Potential for Mcl-PHA Production from Nonanoic and Azelaic Acids

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2017-0554.R1
Manuscript Type:	Article
Date Submitted by the Author:	05-Oct-2017
Complete List of Authors:	Gillis, James; Queen's University, Chemical Engineering Ko, Kenton; Queen's University, Biology Ramsay, Juliana A.; Queen's University, Chemical Engineering Ramsay, Bruce A.; Queen's University, Chemical Engineering;
Is the invited manuscript for consideration in a Special Issue? :	N/A
Keyword:	mcl-PHA, azelaic acid, nonanoic acid, acrylic acid, Pseudomonas

SCHOLARONE™
Manuscripts

Potential for Mcl-PHA Production from Nonanoic and Azelaic Acids

James Gillis¹, Kenton Ko², Juliana A. Ramsay¹, and Bruce A. Ramsay^{1*}

¹ Department of Chemical Engineering, Queen's University, Kingston, Ontario, Canada
K7L 3N6

² Department of Biology, Queen's University, Kingston, ON K7L 3N6, Canada

* Corresponding author. Tel: +1 6135336000 Ext: 75293; Fax: +1 6135336637.

E-mail address: Bruce.Ramsay@queensu.ca

Running title: Mcl-PHA from Nonanoic and Azelaic Acids

Draft

ABSTRACT

Greater than 65 % of canola and high oleic soy oil fatty acids is oleic acid, which is readily converted to nonanoic (NA) and azelaic (AzA) acids by ozonolysis. NA is an excellent substrate for medium-chain-length polyhydroxyalkanoate (mcl-PHA) production but AzA has few uses. *Pseudomonas citronellolis* DSM 50332 and *Pseudomonas fluorescens* ATCC 17400, both able to produce mcl-PHA from fatty acids and able to grow on AzA as the sole source of carbon and energy were assessed for the accumulation of mcl-PHA from AzA and NA. In N-limited shake-flasks using NA, *P. citronellolis* produced 32% of its dry biomass as mcl-PHA containing 78% 3-hydroxynonanoate with 22 % 3-hydroxyheptanoate. *P. fluorescens* produced only 2% PHA.. N-limited *P. citronellolis* on AzA produced 20% dry weight PHA containing 75% 3-hydroxydecanoate and 25% 3-hydroxyoctanoate, indicative of *de novo* synthesis. Although selective pressure, including β -oxidation inhibition, under well-controlled (chemostat) conditions was applied to *P. citronellolis*, no side-chain carboxyl groups were detected. It was concluded that one or more of FabG and PhaJ or the PHA synthase cannot catalyze reactions involving ω -carboxy substrates. However, a process based on oleic acid could be established if *P. putida* was engineered to grow on AzA.

Key words: mcl-PHA, azelaic acid, *Pseudomonas*, nonanoic acid, acrylic acid

1. Introduction

Medium-chain-length poly-3-hydroxyalkanoic acids are bacterially accumulated polyesters with great potential as compostable thermoelastomers in high value commercial applications (Chen, 2009, Rai et al. 2011). Oleic acid is the predominant fatty acid of many tall and vegetable oils, particularly canola and high-oleic soy, that are likely

to be major feedstocks for renewable oil biorefineries in the temperate climates where these oils are produced in large quantity. Oleic acid is an excellent substrate for growth and medium-chain-length polyhydroxyalkanoate (mcl-PHA) accumulation by bacteria such as *Pseudomonas putida* (Lee et al. 2000). However, since such substrates must be activated via fadD (acyl-CoA synthase) for polymerization, substrates are subject to β -oxidation producing mcl-PHA containing a mixture of subunits. This reduces crystallinity compared to homopolymeric mcl-PHA, resulting in a melting point that is too low for many applications and poor mechanical strength. In contrast, nonanoic acid (NA) is one of the best substrates for high yield production of mcl-PHA (Sun et al. 2009) and can be used to produce materials with substantially superior mechanical properties and a melting point above 60 C (Jiang et al. 2012). NA is commercially produced by ozonolysis of oleic acid to make an equimolar mixture of NA and azelaic (AzA) acids (Rupp et al., 2009) (Fig. 1). Although small quantities of AzA are used in the production of the specialty polymer Nylon-6,9 and in acne medications (Elsaie and Choudhary, 2009), this dicarboxylic acid has few current uses. Since substrate cost is a major factor in the production of mcl-PHA, we investigated the possibility of using AzA and NA for mcl-PHA production.

Figure 1

Nonanoic acid can be metabolized by many bacteria via β -oxidation. AzA and other dicarboxylic acids are metabolized by some pseudomonads and related organisms. They include *Alcaligenes eutrophus* (more commonly referred to as *Ralstonia eutropha*,

but now officially called *Cupriavidus necator*) H16 (Snong et al. 1993), most *P. aeruginosa*, some strains of *P. fluorescens*, *P. stutzeri*, *P. multivorans* (now *Burkholderia cepacia*) and *P. acidovorans* (Stanier et al. 1966) and *P. citronellolis* ATCC 13674 (same as DSM 50332) (Choi and Yoon, 1994). Degradation of dicarboxylic acids has been shown to occur via β -oxidation in *P. aeruginosa* 8602 (Chapman and Duggleby, 1967) and *P. fluorescens* St. 18 (Hoet and Stanier, 1970) and is generally accepted as the route by which capable pseudomonads degrade dicarboxylic acids. *P. fluorescens* ATCC 17400 is a psychrophilic bacterium that can grow on a diverse range of compounds as sole sources of carbon and energy (Stanier et al. 1966). Several studies have focused on its ability to produce iron-chelating compounds (siderophores), but there are no publications in the context of PHA production. *P. citronellolis* has the unusual ability to grow on recalcitrant branched hydrocarbons (Fall et al. 1979) and terpenes as it possesses an acyclic terpene utilization pathway (Förster-Fromme and Jendrossek, 2010). *P. citronellolis* ATCC 13674 is the only organism shown to grow on α,ω -dicarboxylic acids and produce mcl-PHAs (Choi and Yoon, 1994). On azelaic acid (nonanedioate), the mcl-PHA contained 28 mol% 3-hydroxyoctanoate (C8), 63 mol% 3-hydroxydecanoate (C10), 7.3 mol% 3-hydroxy-1-dodecenoate (C12:1) and 1.6 mol% 3-hydroxy-1-tetradecenoate (C14:1) monomers, indicative of the biodegradation of the dicarboxylic acid leading to PHA production by fatty acid *de novo* synthesis. Hence *P. citronellolis* DSM 50332 and *P. fluorescens* ATCC 17400 were evaluated for growth and mcl-PHA production on AzA. The aim was to identify the best use of the oleic acid ozonolysis products, nonanoic and azeleic acids, in the production of mcl-PHA and to determine whether even trace amounts of carboxylated side-chains could be produced from azeleic acid.

2. Materials and Methods

2.1 Culture maintenance and shake-flask batch experiments

Cultures (*P. putida* KT2440, *P. citronellolis* DSM 50332 (same as ATCC 13674) and *P. fluorescens* ATCC 17400) were preserved on polymer beads and stored at -80°C and were maintained on nutrient agar plates at 4°C. In shake flask experiments to evaluate growth and PHA production on AzA or NA, a 2.5% (v/v) inoculum prepared on 1.0 g/L of the carbon source was added to 100 mL of medium in 500 mL Erlenmeyer flasks (pH of 7.0 before inoculation), then incubated at 28°C and 200 rpm on a rotary shaker (New Brunswick Scientific). Each Liter of medium for the inoculum and experimental flasks contained 0.50 g (NH₄)₂SO₄, 0.39 g MgSO₄, 6.35 g Na₂HPO₄, 2.70 g KH₂PO₄ and appropriate carbon source(s).

2.2 Toxicity of *P. citronellolis* to acrylic acid (AA)

Erlenmeyer shake flasks (500 mL) contained 100 mL of medium with acrylic acid (AA) at different concentrations with (a) glucose (9 g/L) and nutrient broth (1 g/L) or (b) nonanoic acid (2.5 g/L) as the sole source of carbon and energy. The medium for the inoculum and the toxicity test contained per Liter, 4.70 g (NH₄)₂SO₄, 0.39 g MgSO₄, 6.35 g Na₂HPO₄, 2.70 g KH₂PO₄, 1.00 g nutrient broth and 9.00 g glucose. A 2.5% (v/v) inoculum was added to each flask and cultivated at 28°C and 200 rpm for 36 h. Optical density was measured at 630 nm using a Unicam UV1 spectrophotometer (Spectronic Unicam, Cambridge, UK).

2.3 Chemostat start-up and operation

Each Liter of shake-flask inoculum medium contained 9.00 g glucose, 4.70 g $(\text{NH}_4)_2\text{SO}_4$, 0.39 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.36 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2.70 g KH_2PO_4 , 1.00 g nutrient broth. The initial medium (batch phase) for the chemostat contained per Liter: 3.90 g glucose, 2.90 g nonanoic acid 6.36 g Na_2HPO_4 , 2.70 g KH_2PO_4 , 4.70 g $(\text{NH}_4)_2\text{SO}_4$, 0.39 g MgSO_4 and 1.40 mL of trace element solution. Each Liter of trace element solution contained 10 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.2 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.3 g H_3BO_3 , 0.2 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.15 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ and 1.00 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The chemostat feed medium contained per Liter: 4.00 g $(\text{NH}_4)_2\text{SO}_4$, 0.503 g MgSO_4 , and in all but P-limited fermentations, 0.234 g Na_2HPO_4 , 0.099 g KH_2PO_4 and 1.4 mL/L trace element solution. Carbon substrates were added as described later. NA was added via computer control from a separate reservoir on a balance. Due to poor water solubility of AzA, it was solubilized in aqueous potassium hydroxide (mass ratio of AzA to KOH of approximately 1.6) and adjusted to pH 7 prior to autoclaving and added to the chemostat medium when cooled. The nitrogen source was supplied and pH controlled automatically with 14% (w/v) NH_4OH . CO_2 in the outlet gas was monitored throughout the batch phase until an outlet gas CO_2 content (Guardian+ carbon dioxide monitor, Topac Inc., USA) of between 1.0 and 1.2% before switching to chemostat mode. Single-stage chemostat fermentations were conducted in 2 L Series 351 Fermenters (Electrolab Ltd., UK) with baffles, 2 sets of flat-blade turbine impellers, a pH electrode (open junction, S8 interface, Cole Parmer Canada Inc.) and a dissolved O_2 probe (Model D100 Series OxyProbe®, Broadley James Corp, USA). Sterile aqueous medium was stored in a 10 L Nalgene® reservoir agitated with a magnetic stir bar. Aeration was 0.75 L/min and the pH was

controlled at 6.85. The dissolved oxygen concentration never fell below 20 % of saturation. The reactor working volume was 1.1 L and the dilution rate (D) was 0.25 h⁻¹. A minimum of two samples were taken at least three hours apart using a peristaltic pump after achieving steady state as monitored by dissolved oxygen concentration and outlet gas CO₂ content after at least 5 volumes of medium had been fed.

2.4 Analytical methods

Growth was monitored by optical density measurements at 630 nm. Cell dry weight was determined after lyophilization of biomass obtained by centrifugation of 5 mL culture broth at 17000 x g for 15 min and washing twice with 5 mL distilled water. Supernatant of the samples and the distilled water washes were saved for analysis of NA and key nutrients. NA was determined by methylation followed by GC-FID analysis with decanoic acid as the internal standard (Ramsay et al. 1991). Phosphate was measured via reduction of phosphomolybdate to molybdene blue (Clesceri et al. 1999) and ammonium by the phenol-hypochlorite method (Weatherburn, 1967). PHA content and composition was determined by modification of the method of (Braunegg et al. 1978) and (Lageveen et al. 1988) in which lyophilized cell samples were suspended with 2 mL of methanol containing 15% (v/v) H₂SO₄ and 0.2% (w/v) benzoic acid (internal standard) and 2 mL of chloroform. Methanolysis was performed at 100°C for 3.5 h with periodic vigorous mixing. After cooling to room temperature, 2 mL distilled water was added, vortexed for 1 min and left overnight for phase separation. The organic phase was analyzed by GC-FID. The PHA standard was prepared by repeated solvent extraction followed by precipitation in cold methanol, and its composition was determined by NMR. For ¹H-

NMR and ^{13}C -NMR analysis, purified samples were dissolved in deuterated chloroform (CDCl_3) to a final concentration of about 25 mg/mL and spectra were recorded on a Bruker Avance 400 MHz spectrometer. AA was analyzed according to Qi et al. (1998) in which 2 mL of supernatant were acidified with 1/10 volume of 2 M HCl containing 0.02% (v/v) isovaleric acid as an internal standard (Baksanova et al., 2002) and 10 μL injected into a Hewlett Packard 5890 GC equipped with a polyethylene glycol column (Carbowax®) and FID. FID and injector temperatures were 275°C and 250°C, respectively.

3. Results and Discussion

3.1 Preliminary screening and selection of microorganisms on NA and AzA

P. citronellolis DSM 50332 and *P. fluorescens* ATCC 17400 were evaluated for growth and PHA production on NA and AzA in shake flask culture and *P. putida* KT2440 on AzA alone. As expected, *P. putida* KT2440, a well known mcl-PHA producer did not grow on AzA as sole source of carbon and energy. Another *P. putida* strain, KCTC 2407, was previously shown not grow on AzA (Kim et al. 2000). Although *P. citronellolis* DSM 50332 (same strain as ATCC 13674 used by Choi and Yoon (1994)) and *P. fluorescens* ATCC 17400 can produce mcl-PHA and also use AzA as a carbon and energy source, likely through β -oxidation, the ability of these strains to accumulate mcl-PHAs has not been well characterized. Neither strain produced PHA in shake-flasks at low NA concentration but did at higher NA concentrations when NH_4^+ was exhausted (Table 1), indicating that nutrient (other than carbon) limitation is required for PHA production. The maximum PHA concentration was accumulated at the highest NA

concentration tested (3 g/L) at 32% of dry weight biomass for *P. citronellolis* but only 2% for *P. fluorescens*. The PHA produced from NA by both strains was about 78 mol% 3-hydroxynonanoate (C9) with the balance being 3-hydroxyheptanoate (C7), indicating that both strains metabolized NA through β -oxidation.

When grown in shake-flasks with AzA as the sole carbon source, both *citronellolis* and *fluorescens* grew well with no evidence of AzA toxicity even at the maximum concentration tested of 8 g/L (Table 2). Growth was N-limited above 1.0 g/L AzA. In the case of *P. citronellolis*, PHA accumulation increased as the AzA concentration increased. *P. fluorescens* did not accumulate any PHA indicating that it cannot directly utilize 3-hydroxyAzA for PHA synthesis and that it cannot produce mcl-PHA from unrelated substrates probably due to a lack of phaG. *P. citronellolis* produced mcl-PHA of up to 20% of its dry weight biomass. The PHA was about 75 mol% 3-hydroxydecanoate (C10) with the balance being 3-hydroxyoctanoate (C8) indicating that all of the PHA was produced via *de novo* fatty acid biosynthesis from acetate. These results are similar to the shake-flask experiments of Choi and Yoon (1994), which yielded 8.2% PHA having a composition of 28 mol% C8 and 63 mol% C10 from *P. citronellolis* grown on 8 g/L AzA under N-limitation. However, in their case, the remaining 9% of PHA monomers were identified by $^1\text{H-NMR}$ as unsaturated 3-hydroxy-1-dodecenoate (C12:1, 7.3 mol%) and 3-hydroxy-1-tetradecenoate (C14:1, 1.6 mol%) monomers which may have been undetected in the lower resolution GC-FID chromatograms.

3.2 Mcl-PHA production by *P. citronellolis* DSM 50332 from NA under phosphate limitation with glucose as co-substrate, no AzA

We wished to determine whether an AzA-utilizing mcl-PHA producer could directly accumulate AzA into mcl-PHA through the initial steps of β -oxidation (as opposed to *de novo* fatty acid biosynthesis) under highly controlled (chemostat) conditions designed to promote mcl-PHA accumulation. Optimal chemostat conditions were determined with a substrate known to be transformed into mcl-PHA, NA. Thus, the capacity of *P. citronellolis* DSM 50332 to accumulate mcl-PHA from the PHA related substrate, NA (2.90 g/L in feed), under phosphate limitation in a chemostat ($D=0.25\text{ h}^{-1}$) was evaluated. Glucose (3.90 g/L in feed) was supplied as a growth substrate. Ammonium was always in excess while phosphate became limiting at phosphate feed concentrations below 0.400 g/L (Fig. 2). Phosphate limitation caused the CO_2 production rate and steady-state biomass concentration to decrease but generally led to increased PHA accumulation with a maximum of 20.0 wt% at an inlet phosphate concentration of 0.225 g/L (Fig. 2). Polymer composition was constant at approximately 65 mol% C9 and 35 mol% C7 for all experimental conditions. These conditions were then used to study PHA production with a combination of NA and AzA in the feed.

Figure 2

3.3 Mcl-PHA production by *P. citronellolis* DSM 50332 from AzA and NA under phosphate limitation with glucose as co-substrate

AzA was introduced as an additional carbon source in the chemostat study of *P. citronellolis* DSM 50332. The objective was to determine if AzA could be used to produce mcl-PHA containing a side-chain carboxyl group by supplying another carbon source (NA) known to produce mcl-PHA via β -oxidation in an effort to stimulate this pathway. Other than the addition of AzA, the conditions were identical to those in the previous section, and the concentration of phosphate was fixed at 0.225 g/L since the highest % PHA was produced at this concentration.

Although about 35% of the AzA fed was utilized (Fig. 3), biomass, CO₂ production rate (CPR), PHA content (about 20% of dry biomass) and composition (65 mol% C9 and 35 mol% C7) remained almost constant (data not shown) as the AzA feed concentration was increased. Phosphate was always limiting, ammonium and glucose were in excess and NA was completely utilized (Fig. 3). Under these conditions, only NA was used as a carbon source for PHA synthesis. No C10 monomer (indicative of *de novo* synthesis) nor carboxylated monomer (indicative of direct incorporation of AzA) were detected.

Figure 3

3.4 Effect of AA on mcl-PHA production by *P. citronellolis* DSM 50332 from AzA and NA under phosphate limitation with glucose as co-substrate

Since some pseudomonads are known to metabolize dicarboxylic acids by β -oxidation (Chapman and Duggleby, 1967; Hoet and Stanier, 1970), inhibition of β -ketothiolase in the β -oxidation pathway by acrylic acid (AA) may increase the likelihood

of producing carboxylated PHA from dicarboxylic acids. β -oxidation inhibition has been previously used to increase the likelihood of incorporation of specific PHA subunits (Olivera et al. 2001; Escapa et al. 2011). In order to determine the AA concentration range that should be studied, the amount of AA required to suppress β -oxidation was evaluated in shake-flasks. Even when supplied with glucose and nutrient broth, as little as 0.10 g/L AA severely inhibited growth (Fig. 4a). Based on these data, another toxicity test was performed employing NA as the sole source of carbon and energy. NA utilization requires β -oxidation. While concentrations above 0.005 g/L AA decreased the growth rate, it was determined that 0.05 g/L AA would substantially inhibit β -oxidation in *P. citronellolis* DSM 50332 while still allowing some β -oxidation which may be necessary for certain cellular functions (Fig. 4b). Increasing the AA concentration to 0.10 g/L virtually eliminated growth with NA as the sole carbon and energy source, indicating that β -oxidation was almost completely suppressed at this concentration.

Figure 4

Another chemostat experiment, almost identical to the previous study, was conducted but the AzA inlet concentration was fixed at 2.50 g/L while the AA feed concentration was varied (Fig. 5). Under these conditions, the residual glucose and AzA concentration in the bioreactor remained unchanged at about 0.5 g/L and 0.7 g/L respectively (data not shown). All NA was consumed until the highest AA inlet concentration tested (0.04 g/L) when NA levels rose to about 0.35 g/L. The steady-state metabolism (as reflected in the CPR data) decreased as the inlet AA concentration was

increased above 0.02 g/L. This corresponded with an increase in mcl-PHA accumulation as NA derived carbon was diverted from production of other biomass components which require β -oxidation. Inhibition of β -oxidation was evident as the ratio of C9 to C7 monomers also increased. However, there was still no accumulation of the carboxylic acid side-chains expected from direct incorporation of 3-hydroxyAzA as determined by GC-FID. There are no other published studies which have examined the effect of β -oxidation inhibition on production of mcl-PHAs from azaleic acid.

Figure 5

3.5 NMR analysis of PHA

In order to confirm the GC-FID results, ^1H -NMR and ^{13}C -NMR analyses were performed on PHA extracted taken from the 0.025 g/L AA steady-state sample to ascertain whether any carboxylated monomers were present. A C9/C7 mcl-PHA standard produced from *P. putida* KT2440 grown on NA was used as a basis for comparison. ^{13}C -NMR and ^1H -NMR both demonstrated that the *P. citronellolis* sample contained C9 and C7 monomers. No side-chain carboxyl groups were detected.

Polar PHAs are typically produced via chemical modification (Li and Loh, 2015). Among the most polar PHAs produced through a totally biological process are those containing 3-hydroxy-7-oxo-octanoate and 8-acetoxy-3-hydroxyoctanoate monomers (Jung et al. 2000) and those containing thioester groups (Escapa et al. 2011). Although AA addition reduced β -oxidation activity enough to enrich the C9 content from by up to 11%, *P. citronellolis* DSM 50332 was unable to produce mcl-PHA containing a side

chain carboxyl group from AzA. AzA could be used for PHA accumulation only via *de novo* synthesis. Assuming that β -oxidation of AzA proceeds exclusively from one terminus, the capacity for producing carboxylated mcl-PHA is contingent on the specificity of the enzymes linking β -oxidation to mcl-PHA production (phaJ) and the PHA synthase. Carboxylated mcl-PHA cannot be synthesized if key enzymes are unable to catalyze reactions involving a substrate with a carboxyl group.

4. Conclusions

Although *Pseudomonas citronellolis* accumulated much more PHA than *P. fluorescens*, it could not incorporate AzA to produce any carboxyl side-chains in its PHA. However, it can degrade AzA, most probably producing acetate which can feed the TCA cycle or produce mcl-PHA via *de novo* synthesis. Unless key enzymes are modified to accept AzA as a substrate, its use in PHA production will be restricted to a carbon and energy source, similar to glucose. While the bacteria that could metabolize AzA accumulated relatively little mcl-PHA (well under 50 % of dry weight), the most productive mcl-PHA accumulator, *P. putida* cannot metabolize this diacid. However, if *P. putida* could be modified to catabolize AzA to acetate by incorporation of fatty acid degradation genes from *P. citronellolis*, AzA could replace glucose which has already been shown to aid in a highly productive process for the production of mcl-PHA from nonanoic acid (Sun et al. 2009). The ozonolysis products of oleic acid could be fed to the fed-batch fermentation process with no requirement for their separation, thus further reducing production cost.

Acknowledgements

This work was supported by the Ontario Ministry of Agriculture, Food and Rural Affairs and by scholarships to J. Gillis from Natural Sciences and Engineering Research Council of Canada, the Ontario Graduate Scholarship Program and Queen's Graduate Awards.

References

- Baksanova, L.E., Val'tsifer, V.A., Budnikov, V.I., Kondrashova, N.B. 2002. Gas chromatographic determination of acrylic acid from aqueous solutions. *Russ. J. Appl. Chem.* 75, 1427-1429.
- Braunegg, G., Sonnleitner, B., Lafferty, R.M. 1978. Rapid gas-chromatographic method for determination of poly-beta-hydroxybutyric acid in microbial biomass. *Eur. J. Appl. Microb. Biotechnol.* 6, 29-37.
- Chapman, P.J., Duggleby, R.G. 1967. Dicarboxylic acid catabolism by bacteria. *Biochem. J.* 103, 7c-9c.
- Chen GQ. 2009. A microbial polyhydroxyalkanoates (PHA) based bio-and materials industry. *Chemical Society Reviews.* 38, 2434-46.
- Choi, M.H., Yoon, S.C. 1994. Polyester biosynthesis characteristics of *Pseudomonas citronellolis* grown on various carbon sources, including 3-methyl-branched substrates. *Appl. Environ. Microbiol.* 60, 3245-3254.
- Clesceri, L.S., Greenberg, A.E., Eaton, A.D. 1999. Standard methods for the examination of water and wastewater 20th edition. American Public Health Association, Washington, DC.
- Elsaie, M.L., Choudhary, S. 2009. Updates on the pathophysiology and management of acne rosacea. *Postgrad. Med.* 121, 178-186.

- Escapa, I.F., Morales, V., Martino, V.P., Pollet, E., Averous, L., Garcia, J.L., Prieto, M.A. 2011. Disruption of beta-oxidation pathway in *Pseudomonas putida* KT2442 to produce new functionalized PHAs with thioester groups. Appl. Microbiol. Biotechnol. 89, 1583-1598.
- Fall, R.R., Brown, J.L., Schaeffer, T.L. 1979. Enzyme recruitment allows the biodegradation of recalcitrant branched hydrocarbons by *Pseudomonas fluorescens*. Appl. Environ. Microbiol. 38, 715-722.
- Förster-Fromme, K., Jendrossek, D. 2010. Catabolism of citronellol and related acyclic terpenoids in pseudomonads. Appl. Microbiol. Biotechnol. 87, 859-869.
- Hany, R., Bohlen, C., Geiger, T., Hartmann, R., Kawada, J., Schmid, M., Zinn, M., Marchessault, R.H. 2004. Chemical synthesis of crystalline comb polymers from olefinic medium-chain-length poly[3-hydroxyalkanoates]. Macromolecules 37, 385-389.
- Hoet, P.P., Stanier, R.Y. 1970. The dissimilation of higher dicarboxylic acids by *Pseudomonas fluorescens*. Eur. J. Biochem. 13, 65-67.
- Jiang X., Sun Z., Marchessault R.H., Ramsay J.A., Ramsay B.A. 2012. Biosynthesis and properties of medium-chain-length polyhydroxyalkanoates with enriched content of the dominant monomer. Biomacromolecules. 13, 2926-32.
- Jung, K., Hany, R., Rentsch, D., Storni, T., Egli, T., Witholt, B. 2000. Characterization of new bacterial copolyesters containing 3-hydroxyoxoalkanoates and acetoxy-3-hydroxyalkanoates. Macromolecules 33, 8571-8575.
- Koller, M., Braunegg, G. 2015. Potential and prospects of continuous polyhydroxyalkanoate (PHA) production. Bioengineering, 2, 94-121.

- Konig, G.J.M., van Bilsen, H.M.M., Lemstra, P.J., Hazenberg, W., Witholt, B., Preusting, H., van der Galien, J.G., Schirmer, A., Jendrossek, D. 1994. A biodegradable rubber by crosslinking poly(hydroxyalkanoate) from *Pseudomonas oleovorans*. *Polymer* 35, 2090-2097.
- Lageveen, R.G., Huisman, G.W., Preusting, H., Ketelaar, P., Eggink, G., Witholt, B. 1988. 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.* 54, 2924-2932.
- Lee, M.Y., Park, W.H. 2000. Preparation of bacterial copolyesters with improved hydrophilicity by carboxylation. *Macromol. Chem. Phys.* 201, 2771-2774.
- Lee, S.Y., Wong, H.H., Choi, J.I., Lee, S.H., Lee, S.C., Han, C.S. 2000. Production of medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation of *Pseudomonas putida* under phosphorus limitation. *Biotechnol. Bioeng.* 68, 466-470.
- Li, Z., Loh, X.J. 2015. Water soluble polyhydroxyalkanoates: future materials for therapeutic applications. *Chem. Soc. Rev.* 44, 2865-2879.
- Olivera, E.R., Carnicero, D., Jodra, R., Miñambres, B., García, B., Abraham, G.A., Gallardo, A., Román, J.S., García, J.L., Naharro, G., Luengo, J.M. 2001. Genetically engineered *Pseudomonas*: a factory of new bioplastics with broad applications. *Environ. Microbiol.* 3, 612-618.
- Qi, Q., Steinbüchel, A., Rehm, B.H. 1998. Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (fadR): inhibition of fatty acid beta-oxidation by acrylic acid. *FEMS Microbiol. Lett.* 167, 89-94.

- Ramsay, B.A., Saracovan, I., Ramsay, J.A., Marchessault, R.H. 1991. Continuous production of poly- β -hydroxyalkanoates with long-side-chains by *Pseudomonas oleovorans*. Appl. Environ. Microbiol. 57, 625-629.
- Rai R, Keshavarz T, Roether JA, Boccaccini AR, Roy I. 2011. Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. Materials Science and Engineering: R: Reports. 72, 29-47.
- Rup, S., Zimmermann, F., Meux, E., Schneider, M., Sindt, M., Oget, N. 2009. The ultrasound-assisted oxidative scission of monoenic fatty acids by ruthenium tetroxide catalysis: Influence of the mixture of solvents. Ultrasonics Sonochem. 16, 266-272.
- Song, J.J., Shin, Y.C., Yoon, S.C. 1993. Polyesters biosynthesis of *Alcaligenes eutrophus* H16 (ATCC 17699) from various mono- and dicarboxylic acids and diols. J. Microbiol. Biotechnol. 3, 123-128.
- Stanier, R.Y., Palleroni, N.J., Doudoroff, M. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43, 159-271.
- Sun, Z., Ramsay, J., Guay, M., Ramsay, B. 2009. Enhanced yield of medium-chain-length polyhydroxyalkanoates from nonanoic acid by co-feeding glucose in carbon-limited, fed-batch culture. J. Biotechnol. 143, 262-267.
- Weatherburn, M.W. 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39, 971-974.

Figure Captions

Fig. 1 Structure of nonanoic and azelaic acids.

Fig. 2 Effect of phosphate limitation on growth and PHA accumulation from nonanoic acid in *P. citronellolis* DSM 50332 in chemostat culture ($D=0.25\text{ h}^{-1}$). Steady state data are plotted versus PO_4 in the feed. Inlet concentrations of 3.90 g/L glucose and 2.90 g/L nonanoic acid were maintained. Error bars indicate standard error from two independent samples.

Fig. 3 *P. citronellolis* DSM 50332 chemostat ($D=0.25\text{ h}^{-1}$) steady state glucose, nonanoic acid, azelaic acid, NH_4^+ and PO_4 concentrations in reactor at different, azelaic acid feed concentrations with inlet concentrations of 3.90 g/L glucose and 2.90 g/L nonanoic acid. Error bars indicate standard error from two independent samples.

Fig. 4 Optical density profiles for *P. citronellolis* DSM 50332 grown in shake-flasks with different concentrations of acrylic acid (AA) in mineral salts medium with (a) glucose (9 g/L) and nutrient broth (1 g/L) or (b) nonanoic acid (2.5 g/L) as sole source of carbon and energy.

Fig. 5 *P. citronellolis* DSM 50332 chemostat ($D=0.25\text{ h}^{-1}$) steady state biomass concentration, carbon dioxide production rate (CPR), PHA content and composition at different inlet concentrations of acrylic acid. Error bars indicate standard error from two independent samples.

Table 1 Growth and PHA production by *P. citronellolis* and *P. fluorescens* on nonanoic acid in shake flasks. Each data point is from a single shake-flask.. The growth curve in each flask was derived from six to ten samples for optical density measurement with substrate, biomass and PHA concentrations determined for the final sample.

		Nonanoic acid in shake flask (g/L)					
		0.0	0.5	1.0	1.5	2.0	3.0
<i>P. citronellolis</i> DSM 50332	NH ₄ (g/L)	0.15	0.06	-	-	-	-
	Biomass (g/L)	0.17	0.73	1.10	1.26	1.21	0.97
	PHA (wt%)	0	0	0	16.3	18.2	31.7
	C ₉ (mol%)				78	80	75
	C ₇ (mol%)				22	20	25
<i>P. fluorescens</i> ATCC 17400	NH ₄ (g/L)	0.06	0.03	0.01	-	-	-
	Biomass (g/L)	0.26	0.68	0.82	1.02	0.66	0.66
	PHA (wt%)	0	0	0	0	0	1.9
	C ₉ (mol%)						79
	C ₇ (mol%)						21

Table 2: Growth and PHA production by *P. citronellolis* and *P. fluorescens* on azelaic acid in shake flasks. Each data point is from a single shake-flask. The growth curve in each flask was derived from six to ten samples for optical density measurement with substrate and biomass and PHA concentrations determined for the final sample.

		Azelaic acid in shake flask (g/L)							
		0.0	1.0	2.0	3.0	4.0	5.0	6.0	8.0
	NH ₄ (g/L)	0.09	0.02	-	-	-	-	-	-
<i>P.</i>	Biomass (g/L)	0.07	0.53	0.96	0.98	1.06	1.10	1.27	1.35
<i>citronellolis</i>	PHA (wt%)	0	0	4.5	14.0	17.8	19.1	19.3	20.1
DSM 50332	C ₁₀ (mol%)			77	77	77	75	75	74
	C ₈ (mol%)			23	23	23	25	25	26
	NH ₄ (g/L)	0.05	0.02	-	-	-	-	-	-
<i>P.</i>	Biomass (g/L)	0.38	0.60	1.06	1.21	1.51	1.62	1.58	1.66
<i>fluorescens</i>	PHA (wt%)	0	0	0	0	0	0	0	0
ATCC 17400	C ₁₀ (mol%)								
	C ₈ (mol%)