicity of the tailing ponds water has been traced to a complex mixture of organic acids, which is believed to be NAs (Mackinnon et al., 1986;

Schramm et al., 2000; Quagraine et al., 2005). Due to the complexity of NAs mixtures and difficulty under current technology in analyzing and isolating individual NA compound within the mixtures, the principal toxic members in the NAs mixtures still remains unknown (Quagraine et.al., 2005) . Rogers et al. (2002b) reported that the most toxic contaminants in the tailing ponds waters are those NAs with low molecular weights. Additionally, Holowenko et al, (2002) reported that the toxicity decreases by the increases the number of cycloaliphatic rings (Holowenko et al., 2002; Mishra, 2009). Considering that the NA fractions with multiple rings and higher molecular weights are less toxic and that lower molecular weight NAs are more bioavailable in Nature, one can conclude that toxicity of oil sand process water decreases with aging (Holowenko et al., 2002; Frank et al., 2008).

Determination of the total concentration of naphthenic acids is not sufficient to explain the toxic effects, many research results demonstrate that the toxicity eventually can be related back to the concentration of individual NAs (Holowenko et al., 2002; McMartin, 2003; Paslawski, 2008;). The current NA concentrations in the tailing ponds are in the range of 40-120 mg/L and expected to increase (Schramm et al., 2000; Holowenko et al., 2002; Mishra, 2009). When the oil sands operations cease (in about 50 years) or even during the processing, the disturbed land and tailing ponds wastewaters will have to be reclaimed, which means the concentration of NAs needs to be reduced to a safe level.

The petroleum industry intends to reduce the toxicity of the mine tailings wastes, partially by natural biodegradation. However, the rate of natural biodegradation is generally slow and not sufficient to cope with the increasing wastewater production. Therefore, enhancing the biodegradation rate has become a challenge and the focal point for engineers and researchers dealing with the treatment of oil sand tailings.

2.4 Treatment Methods for Naphthenic Aicds Contaminated Water

Over the past few decades, various methods have been developed to treat NAs contaminated oil sands process water (OSPW). These methods include chemical oxidation (ozonation), adsorption of NAs on activated carbon, membrane filtration (ultrafiltration), and bioremediation (Allan, 2008). Among these methods, bioremediation is considered as one of the most cost-effective and environmental friendly methods (Scott et al., 2008). Chemical oxidation, photocatalysis, and bioremediation as the most studied NAs treatment technologies will be briefly discussed in this section.

2.4.1 Chemical Treatment

Chemical treatment typically refers to use of chemical agents to destroy or to convert the contaminants to less toxic compounds (Gore, 2006). Chemical treatment has a short reaction time, but disadvantages in high operating costs and the potential causing "secondary contamination" through producing hazardous by-products. Chemical oxidation of wastewater has been used for decades, and has been proven to be effective in removing NAs from OSPW.

Chemical oxidation processes degrade pollutants through a series of ionic or radical reactions, where the oxidant compound either accepts electrons or donates an electron-accepting group (Allen, 2008). Common oxidants used in wastewater treatment include chlorine (Cl_2), hydrogen peroxide (H_2O_2), ozone (O_3), and permanganate (MnO_4^-) (Singer et al., 1999; Allen, 2008). Chemical oxidation is normally applied to the more persistent and recalcitrant pollutants that are not amenable to biological treatment.

Ozonation (O_3) has been widely investigated for the removal of NAs. Scott et al. (2008) demonstrated that ozonation of sediment-free oil sands process wastewater for 50 minutes led to

a non-toxic effluent and decreased the NAs concentration by approximately 70%. After 130 min of ozonation, the NAs concentration was reduced to 2 mg/L from the initial 59 mg/L. These results show ozonation is superior to biodegradation in term of rate of degradation as well the possibility of removing NAs by using powerful chemical agents. However, relatively high costs of generating ozone would be an important factor which should be considered when evaluating whether ozonation could be incorporated as part of a feasible petroleum wastewater management strategy (Scott et al., 2008).

2.4.2 Photocatalysis

Photocatalysis is one of the most promising alternatives for wastewater treatment (Doll et al., 2005). Photocatalysis is a photochemical process where the pollutants are oxidized by the radicals produced through the photo-excitation of a valence electron on the surface of a catalyst (Bahnemann, 2004). Titanium dioxide (TiO_2) is the most common catalyst used in the photocatalytic process due to its activity, non-toxic characteristic, and stability in aqueous environments (Hsien et al., 2000; Mishra, 2009). Titanium dioxide generates electron-hole pairs when it is subjected to radiation exceeding the materials' band gap. The energetic electrons and the holes carrying the positive charges in the valence band are highly reactive, which facilitate the reduction and/or oxidation reactions of adsorbed molecules on TiO_2 surfaces (Fujishima et al., 2008).

Protosawicki et al. (2002) reported that the suitable ultraviolet wavelengths for most waste water treatments are the UV-B (280 to 315 nm) and UV-C (200 to 280 nm) sub ranges and UV_{254} radiation has the most potential for remediation of naphthenic acids (Dutta et al.,

2000; McMartin et al., 2004). Normally, photocatalyst particles are either immersed in the contaminated water as slurry or fixed as a bed in a proper reactor (Devipriya et al., 2005).

Many lab-scale studies have reported the successful photocatalytic oxidation of aliphatic and aromatic carboxylic acids, including naphthenic acids. For example, McMartin (2003) reported that the use of photolysis as a pre-treatment of NAs prior to the biological treatment increases the bioavailability of NAs to microorganisms, which is beneficial for the latter biodegradation stage. Headley et al. (2009) studied the photocatalytic oxidation of commercial Fluka NAs mixture and a model NA (4-methyl-cyclohexane acetic acid, 4MCHAA) and reported that under natural sunlight irradiation over the TiO_2 suspension, 75% of NAs mixture (64 mg/L) and 100% of 4MCHAA (1.5 mg/L) were degraded within 8 hrs. However, no degradation occurred under dark conditions, regardless of presence or absence of TiO_2.

Photocatalytic oxidation of NAs contaminated OSPW has been investigated. However, this area of research is not well developed as others. One of the key disadvantages of this technology is that photocatalytic degradation rates are strongly dependent on the efficiency of adsorption of pollutants on the catalysts. The presences of certain ionic compounds such as chloride (Cl^-), or bicarbonate (HCO_3^-) in the OSPW may compete for the adsorption site, which reduces the activity of catalyst with respect to NAs (Bessa et al., 1999). Therefore, commercial application of photocatalytic processes requires a source of an inexpensive and highly active photocatalyst to be competitive with the conventional and well established water treatment processes (Freudenhammer et al., 1997; Bahnemann, 2004; Allen, 2008).

2.4.3 Bioremediation

Bioremediation is a treatment process relying on the use of microorganisms to remove organic pollutants from the biosphere to minimize the unwanted environmental impacts (Prince, 2009). As a cleanup technology, it has been successfully used to treat contaminated air, water, soils and sediments. Compared to conventional treatment methods, bioremediation is low cost and could be considered as a permanent solution since in most cases the contaminants can be completely destroyed or metabolized by the microorganisms.

Along with rapid expansion of the oil sands industry in Northern Alberta, Canada, interests in the application of bioremediation technology to eliminate petroleum pollutants has became intensified in recent years. The bioremediation process can be carried out either *in-situ* or *ex-situ*. *Ex*-situ bioremediation conducted in a bioreactor optimizes the microbial growth and activity by controlling the environmental conditions resulting in efficient conversion of the contaminants to less harmful compounds.

Preliminary analyses have shown that indigenous microorganisms found in the local tailing ponds are able to effectively metabolize commercially available surrogate NAs (Biryukova et al., 2007). However, NAs extracted from the local tailing ponds are observed to be degraded at a slow rate (Tanapat, 2001; Quagraine et al, 2005). Published works have revealed the effect of chemical structures of NAs on the biodegradation. For example, Tanapat (2001) demonstrated the significant impacts of chemical structure on biodegradation rate by studying several commercially available NAs isomers including *cis*- and *trans*- isomer of 4-methylcyclohexane acetic acid, 4-methyl-1-cyclohexane carboxylic acid, and 3-methylcyclohexane carboxylic acids. NAs extracted from the oil sands tailing wastes have much

greater structural complexity than commercial compounds, which may partially explain their greater resistances to the natural biodegradation.

Quail et al. (1991) demonstrated that the rate of biodegradation can be greatly improved by treating the pollutants using optimum environmental conditions and a better designed and controlled bioreactor. Biodegradation of NAs in laboratory studies provides rapid degradation compared to *in-situ* approaches through optimizing the environmental conditions to promote the growth and activity of the microorganisms (Mandelstam et al. 1968; Tanapat 2001; Paslawski 2008). For example, Mandelstam et al. (1968) reported that the rate of biodegradation of NAs was greatly enhanced in the temperature range of 30 ˚C to 37 ˚C compared to lower temperatures. Paslawski (2008) investigated the enhancement of biodegradation of model NAs (*trans*- 4-methyl-1-cyclohexane carboxylic acid) and reported that the biodegradation rate can be significantly improved by varying the environmental conditions (temperature and pH) and reactor configuration. Overall, the factors that may potentially affect the rate of NAs biodegradation include salinity, temperature, nutrient availability, pH, dissolved oxygen and bioreactor design (Shuler et al., 2002; Quagraine et al., 2005; Paslawski, 2008).

2.5 Bioreactors

The bioremediation of contaminated waters is typically carried out either *in- situ* or *ex-situ*. *In-situ* process is generally slow and can not be effectively controlled while the *ex-situ* process has proved to efficiently accelerate the biodegradation rate by treating the contaminants in a controlled environment (i.e. bioreactor). A wide variety of bioreactors have been invented over the past few decades. Three representative bioreactors will be discussed in this section, including bioreactors with internal mechanical agitation such as a stirred-tank bioreactor, bubble column

where the agitation is achieved by gas sparging, and loop reactor, in which circulation is induced by the motion of injected air/gas or by a mechanical pump such as circulating packed bed bioreactor.

2.5.1 Stirred-tank Bioreactors

The stirred tank bioreactor, equipped with a rotating impeller and baffles, is the most common type of aerobic bioreactor in use today. The main virtues of such a bioreactor are its ability to provide a high K_La (volumetric mass-transfer coefficient) value for gas transfer and for being extremely adaptable to a wide range of conditions (Shuler et al., 2002). The air can be supplied up to ~1.5 vvm (volume of air/min per unit volume of fluid), which satisfies most aerobic bioreactions (Nienow, 2000). The customized internal configuration can provide a specific circulation pattern and good bulk mixing. The Rushton impeller, a disc with typically 6 to 8 blades with the diameter approximate 30 % to 40% of the tank diameter, are commercially used to improve the hydrodynamics and ensure the efficient mixing of liquids with viscosities up to 2000 centipoise (Shuler et al., 2002).

However, stirred bioreactors often encounter foaming problems, which makes the exhaust filters wet, increases the pressure drop, reduces the air flow, and provides alternative pathways for contaminating cells to enter the bioreactor (Shuler et al., 2002). Furthermore, the presence of mechanical agitation may not be suitable for some shear sensitive cells. Stainless steel is the standard material of construction of stirred tanks while glass is commonly used at the laboratory scale. Moreover, most stirred tank bioreactors are built with a height-to-diameter ratio of 2 to 3 (Shuler et al., 2002). Under aeration conditions, the exhaust gas is discharged from the top of the tank. A schematic diagram of stirred-tank bioreactor is showed in Figure2.2.

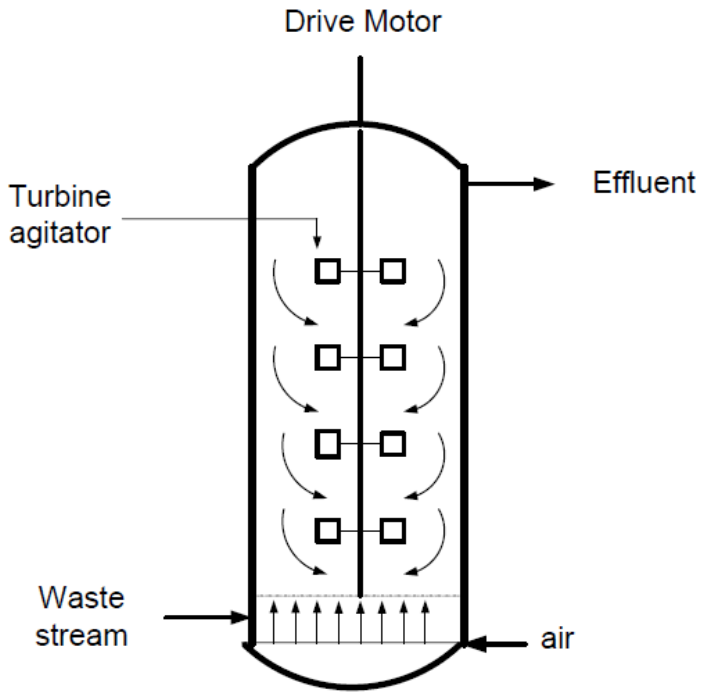


Figure 2.2: Schematic diagram of stirred-tank bioreactor

Stirred-tank reactors can be run in either batch or continuous mode. Under continuous mode, the substrate is continuously fed into the system while the product is continuously or semi-continuously withdrawn from the reactor. Paslawski et al. (2009) used a continuous stirred-tank reactor to study the kinetics of biodegradation of a model NA (trans-4-methyl-1-cyclohexane carboxylic acid) and achieved a maximum biodegradation rate of 9.6 mg/L-h.

2.5.2 Bubble Column Bioreactors

The bubble column or airlift bioreactor is simply an air driven system, in which the liquid is partially mixed by dispersed bubbles due to the injection of air from a sparger located at the bottom of the column. Compared to other conventional bioreactors, the bubble column is clearly

the simplest type to construct and encompasses several advantages including: 1) extremely suitable for low-viscosity Newtonian broths; 2) simple design with no moving parts or agitator shaft, which reduces the potential for contamination; 3) lower shear rate, which provides a suitable environment for shear- sensitive cell growth such as animal cells. 4) higher energy efficiency, it greatly reduces energy consumption due to the absence of mechanical agitation (Nienow, 2000; Shuler et al., 2002; Williams, 2002;).

However, there are also some associated disadvantages. For example, there is poor capability for handling highly viscous broths, and inadequate mixing at low air flow rates (Shuler et al., 2002). Besides having less vigorous mixing capabilities than stirred-tank bioreactors, bubble column operation is also limited by foaming and bubble coalescences. It is understood that the range of appropriate gas flow varies with the nature of the broth. However, due to bubble coalescence, bubble column works over a rather narrow range of gas flow rate. Thus, less sufficient gas dispersion in the column is likely to occur that reduces the K_La (volumetric mass-transfer coefficient) values for gas transfer (Shuler et al., 2002). This can be a major concern for an aerobic biological process. The schematic diagram of bubble column is shown in Figure 2.3.

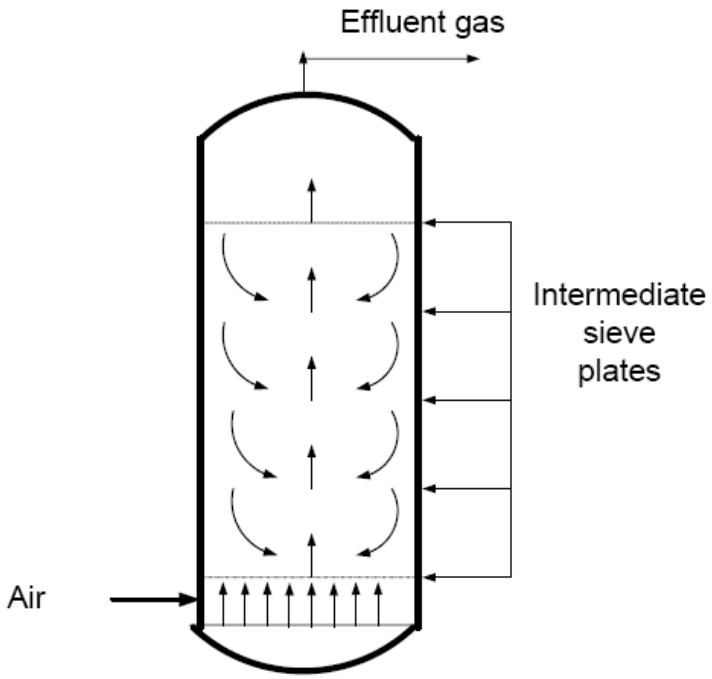


Figure 2.3: Schematic diagram of bubble column

2.5.3 Circulating Packed Bed Bioreactors

Air-lift loop reactors have characteristics between those of bubble columns and stirred tanks. Based on the pattern of liquid circulation in the reactor, they can be categorized into either internal (draught) or external (loop) type. The design of airlift-loop bioreactors is similar to bubble columns with an additional draft tube devised either inside of the column or external loop (down comer) attached to the column, which is used to control the circulation of air and medium (Shuler et al., 2002). Compared to bubble columns, loop reactors can handle somewhat more viscous fluids and create a more homogeneous environment, especially in continuous mode of operation (Nienow, 2000; Nikakhtari, 2005).

In an air-lift loop system, gas disengages from the liquid at the top and the degassed liquid (denser than gassed liquid) descends through a draft tube or down comer. At the bottom of the reactor, the descending fluid again encounters the gas stream and is carried back up to the top to accomplish the circulation. Therefore, coalescence is not so much of a problem in the loop reactor due to the presence of internal or external circulation (Shuler et al., 2002). Additionally, it was found that the characteristics of coalescing and non-Newtonian fluids in external loop are distinctly different from internal loop airlift reactors (Nienow, 2000). In other words, the difference of the two-phase flow pattern in the two types of loop reactors may lead to different efficiency of oxygen mass transfer rates. Other advantages associated with airlift-loop reactors include: 1) low friction with an optimal hydraulic diameter for both the riser and downcomer; 2) energy efficiency as no pump or impellers are required for circulating and mixing, which reduces the operating costs, 3) effective control of heat and mass transfer (Shuler et al., 2002; Williams, 2002). Schematic diagrams of loop-airlift bioreactors a) draught tube and b) external loop are shown in Figure 2.4.

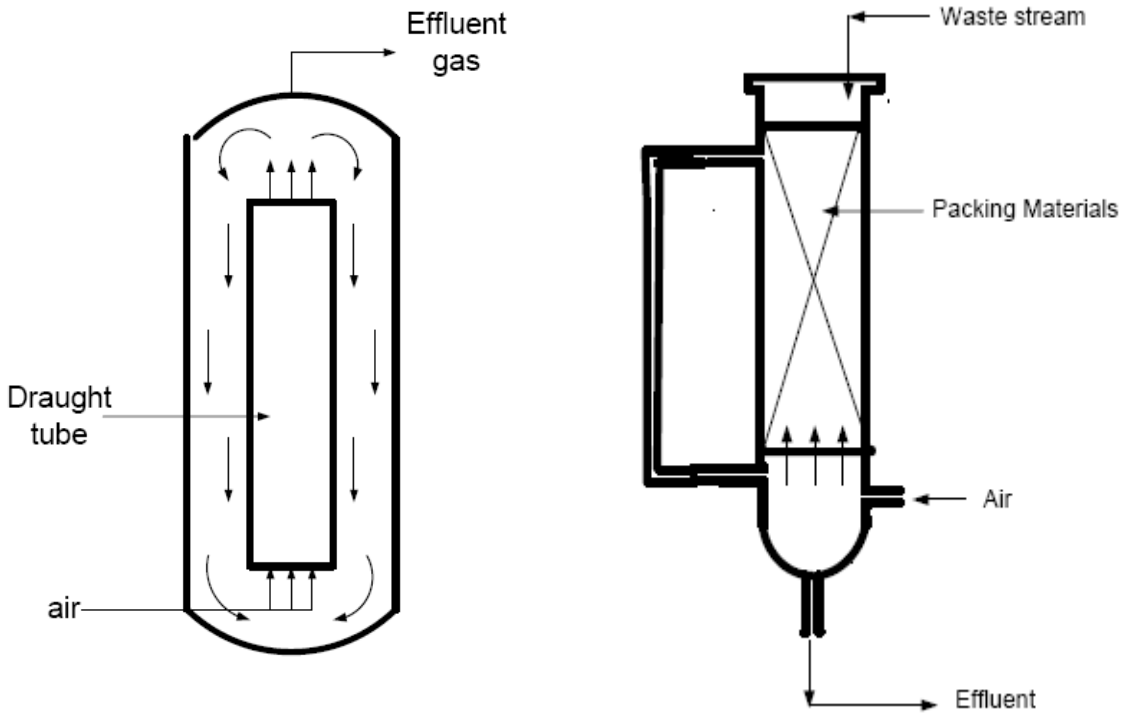


Figure 2.4: Schematic diagrams of loop airlift bioreactor: (a) draught tube, (b) external loop

The circulating packed bed bioreactor (CPBB) is a novel bioreactor configuration. It is a modified external loop airlift bioreactor (ELAB) in which packing material is placed in the riser section. For aerobic biodegradation, microorganisms are able to aggressively degrade the water soluble toxic organic with sufficient oxygen present in the environment. Meng et al. (2002a) reported that including the packing in an external loop airlift bioreactor increases gas-hold up, reduces bubble size, and decreases liquid circulating rate, which all contributed to the significant improvement in oxygen mass transfer rates in the reactor. A wide variety of packing materials, including nylon, HDPE (high density polyethylene), crushed glass, stainless steel mesh, porcelain and acrylic Raschig rings, has been investigated to improve oxygen mass transfer rates.

Nikakhtari (2005) reported that the oxygen mass transfer coefficient can be improved by an average factor of 2.45 using stainless steel mesh packing (porosity of ~0.90) in the ELAB.

The packing inside of the ELAB not only enhances the oxygen mass transfer but also provides a solid support surface for cell immobilization that improves the biomass hold-up in the bioreactor. Paslawski et al. (2009) used a stainless steel coiled mesh in a bubble column to enhance the performance of biodegradation of a model NA and reported biodegradation rate increases up to 95 times of that in a system with freely suspended cells. The schematic diagram of a modified ELAB, a circulating packed bed bioreactor, is shown in Figure 2.5.

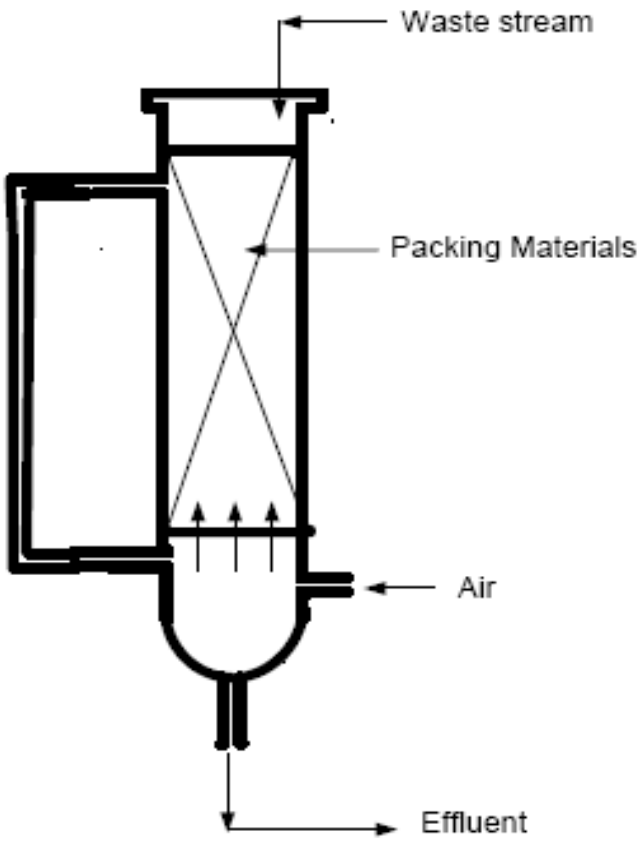


Figure 2.5: Schematic diagram of the circulating packed bed bioreactor.

3 RESEARCH OBJECTIVES

A review of the available literature reveals that the majority of earlier works have focused on the bioremediation of the whole NAs mixture with very limited success. Biodegradation studies of individual NA are limited. Additionally, biodegradation can be greatly improved by treating the pollutants in a well designed and controlled bioreactor (Quail et al., 1991), where the microbial growth and activity are optimized by controlling the environmental conditions resulting in efficient conversion of the contaminants to less harmful compounds. Therefore, further investigation on the *ex-situ* process is necessary to enhance the biodegradation of NAs. The specific objectives of this work are:

1. Biodegradation studies of several representative NAs (*trans*-4-methyl-1-cyclohexane carboxylic acid (*trans*-4MCHCA), 4-methyl-cyclohexane acetic acid (*cis*- and *trans*-4MCHAA), and octanoic acid under batch and continuous modes of operation to assess the impact of structure and to evaluate the relevant biodegradation kinetics.
2. Comparing the biodegradation of model NAs using the novel circulating packed bed bioreactor (CPBB) in respect to those obtained in the conventional batch reactor, continuous stirred tank reactor (CSTR), and the packed-bed bioreactor.
3. Investigation of temperature effects on the rate of biodegradation of 4-methyl-cyclohexane acetic acid (4MCHAA).
4. Evaluation of the potential for enhanced biodegradation of ring-structural NAs (*trans*-4MCHCA and 4MCHAA) through co-metabolism of NAs with octanoic acid as a co-substrate.

4 MATERIAL AND METHODS

4.1 Selection of Model Naphthenic Acids

Based on the current literatures, naphthenic acids (NAs) used in laboratory investigations to assess the biodegradation potential are generally classified into three categories: surrogate naphthenic acids which is individual (pure) naphthenic acid fitting the formula C_nH2_{n+Z} O_2, commercially available mixture of NAs (i.e. Fluka or Kodak), and NAs extracted from the oil sands tailing ponds water (Clemente et al., 2005).

Based on the commercial availability and variation in structure, *trans*-4-methyl-1-cyclohexane carboxylic acid (referred to herein *trans*-4MCHCA, CAS NO. 13064-83-0), 4-methylcyclohexane-acetic acid (mixture of *cis*- & *trans*- isomers, referred to herein 4MCHAA, CAS NO. 6603-71-0), and octanoic acid (CAS NO. 124-07-2) were selected as model compounds to evaluate the kinetics of biodegradation in the designed circulating packed bed bioreactor (CPBB). All model NA compounds used in the study were purchased from Sigma-Aldrich Co. (~97 % purity).

At room temperature, *trans*-4MCHCA appears as a white crystalline solid, 4MCHAA appears as a white solid with a waxy texture, and octanoic acid is a clear, colorless to slightly yellow, oily liquid. The biodegradation of candidate NAs have been previously studied in a shake flask, CSTR, and packed-bed bioreactor with no circulation (Tanapat 2001; Paslawski, 2008). The molecular structure of *trans*-4MCHCA, 4MCHAA, and octanoic acid are shown in Figures 4.1, 4.2 and 4.3, respectively.

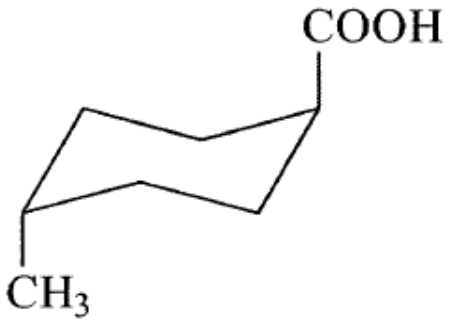


Figure 4.1: Molecular structure of *trans*-4-methyl-1-cyclohexane carboxylic acid, *trans*-4MCHCA (Sigma Aldrich Co., 2009)

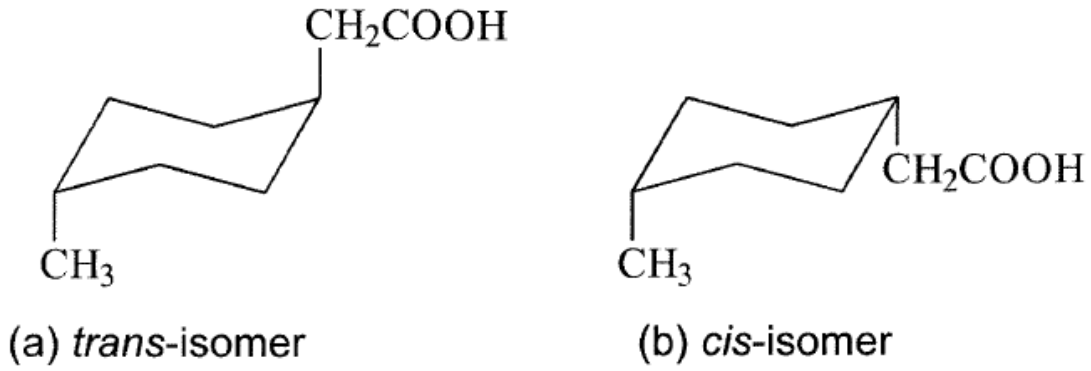


Figure 4.2: Molecular structure of *trans- isomer* (a) and *cis*- isomer (b) of 4-methylcyclohexane-acetic acid, 4MCHAA (Sigma Aldrich Co., 2009)

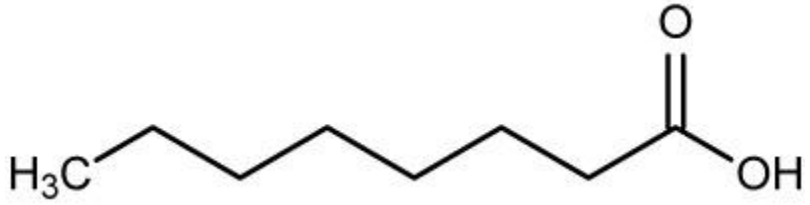


Figure 4.3: Molecular structure of octanoic acid (Sigma Aldrich Co., 2009)

4.2 Microbial Cultures and Medium

4.2.1 Microbial Consortium

The indigenous microorganisms isolated from a contaminated tailing pond soil of an industrial site were initially grown and maintained in modified McKinney's medium using Fluka commercial NAs (Sigma-Aldrich , CAS No. 1338-24-5) as substrate (Paslawski, 2008). This culture was then inoculated (10% v/v) into McKinney's medium containing 100 mg/L of *trans*-4MCHCA. All cultures were maintained in shake flasks at 25 °C and used as inocula for batch and continuous runs carried out throughout this study.

The dominant species of the developed microbial consortium was identified by growing the culture on aseptic agar. The agar mixture was prepared by dissolving 3g of Difco® Bacto agar, 3g Difco® Bacto tryptose phosphate, and 250mg of *trans*-4MCHCA in 100 ml of McKinney's medium. This prepared mixture was then sterilized and poured into Petri dishes. After streaking the microbial culture on agar plates, they were placed in an incubator at 32 °C. The bacterial growth became visible in 2-3 days. Microbial identification had been conducted at a commercial laboratory (EPCOR-Quality Assurance Lab, Edmonton, Canada) and indicated that the consortium was comprised of two bacterial species, which were *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans xylosoxidans (Alcaligenes).*

Pseudomonas aeruginosa is a well-known microorganism due to its capability to metabolize recalcitrant chemicals and it is commonly used to treat persistent environmental contaminants. Additionally, *Alcaligenes has* been shown to effectively degrade single NA compounds (Paslawski, 2008).

4.2.2 Medium

McKinney's modified medium containing essential mineral nutrients to support the growth and activity of microorganisms was used for the purpose of candidate NAs biodegradation throughout this study. The medium composition was selected on the basis of earlier works (Paslawski, 2008; Hill, 1974). The medium was prepared in 2 L batches of reverse osmosis (RO) water and had the following composition: KH_2PO_4 (840 mg/L); K_2HPO_4 (750 mg/L); $(NH_4)_2SO_4$ (474 mg/L); NaCl (60 mg/L); $CaCl_2$ (60 mg/L); $MgSO_4 \cdot 7H_2O$ (60 mg/L); $Fe(NH_4)_2(SO4)_2 \cdot 6H_2O$ (20 mg/L).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); $CoCl_3$ (400 mg/L); $ZnSO_4 \cdot 7H_2O$ (200 mg/L); $MnCl_2$ (60 mg/L); $NaMoO_4 \cdot 2H_2O$ (60 mg/L); $NiCl_2$ (40 mg/L); and $CuCl_2$ (20 mg/L).

4.3 Experimental Systems for Biodegradation Study

4.3.1 Specification of Circulating Packed-bed Bioreactor (CPBB)

Batch and continuous experiments were carried out in two circulating packed-bed bioreactors. Both CPBBs were constructed of clear glass. The specifications of 1st and 2nd CPBB are listed in Table 4.1. Each CPBB had inlet and outlet ports which allowed batch and continuous operation of the bioreactor. Stainless steel mesh was used as carrier matrix for establishment of biofilm. The steel packing material used in CPBBs had a porosity of ~80% (fresh packing). The representative photograph of the CPBB prior to experiments is presented in Figure 4.4.

Table 4.1: Specifications of 1st and 2nd circulating packed bed bioreactor (CPBB)

Parameter	1st CPBB	2nd CPBB
Riser height, h_r (cm)	35.0	35.0
Riser Diameter, D_r (cm)	4.5	4.1
Down comer height, h_d(cm)	32.0	32.0
Down comer diameter, D_d (cm)	0.5	0.5
Porosity, η (unitless)	0.801	0.802
Volume of free liquid at completion / working volume, V_w (ml)	450	375
Reactor volume (without packing), V_R (ml)	562	468

Figure 4.4: The representative photograph of the bioreactor prior to formation of biofilm.

4.3.2 Development of Biofilm

The biofilm were developed in both CPBBs using a procedure developed by Walls (1993), separately. The biofilm was developed in the 1st CPBB firstly, where the sterile medium containing substrate (50 mg/L *trans*-4MCHCA) was trickled at a flow rate of 0.0375 L/h over the stainless steel mesh packing for a month. Simultaneously, partially degraded effluent was recycled back into the reactor at a rate of 2.4 L/h, which provided a continuous source of viable microorganisms and substrate. Upon completion of this period of operation, visual examination indicated formation of a substantial amount of biofilm on the packing. The bioreactor was then drained and filled with fresh medium with desired NAs at the specified concentrations in order to initiate batch or continuous experiments.

The development of biofilm in the 2nd CPBB was similar to the procedure described above. However, the inoculum used in the 2nd CPBB was from the effluent of 1st CPBB, and biofilm was grown on octanoic acid instead of *trans*-4MCHCA. Much higher feed substrate concentrations (500mg/L octanoic acid) were implemented in the 2nd CPBB, where the setting of trickling and recycling flow rates however was the same as the 1st CPBB. Due to aggressive feeding and an easy-degraded growth substrate of octanoic acid itself, the biofilm was able to form within 2 weeks. A representative photograph indicating the CPBB with fully developed biofilm is shown in Figure 4.5.

Figure 4.5: The representative photograph of the bioreactor after formation of biofilm.

4.4 Experimental Procedure

4.4.1 Batch Experiments

For batch experiments the CPBBs were charged with medium containing the desired concentrations of the designated naphthenic acids. Air was introduced into the bottom of the bioreactors. The progress of biodegradation was monitored by sampling of the liquid at the desired time intervals. Samples were tested for residual concentration of NAs. All batch experiments for biodegradation of *trans*-4MCHCA, 4MCHAA, mixture of *trans*-4MCHCA and 4MCHAA, and assessment of temperature effect were carried out in the 1st CPBB. Batch biodegradation of the mixture of octanoic acid with *trans*-4MCHCA or 4MCHAA were conducted in the 2nd CPBB.

The range of concentrations evaluated for biodegradation of *trans*-4MCHCA and 4MCHAA were from 50 mg/L to 500 mg/L (50, 100, 250, and 500 mg/L) and 25 mg/L to 350 mg/L (25, 50, 75, 100, 250, and 350 mg/L) respectively, which spanned concentrations that were higher than the actual concentrations observed in tailing pond waters (40-120 mg/L) (Paslawski, 2008; Clemente et al., 2005). The reason for applying higher concentrations than those in the tailing ponds was to evaluate the ultimate capability of this system, as application of this system was not limited to treatment of NAs in oil sand tailings. The applied temperature was (25±2 ºC). The injected airflow was controlled in the range of 0.5 to 1.2 L/min (corresponding to 1.1 to 2.7 vvm) which ensured oxygen was not a limiting factor for microbial growth.

Batch experiments were also conducted on the mixture of *trans*-4MCHCA and 4MCHAA at room temperature where *trans*-4MCHCA initial concentration was set as approximate 100 mg/L and 4MCHAA concentrations were increased incrementally from 25 mg/L to 70 mg/L (25, 50, and 70 mg/L). Upon completion of room temperature experiments, additional batch experiments were carried out with pure 4MCHAA (100 mg/L) at various temperatures of 15, 20, 25, 30, and 35 ºC to assess temperature effects on biodegradation rates.

Furthermore, additional batch experiments were carried out in the 2nd CPBB to investigate effect of co-substrate on enhancement of biodegradation of *trans*-4MCHCA and 4MCHAA. During these experiments, octanoic acid served as a co-substrate for biodegradation of *trans*-4MCHCA or 4MCHAA. In these experiments initial octanoic acid concentration was kept constant as approximately 500 mg/L and varying *trans*-4MCHCA concentrations (50, 100, and 250 mg/L) and 4MCHAA concentrations (50, 100, and 330 mg/L) were used. All experiments were conducted at room temperature (25± 2 ºC) condition. Airflow was controlled in the range of 0.5 to 1.2 L/min

To assess the reproducibility of the results, some of the batch experiments were repeated at specified conditions. These include experiments aiming to study the effects of initial substrate concentration, temperature, and co-substrate biodegradation.

4.4.2 Continuous Experiments

The circulating packed bed bioreactors (CPBBs) were also used to study the continuous removal of NAs under aerobic condition at room temperature (25 $\pm$ 2ºC). Similar to batch studies, the 1st CPBB was used for continuous experiments of pure *trans*-4MCHCA and 4MCHAA while the 2nd CPBB was used for biodegradation of pure octanoic acid.

During continuous experiments, sterile medium containing the model NA at the desired concentration was continuously fed into the CPBB at the top using a peristaltic pump. Simultaneously, the effluent was removed from the bottom of the reactor using an overflow tube. The flow rates were set using a calibrated pump and were measured daily by weighing the effluent collected over a specified period. Furthermore, a feed reservoir tank (1L) was placed on a magnetic stirrer with vigorous mixing to ensure the complete dissolution of NAs in the feed stream.

Two initial substrate concentrations (50 mg/L and 100 mg/L) were selected to study continuous biodegradation of *trans*-4MCHCA while one constant concentration (100 mg/L) was used for continuous biodegradation of 4MCHAA. Experiments with pure octanoic acid were conducted at a concentration of 615 $\pm$30 mg/L.

Initially, biodegradation of *trans*-4MCHCA was studied where the CPBB was operated with a medium containing 50 mg/L of *tran*-4MCHCA at incrementally increased flow rates of 6.25, 12.5, 25.0, 50.0, and 100 ml/min (corresponding to residence time of 1.2, 0.6, 0.3, 0.15, and

0.075 h; Loading rate of 39.2,85.2,151,320, and 642 mg/L-h,).Then the flow rate was maintained at 100 ml/min and feed concentration was increased to100 mg/L (corresponding to a residence of 0.075 h and loading rate of 1230 mg/L-h). At each flow rate sufficient time (at least 8 residences times) was given for establishment of steady state conditions which was verified by stability in the residual substrate concentrations (variation less than 10%). Throughout the experiments, the optical densities of the liquid samples from the top and bottom of the bioreactor were measured. Substrate concentrations in the reservoir tank and the delivery point to the bioreactor were checked frequently to ensure no contamination occurred in the feed reservoir tank or within the tube to the reactor. Furthermore, to assess the reproducibility of the experimental results, duplicate experiments were conducted at the flow rate of 25 ml/min.

Continuous biodegradation of 4MCHAA was also studied in the 1st CPBB, where the bioreactor was operated with a feed containing 50 mg/L of 4MCHAA. Since 4MCHAA was much more persistent and recalcitrant than *trans*-4MCHCA, lower flow rates were applied for this study. The flow rate was increased step-wise 56.3, 97.8, 135 and 167 ml/h (corresponding to residence time of 8.0, 4.6, 3.3, and 2.7 h).

The 2nd CPBB was operated with a medium containing 615$\pm$30 mg/L octanoic acid. The reactor was initially operated continuously at a flow rate of 52.4 ml/h which was gradually increased. The applied flow rates were 168, 283, 403, 476, 583 ml/h with the corresponding residence times being 7.06, 2.20, 1.30, 0.923, 0.78, and 0.63 h. Due to molecular structure of octanoic acid, consisting of a saturated hydrocarbon chain and carboxyl group, this substrate was not as persistent as other NAs. Therefore, higher loading rates were applied for this study. Furthermore, a duplicate steady state condition was examined at the flow rate of 4.47 ml/min. All other conditions and procedures were similar to those described earlier. The schematic diagram

and photograph of the experimental system used in these experiments are shown in Figure-4.6 and 4.7.

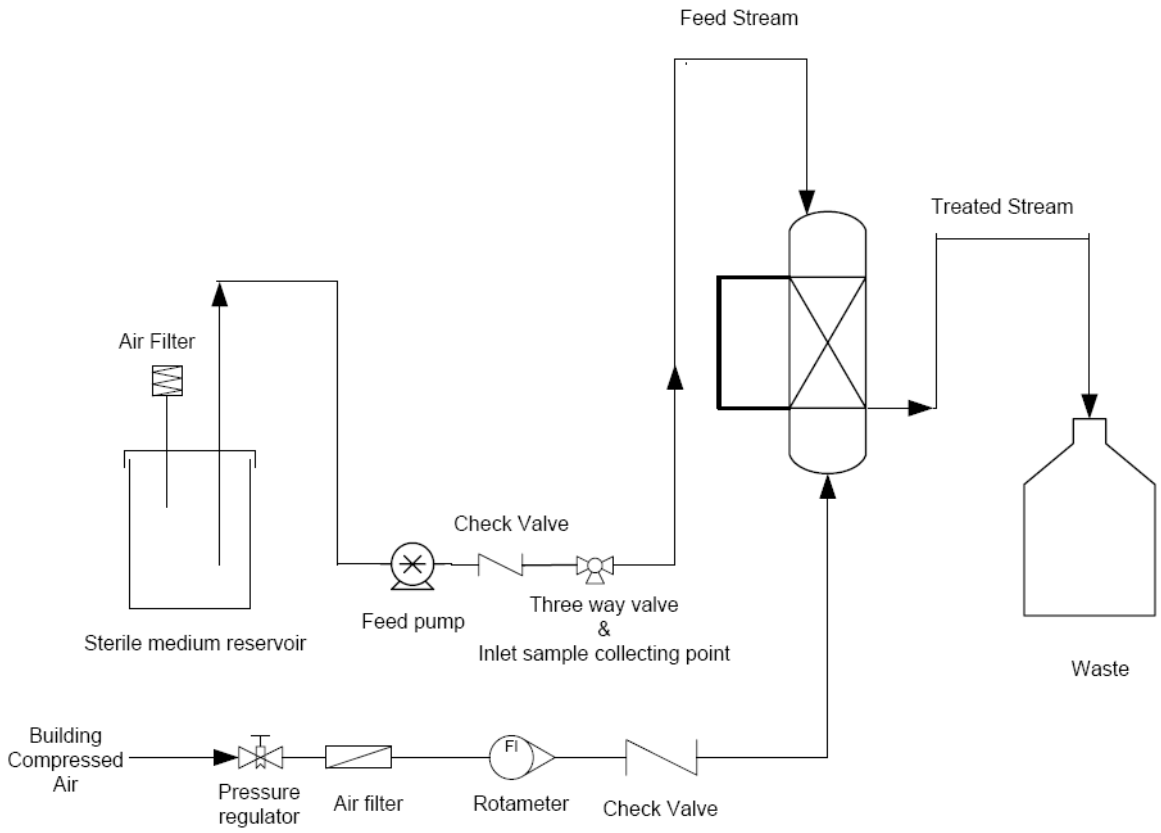


Figure 4.6: Process flow diagram of experimental system.

Figure 4.7: Photograph of experimental setup

4.5 Analytical Methods

4.5.1 Measurement of Naphthenic Acids Concentration

NAs are in general difficult to analyze. Several methods have been successfully developed for the purpose of quantitative or qualitative analyses of naphthenic acids in water and biological medium over the past few decades. Techniques include high-performance liquid

chromatography (HPLC), fourier transform infrared (FTIR) spectroscopy, negative ion electrospray ionization-mass spectrometry (ESI-MS), gas chromatography with a flame ionization detector (GC-FID), gas chromatograph-mass spectrometry (GC-MS), liquid secondary ion mass spectrometry (LSI-MS), electrospray ionization (ESI), and quantitative quadrupole time of flight –MS (QTOF_MS) (Paslawski, 2008; Bataineh et al., 2006; Clemente et al., 2005; Barrow et al.,2004). All of the above methods have unique strengths as well limitations.

In an earlier work in our laboratory, Paslawski (2008) successfully used gas chromatography with a flame ionization detector (GC-FID) to accurately determine NAs concentrations in aqueous solutions. In this work, due to its simplicity, GC-FID was also used for analysis of the candidate NAs. The analytical equipment used in this study was a Varian- 430 gas chromatograph, where helium served as carrier and makeup gas and hydrogen and air were used as combustion gas in the FID. A HP-INNOWAX high resolution gas chromatography column (19091N-133) was used. The column had the following specifications: length of 30m, inside diameter of 0.250 mm, and film thickness of 0.25 μm. The operating conditions for the system were as follows:

- H_2 flow rate: 30 ml/min
- He flow rate: 29 ml/min
- Air flow rate: 300ml/min
- Injector split ratio: 1to10
- Column oven initial temperature: 90 °C
- Injector temperature: 220 °C
- Detector temperature: 250 °C
- Column oven temperature program: 90 °C ramped to 210 °C at a rate of 40 °C/min

A linear calibration curve was developed to convert gas chromatography readings to the actual NAs concentrations (mg/L) in the sample. To develop the calibration curve, the candidate NA compounds were dissolved into the sterile McKinney's modified medium. This solution was then diluted into five different concentration solutions, which were used as the standards. The

standard concentrations were 0.932 mg/L, 12.4, 24.8, 49.6, and 99.2 for *trans*-4MCHCA, 0.673, 13.5, 26.9, 53.8, and 108 mg/L for 4MCHAA, and 16.8, 37.3, 74.7, 149, 299, and 580 mg/L for octanoic acid. Each standard solution was injected four times and Millipore water was run between samples to prevent the possible accumulation of the substrate in the column and ensure the accuracy of readings. The developed linear calibration curves for *trans*-4MCHCA, 4MCHAA, and octanoic acid are shown through Figure A.1 to A.3 in the Appendices. The elution order of investigated NAs was octanoic acid (3.40min), *trans*-4MCHCA (3.72min), *cis*-4MCHAA (4.10min), and *trans*-4MCHAA (4.27min). The representative GC-FID chromatogram of these three model NAs is shown in Figure B.1

Measurement of Biomass Hold-up

Upon completion of all experiments, both CPBBs were drained and the stainless steel mesh was removed from the column. The drained liquid volume was measured as the working volume in each reactor. Then, the liquid was filtered, and the residues were dried in a vacuum oven at 65 °C with a vacuum pressure of -70 kPa over a week to determine the weight of suspended biomass in the reactor. Meanwhile, the wet biofilm packing material from each reactor was weighted and then loaded into the same vacuum oven with the same conditions as stated above. After one week, the packing was carefully taken out of the oven and weighted again to obtain the dry mass of biofilm.

4.5.2 Statistical Methods

During batch and continuous experiments, samples were periodically taken in duplicate. Each sample was analyzed three times to determine the biomass and substrate concentrations.

The mean values with one standard deviation were reported throughout this thesis. The standard deviations were calculated using Microsoft Exel^{TM} and presented as error bars. With a normal distribution, this interval has a 68.26% confidence interval, which is commonly used in biological studies (Nikakhtari, 2005). Also, the reproducibility of the experimental data was assessed by repeating a number of experiments as stated previously.

5 RESULTS AND DISCUSSION

5.1 Biodegradation of *trans* - 4-methyl -1-cyclohexane Carboxylic Acid

This section presents the results of biodegradation of *trans*-4-methyl-1-cyclohexane carboxylic acid (*tran*s-4MCHCA) in the circulating packed-bed bioreactor (CPBB). The effects of initial concentrations and volumetric loading rates of *trans*-4MCHCA on biodegradation rate were studied by running the 1st circulation packed bed bioreactor (CPBB) under batch and continuous mode, respectively. The obtained data were used to assess the performance of the CPBB under various conditions.

The extent of biofilm (dry weight of attached biomass) in the 1st CPBB was measured subsequent to completion of the entire research. The total dry biomass weighed 2.83g, which was 4.78 % of clean dry packing material (g of dry mass per g of clean dry packing material). The free liquid volume present in the 1st CPBB was found to be 450 ml, which was 80 % of the total volume of the bioreactor. The calculated biomass concentration in the 1st CPBB was 6.29 g/L. This value was used to determine the relevant specific biodegradation for the experiments conducted in the 1st CPBB in order to demonstrate the enhancement of CPBB on biodegradation of NAs in respect to conventional batch reactor, freely suspended cell reactor (CSTR), and packed bed reactor.

5.1.1 Batch Biodegradation of *trans*-4MCHCA

Four selected initial substrate concentrations (50, 100, 250, and 500 mg/L) were studied. Substrate concentration profiles are shown in Figure 5.1 (Panels: A to D). A short lag phase of approximately 0.651 to 1.30 h was observed for initial concentrations of 50 mg/L and 250 mg/l,

while no lag phase was seen at 106 mg/L and 503 mg/L. During the exponential phase, regardless of substrate initial concentration, cells exhibited their metabolic activity which resulted in a continuous and linear decrease in substrate concentration until the substrate was completely consumed. Additionally, no substrate inhibitory effect was observed at any of the tested concentrations.

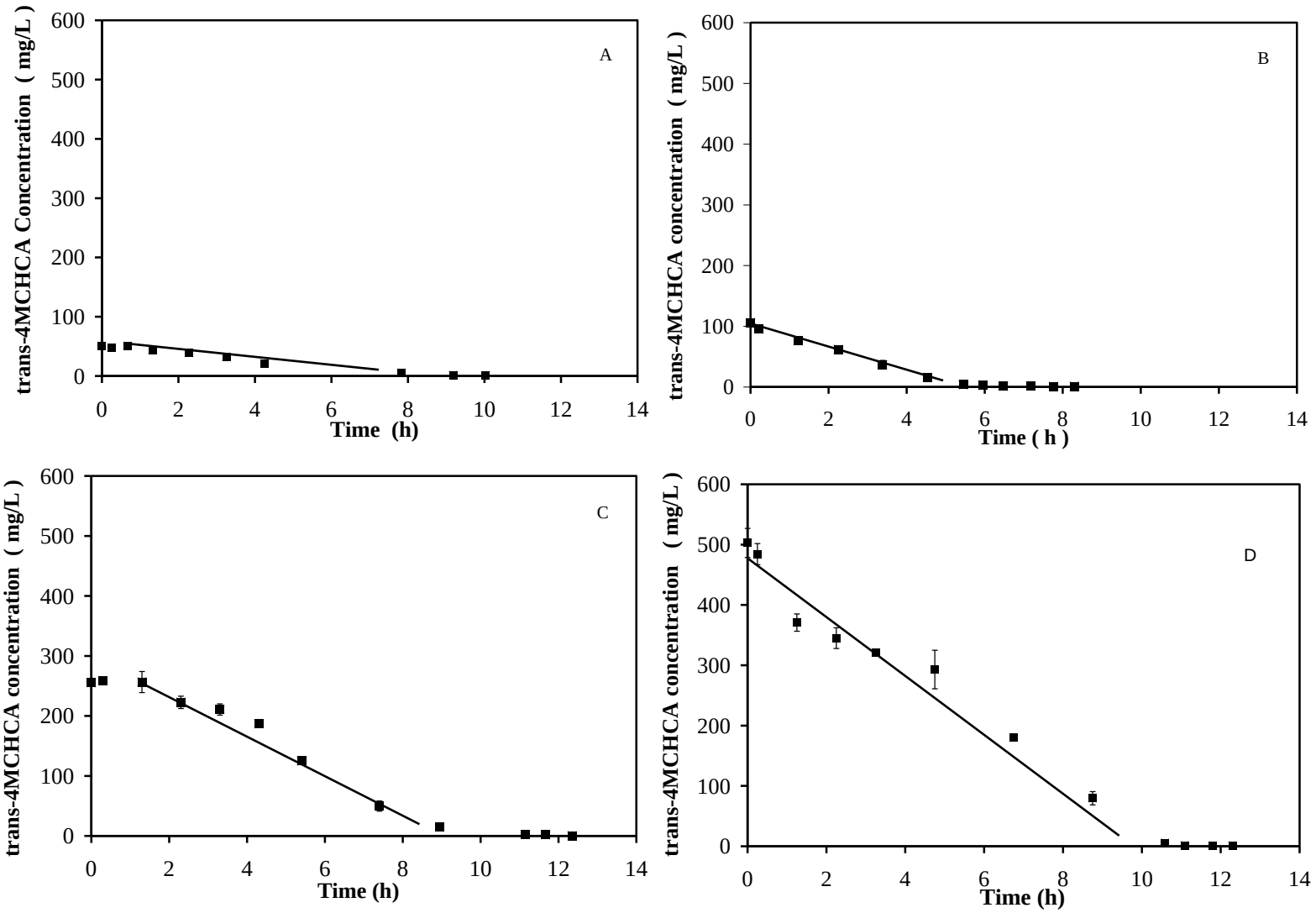


Figure 5.1: Substrate biodegradation as a function of time. *trans*-4MCHCA concentrations of 50.0 (A), 100 (B), 250 mg/L (C) and 500 mg/L (D). Error bars represent one standard deviation and may not be visible for some cases

The biodegradation rates at various concentrations were determined using the slopes of the linear part of the concentration profiles (lag phase excluded). The biodegradation rate of *trans*-4MCHCA as a function of its initial concentration is shown in Figure 5.2.

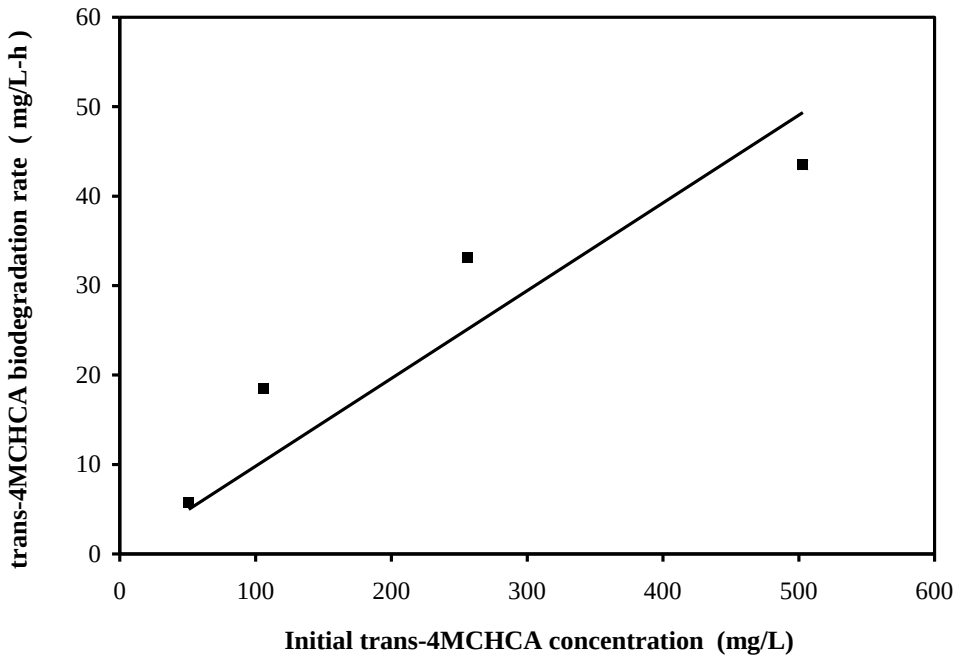


Figure 5.2: Biodegradation rates as functions of initial *trans*-4MCHCA concentrations.

As can be seen biodegradation rates ($r_{trans\text{-}4MCHCA}$) increased with increases in initial substrate concentrations and followed a linear trend as represented by Equation 5.1:

$$r_{trans\text{-}4MCHCA} = 0.0981 \times S_i \qquad (R^2=0.800) \qquad (5.1)$$

Similar biodegradation studies were conducted by Paslawski (2008) in shake flasks with free cells, where direct relationship between biodegradation rate and initial substrate concentration was reported according to Equation 5.2:

$$r_{trans\text{-}4MCHCA} = (0.00800 \pm 0.00113) \times S_i \qquad (5.2)$$

In the present work the maximum biodegradation rate of 43.5 mg/L-h were observed at the highest concentration of 500 mg/L. Biodegradation rates obtained at all tested concentration levels were significantly higher than those reported previously. For example, Paslawski (2008), using a batch reactor, reported a biodegradation rate of 4.75 mg/L-h at an initial concentration of 500 mg/L, which was 9.2 times less than the value obtained in this study. A summary of calculated *trans*-4MCHCA biodegradation rates at various initial substrate concentrations together with literature values are presented in Table 5.1.

Table 5.1: Summary of biodegradation rates and literature values at various initial *trans*-4MCHCA concentrations at 25 ^{0}C and pH of 6.3 to 6.7.

Initial Substrate concentration (present work) (mg/L)	Biodegradation rate (present work) (mg/L-h)	Biodegradation rate (Paslawski, 2008) (mg/L-h)
50	5.770	0.46
100	18.501	1.16
250	33.067	1.30
500	43.504	4.75

pH was measured at the start and end of each experiment. It was found that the value of pH in the bioreactor did not change by more than 0.5 and fell in the range of 6.3 to 6.7. Overall, the results of this study indicate that the biodegradation rate of the candidate naphthenic acid is strongly influenced by its initial concentration. Significantly higher biodegradation rates obtained in this study also revealed the advantages of the CPBB for creating good environmental conditions for microbial growth resulting in the enhancement of biodegradation rate.

5.1.2 Continuous Biodegradation of *trans*-4MCHCA

The effect of volumetric loading rate of *trans*-4MCHCA on the biodegradation rate was examined in the 1st CPBB, where the performance of the designed bioreactor was assessed in terms of conversion ratio and biodegradation rates of *trans*-4MCHCA.

The reactor was initially fed with medium containing 50 mg/L of *trans*-4MCHCA and operated with a flow rate of 6.25 ml/min (equivalent loading rate of 39.2 mg/L-h) for approximate 209 hours, where the establishment of steady state conditions was verified by stability in the residual substrate concentration (less than 10% variation over 8 retention times of 1.20 h). The highest conversion of 83.0% was achieved at the above flow rate with the corresponding *trans*-4MCHCA removal rate of 39.2 mg/L-h. The flow rate was then incrementally increased (12.5, 25, 50, and 100 ml/min), while feed substrate concentration was kept constant at 50 mg/L. At the end of experiments with 50 mg/L, the highest flow rate of 100 ml/min was repeated with an increased feed concentration of 100 mg/L (corresponding to a loading rate of 1230 mg/L-h). Over this period, the *trans*-4MCHCA biodegradation rate was increased linearly with increases in flow rate or loading rate till it reached the maximum biodegradation rate of 209 mg/L-h, which had a corresponding flow rate and the loading rate of 50 ml/min and 320 mg/L-h. Application of a higher flow rate (100ml/min) or loading rates (642 mg/L-h and 1230 mg/L-h) led to decreases of biodegradation rates of *trans*-4MCHCA. During the period of incremental increase of flow rate, the residence time decreased from 1.2 h to 0.075 h and the residual steady state substrate concentrations increased from 7.98 mg/L to 87.3 mg/L. The minimum conversion of 9.16% was observed at the maximum loading rate of 1230 mg/L-min. Also, pH in the reactor was found ~6.4. Steady state biodegradation rate of *trans*-4MCHCA and the associated conversion as functions of the volumetric loading rate of *trans*-4MCHCA are

shown in Figure 5.3. To assess the reproducibility of the experimental results, the bioreactor was operated again at the flow rate of 25 ml/min (corresponding loading rate of 151 mg/L-h) which yielded almost similar results where variation in removal rate and conversion were 3.2% and 8.6%, respectively.

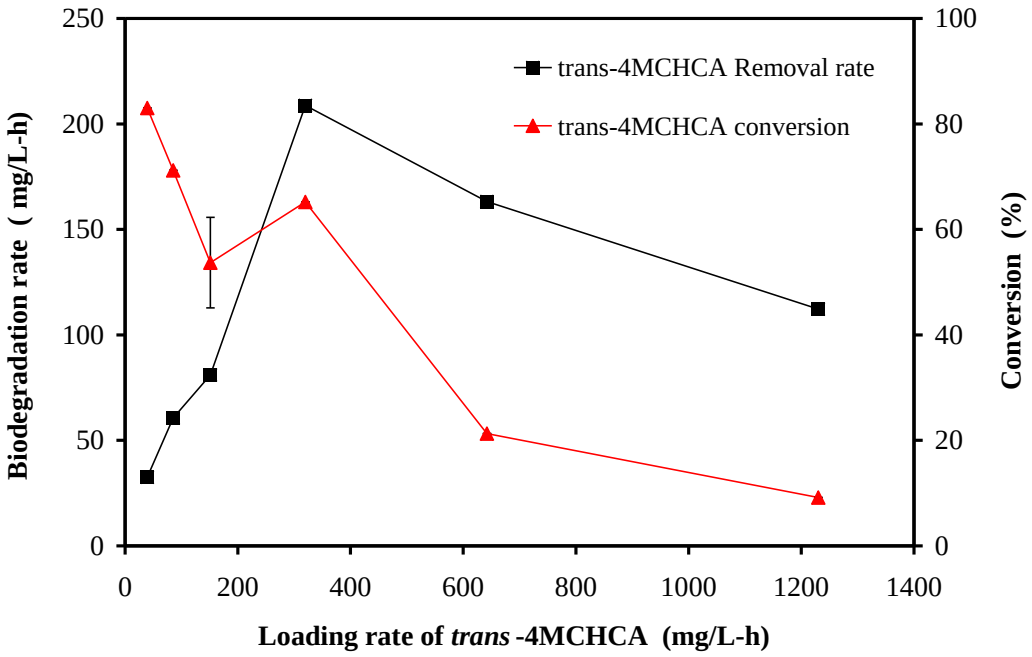


Figure 5.3: The effect of *trans*-4MCHCA loading rates on the performance of the CPBB. Error bars represent one standard deviation and may not be visible for some cases

Paslawski et al. (2009) studied continuous biodegradation of *trans*-4MCHCA in both CSTR and packed-bed reactor, and was able to achieve a maximum biodegradation rate of 9.6 mg/L-h (corresponding to a residence time of 38.4 hours) and 918 mg/L-h (corresponding to a residence time of 0.6 hours; specific biodegradation rate of 8.83 mg substrate /mg biomass -h), respectively. The maximum biodegradation rate obtained in this study was 209 mg/L-h (corresponding to a residence time of 0.15 hours; specific biodegradation rate of 33.2 mg

substrate /mg biomass-h), which was approximate 22 times over that observed in the CSTR in term of biodegradation rate and 3.8 times than packed-bed reactor in term of specific biodegradation rate. Compared to a freely suspended cell bioreactor (CSTR) and packed-bed reactor, the CPBB with established biofilm was able to provide a higher concentration of biomass and form a much more homogeneous environment inside of reactor, which led to an efficient removal of soluble NAs and demonstrated that enhancement of biodegradation of NAs can be achieved by improving the configuration of bioreactor.

5.2 Biodegradation of 4-methylcyclohexane Acetic Acid (4MCHAA)

Acclimation experiments were conducted prior to the actual experiments to ensure the developed microbial consortium was able to utilize 4MCHAA as sole substrate. The following batch and continuous experiments at various initial substrate concentrations and loading rates were performed to assess the effects of these parameters on the biodegradation rate. Additionally, effect of temperature was also studied. All experiments were conducted in the 1st CPBB.

5.2.1 Acclimation of the Microbial Consortium for Utilization of 4MCHAA

Preliminary work conducted in our laboratory indicated that 4MCHAA was not biodegraded by the microbial consortium grown on *trans*-4MCHCA. Acclimation of the developed microbial consortium was necessary in order to improve 4MCHAA biodegradation efficiency, where non-specific enzymes could be induced for mutation or the appearance of new genotypes after the exposure of bacteria to the compound of interest (Stephenson et al., 1984; Torstensson et al., 1975; Schmidt et al., 1983; Walker et al., 1956). Most importantly, species

with the ability to metabolize 4MCHAA become dominant in the mixed cultures. Acclimation was initiated by operating the 1st CPBB under batch mode, where the medium contained both *trans*-4MCHCA and 4MCHAA as growth substrates. Since acclimation of NAs-degrading microorganisms was generally difficult due to the toxicity of the substrate, careful adjustment of the cultivation conditions was undertaken. The bacteria were first fed with a rather low substrate concentration, and then the substrate concentration was increased incrementally. This allowed development of tolerance and utilization of substrate at higher concentrations. Therefore, during the acclimation period, the initial 4MCHAA concentrations gradually increased from 25 mg/L to 70 mg/L (25, 50, 70 mg/L), while the initial *trans*-4MCHCA concentration was held constant as 100 mg/L. Manonmani et al. (2000) reported that continuous exposure of bacteria to increasing concentrations of substrate markedly improved their degrading abilities.

Over this period, the reactor was incubated at room temperature (25 °C) and sampled periodically to measure residual substrate concentrations. Also, pH was found in the range of 6.3 to 6.5. Substrate utilization curves at three tested concentrations are shown in Figure 5.4 (panels A, B, C). The corresponding substrate biodegradation rates were also calculated and are presented in Table 5.2

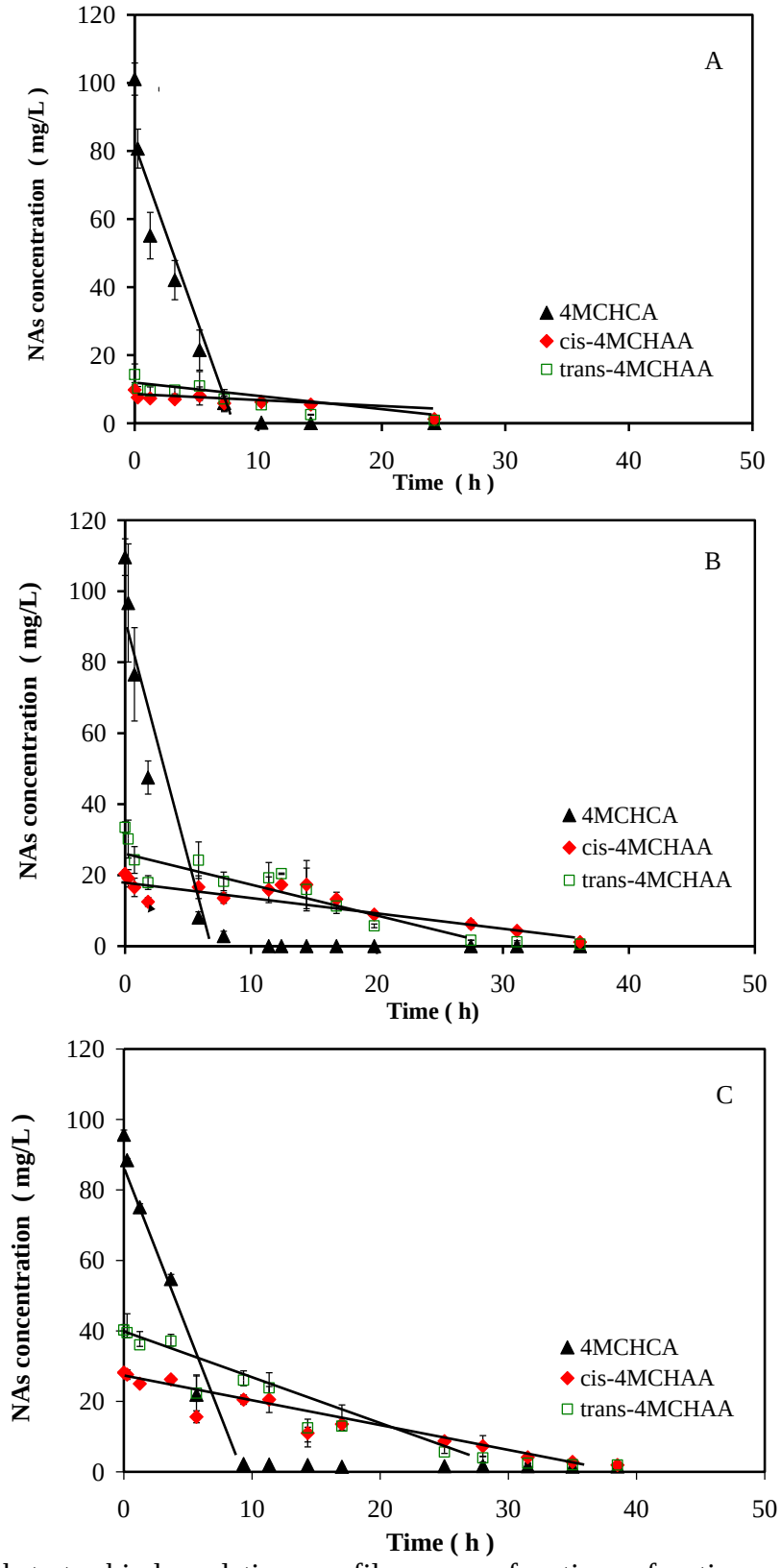


Figure 5.4: Substrate biodegradation profiles as a function of time at various initial concentrations, where initial *trans*-4MCHCA concentration maintained as 100 mg/L and *trans*-4MCHCA concentration varied as 25 mg/L (panel A), 50 mg/L (panel B), and 70 mg/L (panel C). Error bars represent one standard deviation which may not be visible in some cases due to small value.

Table 5.2: Summary of biodegradation rates of 4MCHAA and *trans*-4MCHCA at various initial concentrations obtained at room temperature (25°C), where initial *trans*- 4MCHCA concentration was kept constant at 100 mg/L. pH ranged from 6.3 to 6.5.

Initial 4MCHAA concentration (mg/L)	Biodegradation rate of *cis*-4MCHAA (mg/L-h)	Biodegradation rate of *trans*-4MCHAA (mg/L-h)	Biodegradation rate of *trans*-4MCHCA (mg/L-h)
25	0.276 (R^2=0.861)	0.499 (R^2=0.848)	11.686 (R^2=0.913)
50	0.438 (R^2=0.823)	0.960 (R^2=0.829)	12.975 (R^2=0.896)
70	0.698 (R^2=0.971)	1.234 (R^2=0.921)	10.135 (R^2=0.907)

The results showed that the acclimated cultures were capable of utilizing 4MCHAA with varying initial concentrations up to 70 mg/L. Biodegradation rate of 4MCHAA increased linearly with increases in its initial concentration. This dependency is expressed by equations 5.3 and 5.4, respectively and represented in Figure 5.4.

$$r_{cis\text{–}4MCHAA} = 0.0097 \times S_i \qquad (R^2 = 0.959) \qquad (5.3)$$

$$r_{trans\text{-}4MCHAA} = 0.0183 \times S_i \qquad (R^2 = 0.977) \qquad (5.4)$$

It was also observed that *trans*-4MCHCA was degraded more rapidly in comparison to 4MCHAA, and biodegradation of *trans*-4MCHAA was faster than *cis*-4MCHAA.Furthermore, the maximum biodegradation rates for *trans*-4MCHCA, *cis*-4MCHAA and *trans*-4MCHAA were 10.1, 0.698 and 1.23 mg/L-h, respectively, and were observed with initial *trans*-4MCHCA concentration of 100 mg/L and 4MCHAA concentration of 70 mg/L.

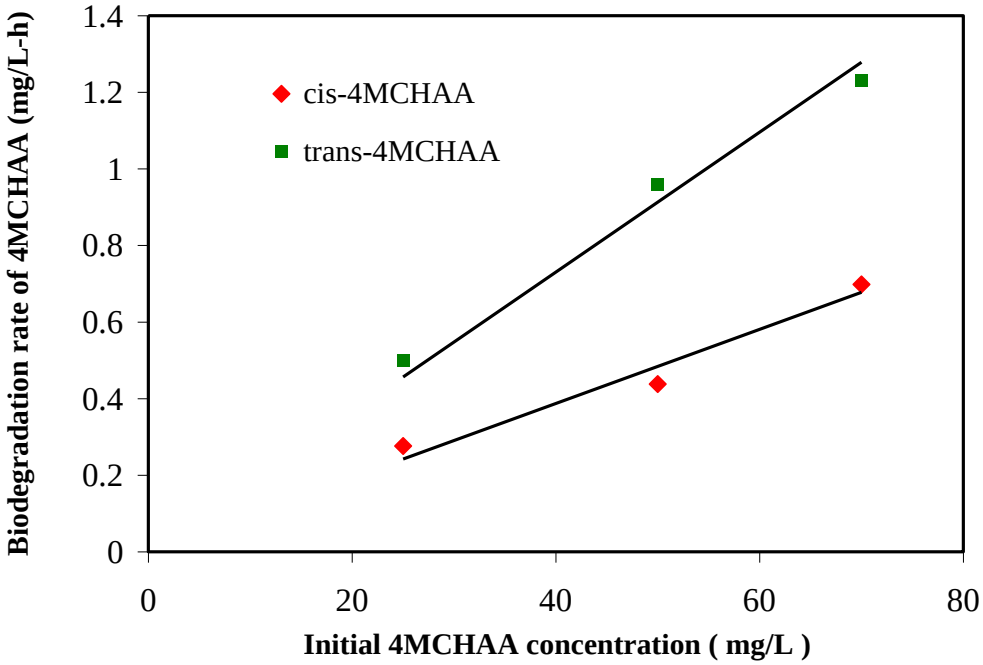


Figure 5.5: Biodegradation rate as functions of initial 4MCHAA concentration.

5.2.2 Batch Biodegradation of 4MHCAA as the Sole Substrate

Following the acclimation of the microbial cultures to biodegradation of 4MCHAA, batch experiments were carried out in the 1st CPBB to study biodegradation of 4MCHAA as the sole substrate, and in particular to investigate the influence of initial substrate concentration on the biodegradation rate of 4MCHAA. The initial concentrations ranged from 25 mg/l to 350 mg/L (25, 50, 75, 100, 250, and 350 mg/L). The substrate utilization profiles are illustrated in Figure 5.6 (Panels A to F), where repeated experiments with initial 4MCHAA concentrations of 243 mg/L and 246 mg/L are also shown together in panel (E) to demonstrate the reproducibility of experimental results. The pH ranged from 6.1 to 6.5 and do not change by more than 0.5 at the start and the end of experiment.

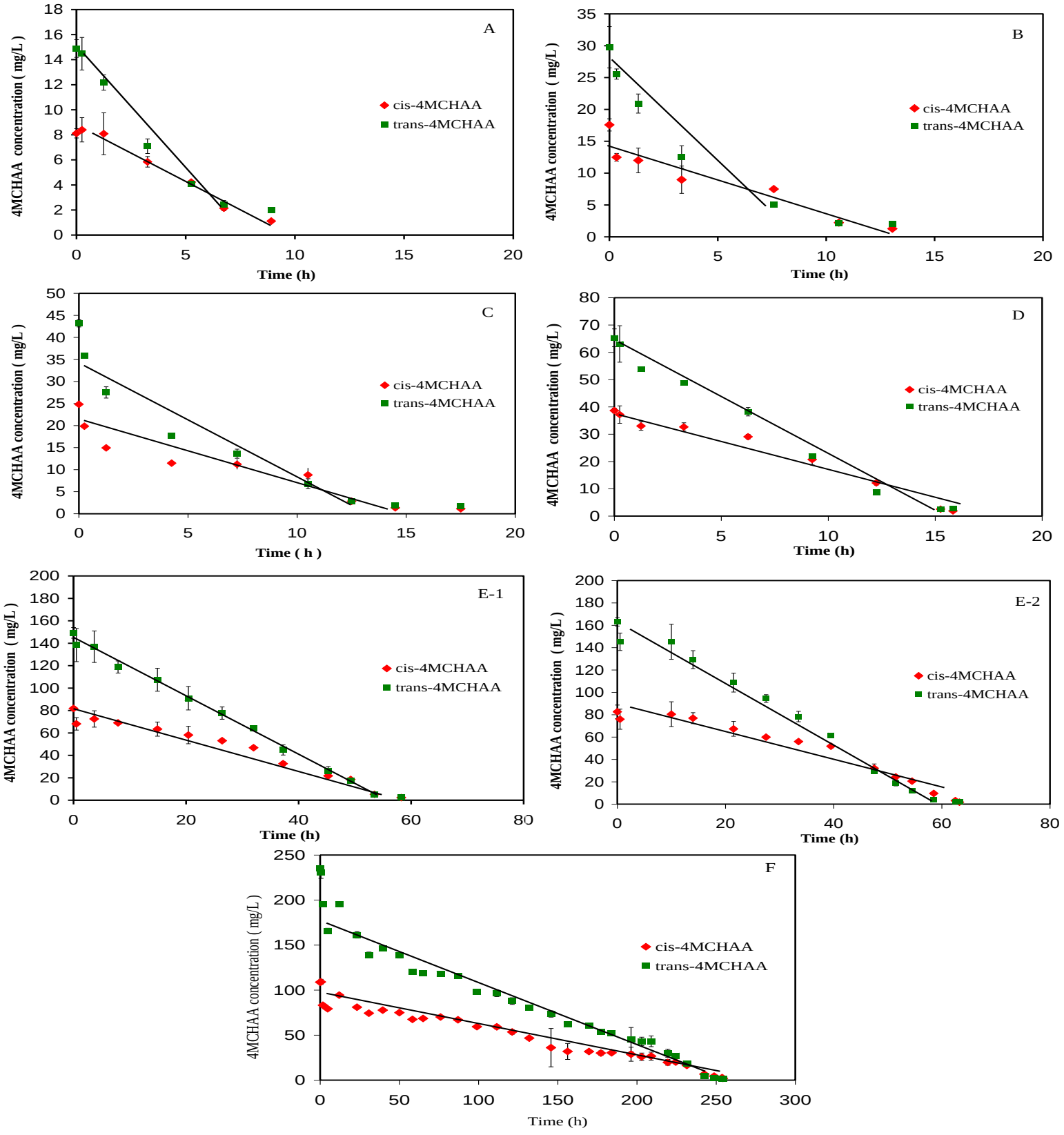


Figure 5.6: Biodegradation of 4MCHAA at initial concentrations of 25(A), 50(B), 75(C), 100 (D), 250 (E), and 350 mg/L (F), where duplicate experiments with initial 4MCHAA concentration of 243 mg/L and 246 mg/L are shown in E-1 and E-2, respectively. Error bars represent one standard deviation which may not be visible in some cases due to small value.

Biodegradation rates, calculated using the slope of the linear part of the concentration profile, at different initial substrate concentrations are presented in Table 5.3. The maximum biodegradation rates for *cis*-4MCHAA and *trans*-4MCHAA were 2.25mg/L-h and 4.17 mg/L-h obtained at an initial concentration of 100 mg/L. A similar biodegradation study was conducted by Tanpat (2001) in a batch reactor, where initial 4MCHAA concentration was 5 mg/L and pH was 7. He reported biodegradation rates of only 0.0105 mg/L-h and 0.00760 mg/L-h for *trans*- and *cis*- isomers of 4MCHAA, respectively. Significant improvement in biodegradation rates achieved in the present study, three orders of magnitude higher, showed the superior performance of the circulating packed-bed bioreactor in terms of enhancement of biodegradation rate and capability of treating much higher concentration of naphthenic acids when compared with a conventional batch bioreactor. The small variance in biodegradation rate (less than 4%) obtained in the repeated experiments proved the reproducibility of the experimental results.

Overall biodegradation rates exhibited the trend that increased with increases of initial 4MCHAA concentrations up to a maximum value 100 mg/L. Further increases in initial substrate concentrations led to decreases of biodegradation rate, indicating potential inhibitory effect of substrate. 4MCHAA biodegradation rates as functions of its initial concentrations are shown in Figure 5.5. Interestingly, a similar trend was also reported by Kumar et al. (2011) when he studied the biodegradation of 1, 4-benzoquinone in a batch system using *Pseudomonas* as the microbial culture.

Table 5.3: Summary of biodegradation rates of 4MCHAA as the sole substrate obtained at various initial concentrations and room temperature (25°C), and pH ranged from 6.1 to 6.5.

Initial substrate concentration (mg/L)	Biodegradation rate of *cis*-4MCHAA (mg/L-h)	Biodegradation rate of *trans*-4MCHAA (mg/L-h)
25	0.867 (R^2=0.981)	1.924 (R^2=0.982)
50	1.050 (R^2=0.902)	2.486 (R^2=0.916)
75	1.305 (R^2=0.884)	2.831 (R^2=0.909)
100	2.247 (R^2=0.973)	4.165 (R^2=0.988)
250[a]	1.265[a] (R^2=0.962)	2.593[a] (R^2=0.996)
250[a]	1.241[a] (R^2=0.934)	2.712[a] (R^2=0.985)
350	0.336 (R^2=0.977)	0.660 (R^2=0.969)

[a] Results obtained in repeated experiments.

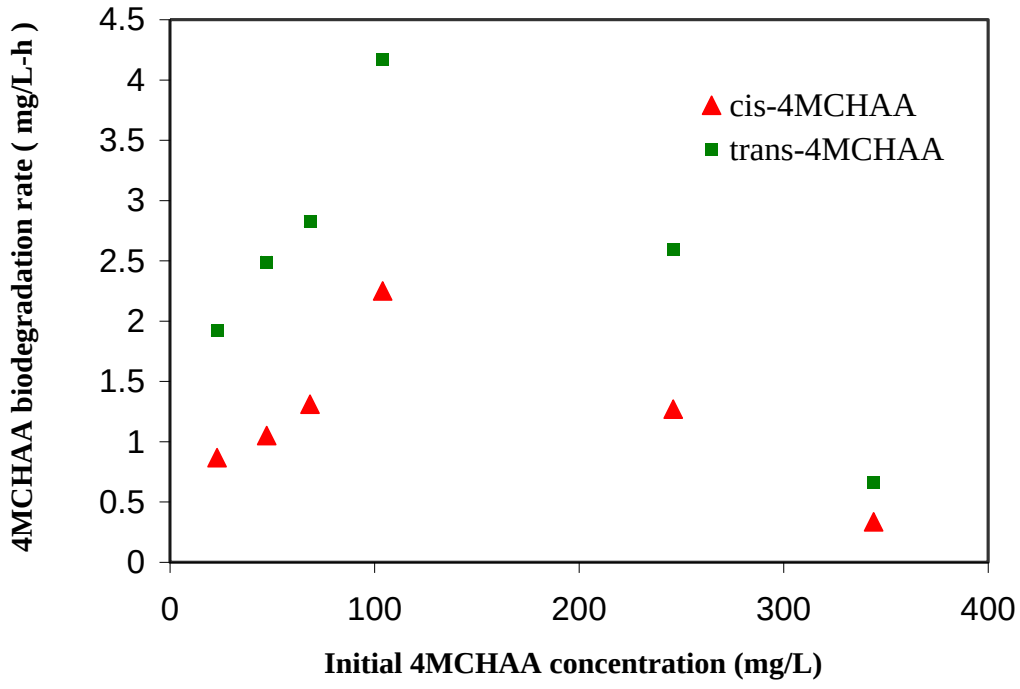


Figure 5.7: 4MCHAA biodegradation rates as functions of its initial concentrations.

During this biodegradation study, regardless of the initial concentration of the 4MCHAA mixture, higher biodegradation rates were observed for *trans*-4MCHAA when compared to *cis*-4MCHAA, which revealed the importance of chemical structure on biodegradation rate. Tanapat (2001) stated that *trans*-isomer degraded more rapidly than *cis*- isomer due to its relatively open geometry. He also explained that intramolecular hydrogen-bonding forces, only existing in the *cis*-isomer, require more energy to be broken down, and this is the main reason for slow degradation of the *cis*-isomer in comparison to the *trans*-isomer.

Additionally, the structural difference between the *trans*-4MCHAA and the *trans*-4MCHCA where the cartboxylic acid was directly attached to the cyclohexane ring (while with 4MCHAA it was attached via $–CH_2$ group) appeared to have a significant effect on biodegradation rate. For example, in batch system, the biodegradation rate of *trans*-4MCHCA (100 mg/L) was 17.6 mg/L-h, while for trans-4MCHAA the rate was 4.17 mg/L-h at the same concentration.

5.2.3 Temperature Effect on Biodegradation Rate of 4MCHAA

To evaluate the temperature effect on biodegradation rate of 4MCHAA, a series batch experiments were conducted in a temperature controlled environmental chamber where the temperatures were increased from 15 °C to 35 °C in 5 °C increments. The initial substrate concentration was set at 100 mg/L for all cases. Substrate utilization profiles at various temperatures are shown in Figure 5.8 (panels A to E). A lag phase of 0.5 h and 4.25 h were observed upon changing the temperature from 25 °C to 30 °C or 20 °C, respectively. After this lag phase, both isomers were degraded simultaneously and faster degradation rates were obtained for *trans*-4MCHAA compared to *cis*-4MCHAA which was similar to the previous observations.

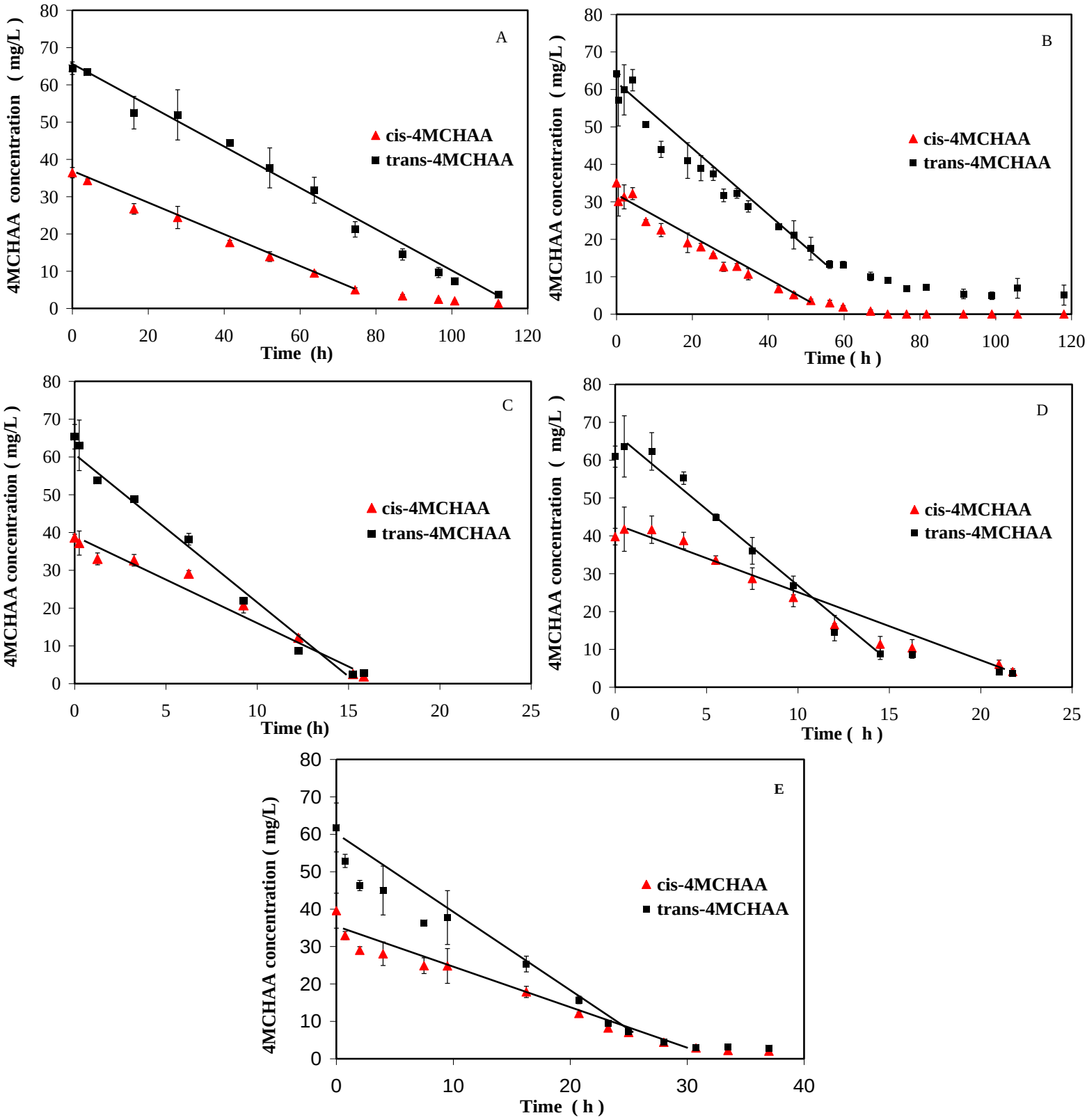


Figure 5.8: Biodegradation of 100 mg/L 4-MCHAA at 15 °C (A), 20 °C (B), 25 °C (C) 30 °C (D), and 35 °C (E). pH in all cases was ~6.5. Error bars represent one standard deviation which may not be visible in some cases due to small value.

As seen in Figure 5.8, the developed consortium were able to degrade 4MCHAA at pH of ~6.5 over the selected temperature range with the optimum temperature (corresponding to the fastest biodegradation rate) being 25 °C. The biodegradation rates of *cis*- and *trans*-4MCHAA reached the maximum values of 2.25 and 4.17 mg/L-h at 25 °C, which was 5.4 and 7.4 times greater than the rates obtained at 15°C. Interestingly, similar trends have been reported for *Pseudomononas* grown with different substrates such as *trans*-4MCHCA and 1, 4-benzoquinone (Paslawski, 2008; Kumar, 2010). A summary of biodegradation rates obtained at various temperatures is shown in Table 5.4.

Table 5.4: Summary of biodegradation rates at different temperatures with an initial 4MCHAA concentration of 100 mg/L and pH ~ 6.5.

Temperature (°C)	Biodegradation rate of *cis*-4MCHAA (mg/L-h)	Biodegradation rate of *trans*-4MCHAA (mg/L-h)
15	0.412 (R^2=0.993)	0.565 (R^2=0.991)
20	0.483 (R^2=0.946)	0.740 (R^2=0.959)
25	2.252 (R^2=0.993)	4.171 (R^2=0.993)
30	1.873 (R^2=0.968)	4.044 (R^2=0.978)
35	1.064 (R^2=0.970)	1.860 (R^2=0.973)

5.2.4 Continuous Biodegradation of 4MCHAA

The 1st CPBB which was earlier used for batch experiments was switched to continuous mode and fed with a medium containing 50 mg/L 4MCHAA at a flow rate of 56.3 ml/h. The flow rate was then increased incrementally up to 167 ml/h (56.3, 97.8, 135 and 167 ml/h). The

effect of the volumetric loading rate of 4MCHAA (or residence time) on the performance of the CPBB in terms of biodegradation rate and conversion was examined.

Figure 5.9 shows the biodegradation rate of 4MCHAA as a function of its loading rate. Conversions at various loading rates are also presented in this figure. It was observed that biodegradation rates of both isomers increased with increases in flow rates up to 135 ml/h (corresponding to loading rates of 4.67 mg/L-h (*cis*-4MCHAA) and 10.7 mg/L-h (*trans*-4MCHAA); residence time 3.3 h), with maximum biodegradation rates for *cis*-4MCHAA and *trans*-4MCHAA being 4.17 and 7.80 mg/L-h, respectively. Further increases of flow rate or loading rate led to a decline of biodegradation rate. It also was seen that increases of loading rates led to direct increases in residual steady state substrate concentrations from 0.862 to 9.36 mg/L and 3.59 to 24.8 mg/l for *cis*-4MCHAA and *trans*-4MCHAA, respectively. The highest conversion of 94.5% (*cis*-4MCHAA) and 88.4% (*trans*-4MCHAA) were achieved at a flow rate of 56.3 ml/h (corresponding residence time: 8.0 h). The recorded pH in the reactor was ~ 6.4.

Compared to batch mode of operation, continuous operation was found more efficient for treatment of 4MCHAA. The maximum biodegradation rate was twice as fast compared to batch operation. Similar to batch results, higher biodegradation rates obtained for *trans*-4MCHAA when compared with *cis*-4MCHAA indicated the structural effects on the biodegradation rate, mainly due to the more open molecular structure of the *trans*- isomer. Furthermore, consistent with batch results the biodegradation rate obtained for 4MCHAA was much lower than that for *trans*-4MCHCA. For instance, with *trans*-4MCHCA the maximum biodegradation rate of 209 mg/L-h was achieved at loading rate of 320 mg/L-h (residence time: 0.15 h), while the biodegradation rates for *cis*-4MCHAA and *trans*-4MCHAA were 4.17 and 7.80 mg/L-h, respectively and were achieved at loading rates of 4.67 and 10.7 mg/L-h, respectively (residence

time: 3.2 h). This observation again revealed the more recalcitrant nature of 4MCHAA. Since no study has previously been reported in the literature on the continuous biodegradation of 4MCHAA, a direct comparison could not be made at this point.

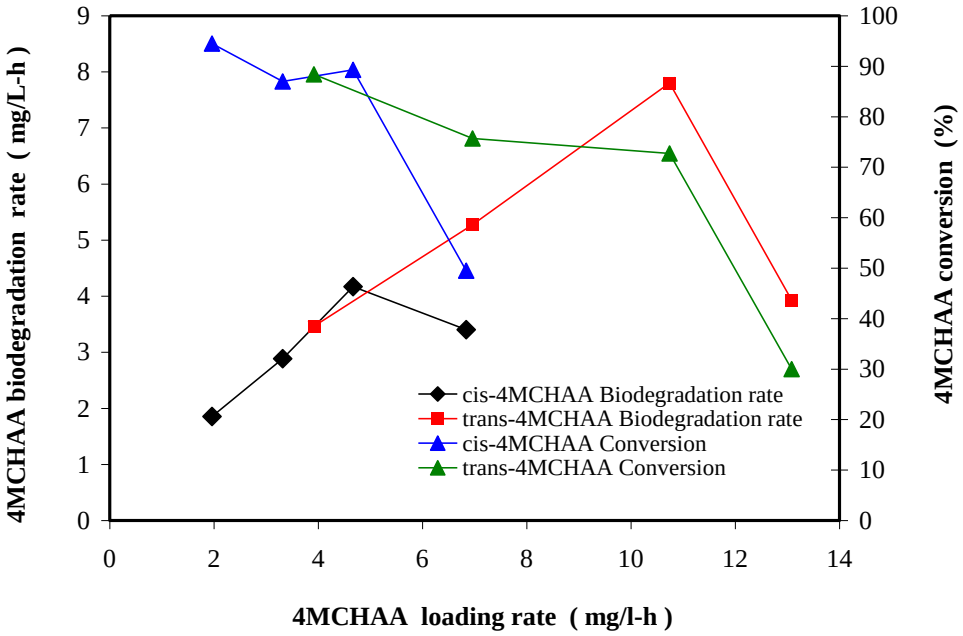


Figure 5.9: The effect of 4MCHAA loading rates on the performance of the CPBB.

5.3 Biodegradation of Octanoic Acid, and Mixtures of Octanoic Acid with *trans*-4MCHCA or 4MCHAA (Co-metabolism)

The developed microbial consortium used in this study has been shown able to degrade the selected single ring structure NAs (*trans*- 4MCHCA and 4MCHAA). However, it is understood that the degradation rate of NAs can be potentially enhanced by using a co-substrate. Octanoic acid, an eight-carbon saturated fatty acid, was selected as a co-substrate for this study and believed to be a suitable candidate. As a relatively inexpensive easy-metabolized substrate, it serves as an additional carbon and energy source, which could help to stimulate the growth of microorganisms and build a more active biofilm leading to increased removal rate of other persistent pollutants. In this study, continuous biodegradation of pure octanoic acid (single

substrate) was conducted firstly. This was followed by batch biodegradation of mixtures of *trans*-4MCHCA and 4MCHAA with octanoic acid in a multi-substrate system to study the co-substrate effect on the rate of biodegradation.

Upon completion of entire experiments conducted in the 2nd CPBB, the bioreactor was disassembled, where the total weight of biomass was measured. The total dry biomass weighed 5.82 g, which was 18.2 % of clean dry packing material (g of dry mass per g of clean dry packing material). The free liquid volume in the 2nd CPBB was 375 ml, which was 80.2 % of the total volume of the bioreactor. The calculated biomass concentration in the 2nd CPBB was 15.5 g/L. This vale was used to determine the relevant specific biodegradation rates.

5.3.1 Continuous Biodegradation of Octanoic Acid

Initially, a medium containing 615 $\pm$ 30mg/L of octanoic acid was continuously fed into the reactor at a flow rate of 52.4 ml/h (corresponding to a loading rate of 95.9 mg/L-h; residence time of 7.06 h). When a removal of 99% was achieved, the flow rates were increased step wise. The tested flow rates were 168, 283, 403, 476, 583 ml/h with the corresponding residence times being 2.20, 1.30, 0.923, 0.78, and 0.63 hours. The biodegradation rate and conversion of octanoic acid as functions of its loading rates obtained in the CPBB are shown in Figure 5.10. As seen the increase in loading rate up to 477 mg/L-h (corresponding to a flow rate of 283 ml/h; residence time of 1.30 h) led to a continuous increase in biodegradation rate of octanoic acid with the highest biodegradation rate and conversion being 352 mg/L-h and 73.8%, respectively. Further increases of loading rate led to the decrease of biodegradation rate. Steady-state residual substrate concentration increased from 8.2 mg/L up to 452 mg/L when loading rate was increased from 95.9 mg/L-h to 933 mg/L-h.

After two weeks operation, a mild NaOH solution was used to remove the excess biomass and prevent the plugging of the bioreactor. The growth of biomass and extent of biofilm when octanoic acid was used as the single substrate was much higher than that observed when *trans*-4MCHCA or 4MCHAA were used as single substrates. To assess the reproducibility of the experimental result, the bioreactor was run at a loading rate of 454 mg/L-h which was close to 477 mg/L-h (corresponding to a flow rate of 283 ml/h; residence time of 1.3 h) applied in the preceding runs. The results obtained in the reproducibility run were relatively close to the initial results. For instance biodegradation rates from the duplicated experiments were found to be 352mg/L-h and 342 mg/L-h and corresponding conversions were 73.8% and 75.2%, with variations for biodegradation rates and conversions being 2.8% and 1.9%, respectively. Additionally, the measured pH and temperature in the reactor was 6.21 to 6.34 and 25 °C, respectively.

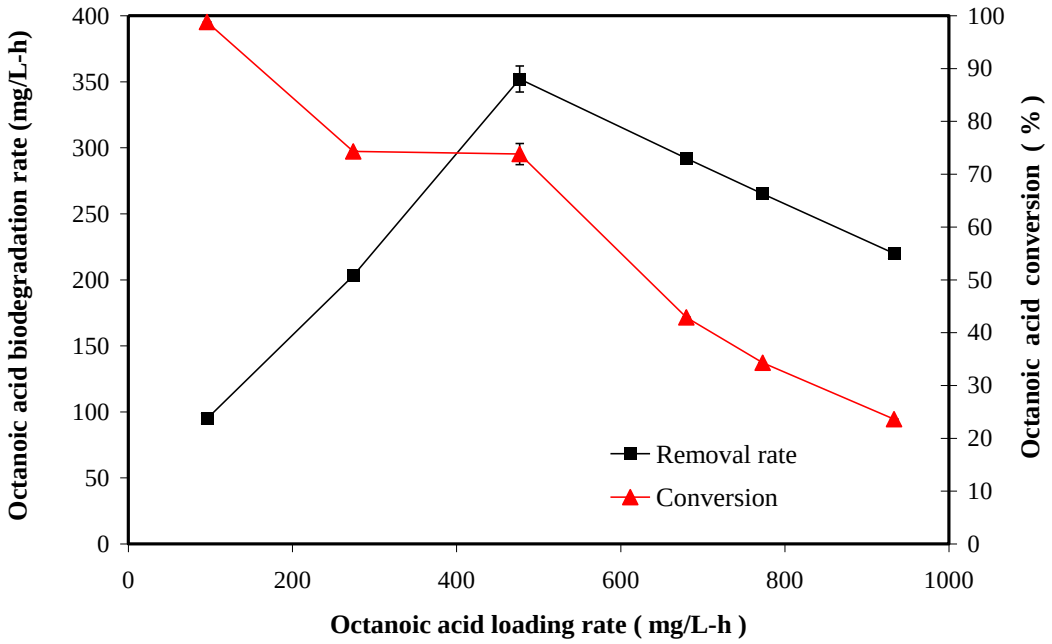


Figure 5.10: The effect of octanoic acid loading rates on the performance of CPBB

The maximum observed removal rate of octanoic acid in this study was 352 mg/L-h observed at a loading rate of 477 mg/L-h and a conversion of 73.8%. Considering that the total biomass concentration measured in the bioreactor was 15.7 g/L and working volume of 375 ml, the corresponding specific biodegradation rate was calculated as 0.0224 mg substrate /mg biomass -h. In contrast, the highest biodegradation rates of *trans*-4MCHCA and 4MCHAA from the previous continuous experiments were 209 mg/L-h (*trans*-4MCHCA), 4.17 mg/L-h (*cis*-4MCHAA), and 7.80 mg/L-h (*trans*-4MCHAA), which corresponded to specific biodegradation rates of 3.32×10^{-2} mg substrate /mg biomass -h, 6.63×10^{-4} mg substrate /mg biomass -h, and 1.24×10^{-3} mg substrate /mg biomass -h, respectively, where the total biomass concentration measured in the 1st bioreactor was 6.29 g/L with working volume of 450 ml.

Higher specific biodegradation rates obtained for octanoic acid compared to 4MCHAA using the same mixed microbial populations indicated the suitability of octanoic acid as a co-substrate which could enhance cell mass (and/or cell number density) and production of non-specific enzymes that promote degradation of other contaminants such as *trans*-4MCHCA or 4MCHAA.

Interestingly, the specific biodegradation rates of *trans*-4MCHCA were slightly higher than octanoic acid in this study. This could be potentially attributed to the acclimation of developed cultures in utilizing *trans*-4MCHCA. The mixed culture used in this study was maintained on *trans*-4MCHCA and subcultured regularly for a long period of time (~5 years). Therefore, as a growth substrate, *trans*-4MCHCA has been completely adopted by the microorganisms. In contrast, acclimation period of microbial populations to octanoic acid was approximate 3 weeks, which could result in slow degradation at this point.

5.3.2 Batch Biodegradation of *trans*-4MCHCA Using Octanoic Acid as the Co-Substrate

Upon competition of continuous biodegradation of octanoic acid, batch experiments were conducted to evaluate the effect of addition of octanoic acid as a co-substrate on biodegradation rate of *trans*-4MCHCA. In these experiments, 515 $\pm$ 18 mg/L octanoic acid and *trans*-4MCHCA at various initial concentrations of 50, 100, and 250 mg/L were utilized. Additional duplicate batch experiments were also performed to assess the reproducibility of the results in which biodegradation of octanonic acid (350 mg/L) and *trans*-4MCHCA at concentrations of 447mg/L and 453 mg/L. Figure 5.10 illustrates the results of substrate utilization in the bioreactor. The biodegradation rates were determined using the slopes of the linear part of the concentration profiles. Data collected before complete exhaustion of octanoic acid were used for the calculation of biodegradation rate. Data obtained in the repeated experiments are compared in panel (D) of Figure 5.10.

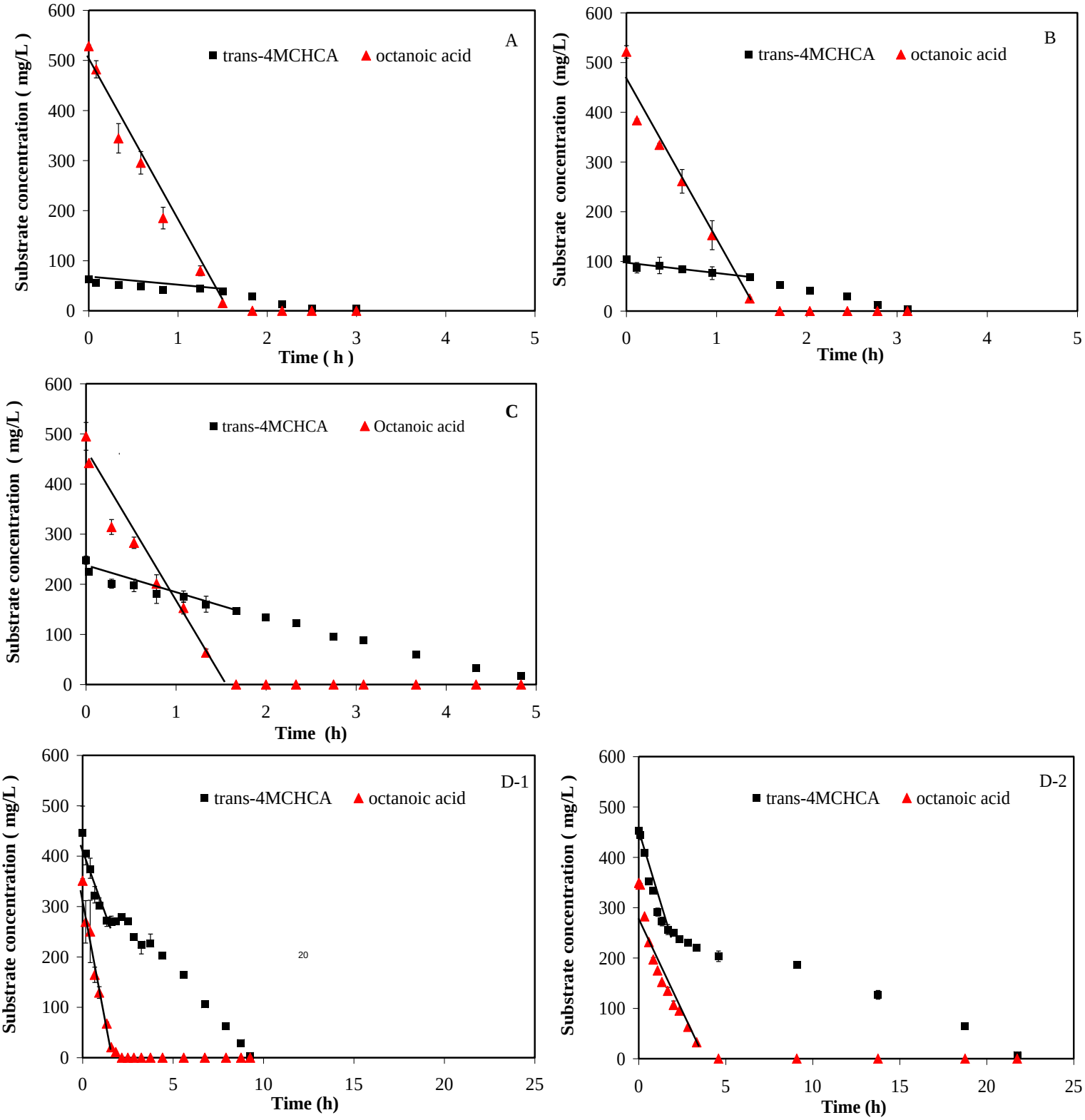


Figure 5.11: Substrate biodegradation as a function of time. Octanoic acid concentration 515 ± 18 mg/L and *trans*-4MCHCA concentrations of 50 (A), 100 (B), and 250 mg/L (C). Duplicate experimental results are presented in the panel D, where octanoic acid concentration set as 350 mg/L and the tested *trans*-4MCHCA concentrations were 447mg/L and 453 mg/L. Error bars represent one standard deviation and may not be visible for some cases

It was observed that biodegradation of octanoic acid and *trans*-4MCHCA occurred simultaneously. However, degradation of octanoic acid was much faster than *trans*-4MCHCA that confirmed that linear NAs are more amenable to biodegradation when compared with those with a ring-structure. Additionally, no lag phase was observed in any cases and pH remained in the range of 5.4 to 6.7. As shown in panel D-1 and D-2 of Figure 5.10, experimental results from duplicate experiments were close, with the calculated biodegradation rates of *trans*-4MCHCA were found to be 112 mg/L-h and 97.2 mg/L-h. This translated to 13.2 % variation indicating the reproducibility of experimental result. Calculated biodegradation rates from these experiments and those obtained in the absence of octanoic acids are presented in Table 5.5.

Table 5.5: Summary of the biodegradation rate of *trans*-4MCHCA in the presence and absence of octanoic acid

4-MCHCA concentration (mg/L)	Enhanced biodegradation rate of *trans*-4MCHCA (mg/L-h) (present work)[a]	Enhanced biodegradation rate of *trans*-4MCHCA (mg/L-h) (present work)[b]	Biodegradation rate of *trans*-4MCHCA (mg/L-h) (early work)[c]	Specific Biodegradation rate of *trans*-4MCHCA (mg substrate / mg biomass-h) (present work)[a]	Specific biodegradation rate of *trans*-4MCHCA (mg susbstrate/ mg biomass-h) (early work)[c]
50	13.580 (R^2=0.864)	35.336 (R^2=0.993)	5.770 (R^2=0.9748)	8.761×10^{-4}	9.173×10^{-4}
100	21.806 (R^2=0.859)	34.839 (R^2=0.992)	18.501 (R^2=0.9942)	1.407×10^{-3}	2.941×10^{-3}
250	55.395 (R^2=0.892)	40.512 (R^2=0.993)	33.067 (R^2=0.9807)	3.573×10^{-3}	5.257×10^{-3}
447[d]	111.660[d] (R^2=0.923)	37.660 (R^2=0.990)	43.504 (R^2=0.9813)	7.203×10^{-3}	6.916×10^{-3}
453[d]	97.195[d] (R^2=0.912)	13.827 (R^2=0.962)		6.270×10^{-3}	

Note: [a] Biodegradation rate calculated based on the data collected before the exhaustion of octanoic acid in the reactor. [b] Biodegradation rate calculated based on the data collected after the exhaustion of octanoic acid in the reactor. [c] Biodegradation rate obtained from early study where trans-4MCHCA used as the sole substrate in the system. [d] Comparison of duplicated experimental results.

As shown in Table 5.5, addition of octanoic acid did not offer significant improvement in specific biodegradation rates of *trans*-4MCHCA. However, it was observed that supplying the medium with octanoic aicd as an additional carbon source increased the biomass hold-up in the bioreactor and resulted in much higher biodegradation rates of *trans*-4MCHCA in all cases. For example, the maximum biodegradation rate of *trans*-4MCHCA was 112 mg/L-h observed at the initial *trans*-4MCHCA concentration of 450 mg/L, supplied with 350 mg/L octanoic acid, which was 2.6 times faster than the maximum value of 43.5 mg/L-h obtained in the absence of octanoic acid.

Similar to previous observations, the biodegradation rate ($r_{trans\text{-}4MCHCA}$) increased proportional to the initial concentration of *trans*-4MCHCA following a linear correlation as expressed in Equation 5.5.

$$r_{trans\text{-}4MCHCA} = 0.1945 \times S_i \qquad (R^2 = 0.975) \qquad (5.5)$$

It indicated no substrate inhibition on the microbial growth for the concentration range tested. Biodegradation rates as functions of initial substrate concentrations are presented in Figure 5.12.

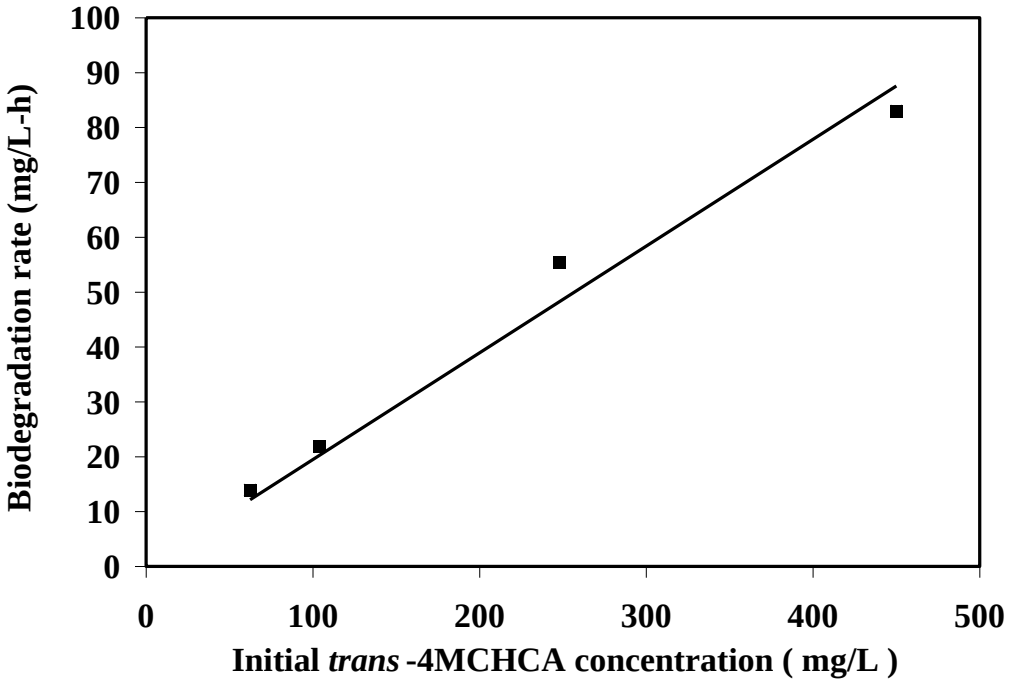
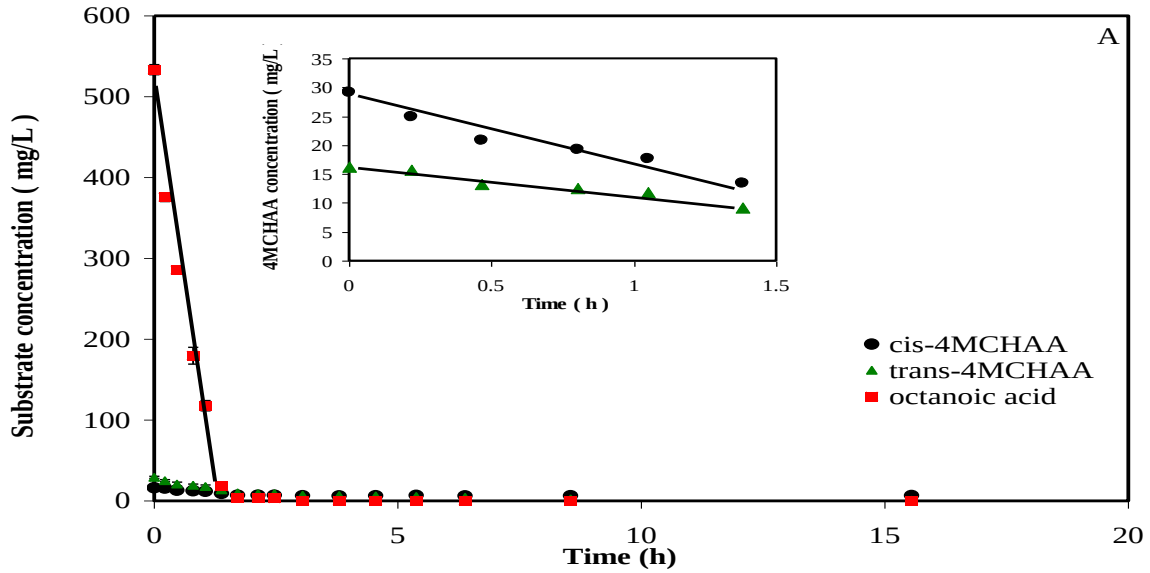
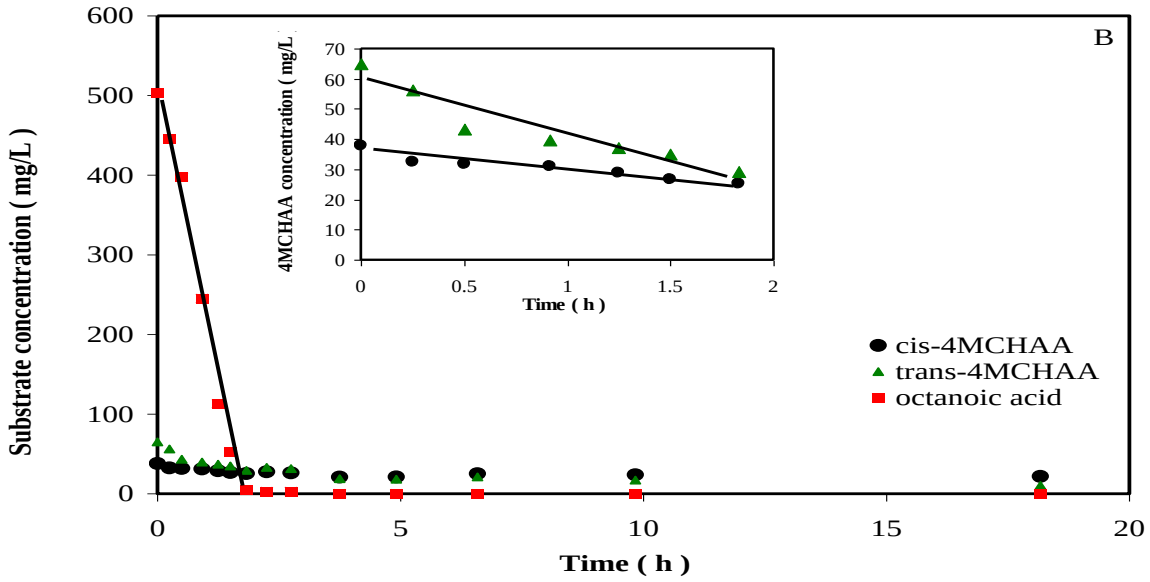


Figure 5.12: Biodegradation rates as functions of *trans*-4MCHCA concentrations.

5.3.3 Batch Biodegradation of 4MCHAA Using Octanoic Acid as the Co-substrate

Batch experiments were also carried out to study biodegradation of 4MCHAA in the presence of octanoic acid as a co-substrate. The initial 4MCHAA concentrations for these experiments were 50,100, and 330mg/L, and the initial octanoic acid concentration was kept constant at 510$\pm$20 mg/L. Figure 5.12 shows the substrate utilization profiles obtained in these experiments. Similar to previous runs with *trans*-4MCHCA, biodegradation of 4MCHAA followed pseudo first – order kinetics and the corresponding biodegradation rates were determined using the slope of linear portion of the concentration profile (lag phase excluded). Only data collected prior to the depletion of octanoic acid in the reactor were used for the calculation of biodegradation rates of 4MCHAA. Calculated enhanced biodegradation rates and specific biodegradation rates of 4MCHAA at various initial substrate concentrations along with comparisons to the early results are presented together in Table 5.6 and 5.7, respectively.





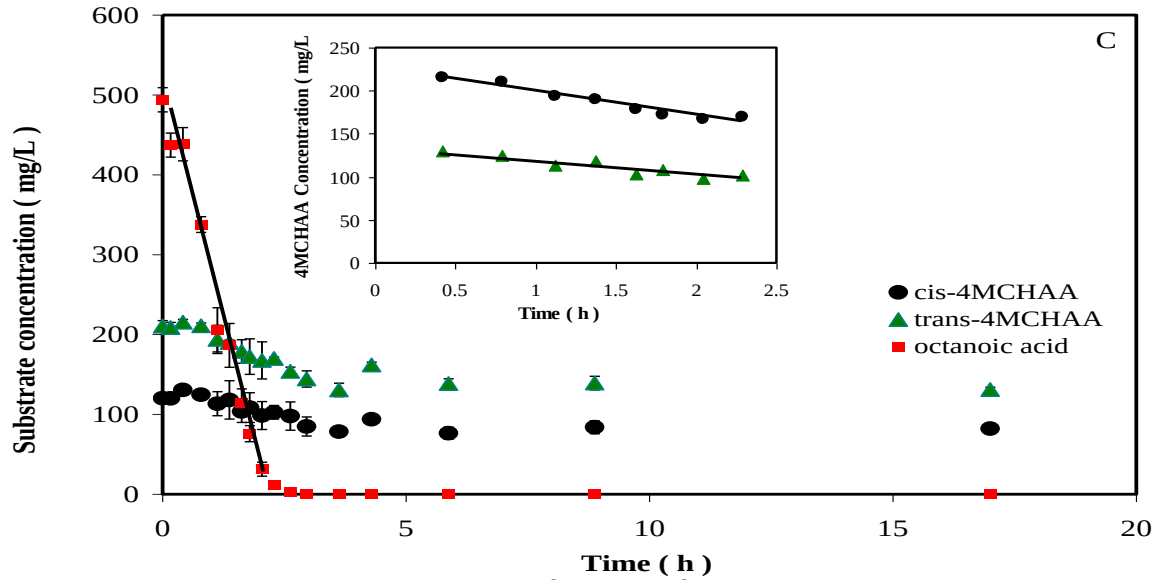


Figure 5.13: Substrate biodegradation as a function of time. Octanoic acid concentration 510 ± 20 mg/L and *trans*-4MCHCA concentrations of 50 (A), 100 (B), and 330 mg/L (C). Error bars represent one standard deviation and may not be visible for some cases.

Table 5.6: Summary of the biodegradation rate of 4MCHAA in the presence and absence of octanoic acid, wher the co-substrate (octanoic acid) concentration set constantly as 510±20 mg/L and varied 4MCHAA concentrations.

4MCHAA concentration (mg/L)	Enhanced biodegradation rate of cis-4MCHAA (mg/L-h) (present work)[a]	Enhanced biodegradation rate of trans-4MCHAA (mg/L-h) (present work)[a]	Enhanced biodegradation rate of cis-4MCHAA (mg/L-h) (present work)[b]	Enhanced biodegradation rate of trans-4MCHAA (mg/L-h) (present work)[b]	Biodegradation rate of cis-4MCHAA (mg/L-h) (early work)[c]	Biodegradation rate of trans-4MCHAA (mg/L-h) (early work)[c]
50	4.891 (R^2=0.957)	10.326 (R^2=0.9543)	0.356 (R^2=0.7794)	1.066 (R^2=0.967)	1.050 (R^2=0.902)	2.486 (R^2=0.915)
100	5.857 (R^2=0.897)	17.517 (R^2=0.887)	0.325 (R^2=0.9374)	1.422 (R^2=0.909)	2.247 (R^2=0.973)	4.165 (R^2=0.987)
330	16.684 (R^2=0.873)	28.376 (R^2=0.947)	1.391 (R^2=0.8861)	2.895 (R^2=0.95)	0.336 (R^2=0.977)	0.660 (R^2=0.968)

Note: [a] Biodegradation rate calculated based on the data collected before the exhaustion of octanoic acid in the reactor.
[b] Biodegradation rate calculated based on the data collected after the exhaustion of octanoic acid in the reactor. [c] Biodegradation rate obtained from early study where trans-4MCHCA used as the sole substrate in the system.

T

Table 5.7: Summary of the specific biodegradation rate of 4MCHAA in the presence and absence of octanoic acid, where the co-substrate (octanoic acid) concentration set constantly as 510±20 mg/L and varied 4MCHAA concentrations.

4MCHAA concentration (mg/L)	Specific biodegradation rate of cis-4MCHAA (mg substrate / mg biomass –h) (present work)[a]	Specific biodegradation rate of trans-4MCHAA (mg substrate / mg biomass –h) (present work)[a]	Specific biodegradation rate of cis-4MCHAA (mg substrate / mg biomass –h) (present work)[b]	Specific biodegradation rate of trans-4MCHAA (mg substrate / mg biomass –h) (present work)[b]	Specific biodegradation rate of cis-4MCHAA (mg substrate / mg biomass –h) (early work)[c]	Specific biodegradation rate of trans-4MCHAA (mg substrate / mg biomass –h) (early work)[c]
50	3.154×10^{-4}	6.661×10^{-4}	2.297×10^{-5}	6.877×10^{-5}	1.669×10^{-4}	3.952×10^{-4}
100	3.779×10^{-4}	1.130×10^{-4}	2.097×10^{-5}	9.110×10^{-5}	3.572×10^{-4}	6.622×10^{-4}
330	1.076×10^{-3}	1.831×10^{-4}	8.974×10^{-5}	1.868×10^{-4}	5.341×10^{-5}	1.049×10^{-4}

Note: [a] Specific biodegradation rate calculated based on the data collected before the exhaustion of octanoic acid in the reactor. [b] Specific biodegradation rate calculated based on the data collected after the exhaustion of octanoic acid in the reactor. [c] Specific biodegradation rate obtained from early study where trans-4MCHCA used as the sole substrate in the system.

As shown in Figure 5.13, the biodegradation of 4MCHAA and octanoic acid occur simultaneously at all tested concentrations (50,100, and 330 mg/L). It was observed that the addition of octanoic acid successfully overcame the substrate inhibition problem. It is understood that at high concentrations of substrate, the microbial growth rate is inhibited by the substrate and depends on the inhibitor concentration. Our earlier results with pure 4MCHAA indicated that microbial growth became inhibited at 4MCHAA concentrations above 100 mg/L and resulted in decreased biodegradation rates. However, in the presence of octanoic acid this inhibition was not observed and the biodegradation rate increased linearly with increases of initial 4MCHAA concentration up to 330 mg/L, the highest tested concentration. The following expression represents the dependency of biodegradation rate on the concentration of *cis*- and *trans*-4MCHAA. Biodegradation rate as a function of initial substrate concentrations is also presented in Figure 5.14:

$$r_{cis\text{–}4MCHAA} = 0.1432 \times S_i \qquad (R^2 = 0.918) \qquad (5.6)$$

$$r_{trans\text{-}4MCHAA} = 0.1505 \times S_i \qquad (R^2 = 0.369) \qquad (5.7)$$

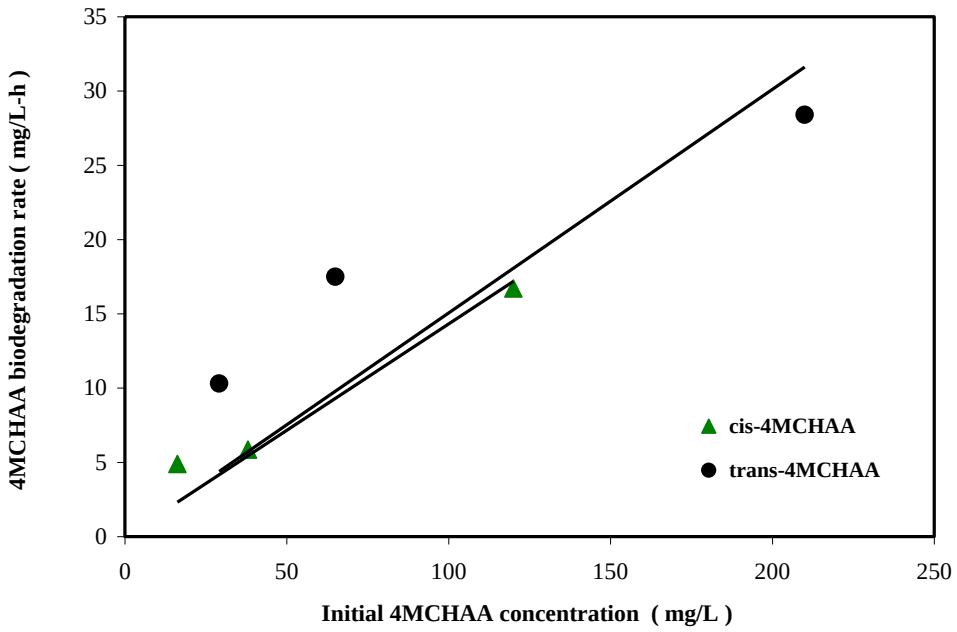


Figure 5.14: Biodegradation rates as functions of 4MCHAA initial concentrations.

It was also observed that supplementation of the medium with 510±20 mg/L octanoic acid as a co-substrate resulted in significant improvement of biodegradation rate as well specific biodegradation rate for 4MCHAA. As a co-substrate, octanoic acid provided an additional carbon source which is much less recalcitrant than 4MCHAA, thus stimulating microbial activity and growth and consequently led to effective removal of 4MCHAA and *trans*-4MCHCA. The maximum biodegradation rates of *cis*-4MCHAA and *trans*-4MCHAA obtained were 16.7 and 28.4 mg/L-h, respectively, almost eight times higher than 2.25 and 4.17 mg/L-h obtained when 4MCHAA was used as the sole substrate from early study. The maximum specific biodegradation rates in the presence of octanoic acid were almost 19 times faster than that with 4MCHAA alone as presented in the Table5.7. Moreover, measured pH during the experiments was found in the range of 5.8 to 6.3 and all experiments were conducted under room temperature (25 °C).

6 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 Conclusions

Bioremediation is a clean technology using microorganisms to transform toxic compounds to less hazardous /non-hazardous forms with less input of chemical, energy and time compared to other remediation technologies. Bioremediation could be considered as one of the most effective, economical, and eco-friendly treatments for NAs contaminated oil sands process water (OSPW). Due to the complexity of NAs mixtures, the present work mainly focused on the batch and continuous biodegradation of model NAs in pure form and in a mixture.

A novel circulating packed bed bioreactor (CPBB) was used to study biodegradation kinetics of the selected naphthenic acids, *trans*-4-methyl-1-cyclohexane carboxylic acid (*trans*-4MCHCA), the *trans*- and *cis*- isomers of 4-methylcyclohexane-acetic acid (4MCHAA), and octanoic acid; using a microbial consortium developed in this study, dominated by *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* (Alcaligenes).

Batch biodegradation results for *trans*-4MCHCA (from 50 up to 500 mg/L) indicated a maximum biodegradation rate of 43.5 mg/L-h at the highest applied concentration of 500 mg/L, which is 9.2 times higher than those reported previously. Biodegradation rates increased as increases of initial substrate concentrations. No substrate inhibitory effect was observed at any of the tested concentrations.

When the CPBB was run continuously, the achieved highest removal rate of *trans*-4MCHCA was 209 mg/L-h corresponding to a specific biodegradation rate of 0.0332 mg substrate /mg biomass- h. This biodegradation rate was approximately 22 times faster than that reported for a continuous stirred tank reactor (CSTR) and 3.8 times faster than that obtained in a

packed-bed bioreactor in term of specific biodegradation rate (Paslawski et al., 2009). Significant improvements of biodegradation rate in different bioreactor configurations demonstrate that *ex*-situ biodegradation of NAs can be enhanced by modifying the bioreactor design (i.e. use of CPBB).

Acclimation of the developed microbial consortium before studying biodegradation of 4MCHAA ensured that species with the ability to metabolize 4MCHAA became dominant in the mixed culture, or the existing species develop such ability. A gradual increase of initial 4MCHAA concentration from 25 to 70mg/L and holding initial *trans*-4MCHCA concentration constant at 100 mg/L was found to be an effective strategy to achieve this objective.

Batch experimental results with 4MCHAA (mixture of *cis*- and *trans*- isomers) indicated that the biodegradation rate was dependent on the 4MCHAA concentration. However, in this case the pattern was different from that observed with *trans*-4MCHCA. The biodegradation rate increased as the substrate concentration increased to 100 mg/L. Further increases of concentrations resulted in reduction of the biodegradation rate indicating a potential inhibitory effect of substrate. The maximum biodegradation rates of *cis*-4MCHAA and *trans*-4MCHAA observed at an initial concentration of 100 mg/L were 2.25mg/L-h and 4.17 mg/L-h, respectively. These values are three orders of magnitude higher than previous literature values obtained in a conventional batch bioreactor (Tanapat, 2001), which again showed the superior performance of the circulating packed-bed bioreactor. Also, higher biodegradation rates obtained for *trans*-4MCHAA compared to *cis*-4MCHAA indicated the structural effects on the biodegradation rate, mainly due to the more open molecular structure of *trans*-isomer of 4MCHAA.

The effect of the volumetric loading rate of 4MCHAA on the performance of the CPBB in terms of biodegradation rates was examined through continuous biodegradation experiments. It was observed that the removal rates of both isomers increased with increases of flow rate up to 135 ml/h (corresponding to a loading rate of 4.67 mg/L-h of *cis*-4MCHAA) and 10.7 mg/L-h of *trans*-4MCHAA; residence time of 3.33 h), with the maximum biodegradation rates being 4.17 and 7.80 mg/L-h, respectively. Further increases of flow rate or loading rate led to a decline of removal rate. The *cis*-4MCHAA and *trans*-4MCHAA conversion ranged from 49.5% to 94.5% and 30.0% to 88.4%, for the highest and lowest applied loading rates of 6.84 and 1.96 mg/L-h (*cis*-4MCHAA) and 13.1 and 5.88 mg/L-h (*trans*-4MCHAA), respectively. Lower biodegradation rates for 4MCHAA obtained in both batch and continuous operations when compared to *trans*-4MCHCA could be potentially attributed to structural differences, namely difference of attachment of the cartboxylic acid to the cyclohexane ring (direct attachment in case of *trans*-4MCHCA and attachment via $–CH_2$ group for 4MCHAA). As discussed earlier, similar to batch results, biodegradation of *trans*-4MCHAA was faster than *cis*-4MCHAA that again could be attributed to the structural differences of these two isomers.

Evaluation of temperature effects (15 to 35°C in 5 °C increments) indicated that 100% conversion of 4MCHAA at an initial concentration of 100mg/L was achieved in all cases. However, batch experiments indicated that biodegradation was strongly influenced by the temperature with 25 °C being the optimum temperature. The maximum biodegradation rate for *cis*-4MCHAA and *trans*-4MCHAA at this optimum temperature was 2.25mg/L-h and 4.17 mg/L-h, respectively, which was 5.4 and 7.4 times faster than that at 15 °C in which the lowest rates were observed 0.412 and 0.565 mg/L-h.

Continuous biodegradation of octanoic acid was investigated in the 2nd CPBB. Experimental results showed that the removal rates of octanoic acid increased with increases in loading rates up to 477 mg/L-h (corresponding to a flow rate of 283 ml/h and a residence time of 1.30 h). Further increases of loading rate led to the decrease of removal rate. The maximum removal rate observed in this study was 352 mg/L-h with a conversion of 73.8%. Much higher specific biodegradation rates were obtained with octanoic acid (using the same microbial populations) when compared to 4MCHAA. This indicates that a linear NA could be more amenable to biodegradation than aromatic NAs. Moreover, it highlighted the potential for using octanoic acid as a co-substrate to stimulate the cell growth and enhance the biomass hold-up in the bioreactor as a means to improve the biodegradation of *trans*-4MCHCA or 4MCHAA.

Biodegradation studies of single ring NAs in the presence of octanoic acid as a co-substrate revealed that addition of octanoic acid (~ 500 mg/L), significantly improved the biodegradation rate of *trans*-4MCHCA and 4MCHAA. The maximum biodegradation rate of *trans*-4MCHCA was 112 mg/L-h, which was 2.6 times faster than the maximum value of 43.5 mg/L-h from the early study where *trans*-4MCHCA was used as a sole substrate in the designed system. Similarly, the highest biodegradation rates of *cis*-4MCHAA and *trans*-4MCHAA were 16.7 and 28.4 mg/L-h, almost 7.4 and 6.8 times higher than the maximum rates of 2.25 and 4.17 mg/L-h in the absence of octanoic acid.

6.2 Recommendations for Future Work

The work presented in this thesis indicated that a single ring naphthenic acid can be treated efficiently by running the CPBB in either batch or continuous operation. Comparison of experimental results to the literature indicated that the biodegradation rate of NAs can be significantly enhanced by varying environmental conditions and using a proper designed bioreactor. However, further research in certain specific areas, as listed below, are required for more effective treatment and practical applications in treatment of oil sand tailings which are comprised of a complex mixture of NAs.

Research with different commercially available pure compounds should be carried out to assess the NAs molecular structural effects on biodegradation kinetics. Particularly, it would be important to investigate the effect of the number of cycloalkane rings and/or position of alky-groups respective to the ring. These studies should also be expanded to investigate biodegradation of commercially available NA mixtures and those which are extracted from the oil sand tailings.

In this study biodegradation of naphthenic acids was investigated under aerobic conditions. There is little information about NAs biodegradation under an anaerobic condition. Further evaluation of the anaerobic process would be beneficial, especially that it could provide a basis for understanding the potential for *in*-situ bioremediation of oil sands naphthenic acid.

The biological degradation of pollutants can be enhanced by a chemical pretreatment step, especially in case of recalcitrant NAs. The designed bioreactor could be used in conjunction with a chemical pretreatment step which requires a thorough understanding of various chemical treatments. Thus, a study on the effects of various oxidants such as ozone, hydrogen peroxide or

chlorine dioxide on oxidation of NAs and identification of intermediates formed as a result of such treatments would be beneficial.

Co-metabolism has been demonstrated to be a feasible strategy to improve degradation efficiency of model NAs in this study. However, more detailed investigations of the effect of co-substrates, especially on biodegradation of more complex NA and NA extracted from the tailing ponds is recommended.

Mathematical models should be developed to predict the performance of the circulating packed bed bioreactor with respect to biodegradation of NAs. Such models would be also useful in the design and control of large scale systems. Development of such models, however, requires a thorough understanding of the intrinsic kinetics and kinetic expressions governing biodegradation of NAs. Thus kinetic studies focusing on microbial growth and biodegradation are recommended.

7 REFERENCES

Allen, E.W. 2008. Process water treatment in the oil sands industry: I. Target pollutants and treatment objectives. J. Environ. Eng. Sci. **7**: 123–138. doi:10.1139/S07-038.

Allen, E.W. 2008. Process water treatment in the oil sands industry:II. A review of emerging technologies. J. Environ.Eng. Sci. **7**: 499–524. doi:10.1139/S08-020.

Bahnemann, D.(2004). Photocatalytic water treatment: solar energy applications. Sol. Energy, 77:445-459. DOI10.1016/j.solener.2004.03.031.

Barrow, M. P., J. V. Headley, K. M. Peru and P. J. Derrick, "Fourier Transform Ion Cyclotron Resonance Mass Spectrometry of Principal Components in Oilsands Naphthenic Acids," J. Chromatogr. A. **1058,** 51-59 (2004).

Bataineh, M., A. C. Scott, P. M. Fedorak and J. W. Martin, "Capillary HPLCQTOF-MS for Characterizing Complex Naphthenic Acid Mixtures and their Microbial Transformation," Anal. Chem. **78,** 8351-8361 (2006).

Bessa, E., Sant'Anna, G.L., and Dezotti, J. 1999. Photocatalysis: An approach to the treatment of oil field produced waters. J. Adv. Oxid. Technol. **4**: 196–202.

Biryukova, O.V., Fedorak, P.M., Quideau S.A. (2006). Biodegradation of naphthenic acids by rhizosphere microorganisms. *Chemosphere*, *67*(10), 2058-2064.

Brient, A., P.J.Wessner, M.N.Doyle (1995). Napthenic acids. In *Kirk-Othmer Encyclopaedia of Chemical Technology*, 4th ed.; Kroschwiz, J.I., Ed. JohnWiley and Sons: New York, 1017–1029.

Clemente, J.S., P.M. Fedorak(2005). A Review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. *Chemosphere*, 60(5), 585-600.

Conrad Environmental Aquatics Technical Advisory Group (CEATAG), (1998). *Naphthenic Acids Background Information Discussion Report*, 65 pp.

Deineko, P.S., E.N.Vasil'eva, O.V. Popova, and S.T.Bashkatova (1994). Naphthenic Acids as Antiwear Additives for Jet Fuels, Chem. Technol. Fuel Oils. 30, 343-345

Devipriya, S., S.Yesodharan (2005). Photocatalytic degradation of pesticide contaminants in water. Sol energy Mat. Sol. Cell, 86:309-348. DOI: 10.1016/j.solmat.2004.07.013

Dokholyan, V.K.; A.K.Magomedov (1983). Effects of sodium naphthenate on survival and some physiological-biochemical parameters of some fishes. J. Ichthyol. *23*, 125–128.

Doll, T. E. and F.H. Frimmel (2005). Removal of selected persistent organic pollutants by heterogeneous photocatalysis in water. *Catalysis Today* 101:195–202.

Dutta, T.K. and S. Harayama (2000). Fate of crude oil by the combination of photooxidation and biodegradation. *Environmental Science and Technology* 34: 1500-1505.

Energy Resources Conservation Board (ERCB) (2011).Public Zone Oil Sands.[online]. Available from http://www.ercb.ca/portal/server.pt?open=512&objID=249&PageID=0&cached=true&mode=2 [cited April 2011].

Frank, R.A., R.Kavanagh, B.K.Burnison, G. Arsenault, J.V.Headley, K.M. Peru, G.V.Kraak and K. R. Solomon (2008). Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. *Chemospere* 72: 1309-1314.

Freudenhammer, H., D.W.Bahnemann, L.Bousselmi, S.U. Geissen, S.U. Ghrabi, F.Saleh, A. Si-Salah, U.Simeon, and A.Vogelpohl (1997). Detoxification and recycling of wastewater by solar-catalytic treatment. Water Sci. Technol. **35**: 149–156. doi:10.1016/S0273-1223(97)00020-6.

Fujishima, A., X. Zhang and D.A. Tryk. 2008. TiO2 photocatalysis and related surface phenomenon. *Surface Science Report* 63: 515-582.

Gore, B. A. (2006). *Environmental research at the leading edge.* New York, USA: Nova Science Publisher Inc.

Headley, J.V. and D.W. McMartin. 2004. A review of the occurrence and fate of naphthenic acids in aquatic environments. *Journal of Environmental Science and Health: Part A* 39(8): 1989-2010

Headley, J.V., J. Du, K.M. Peru and D.W. McMartin (2009). Electrospray ionization mass spectrometry of the photodegradation of naphthenic acids mixtures irradiated with titanium dioxide. *Journal of Environmental Science and Health: Part A* 44: 591-597.

Headley, J.V., K.M. Peru, D.W. McMartin and M. Winkler. 2002a. Determination of dissolved naphthenic acids in natural waters using negative-ion electrospray mass spectrometry. *Journal of the AOAC International* 85: 182-187.

Headleyl,J.V., S.Tanapat, G.Putz, and K.M. Peru, (2002). Biodegradation kinetics of geometric lsomers of model naphthenic acids in athabasca river water. *Canadian Water Resources Journal*, *27*(1)

Herman, D.C., P.M. Fedorak and J.W. Costerton. 1993. Biodegradation of cycloalkane carboxylic acids in oil sands tailings. *Canadian Journal of Microbiology* **39**: 576-580.

Holowenko, F.M., M.D. MacKinnon, P.M.Fedorak (2002). Characterization of naphthenic acids in oil sands wastewaters by gas chromatography–mass spectrometry. Water Res. 36, 2843–2855.

Holowenko, F.M., M.D. MacKinnon, P.M. Fedorak (2001). Naphthenic Acids and Surrogate Naphthenic Acids in Methanogenic Microcosms. Water Res. 35**,** 2595-2606.

Hsien, Y. H., K.H. Wang, R.C. Ko and C.Y. Cang. 2000. Photocatalytic degradation of wastewater from manufactured fiber by titanium dioxide suspensions in aqueous solution: a feasibility study. *Water Sci. Tech.* 42:95–99.

Kumar, P., Nemati, M. and Hill, G.A. (2011). Biodegradation kinetics of 1,4-benzoquinone in batch and continuous systems, *Biodegradation*, . Available online at: http://dx.doi.org/doi:10.1007/s10532-011-9465-1

Lo, C. C., B.G.Brownlee, N.J.Bunce (2003). Electrospray-Mass Spectrometric Analysis of Reference Carboxylic Acids and Athabasca Oil Sands Naphthenic Acids, Anal. Chem. **75,** 6394-6400

MacKinnon, M.D. (1989). Development of the tailings pond at Syncrude's oil sands plant: 1978–1987. AOSTRA J. Res. 5, 109–134.

Mackinnon, M., H. Boerger (1986). Description of two treatment methods for detoxifying oil sands tailings pond water. Water Pollut. Res. J. Can. *21*, 496–512.

Mandelstam, J.; McQuillen K., (1968). Biochemistry of bacterial growth. John Wiley&Sons Inc. New York.

Manonmani, H.K. D. H. Chandrashekaraiah, N. Sreedhar Reddy, C. D. Elcey, and A. A. M. Kunhi, Initials. (2000). Isolation and acclimation of a microbial consortium for improved aerobic degradation of α-hexachlorocyclohexane. *J. Agric. Food Chem*, *48*(9), 4341-4351.

McMartin, D. W., (2003). Persistence and Fate of Acidic Hydrocarbons in Aquatic Environments: Naphthenic Acids and Resin Acids, Ph.D. Thesis, Univ. of Saskatchewan, Saskatoon, Canada.

McMartin, D.W., J.V. Headley, D.A. Friesen, K.M. Peru and J.A. Gillies. 2004. Photolysis of naphthenic acids in natural surface water. *Journal of Environmental Science and Health* A 39 (6): 1361–1383.

Meng, A.X. Hill, G.A.; Dalai, A.K. (2002[a]). Hydrodynamic characteristics in an external loop airlift bioreactor containing a spinning sparger and a packed bed. *Ind. Eng Chem.Res41,* 2124-2128.

Mishra, S.,(2009). Microwave Assisted Photocatalytic Treatment of Naphthenic Acids in Water, Ph.D. Thesis, Univ. of Saskatchewan, Saskatoon, Canada.

National Energy Board of Canada (NEB). (2009). Estimated Production of Canadian Crude Oil and Equivalent. [Online]. Available from http://www.neb.gc.ca/clfnsi/rnrgynfmtn/sttstc/crdlndptrlmprdct/stmtdprdctn-eng.html. Retrieved 2009-01-27[cited April 2011]

National Energy Board of Canada (NEB). (2011). Oil Sands. [Online]. Available from http://www.ercb.ca/portal/server.pt?open=512&objID=249&PageID=0&cached=true&mode=2 [cited April 2011]

National Energy Board of Canada (NEB). (2009). Total Crude Oil Exports (m3 and bbl) - Annual. [Online]. Available from http://www.neb.gc.ca/clfnsi/rnrgynfmtn/sttstc/crdlndptrlmprdct/ttlcrdlxprt-eng.html [cited April 2011]

Nienow, A.W. (2000). Aeration biotechnology. *Kirk-othmer encyclopedia of chemical technology*. Wiley-Interscience. .

Nikakhtari, H., (2005). Bioremediation of Industrial VOC Air Pollutants, Ph.D. Thesis, Univ.of Saskatchewan, Saskatoon, Canada (2005).

Paslawski, J. C.,(2008).The kinetics of biodegradation of trans 4-methyl-1-cyclohexane carboxylic acid, Ph.D. Thesis, Univ. of Saskatchewan, Saskatoon, Canada (2008).

Paslawski, J.C., M.Nemati, G.A.Hill, J.V. Headley(2009). Biodegradation kinetics of trans-4-methyl-1-cyclohexane carboxylic acid in continuously stirred tank and immobilized cell bioreactors. *Journal of Chemical Technology and Biotechnology*, *84*(7), pages 992–1000.

Paslawski, J.C., M. Nemati, G.A. Hill, J.V. Headley (2009).Model for biodegradation of naphrthenic acid in a immobilized cell reactor. *Journal of Chemical Technology and Biotechnology*, *87*(3), pages 507–513.

Prince, Roger C. (2009). Bioremediation. *Kirk-Othmer Encyclopedia of Chemical Technology*, Retrieved from http://mrw.interscience.wiley.com/emrw/9780471238966/kirk/article/biorprin.a01/current/html?hd=All,bioremediation doi: 10.1002/0471238961.0209151816180914.a01.pub2

Quagraine, E.K., H.G.Peterson, J.V.Headley(2005). In situ bioremediation of naphthenic acids contaminated tailing pond Waters in the Athabasca Oil Sands Region----Demonstrated Field Studies and Plausible Options: A Review. *Journal of Environmental Science and Health*, 40, 685-722.

Quail, B. E., Hill, G. A., (1991) A Packed-Column Bioreactor for Phenol Degradation: Model and Experimental Verification, J. Chem. Tech. Biotechnol. **52,** 545-557.

Rogers, V. V., K. Liber and M.D. MacKinnon. 2002a. Isolation and characterization of naphthenic acids from Athabasca oil sands tailings pond water. *Chemosphere* 48: 519-527.

Rogers, V.V., M.Wickstrom, K.Liber, M.D. MacKinnon (2002b). Acute and subchronic mammalian toxicity of naphthenic acids from oil sands tailings. Toxicol. Sci. *66*, 347–355.

Schmidt, E., M. Hellwig, and H.-J. Knackmuss. 1983. Degradation of chlorophenols by a defined mixed microbial community. Appl. Environ. Microbiol. 46:1038-1044.

Schramm, L.L.; E.N.Stasiuk,M. MacKinnon (2000). Surfactants in Athabasca oil sands slurry conditioning, flotation recovery, and tailings processes. In *Surfactants: Fundamental and Applications in the Petroleum Industry*; Schramm, L.L., Ed. Cambridge University Press: Cambridge, UK. 365–430.

Schramm, L.L.; Stasiuk, E.N.; MacKinnon, M.(2000). Surfactants in Athabasca oil sands slurry conditioning, flotation recovery, and tailings processes. In *Surfactants: Fundamentals and Applications in the Petroleum Industry*; Schramm, L.L., Ed. Cambridge University Press: Cambridge, UK, 365–430.

Scott, A. C., M. D. MacKinnon and P. M. Fedorak. (2005). Naphthenic acids in Athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. *Environ. Sci. Technol* 39: 8388-8394.

Scott, A.C., W.Zubot, M.D. Mackinnon, D.W.Smith, P.M. Fedorak (2008). Ozonation of oil sands process water removes naphthenic acids and toxicity . *Chemosphere*, 71(1), 156-160.

Shell Canada. 2005. The Athabasca Oil Sands Project. 2004 Sustainability Report. [online]. Available from http://www.shell.com/static//caen/downloads/about_shell/what_we_do/aosp_sd_report.pdf [cited 1 February 2006].

Shuler, M.L., Kargi, F., (2002). *Bioprocess engineering basic concepts* . Upper Saddle River, NJ, USA : Prentice Hall PTR.

Singer, P.C., and Reckhow, D.A.(1999). Chemical Oxidation. In Water Quality ad Treatment. A Handbook of Community Water Suppliers. American Water Association , McGraw-Hill, Inc., New York. No.12, p.12.1-12.51

Stephenson, T., J. N. Lester, and R. Perry. 1984. Acclimatisation to nitrilotriacetic acid in the activated sludge process. Chemosphere 13:1033-1040.

Suncor Energy. (2005). Suncor Energy 2005 Report on Sustainability.[online]. Available from http://www.suncor.com/data/1/rec_docs/616_Suncor%20SD%20Report_2005%20.pdf [cited February 2011].

Syncrude Canada. (2010). 2010 Annual Tailings Plan Submission Syncrude Aurora [online]. Available from http://www.ercb.ca/docs/products/TailingsPlans/Syncrude_2010_AuroraNorth_Submission.pdf [cited 16 April 2011]

Syncrude Canada. (2004). Syncrude Canada Limited 2004 Sustainability Report. [online]. Available from http://sustainability.syncrude.ca/sustainability2004/download/SyncrudeSD2004.pdf [cited February 2011]

Tanapat, S., (2001)" Comparison of the kinetics of biodegradation of geometric isomers of naphthenic acids (NAs) in Athabasca river water" M.Sc. Thesis, Univ. of Saskatchewan, Saskatoon, Canada .

Torstensson, N. T. L., J. Stark, and B. Goransson. 1975. The effect of repeated applications of 2,4-D and MCPA on their breakdown in soil. Weed Res. 15:159-164.

U.S. Energy Information Adminstration (USEIA) (2011). Crude Oil and Total Petroleum Imports Top 15 Countries. [online] available from http://www.eia.gov/pub/oil_gas/petroleum/data_publications/company_level_imports/current/import.html [cited from April 2011]

Williams, J.A. (2002, March). Keys to bioreactor selections . *Chemical Engineering Progress*, 98(34-41), Retrieved from http://www.aiche.org/uploadedFiles/SBE/DepartmentUploads/KEYSTO7E1.pdf

Walker, R. L., and A. S. Newman. 1956. Microbial decomposition of 2,4 dichlorophenoxyacetic acid. Appl. Microbiol. 4:201-206.

8 APPENDICES

A. Calibration Curves for Analytical Methods

Utilization of GC-FID for direct analysis of model NAs in water and biological media requires a liner calibration curve to convert GC reading (uv.min) into actual concentration (mg/L). During the research standard solutions were prepared for each candidate NA. The generated calibration curves were updated regularly to ensure the accuracy of experimental results. The representative calibration curves for the model NA are presented through Figure A.1, to A.3.

a. Representative Calibration Curve for *trans*-4MCHCA

The calibration curve for the measurement of trans-4MCHCA is shown in the Figure B.2. The equation of the best fit line was the following:

$$C_{biomass} = 0.0347 \times \mathrm{Re}ading - 1.2032 \quad (R^2=0.997) \qquad \text{(A-1)}$$

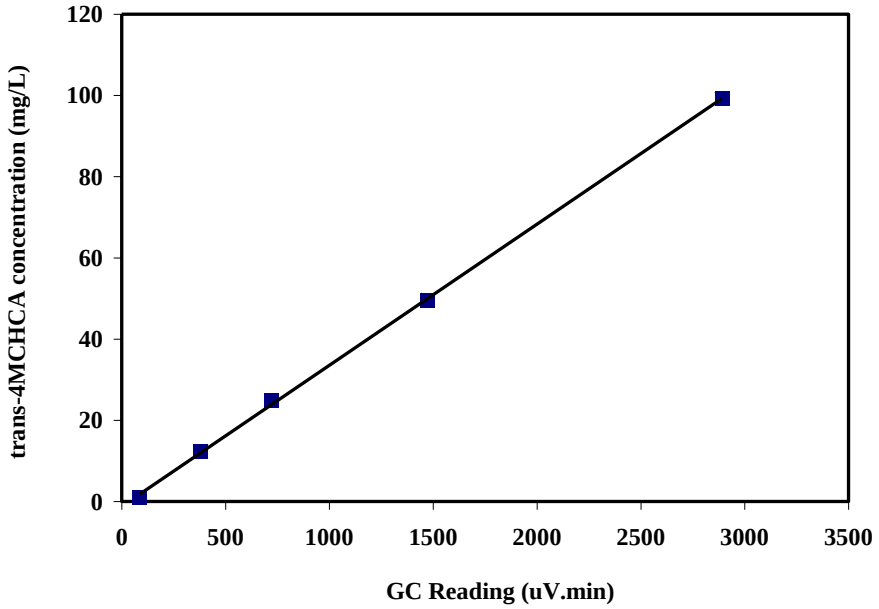


Figure A.1: The representative calibration curve for trans-4MCHCA concentration measurement. Error bars represent standard deviation in GC readings and may not visible as the associated error is small.

b. Representative Calibration Curve for 4MCHAA

Calibration curves for the measurement of 4MCHAA are shown in the Figure B.3. Due to the presence of two isomers (trans-and cis-) in this compound, two calibration curves are require for the determination of the concentration of individual isomer. The equation of the best fit lines for trans- and cis- 4MCHAA are shown in the Equation A.2 and A.3, respectively.

$$C_{trans\text{-}4MCHAA} = 0.0254 \times reading + 1.4761 \quad (R^2=0.9967) \qquad (A.2)$$

$$C_{cis\text{-}4MCHAA} = 0.0254 \times reading + 0.8215 \quad (R^2=0.9971) \qquad (A.3)$$

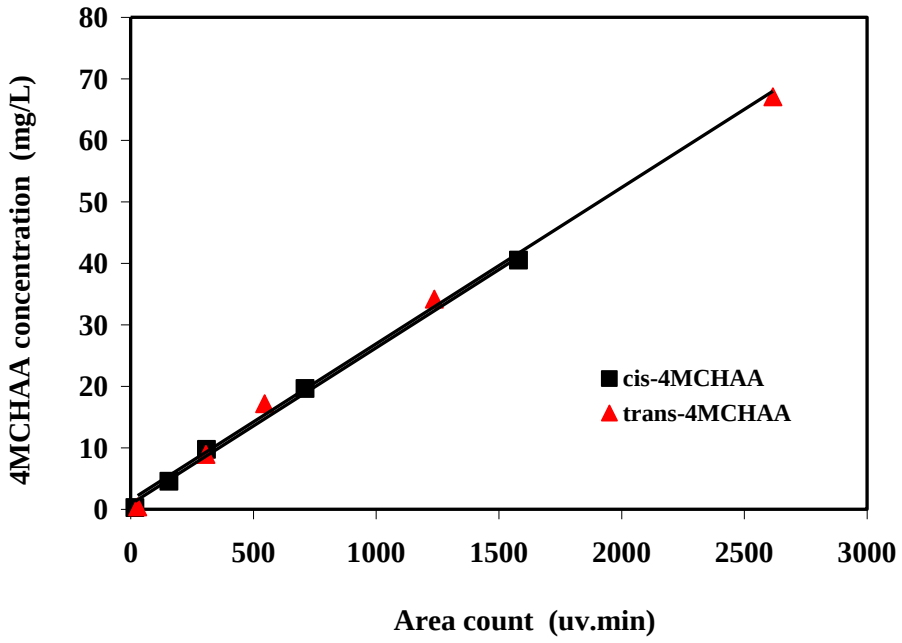


Figure A.2: The representative calibration curves for *trans*- and *cis*- 4MCHCA concentration measurement. Error bars represent standard deviation in GC readings and may not visible as the associated error is small.

c. Representative Calibration Curve for Octanoic Acid

The calibration curve for the measurement of octanoic acid is shown in the Figure B.4. The equation of the best fit lines was following:

$$C_{OctanoicAcid} = 0.046 \times reading - 0.954 \quad (R^2=0.9954) \qquad (B.5)$$

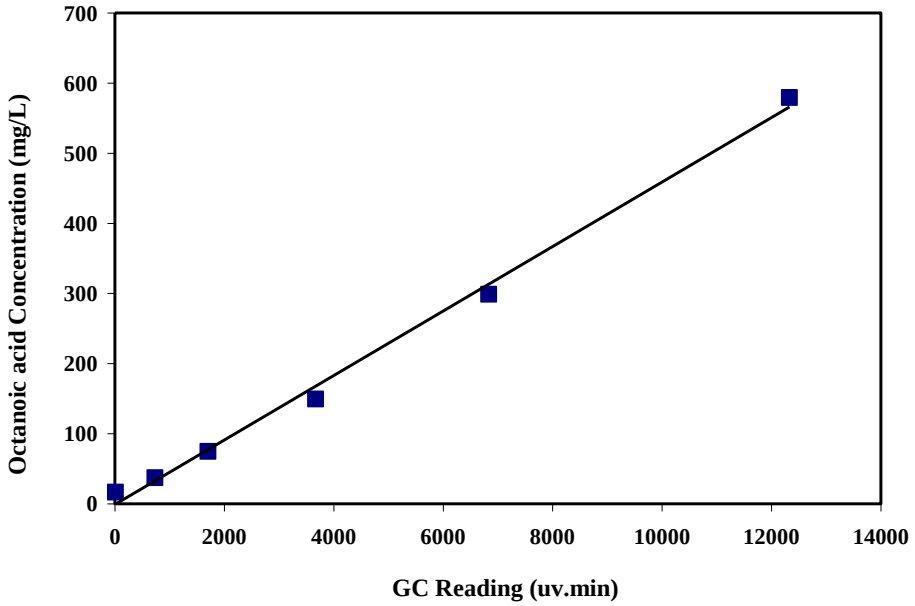


Figure A.3: The representative calibration curve for octanoic acid concentration measurement. Error bars represent standard deviation in GC readings and may not visible as the associated error is small

B. Quantification of *tans*- and *cis*- isomer of 4MCHAA Mixture

Headley et al. (2002) reported that the *trans*-isomer eluted before the *cis* –isomer for 4MHCAA in a non-polar column (RXI-5MS column from Restek Corporation, 2010; Cat. # 13423). However, it was found that this elution order was reversed when using a polar column applied for this work (HP-INNOWAX purchased from Agilent Technologies Canada Inc, 2010; Part # 19091N-133), where identifications were based on comparison of their measured relative abundances. The elution time of all the investigated NAs was 3.40min (octanoic acid), 3.72min

(*trans*-4MCHCA), 4.10min (*cis*-4MCHAA), and 4.27min (*trans*-4MCHAA). The representative GC-FID chromatogram of these three model NAs is shown in the Figure B.1

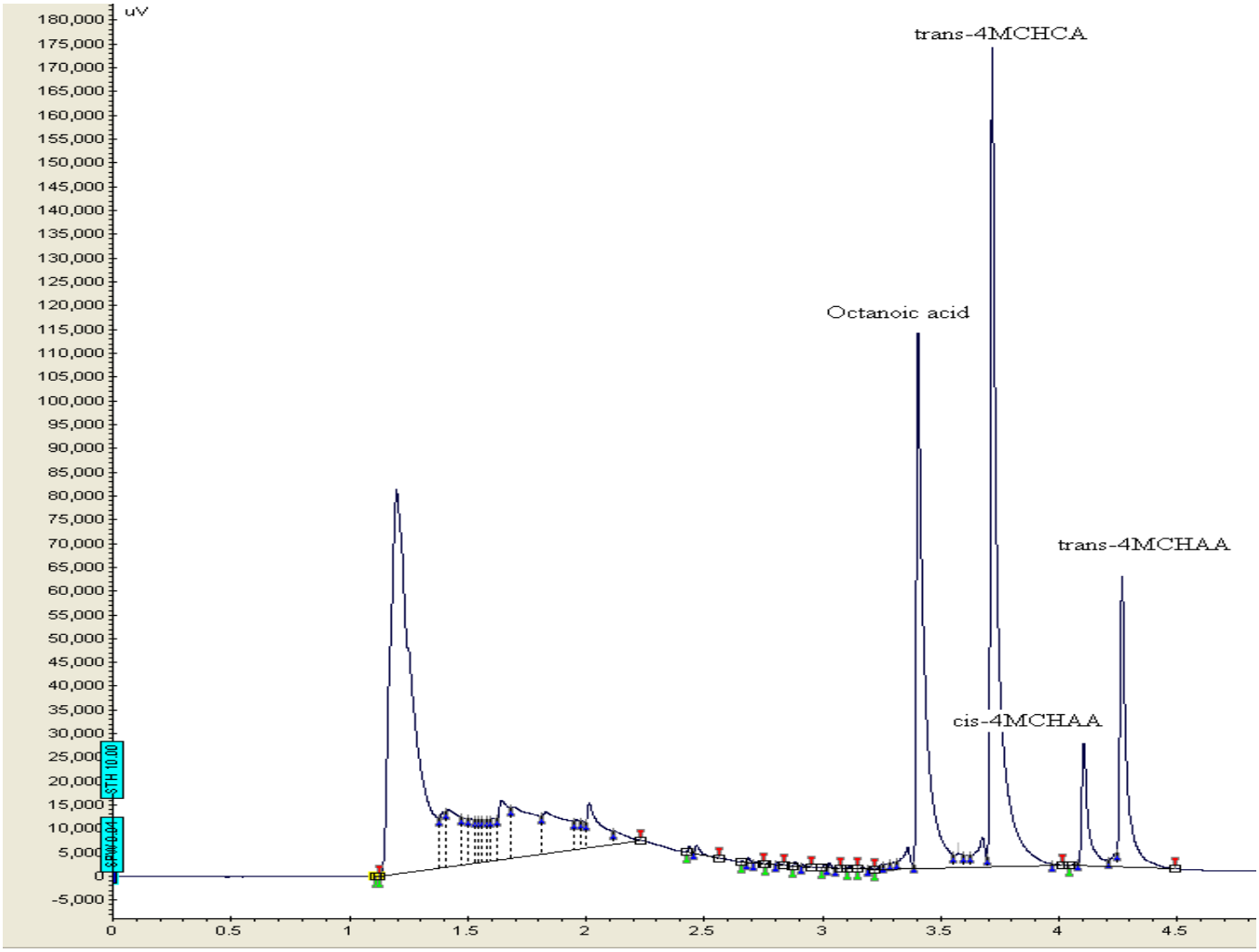


Figure B.1: The representative GC/FID chromatogram of the three NAs investigated.

Furthermore, mass percentage of individual isomers in this product were unavailable from material data sheets, quantifying the composition prior to investigation was essential. *cis*- and *trans*- isomers of 4MCHCAA appear as two adjacent peaks in the GC-FID analysis, where the corresponding concentrations of individual isomers were determined by area count of each

peak. Therefore, the weight percentages of *cis*-4MCHAA and *trans*-4MCHAA were calculated by the following Equations B.1 and B.2, respectively.

$$mass_{cis-4MCHAA}\,\% = \frac{AreaCount_{4MCHAA1}}{AreaCount_{4MCHAA1} + AreaCount_{4MCHAA2}} \qquad \text{(B.1)}$$

$$mass_{trans-4MCHAA}\,\% = \frac{AreaCount_{4MCHAA2}}{AreaCount_{4MCHAA1} + AreaCount_{4MCHAA2}} \qquad \text{(B.2)}$$

Using the above two equations, analytical results indicated that the mixture of 4MCHAA consisted of 30-40 % (35% average) *cis*-4MCHAA and 60-70% (65% average) of *trans*-4MCHAA. The consistency and reproducibility of this result has been proved during this study and used to monitor the quantitative concentrations of the compounds.