PRODUCTION OF VALUED BIOPOLYMER, POLYHYDROXYBUTYRATE (PHB), FROM GLYCEROL: EFFECTS OF IMPURITIES ON SELECTED SALT-TOLERANT MICROBIAL STRAINS

A Thesis

by

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ABSTRACT

Polyhydroxybutyrate (PHB) is a type of bioplastics with similar chemical and physical characteristics to petroleum-based plastics. However, the production cost of PHB is high, in part, due to expensive growth substrate. In this study, we explore the feasibility to reduce the production cost of PHB by using a cheap carbon source like crude glycerol, a waste stream from biodiesel industry. Crude glycerol contains undesirable impurities like salts and fatty acids that might be inhibitory to bacterial growth and/or PHB production.

Thus, this study examined the ability of salt-tolerant strains to grow on glycerol and the effects of glycerol impurities on the growth and PHB production by the glycerol-grown strains. Among four salt-tolerant bacteria (*Azohydromonas lata*, *Pseudomonas oleovorans*, *Burkholderia* sp., and *Zobellella denitrificans* ZD1), *A. lata* was unable to grow on 5 g/L of glycerol in the presence or absence of 0.5 g/L of NaCl. *Z. denitrificans* ZD1 accumulated the highest PHB content (84% g-PHB/g-dry cell weight) in 24 hours; while *P. oleovorans* and *Burkholderia* sp. accumulated 38% (g-PHB/g-dry cell weight) and 45% (g-PHB/g-dry cell weight) in 46 hours and 40 hours, respectively. The growth of *P. oleovorans* was slightly enhanced by the presence of fatty acids (stearate, oleate, and linoleate); however, no impacts on the growth and PHB content were observed for *Burkholderia* sp. When fatty acids was increased to 1% (w/w), delayed cell growth, lower maximum cell density (from 0.98 g/L to 0.68 g/L) and lower PHB content (from 84 to 71% (g-PHB/g-dry cell weight) were observed for *Z. denitrificans* ZD1. Effects of

nitrogen source, electron acceptors, initial pH, and C/N ratios suggested that *Z. denitrificans* ZD1 was a favorable strain for PHB production using glycerol. *Z. denitrificans* showed the highest PHB content in the dry cell weight, 84%, the shortest utilization period, 24 hours, even under the influence of fatty acids. The strain also can accumulate PHB using nitrate as a nitrogen source and electron acceptor.

DEDICATION

I dedicate this thesis to my parents, sisters, brothers, and friends who stood next to me all the time, even when they were far away from me; especially to my mother who raised me to be able to write these words; to my father who just died a few months ago and wished to see me in the graduation ceremony. I also dedicate this thesis to my wife who supported me with all her efforts and surrounded my life with love; and to my handsome little baby who brings me more joy than I could have ever imagined.

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CHAPTER I

INTRODUCTION

Introduction

Bioplastic production is marking a new era for biotechnological applications. In recent decades, the use of bioplastics produced from microorganisms has been suggested to replace petroleum-based plastics. This would help to reduce the negative environmental effects of petroleum-based plastics such as undegradable by nature, digestible by marine animals, and absorbable by human bodies. Comparatively, bioplastics have many advantages over petroleum-based plastics such as they are biodegradable, renewable, and not toxic. The concept behind the production of bioplastics is to use microorganisms such as bacteria or archaea to feed on industrial wastes to produce bioplastics. These wastes contain one type or several types of carbons. Under certain growth conditions such as nutrient limitation and carbon excess, some microorganisms will accumulate bioplastic, polyhydroxybutyrate (PHB), inside their cells.[1] PHB is a short-chain length biopolymer (3-5 carbon atoms) that has for some extend similar physical and chemical characteristics to petroleum-based polymers.[2] However, PHB is still very expensive to produce compared to petroleum-based plastics. PHB has a production price of approximately $3.1-4.4 US$/Kg, which is 5-10 times higher than petroleum-based plastics.[3, 4] Therefore, scientists and researchers are trying to find different methods to maximize PHB production and reduce its selling price.

Several inexpensive and sustainable carbon sources, such as paper mill wastewater, activated sludge, ground coffee wastes, and biodiesel production wastes[5-9],

have been explored to achieve the metabolic bioconversion to produce bioplastics. By reducing the price of carbon substrates used in bioplastic production, the selling price of bioplastic would decrease and could compete with petroleum-based plastics on the market place.[10, 11] In this study, glycerol was the carbon substrate used for PHB accumulation by different bacteria. Crude glycerol is a waste byproduct from the biodiesel industry. In recent decades, there has been a huge interest in using crude glycerol as a carbon substrate due to the dramatic decline in its selling price, 0.118-0.149 US$/Kg.[3, 4] However, crude glycerol usually comes with some impurities such as alcohols, salts, free fatty acids, and water that weaken the bacterial utilization process.[12, 13] Free fatty acids (stearic, oleic, and linoleic) are the main crude glycerol impurities, their effects on the utilization process and PHB production were tested. Free fatty acids are long hydrocarbon chains that contain either single or multiple double bonds between the carbons. Therefore, understating how these impurities are affecting the utilization process is essential to building a biological viable technology for the production of bioplastics.

In this research, four strains: *Azohydromonas lata, Pseudomonas* oleovorans, *Burkholderia* sp., and *Zobellella denitrificans* ZD1 were chosen to produce PHB from glycerol. These strains are all gram-negative aerobic bacteria with the exception of *Zobellella denitrificans* ZD1, which has facultative anaerobic behavior. The reason for selecting these bacterial strains is that they are all tolerant to high salt concentration that is a typical impurity in crude glycerol, about 10% (w/w).[9, 14-18] Several studies have reported the ability of *A. lata*[14, 15], *P. oleovorans*[9, 16, 19], and *Burkholderia* sp.[17] to grow on glycerol, with the exception of *A. lata* that was reported to have poor growth on glycerol.

The studies have also shown that *P. oleovorans* and *Burkholderia* sp. utilized either pure or crude glycerol to accumulate PHB. *Z. denitrificans* ZD1 is another salt-tolerant strain that was isolated by Lin and Shieh[18] from sediment samples collected from various mangrove ecosystems in Taiwan. There are no research studies that report the ability of *Z. denitrificans* ZD1 to either use glycerol or accumulate PHB. However, Ibrahim and Steinbuchel[20] reported that *Zobellella denitrificans* MW1 was capable of using glycerol as a sole carbon source with NaCl salt to produce PHB while growing with optimum cell accumulation of 87% PHB. According to their study, the 16S rRNA gene sequence of *Z. denitrificans* MW1 exhibited 98.5% similarity to *Z. denitrificans* ZD1. Furthermore, much research has been performed to evaluate the effect of saturated and unsaturated fatty acids on the growth of other bacterial strains and their production of value-added products.[21-24] The literature contained altered results regarding the effect of fatty acids. Usually fatty acids are incorporated into the chains of cellular lipids and membranes.[23, 25] But, fatty acids with their long chains could also damage the bacterial cell membrane, interfere with metabolic pathways, and eventually destruct the bacteria.[22, 26] In contrast, there are no research studies that reported the impact of these fatty acids present in the utilization of glycerol and production of PHB by the aforementioned strains.

The overall goal of this research is to examine the feasibility of using glycerol as a main carbon substrate by different salt-tolerant bacterial strains *A. lata, P. oleovorans*, *Burkholderia* sp., and *Z. denitrificans* ZD1 to accumulate PHB. The research specifically studies the effect of fatty acids presence such as stearic, oleic, and linoleic in glycerol on

the bacterial growth and accumulation of PHB by these strains. To accomplish these goals, experiments were performed to achieve specific objectives as described below:

Objective 1: Accumulate PHB by salt-tolerant bacterial strains *A. lata, P. oleovorans, Burkholderia* sp., and *Z. denitrificans* ZD1 with glycerol used as a sole carbon substrate.

Hypothesis: These salt-tolerant strains can grow on glycerol with salt while accumulating PHB.

Task 1a: Examine the ability of these strains to grow in mineral salts medium containing glycerol as a sole carbon substrate

Task 1b: Examine the ability of these strains to accumulate PHB in mineral salts medium containing glycerol as a sole carbon substrate

Objective 2: Test the effects of fatty acids in glycerol on the bacterial growth and PHB accumulation by salt-tolerant bacterial strains *A. lata, P. oleovorans, Burkholderia* sp., and *Z. denitrificans* ZD1.

Hypothesis: fatty acids such as stearic, oleic, and linoleic in glycerol can inhibit or enhance the growth and PHB accumulation by these salt-tolerant strains.

Task 2a: Observe the effects of fatty acids in glycerol on the bacterial growth

Task 2b: Observe the effects of fatty acids in glycerol on the PHB accumulation

Objective 3: Investigate the effects of critical process variables on the growth and PHB accumulation by the best performing strain.

Hypothesis: Process variables can have different effects on the growth and PHB accumulation

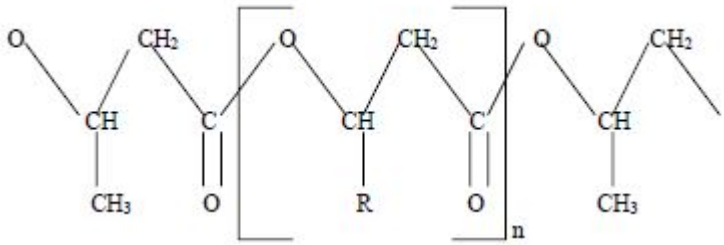
Task 3a: Study the effects of nitrogen source, electron acceptor, initial pH, and initial C/N ratios on the growth and PHB accumulation by the best performing strain

CHAPTER II

LITERATURE REVIEW

Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are biodegradable biobased polymers that consist of 150 types of monomers.[27, 28] Polyhydroxybutyrate (PHB) is the most common type of PHAs thermoplastic polymers due to its similarity with petroleum-based polymers. PHB is biodegradable aerobically to water and carbon dioxide and anaerobically to methane.[29] Therefore, it is considered as an alternative to petroleum-based plastics. Fig. 1 is a typical molecular structure of PHAs, including PHB.[29, 30] The monomers vary based on the molecular weight and the length of the chain. Mainly, PHAs are divided into two groups based on the chain length of the molecule. The first group is called short-chain-length PHAs (SCL-PHAs), which consist of 3-5 carbon atoms. An example of SCL-PHAs is poly(3-hydroxybutyrate) (PHB), which can be produced by several bacteria such as *Pseudomonas oleovorans*, *Burkholderia* sp., and *Azohydromonas lata*.[9, 17, 31] The second group is called medium-chain-length PHAs (MCL-PHAs), which consist of 6-14 carbon atoms such as poly(3-hydroxyundecanoate) and poly(3-hydroxyoctanoate).[1]



n varies from 600 to 35000

R= hydrogen	Poly(3-hydroxypropionate)
R=methyl	Poly(3-Hydroxybutyrate)
R=ethyl	Poly(3-hydroxyvalerate)
R=propyl	Poly(3-hydroxyhexanoate)
R=pentyl	Poly(3-hydroxyoctanoate)
R=nonyl	Poly(3-hydroxydodecanoate)

Fig. 1 Typical molecular structure and weight of PHA and its monomers. Original figure from Lee[30] and modified by Khanna et al.[29]

Polyhydroxybutyrate (PHB)

PHB is classified as one of the short-chain-length PHAs monomer, which was first described by Lemoigne[32] in 1926 when he noticed a significant decrease in pH due to the production of β-hydroxybutyric acid during the autolyzation of *Bacillus subtilis* in distilled water. PHB is accumulated in the bacterial cells as energy storage through the fermentation of sugars, animals and vegetable oils.[29] MCL-PHAs provide elastic and adhesive behaviors. In contrast, SCL-PHAs, like PHB, have physical properties that are similar to petroleum-based polymers; therefore, they are considered to be promising monomers for the bioplastic production industry. Specifically, PHB is highly stiff and relatively brittle. It also has a crystallization degree of 50-70% and melting point of 175 °C, which is slightly lower than crotonic acid's melting point, 185 °C.[2] Aside from its melting point, it has a molecular weight that ranges from 10,000-3,000,000 Da based on

the carbon substrate and strain used for fermentation.[33] These properties of PHB support its usage in numerous useful applications such as packaging, production of films, and biomedicals including patch materials and bone implements.[29]

Carbon substrate

Carbon substrates used in biological utilization play an important role in the production of value-added products, like PHB. In addition to reducing production costs, they help produce a higher amount of PHB and reduce the utilization period. Some of the carbon substrates that have been used in earlier research include the following: starch, sucrose, n-octane, glycerol, etc.[9, 14, 15, 19, 31, 34, 35] Table 1 provides a list for some of the carbon substrates used by different strains to accumulate PHB. The table sheds light on some of the optimum growth conditions, percent of PHB accumulated in the dry cells, PHB produced compared to substrate used, and article references. Usually carbon substrates derived from industrial wastes come with impurities that need to be purified to achieve a higher PHB production. This purification process adds on another cost element to the production cost of PHB.[29] Thus, researchers are trying to discover new utilization techniques, new carbon substrates, and new bacterial strains to overcome this problem.

Table 1. List of bacteria capable of using different carbon substrates to accumulate PHB biopolymer

Bacteria	pH	Temperature °C	%PHB/DCW[a]	PHB yield[b] (gP/gS)	References
Azohydromonas lata (Alcaligenes latus)	7 6.8	33 30	63 (Sucrose) 87 (Sucrose)	0.35 (Sucrose) N.A.[e]	Grothe and Chisti (2000) Wang and Lee (1997)[14, 36]
Bacillus megaterium	7	30	62 (PG[c]) 53 (CG[d])	0.3 (PG) N.A.	Naranjo et al. (2013)[4]
Burkholderia sp. USM (JCM15050)	7	37	54-60 (PG) 31 (CG)	N.A. N.A.	Chee et al. (2010)[17]
Cupriavidus necator JMP 134 (DSM 545)	6.8	34	51.2 (PG) 38.1 (CG)	0.36 (PG) 0.34 (CG)	Cavalheiro et al. (2009)[37]
Haloferax mediterranei	7.2	38	60 (Starch)	0.33 (Starch)	Lillo and Rodriques-Valera (1990)[34]
Paracoccus denitrificans	7	35	65 (PG[b])	N.A.[d]	Mothes et al. (2007)[38]
P. oleovorans (ATCC 29347) *P. oleovorans* (NRRL B-14682)	7 7 7	30 30 30	25 (n-octane) 13-27 (CG) 38 (PG)	N.A. N.A. N.A.	Lageveen (1988) Ashby (2004) Ashby (2011)[9, 19, 35]
Zobellella denitrificans MW1	7.3	41	80.4 (PG)	N.A.	Ibrahim et al. (2009)[20]

[a] DCW represents dry cell weight. [b] PHB yield (gram of PHB was produced per gram of substrate was utilized). [c] PG represents pure glycerol. [d] CG represents crude glycerol. [e] N. A. represents information is not available.

Glycerol

Glycerol was the carbon substrate used in this research. It provides an alternative cheap carbon substrate for bioplastic production. Specifically, crude glycerol is a waste byproduct produced through the transesterification process of animal fats or vegetable oils to produce biodiesel as an alternative fuel.[9, 39, 40] Fig. 2 represents the transesterification process, which occurs through the reaction of one mole of generic triglyceride with three moles of alcohol such as methanol, butanol or ethanol to produce one mole of glycerol and three moles of fatty acid methyl esters, biodiesel, in the presence of a catalyst, which can be an alkali, acid, or enzyme.[41] There is approximately 10 kg of crude glycerol produced with every 100 kg of biodiesel.[42] After purification, glycerol is used in food, cosmetics, drugs, pharmaceutical, and textiles.[37] It is also used as a carbon substrate for PHB production by some microbial strains.[9, 12, 13, 17, 19, 29] However, crude glycerol produced from the biodiesel industry comes with some impurities that could inhibit the growth of these strains and limit their PHB production. These impurities in crude glycerol are described further in the next section.

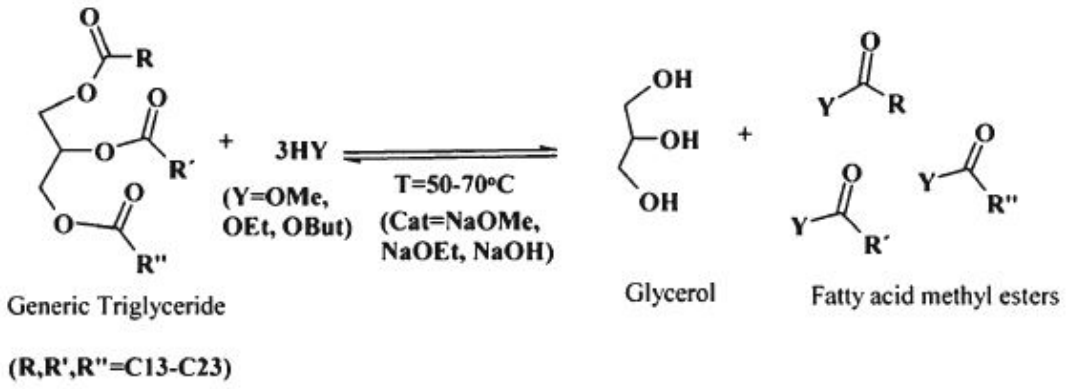


Fig. 2 Typical reaction scheme for the transesterification of animal fats or vegetable oils to glycerol and biodiesel. Luque et al.[41]

Glycerol impurities

As mentioned earlier, crude glycerol produced from the biodiesel industry contains several impurities at different concentrations that might improve or worsen the utilization process for PHB production. These impurities in crude glycerol are water, alcohol, free fatty acids, heavy metals, and salts.[9, 12, 43] Alcohol such as methanol or ethanol ends up in glycerol due to its usage in the transesterification process of animal fats or vegetable oils. Also, fatty acid salts and inorganic salts are formed by the addition of an alkaline catalyst like sodium hydroxide or potassium hydroxide during saponification.[43] Table 2 below lists typical impurities in crude glycerol with their concentration ranges, production processes, treatment methods, and article references.

Table 2. Typical impurities in crude glycerol produced from the biodiesel industry

Impurity	Concentration range[a] (w/w)	Production process	Treatment method	References
Water	14% 6-22%	N.A.[b]	N.A.	Hajek and Skopal (2010) and Ashby et al. (2011)[9, 43]
Alcohol: Methanol	Alcohol: Methanol: 4.6% and 12-15% Methanol: 1%	Neutralization of base with acid	Distillation of glycerol	Chee et al. (2010) and Ashby et al. (2011)[9, 17]
Free fatty acids (FFAs): Stearic Oleic Linoleic	FFAs: 21.8% and 23-25% Stearic 4.4% and 16% (w/wFFAs) Oleic 43.8% and 45% (w/wFFAs) Linoleic 14.3% and 12% (w/wFFAs)	Transesterification process and the addition of an alkali-catalyst	Saponification	Hajek and Skopal (2010), Chee et al. (2010), Chatzifragkou (2010) and Venkataramanan et al. (2012)[17, 22, 23, 43]
Salts: NaCl, KCl, and K_2SO_4	Salts: 3-10%	Neutralization of base with acid	Ion exchange on strong acid resin Amberlite-252	Carmona et al. (2008) and Venkataramanan et al. (2012)[23, 44]

[a] Concentration range is presented as the percentage of weight of impurity to the total weight of crude glycerol. [b] N. A. represents information is not available.

The purity level of crude glycerol ranges between 40 to 90% based on the transesterification process, biodiesel separation condition, and the origin of the feedstock such as soybean, rapeseed oil, or animal fats.[12, 13, 45] Fig. 3 is a flow chart, which shows the alkali-catalyzed biodiesel production processes that produce crude glycerol along with its impurities. The figure also provides different types of crude glycerol with their purity levels.[45] As mentioned earlier, in the case of using alkali as a catalyst such as sodium or potassium hydroxide, high amount of salts such as sodium or potassium chloride are generated. It also produces high amounts of saturated and unsaturated fatty acids such as palmitic, stearic, oleic, and linoleic acids at altered concentrations.[19, 23] In addition, a high concentration of alcohol is found to be present in crude glycerol due to its usage in the transesterification process. These impurities have an influence on the growth and utilization process of the microbial communities to produce added value products, including PHB.[9, 19, 41]

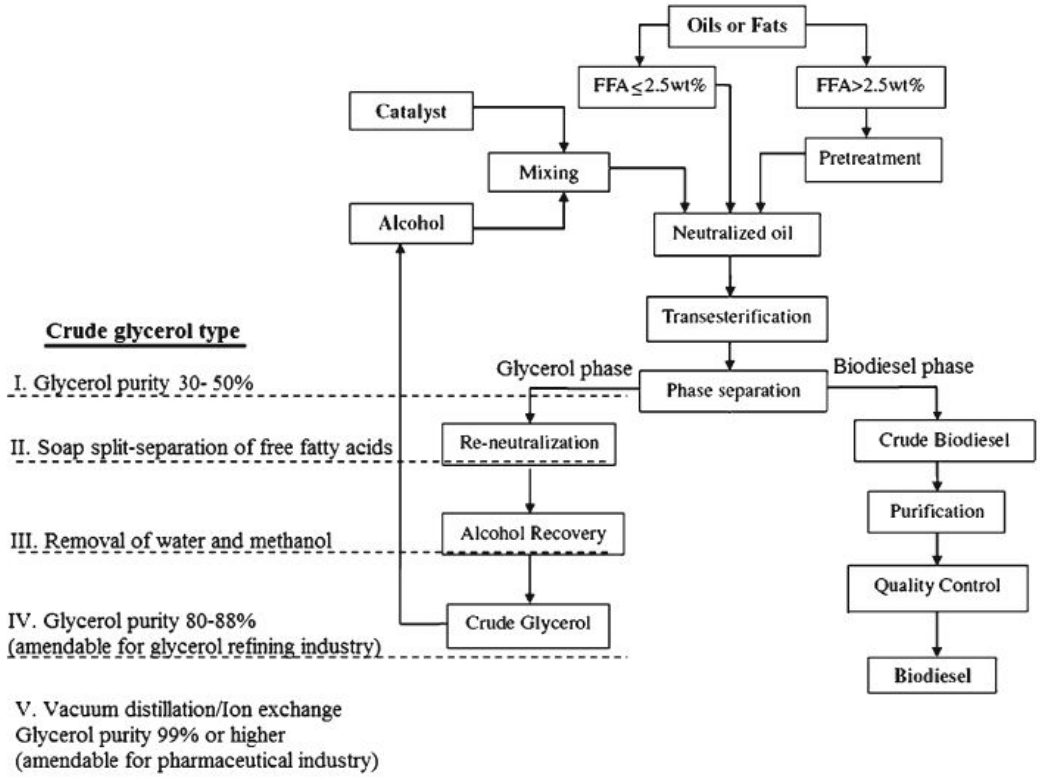


Fig. 3 Flow chart of alkali-catalyst transesterification process. Original chart from Leung et al.[45] and modified by Chatzifragkou and Papanikolaou.[12]

Crude glycerol impurities have different effects on the biological utilization process. For instance, salts like sodium chloride or potassium sulfate had no effects on the utilization process of crude glycerol, when *Clostridium pasteurianum* ATCC 6013 was used.[23] Ibrahim and Steinbuchel[20] reported that sodium chloride at 20 g/L enhanced the metabolism of *Zobellella denitrificans* MW1 to glycerol.[20] However, sodium chloride at higher concentrations had a negative effect on the accumulation of PHB.[20, 22] A similar observation regarding sodium chloride inhibition was reported by Chatzifragkou et al.[22] when *Clostridium butyricum* VPI 1718 was used for the production of 1,3-Propanediol. However, other phosphate salts such as Na_2HPO_4 and K_2HPO_4 had no inhibitory effect on

Clostridium butyricum VPI 1718. Similarly, alcohol such as methanol with 2.5 g/L and 5.0 g/L concentrations had no impact on the fermentation of *Clostridium pasteurianum* ATCC 6013 to crude glycerol.[23] In contrast, methanol caused a termination "end-capping" of the PHB chain length, weakening the mechanical properties of the polymer itself.[9]

Fatty acids

Fatty acids in crude glycerol also play an important role in the utilization process and metabolic conversion. They consist of long connected chains of carbon and hydrogen atoms with single or double bonds between the carbons. Fatty acids are divided into two main groups, saturated and unsaturated fatty acids, based on the bond type between the carbon atoms. Saturated fatty acids such as lauric, palmitic, and stearic consist of only single bonds between the carbon atoms, while unsaturated fatty acids such as oleic, vaccenic, and linoleic contain one or more double bond. When a fatty acid like stearic acid was given a symbol (18:0), it means that the fatty acid contains eighteen carbon atoms with zero double bonds between the carbons. So, the first number represents the number of carbons in the molecule, and the second number represents the number of double bonds between the carbons. As mentioned in table 2, fatty acids present in crude glycerol at different concentrations. However, stearic (18:0), oleic (18:1), and linoleic (18:2) acids are found to be the most dominant types of fatty acids when crude glycerol was analyzed by gas chromatography-mass spectrometry (GC-MS).[22, 23]

Much research has been performed to evaluate the effect of these fatty acids on the bacterial growth and value-added products.[21-24] All research contained different results regarding their effects. Usually fatty acids are incorporated into the chains of cellular lipids

and membranes.[23, 25] However, fatty acids with their long chains can damage the bacterial cell membrane, interfere with metabolic pathways, and eventually destruct the bacteria.[22, 26] Therefore, the general effect of these fatty acids depend on the type of fatty acid, its concentration, number of double bonds between the carbon atoms, and the type of strain used for utilization.[12, 19, 22, 23] For instance, gram-negative bacteria usually have less tolerance behavior to fatty acids compared to gram-positive bacteria.[46] Chatzifragkou et al.[22] found that the single double bond that exists in oleic acid inhibited the growth and 1,3-Propanediol production by *Clostridium butyricum* VPI 1718, while stearic acid with only single bonds between the carbon atoms had no effect. The same inhibitory effect was reported by Venkataramanan et al.[23] when *Clostridium pasteurianum* ATCC 6013 was used for the fermentation of crude glycerol. The inhibitory effect of fatty acids increased with the increase number of double bonds between the carbon atoms. Linoleic and oleic acids had the highest negative effect.

Bacterial metabolism

The bioconversion of waste carbons into value-added products is a very significant biotechnological development. For production of bioplastic, several strains have been found to use different carbon substrates, including glycerol. Table 3 sheds light on some of the most important value-added products, including PHB that are produced by the utilization of glycerol. It provides information regarding the type of strains, metabolic products, productivity, and article references.

Table 3. List of microbial strains capable of producing value-added products using glycerol as a carbon source

Strain	Metabolic product	Productivity[a]	Reference
Bacillus megaterium	PHB	62% (PG) and 53% (CG)	Naranjo et al. (2013)[4]
Blakeslea trispora	*β*-Carotene	15 mg/g of dry biomass (PG)	Mantzouridou et al. (2008)[24]
Clostridium butyricum VPI 1718	1,3-Propanediol (PDO)	14.2 g/L (PG[b])	Chatzifragkou et al. (2010)[22]
Clostridium pasteurianum ATCC 6013	PDO Butanol Ethanol Butyrate	0.17 (g/g) (PG) 0.28 (g/g) (PG) 0.04 (g/g) (PG) 0.04 (g/g) (PG)	Venkataramanan et al. (2011)[23]
Cupriavidus necator JMP 134	PHB	51.2% (PG) and 38.1% (CG[c])	Cavalheiro et al. (2009)[37]
Paracoccus denitrificans	poly(3-hydroxybutyrate) (PHB)	70% (PG)	Mothes et al. (2007)[38]
P. corrugata 388	3-hydroxyoctanoic acid, 3-hydroxydecanoic acid and 3- hydroxytetradecadic acid	39 mol%, 26 mol% and 15 mol% (CG)	Ashby et al. (2004)[19]
Schizochytrium limacinum SR21	Docosahexaenoic acid (DHA)	4 g/L (CG)	Yokochi et al. (1998)[47]
Yarrowia lipolytica *Yarrowia lipolytica* N15	Citric acid Citric acid	35 g/L 98 g/L (PG) and 71 g/L (CG)	Papanikolaou et al. (2002) Kamzolova et al. (2011)[48, 49]

[a] Productivity represents the percentage of PHB content in the dry cell weight (DCW), concentration of metabolic product produced, or mass produced compared to mass of consumed substrate. [b] PG represents pure glycerol. [c] CG represents crude glycerol.

As mentioned earlier, polyhydroxybutyrate (PHB) can be accumulated in the bacterial cells as an energy storage. Fig. 4 is a typical metabolic pathway that usually both prokaryotic and eukaryotic microorganisms take to produce several value-added products from glycerol such as PHB, 1,3-Propanediol, TAGs, succinic acid, butyric acid, acetic acid, citric acid, hydrogen, and ethanol. The red arrows show the pathway that the microorganisms could take to accumulate PHB.

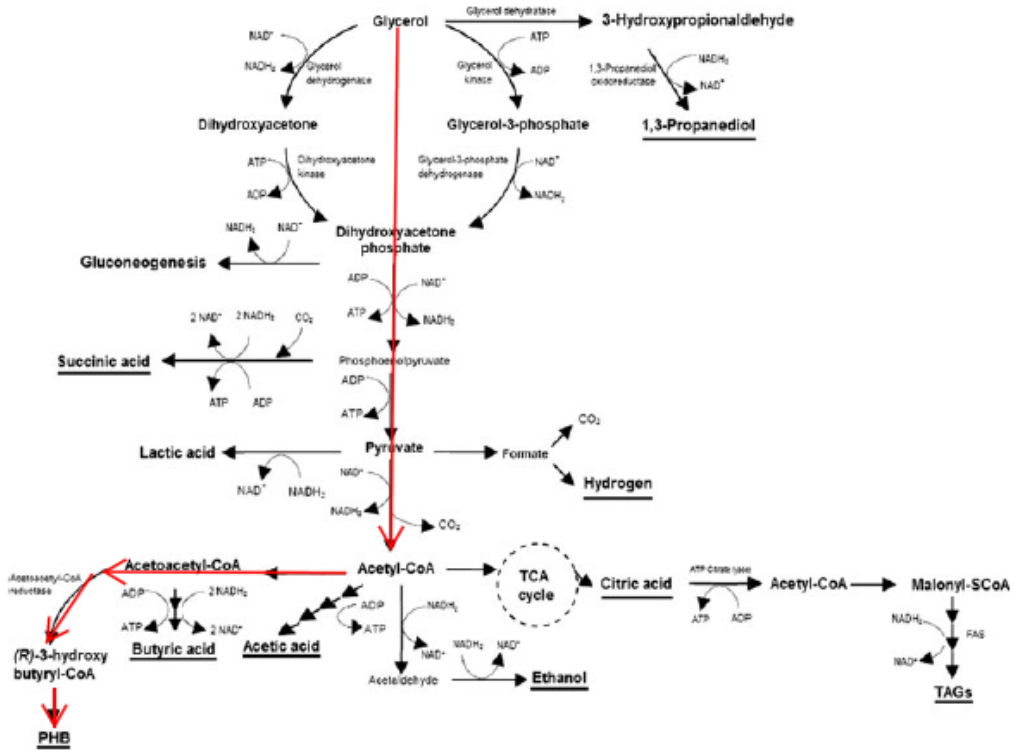


Fig. 4 Typical metabolic pathways for both prokaryotic and eukaryotic microorganisms to produce value-added products from glycerol. Adapted from Rivaldi et al.[50] and modified by Chatzifragkou and Papanikolaou.[12]

Microorganism strains

In this research, four salt-tolerant bacterial strains were chosen to produce PHB from glycerol: *Azohydromonas lata, P.* oleovorans, *Burkholderia* sp., and *Zobellella denitrificans* ZD1. All of these strains are gram-negative aerobic bacteria with only *Zobellella denitrificans* ZD1 has facultative anaerobic behavior. The reason for selecting these bacterial strains in this research is their high tolerance to inorganic salts like sodium chloride or potassium chloride that crude glycerol contains, 10% (w/w).[15, 17, 18] There are several research studies that discuss the ability of some of these strains like *P.* oleovorans and *Burkholderia* sp. to accumulate PHB by utilizing glycerol.[9, 17, 19, 20, 31, 34, 51] On the other hand, there is a lack of research regarding the utilization of glycerol to produce PHB by *Azohydromonas lata* and *Zobellella denitrificans* ZD1. Therefore, this research studied the capability of all these strains to grow on glycerol and accumulate PHB within their cells. It also tested the effect of fatty acids presence in glycerol at different concentrations on the bacterial growth, PHB production, and PHB yield.

Azohydromonas lata

Azohydromonas lata is a gram-negative aerobic bacteria isolated from soil samples collected from the Berkeley campus of the University of California.[51] The cells have short to coccoid rods shape with 1.10 to 1.40 by 1.60 to 2.40 μm in size. The colonies are usually rounded with grayish color. This strain showed growth associated PHB production using sucrose as a sole carbon source.[14, 31] However, a higher PHB content was observed under nitrogen-limitation conditions, 87% PHB/DCW compared to 50% PHB/DCW under nitrogen-sufficient conditions.[14] It also reduced nitrate to nitrite, but no complete

denitrification was observed. The same observation was reported by Ugwa et al.[15] when *Azohydromonas lata* was used to produce (R)-3-hydroxybutyric acid. The cells also accumulated PHB up to 42%.

Pseudomonas oleovorans

Pseudomonas oleovorans is a gram-negative aerobic bacteria isolated from mixed soil samples.[52] It is capable of degrading octane to octanoic acid by alkane monooxygenses process.[52, 53] The strain cells have a rod shape with yellow fluorescent pigment.[16] Several studies have been performed on this strain regarding the utilization of glycerol to produce PHB.[9, 19, 35] Ashby et al.[19] reported that *P. oleovorans* synthesized PHB up to 27% of the bacterial DCW from soy-based biodiesel production containing glycerol, fatty acids, and fatty acid methyl esters. The same group in 2011[9] also studied very specifically the fermentation of both pure glycerol and crude glycerol to accumulate PHB. An average yield of 0.80 to 1.20 g/L was observed after 48 hours.

Burkholderia sp.

Burkholderia sp. is another gram-negative aerobic bacteria isolated from oil polluted wastewater samples.[17] The cells have rod shape with 0.50 to 0.90 by 1.00 to 2.00 μm in size. The colonies are circular with a beige color. By using sucrose density gradient ultracentrifugation and GC analysis, *Burkholderia* sp. found to accumulate PHB and it was growth associated. The strain used several carbon substrates, 0.5% (v/v); however, a better utilization was observed with vegetable oils and glycerol. The strain accumulated PHB up to 70% of DCW under nitrogen-limitation condition.[17]

Zobellella denitrificans ZD1

Zobellella denitrificans ZD1 is also another gram-negative bacteria isolated from sediment samples collected from various mangrove ecosystems in Taiwan.[18] It is heterotrophic with facultative anaerobic behavior and has straight rods shape ranging from 1.60 to 2.60 by 0.6 to 0.80 μm in size. The colonies are circular with off-white color. A complete denitrification was observed for this strain. There are no studies have reported the utilization of glycerol by this strain, or a production of PHB, however there is a study performed by Ibrahim and Steinbuchel[20] reported a utilization of glycerol by *Zobellella denitrificans* MW1. The 16S rDNA sequence of *Zobellella denitrificans* MW1 had 98.5% similarity to *Zobellella denitrificans* ZD1. *Zobellella denitrificans* MW1 accumulated PHB while growing up to 80.4% of DCW when 20 g/L of glycerol was used as a sole carbon source.

Cost analysis

The production cost of PHB bioplastic is the most significant aspect for its adaptation by the plastic industry. The PHB production costs approximately 5-10 times higher than a regular petroleum-based polyester, which costs between 0.25-0.5 US$/Kg.[29, 54] The main producers of PHB in the world are Biotechnology Co., PHB Industrial S/A Company, and Mitsubishi GAS Chemical with a PHB production price of 3.75-6.25, 3.12-3.75, and 2.75 US$/Kg, respectively.[3, 55] In addition, the sale price of PHB fluctuates between $3.1-4.4 US$/Kg in recent years when glucose or sucrose are used as carbon substrates.[4] Therefore, there are three main factors that construct the PHB production cost. These factors are substrate selection, utilization process, and isolation and purification

process of PHB.[3, 4] Each factor consists of key elements contributing to the total PHB production cost such as selecting the right microorganism for utilization, providing suitable environmental conditions (pH, aeration, temperature, etc.), choosing a cheap carbon and nitrogen substrate, and selecting the cheapest PHB extraction and purification technique.[4, 11]

Selecting the carbon substrate is the main element in the PHB production cost that contributes to 25-45% of the total production cost.[56] Therefore, selecting the proper carbon substrate is very important to reduce the PHB sale price. Because of high biodiesel production in recent decades, the price of crude glycerol that is generated as a byproduct from the biodiesel industry has declined dramatically to 0.118-0.149 US$/Kg depending on the purity level of the crude glycerol.[3, 4] This caused the PHB production industry to focus on using this byproduct as a carbon substrate for bacterial utilization. In a study developed by Naranjo et al.[4], the production price of PHB decreased to 2.6 US$/Kg when crude glycerol was used instead of glucose or sucrose as a carbon substrate, leading to a 10-20% profit margin. Because of the purification process of crude glycerol, another cost element has been added to the total production cost. This cost element represents around 4.8-5.6% of total production cost of PHB.[3] In a study performed by Posada et al.[3], the total production cost of PHB ranged between 2.11-2.44 US$/Kg with 88% glycerol purity. However, they were able to decrease this price to 1.94-2.38 US$/Kg when crude glycerol was purified to 98% with a downstream process involving heat pretreatment, enzymatic-alkaline digestion, centrifugation, washing, evaporation, and spray drying. Specifically, using purified crude glycerol would increase the utilization efficiency by the

microorganisms, and therefore decrease the production price. However, crude glycerol might also add another cost element for purification.

Choi et al.[11] summarized the factors that affect the total PHB production price into several categories: PHB productivity, PHB content, PHB yield, price of raw materials, and recovery method. They used previous research studies to evaluate the effect of these factors and provide a table for comparison. PHB productivity is one of the main factors that affects the total production costs. The higher the PHB productivity, the lower production cost achieved because all direct-fixed-capital-dependent, labor-dependent, and utilities costs decreased. The most important factor mentioned in the paper is the PHB content in the cell. With higher PHB content, the required amount of digesting agents for PHB separation decreases; therefore, decreasing the cost of waste disposal. In addition, the equipment-related costs would decrease with high PHB content because a smaller amount of cells need to be produced to obtain the same amount of PHB. As mentioned earlier in this paper, carbon substrate plays an important role in determining the total PHB production price.

Choi et al. [11] also evaluated the PHB yield based on a carbon substrate. PHB yield would decrease the overuse of carbon substrate for utilization; therefore, decreasing the waste and production price. Other important factors that affect the total PHB production price are providing a suitable oxygen content, selecting quality nitrogen sources, and selecting efficient recovery methods for extraction and quantification of PHB. For instance, the recovery methods that involve dealing with toxic and expensive chemicals such as solvent extraction and hypochlorite digestion, enzymatic digestion, alkaline

digestion, or dispersion of chloroform and sodium hypochlorite solution would add extra efforts and costs in total PHB production price to eliminate their toxic impacts on the environment.

CHAPTER III

EXPERIMENTAL SECTION

Bacterial strains and culture conditions

Azohydromonas lata (JCM 20724), *Burkholderia* sp. (JCM 15050), and *Zobellella denitrificans* ZD1 (JCM 13380) were purchased from Riken BRC Microbe Division, Japan Collection of Microorganisms (JCM). *P. oleovorans* was purchased from America Type Culture Collection (ATCC 29347). The cells in minimal P1[57] medium with 5 g/L of glycerol and glycerol (10%) were stored at -80 °C. The P1 medium contained 7.57 mM ammonium and 0.5 g/L of NaCl. The pH of the medium was also adjusted to 7.3-7.5 by adding concentrated H_2SO_4 before autoclaving.

Strain activation and storage

Freeze-dried strains were revived by adding 0.3-0.5 mL of suitable rehydration fluid into the ampoule according to each strain specifications. The rehydration fluid with the cells was then spread on suitable agar plates and incubated under 30 °C for 1-2 days. After confirming the strains purity, the subculture of each strain was incubated in P1 liquid medium containing 5 g/L glycerol, 7.57 mM ammonium, and 0.5 g/L NaCl at 30 °C and 150 rpm. At exponential growth stage, 800 µL of the previously prepared liquid cultures were drawn and added to 200 µL of glycerol in a cryovials. The cryovial of each strain was then stored at -80 °C as stocks.

Chemicals

Pure glycerol (≥ 99 % pure) was obtained from Sigma-Aldrich, St. Louis, MO. Sodium salt of fatty acids (stearate, oleate, and linoleate) were used in all of the

experiments. All of these fatty acids contain the same number of carbon atoms, 18. Sodium stearate was purchased from Alfa Aesar Company, Ward Hill, MA. Sodium oleate (≥ 97% pure) and sodium linoleate (≥ 95% pure) were purchased from TCI America, Portland, OR. Pure PHB was purchased from Sigma-Aldrich, St. Louis, MO. All other chemicals used in this research were either obtained from Fisher Scientific, Fair Lawn, NJ or Sigma-Aldrich, St. Louis, MO.

Cell growth experiments

Cell growth experiments were conducted in 250-mL Erlenmeyer flasks containing 50 mL of P1 medium, 5 g/L of glycerol, 0.5 g/L of NaCl, and one of three fatty acids (0.5 or 1% weight of fatty acid/weight of glycerol). The fatty acids chosen for these experiments were stearate (18:0), oleate (18:1), and linoleate (18:2), due to their high presence in crude glycerol. All of these fatty acids contain the same number of carbon atoms, 18; however, they all vary in the number of double bonds between the carbons. The same number of carbon atoms ensured the same C/N ratio in all of the experiments; therefore, a clear comparison can be made between the results. The flasks were inoculated with pregrown cells. To prepare the pregrown cells, cells were activated from -80 °C stocks by streaking on Reasoner's 2A (R2A) agar plates. The R2A agar plates were incubated at 30 °C for 2-3 days and then re-streaked again in R2A agar plates to ensure purity. Individual colonies from the re-streaked R2A agar plates were picked to inoculate into 25 mL of P1 medium containing 5 g/L glycerol and 0.5 g/L NaCl under 30 °C and 150 rpm to ensure adaptation of those strains to use glycerol as a carbon source. The glycerol-pregrown cells (with optical density (OD) between 0.9-1.0 at 600 nm) were then

used as an inoculum (4% (v/v)) in 250-mL Erlenmeyer flasks containing 50 mL of P1 medium, glycerol (5 g/L), NaCl (0.5 g/L), and fatty acids (0.5 or 1%) to initiate cell growth experiments.

The inoculated flasks were incubated at 30 °C and 150 rpm and liquid samples were withdrawn from the flasks periodically for OD_{600} measurements using an Agilent 8453 UV-Visible Spectrophotometer. The liquid samples were also analyzed for the changes of glycerol concentrations based on chemical oxygen demand (COD) analysis. Liquid samples collected during stationary growth phase were used for PHB, pH, and nitrogen analysis.

The best performing strain, based on the highest PHB content and yield, was selected for further experiments. The additional experiments were designed to examine effects of nitrogen sources, electron acceptors, carbon over nitrogen (C/N) ratios, and pH on cell growth and PHB accumulation. The experiments were set up similarly as previously described, except for change of one of the parameters in nitrogen sources, electron acceptors, C/N ratios and pH. In nitrogen source effect experiments, two different nitrogen sources, $(NH_4)_2HPO_4$ and KNO_3, were used. The change was made by replacing the ammonium in the PI medium with nitrate (7.57 mM). Two different electron acceptors, O_2 and NO_3^-, were used. In electron acceptor effect experiment, oxygen was purged from the flasks and nitrate (7.57 mM) was added into P1 medium. For pH effect experiments, the initial pH values of the medium were adjusted to 6, 7, 8, and 9. Initial C/N ratio of 1, 4, 21.5, and 40 were used. All experiments were conducted in duplicate.

Extraction and quantification of PHB

The collected samples were centrifuged using a SORVALL LEGEND XTR centrifuge at 4,500 g for 10 minutes at 4 °C, the supernatant was discarded, and the pellets were washed twice with deionized water. The pellets were then dried in pre-weighted 2-mL centrifuge tubes using SAVANT DNA120 SpeedVac Concentrator for 8 hours with heating at 43 °C for 6 hours. The dry cell weights (DCW) of the pellets were determined by the weight difference before and after drying the pellets.

The PHB concentrations of the dry cell pellets were determined using spectrophotometric assay adapted from Law and Slepecky[58] with some modifications. Briefly, the PHB in the cells was converted to crotonic acid by heating the dried cells in a glass culture tube containing 1 mL of concentrated sulfuric acid. Glass culture tubes were used to prevent the formation of any plasticizers. The tubes were then heated at 70 °C for 4 hours using a HACH DRB200 Digital Reactor Block. After 4 hours of heating, 7 mL of 4 M sodium hydroxide was added to the tube to raise the pH and dilute the solution to a total volume of 8 mL. After the solution cooled down to room temperature, 1 mL sample was withdrawn and centrifuged using Fisher Scientific accuSpin Micro R at 13,000 rpm for 5 minutes at 4 °C. Only 500 µL of the top layer supernatant was used for spectrophotometric assay. The 500 µL supernatant was diluted with 1500 µL DI water to bring the absorbance measurement to the calibration range. The crotonic acid was determined by absorbance at wavelength of 235 nm. The PHB concentration was determined by comparing the absorbance to a standard curve developed using standard

solutions containing known amounts of PHB, ranging from 0.9 to 10 mg (see supporting information, Fig. S1).

Chemical analysis

The concentration of glycerol in the growth medium were determined using chemical oxygen demand (COD) analysis. Mercury-free COD reagent vials (CHEMetrics Inc., Calverton, VA) were used to measure the COD with a detection limit of 100 mg/L. Glycerol concentration was converted using a factor of 1 g/L glycerol equal to 1.22 COD g/L. Briefly, liquid samples were filtered, diluted, before adding into the COD vials, and the vials were heated for 2 hours at 150 °C using a HACH DRB200 Digital Reactor Block. The absorbance of digested samples were measured at 620 nm using an Agilent 8453 UV-Visible Spectrophotometer. The COD concentrations of samples were determined using a standard COD curve ranging from 0 mg/L to 1,000 mg/L (see supporting information, Fig. S2).

Ammonia-nitrogen (NH_3-N) concentrations of the samples were measured using phenate method with a detection limit for ammonia of 0.01 mg/L.[59, 60] In this method, ammonia in the samples reacts with hypochlorite and phenol to form a blue compound, indophenol, which was then measured using an Agilent 8453 UV-Visible Spectrophotometer at 640 nm. The NH3-N concentrations were determined using a standard curve ranging from 0.01 mg/L to 5 mg/L (see supporting information, Fig. S3).

CHAPTER IV

RESULTS AND DISCUSSION

Strains capable of growing on glycerol containing salts

To assess the ability of these strains to grow on crude glycerol for PHB production, these salt-tolerant strains were grown on P1 medium agar plates containing 2.5, 5, 10, or 20 g/L of glycerol in the presence or absence of 0.5 g/L of NaCl. All the strains were able to form colonies on the plates. However, *A. lata* showed no sign of growth with glycerol in liquid P1 medium regardless of salt (Fig. 5). Therefore, this strain was not selected for further experiments in this study.

On the other hand, the other three strains (*P. oleovorans*, *Burkholderia* sp., and *Z. denitrificans* ZD1) were able to grow on 5 g/L of glycerol as a sole carbon substrate in P1 medium with 0.5 g/L of NaCl (Fig. 5). As shown in Fig. 5, these three strains grew at different rates to reach different highest optical density (OD): OD = 3.3 for *P. oleovorans*, OD = 6.0 for *Burkholderia* sp., and OD = 2.5 for *Z. denitrificans* ZD1. The time for *Z. denitrificans* ZD1 reached to the stationary growth phase was about 24 hours earlier than that for *P. oleovorans*, and about 16 hours earlier than *Burkholderia* sp. Reaching the stationary growth phase quicker means a shorter utilization period; however, it does not correlate with high cell concentrations or PHB productivity. *Z. denitrificans* ZD1 had the highest PHB percentage of their DCW, 84% (w/w), followed by *Burkholderia* sp. and *P. oleovorans* with 45% and 38%, respectively.

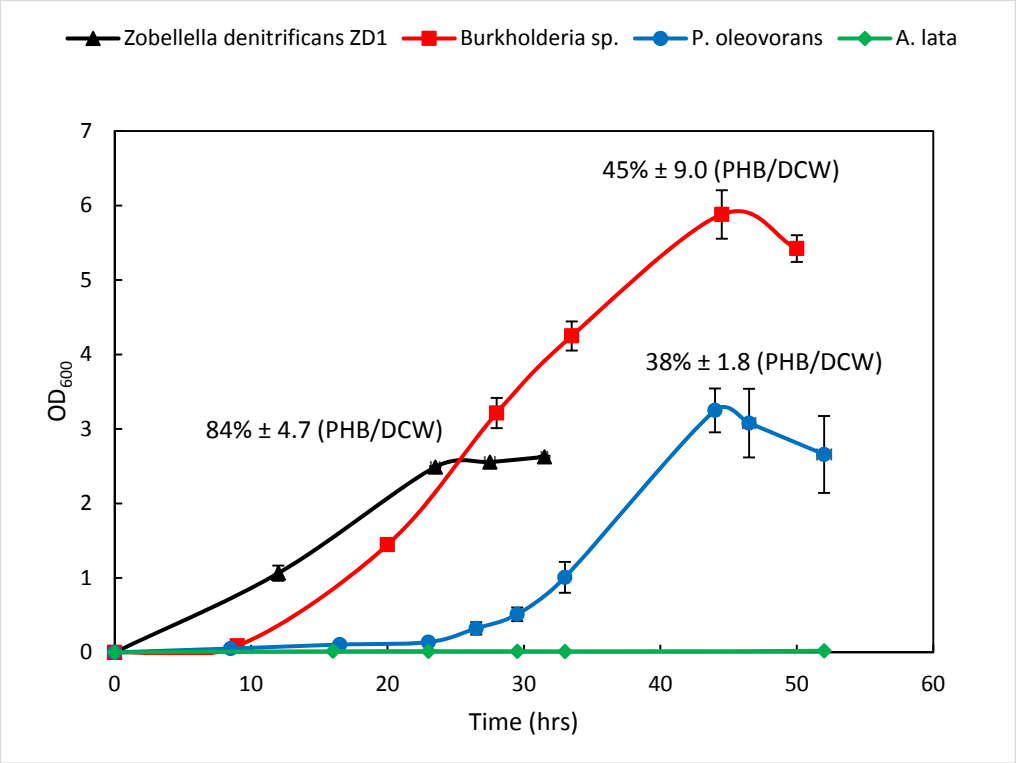
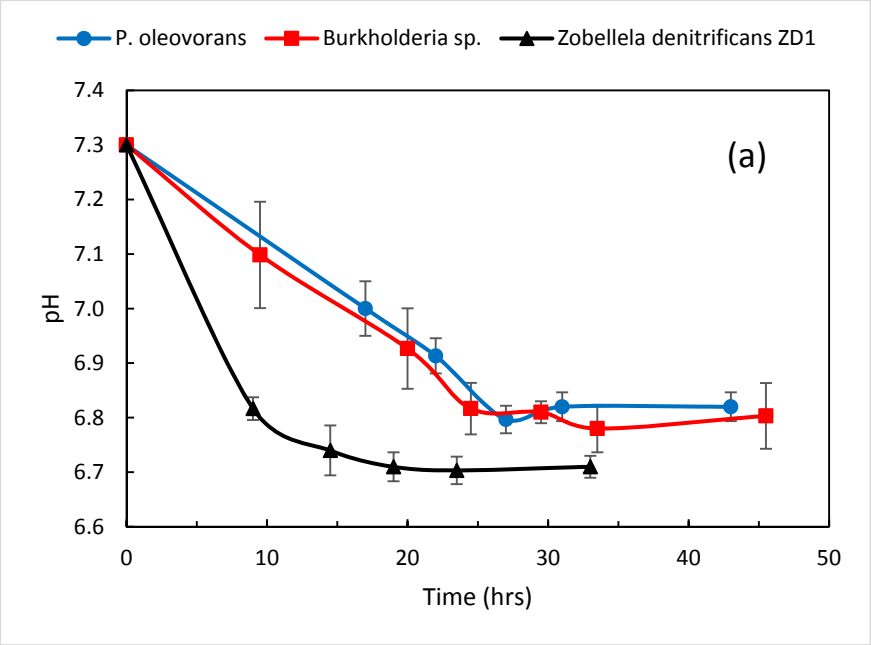


Fig. 5 Growth curves of *Azohydromonas lata*, *Burkholderia* sp., *Pseudomonas oleovorans*, and *Zobellella denitrificans* ZD1 in batch reactors containing P1 medium with 5 g/L of pure glycerol as a sole carbon source and 0.5 g/L of NaCl. The percent of PHB content in their DCW after reaching the stationary growth. Error bars represent standard deviation of triplicate measurements.

The changes of pH, glycerol, and nitrogen during the growth of these three strains in glycerol (5 g/L) and NaCl (0.5 g/L) were also monitored overtime (Fig. 6a-6c). Initial pH decreased overtime as cell grew. For *Z. denitrificans* ZD1, the pH in the medium decreased from 7.3 to 6.7 in 19 hours the time for the cell entering the stationary growth phase. Similarly, pH decreased from 7.3 to approximately 6.8 for *P. oleovorans* and *Burkholderia* sp., but the process took about 30 hours (Fig. 6a).

Fig. 6b shows that *P. oleovorans* consumed highest amount of added glycerol (79%), followed by *Burkholderia* sp. (72%), and then by *Z. denitrificans* ZD1 (70%). Noted that the remaining glycerol for each strain was similar, in a range of 1.1-1.5 g/L. However, *Z. denitrificans* ZD1 consumed glycerol at a faster pace than those by *P. oleovorans* and *Burkholderia* sp. As expected, with a C/N ration of 21, the NH_3-N concentration in the growth medium was almost depleted from 0.11 g/L to zero before cells reached stationary growth (Fig. 6c). The profiles of pH, the remaining glycerol and nitrogen source suggested that each strain reached the stationary growth phase due to the total depletion of nitrogen.

The growth results (Figs. 5 and 6) suggested that *Z. denitrificans* ZD1 was the most efficient strain than other two strains to utilize glycerol as a growth substrate for PHB production. It also had the most glycerol remained available for utilization; therefore, the strain would continue to grow if sufficient amount of nitrogen was supplied. Thus, *Z. denitrificans* ZD1 was selected more detailed experiments to determine optical growth conditions and PHB yields.



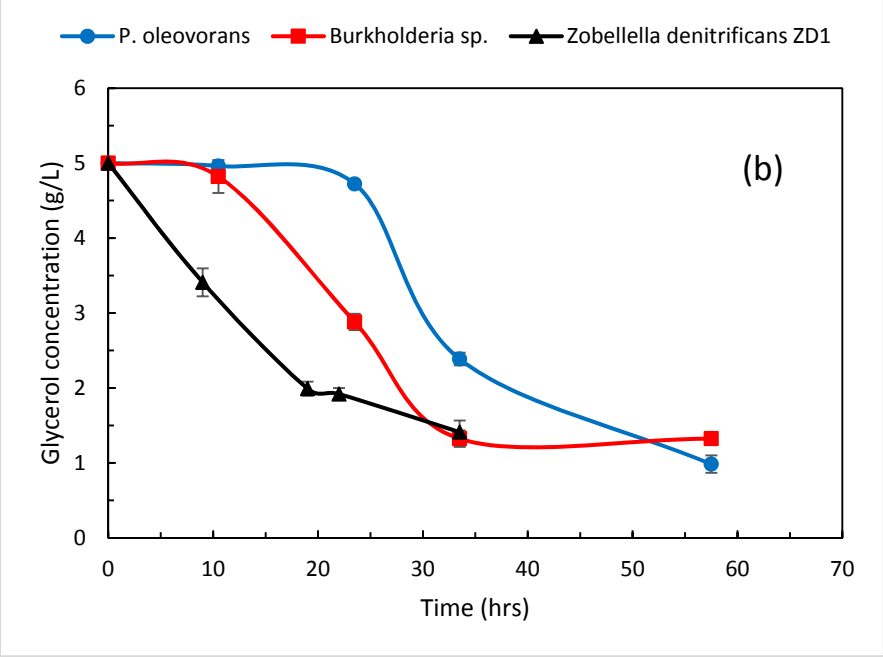


Fig. 6 pH profile (a), glycerol profile (b), and ammonia-nitrogen profile (c) for each strain growing in P1 medium containing initially 5 g/L of glycerol as a sole carbon source, 7.57 mM of ammonium, and 0.5 g/L of NaCl. Error bars represent standard deviation of duplicate measurements.

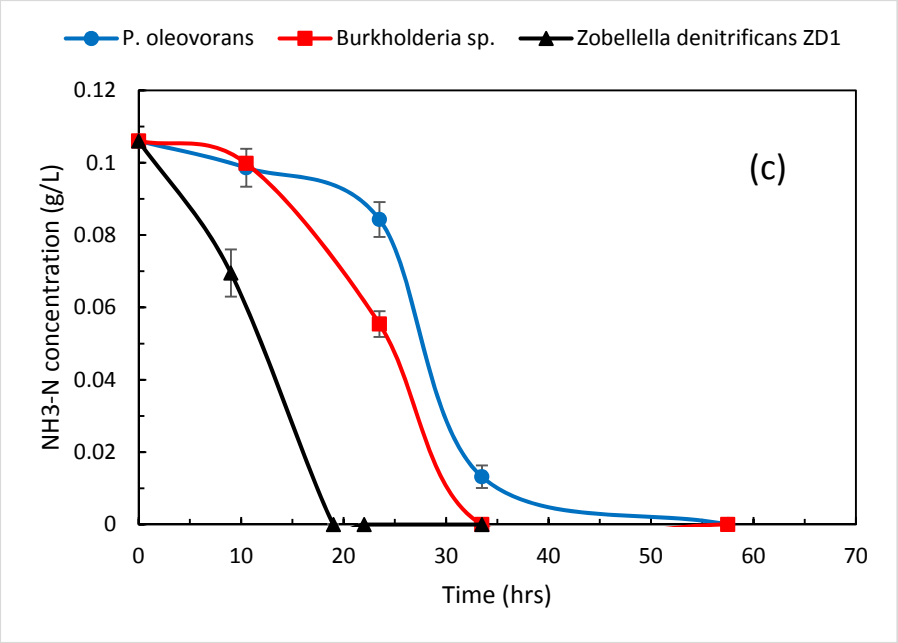


Fig. 6 Continued

While *Burkholderia* sp. was able to reach a higher DCW (OD_{600} = 6.0) compared to that of *Z. denitrificans* ZD1 (OD_{600} = 2.5), both *Z. denitrificans* ZD1 and *Burkholderia* sp. produced the highest PHB yield, around 0.24 g-PHB/g-glycerol (Fig. 7). This indicates that high cell concentration in the solution does not correlate to high PHB concentration or yield. Likewise, high cell concentration in the solution does not correlate to high PHB content. In other words, there might be more cells in the solution for one strain, but its PHB content could be lower than the other strain. As a result, *Z. denitrificans* ZD1 had the highest PHB content, 84% after only 24 hours compared to 45% PHB by *Burkholderia* sp. after 36 hours. This indicates that *Z. denitrificans* ZD1 could be a promising strain for

PHB production of glycerol if a high amount of cell concentration is achieved in the reactor.

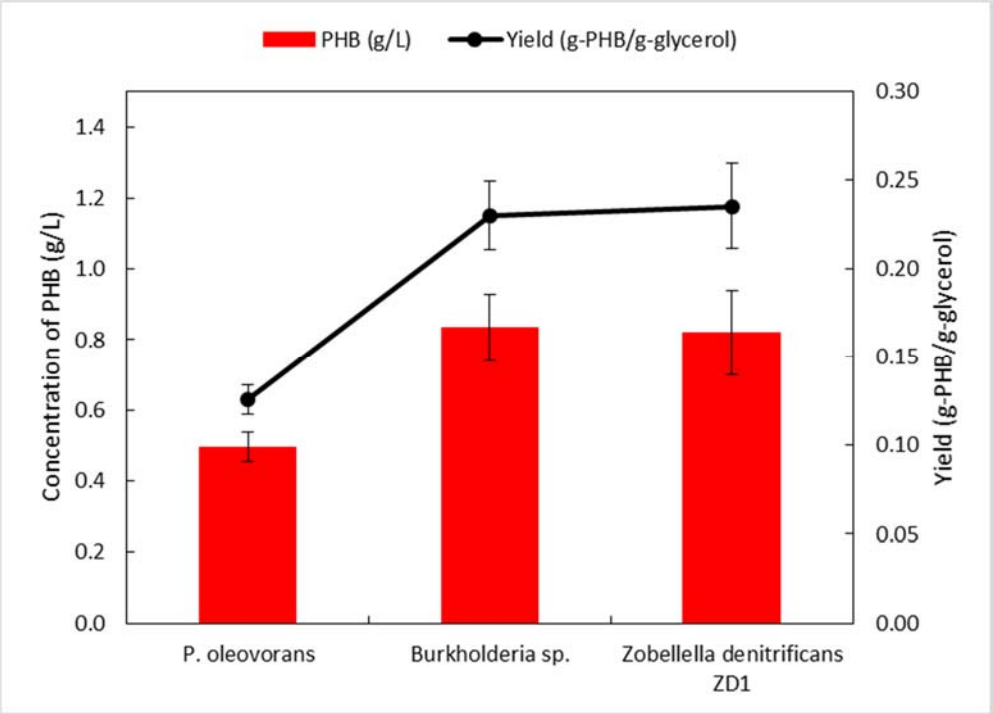


Fig. 7 PHB concentrations and PHB yields after reaching the stationary growth phase by each strain in P1 medium containing initially 5 g/L of glycerol (G) as a sole carbon source and 0.5 g/L of NaCl. Error bars represent standard deviation of triplicate measurements.

Both *P. oleovorans* and *Burkholderia* sp. were already know to use glycerol to accumulate PHB within their cells (Table 4). *P. oleovorans* accumulated the lowest PHB content in this study, 38%, which is slightly higher than the PHB content reported previously (Table 4). Ashby et al.[19] used soy-based biodiesel production (CSBP)

containing glycerol, fatty acids, and fatty acids methyl esters for fermentation by *Pseudomonas oleovorans* NRRL B-14682. The dry cells contained 13-27% of PHB. The higher the concentration of CSBP, the higher the production of PHB. The same group in 2011[9] also demonstrated the ability of *P. oleovorans* NRRL B-14682 to utilize both pure and crude glycerol containing methanol, fatty acids methyl esters, and water to produce PHB. A higher advantage for PHB accumulation was noticed with pure glycerol, i.e., 38% of its DCW after 72 hours, which is similar to that produced by *P. oleovorans* (ATCC 29347) in this research, even with the addition of 0.5 g/L of NaCl.

Burkholderia sp. accumulated about 45% of PHB per DCW, which is similar to that reported by Chee et al.[17] (Table 4). By using 0.5% (v/v) of pure glycerol, this strain was able to accumulate PHB as high as 54-60% of its DCW. When grown in crude glycerol containing impurities such as free fatty acids, methanol, and spent catalyst, *Burkholderia* sp. accumulated less PHB, i.e., 31% of its DCW after 70 hours.[17]

Z. denitrificans ZD1 accumulated the highest PHB content, 84% of its DCW. This high PHB accumulation ability is similar to that reported by that of another strain, *Zobellella denitrificans* MW1 (Table 4). *Z. denitrificans* MW1[20], similar strain to *Z.* denitrificans ZD1, was able to accumulate PHB up to 80.4% of its DCW when pure glycerol used as a sole carbon source. While *Z. denitrificans* MW1 can grow on glycerol concentration as high as 20 g/L, the strain produced lower PHB content (80.4%) when compared to that grown on 5 g/L glycerol only. Interestingly, when 20 g/L of NaCl was added to glycerol, *Z. denitrificans* MW1 accumulated higher PHB (i.e., 87% PHB per DCW), which is slightly higher than that of *Z. denitrificans* ZD1 observed in this study.

Table 4. List of bacterial strains reported in the literature and used in this research that are able to utilize glycerol to accumulate PHB

Bacterial strain	Carbon substrate	Impurities present/added[a]	%PHB/DCW	DCW concentration (g/L)	PHB yield (g/g)[b]	Scale	Utilization period (hrs)	Temperature (°C)	pH	Reference
Bacillus megaterium	Pure glycerol	-	62.4	7.7	0.3	Batch	42	33	Controlled at 7	Naranjo et al. (2013)[4]
Burkholderia sp. (JCM15050)	Pure glycerol		54-60 ± 4	2.0-2.5 ± 0.4	N.A.	Batch	72	37	N.A.[c]	Chee et al. (2010)[17]
	Crude glycerol	Wastewater, spent catalyst, salts, fat soaps, free fatty acids, and methanol	31 ± 3	1.9 ± 0.4	N.A.	Batch	72	37	N.A.	
Burkholderia sp. (JCM 15050)	Pure glycerol	NaCl	45 ± 9.0 (only NaCl)	1.89 ± 0.15 (only NaCl)	0.23 ± 0.03 (only NaCl)	Batch	36	30	pH_i=7.3 pH_f=6.8	This study
Cupriavidus necator JMP 134	Pure glycerol	Salts (NaCl or K_2SO_4) (3 g/L)	70 (no salt) and 48 (NaCl salt)	22-25	0.37 (no NaCl) 0.14 (NaCl)	Fed-batch	N.A.	35	N.A.	Mothes et al. (2007)
Cupriavidus necator DSM 545	Pure glycerol		62	82.5	0.36	Fed-batch	33.5	34	Controlled at 6.8	Cavalheiro et al. (2009)[37, 38]
	Crude glycerol	3% Na^+, 0.2% MeOH, and 1.5% (w/w) MONGs[d]	50	76.2	0.34	Fed-batch	33.5	34		

Table 4. Continued

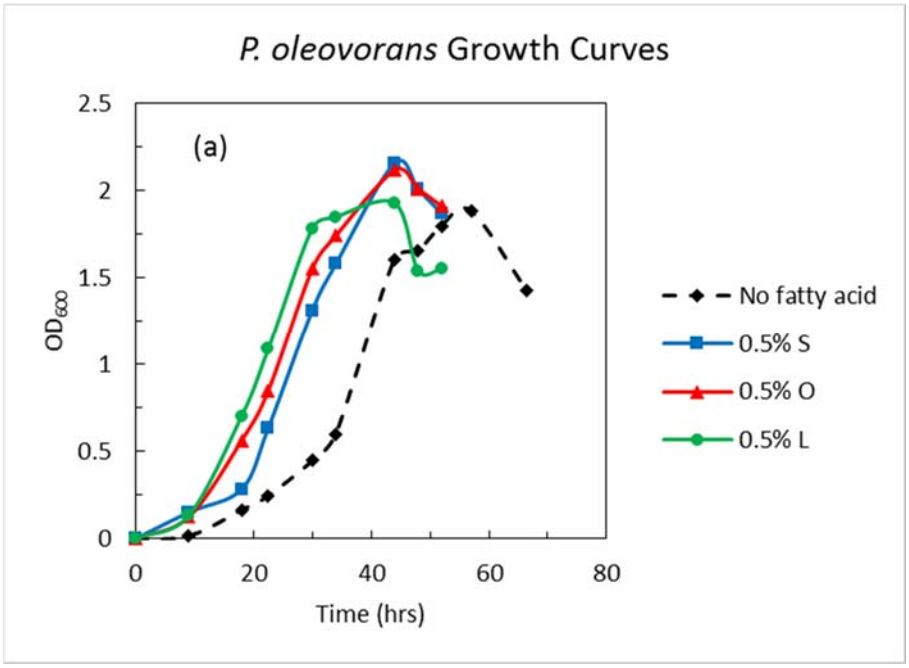
Bacterial strain	Carbon substrate	Impurities present/added[a]	%PHB/DCW	DCW concentration (g/L)	PHB yield (g/g)[b]	Scale	Utilization period (hrs)	Temperature (°C)	pH	Reference
Paracoccus denitrificans	Pure glycerol	Salts (NaCl or K_2SO_4) (3 g/L)	65 (no salt) 48 (NaCl salt)	N.A.	N.A.	Fed-Batch	N.A.	35	N.A.	Mothes et al. (2007) [38]
Pseudomonas oleovorans NRRL B-14682	Pure glycerol Crude glycerol	Methanol, fatty acids, and MONGs	38 13-27	3.0 2.54	N.A. N.A.	Batch Batch	72 72	30 30	N.A. N.A.	Ashby et al. (2011) [9]
Pseudomonas oleovorans ATCC 29347	Pure glycerol	NaCl	38 ± 1.8 (only NaCl)	1.33 ± 0.14 (only NaCl)	0.13 ± 0.01 (only NaCl)	Batch	46	30	pH_i=7.3 pH_f=6.8	This study
Zobellella denitrificans MW1	Pure glycerol	Salt (20 g/L)	80.4 ± 3.0 (no salt) 87 ± 3.7 (salt)	3.6 ± 0.1 (no salt) 4.78 ± 0.6 (salt)	0.22 (no salt) 0.37 (salt)	Batch	96	41	pH_i=7 pH_f=6.02 (no salt) pH_f=4.80 (salt)	Ibrahim et al. (2009) [20]
Zobellella denitrificans ZD1	Pure glycerol	NaCl	84 ± 4.7 (only NaCl)	0.98 ± 0.15 (only NaCl)	0.24 ± 0.03 (only NaCl)	Batch	24	30	pH_i=7.3 pH_f=6.7	This study

[a] Types of impurities are either added to pure glycerol or already presented in crude glycerol. [b] PHB yields were calculated based on gram of PHB produced per gram of glycerol consumed. [c] N.A. represents information is not available information. [d] MONGs = Material organic not glycerol and is composed of unrecovered alkyl esters and unreacted free fatty acids, tri-, di-, and monoacylglycerols.

Effect of fatty acids on glycerol-grown cells and their PHB production

The effects of saturated and unsaturated fatty acids on the bacterial growth and PHB production were examined using P1 medium containing 5 g/L of glycerol, 0.5 g/L of NaCl, and fatty acids (0.5 and 1%). The additional of 0.5% fatty acids to 5 g/L of glycerol excreted different effects on the bacterial growth for these three strains (Fig. 8). The addition of fatty acids slightly improved the growth of *P. oleovorans* (Fig. 8a). There was no significant difference in the growth of *Burkholderia* sp. Even though there was no large effect of 0.5% fatty acids on *Z. denitrificans* ZD1, the optical densities of bacterial growth curves slightly decreased (Fig. 8c). However, the impact of each individual 0.5% fatty acid on the bacterial growth curves and PHB production could not be clearly observed. Therefore, higher fatty acids concentration in glycerol was used.

Interestingly, with 1% fatty acids added, the impacts of the types of fatty acids on the growth profiles and utilization periods of glycerol by *P. oleovorans* and *Z. denitrificans* ZD1 were very different (Fig. 9a and 9c). The optical densities of *P. oleovorans* increased slightly in the presence of all three fatty acids, particularly for oleate and linoleate (Fig. 9a). For *Burkholderia* sp., once again, no effects were observed regardless of the types of fatty acids. The strain reached the stationary growth phase in all cases at the same time, 40 hours (Fig. 9b). Different from *P. oleovorans*, *Z. denitrificans* ZD1 was negatively affected by the presence of 1% fatty acids in glycerol (Fig. 9c). The utilization period stretched longer when 1% fatty acids presented in the solution, stretching from 24 hours to approximately 42 hours with the presence of 1% linoleate in glycerol; linoleate had the highest impact, followed by oleate, and then stearate.



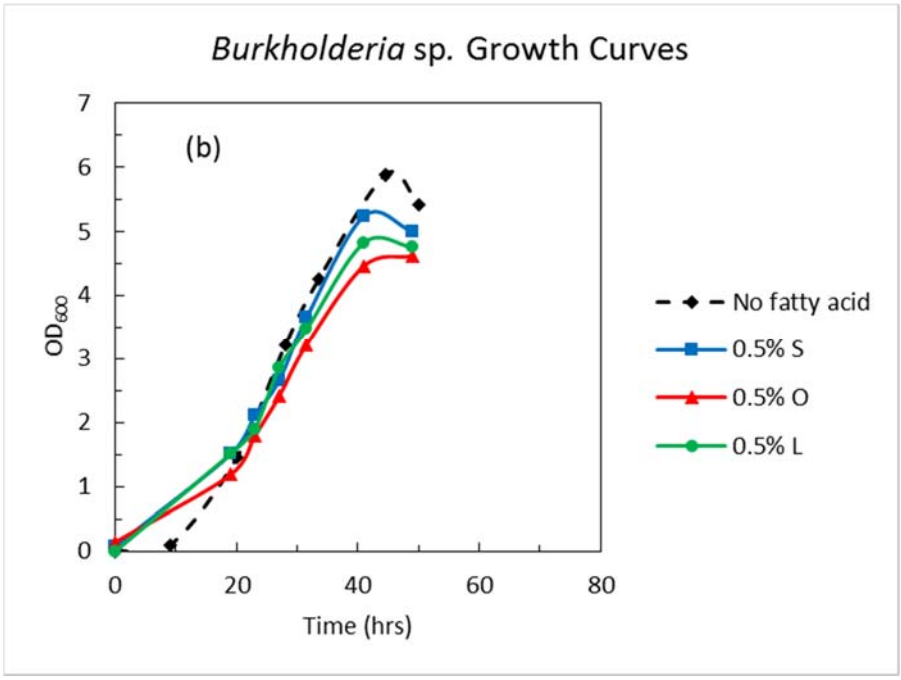


Fig. 8 Effects of 0.5% (w/w) fatty acids on bacterial growth of *P. oleovorans* (a), *Burkholderia* sp. (b), and *Z. denitrificans* ZD1 (c) in P1 medium containing 5 g/L of glycerol and 0.5 g/L of NaCl. Symbols: stearate (S), oleate (O), linoleate (L). The growth curves are based on average values (information for error bars are available in Fig. S4 in supporting materials).

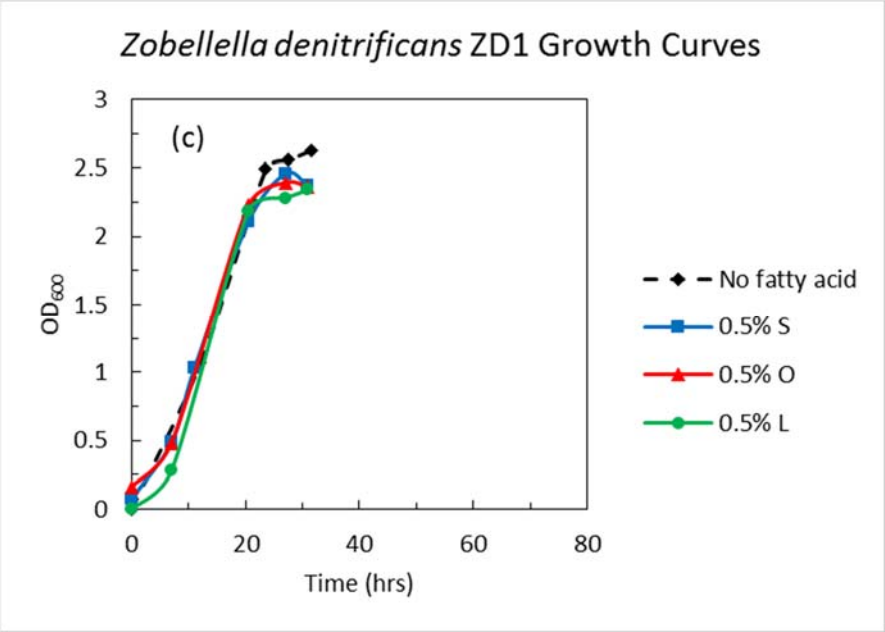


Fig. 8 Continued

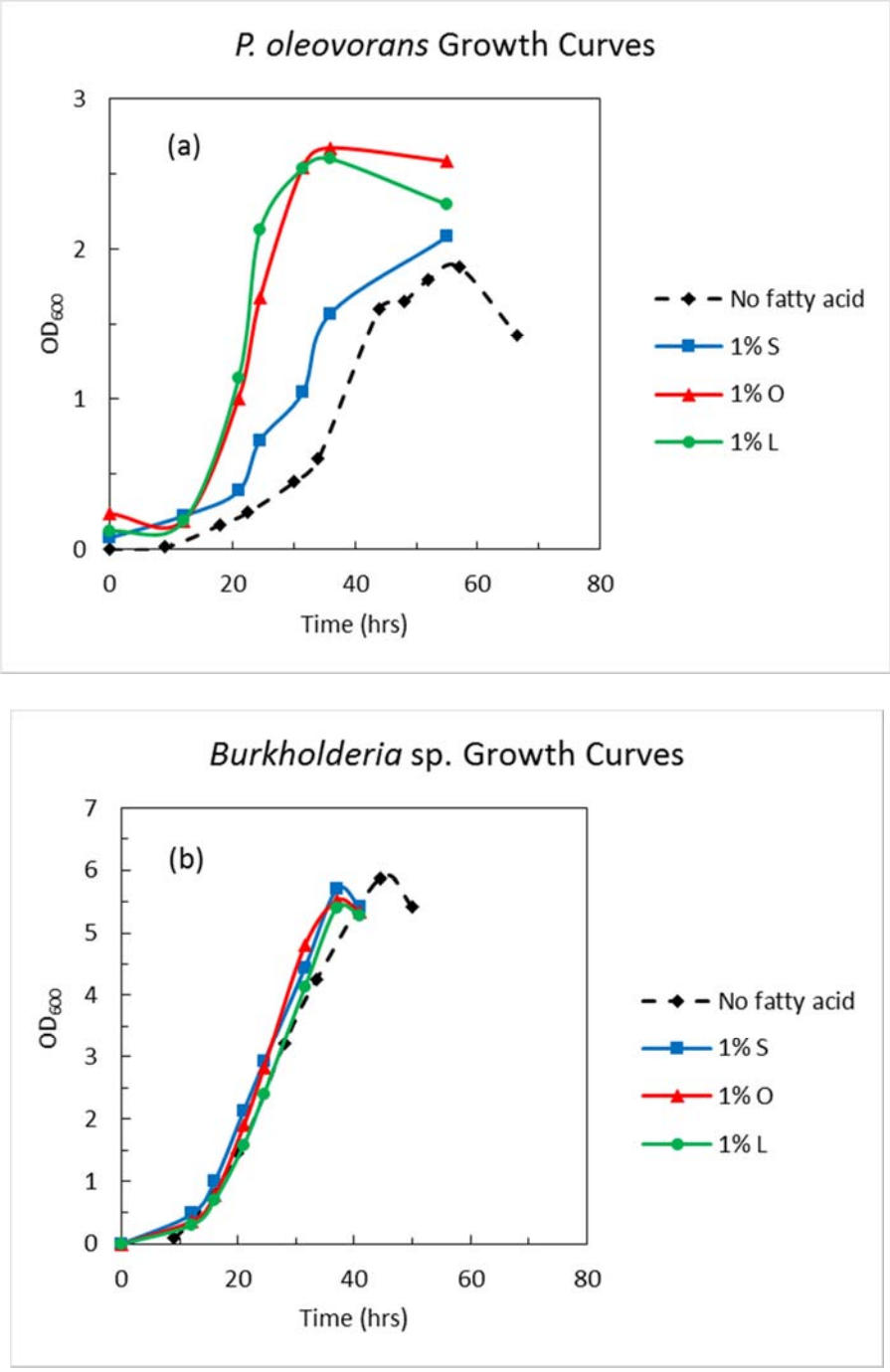


Fig. 9 Effects of 1% (w/w) fatty acids on bacterial growth of *P. oleovorans* (a), *Burkholderia* sp. (b), and *Z. denitrificans* ZD1 (c) in P1 medium containing 5 g/L of glycerol and 0.5 g/L of NaCl. Symbols: stearate (S), oleate (O), linoleate (L). The growth curves are based on average values (information for error bars are available in Fig. S5 in supporting materials).

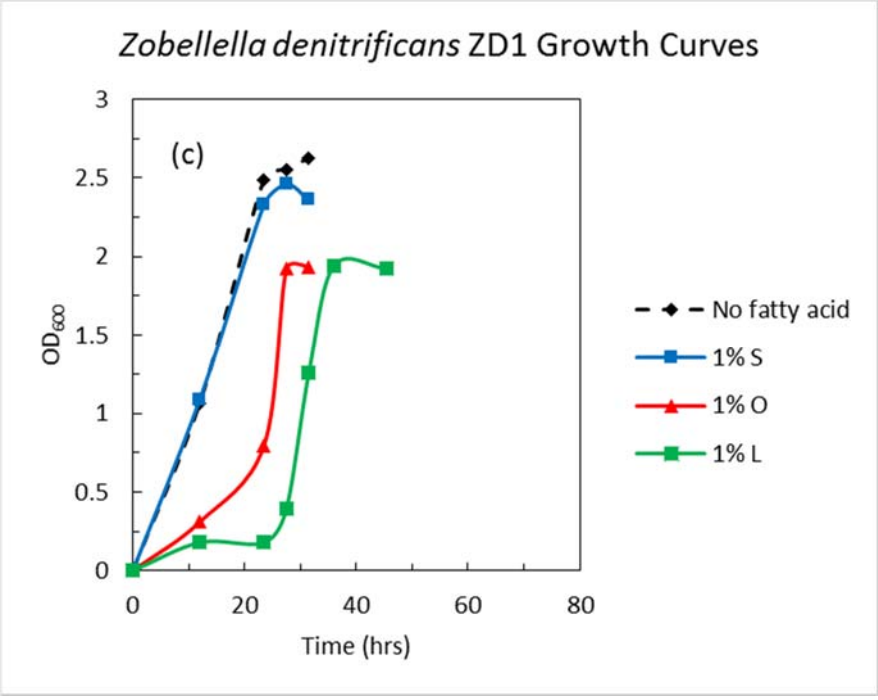


Fig. 9 Continued

The impact of fatty acid on the %PHB/DCW and total PHB yield were more noticeable in samples spiked with 1% (Table 5 and Fig. 10a-c) than 0.5% fatty acid (Table S1 in the supporting information). For *P. oleovorans*, the PHB content in DCW slightly increased from 38% to 41% with the addition of 1% fatty acids (Table 5). When 1% oleate was added to pure glycerol, DCW concentration increased from 1.33 g/L to 1.55 g/L (Table 5). Similarly, PHB concentration and PHB yield with the 1% addition of oleate increased from 0.50 g/L to 0.62 g/L and from 0.13 (g-PHB/g-glycerol) to 0.16 (g-PHB/g-glycerol), respectively (Fig. 10a). According to Ashby et al.[19], *P. oleovorans* was capable of utilizing soy-based biodiesel containing glycerol, fatty acids, and residual methyl esters with a favorable utilization of glycerol to accumulate an amount of PHB up to 27% of its

DCW. Interestingly enough, they noticed a decrease in oleic and linoleic acid concentrations in the culture supernatant, suggesting that *P. oleovorans* might be able to utilize both fatty acids. This observation can be explained by the ability of *P. oleovorans* to break oily molecules, especially glycerides and fatty acids.[9, 19] Accordingly, *P. oleovorans* showed enhanced growth by breaking the long fatty acid chain at the double bonds into shorter chains. This observation might be especially true with oleate because it has a double bond right at the middle of the molecule, providing similar characteristics to octane, a primary carbon source for *P. oleovorans*.[61]

For *Burkholderia* sp., there was no impact of the addition of 0.5% or 1% fatty acids in glycerol on the DCW concentrations, PHB concentrations, and PHB yields (Table 5, Fig. 10b, and Table S1). On the other hand, *Z. denitrificans* ZD1 was clearly affected by the addition of 1% fatty acids (Table 5 and Fig. 10c). The PHB content in the DCW decreased from 84% to 71% when 1% oleate or linoleate was added (Table 5). Moreover, PHB concentrations decreased dramatically from 0.82 g/L to 0.49 g/L with the addition of 1% linoleate to glycerol (Fig. 10c). As a result, the PHB yield clearly decreased by 41% with 1% linoleate added to the medium compared to only pure glycerol (Fig. 10c). This decrease might be due to the number of double bonds between the carbons. A higher number of double bonds caused a higher level of growth inhibition by expanding the growth utilization period to 42 hours and decreasing both cell and PHB concentrations. The double bond in oleate and linoleate introduces a kink in the molecule that could stop the diffusion of nutrients and metabolites through the cell membrane.[23, 62, 63] Venkataramanan et al.[23] reported related results regarding the effect of fatty acids in

glycerol fermentation. They reported that a higher degree of unsaturation (double bond) caused a stronger inhibitory effect on the glycerol utilization by *Clostridium pasteurianum* ATCC 6013. Likewise, Chatzifragkou et al.[22] found that the reason behind the inhibition of *Clostridium butyricum* VPI 1718 in glycerol was the double bond in oleic acid compared to a normal growth with the presence of stearic acid.

Although it is true that the PHB content for *Z. denitrificans* ZD1 decreased to almost 71% with the addition of 1% fatty acids, it was still higher than the PHB contents for the other strains (Table 5). Also, the strain still had shorter utilization periods compared to the other strains. It reached the stationary growth phase in 24 hours even after the addition of 1% fatty acids to glycerol (Fig. 9c). The only exception was with the addition of 1% linoleate, which caused the strain to take longer time, 40 hours, to reach the stationary growth phase (Fig. 9c). The only issue with *Z. denitrificans* ZD1 is the failure to reach high cell and PHB concentrations compared to the other strains (Table 5). However, this problem could be tackled easily in real life applications. Controlling the growth parameters such as pH, temperature, dissolved oxygen, glycerol concentration, and nitrogen concentration would increase the growth and PHB concentration.

Table 5. Effect of 1% fatty acids presence in glycerol on the bacterial growth and PHB accumulation

Bacterial Strain	No fatty acid			1% S			1% O			1% L		
	DCW (g/L)	**%PHB**	**Yield[a]**	**DCW (g/L)**	**%PHB**	**Yield[a]**	**DCW (g/L)**	**%PHB**	**Yield[a]**	**DCW (g/L)**	**%PHB**	**Yield[a]**
P. oleovorans	1.33 ± 0.14	38 ± 1.8	0.13 ± 0.01	1.37 ± 0.03	41 ± 1.6	0.14 ± 0.01	1.55 ± 0.06	40 ± 4.3	0.16 ± 0.02	1.33 ± 0.18	38 ± 2.1	0.13 ± 0.02
Burkholderia sp.	1.89 ± 0.15	45 ± 9.0	0.23 ± 0.03	1.90 ± 0.20	46 ± 6.5	0.24 ± 0.01	1.97 ± 0.25	45 ± 7.6	0.24 ± 0.01	1.91 ± 0.26	47 ± 13	0.24 ± 0.04
Z. denitrificans ZD1	0.98 ± 0.15	84 ± 4.7	0.24 ± 0.03	0.83 ± 0.07	80 ± 4.5	0.19 ± 0.02	0.70 ± 0.03	71 ± 3.0	0.14 ± 0.01	0.68 ± 0.03	71 ± 6.9	0.14 ± 0.02

All strains were grown in P1 medium containing initially 5 g/L of glycerol (G) and 0.5 g/L of NaCl. Addition of 1% (w/w) of each type of fatty acids such as stearate (S), oleate (O), linoleate (L) to glycerol (G) used as an additional impurity to 0.5 g/L of NaCl salt. Error represent standard deviation of triplicate measurements.

[a] PHB yields were calculated based on gram of PHB produced per gram of glycerol consumed.

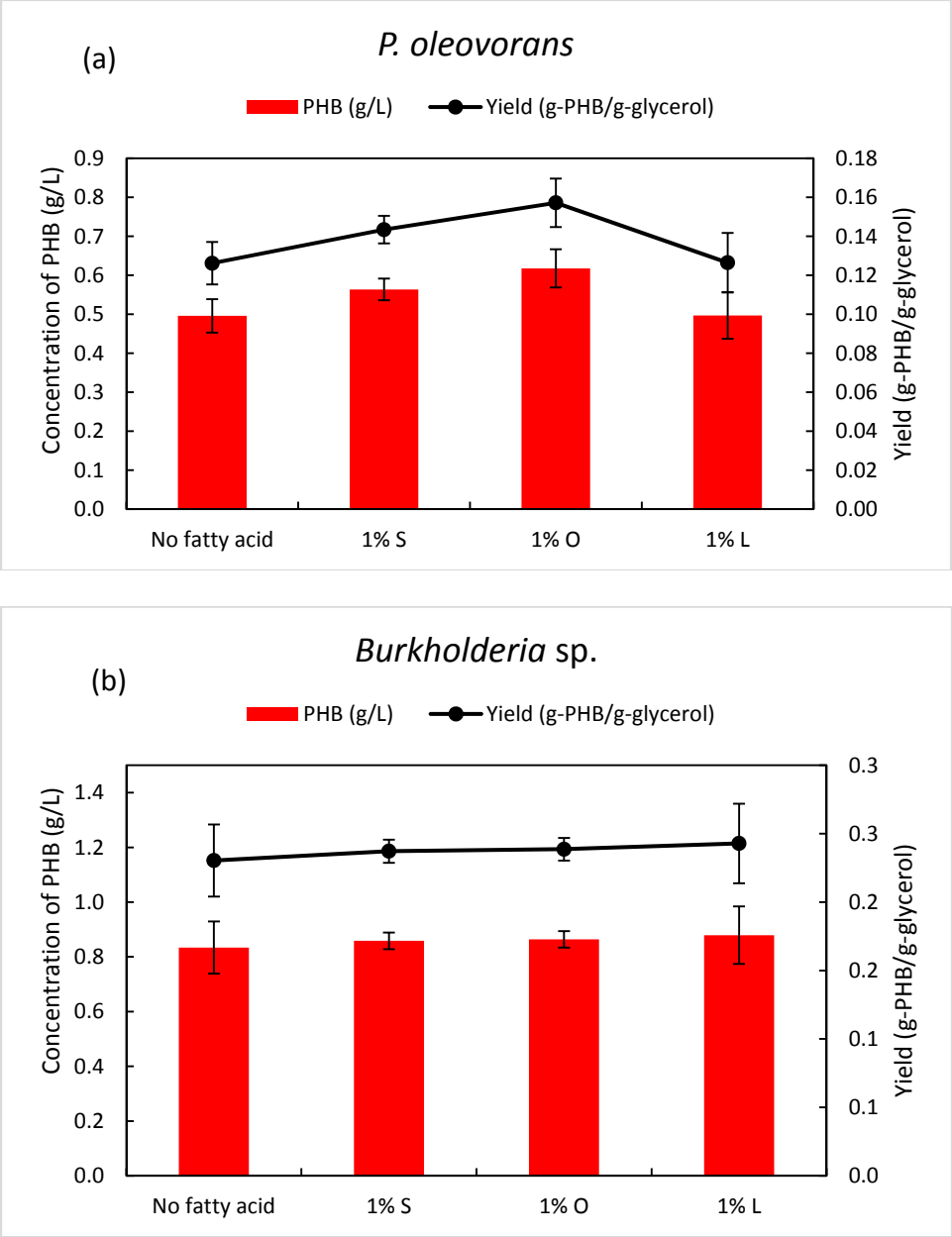


Fig. 10 PHB concentrations and yields for *P. oleovorans* (a), *Burkholderia* sp. (b), and *Z. denitrificans* ZD1 (c) grown in P1 medium containing 5 g/L of glycerol (G), 0.5 g/L of NaCl, and 1% fatty acids such as stearate (S), oleate (O), and linoleate (L) until reaching the stationary growth phase. Error bars represent standard deviation of triplicate measurements.

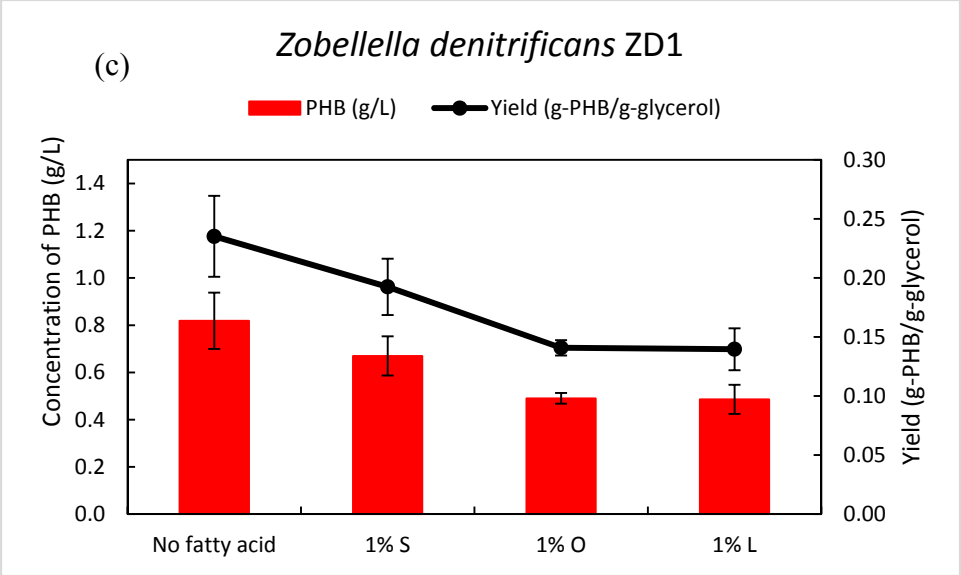


Fig. 10 Continued

Optimization of PHB production by Z. denitrificans ZD1

Based on growth and PHB accumulation, *Z. denitrificans* ZD1 appears as the best candidate to produce PHB by using glycerol. The strain reached the stationary growth phase faster than the other strains even with the addition of fatty acids; therefore, increasing the daily PHB production and decreasing the PHB production price associated with the utilization period. *Z. denitrificans* ZD1 also had the highest PHB content in its DCW, 84%, so high PHB production could be reached when a high amount of cell concentration is achieved. The strain also has a facultative anaerobic behavior; therefore, it could produce PHB without the need of supplying oxygen that is very expensive. The only problem with this strain is that it could not reach cell and PHB concentrations as high as the other strains under the same conditions. Therefore, further analysis was performed

on this strain to study the effects of different variables such as nitrogen source, electron acceptor, initial pH, and initial C/N ratio to optimize the growth and PHB production.

Effect of nitrogen source and electron acceptor

Z. denitrificans ZD1 showed a better growth ability in 5 g/L glycerol when 7.57 mM of nitrate was used as a nitrogen source instead of 7.57 mM of ammonium, while keeping the molar C/N ratio at 21.5 (Fig. 11a). Even though the cell concentration was higher with nitrate, 1.21 g/L, the PHB content in the DCW decreased from 84% with ammonium to 73% with nitrate (Table 6).

Lin and Shieh[18] reported the ability of *Z. denitrificans* ZD1 to use nitrate as an electron acceptor. In this study, Fig. 11a also shows that the strain used nitrate as an electron acceptor and grew in anaerobic condition, whereas the strain could not grow in anaerobic condition with ammonium. When nitrate was used as an electron acceptor instead of oxygen, *Z. denitrificans* ZD1 was able to accumulate PHB, but with a slower growth and a lower PHB content (Fig. 11a and Table 6). The cell concentration and PHB content decreased from 1.21 g/L to 0.20 g/L and from 72% to 55%, respectively. To our knowledge, this is the first study to report of PHB accumulation by bacterial strain in the absence of oxygen. Our results showed that *Z. denitrificans* ZD1 is an ideal candidate to produce PHB with nitrate as an electron acceptor instead of oxygen. Such as approach would be able to decrease the price associated with oxygen supply.

Effect of initial pH

The initial pH of P1 medium was studied at 6, 7, 8, and 9 for the growth ability and PHB accumulation by *Z. denitrificans* ZD1. Fig 11b shows that the strain was unable to grow at pH of 6 or 9 after 24 hours of incubation. On the other hand, the strain grew at pH of 7 and 8 with a higher advantage at pH of 7. The PHB content slightly decreased from 84% with a pH of 7 to 81% with a pH of 8 (Table 6).

Effect of C/N ratios

The effects of C/N ratios (mol/mol) of 1, 4, 21.5, and 40 were examined using *Z. denitrificans* ZD1 in P1 medium with an initial pH of 7.3. The initial glycerol concentration was maintained at 5 g/L, while ammonium concentration was changed to reach the aforementioned C/N ratios. The growth and DCW concentration increased with the decrease of C/N ratio due to the higher availability of nitrogen (Fig. 11c). In contrast, the PHB content and yields increased with the increase of C/N ratios until 21.5 (Table 6). The PHB content increased from 47% for C/N of 1 to 84% for C/N of 21.5. Similarly, the PHB yield increased from 0.17 (g-PHB/g-glycerol) to 0.24 (g-PHB/g-glycerol) with C/N of 21.5 (Table 6). At higher C/N ratio, 40, there was no significant change in the PHB content compared to a C/N ratio of 21.5; however, the cell and PHB concentrations decreased dramatically from 0.98 g/L to 0.42 g/L and from 0.82 g/L to 0.33 g/L, respectively.

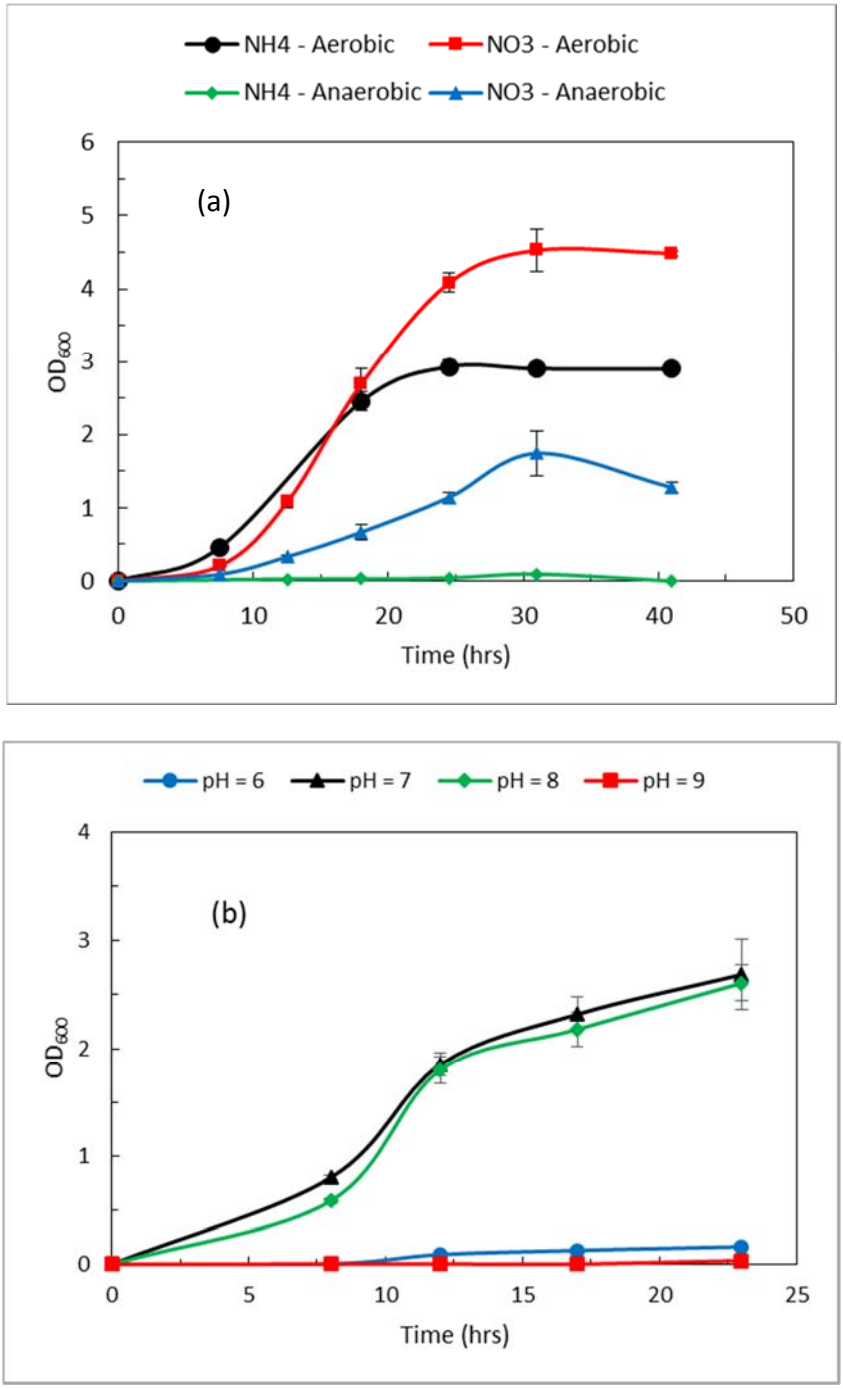


Fig. 11 Growth curves of *Zobellella denitrificans* ZD1 under different nitrogen sources (a), different initial pH conditions (b), and different initial molar C/N ratios (c).

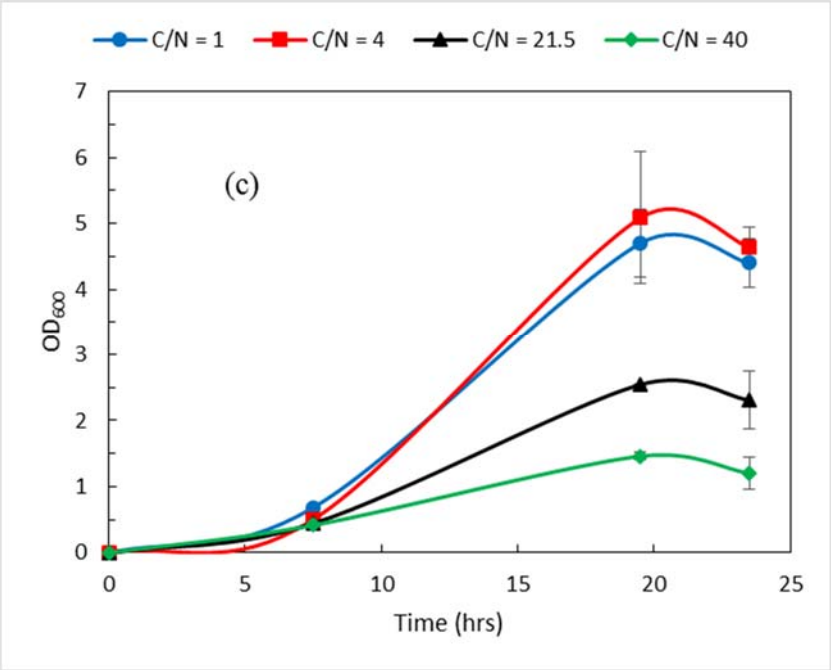


Fig. 11 Continued

Table 6. Effect of several variables on the growth and PHB accumulation by *Zobellella denitrificans* ZD1.

Variables	DCW (g/L)	%PHB	PHB (g/L)	Yield[a]
Nitrogen source				
$(NH_4)_2HPO_4$	0.98 ± 0.15	84 ± 4.7	0.82 ± 0.12	0.24 ± 0.03
KNO_3	1.21 ± 0.03	72 ± 1.0	0.87 ± 0.03	N.A.[c]
Electron acceptor				
O_2[b]	No growth			
NO_3^-	0.20 ± 0.02	55 ± 2.0	0.11 ± 0.02	N.A.
Initial pH				
6	No growth			
7	0.98 ± 0.03	84 ± 0.58	0.83 ± 0.02	N.A.
8	0.96 ± 0.01	81 ± 0.87	0.78 ± 0.02	N.A.
9	No growth			
Initial C/N ratio				
1	1.82 ± 0.06	47 ± 1.0	0.85 ± 0.01	0.17 ± 0.01
4	1.58 ± 0.32	56 ± 6.5	0.79 ± 0.06	0.16 ± 0.01
21.5	0.98 ± 0.15	84 ± 4.7	0.82 ± 0.12	0.24 ± 0.03
40	0.42 ± 0.02	81 ± 3.4	0.33 ± 0.01	0.09 ± 0.01

Zobellella denitrificans ZD1 was grown in P1 medium containing initially 5 g/L of glycerol and 0.5 g/L of NaCl. The initial molar C/N ratio was 21.5, except for the initial C/N ratio study. The initial pH of the medium was 7.3, except for the initial pH study.

[a] PHB yields were calculated based on gram of PHB produced per gram of glycerol consumed.
[b] $(NH_4)_2HPO_4$ was used as a nitrogen source in this experiment.
[c] represents information is not available information.

CHAPTER V

CONCLUSIONS

This research observed cell growth and PHB accumulation by three salt-tolerant strains *P. oleovorans*, *Burkholderia* sp., and *Z. denitrificans* ZD1, when grown on a cheap carbon source, glycerol with and/or without fatty acids. While colonies of *A. lata* were observed on glycerol-containing plates, *A. lata* was unable to grow in liquid medium containing glycerol. However, the ability of these strains to utilize glycerol and accumulate PHB in their cells varied based on the strain, fatty acid concentrations, and environmental growth conditions. The effects of saturated and unsaturated fatty acids on cell growth using glycerol showed improved cell growth of *P. oleovorans*, no impacts on *Burkholderia* sp., and negative impacts on *Z. denitrificans* ZD1.

Results of study on effects of nitrogen source, electron acceptor, initial pH, and C/N ratios suggested that *Z. denitrificans* ZD1 was a favorable strain for PHB production using glycerol. *Z. denitrificans* ZD1 showed the highest PHB content in the DCW, 84%, the shortest utilization period, 24 hours, even under the influence of fatty acids. The strain also can accumulate PHB using nitrate as a nitrogen source and electron acceptor. This is the first study, to our knowledge, showing PHB accumulation by facultative microorganism, *Z. denitrificans* ZD1, under nitrate reducing condition.

REFERENCES

1. Anderson, A.J. and Dawes, E.A. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.* **1990,** *54* (4), 450-472.

2. Van der Walle, G.; De Koning, G.; Weusthuis, R.; Eggink, G. Properties, modifications and applications of biopolyesters. *Biopolyesters* **2001**, 263-291.

3. Posada, J.A.; Naranjo, J.M.; López, J.A.; Higuita, J.C.; Cardona, C.A. Design and analysis of poly-3-hydroxybutyrate production processes from crude glycerol. *Process Biochemistry* **2011,** *46* (1), 310-317.

4. Naranjo, J.M.; Posada, J.A.; Higuita, J.C.; Cardona, C.A. Valorization of glycerol through the production of biopolymers: The PHB case using *Bacillus megaterium*. *Bioresour. Technol.* **2013,** *133*, 38-44.

5. Jiang, Y.; Marang, L.; Tamis, J.; van Loosdrecht, M.C.; Dijkman, H.; Kleerebezem, R. Waste to resource: converting paper mill wastewater to bioplastic. *Water Res.* **2012,** *46* (17), 5517-5530.

6. Chua, A.S.; Takabatake, H.; Satoh, H.; Mino, T. Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: effect of pH, sludge retention time (SRT), and acetate concentration in influent. *Water Res.* **2003,** *37* (15), 3602-3611.

7. Rodgers, M. and Wu, G. Production of polyhydroxybutyrate by activated sludge performing enhanced biological phosphorus removal. *Bioresour. Technol.* **2010,** *101* (3), 1049-1053.

8. Obruca, S.; Benesova, P.; Petrik, S.; Oborna, J.; Prikryl, R.; Marova, I. Production of polyhydroxyalkanoates using hydrolysate of spent coffee grounds. *Process Biochemistry* **2014,** *49* (9), 1409-1414.

9. Ashby, R.D.; Solaiman, D.K.; Strahan, G.D. Efficient utilization of crude glycerol as fermentation substrate in the synthesis of poly (3-hydroxybutyrate) biopolymers. *J. Am. Oil Chem. Soc.* **2011,** *88* (7), 949-959.

10. Choi, J. and Lee, S.Y. Process analysis and economic evaluation for poly (3-hydroxybutyrate) production by fermentation. *Bioprocess Eng.* **1997,** *17* (6), 335-342.

11. Choi, J. and Lee, S.Y. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Appl. Microbiol. Biotechnol.* **1999,** *51* (1), 13-21.

12. Chatzifragkou, A. and Papanikolaou, S. Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes. *Appl. Microbiol. Biotechnol.* **2012,** *95* (1), 13-27.

13. Samul, D.; Leja, K.; Grajek, W. Impurities of crude glycerol and their effect on metabolite production. *Annals of Microbiology* **2014,** *64* (3), 891-898.

14. Wang, F. and Lee, S.Y. Poly(3-Hydroxybutyrate) Production with High Productivity and High Polymer Content by a Fed-Batch Culture of *Alcaligenes latus* under Nitrogen Limitation. *Appl. Environ. Microbiol.* **1997,** *63* (9), 3703-3706.

15. Ugwu, C.U.; Tokiwa, Y.; Ichiba, T. Production of (R)-3-hydroxybutyric acid by fermentation and bioconversion processes with *Azohydromonas lata*. *Bioresour. Technol.* **2011,** *102* (12), 6766-6768.

16. Hauser, E.; Kampfer, P.; Busse, H.J. *Pseudomonas psychrotolerans* sp. *Int. J. Syst. Evol. Microbiol.* **2004,** *54* (5), 1633-1637.

17. Chee, J.; Tan, Y.; Samian, M.; Sudesh, K. Isolation and characterization of a *Burkholderia* sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty acids and glycerols. *Journal of Polymers and the Environment* **2010,** *18* (4), 584-592.

18. Lin, Y.T. and Shieh, W.Y. *Zobellella denitrificans* gen. nov., sp. nov. and *Zobellella taiwanensis* sp. nov., denitrifying bacteria capable of fermentative metabolism. *Int. J. Syst. Evol. Microbiol.* **2006,** *56* (6), 1209-1215.

19. Ashby, R.D.; Solaiman, D.K.; Foglia, T.A. Bacterial poly (hydroxyalkanoate) polymer production from the biodiesel co-product stream. *Journal of Polymers and the Environment* **2004,** *12* (3), 105-112.

20. Ibrahim, M. and Steinbüchel, A. *Zobellella denitrificans* strain MW1, a newly isolated bacterium suitable for poly (3-hydroxybutyrate) production from glycerol. *J. Appl. Microbiol.* **2010,** *108* (1), 214-225.

21. Maczulak, A.E.; Dehority, B.A.; Palmquist, D.L. Effects of long-chain Fatty acids on growth of rumen bacteria. *Appl. Environ. Microbiol.* **1981,** *42* (5), 856-862.

22. Chatzifragkou, A.; Dietz, D.; Komaitis, M.; Zeng, A.; Papanikolaou, S. Effect of biodiesel-derived waste glycerol impurities on biomass and 1, 3-propanediol production of *Clostridium butyricum* VPI 1718. *Biotechnol. Bioeng.* **2010,** *107* (1), 76-84.

23. Venkataramanan, K.P.; Boatman, J.J.; Kurniawan, Y.; Taconi, K.A.; Bothun, G.D.; Scholz, C. Impact of impurities in biodiesel-derived crude glycerol on the fermentation by

Clostridium pasteurianum ATCC 6013. *Appl. Microbiol. Biotechnol.* **2012,** *93* (3), 1325-1335.

24. Mantzouridou, F.; Naziri, E.; Tsimidou, M.Z. Industrial glycerol as a supplementary carbon source in the production of β-carotene by *Blakeslea trispora*. *J. Agric. Food Chem.* **2008,** *56* (8), 2668-2675.

25. Khuller, G. and Goldfine, H. Replacement of acyl and alk-1-enyl groups in *Clostridium butyricum* phospholipids by exogenous fatty acids. *Biochemistry* **1975,** *14* (16), 3642-3647.

26. Khulusi, S.; Ahmed, H.A.; Patel, P.; Mendall, M.A.; Northfield, T.C. The effects of unsaturated fatty acids on *Helicobacter pylori* in vitro. *J. Med. Microbiol.* **1995,** *42* (4), 276-282.

27. Abe, H.; Doi, Y.; Fukushima, T.; Eya, H. Biosynthesis from gluconate of a random copolyester consisting of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoates by *Pseudomonas* sp. 61-3. *Int. J. Biol. Macromol.* **1994,** *16* (3), 115-119.

28. Lee, E.; Jendrossek, D.; Schirmer, A.; Choi, C.; Steinbüchel, A. Biosynthesis of copolyesters consisting of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids from 1, 3-butanediol or from 3-hydroxybutyrate by *Pseudomonas* sp. A33. *Appl. Microbiol. Biotechnol.* **1995,** *42* (6), 901-909.

29. Khanna, S. and Srivastava, A.K. Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry* **2005,** *40* (2), 607-619.

30. Lee, S. Y. Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.* **1996,** *49* (1), 1-14.

31. Penloglou, G.; Kretza, E.; Chatzidoukas, C.; Parouti, S.; Kiparissides, C. On the control of molecular weight distribution of polyhydroxybutyrate in *Azohydromonas lata* cultures. *Biochem. Eng. J.* **2012,** *62*, 39-47.

32. Lemoigne, M. Products of dehydration and of polymerization of β-hydroxybutyric acid. *Bull.Soc.Chem.Biol* **1926,** *8*, 770-782.

33. Macrae, R.M. and Wilkinson, J.F. Poly-beta-hyroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. *J. Gen. Microbiol.* **1958,** *19* (1), 210-222.

34. Lillo, J.G. and Rodriguez-Valera, F. Effects of Culture Conditions on Poly(beta-Hydroxybutyric Acid) Production by *Haloferax mediterranei*. *Appl. Environ. Microbiol.* **1990,** *56* (8), 2517-2521.

35. Lageveen, R.G.; Huisman, G.W.; Preusting, H.; Ketelaar, P.; Eggink, G.; Witholt, B. Formation of Polyesters by *Pseudomonas oleovorans*: Effect of Substrates on Formation and Composition of Poly-(R)-3-Hydroxyalkanoates and Poly-(R)-3-Hydroxyalkenoates. *Appl. Environ. Microbiol.* **1988,** *54* (12), 2924-2932.

36. Grothe, E. and Chisti, Y. Poly (β-hydroxybutyric acid) thermoplastic production by *Alcaligenes latus*: behavior of fed-batch cultures. *Bioprocess Eng.* **2000,** *22* (5), 441-449.

37. Cavalheiro, J.M.; de Almeida, M Catarina MD; Grandfils, C.; Da Fonseca, M. Poly (3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. *Process Biochemistry* **2009,** *44* (5), 509-515.

38. Mothes, G.; Schnorpfeil, C.; Ackermann, J. Production of PHB from crude glycerol. *Engineering in Life Sciences* **2007,** *7* (5), 475-479.

39. Barbirato, F.; Himmi, E.H.; Conte, T.; Bories, A. 1, 3-Propanediol production by fermentation: an interesting way to valorize glycerin from the ester and ethanol industries. *Industrial Crops and Products* **1998,** *7* (2), 281-289.

40. Hirschmann, S.; Baganz, K.; Koschik, I.; Vorlop, K. Development of an integrated bioconversion process for the production of 1, 3-propanediol from raw glycerol waters. *Landbauforschung Völkenrode* **2005,** *55* (4), 261-267.

41. Luque, R.; Herrero-Davila, L.; Campelo, J.M.; Clark, J.H.; Hidalgo, J.M.; Luna, D.; Marinas, J.M.; Romero, A.A. Biofuels: a technological perspective. *Energy & Environmental Science* **2008,** *1* (5), 542-564.

42. Yazdani, S.S. and Gonzalez, R. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. *Curr. Opin. Biotechnol.* **2007,** *18* (3), 213-219.

43. Hájek, M. and Skopal, F. Treatment of glycerol phase formed by biodiesel production. *Bioresour. Technol.* **2010,** *101* (9), 3242-3245.

44. Carmona, M.; Warchoł, J.; Lucas, A.d.; Rodriguez, J.F. Ion-exchange equilibria of Pb2 , Ni2 , and Cr3 ions for H on Amberlite IR-120 resin. *Journal of Chemical & Engineering Data* **2008,** *53* (6), 1325-1331.

45. Leung, D.Y.; Wu, X.; Leung, M. A review on biodiesel production using catalyzed transesterification. *Appl. Energy* **2010,** *87* (4), 1083-1095.

46. Nieman, C. Influence of trace amounts of fatty acids on the growth of microorganisms. *Bacteriol. Rev.* **1954,** *18* (2), 147-163.

47. Yokochi, T.; Honda, D.; Higashihara, T.; Nakahara, T. Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21. *Appl. Microbiol. Biotechnol.* **1998,** *49* (1), 72-76.

48. Papanikolaou, S.; Muniglia, L.; Chevalot, I.; Aggelis, G.; Marc, I. *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. *J. Appl. Microbiol.* **2002,** *92* (4), 737-744.

49. Kamzolova, S.V.; Fatykhova, A.R.; Dedyukhina, E.G.; Anastassiadis, S.G.; Golovchenko, N.P.; Morgunov, I.G. Citric acid production by yeast grown on glycerol-containing waste from biodiesel industry. *Food Technology and Biotechnology* **2011,** *49* (1), 65.

50. Rivaldi, J.D.; Sarrouh, B.F.; da Silva, S.S. Development of biotechnological processes using glycerol from biodiesel production. *Current research topics in applied microbiology and microbial biotechnology. World Scientific, Singapore* **2009,** 429-433.

51. Palleroni, N.J. and Palleroni, A.V. *Alcaligenes latus*, a new species of hydrogen-utilizing bacteria. *Int. J. Syst. Bacteriol.* **1978,** *28* (3), 416-424.

52. Baptist, J.N.; Gholson, R.K.; Coon, M.J. Hydrocarbon oxidation by a bacterial enzyme system: I. Products of octane oxidation. *Biochim. Biophys. Acta* **1963,** *69*, 40-47.

53. Kim, M.H.; Wang, N.; McDonald, T.; Chu, K. Biodefluorination and biotransformation of fluorotelomer alcohols by two alkane-degrading *Pseudomonas* strains. *Biotechnol. Bioeng.* **2012,** *109* (12), 3041-3048.

54. Poirier, Y.; Nawrath, C.; Somerville, C. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. *Biotechnology* **1995,** *13* (2), 142-150.

55. Shen, L.; Haufe, J.; Patel, M.K. Product overview and market projection of emerging bio-based plastics PRO-BIP 2009. *Report for European Polysaccharide Network of Excellence (EPNOE) and European Bioplastics* **2009,** *243*.

56. Nath, A.; Dixit, M.; Bandiya, A.; Chavda, S.; Desai, A. Enhanced PHB production and scale up studies using cheese whey in fed batch culture of *Methylobacterium* sp. ZP24. *Bioresour. Technol.* **2008,** *99* (13), 5749-5755.

57. Schwartz, R.D. Octene epoxidation by a cold-stable alkane-oxidizing isolate of *Pseudomonas oleovorans*. *Appl. Microbiol.* **1973,** *25* (4), 574-577.

58. Law, J.H. and Slepecky, R.A. Assay of poly-beta-hydroxybutyric acid. *J. Bacteriol.* **1961,** *82*, 33-36.

59. Solorzano, L. Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnol. Oceanogr.* **1969,** *14* (5), 799-801.

60. Parsons, T.R. *A Manual of Chemical & Biological Methods for Seawater Analysis.* Elsevier, 2013.

61. De Smet, M.J.; Eggink, G.; Witholt, B.; Kingma, J.; Wynberg, H. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. *J. Bacteriol.* **1983,** *154* (2), 870-878.

62. Furusawa, H. and Koyama, N. Effect of fatty acids on the membrane potential of an *alkaliphilic Bacillus*. *Curr. Microbiol.* **2004,** *48* (3), 196-198.

63. Thompson, L.; Cockayne, A.; Spiller, R.C. Inhibitory effect of polyunsaturated fatty acids on the growth of *Helicobacter pylori*: a possible explanation of the effect of diet on peptic ulceration. *Gut* **1994,** *35* (11), 1557-1561.

APPENDIX

Quantification of PHB

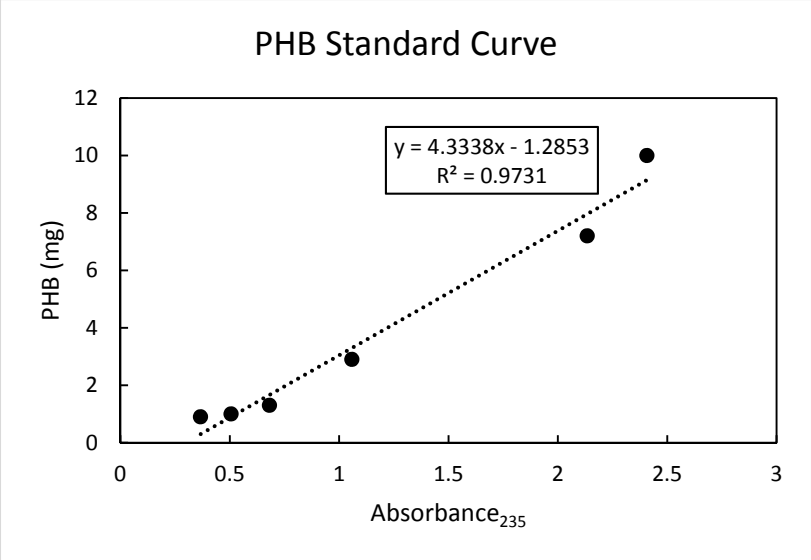


Fig. S1 Standard curve for PHB quantification

Chemical oxygen demand (COD) analysis

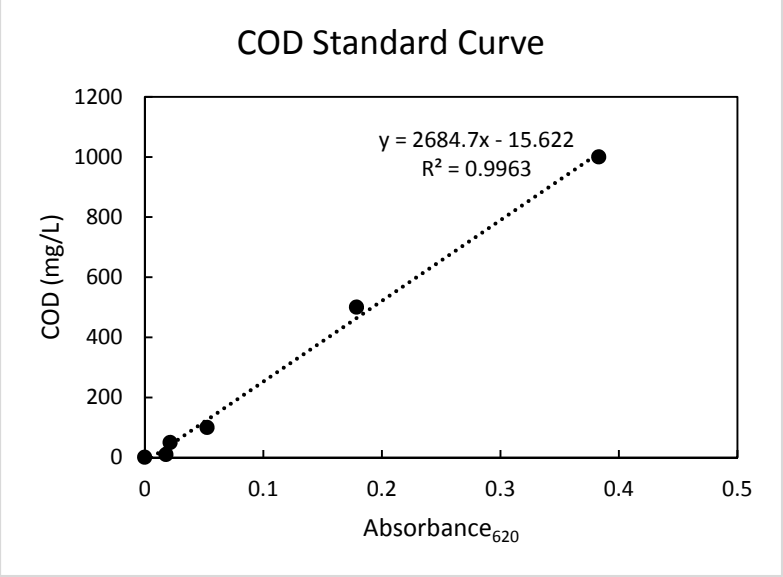


Fig. S2 COD standard curve

Ammonia-nitrogen (NH_3-N) analysis

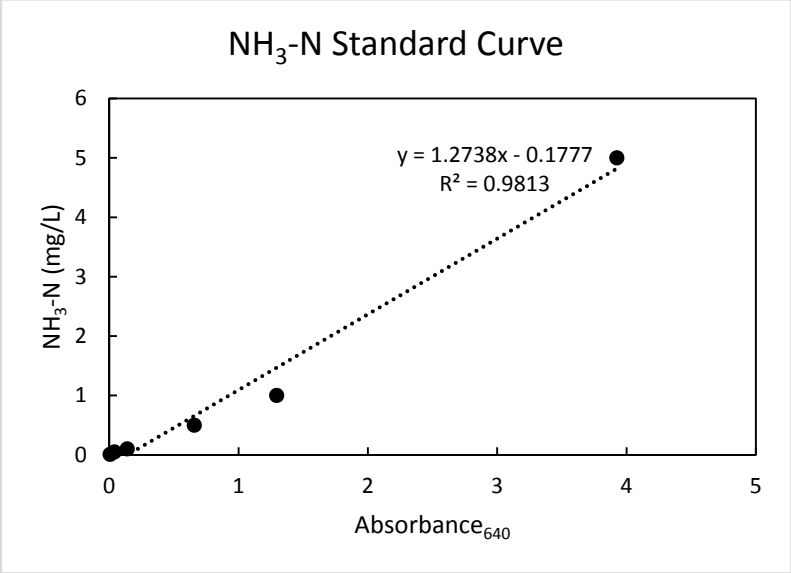


Fig. S3 Ammonia-nitrogen standard curve using phenate method

Effect of 0.5% fatty acids on glycerol-grown cells and their PHB production

Table S1. Effect of 0.5% fatty acids presence in glycerol on the bacterial growth and PHB production

Bacterial Strain	**No fatty acid**			**0.5% S**			**0.5% O**			**0.5% L**		
	DCW (g/L)	**%PHB**	**Yield [a]**	**DCW (g/L)**	**%PHB**	**Yield [a]**	**DCW (g/L)**	**%PHB**	**Yield [a]**	**DCW (g/L)**	**%PHB**	**Yield [a]**
P. oleovorans	1.33 ± 0.14	38 ± 1.8	0.13 ± 0.01	1.27 ± 0.08	38 ± 3.0	0.12 ± 0.01	1.18 ± 0.10	41 ± 5.0	0.12 ± 0.02	1.19 ± 0.05	38 ± 2.3	0.17 ± 0.01
Burkholderia sp.	1.89 ± 0.15	45 ± 9.0	0.23 ± 0.03	1.85 ± 0.21	46 ± 11	0.23 ± 0.03	1.85 ± 0.19	46 ± 11	0.23 ± 0.03	1.86 ± 0.20	47 ± 12	0.24 ± 0.03
Z. denitrificans ZD1	0.98 ± 0.15	84 ± 4.7	0.24 ± 0.03	1.02 ± 0.16	79 ± 7.3	0.23 ± 0.04	0.97 ± 0.14	83 ± 5.2	0.23 ± 0.03	0.99 ± 0.15	82 ± 4.2	0.23 ± 0.04

All strains were grown in P1 medium containing initially 5 g/L of glycerol (G) and 0.5 g/L of NaCl. Addition of 0.5% (w/w) of each type of fatty acids such as stearate (S), oleate (O), linoleate (L) to glycerol (G) used as an additional impurity to 0.5 g/L of NaCl salt. Error represent standard deviation of triplicate measurements.

[a] PHB yields were calculated based on gram of PHB produced per gram of glycerol consumed.

Growth Curves of salt-tolerant strains with the addition of fatty acids to glycerol

Samples were diluted when the OD measurements were greater than one to ensure appropriate representation of bacterial growth curves.

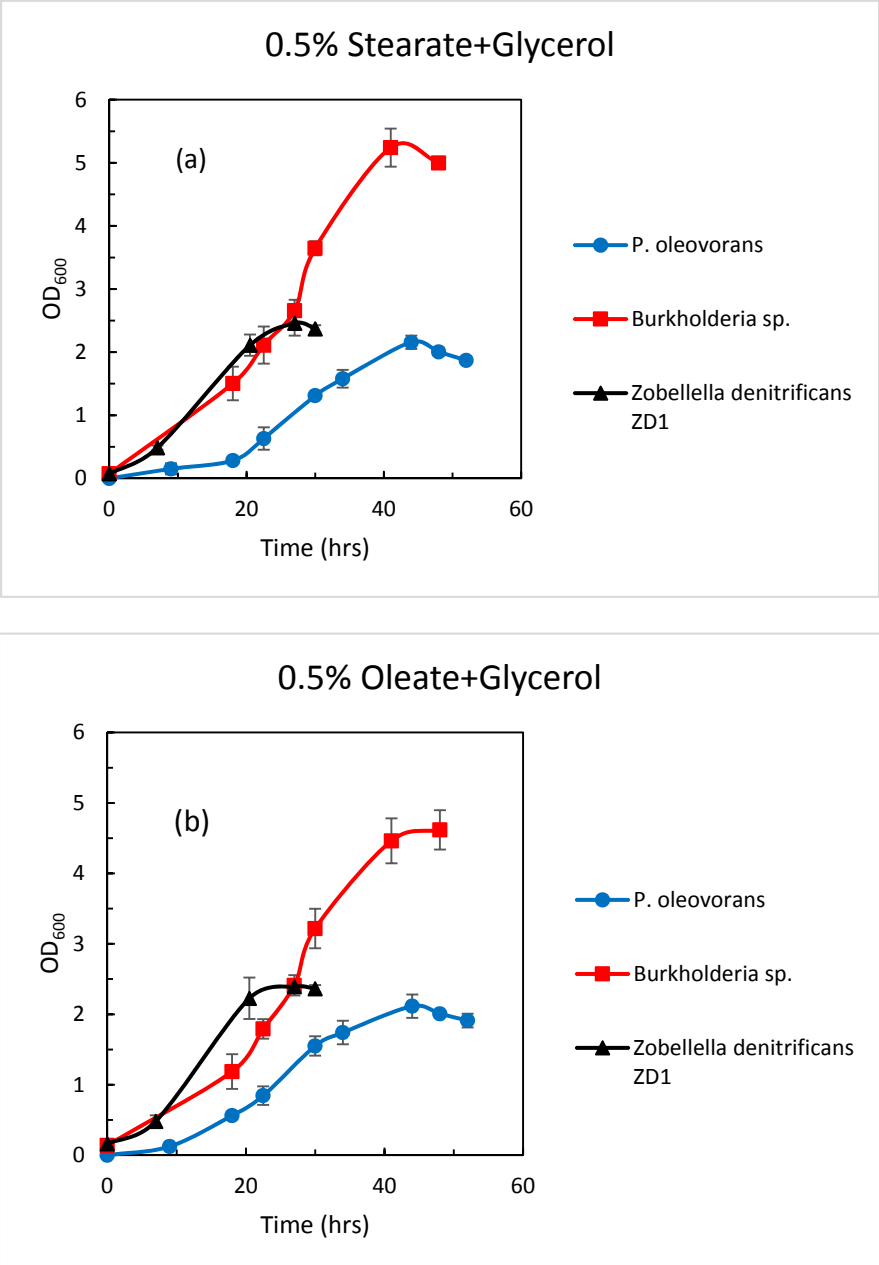


Fig. S4 Effect of 0.5% (w/w) fatty acids such as stearate (a), oleate (b), and linoleate (c) in P1 medium containing initially 5 g/L of glycerol (G) and 0.5 g/L of NaCl on the bacterial growth. Error bars represent standard deviation of triplicate measurements.

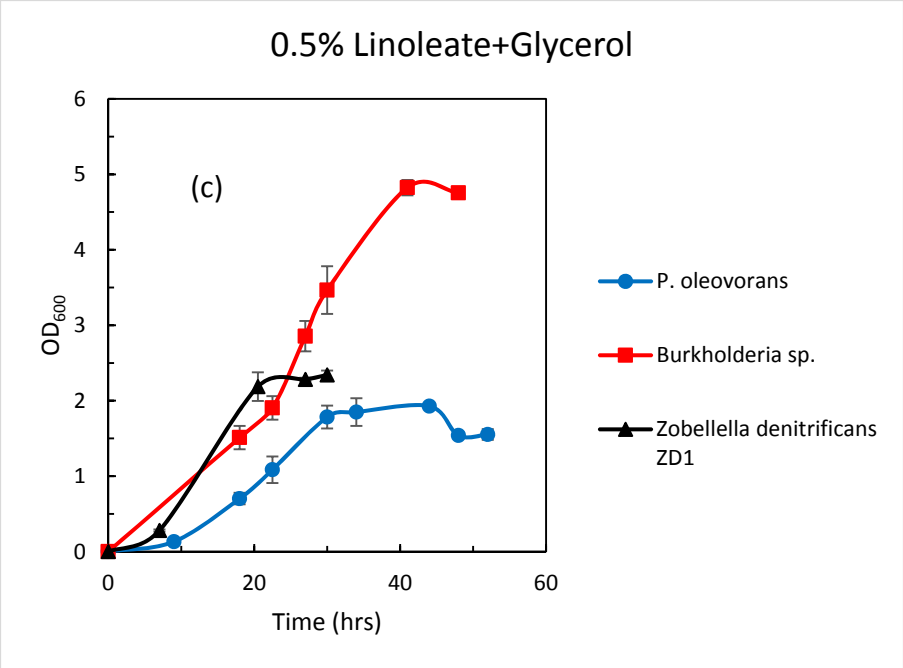


Fig. S4 Continued

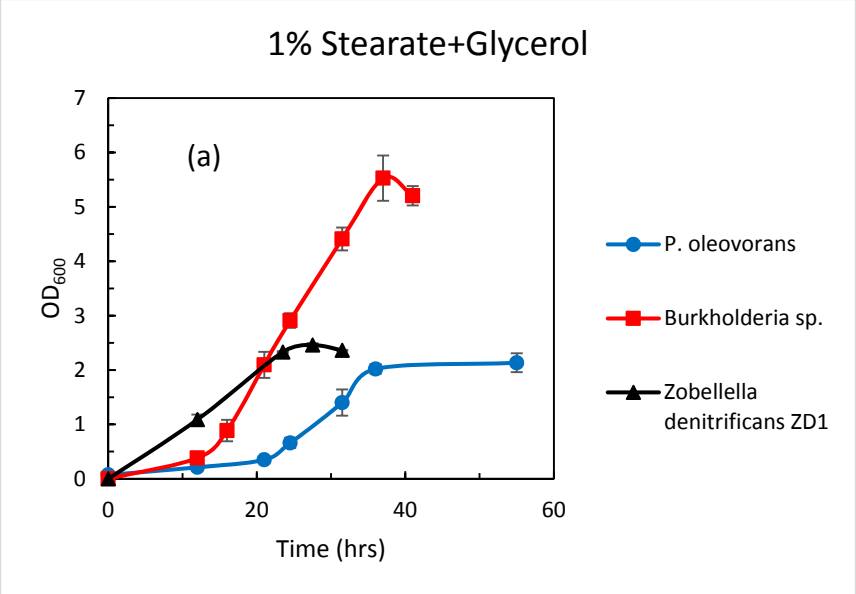
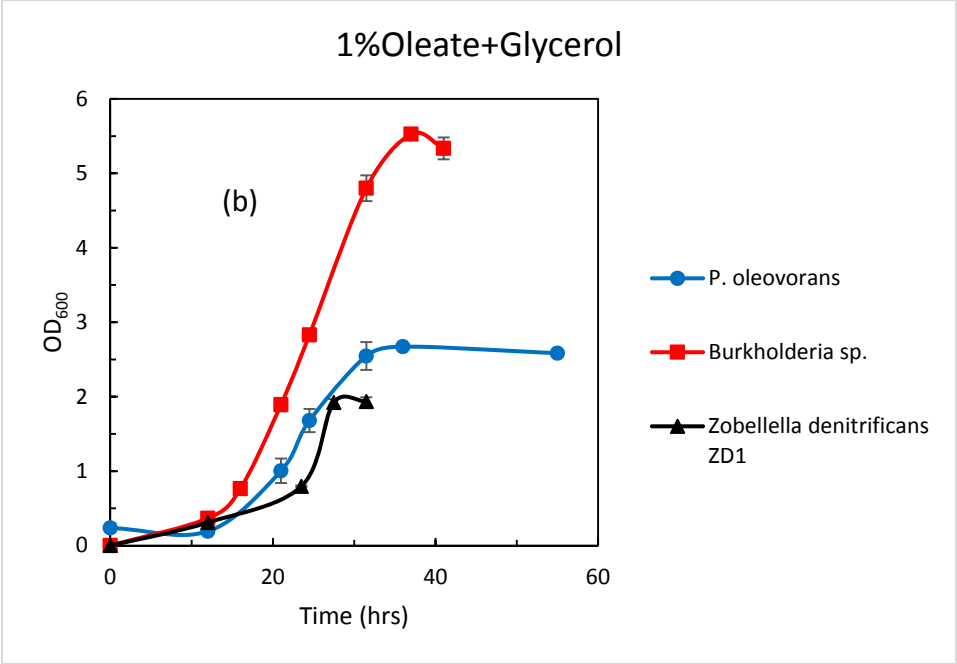


Fig. S5 Effect of 1% (w/w) fatty acids such as stearate (a), oleate (b), and linoleate (c) in P1 medium containing initially 5 g/L of glycerol (G) and 0.5 g/L of NaCl on the bacterial growth. Error bars represent standard deviation of triplicate measurements.



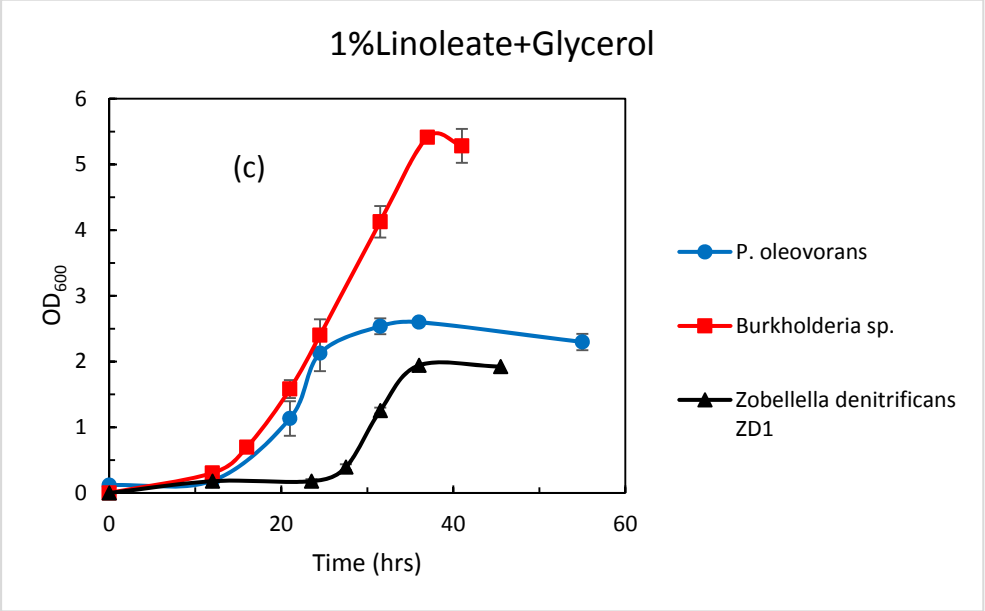


Fig. S5 Continued