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Bacterial Production of PHAs from Lipid-Rich by-Products

Lorenzo Favaro[1], Marina Basaglia[1], Jesus Enrique Gamero Rodriguez[1], Andrea Morelli[2], Omodele Ibraheem[3], Valentino Pizzocchero[1], Sergio Casella[1*]

1- Department of Agronomy Food Natural Resources Animals and Environment (DAFNAE), Universita di Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro (PD), Italy.

2- Department of Chemistry and Industrial Chemistry, Universita of Pisa, Via G. Moruzzi 13, 56124 Pisa, Italy.

3- Department of Biochemistry, Federal University Oye-Ekiti, Ekiti State, Nigeria.

Abstract

Background and Objective: Due to oil shortage and environmental problems, synthetic plastics will surely be replaced by alternative, biodegradable materials. A possible good example could be polyhydroxyalkanoates, and the inexpensive agricultural fatty by-products could be usefully converted to polyhydroxyalkanoates by properly selected and/or developed microbes.

Material and Methods: Among the more common by-products available, a variety of lipid-rich residues have been explored as substrate, such as crude glycerol from biodiesel, biodiesel obtained from fatty residues, and, from slaughterhouse, bacon rind, udder and tallow. In this paper, several new isolates and collection PHA-producing microbes have been screened for both lipolytic activities and polyhydroxyalkanoates production. The soil proved to be the most promising mining place to find new interesting microbial species, even better than more specific and selective environments such as slaughterhouses.

Results and Conclusion: Remarkably, two of the collection strains used here, known to be polyhydroxyalkanoates producers, resulted as really promising, being able to grow directly on all the substrates tested and to produce variable amounts of the polymer, including the co-polymers P (3HB-co-3HV).

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***Corresponding author:**
Sergio Casella,
Department of Agronomy Food Natural Resources Animals and Environment (DAFNAE)
Agripolis Universit di Padova
Viale dell'Universita, 16
35020 Legnaro, PADOVA, Italy.

Tel: +49-8272922
Fax: +49-8272929
E-mail: sergio.casella@unipd.it

1. Introduction

In the future, because of oil shortage and environmental problems, synthetic plastics will surely be replaced by alternative, biodegradable materials. A possible good example could be polyhydroxyalkanoates (PHAs) [1]. These relatively new biopolymers are polyesters potentially usable in a number of different applications because of having properties similar to petrochemical thermo-plastics and/or elastomers [2,3,4]. Nevertheless, their substitution over the conventional plastics is limited by their expensive manufacturing. Therefore, the search for suitable and cheap feedstocks for PHAs is one of the main issues in their entire production chain, representing up to 50% of the process costs [5,6]. Inexpensive and abundant by-products have been exploited for the production of polymers, fuels, enzymes and bulk chemicals [7-13].

The use of industrial or agricultural residues can effectively decrease also PHAs cost [14-18]. Waste fats and oils from agricultural and food industries are also potentially useful feedstocks for fermentation processes to produce bioplastics, fuels and surfactants [19-21]. Only lately triglycerides from agricultural and food processing industries were considered as carbon substrates for PHAs production [22-24]. In this perspective, fatty residues from slaughterhouse plants could serve as cheap carbon source for the microbial PHAs production. These by-products are continuously produced in huge quantities worldwide,

resulting in expensive management problems. For instance, the yearly volume of animal lipids from the slaughtering processing has been calculated with more than 500,000 ton [25,26].

However, the cost-effective production of PHAs from animal lipids has not yet been achieved because microbes with the combination of substrate-utilization and PHAs production are not currently available. To obtain PHAs from waste oils or fats from food industries, which is the aim of the present research, bacteria should be both outstanding PHAs producers and proficient in hydrolysing triglycerides and using the resulting acyl chains to grow and/or accumulate PHAs. Theoretically, such bacteria could be obtained mostly applying two research approaches: 1) the engineering of non-lipolytic micro-organisms with native and excellent aptitude for high PHAs product yields and accumulation or 2) the search for novel natural strains capable of efficiently converting lipids into PHAs.

Within the first strategy, we lately developed a recombinant lypolytic strain of *Delftia acidovorans* DSM39 capable of processing several slaughterhouse by-products, such as lard, udder and tallow, into valuable PHAs with high molar fractions of 4-hydroxybutyrate (4HB) [27].

Regarding the second approach, few microbial strains have been recently isolated from soil and slaughterhouse wastewater as encouraging lipolytic and PHAs producing microbes [28]. Nonetheless, PHAs levels and yields were still far from the standards required for industrial applications and further studies are required to pave the way for their efficient exploitation as PHAs producers from fatty waste streams.

In this study, we evaluated the direct use of waste animal fats as carbon feedstocks for PHAs using collections strains as well as novel soil isolates as biocatalysts. Various industrially relevant fats (crude glycerol from biodiesel, biodiesel obtained from fatty residues, bacon rind, udder and tallow waste oil) were used as substrate for both lipase activities and PHAs production. Among the fourteen tested strains, two collection microbes, namely *Cupriavidus* (*C.*) *necator* DSM 545 and *Pseudomonas* (*P.*) *oleovorans* DSM1045, have been selected for their great promise and further investigated to assess their real potential to process several fatty by-products into PHAs.

2. Materials and methods

2.1 Isolation of lipolytic Bacteria, culture media and growth conditions

Within a wilder isolation programme started from 2011, indigenous bacteria from soil of the experimental station of the University of Padova and from waste waters of different slaughterhouses were isolated by plate dilution standard methods [28]. Agar plates of minimal salts medium (MSM) containing commercial corn oil (2.5% w v^{-1}) or commercial lard (2.5% w v^{-1}) as only carbon source and arabic gum (1.0% w v^{-1}) as emulsifier were used for strain selection. Isolated colonies were then streak-plated onto both solidified MSM and nutrient medium containing corn oil and Rhodamine B (Sigma) (0.001% w v^{-1}) and incubated at 30 and 37°C. Lipolytic activity associated with bacterial colonies was visualised using a hand-held UV transilluminator (Model UVGL55; UVP Inc., CA, USA) at a wavelength between 350 and 365 nm [29,30].

For the identification of the isolated bacteria, genomic DNA was extracted and purified as described, its purity assessed, and universal primers R1n (5'-GCTCA-GATTGAACG-CTGGCG-3') e U2 (5'-ACATTTC ACAACACGAGCTG-3') used to amplify a 16S rDNA fragment of about 1-kb [31,32]. QIAquick PCR Purification kit (Quiagen) was used for PCR product purification which was then resuspended in 30 µl deionised water. The dideoxy chain termination method was subsequently used for DNA sequencing by an ABI Prism 3100 DNA Analyzer, using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) according to the manufacturer. 16S rDNA sequence similarities were searched in GenBank and RDP databases [33].

For growth and polymer accumulation the selected isolates were cultured by one-step procedure [34]. The production of the necessary biomass was obtained in NB or in minimal salts medium MSM or DSMZ81 (Deutsche Sammlung Mikroorganismen und Zellkulturen GmbH, http://ww-w.dsmz.de/, Germany) containing 3% fatty substrates as unique carbon source and 1 gl^{-1} NH_4Cl. The lipid-rich by-products were glycerol, crude glycerol from biodiesel and, from slaughterhouse, biodiesel obtained from fatty residues, lard, bacon rind, udder and tallow (produced as waste streams and kindly provided by the animal-processing company U. Reistenhofer GmbH, Austria). Solid carbon sources were finely minced and added to the medium before sterilization. All liquid cultures were grown at 30 or 37°C in shaking flasks (150 rpm). When necessary, media were solidified by the addition of 1.5% w v^{-1} agar.

2.2 Biomass and cellular protein measurements

The bacterial biomass was measured by drying to constant mass at 80°C a washed pellet obtained from 10 ml of the bacterial suspension. The Coomassie plus protein assay reagent kit from Pierce (Rockford, IL, USA) was adopted to determine cell protein, by using bovine serum albumin as a standard.

2.3 Lipase assay

A titrimetric assay was used to measure lipase activity of both the new isolates and collection strains [35]. The supernatant of the bacterial cultures (5 ml) was added to 50 ml of 5% v v^{-1} olive oil or corn oil emulsion in 50 mM Tris-HCl buffer (pH 8), containing 5% w v^{-1} of arabic gum, and the mixture incubated at 37°C for 3 h. Every 30 min 5 ml were taken and analysed. The reaction was stopped by

adding 10 ml ethanol and the released fatty acids were titrated against 0.05 M NaOH using phenolphthalein as an indicator. The amount of fatty acid released was calculated by using the difference in titer values between samples and blank. The amount of enzyme releasing 1 µmol of fatty acid per minute under assay conditions was defined as one unit of lipase activity.

2.4 Analyses of PHA

The ability to accumulate PHA was tested by a preliminary plate Nile Red staining as previously described [27]. The concentration of PHAs was determined in centrifuged cells as previously described [36]. A Thermo Finnigan Trace GC, equipped with FID detector and AT-WAX capillary column (30m × 0.25mm × 0.25 µm, Alltech Italia s.r.l., Milan, Italy) and helium as gas carrier, at flow rate 1.2 ml min, were used to quantify 3-hydroxyalkyl esters. The split/splitless injector, detector and oven temperatures were 250, 270 and 150°C, respectively. GC-temperature programme was as previously described [37]. Benzoic acid served as internal standard, and the external standards, purchased from Sigma-Aldrich (Italy), were: Poly (3-hydroxybutyric acid) (PHB), Poly (3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) P (3HB-*co*-12 mol% 3HV). Results were expressed as percentage of PHAs on cell dry mass.

PHA monomer composition was determined by nuclear magnetic resonance (^{1}HNMR), by using a Varian Gemini 200 (200 MHz) spectrometer interfaced with a Sparc4 (Sun) console and software VNMR6.1B [38]. Spectra were processed by using Mestre software. NMRspectra were recorded on 2% w v^{-1} solutions, by using $CDCl_3$ as solvent.

3. Results and discussion

Within a long term isolation programme started from 2011, a number of new isolates and collection PHA-producing microbes were screened for lipolytic activities [28]. After a base investigation and subsequent first selection on their ability to produce PHA from simple and related carbon sources, a variety of lipid-rich residues have been explored as substrate, such as glycerol, crude glycerol from biodiesel and, from slaughterhouse, biodiesel obtained from fatty residues, lard, bacon rind, udder and tallow. Interestingly, the most active isolates, in terms of PHA production, never resulted to be those isolated from slaughterhouse (data not shown), thus indicating that within such a specific environment, exceptionally rich in carbon (from fats) and nitrogen (from animal protein degradation) sources, the bacteria find constantly suitable conditions to grow and reproduce and did not evolve the specific need to accumulate reserve polymers. In other words, they are only easily surviving thanks to their lipase activities. On the other hand, the soil represents an extremely wide environment where microorganisms are induced to rapidly evolve different metabolic activities while competing with high numbers of different microbial species, and where many more essential growth components could not be continuously found. Among the soil isolates, indeed, some strains gave appreciable PHA values when incubated in the presence of simple fats and glycerol, and further, it was also possible to select bacterial strains able to produce the polymer from biodiesel and lard (Table. 1).

In the same Table. 1, the preliminary results obtained by the collection strains are also reported. While *Acinetobacter venetianus* DSM 23050 showed the highest lipase activity, this strain was unable to produce appreciable amounts of polymers from glycerol and biodiesel.

However, as observed by further growth and polymer production experiments performed in flasks (data not shown), the efficiency of the new isolates as compared to previously selected known collection strains, was definitely lower.

On this basis, two strains, *C. necator* DSM 545 and *P. oleovorans* DSM 1045 have been selected for their ability to grow on such feedstocks and exhibiting the highest PHAs yields.

C. necator DSM 545 was grown firstly in DSMZ81 solid and liquid medium using crude glycerol phase (CGP), biodiesel and pure glycerol, stearic acid methyl ester, palmitic acid methyl ester and myristic acid methyl ester. The aim of testing bacterial growth in these last three acid methyl esters was to check if the strain could use the saturated fraction of the biodiesel, mainly composed by a mixture of them. As shown in Table. 2, *C. necator* DSM 545 can use pure glycerol and biodiesel to grow and produce PHA and, with less efficiency, crude glycerol and the three methyl esters.

In a second set of experiments (Table 3), different combinations of substrates were tested, as well as other carbon sources. The results indicated that *C. necator* DSM 545 is able to use all the carbon sources for growth and PHA accumulation. The addition of biodiesel to glycerol can serve as co-substrate to introduce more hydroxyl-valerate monomers units into the polymer, probably due to its possible and variable margaric acid content [39].

Table 1. A selection of bacterial collection strains and new soil isolates showing promising lipase activity and a preliminary test on different lipid-rich containing substrates for their direct production of polyhydroxyalkanoates.

			PHAs*			
	Strains	Lipase **	Glycerol	Biodiesel	Oil	Lard
	Collection strains					
DSM 63	*Hydrogenophaga palleroni*	0.32	++	+	+	+
DSM 1045	*Pseudomonas oleovorans*	0.27	++	++	++	++
DSM 3456	*Pseudomonas fragi*	0.18	+	+	++	+
DSM 545	*Cupriavidus necator*	0.19	++	++	++	++
DSM 13225	*Diaphorobacter* sp.	0.13			+	+
DSM 23050	*Acinetobacter venetianus*	0.57			++	+
	Soil isolates					
SC-S1	*Leifsonia* sp.	0.30	+	+	+	+
SC-S5	*Enterobacter* sp..	0.19	+	+	+	+
SC-N1	*Pseudomonas* sp.	0.37	++	+	+	+
SC-N22	*Pseudomonas* sp.	0.52	++	+	+	+
SC-93	*Bacillus* sp.	0.39	+	+	+	+
SC-95	*Staphylococcus* sp.	0.25	+	+	+	+
SC-96	*Staphylococcus* sp.	0.55	+	+	+	+
SC-97	*Rhodococcus* sp.	0.20	+	+	+	+

* Nile Red test, PHAs= polyhydroxyalkanoates.

** Specific activity: µmol (minute × ml)$^{-1}$: one unit of lipase activity was defined as the amount of enzyme that released 1 µmol of fatty acid per minute under assay conditions.

Table 2. Growth and PHA production of *Cupriavidus necator* DSM 545 on different carbon sources. The accumulation step was maintained for 48 h. The values represent the means of three replicates and SD is always below 7%.

Carbon source	CDM (g l^{-1})	PHA (g l^{-1})	PHA (% of CDM)
Pure glycerol	9.2	4.8	52.9
Crude glycerol	5.1	2.1	41.4
Biodiesel	11.6	5.9	51.6
Stearic acid methyl ester	2.9	0.8	29.3
Palmitic acid methyl ester	2.8	0.9	32.3
Myristic acid methyl ester	3.2	1.2	37.1

Table 3. Polyhydroxyalkanoates production of *Cupriavidus necator* DSM 545 on further combinations of carbon sources. The accumulation step was maintained for 72 h. The values represent the means of three replicates ± SD.

Sample no.	Carbon source	PHA (% of CDM)
1	Pure glycerol	90±2
2	Pure glycerol + 2.5% v v^{-1} biodiesel	67±12
3	Crude glycerol	25±11
4	Crude glycerol + 2.5% v v^{-1} biodiesel	37±8
5	Oleic acid	41±9
6	Bacon rind	11±2
7	Udder	55±13
8	Tallow	23±2
9	Waste oil	21±7
10	Enzymatic digested waste oil	81±17

Such an effect was confirmed by ^{1}H-NMR analysis spectra of some samples, as shown in Fig. 1. First of all, the graphs analysis indicates that all the polymers produced from the different substrates that have been tested (samples n. 1, 2, 7 and 10 of Table. 3) are P (3HB-co-3HV) copolymers and their 3HV content varies from 0.99 to 2.48. Indeed, leaving aside the signals observed at about 7.2 ppm due to $CDCl_3$ impurities and the peaks at 5.25 ppm, 2.55 ppm and 1.30 ppm ascribable to 3-hydroxybutyrate, the presence of 3-hydroxyvalerate in the copolymers was revealed by the peaks at 0.95 ppm and 1.65 ppm.

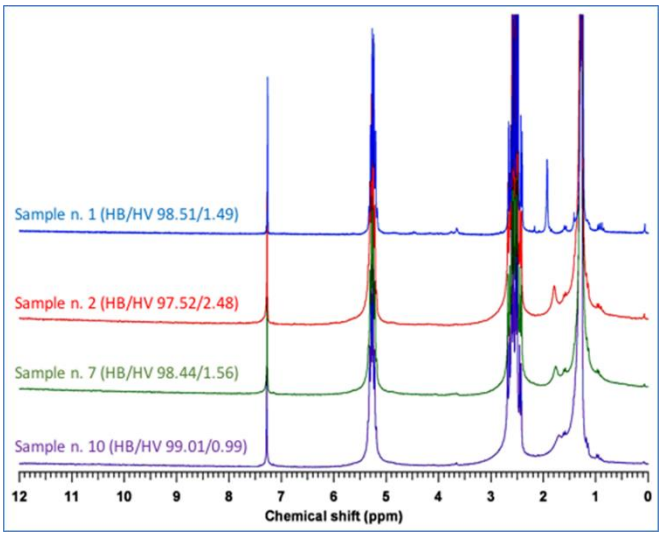

Figure 1. ^{1}H-NMR analysis spectra of polymers extracted from *C. necator* DSM 545 grown on pure glycerol (Sample 1), pure glycerol + 2.5% v v^{-1} biodiesel (Sample 2), udder (Sample 7) and enzymatic digested oil (Sample 10) as reported in Table 3.

In a similar and parallel series of growth and PHA production tests, *P. oleovorans* DSM 1045 was used. Even in this case, the strain was able to produce both biomass and polymers (Table 4), although with amounts lower than those obtained by *C. necator*.

Table 4. Growth and polyhydroxyalkanoates production of *Pseudomonas oleovorans* DSM 1045 on different carbon sources. The accumulation step was maintained for 48 h. The values represent the means of three replicates and SD is always below 5%.

Carbon source	CDM (g l^{-1})	PHA (g l^{-1})	PHA (% of CDM)
Pure glycerol	6.3	2.9	46.7
Crude glycerol	4.0	0.8	20.2
Biodiesel	9.5	2.5	16.3
Stearic acid methyl ester	4.7	1.0	21.3
Palmitic acid methyl ester	5.8	1.6	27.5
Myristic acid methyl ester	4.9	1.7	35.6

However, this observation has to be only partially considered because all the experiments were conducted in flasks and, once again, in a second set of experiments, the values appeared to be higher (Table 5).

Although more sporadically, even in the case of *P. oleovorans* the PHAs obtained were co-polymers containing 3HV fractions (data not shown). This observation, on both *P. oleovorans* and *C. necator*, represents an interesting starting point to deeply investigate on the different structures of the polymer potentially produced by these strains, obtainable by calibrating both the composition of the growth media and the incubation conditions.

4. Conclusion

This research can be considered as preliminary within a wider programme of isolation, characterization, selection and development of superior microbial strains. Once again, the soil proved to be the most promising mining place to find new interesting microbial species, even better than more specific and selective environments such as slaughterhouses. However, newly isolated strains are almost never ready at once to be used for any possible application.

Table 5. Polyhydroxyalkanoates production of *Pseudomonas oleovorans* DSM 1045 on further combinations of carbon sources. The accumulation step was maintained for 72 h. The values represent the means of three replicates ±SD.

Sample n.	Carbon source	PHA (% of CDM)
1	Pure glycerol	71±12
2	Pure glycerol + 2.5% v v^{-1} biodiesel	46±17
3	Crude glycerol	26±9
4	Crude glycerol + 2.5% v v^{-1} biodiesel	21±7
5	Oleic acid	32±2
6	Bacon rind	8±2
7	Udder	15±3
8	Tallow	13±2
9	Waste oil	21±6
10	Enzymatic digested waste oil	76±14

The collection strains used here were known to be PHAs producers, but their ability to utilize fats as substrate was

almost unknown. Remarkably, two of them resulted as really promising, being able to grow directly on all the substrates tested and to produce variable amounts of the polymer. Even more notable is the finding that these strains, especially *C. necator* DSM 545, could produce co-polymers (P(3HB-co-3HV)) even starting from glycerol as the only carbon source.

Some combinations of strain/substrate/incubation conditions indicated that both growth and polymer production are very variable, especially if performed in small scale experiments such as flasks. As always observed in a scale-up from flask to bioreactor until big size plant, the production could be considerably improved, for instance by using a double step process, thus suggesting that the results here obtained, also starting directly from slaughterhouse residues, could be significantly upgraded [40].

Finally, it is interesting to note that the substrates here tested could be used for the production of both *scl*- and *mcl*-PHAs. Indeed, *C. necator* is known to possess class I synthases that utilize short chain length hydroxyalkanoate monomers, while class II synthases are typical of pseudomonads able to polymerize medium chain length PHAs [41,42].

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6. Conflict of Interest

The authors declare no conflict of interest.

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تولید باکتریایی پلی‌هیدروکسی‌آلکانوآت‌ها از فرآورده‌های جانبی غنی از لیپید

لورنزو فاوارو[۱]، مارینا باساگلیا[۱]، جیسس انریکه گامرو رودریگز[۱]، آندرآ موریلی[۲]، اومودلا ایبراهیم[۳]، والنتینو پیزوکرو[۱]، سرجیو کاسلا[۱*]

۱- گروه زراعت، مواد غذایی، منابع طبیعی، حیوانات و محیط زیست، دانشگاه پادووا، آگریپلیس، دانشگاه ویاله ۱۶، ۳۵۰۲۰ لگنارو، ایتالیا.

۲- گروه شیمی و مهندسی شیمی، دانشگاه پیزا، ویا جی موروزی ۱۳، ۵۶۱۲۴ پیزا، ایتالیا.

۳- گروه بیوشیمی، دانشگاه دولتی ایالت اوی-اکیتی، ایالت اکیتی، نیجریه.

چکیده

سابقه و هدف: به علت کمبود نفت و مشکلات زیست محیطی، تولید پلاستیک مطمئناً با مواد زیست‌تجزیه‌پذیر جایگزین خواهد شد. یک نمونه خوب پلی‌هیدروکسی‌آلکانوآت‌ها[۱] و فرآورده‌های جانبی ارزان کشاورزی می‌توانند به کمک میکروب‌های به درستی انتخاب شده و یا توسعه یافته به ترکیب مفید پلی‌هیدروکسی‌آلکانوآت‌ها تبدیل شوند.

مواد و روش‌ها: از بین فرآورده‌های جانبی در دسترس معمول، انواعی از باقیمانده‌های غنی از لیپید مانند گلیسرول خام حاصل از بیودیزل، بیودیزل حاصل از باقیمانده چربی های به دست آمده از کشتارگاه‌ها و حاصل از پیه، پوست و پستان خوک به عنوان رشدمایه[۲] مورد بررسی قرار گرفتند. در این مقاله، چندین میکروب جدید تولیدکننده پلی‌هیدروکسی‌آلکانوآت[۳] جداسازی و گردآوری شده به منظور تولید پلی‌هیدروکسی‌آلکانوآت‌ها و فعالیت لیپولیتیکی‌شان غربالگری شدند. ثابت شد که خاک امیدوارکننده‌ترین محل معدنی برای پیدا کردن گونه‌های میکروبی جدید، حتی بهتر از محیط‌های انتخابی و اختصاصی‌تر مانند کشتارگاه‌ها می‌باشد.

یافته‌ها و نتیجه‌گیری: به طور قابل ملاحظه‌ای دو گونه از گونه های گردآوری شده مورد استفاده در این تحقیق به عنوان تولیدکننده‌های پلی‌هیدروکسی‌آلکانوآت‌ها شناخته شده‌اند. در نتیجه به طور امیدوارانه‌ای، قادر به رشد مستقیم روی تمام زیست‌مایه‌های مورد آزمون و تولید مقادیر متغیری بسپار[۴] از جمله زیست‌بسپارهای P(3HB-co-3HV) می‌باشند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- *کاپریاویدوس نکاتور*
- فعالیت لیپاز
- ضایعات لیپیدیریا
- *سودوموناس اولئووارانس*
- پلی هیدورکسی آلکانوآت ها

***نویسنده مسئول**

سرجیو کاسلا، گروه زراعت، مواد غذایی، منابع طبیعی، حیوانات و محیط زیست، دانشگاه پادووا، آگریپلیس، دانشگاه ویاله ۱۶، ۳۵۰۲۰ لگنارو، ایتالیا.

تلفن: ۸۲۷۲۹۲۲-۴۹+
دورنگار: ۸۲۷۲۹۲۹-۴۹+
پست الکترونیک:
sergio.casella@unipd.it

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۱ (PHAs)
۲ (Substrate)
۳ PHA
۴ (Polymer)