

EFFECT OF QUATERNARY AMMONIUM IONIC LIQUIDS ON FERMENTATION
OF CELLULOSE BY BACTERIA

by

SEYEDMEHDI EMADIAN

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OF CELLULOSE BY BACTERIA

APPROVED BY:

Assist. Prof. Ulaş TEZEL
(Thesis Supervisor)

Prof. Ayşen ERDİNÇLER

Assoc. Prof. Adile Evren TUĞTAŞ

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dedicated to my family

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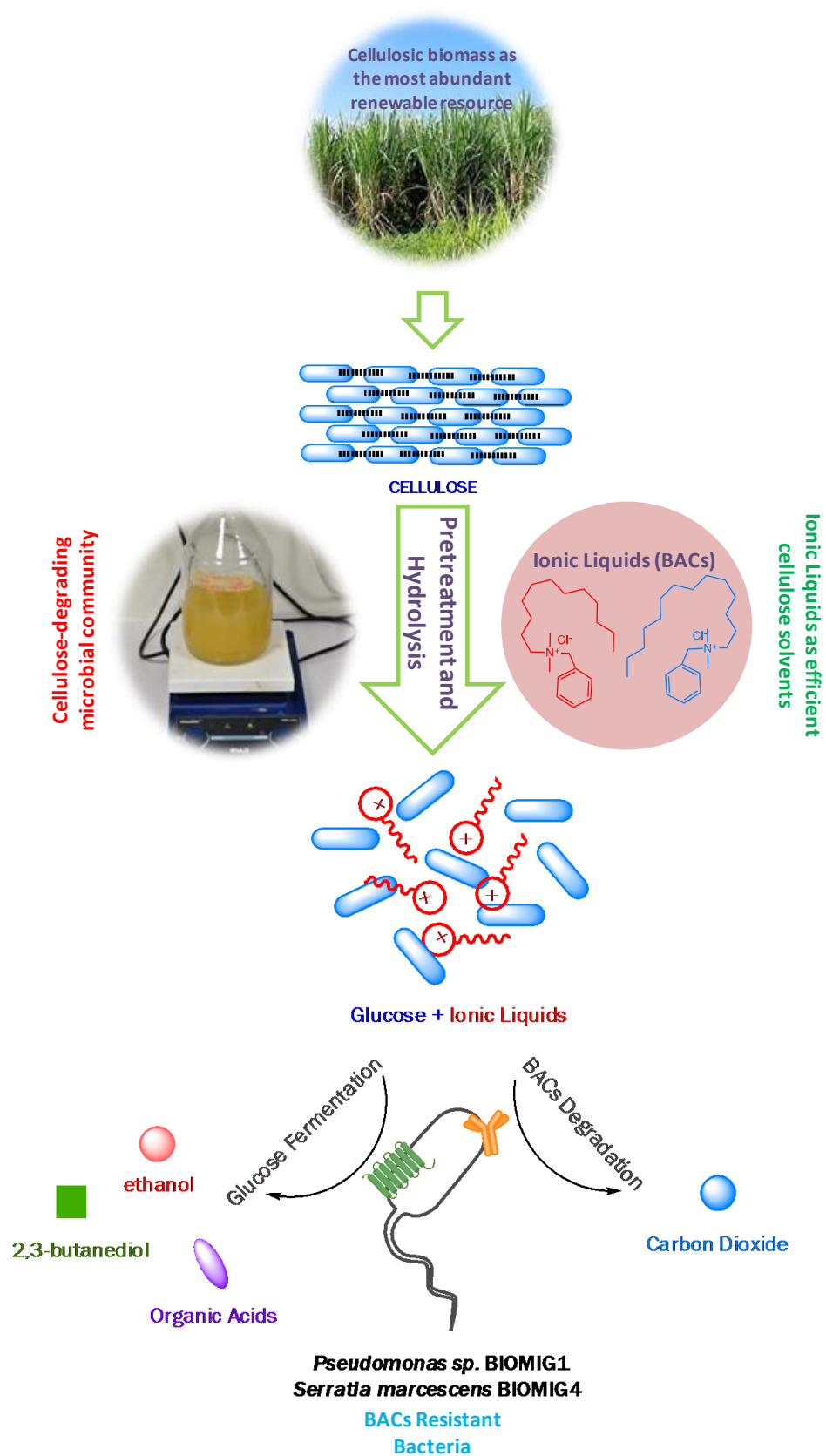
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EFFECT OF QUATERNARY AMMONIUM IONIC LIQUIDS ON FERMENTATION OF CELLULOSE BY BACTERIA

Biofuel and chemical production from lignocellulosic biomass gained significant attention due to high demand of fuels and chemicals. The polysaccharides in lignocellulosic biomass are converted to monosaccharides through enzymatic hydrolysis. The monosaccharides are converted to bioethanol and bio-based chemicals via fermentation. However, the recalcitrance of lignocellulosic biomass to hydrolysis demands a pretreatment process. Current pretreatment methods either have high energy demand or produce wastestreams that are harmful to the environment. Recently, ionic liquids (ILs), which are salts in liquid state, are introduced and utilized as green solvents for cellulose dissolution. However, recent studies showed that ILs are not as “green” as they thought to be in the past. ILs are not only inhibitory to biological processes following the pretreatment, but also they cause ecotoxicity and proliferate antimicrobial resistance among bacteria when released into the environment. The main objective of this research is to couple pretreatment, hydrolysis and fermentation for production of value-added alcohols and organic acids from cellulose in a single phase; utilizing benzalkonium chlorides (BACs), an extensively used quaternary ammonium IL as the reaction medium, cellulose degrading microbial community developed from soil and BACs resistant/degrading *Pseudomonas* sp. BIOMIG1 as biocatalysts. A microbial community capable of cellulose degradation was developed and the cellulose degradation was tested both in absence and presence of BACs. The soil microbial community utilized 1000 mg/L cellulose aerobically within 6 days. Cellulose utilization by the microbial community was inhibited at and above 50 mg BAC/L. On the other hand, when *Pseudomonas* sp. BIOMIG1 was supplemented to the community, BACs was utilized at up to 500 mg BAC/L. In addition, the co-culture of BACs resistant *Pseudomonas* sp. BIOMIG1 and *Serratia marcescens* BIOMIG4 converted glucose mainly to 2,3 butanediol, ethanol and various acids. In conclusion, a biological system that can convert cellulose to value added organic chemicals in the presence of a quaternary ammonium IL and alleviate the environmental consequences of IL was developed in this study.

BAKTERİLERİN SELÜLOZ FERMANTASYONUNA DÖRDÜNCÜL AMONYUM İYONİK SIVILARININ ETKİSİ

Yakıt ve kimyasallara karşı yükselen talep nedeniyle lignoselülozik biyokütleden biyoyakıt ve kimyasal üretimi ciddi bir önem kazandı. Lignoselülozik biyokütle içindeki polisakkaritler, enzimatik hidroliz yoluyla monosakkaritlere dönüştürülür. Monosakkaritler fermantasyon yoluyla biyoetanol ve biyo-bazlı kimyasallara dönüştürülür. Fakat lignoselülozik biyokütlenin hidrolizinin idaresinin güç olması, bu iş için bir ön-işlem süreci gerektirir. Mevcut ön-işlem süreçleri ya yüksek enerji gerektirmekte ya da çevreye zararlı atık akımları üretmektedir. Son zamanlarda iyonik sıvılar, yani sıvı haldeki tuzlar, selüloz dissolüsyonunda yeşil çözücü maddeler olarak kullanılmaya başlamıştır. Ancak son çalışmalar iyonik sıvıların geçmişte düşünüldüğü kadar “yeşil” olmadığını göstermektedir. İyonik sıvılar yalnızca ön-işlemi takip eden biyolojik süreçlere olumsuz yönde etki etmekle kalmayıp, çevreye salındıklarında ekotoksiteye ve bakteriler arasında antibiyotik direncinin artmasına neden olmaktadır. Bu çalışmanın ana amacı, selülozdan değerli alkol ve organik asit fermantasyonunu hidroliz ve ön-işlemlerle birlikte tek bir fazda yürütmektir: bunu yaparken benzalkonyum klorürlerden yararlanılacaktır (BAK); ki bunlar dördüncül amonyum iyonik sıvısı olarak yaygın bir şekilde reaksiyon ortamı olarak kullanılmaktadır, ayrıca topraktan geliştirilen selüloz parçalayan mikrobiyal topluluk ve bunun yanında BAK dirençli/BAK parçalayan *Pseudomonas* türü BIOMIG1 katalist olarak kullanılacaktır. Bu çalışma kapsamında, selüloz parçalama özelliğine sahip bir mikrobiyal topluluk geliştirilmiştir ve selüloz parçalanması BAK varlığında ve yokluğunda test edilmiştir. Toprakten geliştirilen mikrobiyal topluluk 1000 mg/L selülozu aerobik olarak 6 gün içinde kullanmıştır. Mikrobiyal topluluğun selüloz kullanımı 50 mg BAK/L ve üzerinde inhibe olmuştur. Öte yandan, *Pseudomonas* türü BIOMIG1 topluluğa eklendiğinde BAK’lar 500 mg BAK/L’ye kadar kullanılmıştır. Buna ek olarak, BAK dirençli *Pseudomonas* türü BIOMIG1 ve *Serratia marcescens* BIOMIG4 ko-kültürü glikozu ağırlıklı olarak 2,3 bütandiol, etanol ve çeşitli asitlere dönüştürmüştür. Sonuç olarak bu çalışmada, dördüncül amonyum iyonik sıvısı varlığında, selülozu değerli organik kimyasallara dönüştüren biyolojik bir sistem geliştirilmiş ve kuaterner amonyum iyonik sıvısının çevresel etkisi giderilmiştir.



Graphical abstract of the research

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units Used
QAC	Quaternary Ammonium Compounds	
BAC	Benzalkonium Chloride	
Xyl	Xylose	
Cl	Chloride	
Ara	Arabinose	
Glc	Glucose	
Man	Mannose	
Gal	Galactose	
X5P	Xylose-5-Phosphate	
G6P	Glucose-6-Phosphate	
F6P	Fructose-6-Phosphate	
G1P	Glucose-1-Phosphate	
G3P	Glyceraldehyde-3-Phosphate	
Pyr	Pyruvate	
SHF	Separate Hydrolysis and Fermentation	
SSF	Simultaneous Saccharification and Fermentation	
VOC	Volatile Organic Compound	
PF ₆	HexaFluorophosphate	
BF ₄	Tetrafluoroborate	
TFO	Trifluoromethansulfonate	
TF ₂ N	bis(trifluoromethylsulfonyl)imide	
Br	Bromide	
I	Iodide	
PO ₄	Phosphate	
HSO ₄	Hydrogen sulfate	
NO ₃	Nitrate	
SO ₄	Sulfate	
SCN	Thiocyanate	
MeSO ₃	Methanesulfonate	

CFCOO	Trifluoroacetate	
CH ₃ COO	Acetate	
HCOO	Formate	
(C ₆ H ₅)SO ₃	Benzenesulfonate	
SbF ₆	Hexafluorostibane	
(CF ₃ SO ₂) ₂ N	Trifluoromethanesulfonylimide	
CO ₂	Carbon Dioxide	
Ac	Acetate	
[bmim]Cl	1-n-butyl-3-imidazolium chloride	
[bmpy]Cl	3-methyl-n-butylpyridinium chloride	
BDTAC	Benzyl dimethyl(tetradecyl) ammonium	
[emim]Ac	1-ethyl-3-methylimidazolium acetate	
[bmim][BF ₄]	1-butyl-3-methylimidazolium tetrafluoroborate	
[bmim][PF ₆]	1-butyl-3-methylimidazolium hexafluorophosphate	
NMR	Nuclear Magnetic Resonance	
C ₁₂ BDMA-Cl	Dodecyl benzyl dimethyl ammonium chloride	
C ₁₄ BDMA-Cl	Tetradecyl benzyl dimethyl ammonium chloride	
COD	Chemical Oxygen Demand	
sCOD	soluble Chemical Oxygen Demand	
CFU	Colony Forming Unit	
DNA	Deoxyribo Nucleic Acid	
PCR	Polymerase Chain Reaction	
GC	Gas Chromatography	
HPLC	High Performance Liquid Chromatography	
NCBI	National Center for Biotechnology	
TSS	Total Suspended Solids	
VSS	Volatile Suspended Solids	
RFLP	Restriction Fragment Length Polymorphism	
MUSCLE	Multiple Sequence Comparison by Log-Expectation	
V	Volume	(L)
V _{head space}	Volume of headspace	(L)
V _{Total gas}	Volume of total gas	(L)
V _{Taken Sample}	Reduced volume because of taking sample	(L)

$[\text{H}_2\text{CO}_3]$	Concentration of carbonic acid	(mM)
P_g	Partial pressure	(atm)
H	Henry's constant	(L.atm.mole ⁻¹)
x_g	Mole fraction of gas in water	
n_g	Mole of gas	(mole)
n_{water}	Mole of water	(mole)
K_a	Acid ionization constant	(mole.L ⁻¹)

1. INTRODUCTION

Plant biomass is generated from carbon dioxide and water through photosynthesis using sunlight as the energy source. The products of photosynthesis are mainly five and six carbon sugars (monosaccharides). These sugars are the building blocks of lignocellulose which makes up the plant cell wall (Corma et al., 2007). Lignocellulosic materials are mainly composed of three components: 1) cellulose, 2) hemicellulose and 3) lignin. About 170 billion metric tons of biomass is produced by photosynthesis every year. Carbohydrates account for 75% of this amount. On the contrary, only 3-4% of this biomass is used commercially for food and non-food purposes. Therefore, biomass carbohydrates are the most abundant renewable resources available (Corma et al., 2007).

Thermochemical and biochemical processes are utilized for fuel and chemical production from biomass. Indeed biochemical processes are more sustainable, environmentally friendly and economically feasible compared to thermochemical processes. Production of fuels and chemicals from lignocellulosic biomass involves three steps: (1) biomass pretreatment to disintegrate lignocellulose into accessible cellulose, hemicellulose, and eliminating other polymers such as lignin; (2) hydrolysis/saccharification of pretreated biomass to convert cellulose to monosaccharides and (3) fermentation of the monosaccharides to value added alcohols and organic acids (Faik, 2013).

The major obstacle to use lignocellulose in production of fuels and chemicals is the pretreatment step which allows accessibility of enzymes to cellulose and hemicellulose. Pretreatment processes that are commonly used include mechanical comminution, pyrolysis, steam explosion, ammonia fiber explosion, CO₂ explosion, ozonolysis, acid hydrolysis, alkaline hydrolysis, oxidative delignification, organosolv process and biological pretreatment (Sun et al., 2002). Among them, pretreatment of lignocellulose with ILs gained more attention since this method is considered as a green treatment method which requires minimum energy, allows catalyst, sugar and byproduct recovery and has low impact on downstream processes.

ILs are molten salts with melting point less than 100 °C. They are comprised of cations (usually organic) and anions (usually inorganic). Their properties such as non-volatility, low flammability and thermal stability in a wide temperature range separate them from other organic solvents (Fang et al., 2014).

Cellulose is insoluble in water and in many organic solvents. Since common organic solvents do not have enough power to dissolve cellulose, heating or stirring the mixture for a long time is necessary which increase the cost of the process (Fang et al., 2014). ILs are promising new solvents for the pretreatment of lignocellulosic material because of their ability to dissolve large amounts of cellulose at considerably mild conditions with close to 100% recovery by facilitating delignification as well as deacetylation of hemicellulose (Luo et al., 2013). Pretreatment follows an enzymatic hydrolysis/saccharification step where lignocellulose is converted to five and six carbon monosaccharides. Hydrolysis consists of three steps: 1) adsorption of cellulase enzymes on to the surface of the cellulose, 2) the biodegradation of cellulose to fermentable sugars and 3) desorption of cellulases from the cellulose. Pentoses and hexoses produced during the hydrolysis of lignocellulose are converted to alcohols, organic acids, hydrogen and carbon dioxide through fermentation.

In conventional lignocellulose processing for the production of biofuels and bio-based chemicals, fermentation step generally is separate from pretreatment and hydrolysis steps since fermentation pathway involves many steps and each step has specific enzymes that require specific optimum conditions. Therefore, conditions generated during the most of the pretreatment and hydrolysis steps do not favor desired fermentation pathways. For instance, pretreatment of lignocellulose with ILs and enzymatic hydrolysis of cellulose and hemicellulose may be performed in a single reactor but microbial fermentation of hydrolysis products to value added alcohols and acids may not simultaneously happen since ILs are inhibitory to many organisms at high concentrations. As an example, Tezel et al. (2006) showed that quaternary ammonium compounds, which are widely used ILs, were inhibitory to fermentation above 100 mg/L. On the other hand, facultative species of *Pseudomonas* genus of Bacteria not only can ferment carbohydrates but also are resistant to ILs at very high concentrations (Lednicka et al., 2000; Cheng et al., 2011; Tandukar et al., 2013).

ILs are regarded as “environmentally friendly” due to their low vapor pressure and volatility compared to the common organic solvents used in the industry. However, their high solubility in water makes them mobile in the environment. Ecotoxicity related to ILs in the environment is now a concern (Petrovic et al., 2010; Pham et al., 2010). In addition, It was shown that exposure to ILs facilitated the dissemination of antibiotic resistance genes through horizontal gene transfer (Wang et al., 2015; Tezel et al., 2015). As a result, although ILs are beneficial in many applications, they may pose risk to environmental and human health when release to the environment.

In this research, production of value added alcohols and acids from cellulosic biomass in a single stage process coupling pretreatment, hydrolysis and fermentation was investigated. The fate of ILs in the proposed process was also delineated. The results of this research would be used to improve the biofuel production in biorefinery industries using ILs without threatening the environmental and human health.

2. LITERATURE SURVEY

2.1. Cellulosic Biomass

Extensive use of fossil fuel results in depletion of crude oil resources. Campbell and Laherrere (1998) estimated that annual oil production in the world will decline to 25 to 5 billion barrels since 1998 to 2050 (Campbell and Laherrere., 1998). As a result of heavy fossil fuel utilization, a large amount of carbon dioxide is emitted to the atmosphere from industry and vehicles. Anthropogenic carbon dioxide is accepted as the main cause of global warming that we are experiencing today. Shifting to renewable resources for energy and fuel productions has been an alternative way to overcome these problems (Sun et al., 2002 and Faraco, 2013). Ethanol which is a biofuel produced from renewable resources, is used in USA for transportation purposes. However, ethanol is more expensive than fossil fuels since it was produced from corn. Ethanol production from agricultural products such as corn is not economically beneficial because of its competition with limited agriculture land and food (Sun et al., 2002). Therefore, lignocellulosic biomass as a potential source for low cost biofuel production is considered. In addition, about 170 billion metric tons of biomass is produced by photosynthesis every year. Carbohydrates account for 75% of this amount. On the contrary, only 3-4% of this biomass is used commercially for food and non-food purposes. Therefore, lignocellulosic biomass carbohydrates are the most abundant renewable resources available for fuel production (Corma et al., 2007).

Agricultural residues such as straw, hull, stem and stalk, wood; municipal solid wastes such as paper, cardboard, yard trash and wood products; wastes from pulp and paper industries and also herbaceous energy crops like switchgrass and barmudgrass are considered as lignocellulosic biomass. Lignocellulosic materials are mainly composed of three components: 1) cellulose, 2) hemicellulose and 3) lignin. Cellulose is the major component that forms 35-50 % of lignocellulosic biomass. Cellulose is a chemically homogenous polymer composed of D-glucose units linked by 1,4- β -D-glucosidic bonds (Figure 2.1.). Hemicellulose is the second major component (20-35 %) which consists of heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose and galactose) and sugar acids. Lignin is a very complex and amorphous phenylpropanoid

polysaccharide which embeds the cellulose fibers in the structure of lignocellulose (Saha et al., 2004; Costa et al., 2011).

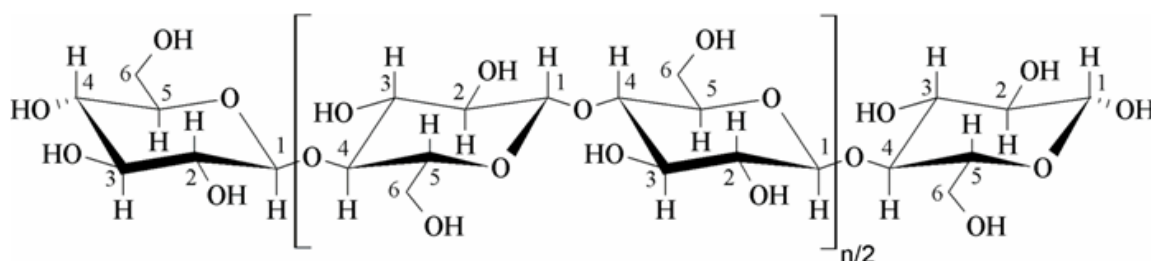


Figure 2.1. Structure of cellulose as the major part of lignocellulose

2.2. Processing of Cellulosic Biomass for Biofuel Production

Production of fuels and chemicals from lignocellulosic biomass involves three steps: (1) biomass pretreatment to disintegrate lignocellulose into accessible cellulose, hemicellulose, and eliminating other polymers such as lignin; (2) hydrolysis/saccharification of pretreated biomass to convert cellulose to monosaccharides and (3) fermentation of the monosaccharides to value added alcohols and organic acids (Faik, 2013).

2.2.1. Pretreatment

Hydrolysis of lignocellulosic biomass can be achieved using supercritical water, subcritical water and strong acids (Faraco, 2013). Using these methods does not require any pretreatment process since water and acids are capable of penetrating the cell walls and break the polysaccharides whereas, enzymatic hydrolysis of lignocellulosic materials needs pretreatment processes since enzymes cannot access to the polysaccharides due to their large size and complex structure (Faraco, 2013). The enzymatic hydrolysis is not efficient without any pretreatment process (Vancov et al., 2012). Therefore pretreatment is necessary to remove the hemicellulose and lignin, decrease the crystallinity of cellulose to make it accessible to enzymes and also increase the material porosity (Sun et al., 2002).

An efficient pretreatment must improve the sugar formation, generate highly digestible cellulose, avoid the loss of carbohydrates, avoid the production of byproducts

which can inhibit the hydrolysis process in order to decrease the energy consumption and the cost of down-stream processes (Sun et al., 2002). Various physical, physicochemical, chemical and biological processes are used for pretreatment of lignocellulosic biomass. Physical processes such as drying and concentration, grinding and granulometric separation; chemical process like alkaline pretreatment, acid pretreatment, organosolv and ozonolysis; physicochemical process such as CO₂ explosion, steam explosion, ammonia fiber explosion and liquid hot water pretreatment and biological processes including ligninolytic abilities of fungi were the most commonly used pretreatments (Sun et al., 2002; Vancov et al., 2012; Faraco, 2013). Every pretreatment process has its own pros and cons based on their effect on the efficiency of down-stream processes. These efficiency parameters involve enzyme loadings, ethanol purity and concentration, waste treatment demands and energy consumption (Vancov et al., 2012).

2.2.2. Enzymatic Hydrolysis and Fermentation

Pretreatment follows an enzymatic hydrolysis/saccharification step where lignocellulose is converted to five and six carbon monosaccharides. The group of enzymes used for hydrolysis called cellulase. Cellulase is composed of endo- β -1,4-D-glucanase which reacts on the amorphous part of cellulose and leads to fragmentation of cellulosic materials; exo- β -1,4-D glucanase that hydrolyzes fragmented cellulose into simple oligosaccharides including cellobiose and cellodextrin and finally β -1,4-D-glucosidase that converts the oligosaccharides to glucose (Cheng et al., 2011). Hydrolysis consists of three steps: 1) adsorption of cellulase enzymes on to the surface of the cellulose, 2) the biodegradation of cellulose to fermentable sugars and 3) desorption of cellulases from the cellulose.

Generally fungal cellulases are used for hydrolyzation of cellulose. Currently, *Trichoderma reesei* is the most extensively used fungus in industry due to its capacity to secrete high level of cellulases. However, conversion is still not optimal due to the heterogeneous composition of plant biomass and novel cellulases are on demand in order to increase the efficiency of hydrolyzation process (Couturier and Berrin, 2013). Some Bacteria also have cellulases which convert cellulose to fermentable sugars. Cellulolytic bacteria are diverse and include species of *Caldocellulosiruptor*, *Butyrivibrio*,

Ruminococcus, *Eubacterium*, *Clostridium*, *Bacteroides*, *Acetivibrio*, *Thermoactinomyces*, *Caldibasillus*, *Bacillus*, *Acidothermus*, *Cellulomonas*, *Micromonospora*, *Actinoplanes*, *Streptomyces*, *Thermobifida*, *Thermomonospora*, *Microbispora*, *Fibrobacter*, *Sporocytophaga*, *Cytophaga*, *Flavobacterium*, *Achromobacter*, *Xanthomonas*, *Cellvibrio*, *Pseudomonas* and *Myxobacter* (<http://www.wzw.tum.de/mbiotec/cellmo.htm>, and the references herein; Dees et al., 1995; Lednicka et al., 2000; Cheng et al., 2011).

In common practice, enzymatic hydrolysis follows the pretreatment when mechanical or chemical pretreatment is employed. Cheng et al., 2011 investigated new cellulases from *Pseudomonas* sp. CL3 isolated from soil which can hydrolase the alkaline pretreated bagasse as substrate to glucose and xylose with the rates of 0.25 and 0.10 g/L.h respectively (Cheng et al., 2011). The enzymatic hydrolysis of a sodium hydroxide pretreated sugarcane resulted in releasing about 16 g/L glucose (Maeda et al., 2011). Alkali (1.0% NaOH) pretreated wheat straw was converted to 343.95 (mg/g substrate) reducing sugars at 50°C (Han et al., 2012).

Pentoses and hexoses produced during the hydrolysis of lignocellulose are converted to alcohols, organic acids, hydrogen and carbon dioxide through fermentation (Figure 2.2.). Fermentation is a biological process which occurs in the absence of external electron acceptor. During the fermentation process, five- and six-carbon sugars that are commonly found in lignocellulosic material include xylose (Xyl), arabinose (Ara), glucose (Glc), mannose (Man) and galactose (Gal) are converted to the phosphorylated forms xylose-5-phosphate (X5P), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) and glucose-1-phosphate (G1P). These molecules are then transformed to glyceraldehyde-3-phosphate (G3P) and pyruvate (Pyr). Pyr serves as the key substrate of fermentation and converted to hydrogen, ethanol, butanol, succinic acid, lactic acid, acetic acid and propionic acid via alcoholic, acidic and mixed acid fermentation pathways (Alper and Stephanopoulos, 2009; El-Mansi et al., 2012). Species of *Clostridium* (Bacteria) and *Saccharomyces* (Yeast) are the major microorganisms used in the fermentation of carbohydrates for alcohol and acid production (Binod et al., 2013).

Two methods are commonly used to produce ethanol and valuable building block chemicals from pretreated lignocellulosic biomass: (1) Separate enzymatic hydrolysis and

fermentation (SHF) and (2) simultaneous saccharification and fermentation (SSF). In SHF process, the pretreated lignocellulose is hydrolyzed to monosaccharides and then fermented to ethanol and other biofuels in different units. Although during SHF, the microorganisms responsible for hydrolysis and fermentation behave in their optimum condition, the glucose and cellobiose as cellulase deactivators are produced during hydrolysis which can alleviate the efficiency of the process. On the other hand, SSF is carried out in a single reactor. Thus, the inhibitory effect of glucose and cellobiose on cellulose is fixed during SSF by spontaneous fermentation of these intermediates to ethanol. The other advantages of SSF toward SHF are the less possibility of contamination and less numbers of vessels required for installing the system (Taherzadeh et al., 2007).

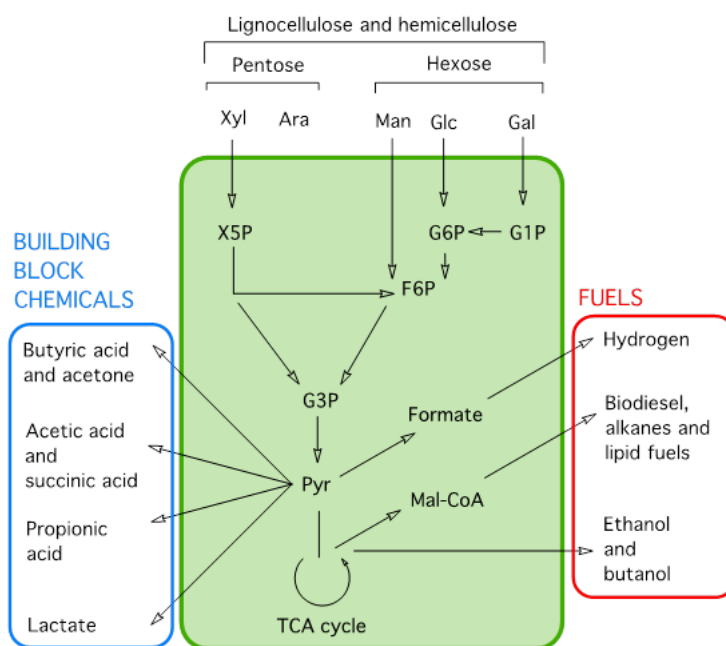


Figure 2.2. Pathways of value added chemicals production from cellulosic biomass by microorganisms through fermentation (adopted from Corma et al., 2007; Alper and Stephanopoulos, 2009; El-Mansi et al., 2012).

2.3. Use of ILs in Dissolution and Fermentation of Lignocellulosic Biomass

2.3.1. Ionic Liquids in General

ILs are molten salts with melting point less than 100 °C. They comprised of cations (usually organic) and anions (usually inorganic) (Figure 2.3.). ILs have interesting features

such as low melting point, non-volatility, high thermal stability and wide liquid range. They are also called green solvents because of their low vapor pressure, therefore eliminating any emission of volatile organic compounds (VOCs). Since these compounds are produced from various anions and cations, a specific ILs with special properties can be made. Therefore, many ILs can be synthesized theoretically, however, the numbers synthesized and reported are very limited (Fang et al., 2014; Wahlstrom, 2014).

ILs are considered as efficient solvents than other molecular solvents. However, depending on the structure of anion and cation, the properties of ILs can be very different. Generally, increase in size of the cation causes the decrease in melting point of the ionic liquid (Holbrey and Rogers, 2003). On the other hand, as the size of the anion increases, the melting point decreases. The viscosity of ILs is also an important factor that affects their solvation. Generally, the viscosity of the ILs increases by increasing alkyl chain length (Dzyuba and Bartsch, 2002). The viscosity of ILs with the most common used (chloride, hexafluorophosphate, tetrafluoroborate trifluoromethanesulfonate, bis(trifluoromethylsulfonyl)imide) anions is $\text{Cl}^- > [\text{PF}_6]^- > [\text{BF}_4]^- > [\text{TfO}]^- > [\text{Tf}_2\text{N}]^-$. ILs have high thermal stability compared to other common organic solvent. Moreover, they are liquid in a wide range of temperatures (from 70 to 300-400 °C) (Fang et al., 2014).

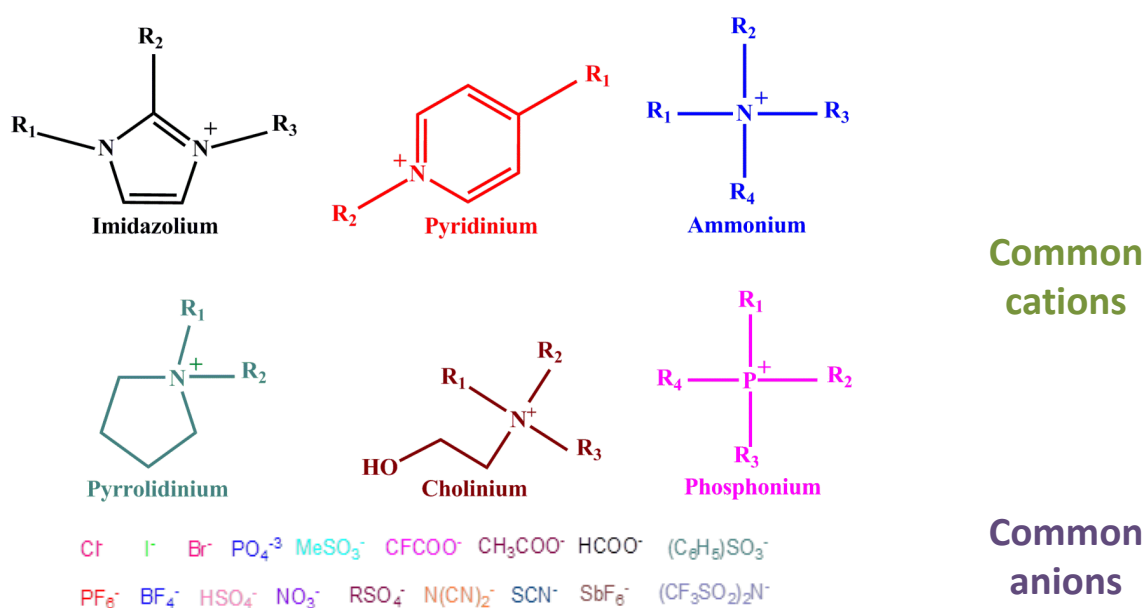


Figure 2.3. Most commonly used cations and anions in ILs structures (Vancov et al., 2012)

2.3.2. Cellulose Pretreatment and Dissolution ILs

Pretreatment processes that are commonly used include mechanical comminution, pyrolysis, steam explosion, ammonia fiber explosion, CO₂ explosion, ozonolysis, acid hydrolysis, alkaline hydrolysis, oxidative delignification, organosolv process and biological pretreatment (Sun et al., 2002). Among them pretreatment of lignocellulose with ILs gained more attention since this method is considered as a green treatment method which requires minimum energy, allows catalyst, sugar and byproduct recovery and has low impact on downstream processes. ILs are promising new solvents for the pretreatment of lignocellulosic material because of their ability to dissolve large amounts of cellulose at considerably mild conditions with close to 100% recovery by facilitating delignification as well as deacetylation of hemicellulose (Luo et al., 2013).

Cellulose dissolution by ILs was first investigated by Swatloski et al. (2002). In their research, cellulose dissolution by imidazolium-based ionic liquid with different cations and anions was studied. 1-butyl-3-methylimidazolium chloride ([bmim]⁺ Cl⁻) could dissolve up to 10% cellulose at 100°C whereas changing the anion to SCN⁻, [BF₄]⁻ and [PF₆]⁻ deteriorate the cellulose dissolution. Moreover cellulose was less soluble when alkyl chain was 6 and 8 carbons (Swatloski et al., 2002). In another study, Li et al., 2011 investigated the complete dissolution of 0.5 g bagasse in 10 g of 1-ethyl-3-methyl-imidazolium acetate [emim]Ac at 175-195°C within 5-15 minutes (Li et al., 2011).

In addition to role of cationic and anionic parts of the ILs, cellulose dissolution in ILs can also be affected by the degree of polymerization (DP) of cellulose. Heinze et al. (2005) investigated the dissolution of different types of cellulose such as Avicel, Spruce sulfite pulp and cotton linters with different DP in 1-n-butyl-3-methylimidazolium chloride [bmim]⁺Cl⁻, 3-methyl-n-butylpyridinium chloride ([bmpy]⁺Cl⁻) and benzyldimethyl(tetradecyl) ammonium chloride (BDTAC). Avicel with the lowest DP was dissolved in [bmpy]⁺Cl⁻ (39% wt/wt). The cellulose with higher DP (cotton linters) was poorly dissolved in all three ILs. Moreover, they verified the results of Swatloski et al. (2002) that ILs with cationic part having longer alkyl chain dissolve less cellulose ([bmim]⁺ Cl⁻ > [bmpy]⁺ Cl⁻ > BDTAC) (Heinze et al., 2005).

NMR spectroscopy shows that cellulose solubility in $[\text{bmim}]^+\text{Cl}^-$ is driven by the hydrogen bond formation between the chloride and the hydroxyl group of cellulose (Remsing et al., 2006). In fact, ILs with higher hydrogen bond basicity such as chloride and acetate, have higher affinity to dissolve cellulose (Vancov et al., 2012; Fang, 2014). Moreover, presence of water in the reaction extremely deteriorates the cellulose dissolution because of the hydrogen bond competition between the water-IL and IL-cellulose (Swatloski et al., 2002; Fang et al., 2014).

To find out the efficiency of ILs dissolution capability, lignocellulose is regenerated using an anti solvent like ethanol and water. Further the difference between carbohydrate content of the regenerated material and the untreated substrate declares the pretreatment efficiency. It was reported that wheat straw fractionation was performed in $[\text{emim}]\text{Ac}$, 1-ethyl-3-methylimidazolium acetate at 120 °C for 6 hours. The regenerated materials were obtained with 81wt% carbohydrate- rich material compared to the initial 62wt% (Costa-Lopez et al., 2013). In a different study, pretreatment of switchgrass, composed of 64.5wt% carbohydrate and 21.8 wt% lignin, with $[\text{emim}]\text{Ac}$ at 160°C for 3 hours resulted in a carbohydrate-rich material with 79.5 wt% carbohydrates and 13.6wt% lignin (Li et al., 2010).

2.3.3. Enzymatic Hydrolysis and Fermentation of Lignocellulosic Biomass in ILs

Less crystalline cellulose produced in pretreatment follows an enzymatic hydrolysis/saccharification step in which cellulose is converted to five and six carbon monosaccharides by cellulase (Cheng et al., 2011). Many microorganisms were identified as cellulase producing microorganisms (Sun et al., 2002). The monosaccharides produced by cellulases are fermented to ethanol, organic acids and other biofuel through fermentation (Poornejad et al., 2014).

Since the cellulose is pretreated by ILs which are efficient solvents, a less crystalline cellulose is the outcome of pretreatment process. Therefore a faster hydrolysis process is expected. Zhao et al. (2009) studied that ionic liquid pretreated cellulose was 2 times more accessible to cellulase than untreated cellulose (Zhao et al., 2009). The enhanced cellulose degradation and ethanol production was obtained from the hydrolysis and fermentation of

cellulose pretreated with ILs in a specific ionic liquid concentration. Ionic liquid with a concentration below of approximately 200 mM produced 90% ethanol yield (Nakashima et al., 2011). In a different study, the simultaneous saccharification and bioethanol production from IL pretreated rice straw was conducted in which the hydrolysis was improved from 25.7% to 75%. Moreover, the ethanol production was enhanced from 35.6% to 79.9% compared to the untreated substrate (Poornejad et al., 2014). Costa- Lopez et al. (2013) studied the enzymatic hydrolysis of regenerated wheat straw which resulted in 76% w/w_{Biomass} glucose yield (Costa- Lopez et al., 2013).

Since ILs may affect the cellulase producing microorganisms, therefore the cellulose is regenerated after pretreatment by IL or, the IL/water mixture is adjusted. (Fang et al., 2014). Depending on the structure of the ILs, their effects on enzymes (cellulases) are different. As an example, an IL with a hydrophobic structure have a strong effect on stabilizing the enzymes whereas the ILs with chloride as their anionic part reduce the cellulase activity since the chloride can make hydrogen bond with cellulases. (Sun et al., 2002; Fang et al., 2014). It was reported that 1-butyl-3-methylimidazolium chloride [bmim]Cl which is one of the most widely used cellulose solvent, denature the cellulase from *Trechidorma reesei* (Tuner et al., 2003). Paljevac et al. (2006) investigated the effects of 1-butyl-3-methylimidazolium chloride [bmim]Cl, 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF₄] and 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF₆] on the activity of cellulase from *Humicola insolens*. [bmim]Cl denatured the cellulase whereas, the cellulase was more active in hydrophobic ILs [bmim][PF₆] and [bmim][BF₄] (Paljevac et al., 2006).

Depending on the percent of water, the cellulose dissolution can be deteriorated (Swatloski et al., 2002) but it is necessary for cellulose hydrolysis which requires an optimum mixture of IL/water. Enzymatic hydrolysis of cellulose in an aqueous solution of IL depends on the ratio of IL/water which affects the cellulases. It was reported that a mixture of 15-20% of water with 1-ethyl-3-methylimidazolium [emim]Ac is able to extract the hemicellulose from a paper-grade kraft pulp after 3 hours , therefore the hydrolysis process was not affected by the presence of water since the IL/water ratio was optimized (Froschauer et al., 2013). Moreover, since the high concentration of ILs can denature the enzymes, thus increasing the water/IL ratio can repair the hydrolysis process. A study

investigated that increasing the IL to water ratio from 3:2 to 1:4 enhanced the cellulase activity (Vancov et al., 2012). Nakashima et al. (2011) studied the direct cellulose fermentation in ionic liquid in which the cellulase-producing yeast was resistance to ionic liquid below a concentration of 200 mM. The cellulose degradation and ethanol fermentation was also significantly enhanced (Nakashima et al., 2011).

The toxicity of ILs is another important factor which affects the hydrolysis and fermentation processes. The toxicity of ILs increases with increasing alkyl chain length. A study performed using imidazolium and pyridinium-based ILs showed that butyl-imidazolium and pyridinium bromides were less toxic whereas hexyl- and octyl-imidazolium and pyridinium bromides had more antimicrobial activity (Docherty and Kulpa, 2004). In a different study it was also reported that the enzymatic hydrolysis of IL pretreated cellulose requires a thorough removal of IL since small amount of IL may deactivate the cellulase (Zhao et al.; 2009). Tezel et al. (2006) showed that quaternary ammonium compounds which are widely used ILs, were inhibitory to fermentation above 100 mg/L.

In addition, effects of ILs as surfactants on cellulose hydrolysis were studied. Some surfactants enhance the cellulose hydrolysis since surfactants tend to absorb on surfaces, alter the cellulose surface properties by lowering the surface tension and further preventing the cellulase deactivation (Helle et al., 1993; sun et al., 2002). Tween Q-86W as a cationic surfactant enhanced the cellulase activity by less than 0.008 wt% whereas anionic surfactants such as Neoplex F-25 denatured the cellulase by more than 0.001 wt% (Ooshima et al., 1986). Helle et al. (1993) obtained seven times increase in cellulose hydrolysis by applying surfactant (Helle et al., 1993). Thus, depending on cellulase producing microorganism, its resistance to the IL and also the structure of anions and cations of IL, the effects of IL on cellulase and further hydrolysis can be different.

3. OBJECTIVES

Quaternary ammonium compounds (QACs), which are cationic surfactants, are used in many ionic liquid applications (Garcia et al., 1999). These compounds are known to disintegrate polymeric substances such as cell membrane by interacting with negatively charged functional groups (Wessels and Ingmer, 2013). In this research, a mixture of two benzalkonium chlorides (BACs) as the most widely used QACs (1:1 w:w), i.e. dodecyl benzyl dimethyl ammonium chloride ($C_{21}H_{38}N^+Cl^-$, 340 g/mole, C_{12} BDMA-Cl) and tetradecyl dimethyl ammonium chloride ($C_{23}H_{42}N^+Cl^-$, 368 g/mole, C_{14} BDMA-Cl) were used as ILs (Figure 3.1.).

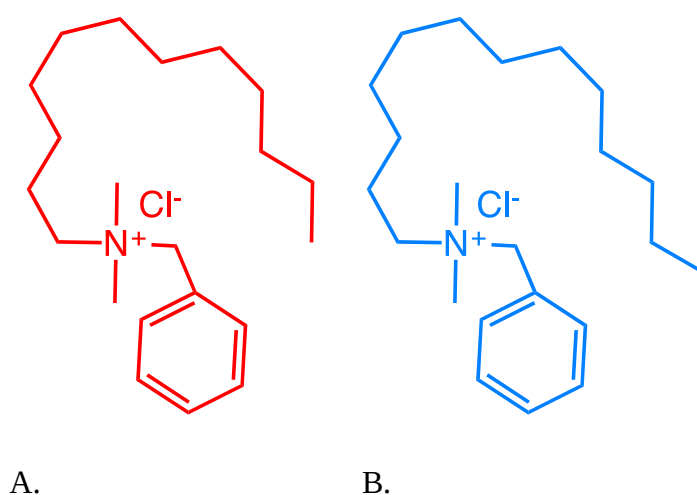


Figure 3.1. Molecular structures of (A) C_{12} BDMA-Cl and (B) C_{14} BDMA-Cl

Relatively recently, Ertekin et al. (2015) isolated a novel *Pseudomonas* sp. BIOMIG1 from soil which can degrade BACs. In addition a soil microbial community able to degrade cellulose was developed.

The overall objective of this research is to enhance dissolution and fermentation of cellulose by a microbial community developed from soil and the co-culture of BAC ILs resistant *Pseudomonas* sp. BIOMIG1 and *Serratia marcescens* BIOMIG4 in BACs as ILs. Given the fact that BIOMIG1 can also degrade QACs, using this strain for fermentation

would avoid any environmentally harmful consequences of utilizing QACs as reaction medium for enhanced dissolution of cellulose.

4. MATERIALS AND METHODS

4.1. General Analytical Methods

4.1.1. pH

All pH measurements were performed potentiometrically with a calibrated microprocessor pH meter model 211 (Hanna Instruments) and a model HI 1230 probe.

4.1.2. Chemical Oxygen Demand

COD was measured using the closed reflux, colorimetric method as described in Standard Methods (Eaton et al., 2005). An aliquot of 3 mL digestion solution composed of 4.9 g $\text{K}_2\text{Cr}_2\text{O}_7$, 6 g HgSO_4 , 6 g Ag_2SO_4 and 500 mL H_2SO_4 was transferred to HACH COD digestion vials (HACH Company, Loveland, CO) and then 2 mL of sample was added to the vial. To measure the soluble COD (sCOD), samples are passed through a 0.45 μm filters and then added to the COD vials. After tumbling the vial for 4-8 times, the content in the vials was digested at 150°C for 2 hours and then cooled down to the room temperature. The COD was measured at 620 nm with a HACH DR/2010 spectrophotometer equipped with a diode array detector, deuterium and tungsten lamps and a 1 cm path length. All samples were prepared in duplicates.

4.1.3. Total Gas Production

Total gas production was measured in closed serum bottles using a 10 mL glass syringe with a sliding plunger.

4.1.4. Gas Composition

The head space gas composition in the bottles, especially H_2 and CO_2 , were determined by an Agilent 6850 Series GC unit (Agilent Technologies Inc., Palo Alto, CA) equipped with a 30 m Carboxen 1010 Plot fused silica, 0.53 mm i.d. column (Supelco Inc.)

and a thermal conductivity detector. Helium was used as the carrier gas at a constant flow rate of 6 mL/min. The 10:1 split injector was maintained at 150°C, the oven was set at 40°C and the detector temperature was set at 150 °C. All gas analyses were performed by injecting a 200 µL gas sample. A calibration curve prepared using known concentrations of the analyte was used to calculate the headspace CO₂ concentration as partial pressure (Figure 4.1.).

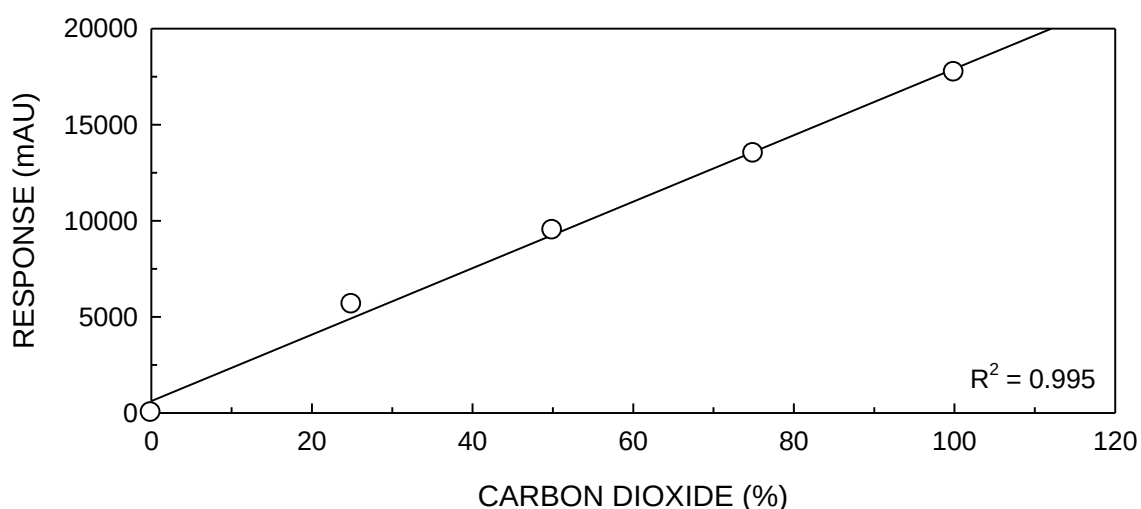


Figure 4.1. Carbon dioxide calibration curve prepared using the gas chromatography

4.1.5. Organic Acids, Alcohols and Glucose

Organic acids (formic, oxalic, citric, malic, pyruvic, lactic, succinic and propionic acids), alcohols (ethanol, and 2,3-butanediol), and carbohydrates (glucose) were measured with an Agilent 1260 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) unit equipped with a Hi-Plex H ion exclusion column (300 × 7.8 mm) (Agilent Inc., USA) and UV/visible diode array and refractive index detectors. A 0.01 N H₂SO₄ solution was used as the mobile phase with a flow rate of 0.6 mL/min and the column was maintained at 65°C. The samples were centrifuged and the supernatant was acidified with 0.01 N H₂SO₄ in a 1:1 ratio. Organic acids were detected by the UV detector at 210 nm wavelength, whereas the glucose and alcohols were detected by the refractive index detector (RID).

A sample of HPLC chromatogram of the observed organic acids, ethanol and 2,3-butanediol are shown in Figure 4.2.

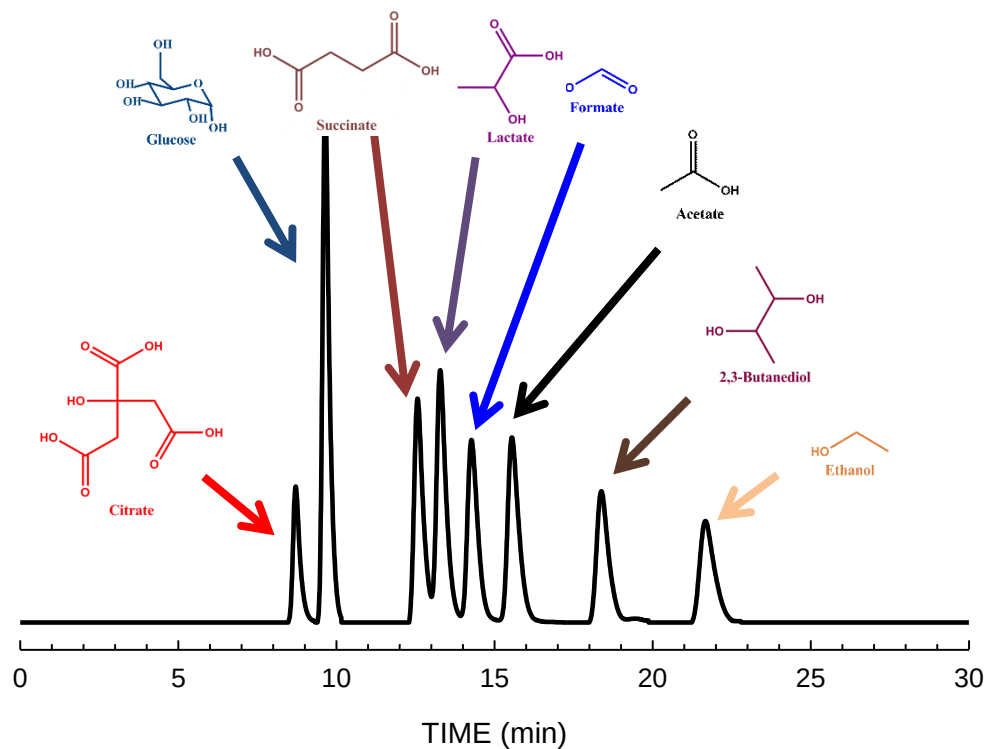
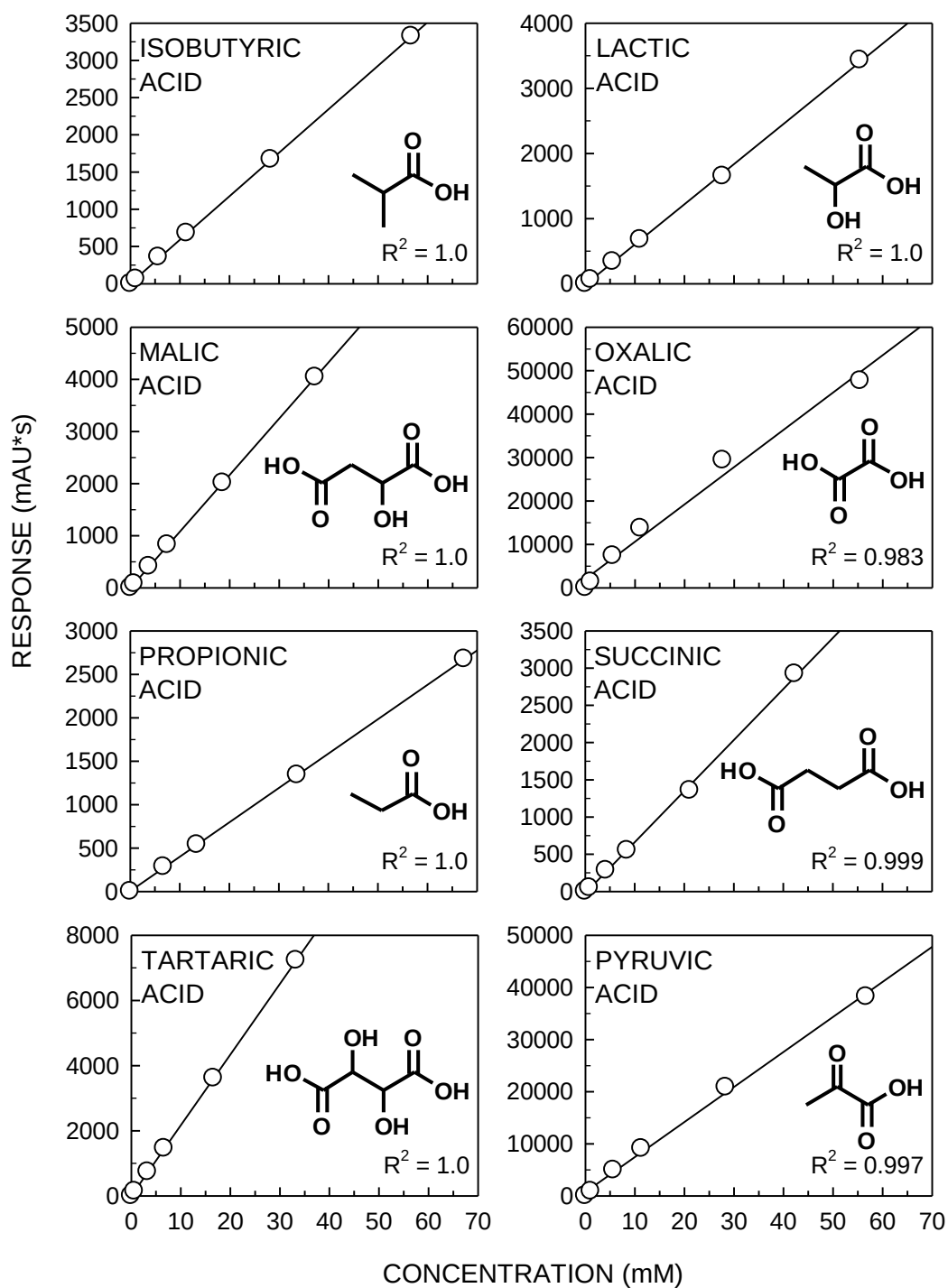


Figure 4.2. A sample of HPLC chromatogram of common observed organic acids, ethanol and 2,3-butanediol

The calibration curves of organic acids were prepared using 10 g/L stock solution of each organic acid acidified with 0.01 N H_2SO_4 . The curves are shown in Figure 4.3. In addition, calibration curve of glucose, ethanol, acetoin and 2,3- butanediol were prepared using 1000 mg/L stock solution of the aforementioned compounds (Figure 4.4.).



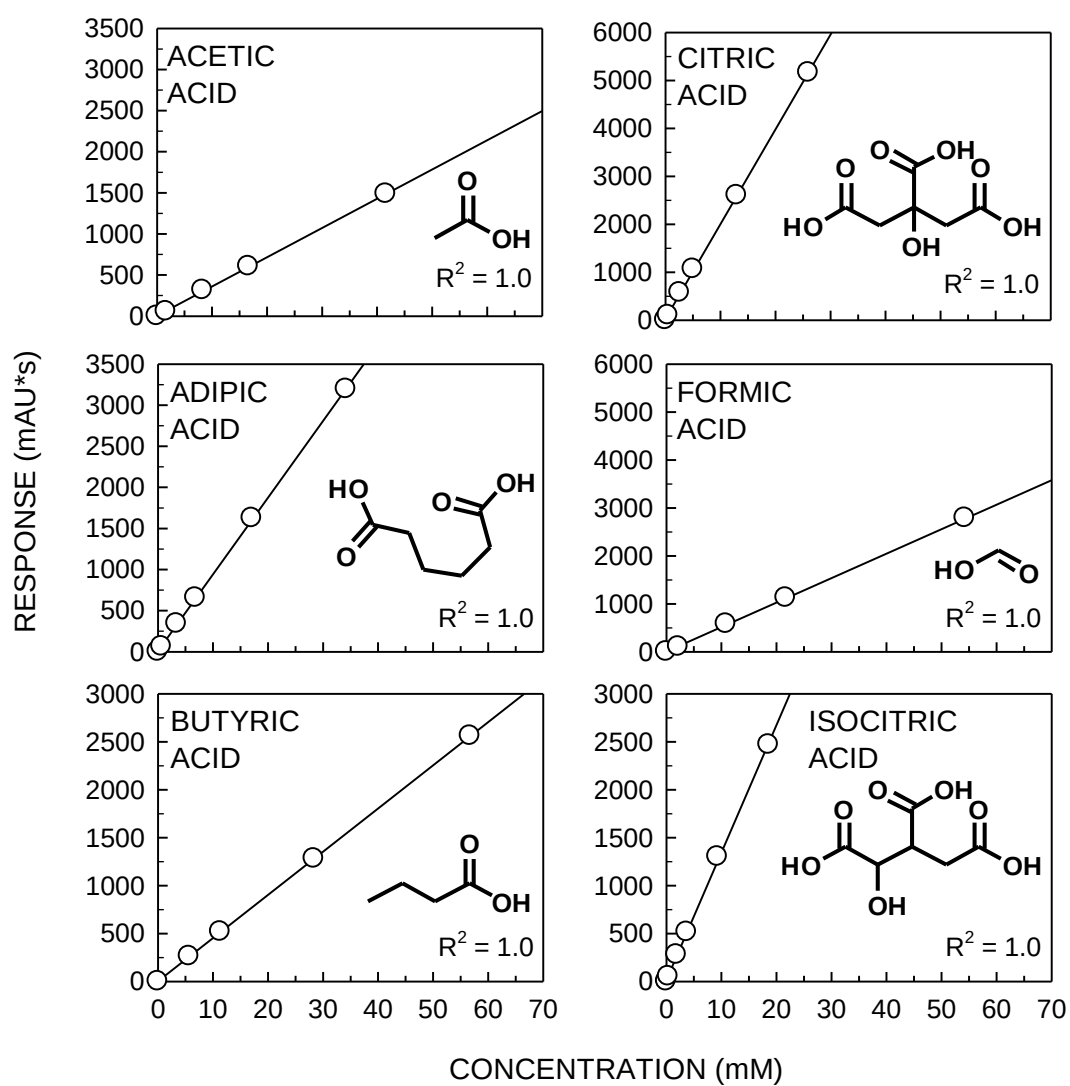


Figure 4.3. Calibration curves of organic acids

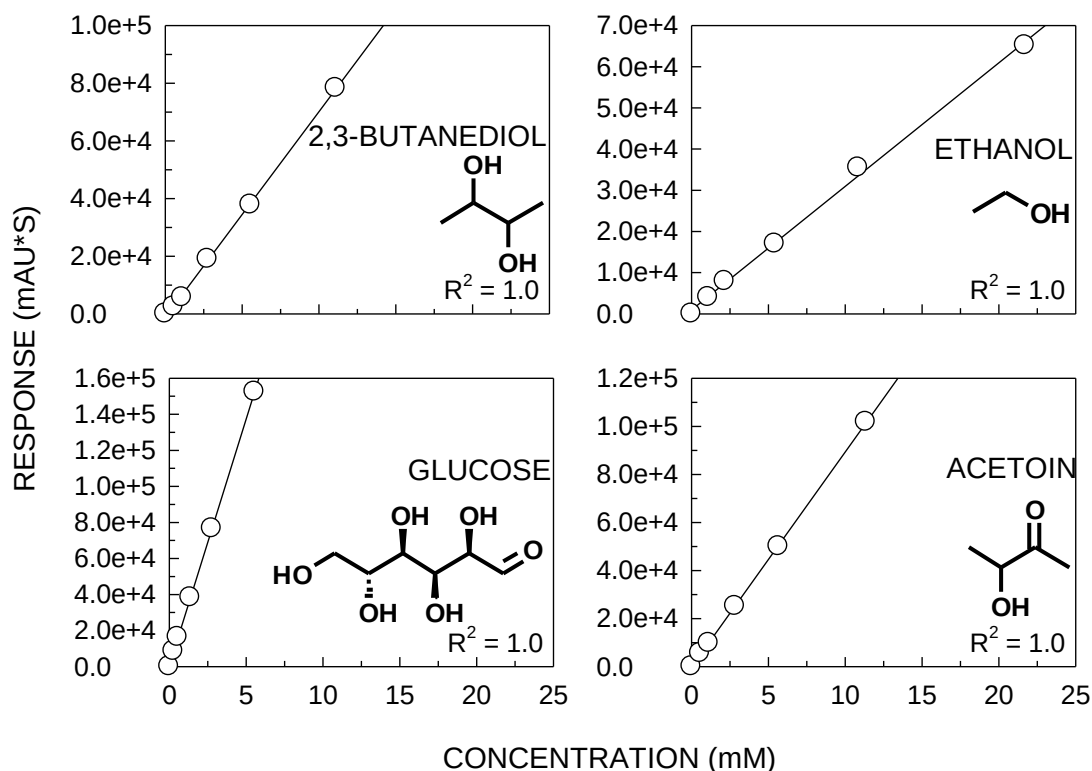


Figure 4.4. Calibration curve of 2,3-butanediol, ethanol, glucose and acetoin

4.1.6. Total Carbohydrates Measurement

The total carbohydrates in samples were measured colorimetrically using the anthrone method. The anthrone agent was prepared by dissolving 0.5 g anthrone in 10 mL absolute ethanol and further adjusting the volume to 250 mL by 75% (V/V) sulfuric acid. The samples were already centrifuged and diluted. About 1 mL of sample was transferred to glass digestion vial. Further, 2 mL of chilled sulfuric acid solution (75% V/V) was added to the vials and agitated. Then, 4 mL of chilled anthrone agent was added to the vials and agitated. Then the vials were placed in a model ECO 25 thermoreactor (VELP Scientifica) and digested at 100 °C for 15 minutes and then cooled down to room temperature. The absorbance was measured at 578 nm with a model P 360 nanophotometer (IMPLEN). All samples were prepared in duplicates and a calibration curve (Figure 4.5.) was prepared using 100 mg/L standard solution of glucose. The total carbohydrates concentration in samples was calculated using the absorbance read and the calibration curve.

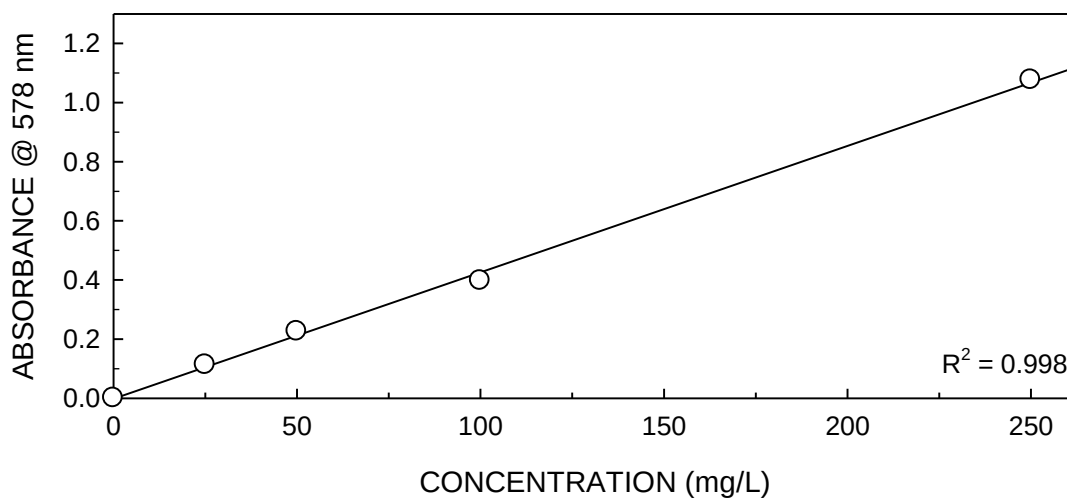


Figure 4.5. Calibration curve prepared for total carbohydrate measurement

4.1.7. BACs Measurement

Benzalkonium chlorides in samples were measured using an Agilent 1260 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a Phenomenex Luna SCX column (250 x 4.6 mm, 5 μ) (Phenomenex, Inc., Torrance, CA) followed by a Polaris C₁₈A column (50 x 4.6 mm, 3.2 μ) (Agilent Technologies, Palo Alto, CA, USA). A Phenomenex SCX SecurityGuard cartridge (4 x 3.0 mm) is used as a precolumn. A 60:40 (v/v) mixture of acetonitrile and 50 mM phosphate buffer (pH 2.5) is used as the mobile phase at a flow rate of 1.0 ml/min and the columns were maintained at 35°C. Detection is achieved with UV-Vis diode array detector at a wavelength of 210 nm.

A sample of HPLC chromatogram of C₁₂BDMA-Cl and C₁₄BDMA-Cl is shown in Figure 4.6. The calibration curve of C₁₂BDMA-Cl and C₁₄BDMA-Cl used in this study is shown in Figure 4.7.

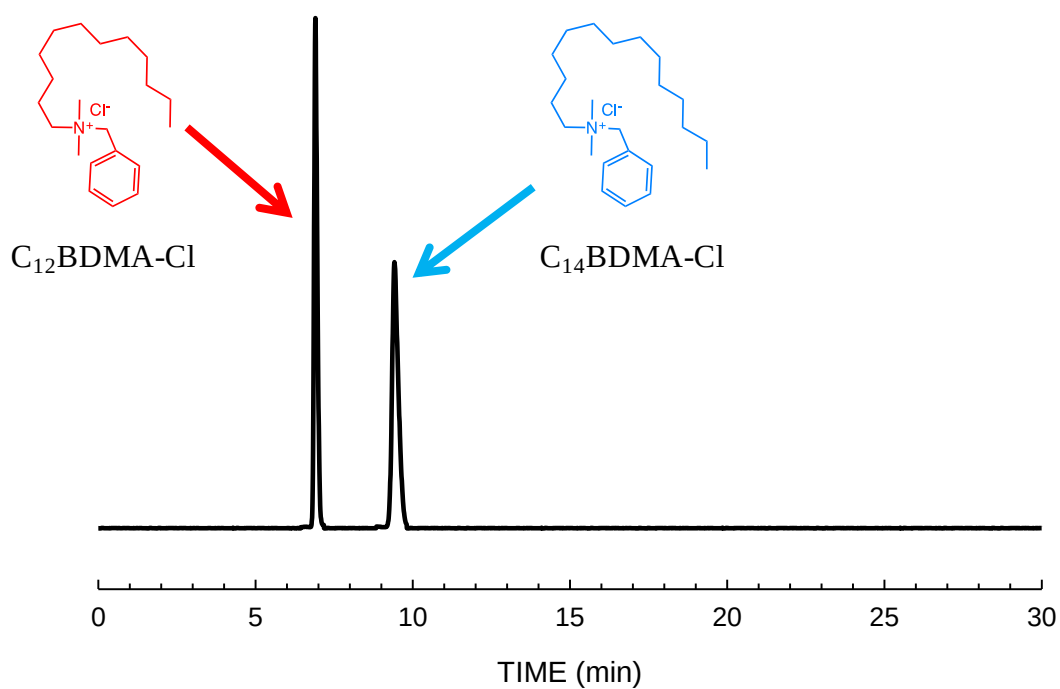


Figure 4.6. HPLC chromatogram of a sample containing $C_{12}BDMA-Cl$ and $C_{14}BDMA-Cl$

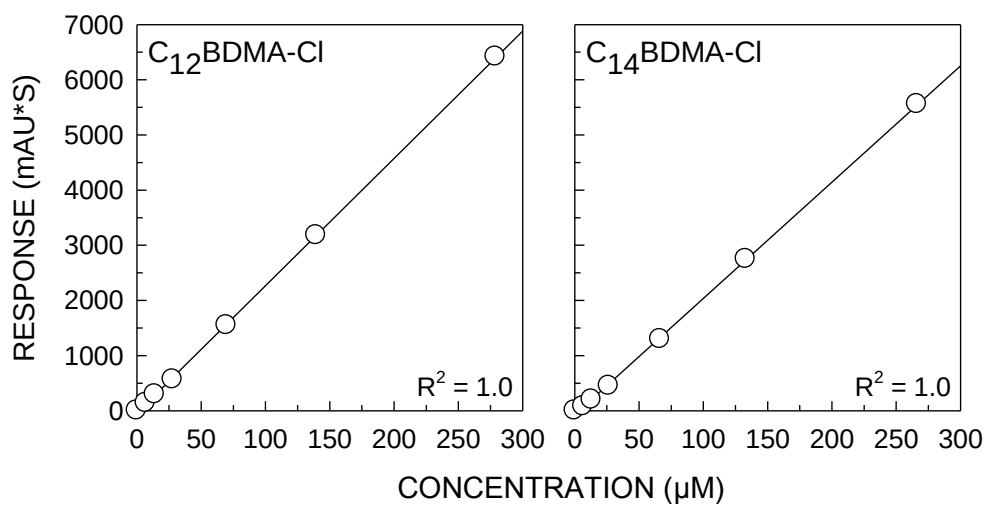


Figure 4.7. Calibration curve of $C_{12}BDMA-Cl$ and $C_{14}BDMA-Cl$ used in this study

4.2. General Procedures

4.2.1. Chemicals

Dodecyl benzyl dimethyl ammonium chloride (C_{12} BDMA-Cl, $C_{21}H_{38}NCl$, 340 g/mole) and tetradecyl benzyl dimethyl ammonium chloride (C_{14} BDMA-Cl, $C_{23}H_{42}NCl$, 368 g/mole) were obtained in high purity from TCI Chemicals (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The D(+)-Glucose was purchased from Merck (Darmstadt, Germany). The cellulose (medium fibers) was obtained from Sigma-Aldrich Chemical Company (St. Louis MO). Mineral salts and organic solvents used in experiments and instrumental analysis were purchased from Merck and Sigma Aldrich Chemicals Company.

4.2.2. Mineral Medium

The composition of the culture medium used to supply necessary nutrients, trace metals and vitamins to the microorganisms is shown in Table 4.1. Moreover, the composition of trace metal stock solution is shown in Table 4.2. Medium was prepared by transferring the first seven ingredients shown in Table 4.1. to a calibrated flask and further adjusting the volume to 1 L with DI water. The content was autoclaved at 121 °C for 15 minutes. After autoclaving, the last two ingredients which were already autoclaved were added to the medium.

Table 4.1. Composition of medium used in this study

Ingredient	Concentration
K ₂ HPO ₄	7.4 g/L
KH ₂ PO ₄	3.0 g/L
NaCl	0.5 g/L
NH ₄ Cl	1.00 g/L
FeCl ₃ .6H ₂ O	0.19 g/L
Yeast extract	0.5 g/L
Trace metal stock solution	1 mL/L
MgSO ₄ .7H ₂ O	0.25 g/L
CaCl ₂	0.01 g/L

Table 4.2. Composition of trace metal stock solution used in this study

Ingredient	Concentration
ZnCl ₂	0.50 g/L
MnCl ₂ .4H ₂ O	0.30 g/L
H ₃ BO ₃	3.0 g/L
CoCl ₂ .6H ₂ O	2.0 g/L
CuCl ₂ .2H ₂ O	0.1 g/L
NiSO ₄ .6H ₂ O	0.2 g/L
Na ₂ MoO ₄ .2H ₂ O	0.3 g/L

4.2.3. Preparation of Cellulose-Agar Plates

The cellulose-agar plates used in this research consist of 1.5% agar (w/v) and 1.5 % cellulose (w/v). To prepare 1 L of this solution, 15 g cellulose and 15 g agar was added to 900 mL deionized water in a 1L glass flask. 100 mL medium (10X) was prepared according to the method described in part 4.2.2. in a 250 mL glass flask. The flasks were placed in autoclave at 121 °C for 15 minutes. After cooling down, the contents of the flasks were mixed and amended with autoclaved Mg and Ca solutions. Further, the solution was poured in sterile petri dishes under sterile condition.

4.2.4. Preparation of BACs Stock Solution

A 10 g/L of BACs stock solution was prepared by mixing 0.5 g C₁₂BDMA-Cl and 0.5 g C₁₄BDMA-Cl in 100 mL sterile medium under laminar hood.

4.2.5. Preparation of 0.85% Saline Solution

To prepare 100 mL of saline solution, 0.85g sodium chloride was added into 100 mL volumetric flask and the volume was adjusted to 100 mL using DI water. The solution was placed in autoclave at 121 °C for 15 minutes for sterilization.

4.3. Microorganisms

4.3.1. *Pseudomonas* sp. BIOMIG1 and *Serratia marcescens* BIOMIG4

The *Pseudomonas* sp. BIOMIG1 and *Serratia marcescens* BIOMIG4 were both isolated from soil (Ertekin et al., 2015). Both of the aforementioned strains are resistant to BACs whereas only BIOMIG1 can degrade BACs and the optimum cell density for BACs biotransformation is 10⁸ CFU/mL. The minimum inhibitory concentration (MIC) of BIOMIG1 for BACs is 1024 mg/L. The MIC of BIOMIG4 is more than 1024 mg/L. The optimum growth temperature of BIOMIG1 is at 28 °C.

4.3.2. Preparation of Cultures

To prepare the culture for the assays, the frozen isolate was taken out from the freezer and get thawed in the room temperature for 2 hours. Then an aliquot of sample was taken by a sterile loop and strake on a LB agar containing 50 mg/L BAC plate (Table 4.3.). The plate was placed in an incubator at 28 °C overnight. Then five single colonies were scratched aseptically on another LB-BAC agar plate using sterile toothpicks and incubated at 28 °C overnight. In the third day, five 50 mL falcon tube including 5 mL LB broth consists of the components in Table 4.4. at 50 mg/L BAC were prepared. Moreover, the plate was taken out from the incubator and half of the patch was transferred to a falcon tube using a sterile wooden applicator. Then, falcon tubes were placed on an orbital shaker

and agitated at 130 rpm overnight. In the fourth day, 1 mL culture in the falcon tube was transferred to a microcentrifuge tube and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet was suspended in 1.5 mL culture medium which was described in section 4.2.2. These culture aliquots were used in cellulose hydrolysis and fermentation experiments.

Table 4.3. Components used for preparation of LB-BAC agar plates in an initial 1L solution

Components	Amount
Tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Agar	15 g
10g/L BACs stock solution	5 mL

Table 4.4. Components used for preparation of 1L LB broth

Components	Amount
Tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g

5. DEVELOPMENT OF CELLULOLYTIC MICROBIAL COMMUNITY AND EFFECT OF BACS ON CELLULOSE DEGRADATION

5.1. Introduction

Cellulosic biomass is the most abundant renewable resource on earth for biofuel productions. Cellulose is the major component of cellulosic biomass since it forms 35-50% of these materials. This polysaccharide consists of linear glucose chains that are connected through 1,4- β -D-Glucosidic bonds (Saha et al., 2004). The crystalline structure of cellulose distracts the hydrolysis process by cellulases. However, crystalline cellulose can be solubilized by multiple activities of microorganisms (Chen et al., 2014).

Cellulolytic bacteria have been isolated from the intestine of the white pine beetle larvae. *Serratia* and *Pseudomonas* were the major strains however some other species such as *Brevundimonas kwangchuensis*, *Brevundimonas vesicularis*, *Pseudoxanthomonas mexicana* and *Methylobacterium populi* were also identified as cellulolytic bacteria (Hu et al., 2014). Huang et al. (2012) isolated different cellulolytic bacteria such as *Ochrobacterium haenatophilum*, *Shinella zoogloeoides*, *Citrobacter freudii* and *Pseudomonas nitroreducens* (Huang et al., 2012). *Paenibacillus cellulositrophicus* which was able to produce cellulase enzyme was isolated from Thai soil (Akarcharanya et al., 2009). *Achromobacter* and *Pseudomonas* isolated from a soil sample were also identified as cellulolytic bacteria (Romano et al., 2013).

The specific objectives of the research reported in this chapter were to: (a) develop a cellulolytic microbial community; (b) identify major species in that community and (c) elucidate the effect of BAC ILs on cellulose degradation by the microbial community.

5.2. Materials and Methods

5.2.1. Development of Cellulose Degrading Soil Microbial Community

A 5 g of soil sample was transferred aseptically into a 500 mL sterile glass bottle with a screw-cap containing 200 mL of sterile mineral medium. 0.2 g cellulose powder amended aseptically to the flask as carbon and energy source. The bottle was placed on an orbital shaker at 130 rpm at room temperature. The total carbohydrate concentration in the bottle was monitored on a daily basis using anthrone method. As soon as the carbohydrate concentration reached a constant value (200 mg/L), a 20 mL of sample taken from the bottle was transferred aseptically into a new bottle containing 180 mL sterile medium and 0.2 g cellulose. This serial dilution was repeated for two times to obtain a specialized cellulose-degrading soil microbial community. 200 mL culture was then scaled up to 1L by transferred aseptically transferring the culture to a sterile 2L glass bottle and adjusting the volume to 1L with sterile medium. 1g cellulose was amended to 1L culture solution to give an initial cellulose concentration of 1000 mg/L. Half of the culture volume was discarded and filled out with a fresh medium and amended with 1 g cellulose every week. The total carbohydrate concentration in the bottle was monitored on a daily basis using anthrone method. The pH of the culture was also measured periodically before adding the cellulose.

5.2.2. Isolation of Dominant Cellulolytic Bacteria in the Enrichment Community

A 100 μ L of cellulolytic microbial community was diluted 10,000 times with saline solution. A 100 μ L sample of diluted sample was aseptically spread on cellulose-agar plate and a plate containing no cellulose. After a period of time, growth of bacteria on the plates containing cellulose was observed. About 18 single colonies grown on the cellulose-plate were aseptically transferred to 50 mL sterile tubes containing 5 mL LB broth and placed on an orbital shaker and agitated at 130 rpm in room temperature overnight. 1mL aliquots of each tube were transferred to microcentrifuge tubes mixed with 180 μ L sterile 80% glycerol solution and stored at -20 °C. The isolates were named from C1-C18.

5.2.3. Identification of the Cellulolytic Bacteria Based on 16SrDNA

In order to extract the DNA of isolates, isolates were grown in 50 mL falcon tubes contacting 5 mL LB broth overnight. An aliquot of 1mL sample was transferred into microcentrifuge tube and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 100 μ L sterile saline solution. Genomic DNA of harvested cells was extracted using BioBasic BS 434 DNA extraction kit following the manufacturer's instructions. Extracted genomic DNA was quantified using Implen® P360 nanophotometer. Its quality was also checked visually on 0.7% Agarose gel after electrophoresis. 16S rDNA of the isolates was amplified by PCR using TaKaRa Premix Taq™ Kit (TaKaRa Bio, Shiga, Japan) with the following primers: 27F forward primer (27F: 5'-AGAGTTTGATCMTGGCTCAG-3') (0.4 μ M), and 1492R reverse primer (1492R: 5'-TACGGYTACCTTGTTACGACTT-3') (0.4 μ M). PCR conditions included 35 cycles at 94 °C (30 sec), 55 °C (30 sec) and 72 °C (1min), with a final extension at 72 °C for 7 min. Amplified 16S rDNA was electroporated on 1% agarose gel (SeaKem LE Agarose, Lonza Inc., Basel, Switzerland), stained with Green DNA Dye (Bio Basic Inc., Onterio, Canada), UV-illuminated (GelDOC2000, BioRad, CA) and purified using EZ-10 PCR Purificaion Kit (Bio Basic Inc., Onterio, Canada). Purified 16S rDNA sample of the isolate was sequenced by MacroGen Inc. Europe (Amsterdam, Netherlands). Forward and reverse sequences were trimmed and assembled using Geneious Software (Biomatters Ltd., Auckland, New Zealand) to yield about 1350 bp length and above 95% quality 16S rDNA sequence. The sequence was then queried against the NCBI database (NCBI, 2012) using MEGABLAST algorithm and closest neighbor sequence to quired sequence was determined. Phylogenetic relationship between our isolates and other cellulolytic species in the literature was performed using Geneious Software following appropriate alignment and tree building algorithms.

5.2.4. Hole Method to Test the Cellulolytic Capability of the Isolates

The hole method was used to visually observe the cellulose degradation by the isolates. In this method the cellulolytic microorganisms are going to make a transparent zone around the hole (McDonald et al., 2012). The cultures of isolates were prepared according to the method described in chapter 4. The holes with same size were made on

cellulose-agar plates with 1.5% cellulose concentration. An aliquot of 5 μ L culture sample was aseptically transferred to each hole. A plate with holes fed just with medium was provided as control plate.

5.2.5. Aerobic and Anaerobic Cellulose Degradation by the Microbial Community

Since the cellulose-degrading microbial community was developed under aerobic condition, the both aerobic and anaerobic cellulose degradation was performed to evaluate the microbial community in cellulose utilization under different conditions. Biodegradation of cellulose by the cellulolytic microbial community was tested under aerobic and anaerobic conditions as follows: To perform the aerobic cellulose degradation, 100 mL of culture was transferred to a 250 mL autoclaved Erlenmeyer and it was capped with caps that allow air penetration in the flasks. 0.1 g cellulose was amended into the flask to maintain 1000 mg/L initial cellulose concentration in the flask. An Erlenmeyer containing just medium and cellulose served as control flask. In addition, biodegradation of cellulose at 10,000 mg/L initial concentration by the cellulolytic community was also tested following the aforementioned procedure but adding 1 g of cellulose into the flasks.

To assess the anaerobic cellulose degradation, 100 mL of culture was transferred aseptically into a 125mL serum bottle. 0.1 g cellulose was added into each serum bottle. The bottles then sealed with rubber stoppers and aluminum caps and flushed with nitrogen gas for 5 minutes to provide the anaerobic conditions. Moreover, a bottle with cellulose and medium was prepared as a control bottle.

The flasks and bottles were prepared in duplicates and were placed on an orbital shaker and agitated at 130 rpm at room temperature. The total carbohydrate, glucose, alcohols and organic acids content of the bottles were monitored on a daily basis for 10 days. The pH was also measured at the end of experiment.

5.2.6. Aerobic Cellulose Degradation by Different Dilutions of Cellulolytic Microbial Community

In these set of experiment, aerobic cellulose degradation by different dilutions of the microbial community was investigated. After the cellulose concentration in 2L flask reached its lowest amount (around 200 mg/L), different amounts of culture was transferred into sterile 250 mL Erlenmeyer flasks as described in the Table 5.1. The flasks were amended with 0.1 g cellulose.

The flasks were placed on an orbital shaker and agitated at 130 rpm in room temperature. The total carbohydrates, glucose, alcohols and organic acids contents of the flasks were measured according to the aforementioned methods. The pH was also measured at the end of experiment.

Table 5.1. Experimental matrix of culture series and controls used in the batch cellulose degradation experiment at different biomass density

Culture Series	Medium (mL)	Culture (mL)	Cellulose (g)	Total Volume (mL)
Control	100	0	0.1	100
Df=0	0	100	0.1	100
Df=2	50	50	0.1	100
Df=5	80	20	0.1	100
Df=10	90	10	0.1	100
Df=100	99	1	0.1	100

*The flasks containing cultures were prepared in duplicates.

5.2.7. Aerobic Cellulose Degradation by Centrifuged Supernatant of Cellulolytic Microbial Community

A 100 mL of culture solution was aseptically transferred to sterile centrifuge vials and centrifuged at 10,000 rpm for 20 minutes. The supernatant was transferred into a 250 mL Erlenmeyer flask. 0.1 g cellulose was added into the flask to give an initial cellulose concentration of 1000 mg/L in the flasks. The flasks were placed on an orbital shaker at

130 rpm for 7 days. A control flask was also prepared without any culture. The total carbohydrates, glucose, organic acids and alcohols content of flasks were followed every day. The pH was also measured at the end of experiment.

5.2.8. Aerobic Cellulose Degradation by Filtered Supernatant of Cellulolytic Microbial Community

A 100 mL of culture supernatant was filtered through a sterile 0.45 μ m filter paper. The filtrate was aseptically transferred into a sterile 250 mL Erlenmeyer flask. The each flask was amended with 0.1 g cellulose fibers and capped with porous stoppers. The flask were placed on an orbital shaker and agitated at 130 rpm in room temperature. A flask with just cellulose and medium was set as control. The total carbohydrate, glucose, alcohols and organic acid concentration were monitored every day for 7 days. The pH was also measured at the end of experiment.

5.2.9. Aerobic Cellulose Degradation by Cellulolytic Microbial Community in the Presence of BACs

Effect of BACs on cellulose degradation by the cellulolytic microbial community was investigated in a series of batch experiments set in 250-mL Erlenmeyer flasks. A sample taken from the cellulolytic culture was diluted twice or 5 times and transferred into 250-mL Erlenmeyer flasks, aseptically. 0.1 g cellulose was added to each flask containing 100 mL culture and amended with an appropriate amount of BACs stock solution to maintain 50, 100, 500 and 1000 mg/L initial concentration (Table 5.2.). The flasks were capped with cellulosic caps and placed on an orbital shaker and agitated at 130 rpm in room temperature. The total carbohydrates, glucose, alcohols and organic acids concentration were monitored in a daily basis. Moreover, the pH of reactors was measured at the end of the retention time.

Table 5.2. Experimental matrix of culture series and controls used in the batch cellulose degradation experiment set either with twice or five times diluted culture at different BAC concentrations

Culture Series	Culture Medium (mL)	Culture (mL)	Cellulose (g)	10 g/L BACs stock solution (mL)	Total Volume (mL)
Control	100	0	0.1	0	100
0 mg BAC/L	0	100	0.1	0	100
50 mg BAC/L	0	99.5	0.1	0.5	100
100 mg BAC/L	0	99	0.1	1	100
500 mg BAC/L	0	95	0.1	5	100
1000 mg BAC/L	0	90	0.1	10	100

*The reactors containing cultures were prepared in duplicates.

5.3. Results and Discussion

5.3.1. Cellulolytic Activity of the Microbial Community

The total carbohydrate concentration in the bottle decreased from 1000 to 200 mg/L within 14 days when the culture was first set up. Given the fact that, cellulose was the only carbohydrate amended into the bottle and its concentration did not change in the control flask without soil inoculum, decrease in the total carbohydrate concentration indicated that cellulolytic microorganisms were present in the soil sample used as the inoculum of the culture. However, total carbohydrate concentration in the bottle did not change further after 14 days of incubation. Given the fact that glucose was not detected in the samples taken from the culture and cellulose particles were not visible at the end of 14 days, carbohydrates measured were assumed to be extracellular polymeric substances produced during cellulose degradation by the community (Figure 5.1.).

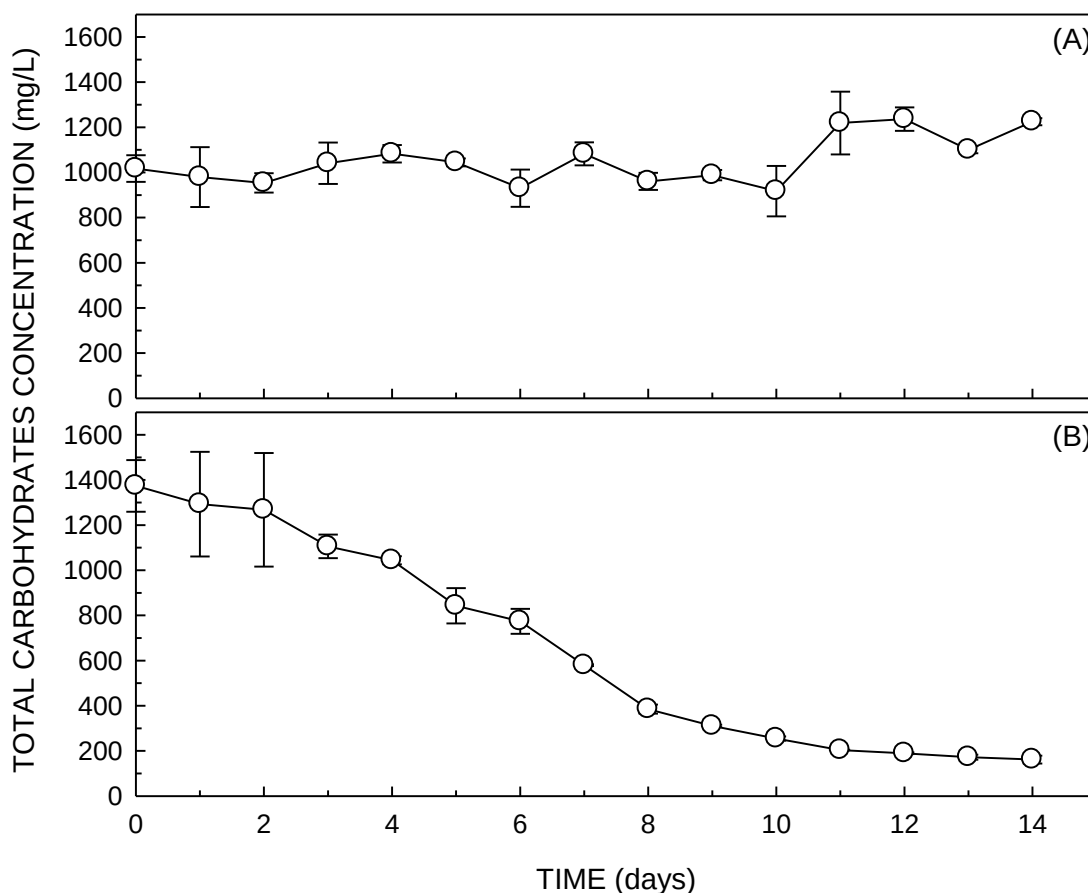


Figure 5.1. Cellulose utilization profile in (A) control and (B) microbial community at the first amendment of cellulose

After various serial dilutions and feeding with cellulose to get a specialized cellulolytic community, culture was scaled up to 1L and operated at fed-batch mode for 150 days as described above (Figure 5.2.). pH of the culture was between 6.9-7.1 and did not change during this course. The total suspended solid (TSS) and volatile suspended solid (VSS) of the culture was 1.675 and 0.97 g respectively. In every feeding total carbohydrate concentration dropped to about 200 mg/L from an initial concentration of 1000 mg/L within a week period (Figure 5.3.). Moreover, no glucose, organic acids or alcohols was detected, which suggested that the cellulose is mineralized to carbon dioxide.



Figure 5.2. Developing cellulose degrading microbial community

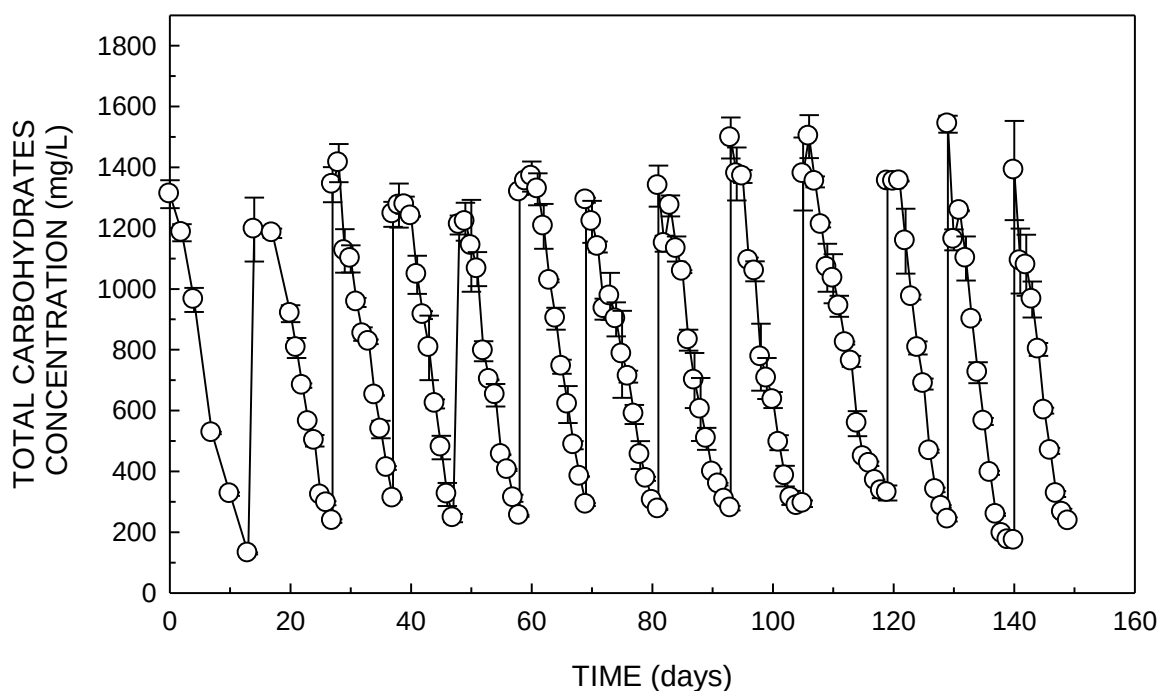


Figure 5.3. Cellulose utilization profile in cellulolytic microbial community during fed-batch operation period

A sample from cellulolytic microbial community was taken and transferred to two sets of flasks containing 1000 mg cellulose/L. One set was incubated aerobically and the other set was kept under anaerobic conditions. Total carbohydrate concentration decreased from 1000 mg/L to about 200 mg/L in the set incubated aerobically whereas carbohydrate concentration did not change in the set incubated anaerobically within 6 days period (Figure 5.4.). Glucose or other monosaccharides were also not detected in the bottles

incubated anaerobically. These results suggested that cellulose hydrolysis occurs aerobically in the culture (Figure 5.4.). According to the literature, most cellulosic materials are degraded aerobically whereas 5-10% is degraded under anaerobic condition (Leschine, 1995). Gupta et al., 2012 reported that the cellulose degrading microbial community developed from the gut of some termites and worms utilized the filter paper aerobically within 7 days incubation period at 37°C (Gupta et al., 2012). In a different study, microbial communities developed from different types of soil from farming and forest areas degraded the CarboxyMethylCellulose (CMC) aerobically at 30°C (Hatami et al., 2008). Lednicka et al. (2000) developed a cellulose microbial community from soil able to utilize the cellulose aerobically at 28°C in 7 days (Lednicka et al., 2000).

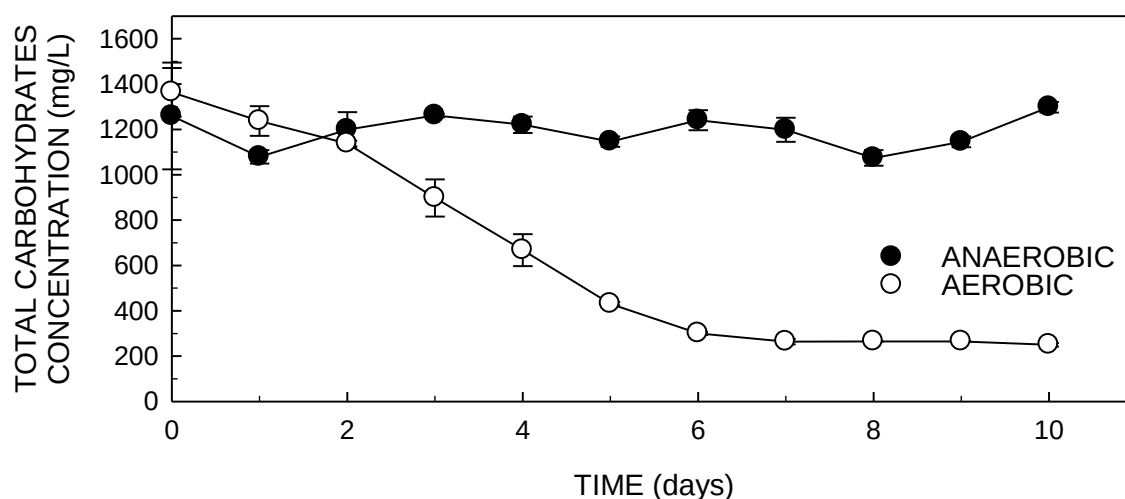


Figure 5.4. Cellulose utilization by soil microbial community under aerobic and anaerobic conditions

In order to test how cellulose concentration affects the biodegradation rate, a sample of cellulolytic microbial community was amended with 10,000 mg cellulose/L. The total carbohydrate concentration decreased from 10,000 to 2,000 mg/L within 12 days, suggesting that increasing the cellulose concentration does not increase the biodegradation rate (Figure 5.5.). Only 80% of the cellulose was degraded at 10,000 mg/L initial cellulose concentration as it was observed at 1000 mg cellulose/L (Figure 5.5.). No glucose, alcohols or organic acids were detected during the incubation period.

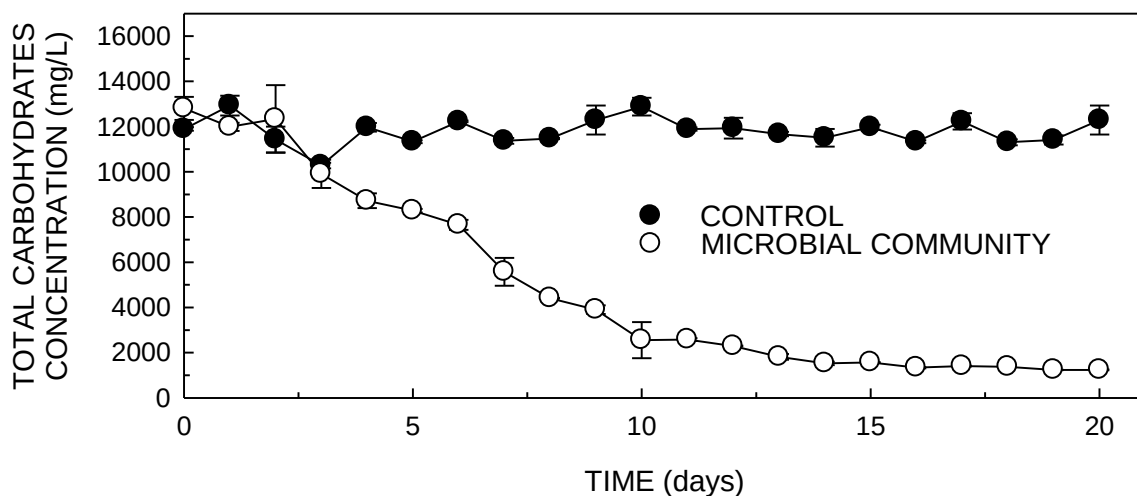


Figure 5.5. Profile of cellulose utilization by microbial community (initial 10,000 mg/L cellulose concentration)

In order to elucidate how biomass concentration affect the biodegradation of cellulose by the cellulolytic microbial community, a set of experiments were performed in Erlenmeyer flasks inoculated with different volumes of culture and amended with 1000 mg cellulose/L. While total carbohydrate concentration did not change in the control series having no culture, 80% of the cellulose was utilized within 6 days in all culture series having different biomass concentration (Figure 5.6.). However, certain lag period was observed in the cultures diluted 10 and 100 times (Figure 5.6.). The pH of each series was at 7.1 and glucose, alcohols and organic acids were not detected in the course of incubation.

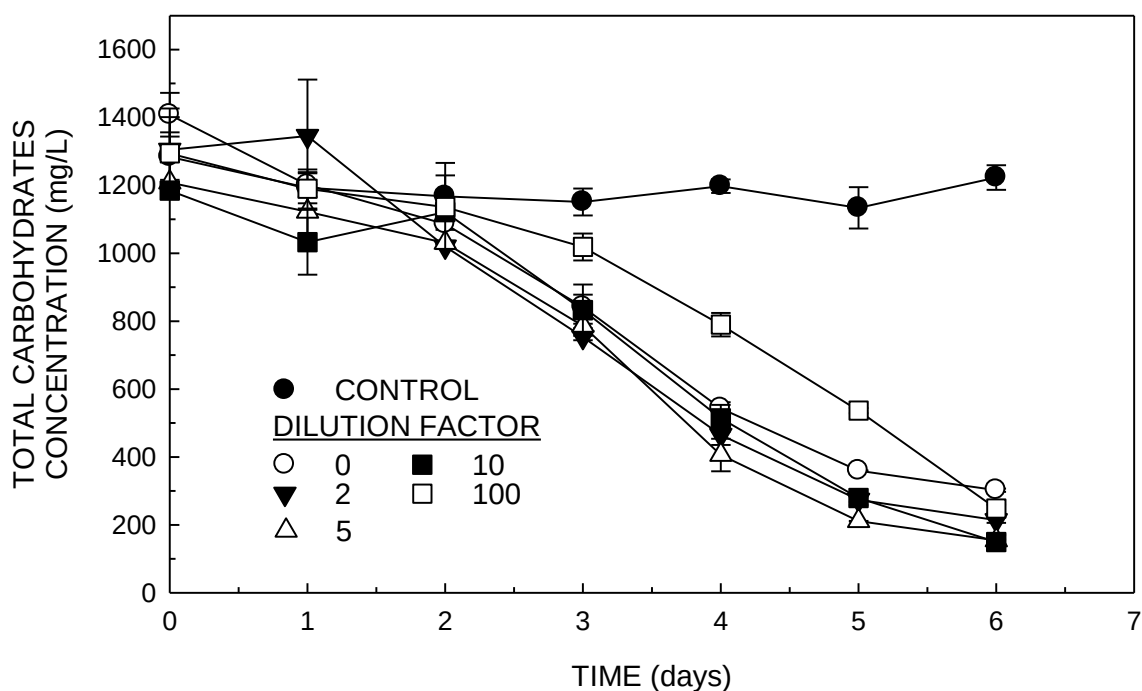


Figure 5.6. Profile of cellulose degradation by 0, 2, 5, 10 and 100 dilution of soil microbial community

Moreover, the supernatant of the cellulolytic microbial community after centrifugation was used to test cellulose biodegradation. Supernatant was also consumed 80% of cellulose within 6 days following a profile similar to the culture except 3 days of delay observed in the initiation of degradation observed in the series set with the supernatant (Figure 5.7.) Glucose, alcohols and organic acids were not detected in the course of the incubation and the pH was constant at 7.0.

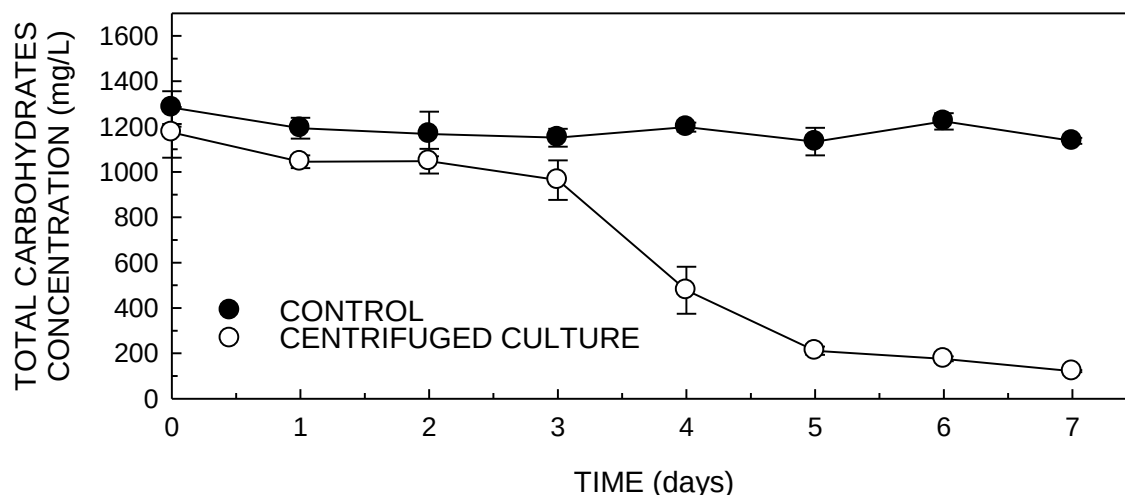


Figure 5.7. Profile of aerobic cellulose utilization by centrifuged soil microbial community

Based on the results obtained in the experiment performed with the culture supernatant, it was hypothesized that cellulases in the cellulolytic community are extracellular. To test this hypothesis, culture supernatant was filtered through 0.45 μm pore size filter, transferred into an Erlenmeyer flask and amended with cellulose at 1000 mg/L concentration. Total carbohydrate concentration did not change and glucose was not detected during the incubation period (Figure 5.8.). This result suggested that cellulose enzymes present in the cellulolytic microbial community was not extracellular or did not pass through the filter used. Since cellulose is an insoluble macromolecule, it is clear that the cellulases must be localized outside of the cells (extracellular) however they may still be bound the cell surface (Ferdousi Begum and Absar, 2009). Cheng et al. (2011) extracted the extracellular cellulases from soil able to efficiently degrade the cellulose (Cheng et al., 2011). Whereas in a different study, intracellular enzymes purified from cellulosic waste samples were responsible for cellulose utilization (Ferdousi Begum and Absar, 2009).

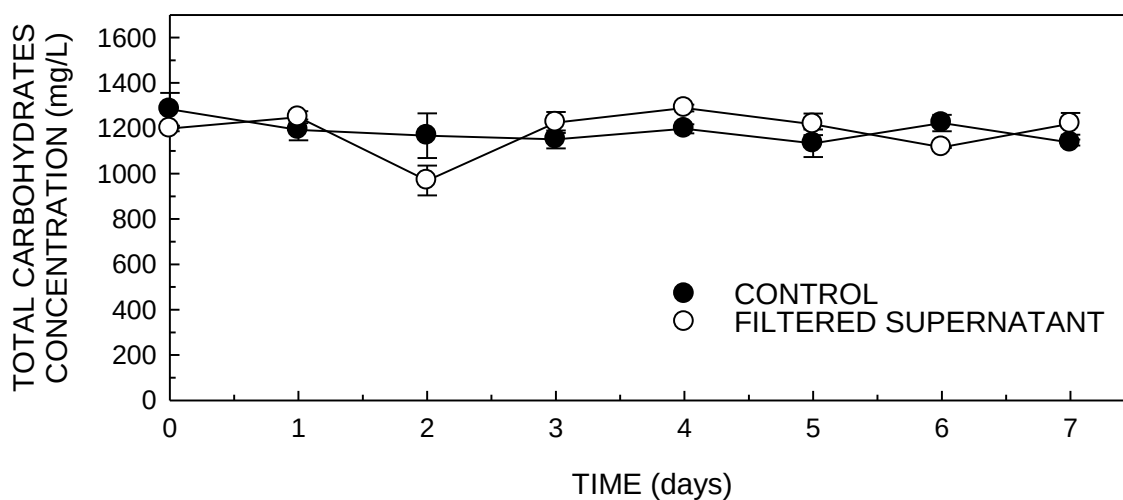


Figure 5.8. Aerobic cellulose utilization by filtered supernatant of soil microbial community

5.3.2. Dominant Cellulolytic Bacteria in the Microbial Community

To isolate the cellulolytic microorganisms in microbial community, a 100 μ L of appropriately diluted sample of community was plated on a cellulose-agar. This method assumes that colonies growing on this agar are bacteria which can degrade cellulose. After about 4 days 18 colonies grew on cellulose-agar plate (Figure 5.9.).



Figure 5.9. Cellulolytic bacteria grew on cellulose-agar plate

DNA of the isolates was extracted and 16S rDNA of each isolate were amplified using PCR. Each 16S rDNA amplicon was digested with MspI endonuclease and restriction fragment length polymorphism (RFLP) patterns of each digest were visualized

on 2% agarose gel (Figure 5.10.). According to the RFLP banding patterns, 5 out of 18 isolates were different species. 16S rDNAs of those species were sequenced, processed and queried against the NCBI nucleotide database.

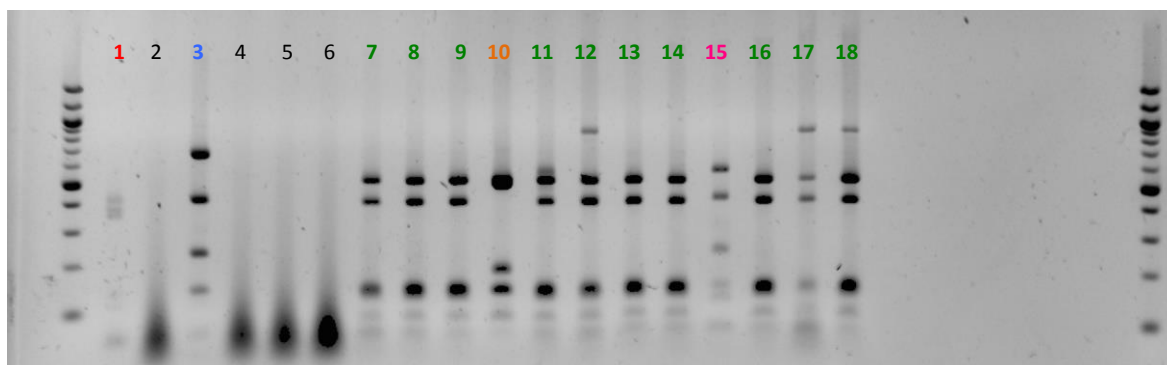


Figure 5.10. RFLP patterns of 16S rDNA amplicons of each isolate

The closest sequence to C1, C3, C8, C10 and C15 belonged to *Ochrobactrum*, *Sinorhizobium*, *Alcaligenes*, *Pseudoxanthomonas* and *Shinella* species respectively. The strain were named *Ochrobactrum* sp. BIOMIG5, *Sinorhizobium* sp. BIOMIG6, *Alcaligenes* sp. BIOMIG7, *Pseudoxanthomonas* sp. BIOMIG8 and *Shinella* sp. BIOMIG9. The 16S rDNA of the isolates was then aligned with sequences of other cellulolytic bacteria from literature using Multiple Sequence Comparison by Log-Expectation (MUSCLE). A similarity matrix was created to compare the sequences. A neighbor joining phylogenetic tree was prepared from a distance matrix created by a maximum likelihood method (Figure 5.12.). Huang et al. (2012) reported *Shinella zoogloeoides* and *Ochrobacterum cytisi* cellulose degrading bacteria among 21 cellulolytic species isolated from the gut of *Holotrichia parallela* larvae (Huang et al., 2012). 11 different cellulolytic bacteria genotypes were obtained from the gut of *Dendroctonus armandi* Larvae. *Pseudoxanthomonas mexicana* accounted 2.2% of the cellulolytic bacteria (Hu et al., 2014). In a different study, a cellulose degrading soil microbial community was developed. *Alcaligenes* sp.CH14 and *Pseudoxanthomonas mexicana* belonged to this community were identified as cellulolytic bacteria (Trujillo-Cabrera et al., 2012).

The hole method was performed to check the capability of 18 isolates in cellulose degradation. In this method, it is assumed that microorganism which can utilize cellulose as carbon source will generate a transparent zone around the hole on cellulose-agar. In this

study, all of the 5 identified isolates were able to grow around the hole which confirms their cellulose degrading ability (Figure 5.11.).

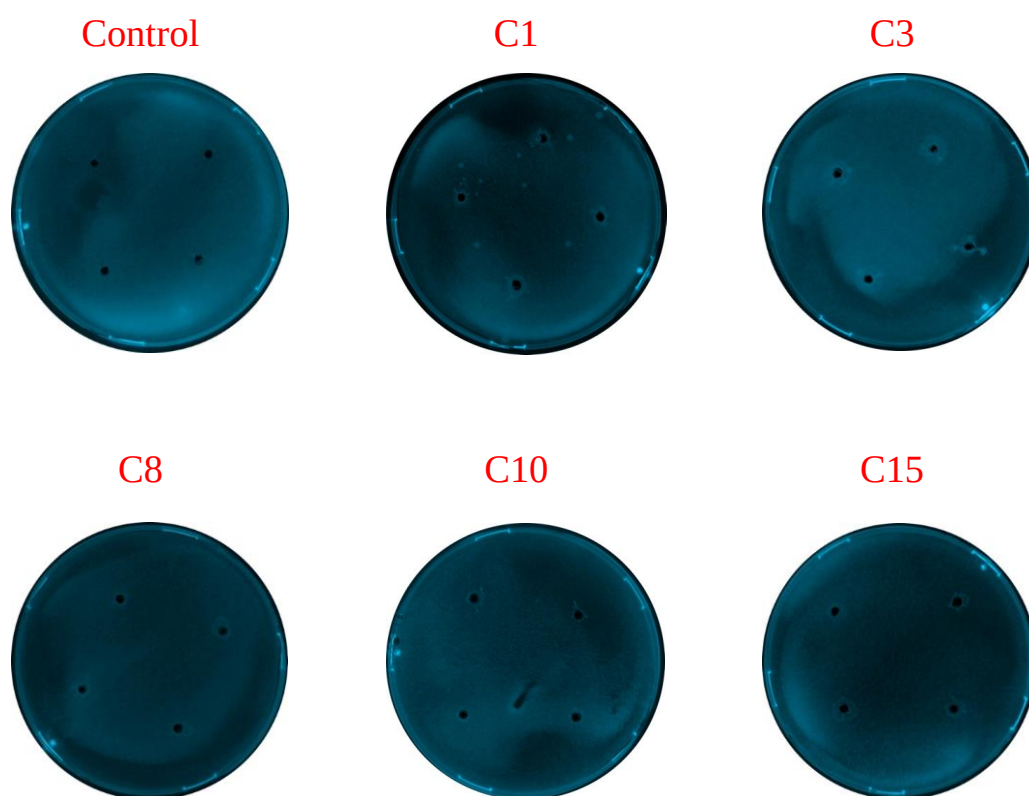


Figure 5.11. Hole method for testing and confirming the cellulose utilization capability of isolates

As a result, the most dominant cellulolytic bacteria in the community was *Alcaligenes sp.* BIOMIG7 and stains of this species were also demonstrated to be cellulolytic. Gunathilake et al. (2013) isolated three different *Alcaligenes sp.* from soil and compost microbial communities. These species were among the five most efficient cellulolytic bacteria obtained from the aforementioned communities (Gunathilake et al., 2013). *Alcaligenes facalis* obtained from the gut of *Prostephanus truncatus* and recorded as cellulolytic bacteria utilizes cellulose at pH 4.5-6.5 under aerobic condition (Vazquez-Arista et al., 1997). Adenan, (2013) reported *Alcaligenes* from the larval gut of *Rhynchophorus ferrugineus* appeared on CMC plate agar and behaved as the cellulolytic bacteria (Adenan, 2013).

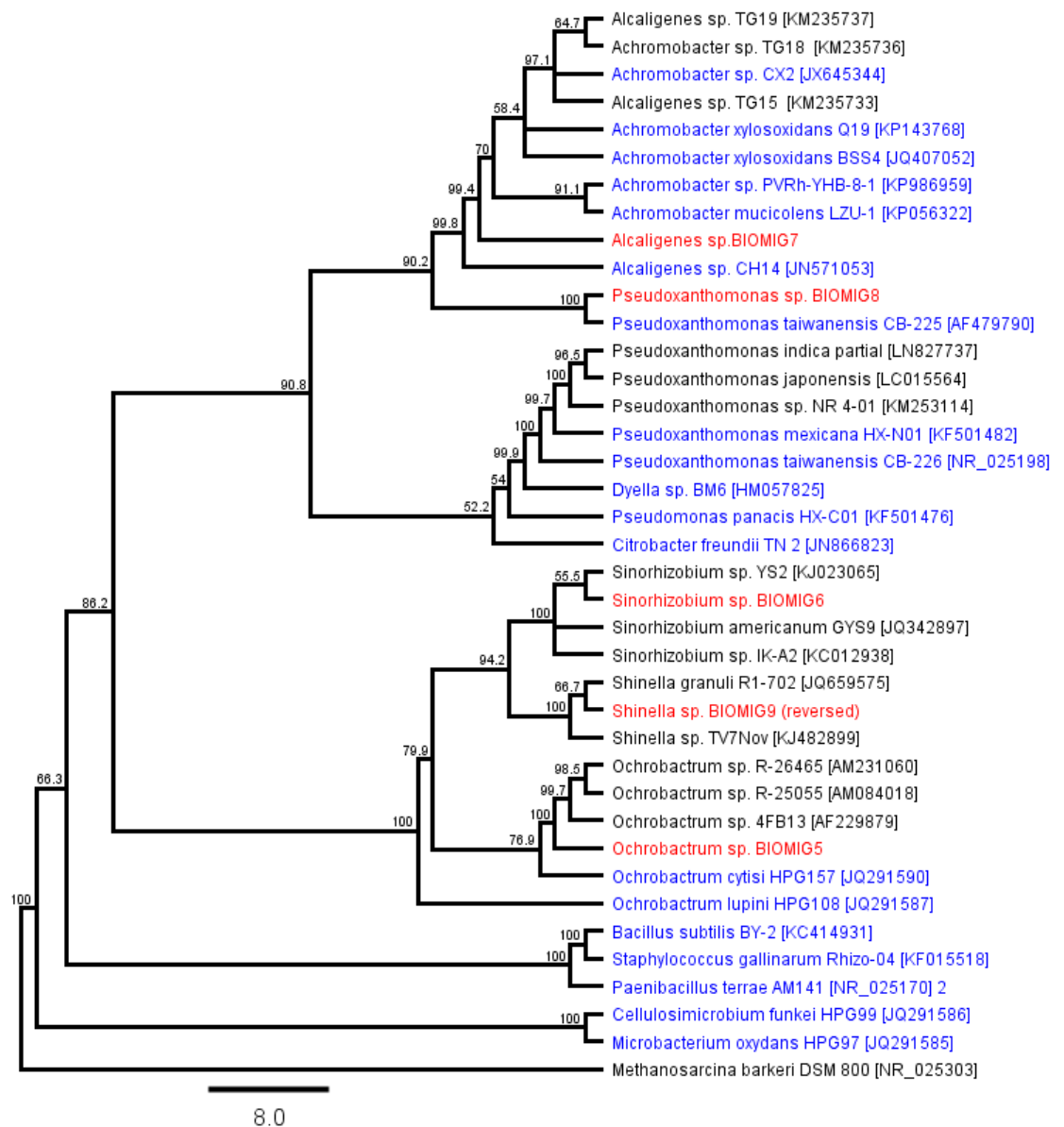


Figure 5.12. Phylogenetic tree of relationships of 16S rDNA sequence of 5 isolates, determined by maximum likelihood followed by neighbor joining tree building method and Hasegawa-Kishino-Yano genetic distance model, relative to other cellulolytic bacteria. The scale bar represents 8.0 substitution per nucleotide position. *Methanosarcina barkeri* was used as the outgroup. (Branches in red are cellulolytic isolates in cellulose-degrading community, Branches in black are nucleotide sequences of species with the closest sequences to 5 isolates and branches in blue are nucleotide sequences of cellulolytic species in literature).

5.3.3. Effect of BAC ILs on Cellulolytic Community

The effect of BACs as ILs was investigated on cellulose biodegradation by the microbial community at 50, 100, 500 and 1000 mg/L BACs concentrations. Total carbohydrate concentration in the culture that received no BACs decreased from 1000 mg/L to 200 mg/L within 6 days (Figure 5.13.B). However, cellulose was not utilized in the BAC receiving cultures even at the lowest BAC concentration tested (Figure 5.13.C-F). Glucose, organic acids and alcohols were detected in none of the culture series during the incubation period. The pH of the cultures was stable at 7.1. BACs were conserved in all flasks in the course of incubation (Figure 5.13.).

It was reported that ILs can denature the enzymes (Nakashima et al., 2011; Vancov et al., 2012). In our study, cessation of utilization of cellulose even in the presence of low concentration of BACs was attributed to inhibition of cellulases responsible for hydrolysis of the cellulose in the culture. Ooshima et al. (1986) also reported that cationic surfactant Tween Q-86W denatured the cellulase at and above 80 mg/L (Ooshima et al., 1986).

Since 5 times dilution of the culture may increase the inhibitory effect of BACs on the microorganism, we repeated the same experiment by diluting the culture twice. The results show that even at higher biomass concentration, the culture could not utilize the cellulose in the presence of BACs (Figure 5.14.).

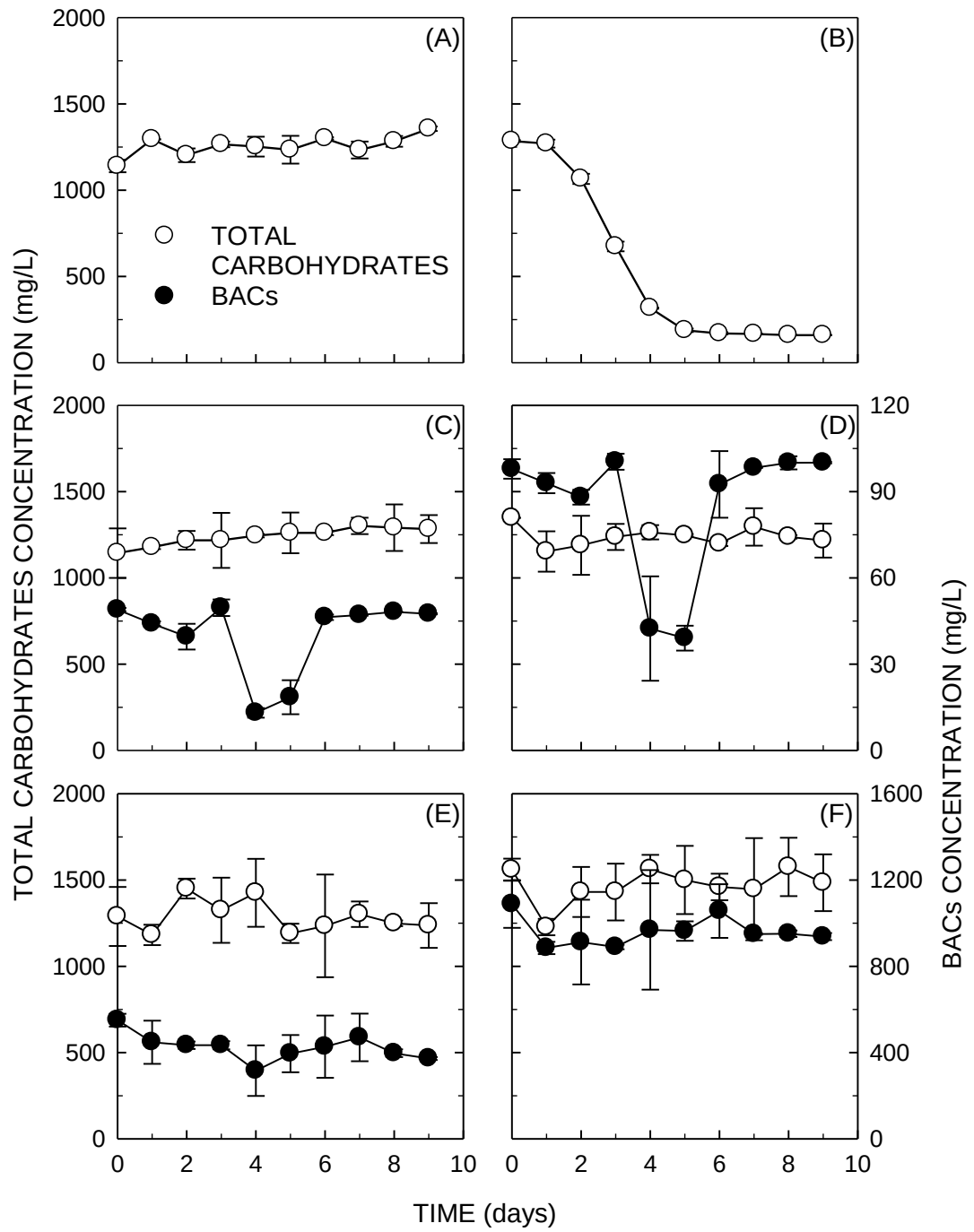


Figure 5.13. Profile of total carbohydrates and BAC ILs utilization in (A) control and flasks containing 5 times diluted cellulolytic microbial community at (B) 0, (C) 50, (D) 100, (E) 500 and (F) 1000 mg BAC/L.

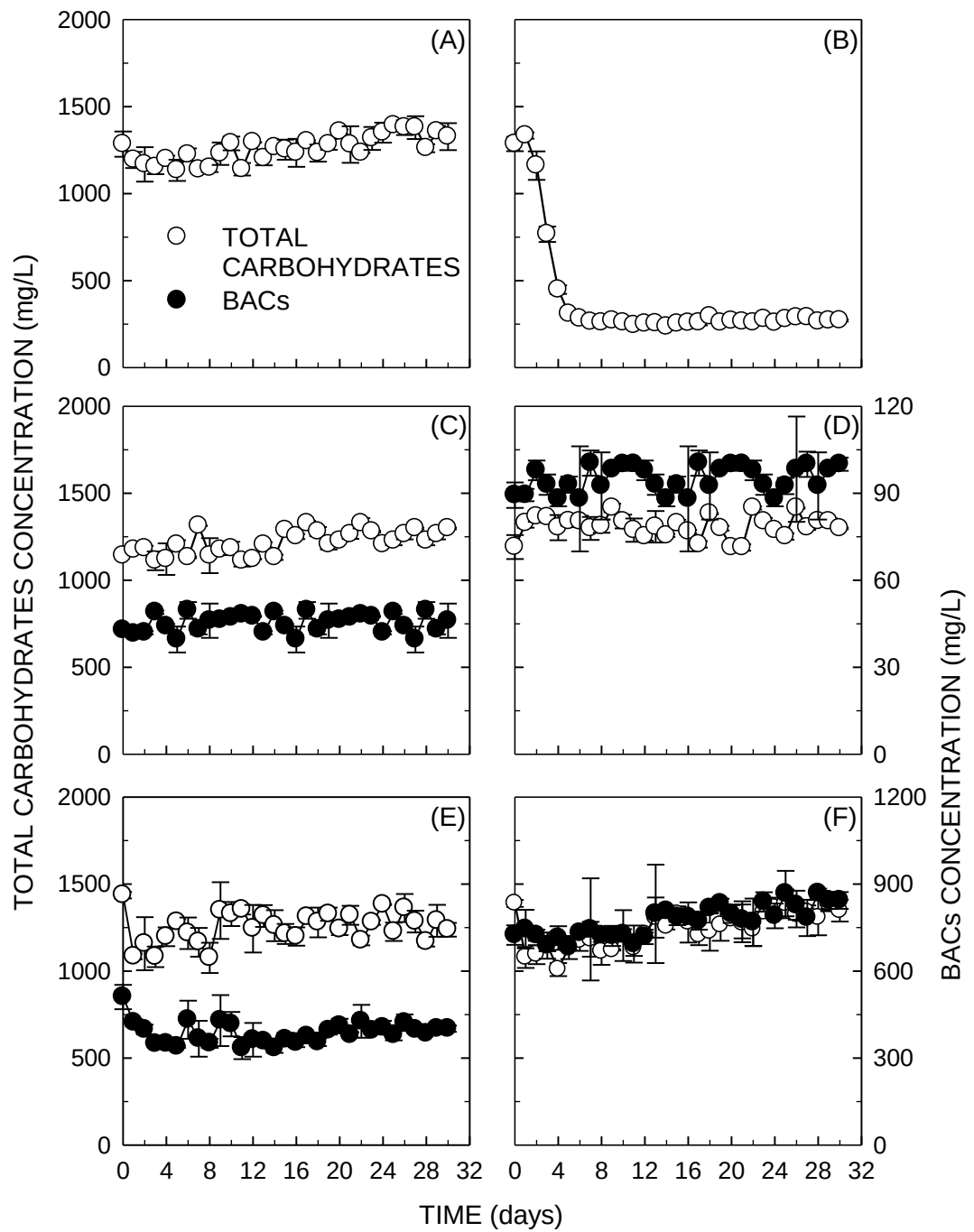


Figure 5.14. Profile of total carbohydrates and BAC ILs utilization in (A) control and flasks containing 2 times diluted cellulolytic microbial community at (B) 0, (C) 50, (D) 100, (E) 500 and (F) 1000 mg BAC/L.

5.4. Summary

A cellulose-degrading soil microbial community was developed from soil which can utilize cellulose (1000 mg/L) in about 6 days under aerobic condition. In addition, the soil microbial community could utilize 10,000 mg/L cellulose in about 12 days. Further, cellulose degradation by filtered supernatant and centrifuged supernatant of soil microbial community was performed. The former could not utilize the cellulose whereas the latter could degrade the cellulose in 6 days.

To isolate the cellulose degrading microorganisms in soil community, a sample of soil community was spread on a cellulose-agar plate. 18 colonies were appeared after 4 days. RFLP result shows that 5 of these colonies were considered as different strains which were named *Ochrobactrum* sp. BIOMIG5, *Sinorhizobium* sp. BIOMIG6, *Alcaligenes* sp. BIOMIG7, *Pseudoxanthomonas* sp. BIOMIG8 and *Shinella* sp. BIOMIG9. After identifying the strains, the hole method was applied to figure out their individual cellulose-utilization. All of the strain were able to make clear zone around the hole in cellulose-agar plate.

In the further step different dilutions of microbial community were tested to check the effect of biomass concentration. 2 and 5 times dilution gave the most consistent results. Later, 2 and 5 times diluted soil microbial community were considered and amended with 0-1000 mg/L BACs to realize the impacts of BACs on the rate of cellulose degradation. The results show that even low BACs concentration inhibited the cellulose degradation since no decline in total carbohydrates concentration was observed.

6. CELLULOSE DEGRADATION POTENTIAL OF BACTERIA RESISTANT TO BAC ILs

6.1. Introduction

Alternative renewable energy production considered as a serious issue when the fossil fuels consumption was increased and the oil crisis faced a new phase of problems. Biofuels from lignocellulosic biomass considered as an acceptable alternative compared to food crops since lignocellulosic materials do not compete with food productions (Gu, 2013; Faraco, 2013). The lignocellulosic biomass composed of three main parts: 1) cellulose, 2) hemicelluloses and 3) lignin in which cellulose is the most abundant (35-50%) and main component for biofuel and value-added chemicals production (Saha et al., 2004).

Cellulose consists of linear glucose units linked by 1,4- β -D-Glucosidic bonds (Saha et al., 2004). In order to produce biofuels, cellulose must be converted to glucose by cellulases and further fermented to biochemicals. A lot of microorganisms were investigated as cellulose producers. Many bacteria species such as *Caldocellulosiruptor*, *Butyrivibrio*, *Ruminococcus*, *Eubacterium*, *Clostridium*, *Bacteroides*, *Acetivibrio*, *Thermoactinomyces*, *Caldibasillus*, *Bacillus*, *Acidothermus*, *Cellulomonas*, *Micromonospora*, *Actinoplanes*, *Streptomyces*, *Thermobifida*, *Thermomonospora*, *Microbispora*, *Fibrobacter*, *Sporocytophaga*, *Cytophaga*, *Flavobacterium*, *Achromobacter*, *Xanthomonas*, *Cellvibrio*, *Pseudomonas*, *Serratia* and *Myxobacter* (<http://www.wzw.tum.de/mbiotec/cellmo.htm>, and the references herein; Dees et al., 1995; Lednicka et al., 2000; Cheng et al., 2011 and Hu et al., 2014).

Several studies reported *Pseudomonas* and *Serratia* strains as cellulolytic bacteria. Hu et al. (2014) isolated cellulolytic bacteria from the intestine of the white pine beetle. 49.5 and 22% of the cellulolytic bacteria in community were identified as *Serratia* and *Pseudomonas* respectively (Hu et al., 2014). *Pseudomonas* strain was isolated from the gut of *Holotrichia parallela* Larvae which was recorded as cellulose degrading bacteria (Huang et al., 2012). Romano et al. (2013) could isolate a cellulolytic *Pseudomonas* strain from a

soil forest (Romano et al., 2013). In a study by Kavitha et al. (2014) aerobic *Pseudomonas aeruginosa* and facultative anaerobe *Serratia marcescens* isolated from termite were identified as cellulolytic bacteria (Kavitha et al., 2014). Sreedevi et al. (2013) isolated cellulase producing *Pseudomonas* from the wood-yards (Sreedevi et al., 2013).

Since cellulose has a crystalline structure which prevents the cellulases to attack to cellulose, pretreatment of cellulose is a necessary process (Saha et al., 2004). Among pretreatments, using ILs to decrease the crystallinity of cellulose gained a lot of attentions due to their excellent feature such as low flammability, non-volatility and high thermal stability. ILs were investigated to be compatible with cellulases depends on their structures, however a high concentration of IL may denature the enzymes. Therefore, an optimum IL/water ratio must be considered to increase the efficiency of the cellulases (Fang et al., 2014).

Relatively recently, Ertekin and Tezel (2014) isolated a novel *Pseudomonas sp.*, strain BIOMIG1 and *Serratia marcescens* strain BIOMIG-4 from soil. These strains are tolerant to C₁₂BDMA-Cl and C₁₄BDMA-Cl (BACs) up to 1024 mg/L. In addition, BIOMIG1 can degrade BACs under aerobic condition.

The objective of the research presented in this chapter is to enhance dissolution of cellulose by using aforementioned BACs as reaction medium and to convert glucose to value-added alcohols such as ethanol and organic acids by *Pseudomonas sp.* BIOMIG1 and *Serratia marcescens* BIOMIG4.

6.2. Materials and Methods

6.2.1. Batch Cellulose Degradation by the Co-culture of *Pseudomonas sp.* BIOMIG1, *Serratia marcescens* BIOMIG4 in BAC ILs

A batch experiment testing the aerobic cellulose utilization by the co-culture of *Pseudomonas* and *Serratia* at different BACs concentrations ranging from 50-1000 mg/L was performed in 250 mL sterile Erlenmeyer flasks. Each flask received 1000 mg/L

cellulose, 10^8 cell/mL BIOMIG1 and BIOMIG4. BACs were amended to give a desired initial concentration in the flasks.

Table 6.1. Experimental matrix of culture series and controls used in the batch cellulose degradation experiment by BIOMIG1 and BIOMIG4 at different BAC concentrations

Culture Series	Culture Medium (mL)	Co-culture (mL)	Cellulose (g)	10 g/L BACs stock solution (mL)	Total Volume (mL)
Control	100	0	0.1	0	100
0 mg BAC/L	97	3	0.1	0	100
50 mg BAC/L	96.5	3	0.1	0.5	100
100 mg BAC/L	96	3	0.1	1	100
500 mg BAC/L	92	3	0.1	5	100
1000 mg BAC/L	87	3	0.1	10	100

*The flasks containing cultures were prepared in duplicates.

6.3. Results and Discussion

6.3.1. Cellulose Degradation Potential of BIOMIG1 and BIOMIG4 in BAC ILs

The effect of BACs as ILs was investigated on cellulose biodegradation by the co-culture of BIOMIG1 and BIOMIG4 at 50, 100, 500 and 1000 mg/L BACs concentrations. Total carbohydrate concentration in the culture that received no BACs did not change which shows that these strains are not cellulolytic bacteria (Figure 6.1.). Moreover, cellulose was not utilized in the BAC receiving cultures even at the lowest BAC concentration tested (Figure 6.1.). Therefore, BACs presence did not affect the dissolution of cellulose for further hydrolysis by the co-culture.

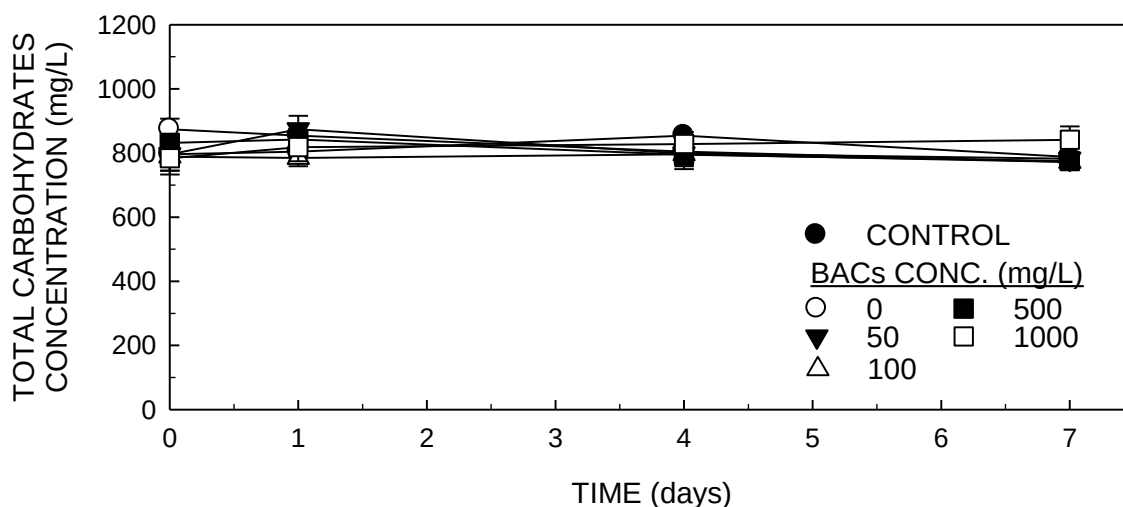


Figure 6.1. Aerobic cellulose utilization by co-culture of BIOMIG1 and BIOMIG4 at different BAC ILs concentration

6.4. Summary

A batch experiment testing the aerobic cellulose utilization by the co-culture of BAC ILs resistant *Pseudomonas sp.* BIOMIG1 and *Serratia marcescens* BIOMIG4 at different BACs concentrations ranging from 50-1000 mg/L was performed in 250 mL sterile Erlenmeyer flasks. The strains did not consume cellulose in the culture that did not receive any BAC ILs. Moreover, cellulose was not utilized in flasks receiving different BAC ILs concentration. The results confirm the non-cellulolytic activity of aforementioned strains. In addition, BAC ILs presence did not favor the cellulose dissolution for the hydrolysis process.

7. *PSEUDOMONAS SP.* BIOMIG1 FACILITATED CELLULOSE DEGRADATION BY CELLULOLYTIC MICROBIAL COMMUNITY IN BACS ILs

7.1. Introduction

Cellulosic biomass is the most abundant renewable resource on Earth which is composed of cellulose, hemicelluloses and lignin. Cellulose, a polymer of glucose monomers plays the most significant role in biofuel and biochemical production (Saha et al., 2004).

Enzymatic hydrolysis of cellulosic biomass to glucose is not easy due to recalcitrant structure of this biomass. To be more accessible to enzymes, cellulosic materials are gone through different pretreatment processes which reduce the crystallinity of cellulose. As a result, cellulases can be more efficient in converting the cellulose to the monomers (Faraco, 2013).

Pretreatment with ILs are proposed as environmentally friendly process since ILs are non-volatile, low-flammable and thermally stable. Many ILs were investigated as pretreatment solvents. After pretreatment, dissolved cellulose regenerated within an antisolvent such as water, ethanol and methanol will be used in downstream processes for biofuel or biobased chemical production (Fang et al., 2014).

Moreover, the dissolved cellulose may be hydrolyzed in IL solution. ILs may have stabilizing or destabilizing influence on enzymes according to their anion and cation structure. Hydrophilic ILs were reported to deactivate the cellulase while they are dissolving the cellulose. On the other hand, hydrophobic ILs like surfactants were realized as enzymes activator. It was reported that [bmim]Cl which is one of the most widely used cellulose solvent, denature the cellulase from *Trechidorma reesei* (Tuner et al., 2003). Paljevac et al. (2006) investigated the effects of [bmim]Cl, [bmim][BF₄] and [bmim][PF₆] on the activity of cellulase from *Humicola insolens*. [bmim]Cl denatured the cellulase

whereas, the cellulase was more active in hydrophobic ILs [bmim][PF₆] and [bmim][BF₄] (Paljevac et al., 2006).

Although ILs are widely used as green solvents, their toxicity and environmental impacts are not completely known. It was investigated that ILs with quaternary ammonium and pyridinium cations have been found to be toxic for bacteria and fungi. ILs may attack the cell membrane and alter its structure (Luo et al., 2014). Moreover, some strains were found to degrade the ILs. *Enterobacter lignolyticus* was investigated to degrade 1-ethyl-3-methylimidazolium. Ertekin and Tezel, (2014) isolated a *Pseudomonas* strain which is able to degrade benzalkonium chlorides.

The *Pseudomonas* sp. BIOMIG1 is able to degrade the BACs aerobically. In addition, results presented in Chapter 5 showed that cellulolytic microbial community is not able to utilize cellulose in BACs even at very low BAC concentrations. We hypothesize that BIOMIG1 may utilize BACs, alleviate its inhibitory effects and facilitate cellulose degradation by the cellulolytic microbial community. Therefore, the objective of the research described in this chapter is to test this hypothesis in a system containing BIOMIG1 and cellulolytic microbial community at BAC concentrations tested in previous experiment described in Chapter 5.

7.2. Materials and Methods

7.2.1. Batch Cellulose Degradation by Cellulolytic Microbial Community with BIOMIG1 in BAC ILs

A batch experiment was performed to test the role of BIOMIG1 on cellulose degradation by the cellulolytic microbial community at a range of BAC concentrations from 50 to 1000 mg/L. A culture of BIOMIG1 was mixed with either two or five times diluted cellulolytic culture. 100 mL of this mixed culture was transferred into 250-mL Erlenmeyer flasks, amended with 1000 mg cellulose/L and BACs to maintain a concentration at 50, 100, 500 and 1000 mg/L (Table 7.1). The flasks then placed on an orbital shaker and agitated at 130 rpm at room temperature. The samples were taken on a

daily basis to analyze the total carbohydrates, glucose, organic acids, alcohols and BACs concentration. The pH of the reactors was also measured at the end of incubation time.

Table 7.1. Feeding Experimental matrix of culture series and controls used in the batch cellulose degradation experiment set either with twice or five times diluted culture and BIOMIG1at different BAC concentrations

Culture Series	Medium (mL)	<i>Pseudomonas</i> Culture (mL)	Diluted Microbial Community (mL)	Cellulose (g)	10 g/L BACs stock solution (mL)	Total Volume (mL)
Control	100	0	0	0.1	0	100
0 mg BAC/L	0	1.5	98.5	0.1	0	100
50 mg BAC/L	0	1.5	98	0.1	0.5	100
100 mg BAC/L	0	1.5	97.5	0.1	1	100
500 mg BAC/L	0	1.5	93.5	0.1	5	100
1000 mg BAC/L	0	1.5	88.5	0.1	10	100

*The flasks containing cultures were prepared in duplicates.

7.3. Results and Discussion

7.3.1. *Pseudomonas* sp. BIOMIG1 Facilitates Biodegradation of Cellulose by Cellulolytic Microbial Community in BAC ILs

The experiment prepared with five times diluted cellulolytic culture lasted 26 days. The pH in the flasks was 7.1 at the end of the incubation period. Glucose, organic acids and alcohols were not detected in the flasks in the course of the experiment. Total carbohydrate concentration did not change in the control flask (Figure 7.1.A) whereas 80% of the carbohydrates were consumed within 6 days in the flask containing the cellulolytic culture and BIOMIG1 (Figure 7.1.B). BACs were completely utilized within one day in the flasks at 50 and 100 mg BAC/L and within 6 days at 500 mg BAC/L (Figure 7.1.). However, cellulose degradation was observed only in the flask containing 100 mg BAC/L initially (Figure 7.1.D), whereas total carbohydrate concentration did not change in the other flasks containing BACs (Figure 7.1.C, E, F).

The experiment prepared with two times diluted cellulolytic culture lasted 20 days. The pH in the flasks was 7.1 at the end of the incubation period. Glucose, organic acids and alcohols were not detected in the flasks in the course of the experiment. Total carbohydrate concentration did not change in the control flask (Figure 7.2.A) whereas 80% of the carbohydrates were consumed within 6 days in the flask containing the cellulolytic culture and BIOMIG1 (Figure 7.2.B). Similar to the previous experiment, BACs were completely utilized within one day in the flasks at 50 and 100 mg BAC/L and within 2 days at 500 mg BAC/L (Figure 7.2.). But this time, cellulose degradation was observed in the flask containing both 50 and 100 mg BAC/L initially (Figure 7.2.C and D), whereas total carbohydrate concentration did not change in the other flasks containing BACs (Figure 7.2.E and F).

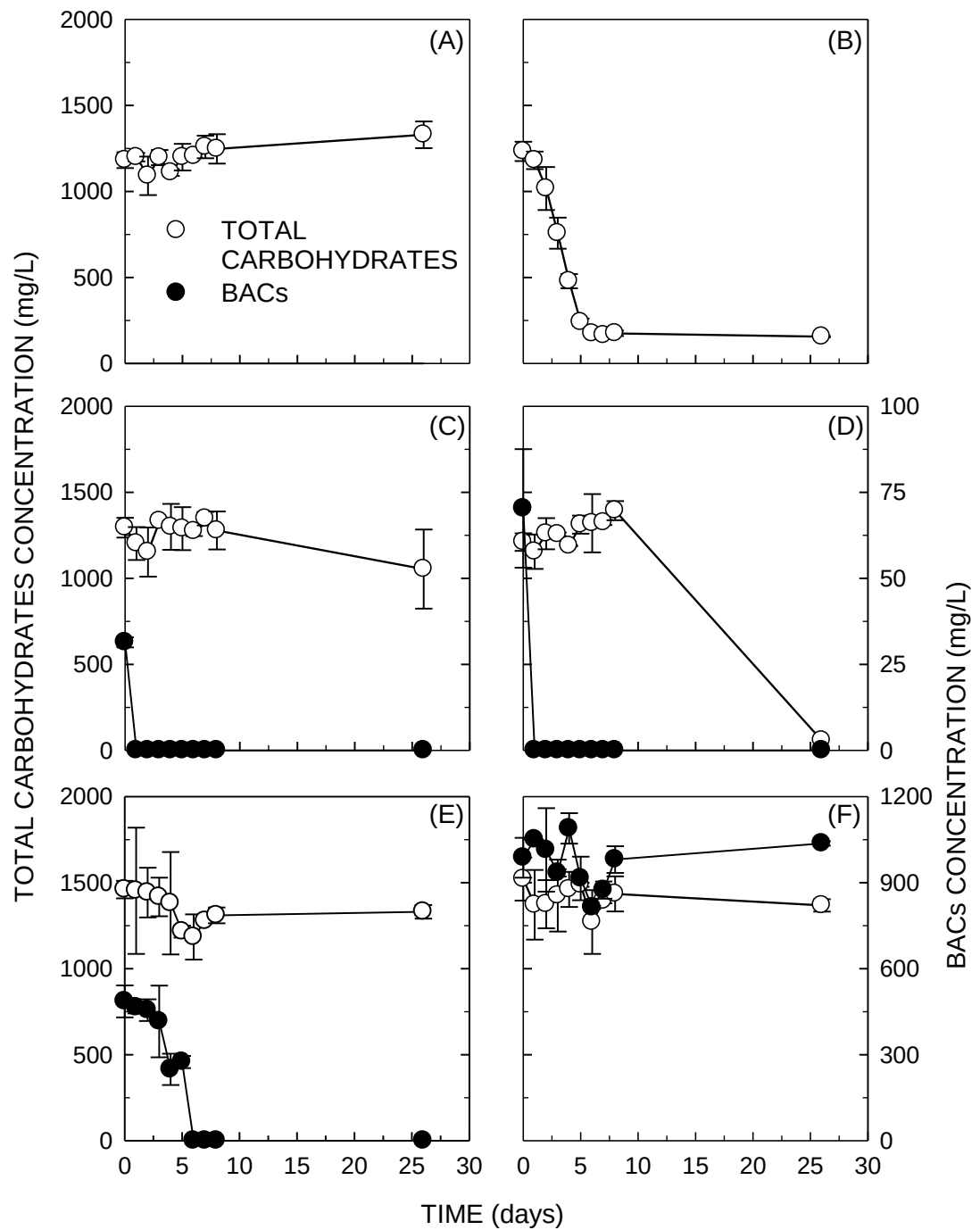


Figure 7.1. Profile of total carbohydrates and BAC ILs utilization in (A) control and flasks containing 5 times diluted cellulolytic culture and BIOMIG1 at (B) 0, (C) 50, (D) 100, (E) 500 and (F) 1000 mg BAC/L

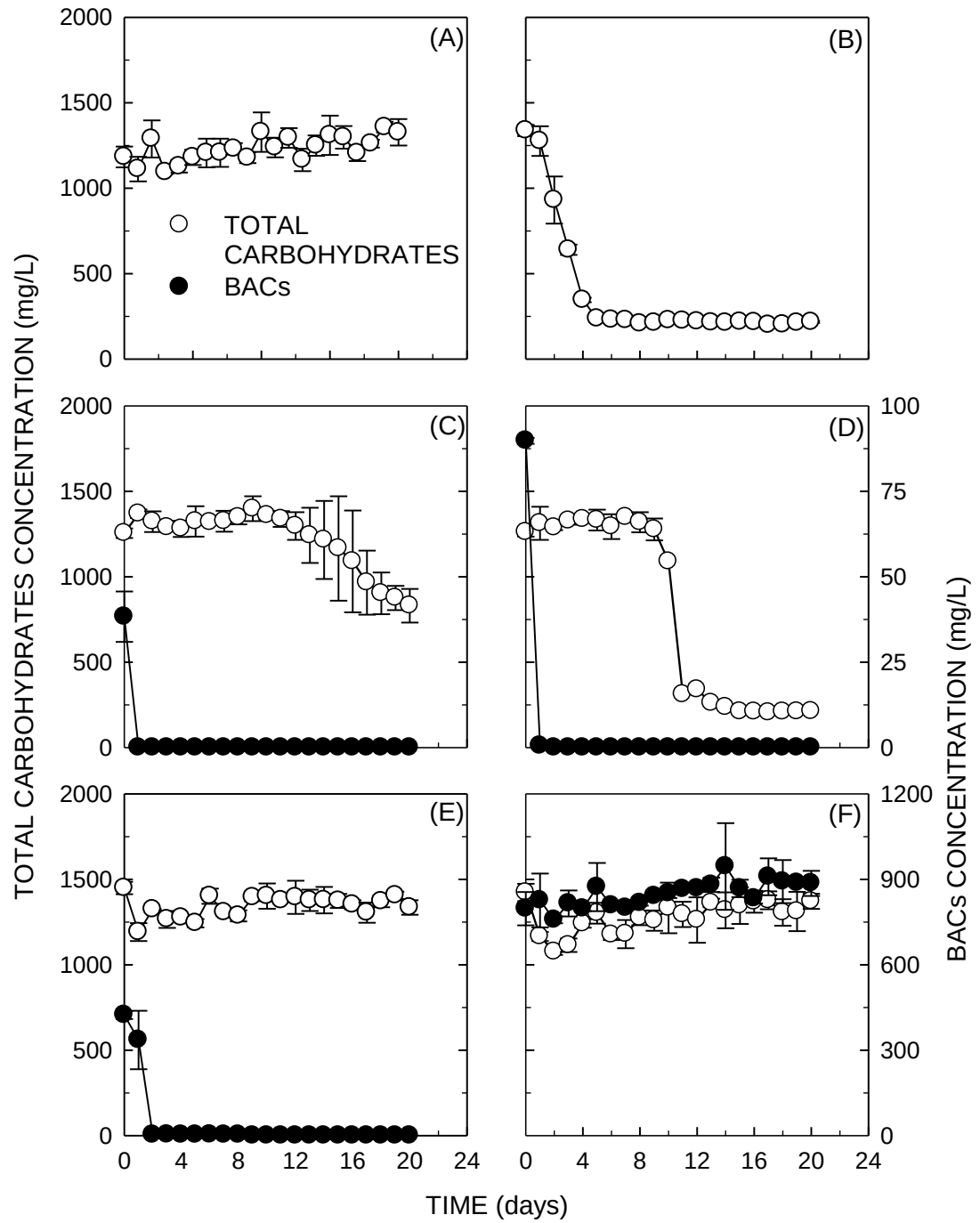


Figure 7.2. Profile of total carbohydrates and BAC ILs utilization in (A) control and flasks containing 2 times diluted cellulolytic culture and BIOMIG1 at (B) 0, (C) 50, (D) 100, (E) 500 and (F) 1000 mg BAC/L

In both experiments, we observed that total carbohydrates were consumed faster at flasks containing 100 mg BAC/L than the ones containing 50 mg BAC/L. This result suggested that BAC ILs enhances the dissolution of cellulose. Based on the results of the

study presented in this chapter, BIOMIG1 can be used to regenerate cellulose from matrix containing BAC ILs by removing the BACs and BAC-free cellulose-containing stream can be used without causing any inhibition problems in down-stream operations.

7.4. Summary

The batch experiments were performed to test the role of BIOMIG1 on cellulose degradation by the cellulolytic microbial community at a range of BAC concentrations from 50 to 1000 mg/L. 5 and 2 times diluted cellulolytic community was tested. In both set, 80% of cellulose was utilized in flasks received no BAC ILs. In the first set, the cellulose was degraded in the flasks containing 100 mg/L BAC ILs although BACs were biotransformed in flasks receiving 50, 100 mg BAC/L in one day and 500 mg BAC/L within 6 days. In the second set tested with 2 times diluted cellulolytic microbial community, total carbohydrates decrease was observed in the flasks containing 50 and 100 mg BAC/L initially. In addition, BACs were completely utilized within one day in the flasks at 50 and 100 mg BAC/L and within 2 days at 500 mg BAC/L. Based on the results of these two sets BAC ILs enhance the dissolution of cellulose. Since BIOMIG1 can aerobically degrade the BACs to carbon dioxide, the cellulose can be regenerated from the BAC ILs matrix and consumed by cellulolytic community.

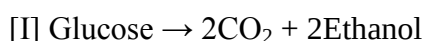
8. GLUCOSE FERMENTATION BY *PSEUDOMONAS SP. BIOMIG1*, *SERRATIA MARCESCENS BIOMIG4* AND THEIR CO-CULTURE IN BAC ILs

8.1. Introduction

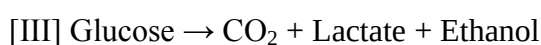
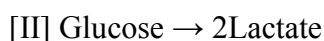
Biofuel and chemical production from biomass gained more attention due to high demand of fuels and chemicals and also the environmental impact of processing of non-renewable resources. Lignocellulosic materials which are the most abundant renewable resources on the earth (Faraco, 2013) compose of three main components 1) cellulose 2) hemicellulose and 3) Lignin. Cellulose part of the lignocellulosic materials is a polymer of glucose (Costa Lopes et al., 2013). Glucose is among the most well-known fermentable substrates which can be converted to valuable organic acids, ethanol and hydrogen depending on the microorganism and the applied conditions.

Basically fermentation is carried out under anaerobic condition in the absence of external electron acceptor. The oxidation of the substrate is coupled to the reduction of another substrate or an intermediate derived from the oxidation, with the difference in redox potential of the substrate and the end product providing energy for adenosine triphosphate (ATP) synthesis through substrate level phosphorylation (El-Mansi et al., 2012). Various substrates such as sugars monomers (hexsoses, pentoses, tetroses), polyols, organic acids, amino acids and purines and pyrimidines can be fermented. Glucose monomers results from the hydrolysis of cellulose part in lignocellulosic materials can be converted to various valuable products by different organisms through fermentation process. During the fermentation, glucose is converted to the phosphorylated form (glucose-6-phosphate (G6P)). This molecule is then transformed to pyruvate which serves as the key substrate of fermentation and converted to hydrogen, ethanol, butanol, succinic acid, lactic acid, acetic acid and propionic acid via alcoholic, acidic and mixed acid fermentation pathways (Alper and Stephanopoulos, 2009; El-Mansi et al., 2012).

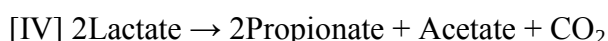
Bacterial fermentation have different types based on the products produced such as ethanol fermentation, lactic acid fermentation, propionate fermentation, mixed acid and butanediol fermentation, butyrate fermentation and homoacetate fermentation. Ethanol which is one the most valuable biofuels can be obtained through the fermentation of glucose by *Clostridium acetobutylicum*, *Klebsiella pneumoniae*, *Leuconostoc mesenteroides*, *Sarcina ventriculi*, *Zymomonas mobilis* [I] (Faraco, 2013).



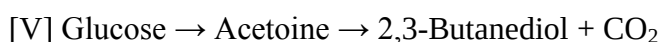
Lactate which is a common products of anaerobic fermentation can be produced by both homofermentative and heterofermentative bacteria. The former produced just lactate [II] whereas the latter can produced CO₂ and ethanol in addition to lactate [III].



Propionate is also a common fermentation product which is produced by propionic acid bacteria such as *Clostridium propionicum*. Moreover, the propionic acid bacteria can ferment the lactate to propionate [IV].



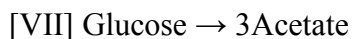
Mixed acids and butanediol fermentation is carried out by facultative anaerobic enterobacteria. Species of *Klebsiella*, *Enterobacter*, *Serratia*, *Erwinia* and *Hafnia* genus of bacteria can produce few acids but considerable amount of butanediol and CO₂.



Butyrate is the typical fermentation product by some *Clostridial* species such as *Clostridium butyricum* that convert glucose to butyrate according to the following reaction:



Acetate is produced during fermentation by many bacterial species however it can be exclusively produced by *Moorella thermoacetica* and *Acetobacterium woodii* through the following reaction:



Aerobic fermentation of glucose which is called aerobic respiration is a process in which oxygen serves as the electron acceptor and will be reduced and the ATP is synthesized by electron transport phosphorylation. The ATP synthesized under aerobic condition is generally more than its synthesis under anaerobic condition. Yeast can ferment the glucose under both conditions, whereas the ATP synthesis under aerobic condition is 38 moles compared to 2 moles under anaerobic condition (Muller, 2001; Jang et al., 2012).

Pseudomonas and *Serratia marcescens* are facultative microorganisms (Hejazi et al., 1997). The rate of growth of *Serratia marcescens* on glucose under anaerobic condition was higher than semi-anaerobic and aerobic conditions. Anaerobic glucose fermentation by *Serratia marcescens* resulted in production of acetic acid, formic acid, CO₂ and ethanol (Misenheimer et al., 1965). *Pseudomonas* species such as *Pseudomonas putida*, *Pseudomonas fluorescence* are recognized as glucose non-fermenting species under anaerobic conditions (Tenover et al., 1990), whereas *Pseudomonas aeruginosa* was distinguished as pyruvate-fermenting specie. 0.56, 0.39 and 0.04 (mole/mole pyruvate) of lactate, acetate and succinate were produced after 23 days by *Pseudomonas aeruginosa* under anaerobic conditions (Eschbach et al., 2004).

Many studies were conducted on the glucose metabolism by bacteria under aerobic condition. Various desired products such as acetic acid, lactic acid, butyric acid, ethanol, carbon dioxide and 2,3-butanediol can be obtained from glucose metabolism by bacteria under aerobic conditions. Aerobic dissimilation of glucose by heterolactic bacteria was studied in which glucose was converted to lactate, acetate and carbon dioxide (Kawai et al., 1970). Moreover, *Thermotoga maritima* metabolized glucose to lactate within 12 hours (Lakhal et al., 2011).

Neish et al. (1947) studied the fermentation of glucose under aerobic condition by four different strains of *Serratia marcescens* at 35 °C. The dissimilation of glucose by all of the strains was almost 100 percent within 5 days. The fermentation products included mainly 2,3-butanediol, ethanol, acetic acid, succinic acid, carbon dioxide and traces of formic and lactic acid (Neish et al., 1947). In a study by Misenheimer et al. (1965) *Serratia marcescens* produced 2-ketogluconic acid at an 12% glucose concentration. Other acids such as acetic acid, formic acid, CO₂ and ethanol were produced below this glucose concentration (Misenheimer et al., 1965). In a different study, *Serratia marcescens* strain H30 was capable of producing 2,3-butanediol, acetoin, acetic acid and ethanol from sucrose under microaerobic condition (Zhang et al., 2010). *Pseudomonas aeruginosa* oxidized the glucose and produced acids under aerobic conditions (Hugh et al., 1953). Eagon et al. (1965) demonstrated that *Pseudomonas natriegens* catabolized the glucose aerobically and produced mainly acetic acid, pyruvic acid and carbon dioxide (Eagon et al., 1965).

ILs have provided an effective alternative for decreasing the cellulose crystallinity which is the most significant bottleneck of enzymatic hydrolysis. The less crystalline cellulose can be hydrolyzed by cellulases to glucose and then converted to organic acids and ethanol through fermentation (Fang et al., 2014). However, ILs may inhibit glucose fermentation. Zhu et al. (2012) reported the effects of different concentrations (0.001 to 1 g/L) of ionic liquid 1-butyl-3-methylimidazolium chloride ([bmim]Cl) on the growth and ethanol fermentation of *Saccharomyces cerevisiae*. By increasing the [bmim]Cl concentration, the ethanol fermentation was deteriorated (Zhu et al., 2012). 1-ethyl-3-methylimidazolium acetate, 1-ethyl-3-methylimidazolium diethylphosphate and 1-ethyl-3-methylimidazolium dimethylphosphate were investigated not to have significant impact on alcohol production by *Clostridium* at <2.5 g/L of IL concentration (Nancharaiah et al., 2011). Mehmood et al. (2015) reported that edition of low IL concentration, 1% of 1-ethyl-3-methylimidazolium acetate and 4% of 1-ethyl-3-methylimidazolium methylphosphate enhanced the ethanol production by 58.2 and 37.9% respectively (Mehmood et al., 2015).

The objective of this research reported in this chapter was to investigate the capability of BACs resistant *Pseudomonas* sp. BIOMIG1, *Serratia marcescens* BIOMIG4

and their co-culture in fermenting the glucose to the valuable products such as organic acids, alcohols and carbon dioxide in the presence of BAC ILs.

8.2. Materials and Methods

8.2.1. Batch Anaerobic Glucose Fermentation Assay

A batch assay was performed to investigate the pathways of glucose fermentation by *Pseudomonas sp.* BIOMIG1, *Serratia marcescens* BIOMIG4 and their co-culture under anaerobic conditions. The assay was conducted in 125-mL serum bottles (100 mL liquid volume + 16 mL headspace). Medium was added to the serum bottles aseptically under laminar flow hood. 10 mL glucose stock solution was added to each bottle to give an initial 10,000 mg/L concentration. Then the bottles were sealed with rubber stoppers and aluminum crimps and flushed with nitrogen for 5 minutes to provide the anaerobic condition. The stock cultures were transferred into the serum bottles using sterile syringes and needles. A serum bottle with just medium and glucose solution served as the control bottle (Table 8.1.). The bottles were placed on an orbital shaker and agitated at 130 rpm at room temperature. The organic acids and alcohols in the liquid phase and gas composition in head space were measured during the incubation period. At the end of incubation period organic acids, alcohols, pH, total gas, produced CO₂, COD and the glucose concentration in the bottles were measured.

Table 8.1. Experimental matrix of culture series and controls used in the batch anaerobic glucose fermentation experiment by BIOMIG1, BIOMIG4 and their co-culture

Culture Series	Medium (mL)	Glucose Stock Solution (mL)	BIOMIG1 Culture (mL)	BIOMIG4 Culture (mL)	Total Volume (mL)
Control	90	10	0	0	100
BIOMIG1	88.5	10	1.5	0	100
BIOMIG4	88.5	10	0	1.5	100
Co-culture	87	10	1.5	1.5	100

*The bottles containing BIOMIG1 culture, BIOMIG4 culture and their Co-culture were prepared in duplicates.

8.2.2. Batch Aerobic Glucose Fermentation Assay

A batch assay was conducted to investigate the aerobic glucose fermentation by *Pseudomonas sp.* BIOMIG1, *Serratia marcescens* BIOMIG4 and their co-culture. The assay was conducted in autoclaved 250-mL Erlenmeyer flasks. Culture medium was added to the flask aseptically under laminar flow hood. 10 mL glucose stock solution was added to each bottle to give an initial 10,000 mg/L concentration. The bottles were then capped with porous caps. Aseptically, the stock cultures were transferred into the flasks. An Erlenmeyer flask containing only medium and glucose solution served as the control flask (Table 8.1.). The flasks were placed on an orbital shaker and agitated at 130 rpm at room temperature. The organic acids and alcohols in the liquid phase were analyzed during the incubation period. At the end of incubation period pH, COD, organic acids, alcohols and the glucose were measured.

8.2.3. Batch Anaerobic and Aerobic Glucose Fermentation Assay in BAC ILs

The effect of BACs on fermentation of glucose by BIOMIG1 and its co-culture with BIOMIG4 was investigated at a range of concentration between 50 and 1000 under both aerobic and anaerobic conditions. Anaerobic assay was performed in 125-mL serum bottles whereas aerobic assay was prepared in 250-mL Erlenmeyer flasks as described above. In addition, BACs were added into the cultures to maintain an initial BAC concentration of 50, 100, 500 or 1000 in the flasks/bottles (Table 8.2. and 8.3.). The flasks and serum bottles were placed on an orbital shaker and agitated at 130 rpm at room temperature. At the end of incubation period pH, COD, organic acids, alcohols and the glucose were measured.

Table 8.2. Experimental matrix of culture series and controls used in the batch aerobic and anaerobic glucose fermentation experiment by BIOMIG1 in BAC ILs

Culture Series	Medium (mL)	BIOMIG1 Culture (mL)	100g/L Glucose Stock Solution (mL)	10 g/L BACs Stock Solution (mL)	Total Volume (mL)
Control	90	0	10	0	100
0 mg BAC/L	88.5	1.5	10	0	100
50 mg BAC/L	88	1.5	10	0.5	100
100 mg BAC/L	87.5	1.5	10	1	100
500 mg BAC/L	83.5	1.5	10	5	100
1000 mg BAC/L	78.5	1.5	10	10	100

*The flasks containing BIOMIG1 culture, BIOMIG4 culture and their Co-culture were prepared in duplicates.

Table 8.3. Experimental matrix of culture series and controls used in the batch aerobic and anaerobic glucose fermentation experiment by the co-culture of BIOMIG1 and BIOMIG4 in BAC ILs

Culture Series	Medium (mL)	BIOMIG1 Culture (mL)	BIOMIG4 Culture (mL)	Glucose Stock Solution (100g/L) (mL)	10 g/L BACs Stock Solution (mL)	Total Volume (mL)
Control	100	0	0	10	0	100
0 mg BAC/L	87	1.5	1.5	10	0	100
50 mg BAC/L	86.5	1.5	1.5	10	0.5	100
100 mg BAC/L	86	1.5	1.5	10	1	100
500 mg BAC/L	82	1.5	1.5	10	5	100
1000 mg BAC/L	77	1.5	1.5	10	10	100

*The flasks containing co-culture were prepared in duplicates.

8.3. Results and Discussion

8.3.1. Anaerobic Glucose Fermentation by BIOMIG1 and BIOMIG4

The capability of *Pseudomonas* sp. BIOMIG1, *Serratia marcescens* BIOMIG4 and their co-culture in anaerobic glucose fermentation to valuable products was tested in 29 days. BIOMIG1 could not ferment the glucose anaerobically whereas decrease in glucose concentration in flasks containing BIOMIG4 and co-culture indicates the glucose

fermentation to the desired products. BIOMIG4 and co-culture fermented 81.7 and 82.2% of glucose during 29 days (Table 8.4. and Figure 8.1.). Neish et al. (1947) reported 93.2% glucose consumption by four *Serratia marcescens* strains in 17 days (Neish et al., 1947).

Table 8.4. Remained glucose concentration at the end of incubation period in reactors containing BIOMIG1, BIOMIG4 and their co-culture under anaerobic condition

Reactor	Remained glucose concentration (mg/L)
Control	9423.4±374
BIOMIG1	10015.73±162
BIOMIG4	1830.83±80
Co-culture	1776.98±162

As it shown in the Table 8.5., the final pH values in control and flasks containing BIOMIG1 are in the neutral range which shows no organic acid production, whereas the pH value in flask containing BIOMIG4 and co-culture shows the production of organic acids.

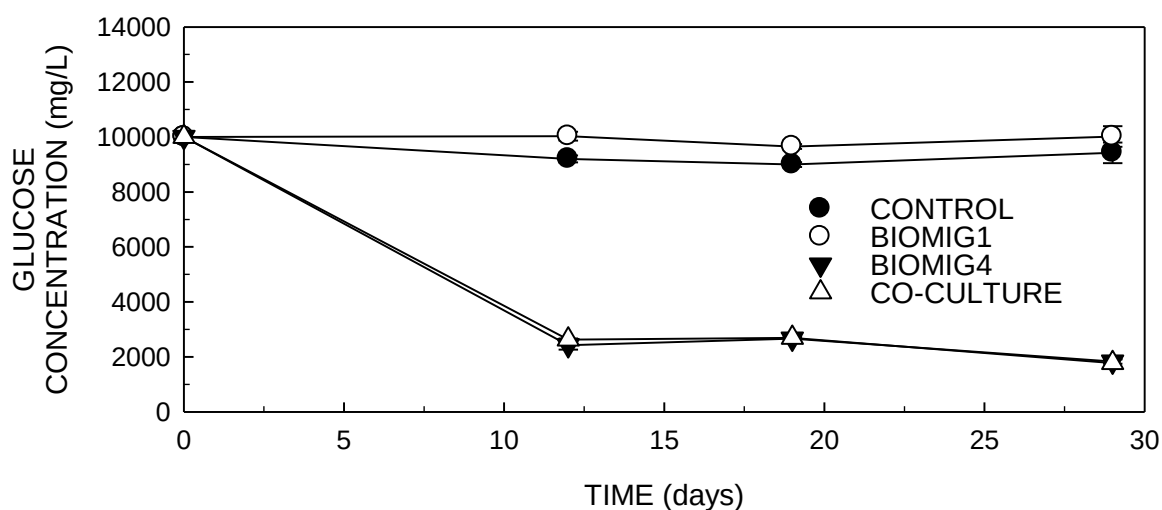


Figure 8.1. Profile of glucose utilization by BIOMIG1, BIOMIG4 and their co-culture under anaerobic condition

Table 8.5. Final pH of flasks for assessing the anaerobic glucose fermentation by BIOMIG1, BIOMIG4 and their co-culture

Culture Series	pH
Control	7.09
BIOMIG1	7.10
BIOMIG4	5.10
Co-culture	5.10

BIOMIG4 and co-culture catabolized the glucose to mainly 2,3-butanediol, ethanol, formic and lactic acids. Neish et al. (1947) reported the production of the aforementioned biofuels by four different *Serratia marcescens* strains. In our study, 42.9 and 37.95 mM 2,3-butanediol and 20.33 and 18.54 mM ethanol was obtained (Table 8.6) which is comparable to the highest production of 2,3-butanediol and ethanol (32.1 and 22.2 mM, respectively) in the study by Neish et al. (1947).

Table 8.6. Concentration of biofuels produced by BIOMIG1, BIOMIG4 and their co-culture under anaerobic condition

Culture Series	Ethanol (mM)	2,3-butanediol (mM)	Total Organic Acids (mM)
Control	0.00	0.00	0.00
BIOMIG1	0.00	0.00	0.1±0.04
BIOMIG4	20.33±1.41	42.92±3.04	34.16±1.39
Co-Culture	18.54±1.09	37.95±4.56	38.08±0.42

The organic acids accumulated in serum bottles containing the BIOMIG4 and co-culture resulted in pH reduction. The most produced organic acids were acetic, lactic, formic and succinic acid. The concentrations of the aforementioned produced organic acids at the end of the experiment period were 0.70 ± 0.032 , 12.39 ± 0.070 , 15.97 ± 0.064 and 3.44 ± 0.024 mM for bottles containing *Serratia* culture, and 0.67 ± 0.001 , 12.47 ± 0.016 , 15.74 ± 0.011 and 2.99 ± 0.006 mM for flasks containing the co-culture, respectively. Pederson and Breed, (1928) accomplished the first study on anaerobic glucose fermentation by *Serratia marcescens* which resulted in production of mainly lactic acid (40%), acetoin (28%), 2,3-Butanediol (18%) acetic acid (5.8%) and ethanol (4.7%) (Pederson and Breed, 1928). Moreover, the four different strains of *Serratia marcescens*

were tested in different periods (17, 12, 9 and 7 days). 9.37, 16.64, 18.48 and 30.11 mM of lactic acid and 28.91, 22.22, 22.13 and 15.35 mM of formic acid were obtained respectively (Neish et al., 1947). In our study, the highest total organic acids production appeared at 3rd week (Figure 8.2.).

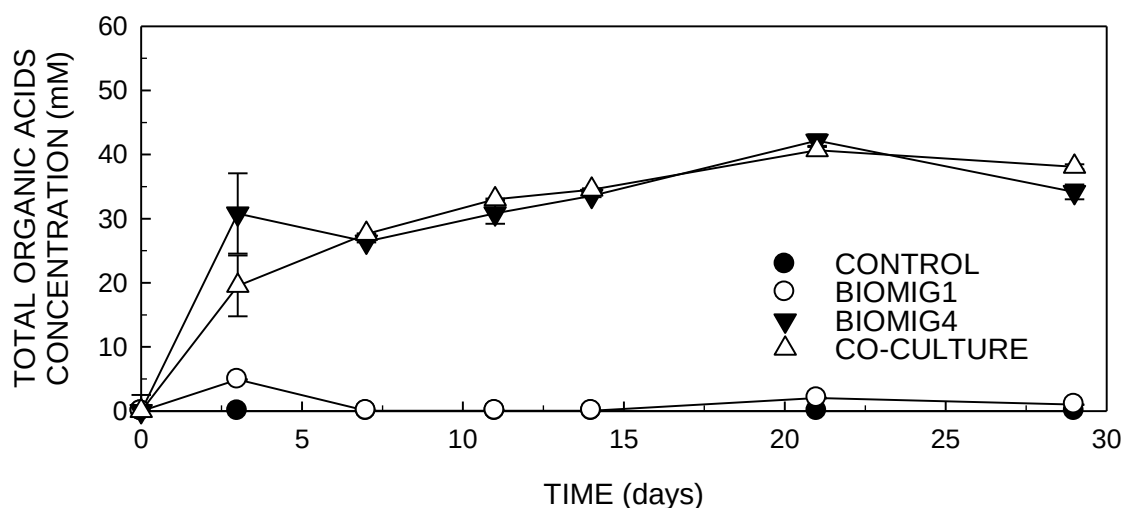


Figure 8.2. Profile of total organic acid production by BIOMIG1, BIOMIG4 and their co-culture under anaerobic condition

At the end of the anaerobic glucose fermentation, the total gas was measured (Table 8.7.). Carbon dioxide was detected in bottles with BIOMIG4 and co-culture (Table 8.8.). *Serratia marcescens* was reported as carbon dioxide producers from glucose (Muller, 2001). In addition, Neish et al, (1947) reported the production of 103 mM carbon dioxide from 100 mM glucose (Neish et al., 1947).

Table 8.7. Total gas production by BIOMIG1, BIOMIG4 and their co-culture under anaerobic condition

Culture Series	Total gas (mL)
Control	0
BIOMIG1	0
BIOMIG4	12.2±2.2
Co-Culture	10.6±0.6

The mass of carbon dioxide accumulated in the headspace of serum bottles was calculated using the ideal gas law $P_g V = nRT$ where P represent the partial pressure of CO₂

in system ($P_g = \% \text{ CO}_2 \cdot 1 \text{ atm}$), $R = 0.08205746 \text{ L.atm.mole}^{-1}.\text{K}^{-1}$, T , the room temperature (23.25°C) and V which was calculated using the following equation,

$$V = V_{(\text{head space})} + V_{(\text{Total gas})} + V_{(\text{Taken samples})}$$

$V_{(\text{head space})}$ = Volume of headspace which was 16 mL for all of the reactors

$V_{(\text{Total gas})}$ = Volume of produced total gas (Table 8.7.)

$V_{(\text{Taken samples})}$ = Reduced volume at the end of the run because of sample taking for organic acids measurements

The dissolved carbon dioxide is in the form of H_2CO_3 in aqueous systems. The $[\text{H}_2\text{CO}_3]$ was founded using the Henry's law. The Henry's constant at room temperature was 1558.45 atm

$$x_g = P_g / H$$

Where x_g is the mole fraction of gas in water, P_g is the partial pressure of gas and H is the henry's constant.

$$x_g = n_g / (n_g + n_{\text{water}})$$

Table 8.8. Accumulated carbon dioxide by BIOMIG1, BIOMIG4 and their co-culture under anaerobic condition

Culture Series	CO ₂ (%)	Mass of CO ₂ (mg)
Control	0.32	0.60
BIOMIG1	0.40	0.75
BIOMIG4	57.10	119.45±0.05
Co-Culture	53.71	110.80±0.01

The COD was conducted to get a perspective of any loss in system. The initial glucose COD applied to each bottle was 10,667 mg/L. Moreover, the COD value of each

bottle was measured at the end of the course. To make the COD balance, COD of remained glucose and whole produced organic acids, ethanol and 2,3-butanediol in bottles were also calculated and compared with the theoretical initial COD. The results regarding the COD balance are shown in Table 8.9. and Figure 8.3. Based on the results, the theoretical COD matched with the COD of remained glucose and the products.

Table 8.9. COD balance of total organic acids, remained glucose, ethanol, 2,3-butanediol and the final COD of bottles containing BIOMIG1, BIOMIG4 and co-culture under anaerobic condition

Culture Series	Organic acids (mg/L)	Remained glucose (mg/L)	Ethanol (mg/L)	2,3-butanediol (mg/L)	Final COD (mg/L)
Control	0.00	10051.94±398.95	0.00	0.00	10290±0
BIOMIG1	104.15±4.90	10683.78±172.81	0.00	0.00	10000±440
BIOMIG4	2054.20±126.46	1952.95±85.34	1951.88±134.97	7553.7±523.6	10320±780
Co-Culture	2515.45±32.13	1895.50±172.81	1780.22±104.92	6679.02±803.26	9090±960

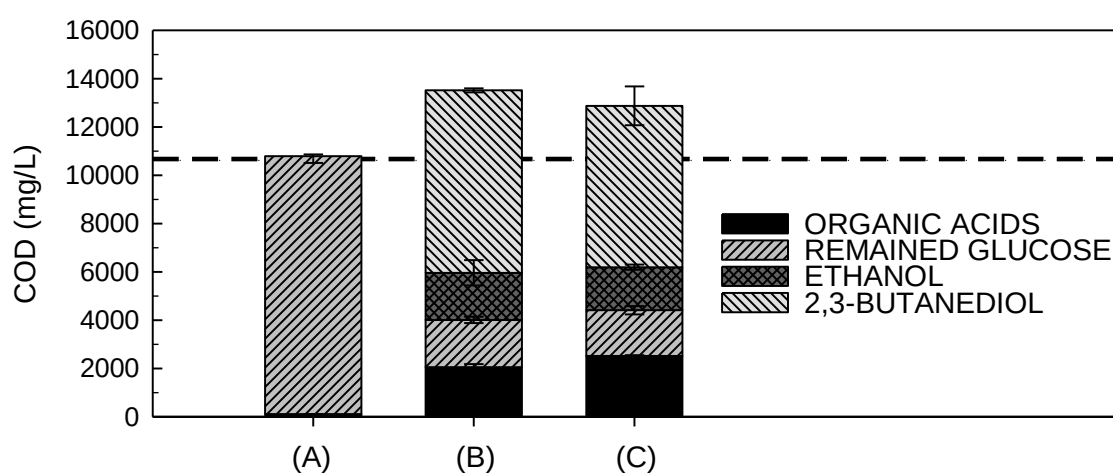


Figure 8.3. COD balance of bottles containing (A) BIOMIG1 Culture, (B) BIOMIG4 Culture and (C) Co-culture under anaerobic condition

The carbon balance was applied and compared to the COD balance results (Table 8.10 and Figure 8.4). The initial carbon applied to the reactors was 400 mg which was attributed to the initial applied glucose (10,000 mg/L). In order to make a carbon balance for each bottle, the present carbon in the forms of organic acids, remained glucose, ethanol,

2,3-butanediol and total produced carbon dioxide (gaseous and dissolved CO₂) were compared with the theoretical applied carbon (400 mg). In an aqueous system, the dissolved CO₂ is converted to the bicarbonates and carbonates. The mass of carbon in carbonate system is founded considering the following reactions.

The data for this part are shown in Table 7.12. and Figure 7.4.



$$K_{a1} = \frac{[\text{HCO}_3^-][\text{H}^+]}{[\text{H}_2\text{CO}_3]}$$

$$K_{a2} = \frac{[\text{CO}_3^{2-}][\text{H}^+]}{[\text{HCO}_3^-]}$$

Where [HCO₃⁻], [CO₃²⁻] and [H⁺] are the molarity of bicarbonate, carbonate and hydrogen ions, respectively.

Table 8.10. Carbon balance of total organic acids, remained glucose, ethanol, 2,3-butanediol and total carbon dioxide of bottles containing BIOMIG1, BIOMIG4 and co-culture under anaerobic condition

Culture Series	Organic acids (mg)	Ethanol (mg)	2,3-butanediol (mg)	Remained glucose (mg)	Total carbon dioxide (mg)
Control	0	0	0	358.09	0.16
BIOMIG1	3.18±0.15	0	0	380.6±6.14	0.2
BIOMIG4	84.11±3.86	48.79±3.37	206.01±14.59	69.57±6.14	33.83±0.014
Co-Culture	95.91±1.12	44.51±2.62	182.2±21.9	67.53±3.07	31.41±0.004

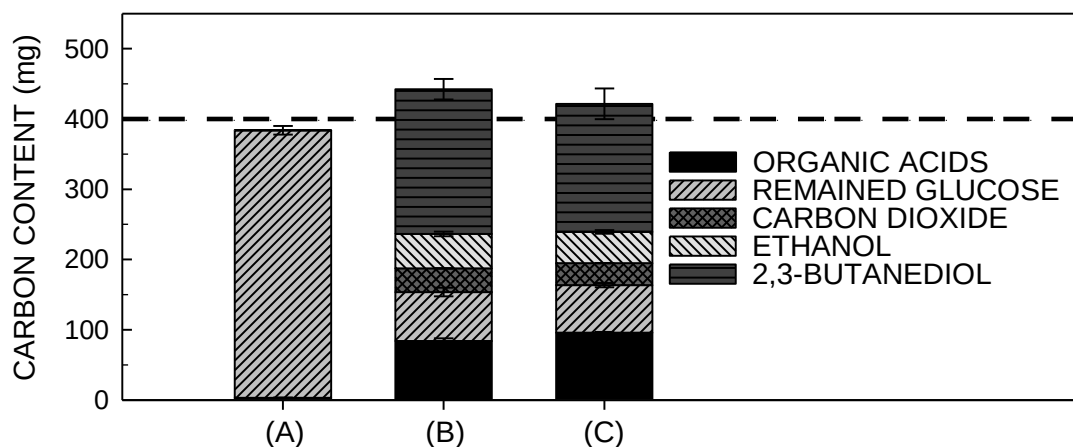


Figure 8.4. Carbon balance of bottles containing (A) BIOMIG1 Culture, (B) BIOMIG4 Culture and (C) Co-culture under anaerobic condition

8.3.2. Aerobic Glucose Fermentation by BIOMIG1 and BIOMIG4

The batch aerobic glucose fermentation by BIOMIG1, BIOMIG4 and their co-culture lasted in 3 days. 78% of glucose was fermented by BIOMIG1 after 3 days whereas no glucose was observed in flask containing BIOMIG4 and co-culture after the first day (Table 8.11. and Figure 8.5.). Therefore, the glucose fermentation by BIOMIG4 and co-culture was also studied during one day. Both of the cultures consumed the glucose in 18 hours (Figure 8.6.). Neish et al. (1947) also studied the aerobic glucose fermentation by *Serratia* which resulted in almost 100% glucose dissimilation after 5 days with initial 100 mM glucose (Neish et al., 1947). The final pH of flasks containing BIOMIG4 and co-culture after 24 hours is shown in Table 8.12.

Table 8.11. Remained glucose concentration in flasks containing BIOMIG1, BIOMIG4 and their co-culture under aerobic condition

Culture Series	Remained glucose Concentration (mg/L)
Control	9315.7
BIOMIG1	1992.4±161.5
BIOMIG4	0
Co-culture	0

Table 8.12. Final pH of flasks for assessing the aerobic glucose fermentation by BIOMIG4 and the co-culture

Culture Series	pH
Control	7.22
BIOMIG4	6.6±0.02
Co-Culture	6.45±0.01

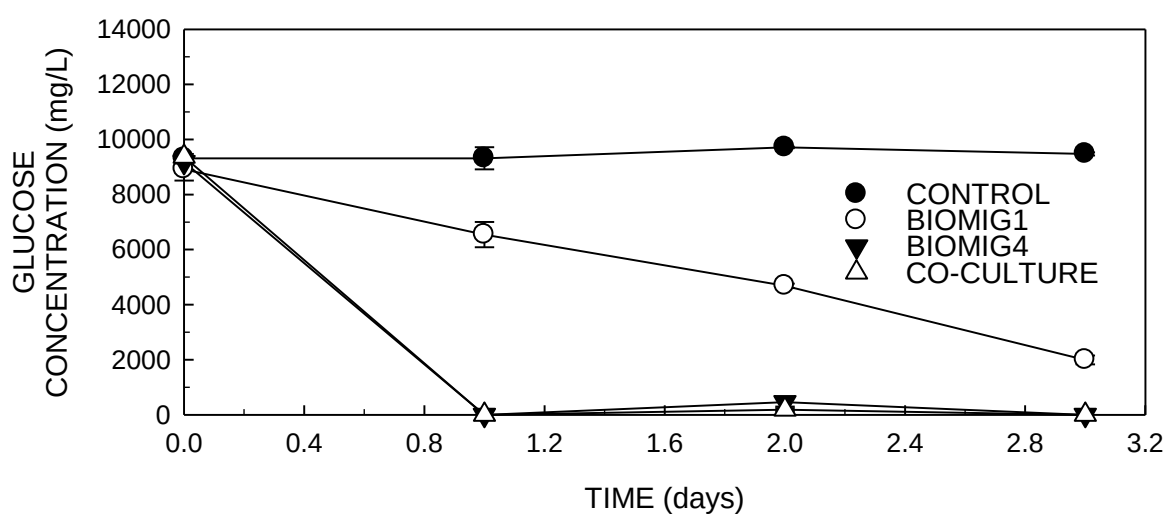


Figure 8.5. Profile of glucose utilization by BIOMIG1, BIOMIG4 and their co-culture under aerobic condition during 3 days incubation period

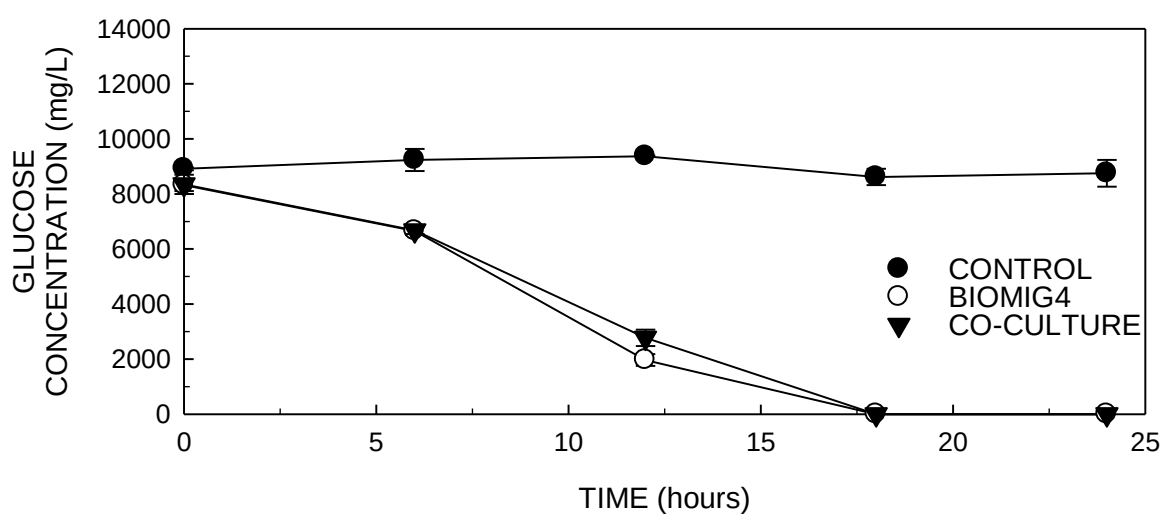


Figure 8.6. Profile of glucose utilization by BIOMIG4 and the co-culture under aerobic condition during 24 hours incubation period

Total acid production by BIOMIG1, BIOMIG4 and co-culture at the end of course was 3.42 ± 0.44 , 28.50 ± 1.19 and 29.40 ± 0.74 mM respectively (Figure 8.7.). Acetic and citric acids were the most produced acids during the glucose fermentation under aerobic condition which were different from the previous assay resulted in mainly lactic and formic acids.

The production of acetic acid reached to 0.342 (mole/mole glucose) at the end of fourth day by co-culture whereas BIOMIG1 produced just 0.017 (mole/mole glucose) acetic acid and no acetic acid was observed in flasks containing BIOMIG4 after 4 days. Glucose fermentation during one day showed that BIOMIG4 could produce 0.594 (mole/mole glucose) acetic acid after just 18 hours. In flasks with co-culture, the total acids were more than the reactors containing BIOMIG4 alone at the 4th day because of increase in acetic acid. Moreover, studying the aerobic fermentation during 1 day showed the increased production of acetic acid in the first 18 hours to a value of 15.02 mM and further higher total acid production (Figure 8.8.). The aerobic glucose fermentation in the study conducted by Neish et al. (1947) resulted in acetic acid production in a range of 1.93 to 4.55 mM during 5 days which was less than our study (Neish et al., 1947). succinic and propionic acids were also obtained.

BIOMIG4 and co-culture produced 32.58 ± 4.58 and 42.26 ± 7.55 mM 2,3-butanediol as the most produced biofuel under aerobic condition which is comparable to 34.7 mM recorded in the study by Neish et al. (1947). In addition, although ethanol is mainly generated anaerobically, 2.1 ± 0.63 and 0.155 ± 0.14 mM ethanol was observed in flasks containing BIOMIG4 and co-culture, respectively.

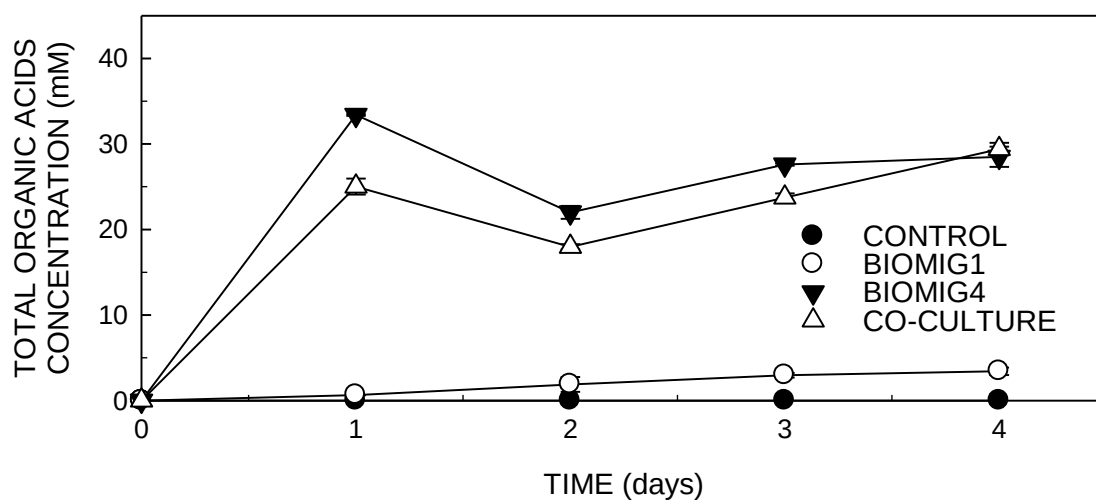


Figure 8.7. Profile of total organic acid production by BIOMIG1, BIOMIG4 and their co-culture during 4 days incubation under aerobic condition

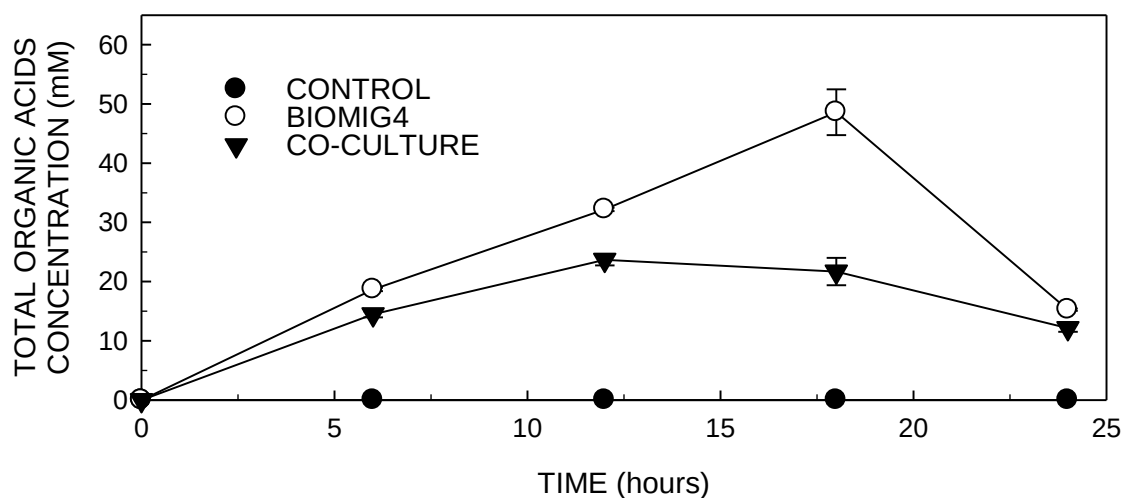


Figure 8.8. Profile of total organic acid production by BIOMIG4 and the co-culture during 24 hours incubation under aerobic condition

The initial glucose COD applied to each bottle was 10,667 mg/L. Moreover, the COD value of each flask was measured at the end of the course. To make the COD balance, COD of remained glucose and whole produced organic acids, ethanol and 2,3-butanediol in reactors were also calculated and compared to the theoretical initial COD. The results regarding the COD balance are shown in Table 8.13. and Figure 8.9. The

theoretical COD did not match with the COD of remained glucose and products which attributed to the production of carbon dioxide.

Table 8.13. COD balance of total organic acids, remained glucose, ethanol, 2,3-butanediol and the final COD of flaks containing BIOMIG1 after 4 days, BIOMIG4 and co-culture after 24 hours under aerobic condition

Culture Series	Organic acids (mg/L)	Remained glucose (mg/L)	Ethanol (mg/L)	2,3-butanediol	Final COD (mg/L)
Control	146.31	9333.95±516.96	0.00	0.00	10840±240
BIOMIG1	285.44±27.88	2125.27±172.32	0.00	0.00	3430±200
BIOMIG4	1598.57±22.83	0.00	201.12±60.58	5733.2±804.84	6232±20
Co-Culture	1224.96±110.42	0.00	14.88±13.44	7437±1328.8	5972±361

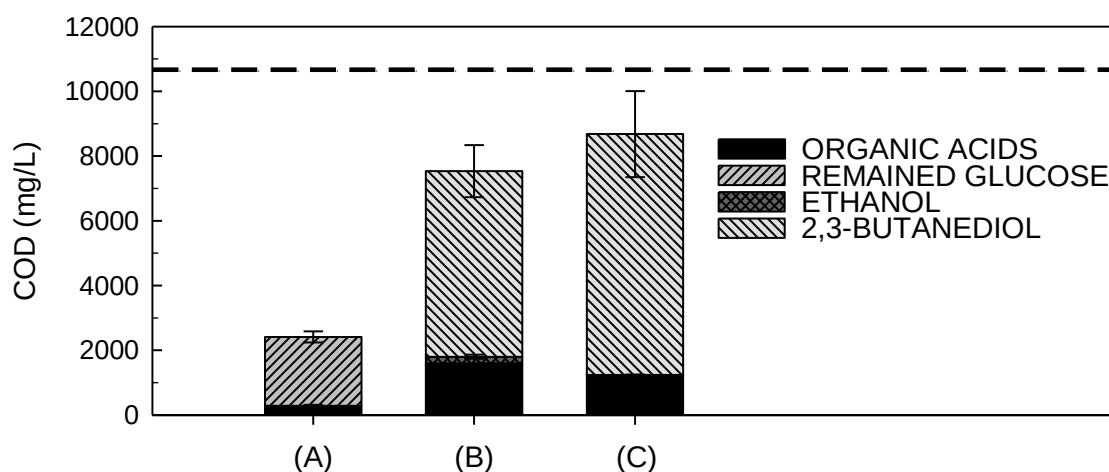


Figure 8.9. COD balance of reactors, (A) BIOMIG1 after 4 days, (B) BIOMIG4 and (C) co-culture under aerobic condition after 24 hours

2,3- butanediol was considered as the most significant biofuel produced by co-culture under both aerobic and anaerobic condition. The pathway of 2,3-butanediol from glucose is predicted (Figure 8.10.). The compounds with green and pink colors were obtained under aerobic and anaerobic conditions, respectively.

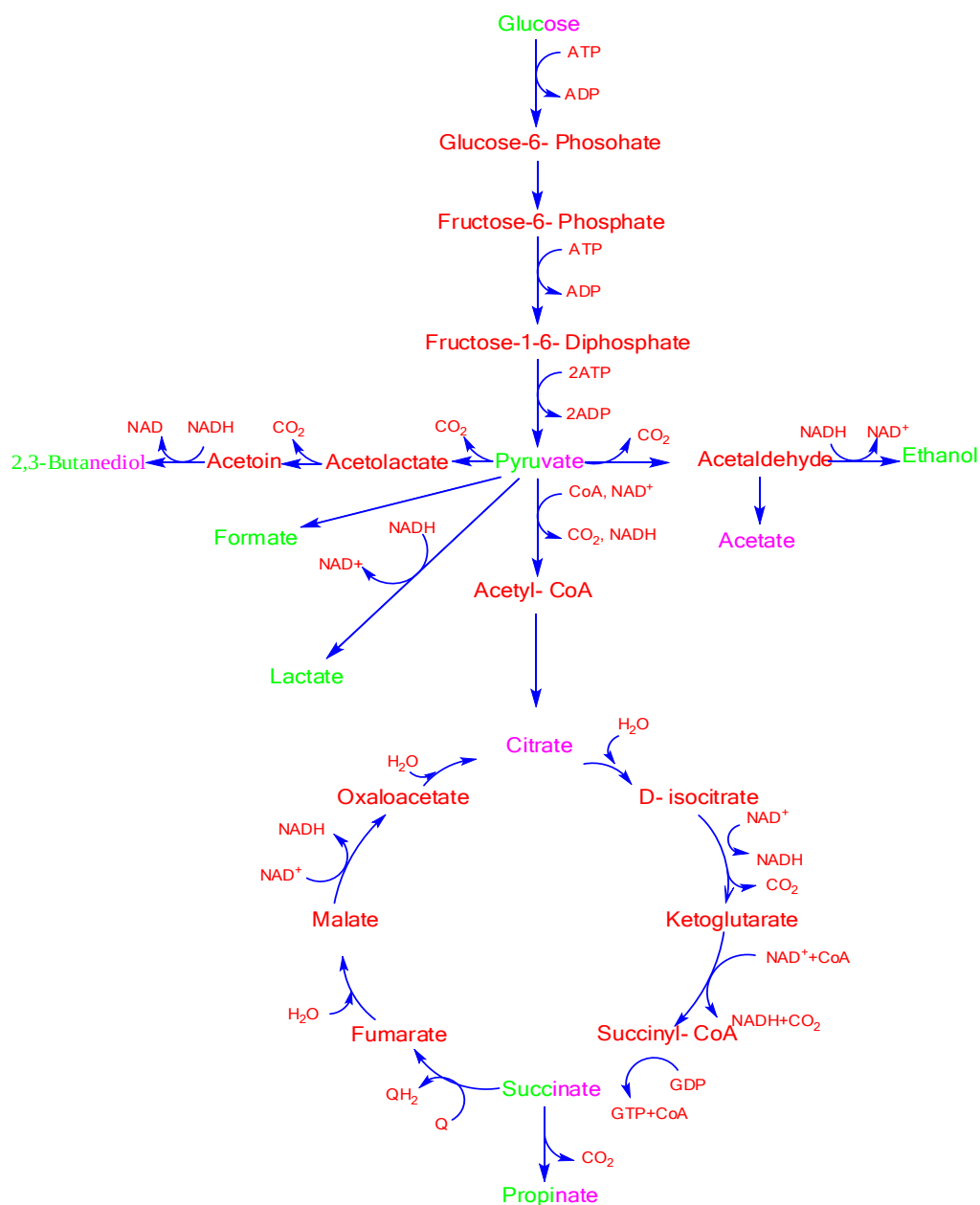


Figure 8.10. Predicted pathway of 2,3-butanediol production from glucose

8.3.3. Effect of BAC ILs on Anaerobic and Aerobic Glucose Fermentation

Anaerobic and aerobic glucose fermentation by BIOMIG1, BIOMIG4 and their co-culture was performed and explained in previous section. Since ILs are used a lot as cellulose solvents, their impact on biofuel production is an important subject. Therefore, effects of different concentration of BACs on glucose fermentation under both aerobic and anaerobic condition were investigated.

Anaerobic glucose fermentation by BIOMIG1 and also its co-culture with BIOMIG4 in 0, 50, 100, 500 and 1000 mg/L BACs was performed during 14 days. The final pH of bottles is shown in Table 8.14. BIOMIG1 was not able to ferment the glucose in 14 days; therefore no organic acids and alcohols were produced. It shows that BACs cannot affect the fermentation process by BIOMIG1 whereas co-culture dissimilated 77.5, 67.3, 64.5, 60.5 and 57.4% of glucose in serum bottles with 0, 50, 100, 500 and 1000 mg/L BACs, respectively (Figure 8.11.). This represents that BACs reduce the glucose utilization rate under anaerobic condition.

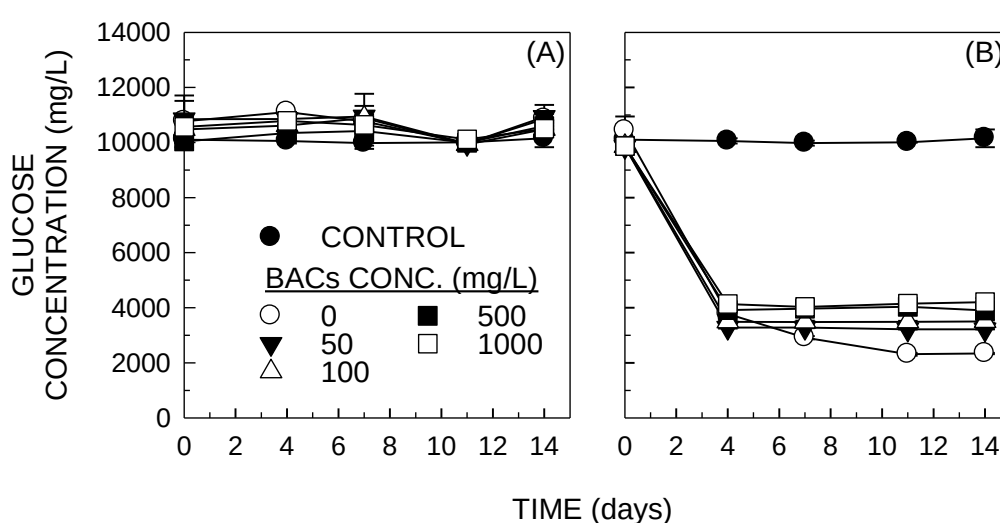


Figure 8.11. Profile of glucose utilization by (A) BIOMIG1 and (B) co-culture at different BAC ILs concentration under anaerobic condition

Table 8.14. Final pH of flasks for assessing the anaerobic glucose fermentation by BIOMIG1 and the co-culture at different BAC ILs concentration

Culture Series	pH	
	BIOMIG1	Co-culture
Control	7.14±0.06	7.14±0.06
0 mg BAC/L	7.15±0.01	5.14±0.01
50 mg BAC/L	7.17±0.01	5.78±0.03
100 mg BAC/L	7.19±0.02	5.85±0.03
500 mg BAC/L	7.25±0.01	5.96±0.03
1000 mg BAC/L	7.21±0.01	5.95

The pH around 7 in bottles with BIOMIG1 confirms that no organic acid was produced. On the other hand in reactors containing co-culture, the pH was decreased due to organic acids production (Table 8.14).

17.7, 18.5, 18.4, 17.2 and 15.8 mM ethanol was generated by co-culture at 0, 50, 100, 500 and 1000 mg BAC/L, respectively which represents that ethanol production was not affected by BACs concentration significantly. In other study 1-ethyl-3-methylimidazolium acetate, 1-ethyl-3-methylimidazolium diethylphosphate and 1-ethyl-3-methylimidazolium dimethylphosphate were investigated not to have significant impact on alcohol production by *Clostridium* at <2.5 g/L of IL concentration (Nancharaiah et al., 2011). It was also reported that ethanol fermentation of *Saccharomyces cerevisiae* was deteriorated by increasing the [bmim]Cl concentration from 0.001 to 1 g/L ionic liquid (Zhu et al., 2012). Compare to these results, our research shows an acceptable outcome in ethanol production. In addition, BAC ILs did not influence the 2,3-butanediol production by co-culture. The 2,3-butanediol was decreased from 37 to 30 mM when BACs concentration increased from 0 to 1000 mg/L (Figure 8.12.).

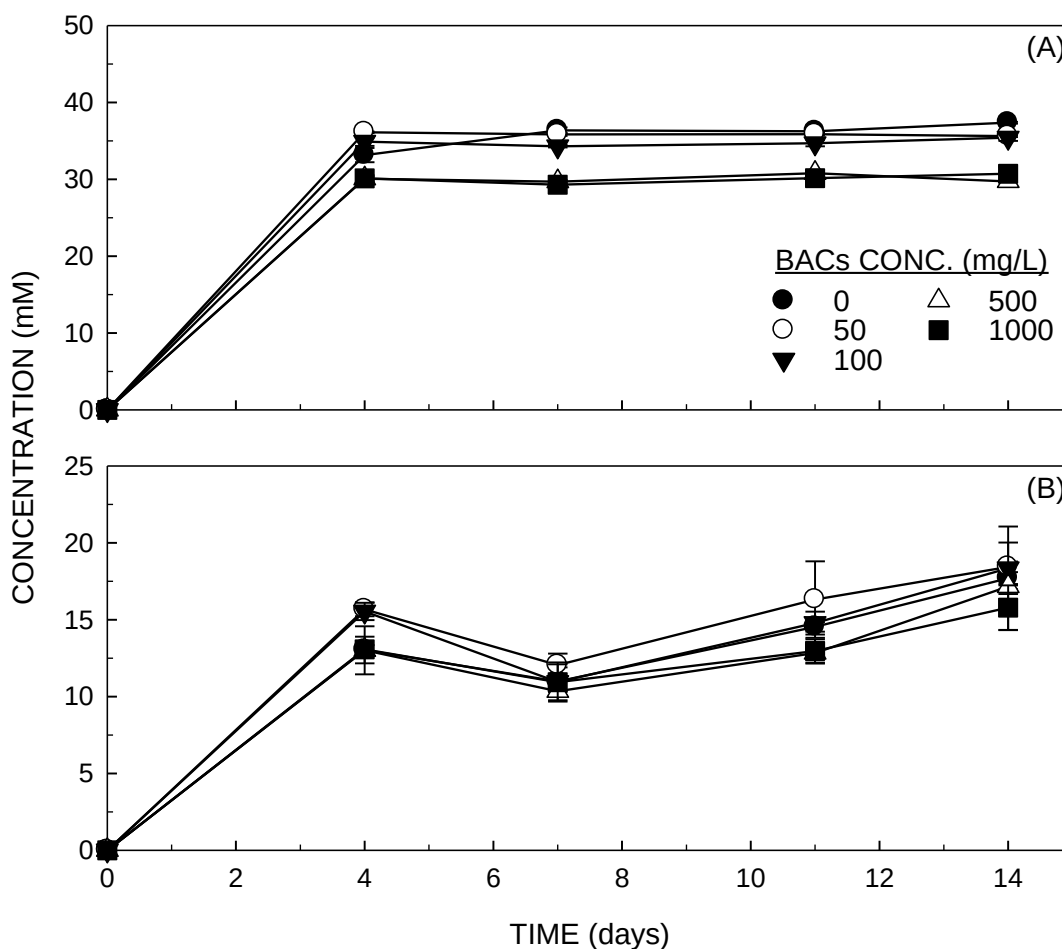


Figure 8.12. Profiles of (A) 2,3- butanediol and (B) ethanol production by co-culture at different BAC ILs concentrations under anaerobic condition

The total organic acids value produced by co-culture was decreased by increase in BAC ILs concentration (Figure 8.13.). Formic and propionic acids were the most obtained organic acids under anaerobic condition. However, formic and lactic acids were produced the most in our test without any BACs. The production of lactic acid in bottles that receives no BACs was about 10 mM, however amending the BACs to the system reduced this value to 1 mM. Therefore, higher BACs concentration may favor the production of propionic acid and suppress the lactic acid generation (Figure 8.14.).

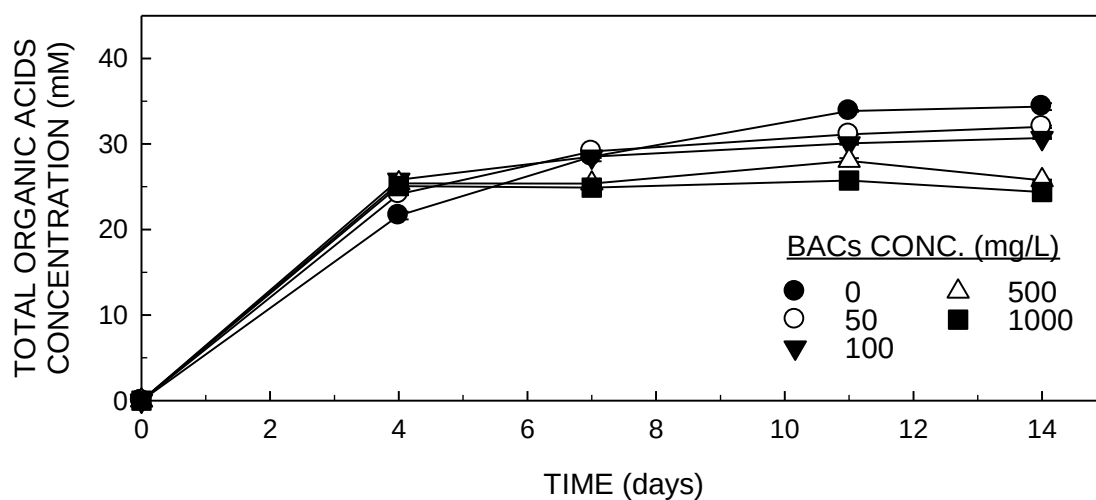


Figure 8.13. Profile of total organic acid production by co-culture at different BAC ILs concentration under anaerobic condition

Since BIOMIG1 is a BAC-degrader strain which can utilize the BACs aerobically therefore, no change in BACs concentration under anaerobic condition proves this fact too (Figure 8.15.).

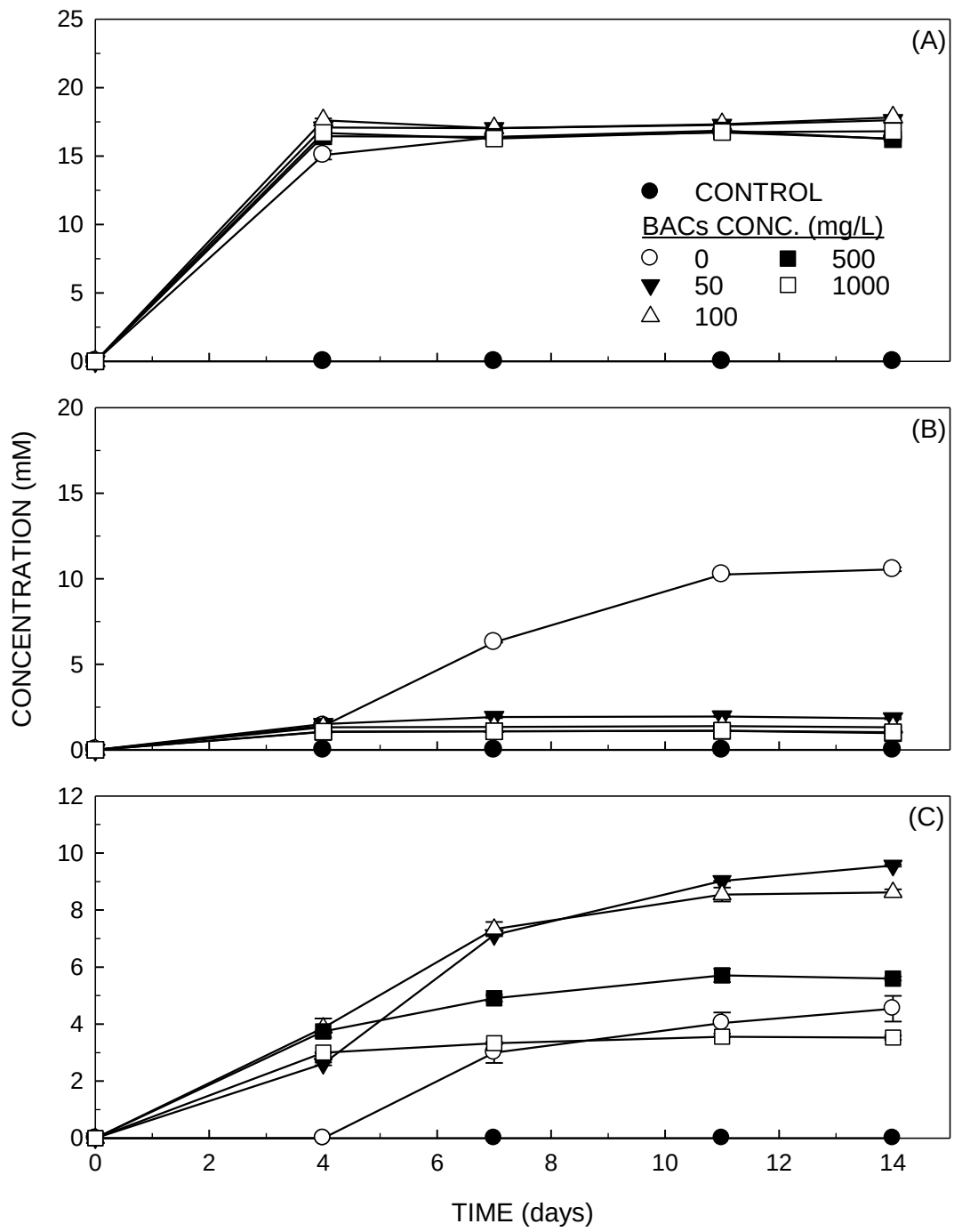


Figure 8.14. Profile of (A) formic, (B) lactic and (C) propionic acids production by co-culture at different BAC ILs concentration under anaerobic condition

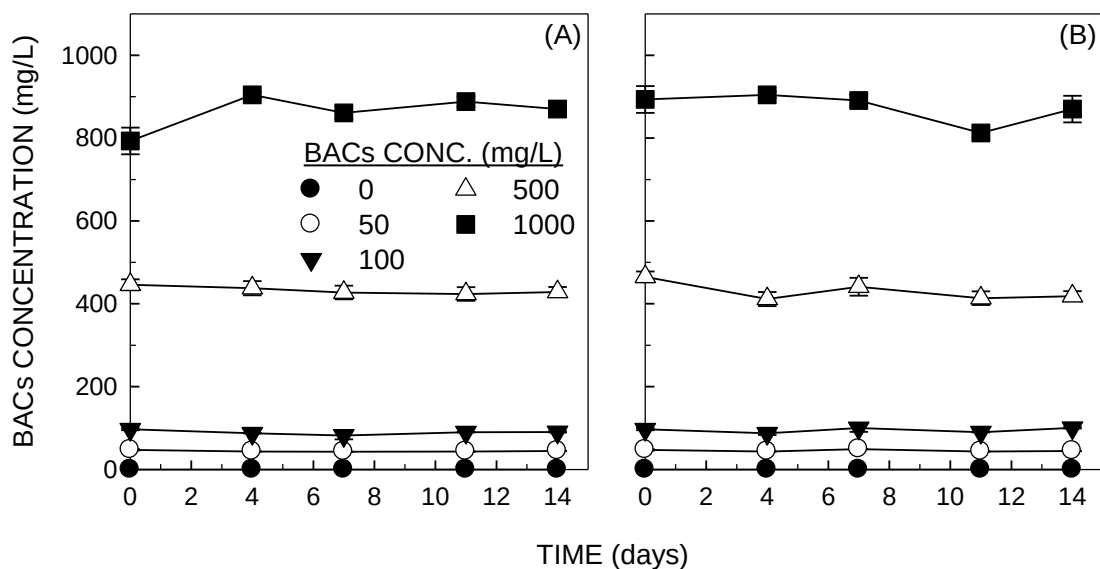


Figure 8.15. Profile of BAC ILs utilization in flasks containing (A) BIOMIG1 and (B) co-culture at different BAC ILs concentration

The COD of all of the products and also the remained glucose was calculated and compared with theoretical COD of 10,667 mg/L which attributed to initial glucose in reactors (Table 8.15. and Figure 8.16.). There is no difference between the theoretical and the calculated COD of the products and remained glucose. This shows that there may not be any other compound produced in the system.

Table 8.15. COD balance of total organic acids, remained glucose, ethanol and 2,3-butanediol in bottles containing co-culture and different BAC ILs concentration under anaerobic condition

Culture Series	Organic acids (mg/L)	Remained glucose (mg/L)	Ethanol (mg/L)	2,3-butanediol
0 mg BAC/L	2119.44±66.03	2494.94±336.6	1662.44±316.63	6564.54±20.00
50 mg BAC/L	1864.27±14.18	2424.05±3.24	1732.62±32.7	6256.83±21.2
100 mg BAC/L	1705.94±16.19	3735.63±50.41	1724.4±157.4	6221.82±73.89
500 mg BAC/L	1311.26±15.24	4165.31±0.85	1612.16±15.82	5220.70±6.06
1000 mg BAC/L	1101.71±36.66	4484.01±155.2	1483.29±136.16	5396.79±154.49

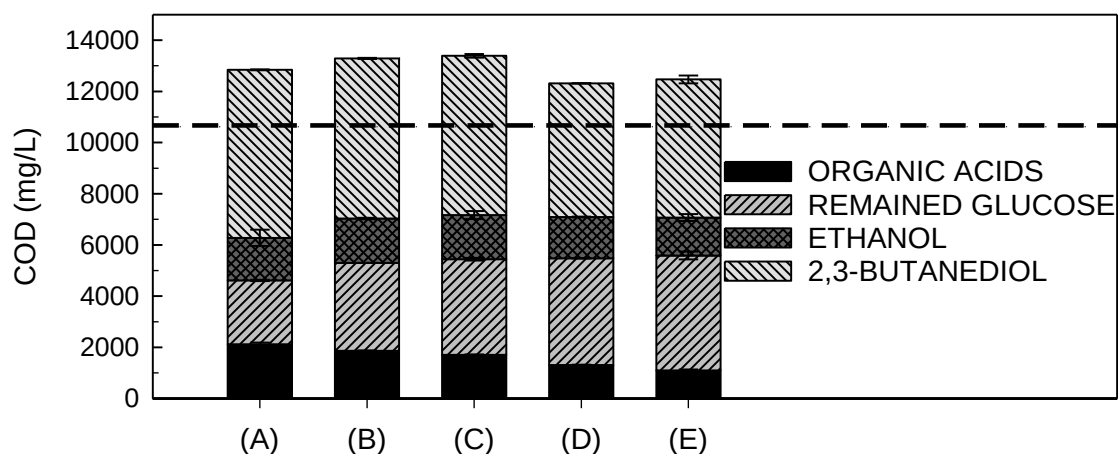


Figure 8.16. COD balance in serum bottles containing co-culture and (A) 0, (B) 50, (C) 100, (D) 500 and (E) 1000 mg BAC/L under anaerobic condition

The total gas (Table 8.16.) and carbon dioxide (Table 8.17.) produced under anaerobic condition was also measured. There results were used to make a carbon balance between the products, remained glucose and theoretical applied carbon content (initial 400 mg in the form of glucose). The results are shown in Table 8.18. and Figure 8.17.

Table 8.16. Total gas production produced by co-culture at different BACs concentration under anaerobic condition

Culture Series	Total gas (mL)
Control	0
0 mg BAC/L	10.6±1.3
50 mg BAC/L	10.2±0.3
100 mg BAC/L	11.6±0.6
500 mg BAC/L	10.8±0.4
1000 mg BAC/L	11.3±0.7

Table 8.17. Carbon dioxide produced by co-culture at different BACs concentration under anaerobic condition

Culture Series	Mass of CO ₂ Produced (mg)
Control	0.03
0 mg BAC/L	117.47±0.03
50 mg BAC/L	122.17±0.05
100 mg BAC/L	132.37±1.03
500 mg BAC/L	128.18±0.73
1000 mg BAC/L	124.65±0.11

Table 8.18. Carbon content of organic acids, remained glucose, ethanol, 2,3-butanediol and total CO₂ in bottles with co-culture and different BAC ILs concentration under anaerobic condition

Culture Series	Organic acids (mg)	Remained glucose (mg)	Ethanol (mg)	2,3-butanediol (mg)	Total carbon Dioxide (mg)
0 mg BAC/L	83.89±2.19	88.85±0.93	40.34±7.69	170.46±0.52	32.04±0.04
50 mg BAC/L	72.70±0.51	122.3±0.12	42.04±0.80	162.47±0.55	33.32±0.11
100 mg BAC/L	67.68±0.63	133.04±1.80	41.84±3.82	161.56±1.92	36.1±1.12
500 mg BAC/L	54.45±0.61	148.34±0.03	39.12±0.38	135.56±0.16	34.96±0.56
1000 mg BAC/L	48.53±1.64	159.69±5.52	35.99±3.30	140.14±4.01	33.99±1.14

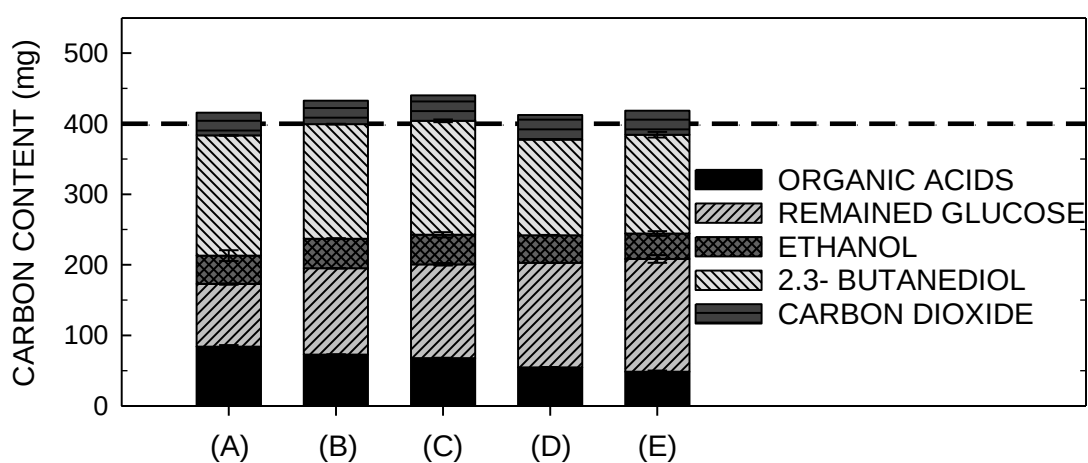


Figure 8.17. Carbon balance in serum bottles containing co-culture and (A) 0, (B) 50, (C) 100, (D) 500 and (E) 1000 mg BAC/L under anaerobic condition

According to Figure 8.17. carbon content of produced ethanol, 2,3-butanediol and carbon dioxide matches with the theoretical applied carbon.

Since the co-culture was able to consume glucose aerobically in just 24 hours, the aerobic glucose fermentation by BIOMIG1 and the co-culture was carried out at 0-1000 mg BAC/L during 24 hours. BIOMIG1 fermented about 24.3, 36.4 and 26.5 % of glucose in 0, 50 and 100 mg BAC/L after 24 hours whereas co-culture utilized all of the glucose in 24 hours (Figure 8.18.).

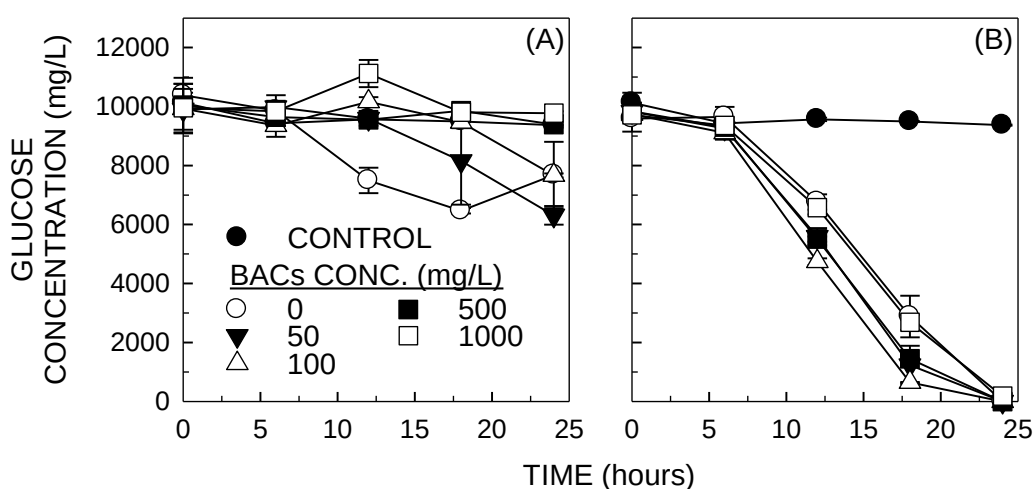


Figure 8.18. Profiles of aerobic glucose fermentation by (A) BIOMIG1 and (B) co-culture at different BAC ILs concentration

Table 8.19. Final pH of flasks for assessing the aerobic glucose fermentation by BIOMIG1 and the co-culture at different BAC ILs concentration

Culture Series	pH	
	BIOMIG1	co-culture
Control	6.89±0.03	6.89±0.03
0 mg BAC/L	6.36±0.02	6.52
50 mg BAC/L	6.22±0.01	6.5
100 mg BAC/L	5.93±0.12	6.47
500 mg BAC/L	6.89	6.45
1000 mg BAC/L	6.89	6.23±0.04

In both sets of flasks, pH values are less than 7 which indicate the organic acids production (Table 8.19.). The total organic acids production in flasks containing BIOMIG1 decreased by increasing in BACs concentration. In addition 1000 mg BAC/L affected the

total organic acids production by co-culture (Figure 8.19.). In both cases, citric and acetic acids were the most observed organic acids. Moreover, propionic acid was also obtained in this set and its concentration was maximum at 500 mg BAC/L. Also a trace of succinic acid was also produced.

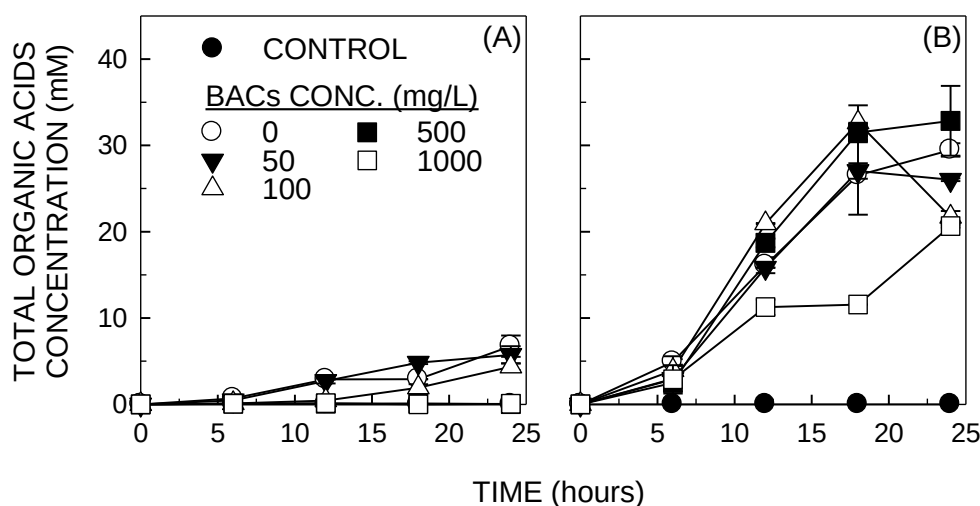


Figure 8.19. Profiles of total organic acids production by (A) BIOMIG1 and (B) co-culture at different BACs concentration under aerobic condition

Maximum acetic acid production by BIOMIG1 was in flask which did not receive any BAC ILs. In fact, increase in BACs concentration reduced the acetic acid production, whereas in flasks containing co-culture, acetic acid had an acceptable value according to BACs concentration. The acetic acid production was 20.4 mM although this value was not observed in the presence of 1000 ppm BACs (Figure 8.20.).

The main organic acid produced by BIOMIG1 and co-culture was citric acid, however the effects of BACs concentration on its production is interesting. The highest citric acid production by BIOMIG1 was 4.92 mM which was in the presence of 50 mg BAC/L. On the other hand, co-culture produced the highest amount of citric acid. 12.7 mM citric acid was produced in the flasks receiving 1000 mg BAC/L. Although BIOMIG1 could not generate any citric acid in high BACs concentration, but its co-culture with BIOMIG4 gave acceptable results (Figure 8.21.).

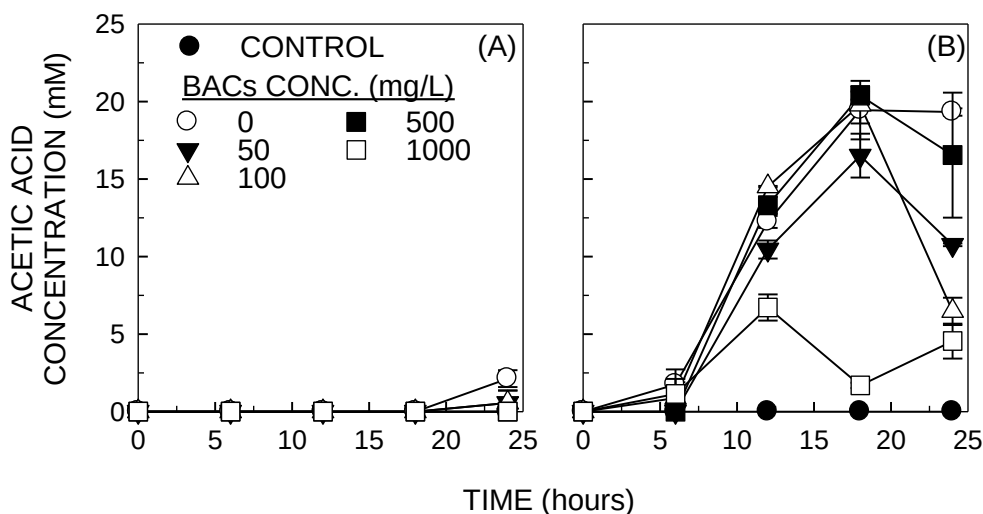


Figure 8.20. Profile of acetic acid production by (A) BIOMIG1 and (B) co-culture at different BACs concentration under aerobic condition

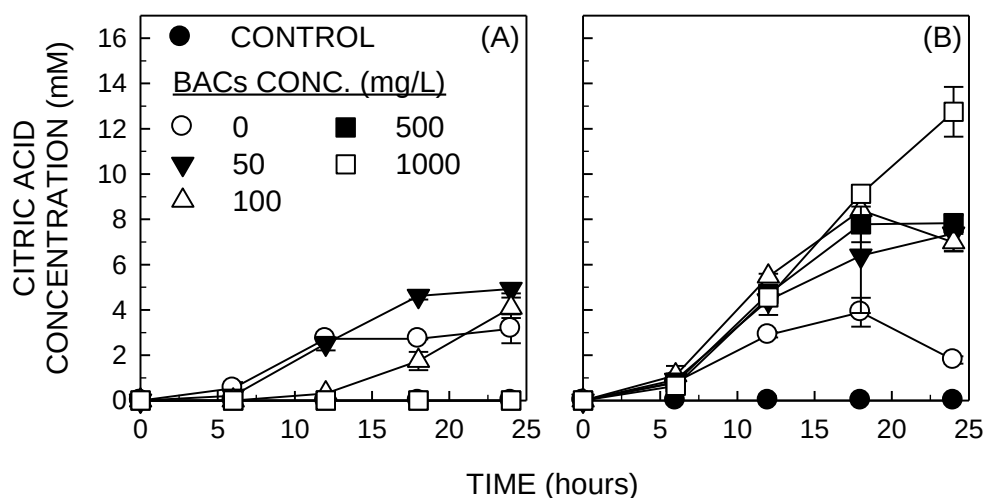


Figure 8.21. Profile of citric acid production by (A) BIOMIG1 and (B) co-culture at different BACs concentration under aerobic condition

Ethanol is the outcome of glucose fermentation under anaerobic condition. Therefore, the low production of ethanol under aerobic condition compared to anaerobic is natural. The highest ethanol value was detected in the presence of 0 and 50 ppm BACs and its production diminished in higher BACs concentration (Figure 8.22.A).

The results in the previous sections represented that 2,3- butanediol was the most produced biofuel among the generated organic acids and alcohols. In the presence of

BACs, 2,3- butanediol is still the main product, however, its production is affected by BACs concentration. Enhancing the BACs concentration caused the decrease in 2,3- butanediol production by co-culture from 45.2 to 21.2 mM (Figure 8.22B).

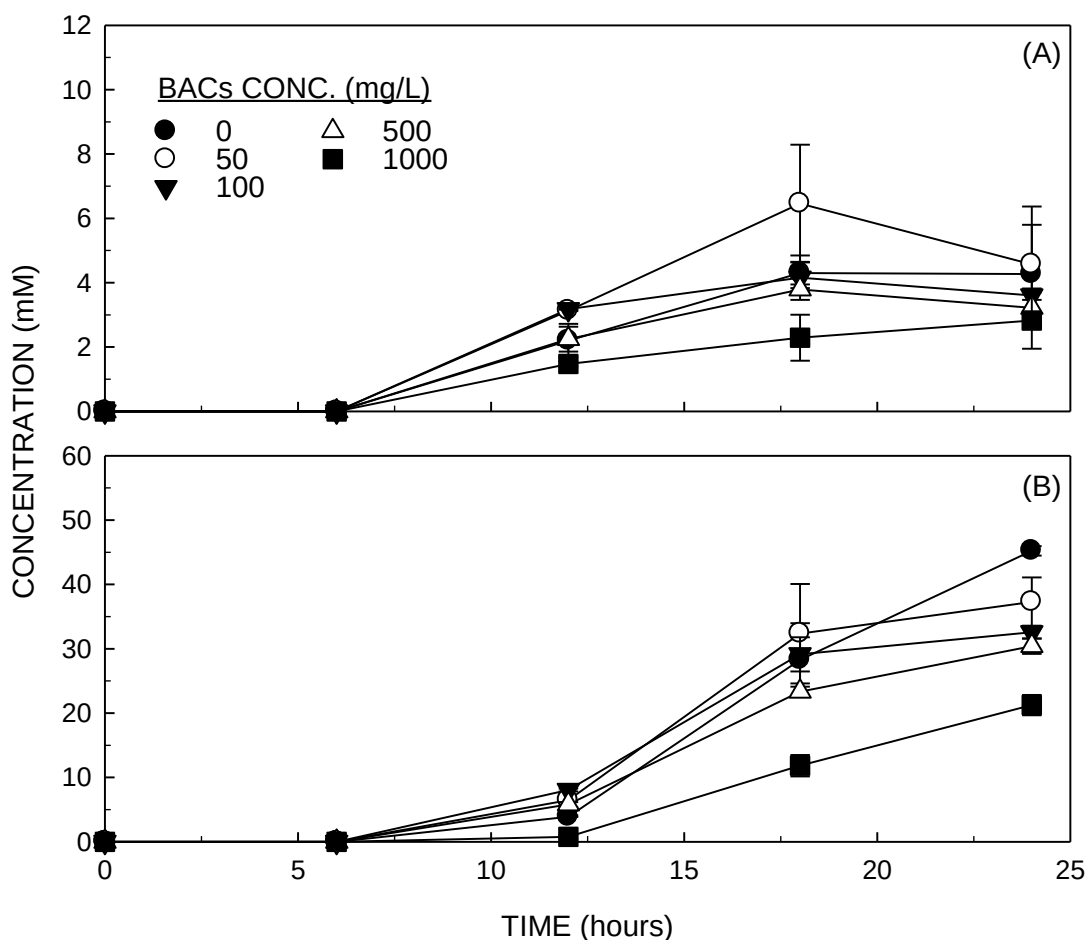


Figure 8.22. Profile of (A) ethanol and (B) 2,3-butanediol by co-culture at different BACs concentration under aerobic condition

BIOMIG1 is also capable of degrading BACs and utilize them as carbon source under aerobic condition. The profiles of BACs concentration shows that 100% of 50 mg BAC/L was degraded by BIOMIG1 and co-culture in 24 hours. In addition, 59.6 and 67.5% of 100 mg BAC/L was utilized, respectively (Figure 8.23.).

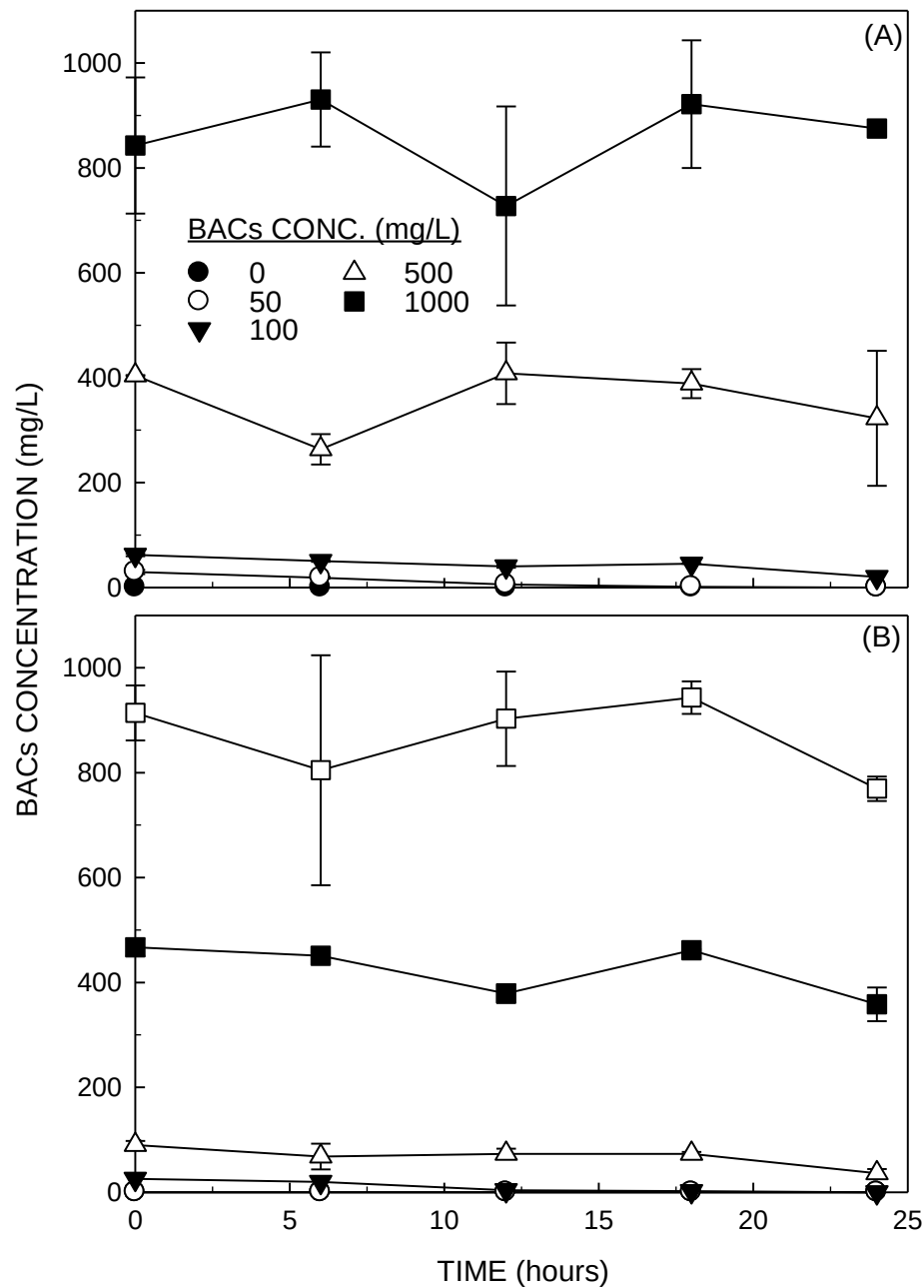


Figure 8.23. Profiles of BACs utilization in the flasks containing (A) BIOMIG1 and (B) co-culture under aerobic condition

At the end of each test, the COD balance was conducted. According to Table 8.20. and Figure 8.24. almost all of the reactors are in balance with the theoretical applied COD (10,667 mg/L as glucose), although some differences in reactors with 0, 50 and 100 ppm BACs is considerable which is caused by generating the carbon dioxide. The COD balance in flasks with co-culture was distracted because of BACs. The big gap between the

theoretical and products COD is related to carbon dioxide production (Table 8.21. and Figure 8.25.).

Table 8.20. COD balance of total organic acids and remained glucose in flasks containing BIOMIG1 and different BAC ILs concentration under aerobic condition

Culture Seies	Organic acids (mg/L)	Remained glucose (mg/L)
0 mg BAC/L	600.61±127.38	8191.78±1199.75
50 mg BAC/L	760.83±80.28	6731.84±336.48
100 mg BAC/L	663.99±120.91	8177.36±70.37
500 mg BAC/L	0	10018.62±114,32
1000 mg BAC/L	0	10427.44±70.27

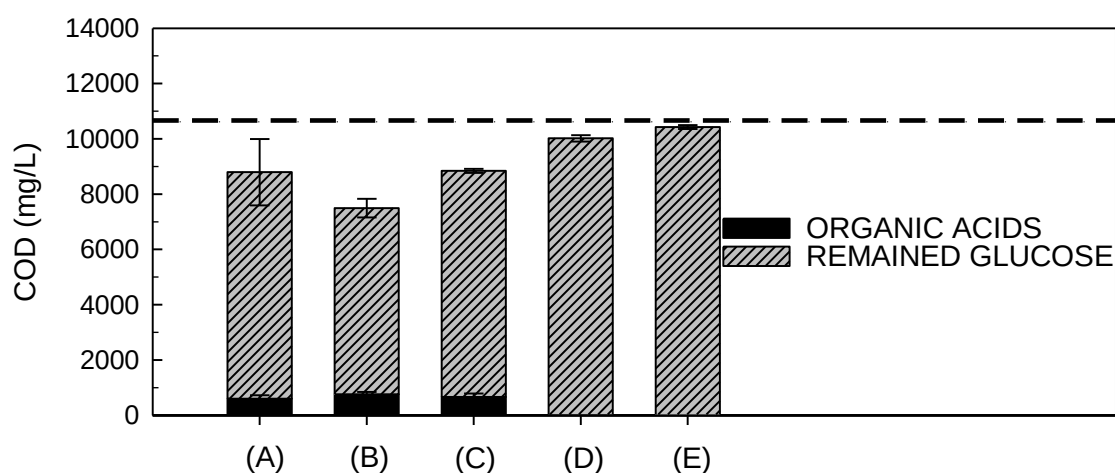


Figure 8.24. COD balance in flasks containing BIOMIG1 and (A) 0, (B) 50, (C) 100, (D) 500 and (E) 1000 mg BAC/L under aerobic condition

Table 8.21. COD balance in flasks containing co-culture and different BAC ILs concentration under aerobic condition

Culture Series	Organic acids (mg/L)	Remained glucose (mg/L)	Ethanol (mg/L)	2,3-butanediol (mg/L)
0 mg BAC/L	2431.23±101.56	0	401.24±143.7	7945.99±130.47
50 mg BAC/L	2726.02±133.25	0	429.43±169.39	6549.3±668.26
100 mg BAC/L	2344.08±133.94	0	338.6±12.86	5724.93±176.05
500 mg BAC/L	3135.02±356.582	0	302.62±56.29	5343.58±212.68
1000 mg BAC/L	2475.20±307.54	188.34±89.1	265.35±82.55	3733.16±358.018

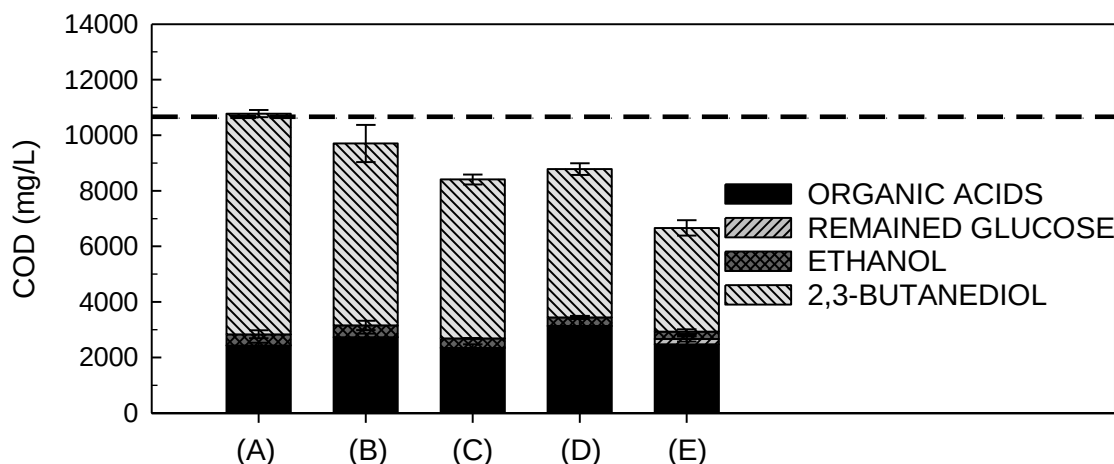


Figure 8.25. COD balance in flasks containing co-culture and (A) 0, (B) 50, (C) 100, (D) 500 and (E) 1000 mg BAC/L under aerobic condition

8.4. Summary

The glucose fermentation by BIOMIG1 and BIOMIG4 was conducted under anaerobic and aerobic conditions. The glucose was completely utilized by BIOMIG4 and co-culture under aerobic condition in less than 24 hours whereas about 82% of glucose was fermented anaerobically by the aforementioned cultures during 29 days. In addition BIOMIG1 showed a low affinity in glucose fermentation. 80% of glucose was utilized by BIOMIG1 in 3 days whereas no glucose was fermented anaerobically. The main product produced in glucose fermentation by BIOMIG4 and the co-culture was 2,3-butanediol under both aerobic and anaerobic conditions. In addition ethanol, formic and lactic acids were also obtained under anaerobic conditions. Acetic and citric acids were the most organic acids produced aerobically.

To evaluate how the BAC ILs affect fermentation, aerobic and anaerobic glucose fermentation by the co-culture of BIOMIG1 and BIOMIG4 which are fermentative bacteria and resistant to BACs was monitored at different BAC ILs concentrations (0-1000 mg/L). About 77.5% of the glucose amended was converted to alcohols and acids within 14 days under anaerobic conditions. This percent was decreased to 57% as the BACs concentration increased to 1000 mg/L. 2, 3-butanediol, ethanol and formic acid were the main products of the fermentation. BACs did not affect the yield of these products significantly at the concentrations tested. Glucose was completely fermented within 24

hours under aerobic conditions. 2, 3- butanediol was the main product and its production was decreased from 45.2 to 21.2 mM as BACs increased from 0 to 1000 mg/L. In addition, acetic acid production was decreased and the citric acid production was increased by increasing the BACs concentration. Since BIOMIG1 is able to degrade the BACs aerobically, 100 and 67.5 % of BACs was degraded in flasks at 50 and 100 mg/L BACs concentration. These results show that BACs concentration affect the fermentation process under aerobic condition whereas no significant change was observed under anaerobic condition.

9. CONCLUSION

In the course of this research a cellulose-degrading microbial community which can mineralize cellulose to carbon dioxide was developed. The cellulolytic strains *Ochrobactrum* sp. BIOMIG5, *Sinorhizobium* sp. BIOMIG6, *Alcaligenes* sp. BIOMIG7, *Pseudoxanthomonas* sp. BIOMIG8 and *Shinella* sp. BIOMIG9 were isolated from this community. BACs were used as ILs to enhance the rate of cellulose dissolution and fermentation; however, the microbial community lost its capability in degrading the cellulose at different BAC ILs concentration (0-1000 mg/L). Therefore, *Pseudomonas* sp. BIOMIG1 was amended as biocatalyst and BAC ILs degrader. Based on the results of cellulose degradation by 2 and 5 times diluted soil microbial community in the presence of BAC ILs, the total carbohydrates were consumed faster in flasks containing 100 mg BAC/L than the ones containing 50 mg BAC/L. This claims that BAC ILs enhance the dissolution of cellulose. In addition, since BIOMIG1 can aerobically degrade the BACs to carbon dioxide, the cellulose can be regenerated from the BAC ILs matrix and consumed by cellulolytic community without any inhibition.

The glucose fermentation by the co-culture of BAC ILs resistant strains, *Pseudomonas* sp. BIOMIG1 and *Serratia marcescens* BIOMIG4 was performed at different BAC ILs concentration (0-1000 mg/L) under both aerobic and anaerobic condition. Based on the results, valuable chemicals including 2,3-butanediol, ethanol, formic and lactic acid under anaerobic condition and 2,3-butanediol, citric and acetic acid were obtained aerobically. Under anaerobic condition, glucose utilization by co-culture was alleviated by increase in BAC ILs concentration whereas ethanol production was not affected. In addition, the 2,3-Butanediol production was decreased from 37 to 30 mM under anaerobic condition compared to its 24 mM reduction under aerobic condition at the highest BACs concentration.

Since the cellulose got degraded when BIOMIG1 amended to the system and also the co-culture of BIOMIG1 and BIOMIG4 fermented the glucose to alcohols and organic acids, the cellulose degradation will be examined at different BACs concentration in a system containing cellulolytic microbial community, BIOMIG1 as BAC ILs Degradar and

BIOMIG4 as biofuel producer. Therefore, a system able to hydrolyse cellulose to glucose and further ferment it to biofuels will be obtained while the BAC ILs as toxic compounds are removed.

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