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Abstract

The growing uses of functional polymers in different domains and the well-established remanence of synthetic polymers underline the necessity to study their microbial biodegradation. A phylloplane bacterial collection was screened for its potential to overcome documented factors limiting polymer biodegradation, i.e. low cleavable long chain linear polymers and low bioavailable hydrophobic compounds. Acrylate-based polymers, namely poly(2-hydroxyethyl methacrylate) (pHEMA), poly(N-isopropylacrylamide) (pNIPAM), poly(acrylamide) (pAM) and poly(acrylic acid sodium salt) (pAS), and a hydrophobic crosslinker Epoxidized Soybean Oil Acrylate (ESOA) were used as selective substrates. OD monitoring of growth with these molecules as sole carbon sources were correlated with substrates alterations determined by ^{1}H NMR, GPC and FTIR. Selected strains belong to Bacillales Family XII. Incertae Sedis, Bacillaceae, Nocardiaceae, Micrococcaceae, Enterobacteriaceae and Pseudomonadaceae. *Bacillus* species mainly developed with ESOA. *Exiguobacterium sibiricum* strains could grow with pHEMA and pNIPAM leading to polymer molecular weight decreases. *Arthrobacter koreensis* showed the highest alteration capacities of pHEMA with molecular weight decreases from 22,000 to 3,148 g/mol and the production of presumed aromatics and carbon chains.

Key words

Biodegradation; Hydrophobic monomer; Long chain backbones; Natural biodiversity; Crops phyllosphere; Acrylate derivatives

1. Introduction

Numerous studies have recently focused on the environmental fate and biodegradation of packaging plastics due to their high stability and their resistance to environmental stresses (Restrepo-Flórez *et al.*, 2014). Nevertheless, other classes of polymeric materials such as engineering and high-performance plastics did not draw as much attention despite several characteristics identified as biodegradation or bioavailability key limiting factors, e.g. long backbones or hydrophobicity (Leja and Lewandowicz, 2009).

Polyacrylate based polymers and notably poly(2-hydroxyethyl methacrylate) (pHEMA), poly(*N*-isopropylacrylamide) (pNIPAM), poly(acrylamide) (pAM) and poly(acrylic acid sodium salt) (pAS) have been widely used in biotechnological and industrial applications. pHEMA, pNIPAM and pAS have found applications in biomedical devices (Huang *et al.,* 2013; García-Millán *et al.*, 2015; Huber *et al.*, 2016; Taddei *et al.*, 2017), cell culture materials (Rzaev *et al.*, 2007) or drug delivery systems (Trigo *et al.,* 1994; Liu *et al.*, 2016). pNIPAM, pAM and pAS are used in water treatment as flocculants or for their sorbent properties (Kenawy *et al.*, 2013; Lee *et al.*, 2014; Nga *et al.*, 2018; Wong *et al.*; 2006). pAS has been employed as water sorbent in agricultural soils (Wenhua *et al.*, 2008).

The areas of use of these functional polymers expose them to shear, oxidative, thermal or biological stresses that may cause wear and a partial or total release in the environment either as dust or water effluents. Besides, direct amendments to prevent post-fire runoff, soil erosion (Prats *et al*., 2014) or heavy metal pollutions as well as waste disposal are sources of soil contaminations (Santos *et al.*, 2013). However, the end of life and environmental fate of functional polymers are still not well determined. Indeed, as biomedical device component, pHEMA biodegradation was mainly studied in mammalians rather than in microorganisms. A previous study of our laboratories reports the isolation of bacterial strains able to grow with pHEMA as sole carbon source from an oil-contaminated soil, but polymer chemical alterations were not characterized (Zhao, 2015). Similarly, few studies extensively investigated pNIPAM toxicity and biodegradation, although the monomer is reported as a toxic compound (U.S. EPA DSSTox Database, Substance ID: DTXSID0033754). *Pantoea* (*Enterobacter) agglomerans, Azomonas macrocytogenes* (Nakamiya and Kinoshita, 1995), *Bacillus cereus* (Wen *et al.*, 2010)*, Bacillus sphaericus* and *Acinetobacter sp.* (Matsuoka *et al.*, 2002) strains were reported able to grow with pAM as sole carbon source. To our knowledge, *Arthrobacter* sp. NO-18 (Hayashi *et al.*, 1993) is the only identified bacteria reported to catabolize pAS as pure culture.

Vegetable oils have been widely investigated as a valuable alternative to hydrocarbons for the development of new plastic materials (Islam *et al.*, 2014; Samarth and Mahanwar, 2015; Hatti-Kaul *et al.*, 2019). In particular, the modification of vegetable triglycerides by epoxidation and the ring-opening with acrylic acid derivatives formed new bio-based monomers such as Epoxidized Soybean Oil Acrylate (ESOA). ESOA can be used for the preparation of polymers with high impact resistance, low electrical properties and high hydrophobicity (Zhang *et al.*, 2017). However, bio-sourcing does not always imply a good biodegradability and the environmental fate of this new generation of oily substrates has mostly to be investigated.

Phyllosphere constitutes a naturally hydrocarbon rich compartment, mainly composed of plant waxes (Riederer and Müller, 2006) consisting in acids, alcohols, esters, aldehydes, ketones and alkanes with very long carbon chains (Kunst and Samuels, 2003; Buschhaus *et al.*, 2007; Buschhaus and Jetter, 2012) and some cyclic terpenoids in the intracuticular wax layer. This specific environment is likely to favor microbial adaptations enabling enhanced access toward hydrophobic substrates and metabolization of complex compounds. Incidentally, several efficient hydrocarbon-degrading bacteria have been isolated from the phyllosphere such as *Acinetobacter*, *Pseudomonas*, *Mycobacterium, Arthrobacter, Bacillus, Rhodococcus* (Yutthammo *et al.*, 2010, Al-Awadhi, 2012). Besides, phyllosphere was not reported to increase human pathogens proportion within microbial communities in contrast to plastics impacted environments (Jacquin *et al.*, 2019; Puglisi *et al.*, 2019).

In this study, we hypothesize that crops phyllosphere natural biodiversity includes bacteria capable of altering functional polymers through their adaptation to low cleavable long linear chains and low bioavailable substrates.

2. Materials and Methods

2.1. Leaves sampling

Entire leaves without any visible lesions were collected from crop fields in the Hauts-de-France region (France) using single-use clean plastic bags and laboratory gloves and were kept at 4°C for 1 month until the isolation step. Four crops were selected and identified through the information about the fields and their morphological properties (Bonnier and De Layens, 1986): cabbage (*Brassica oleracea* L.), corn (*Zea mays* L.), rape (*Brassica napus* L.) and sugar beets (*Beta vulgaris* L.).

2.2. Suspension dilution isolation technique

50 disks of 1 cm^2 were cut out from the leaves limb of each plant variety with a sterile round cutter. Disks were soaked in 50 ml of sterile water in a 100 ml flask (adapted from Dickinson *et al.*, 1975). The flask was agitated 1h at room temperature on an orbital shaker at 150 rpm and then sonicated 1 min at 47 Hz. 100 µl of 10-fold serial diluted suspensions (10^{-1}, 10^{-2} and 10^{-3}) were plated on Tryptic Soy Agar (TSA) containing 5 mg/L of cycloheximide to inhibit fungal development. The Petri dishes were incubated at 35°C over a week. Single colonies were isolated every day on fresh TSA medium by the streaking method and kept at 4°C after that colonies developed.

2.3. Foliar imprints isolation technique

For each plant variety, a disk of 1 cm^2 was cut into a leaf with a sterile round cutter. The upper face was pressed directly on a TSA plate with sterile tweezers. Successive imprints were done on a same Petri dish (adapted from Dickinson *et al.*, 1975). The same protocol was applied to the lower face of the leaf. This protocol was repeated three times per plant. Petri dishes were incubated over a week at 35°C. Single colonies were daily sampled from the imprints, isolated on TSA medium by the streaking method and conserved at 4°C.

2.4. Sequencing and Phylogenetic Analysis

DNA was extracted from isolated bacteria freshly cultured on LB medium according to Serghini *et al.* (1989) and quantified by OD at 260 nm. 250 ng of each DNA sample was submitted to hot-start amplification (40 cycles: 2 min 94°C, 1min 55°C, 1 min 72°C; final elongation: 7 min 72°C) in a Perkin-Elmer/Cetus 480 DNA Thermal Cycler. The 16S 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534-R (5'-ATTACCGCGGCTGCTGG- 3') PCR primers encompassing V1 to V3 hypervariable regions of 16S rDNA (Lane, 1991; Turner *et al.*, 1999) were obtained from Eurofins Genomics (Ebersberg, Germany). The other PCR components were purchased from New England Biolabs France (Evry, France) (Muyzer *et al.*, 1993). PCR amplification products were verified on a 1% agarose gel. Sanger sequencing data were generated by Eurofins Genomics (Ebersberg, Germany) and were compared to the Ribosomal Database Project (RDP release 11, http://rdp.cme.msu.edu) and GenBank at NCBI (https://blast.ncbi.nlm.nih.gov) with Basic Local Alignment Search Tool (BLAST) (D'Aquila *et al.*, 1991), allowing strains identification. The sequences have been registered in Genbank under accession numbers MT159394-MT159410.

2.5. Microplate OD variation measurement screening method

10 ml of TSB (Tryptic Soy Broth) in 50 ml conical bottom tubes were inoculated with colonies of each isolate and incubated 24h at 30°C on a rotary shaker (180 rpm). After centrifugation (3000 rpm, 10 min), pellets were washed twice by manual shaking with Mineral Medium (MM), (250 mg of KCl, 6.464 g of NaH_2PO_4, $2H_2O$, 10.408 mg of Na_2HPO_4, $2H_2O$, 244 mg of $MgSO_4$, $7H_2O$, 1 g of NO_3NH_4, 10 µg of $ZnSO_4$, $7H_2O$, 1 µg of $MnCl_2$, $4H_2O$, 10 µg of $FeSO_4$, $7H_2O$, 5 µg of $CuSO_4$, 1 µg of $CaCl_2$, 2 µg of MoO_3 per liter, pH 7) for 10 minutes to eliminate nutrients, particularly carbon sources remaining from TSB. Final pellets were suspended in MM by manual shaking and successive pipetting until disappearing of aggregates and diluted to reach an absorbance of 0.4 at 600 nm. 10 µl of this suspension were inoculated to microplates (96 well Nunc™ edge 2.0 micro-plates, ThermoFisher Scientific) already containing 190 µl of mineral medium supplemented with the different carbon sources (carbon equivalent concentration: 2 g/L, table 1). The selective substrates pHEMA (20,000 g/mol), pNIPAM carboxylic acid terminated (5,000 g/mol), pAM (40,000 g/mol), pAS (5,100 g/mol, 8-14 % water) and ESOA (purity ≥ 99 %, contains 4,000 ppm monomethyl ether hydroquinone as inhibitor) were purchased from Merck (France). ESOA was dispersed in the mineral medium by sonication, 2 minutes, 40 kHz (Branson Bransonic® CPXH 5800, Emerson). Negative controls

(inoculated MM without carbon source) and positive controls (inoculated MM containing 5 g/L of glucose) were included and culture conditions were conducted in triplicates. Triplicates of non-inoculated MM with glucose were also used as blank to check the absence of contamination.

Micro-cultures were incubated at 30°C under constant agitation and bacterial growth was estimated after 7 days of incubation measuring absorbance at 600 nm (Multiskan GO microplate reader, SkanIt program, ThermoFisher Scientific). Isolates showing the highest absorbance variations with the best diversity of substrates were selected for further studies. Results of the selected strains were processed through Principal Component Analysis with a Pearson correlation model using the XLSTAT software.

2.6. Macro-cultures and growth kinetic monitoring

$OD_{600\ nm}$ evolution kinetics were performed on the selected isolates using macro-cultures. Inocula were prepared and calibrated as described in section 2.5. 5 ml of mineral medium containing the selected substrates (table 1) were distributed in 15 ml bottom tubes, inoculated with 250 µl of adjusted bacterial suspension. 200 µl of these macro-cultures were immediately transferred in a 96-well microplate in triplicate and incubated 4 days at 30°C under constant agitation in order to follow the bacterial growth kinetics in parallel to the macro-cultures incubation. Growth kinetics were followed at 600 nm (Day 1: every 15 minutes, Day 2 : every 30 minutes, Day 3 and 4 : every hour). In parallel, the leftover macro-cultures were incubated at 30°C under constant agitation (180 rpm). After 4 days, macro-cultures were centrifuged 10 min at 3000 rpm, supernatants were kept for analytical measurements and pellets were freeze dried to determine the dry biomass weights. To assess abiotic polymer modifications and losses, polymers in MM were incubated in the same conditions.

Growth kinetics of cultures were characterized determining maximal ΔOD_{600nm} over 7 days and exponential growth rate µ, defined by the equation below

$$\mu = \frac{\ln OD_{600nm\ ty} - \ln OD_{600nm\ tx}}{\ln t_y - \ln t_x}$$

where t_x and t_y are exponential period boundaries. Results are presented as averages over triplicates with the corresponding standard deviations as error bars.

Growth kinetics of the strains studied for polymers alteration are presented as curves of the average OD_{600nm} of triplicates according to time with corresponding standard deviations as error bars.

2.7. Polymer structure analyses

ATR-FTIR spectra (4000-600 cm^{-1}, 200 scans, 4 cm^{-1} resolution) were measured on cultures and controls supernatants using a Thermo Nicolet 6700 spectrophotometer (ThermoFisher scientific) equipped with a ZnSe ATR (attenuated total reflectance) system and MCT-B detector.

^1H NMR was performed on all the polymers and culture supernatants. NMR spectra were recorded on Bruker Avance 400 MHz spectrometer using a direct BBFO probe-head. 600 µl of the culture supernatants were freeze dried and the dry samples were solubilized in 600 µl D_2O for pHEMA, pNIPAM and $CDCl_3$ for ESOA. Pyridine was used as an internal standard at a concentration of 10 µM.

The polymers molecular weight was determined by gel permeation chromatography. pHEMA and pNIPAM were analyzed in DMF + 0.1 % LiBr (w:v) and pure THF (Biosolve) respectively. 600 µl of the samples were freeze dried and resuspended in 300 µl of the corresponding analytical solvent. Polymer suspensions were centrifuged 10 min at 3000 rpm to pellet the insoluble materials. Size exclusion separations were performed on a HPLC–UV/RI/MALS coupling consisting of a LC U3000 system (DIONEX) connected to a refractive index (RI) detector RI-101 (Shodex) or a DAWN 8+ MALLS detector (Wyatt) UV. RI data were processed with the Chromeleon Data System (DIONEX) and MALLS data with ASTRA software (Wyatt). Sample extracts were separated on a 2 PLgel Mixed-C column (300x 7.5 mm, 5 µm, Agilent) thermostated at 30 °C. Flow rate was 1 ml/min and separation was performed during 30 min. For RI detection, calibration was performed by poly(methyl methacrylate) standard (Sigma-Aldrich).

3. Results

3.1. Selection and identification of phylloplane bacterial isolates

190 phylloplane bacterial strains were isolated from four crops: 58 from sugar beet, 51 from cabbage, 42 from rapeseed and 39 from corn. Four polyacrylic water-soluble linear polymers (pHEMA, pNIPAM, pAA and pAM) and a hydrophobic triglyceride-based monomer (ESOA) were used as selective substrates to obtain bacterial strains able to grow on long chains compounds with low susceptibility to enzymatic degradation or with low bioavailability. After 7 days of incubation with one of these substrates as sole carbon source, absorbance variations were recorded and used as a first selective criterion.

98 isolates were selected, because they triggered positive $\Delta OD_{600\ nm}$ with at least one substrate. Considering all selective substrates, proportions of isolates per plant displaying a significant positive $\Delta OD_{600\ nm}$ ranged from 10 to 22 % of the total isolates collection. Selection on ESOA allowed to obtain the highest number of strains (44 isolates), and selection on pNIPAM the lowest (20 isolates). Cabbage, rapeseed and sugar beet were the main sources of isolates growing with ESOA. About 15 % of the bacterial bank showed a positive $\Delta OD_{600\ nm}$ with pHEMA, pAS and pAM, with isolates from the 4 plant species. pHEMA and pAS revealed similar profiles with percentages of isolates from cabbage and sugar beet around 7 % each, and from corn and rapeseed around 2 %. pAM showed a more equilibrated profile between origins with close percentages of isolates from sugar beet, rapeseed and corn (between 2.6 and 4.2 %) and a slightly higher percentage of isolates from cabbage (7 %). Isolates from sugar beet and cabbage were overrepresented regarding growth results with pNIPAM (around 5 % each). Regarding all substrates, sugar beet and cabbage were the most represented degradative strains providers.

17 isolates out of 98 were further selected on the basis of their versatility towards the substrates (absorbance increase for the same strain with different xenobiotics) or the intensity of the growth (highest $\Delta OD_{600\ nm}$ with at least one substrate). In order to prevent the study of artifacts or of non-reproducible growths, $OD_{600\ nm}$ kinetics of the seventeen selected isolates with the substrate(s) suspected to be degraded were checked to fit with classical bacterial growth curves before further characterizations. These isolates were identified through sequencing of their 16S rDNA and belong to 3 phyla (Firmicutes, Actinobacteria and Proteobacteria) covering 6 families (Bacillales Family XII. Incertae Sedis, Bacillaceae, Nocardiaceae, Micrococcaceae, Enterobacteriaceae and Pseudomonadaceae).

3.2. Growth of the selected strains according to the nature of the selective substrate

A principal component analysis (PCA) was performed with the 17 selected strains as an attempt to identify key parameters driving their capacity to grow with the selective substrates (Figure 1). This data analysis was done on $\Delta OD_{600\ nm}$ obtained after 7 days of incubation with all selective substrates.

The PCA indicates a correlation between pHEMA and pNIPAM through the bacterial strains able to grow with them as sole carbon source. Both substrates present side chains on their hydrocarbon backbone, which could be more readily cleaved and used as carbon source. ESOA and pAM appear also correlated, although they do not share any chemical or physical characteristics. The angle formed between pAS and other variables suggests that the values recorded for this substrate are rather independent.

The PCA allowed to distinguish main groups corresponding to the family branches of the phylogenetic classification. This observation suggests that the degradative capacities for the different tested substrates are rather driven by the phylogeny of the bacteria than by their plant of origin. *Exiguobacterium sibiricum* strains, which belongs to the Bacillales Family XII. Incertae Sedis, formed an ellipse corresponding to higher $\Delta OD_{600\ nm}$ with pHEMA and pNIPAM as sole carbon source. Micrococcaceae strains generally showed positive $\Delta OD_{600\ nm}$ with at least 3 substrates including ESOA and pHEMA, which explains their central position. While being a water-soluble polymer, pHEMA also possesses a non-polar methyl group. Thus, changes in the cell membrane hydrophobicity or production of biosurfactants might be common mechanisms to access pHEMA and ESOA in these strains. Nocardiaceae contained two *Rhodococcus* species, whose growths were more favored on

pAM and ESOA, though *R. fascians* showed also a positive $\Delta OD_{600\ nm}$ with pHEMA and appears more central.

3.3. Growth profiles analyses

Growth kinetics were recorded to further characterize growth parameters and in particular maximal growth and exponential growth rate (Figure 2).

The growth using ESOA as unique carbon source was confirmed for 10 out of the 17 bacterial strains. For half of them, growths occurred only with ESOA. Four strains (*Exiguobacterium sibiricum* 2*, Arthrobacter koreensis, Rhodococcus fascians* and *Erwinia persicina*) could grow with both ESOA and pHEMA. *Micrococcus luteus* displayed growth kinetics with ESOA and pNIPAM. The maximal growths were generally higher with ESOA than for the other substrates, which could be in relation with a more efficient use of this molecule. Exponential growth rates observed with ESOA were also higher in average and did not rely upon the maximal absorbance. This could imply diversity in the metabolic pathways enabling the use of this substrate or in the efficiency to have access to it. All the bacterial families were represented among ESOA degraders underlying the fact that oily substrates may be more commonly catabolized. *Bacillus* species were overrepresented (40 %), which is in accordance with the literature widely describing this genus for oily substrate degradation. *Bacillus sp.* 3 showed the highest maximal growth, but had the lowest growth rate. This may imply a low efficiency in the use of ESOA or a limited access rate to it. On the contrary *Bacillus thuringiensis* and *Bacillus sp.* 1 exhibited both high maximal growths and μ values, indicating a good access to the substrate and a high catabolic activity. The highest growth rate was recorded for *E. sibiricum* 2, which was the only *E. sibiricum* strain able to grow with ESOA.

pHEMA led to 10 reliable growth kinetics over the 17 selected strains. 60 % of the strains were identified as *E. sibiricum*. Among them, four grew on pHEMA only, five also with pNIPAM and one also with ESOA. *E. sibiricum* 1 and 6 exhibited the highest maximal growth together with the highest exponential growth rate. The other *E. sibiricum* strains showed lower growth capacities, underlying an intraspecies diversity.

5 strains could grow with pNIPAM as unique source of carbon. The variations of their maximal growth and their exponential growth rate were well correlated for this substrate. As for pHEMA, the species *E. sibiricum* was well represented (3 out of 5 pNIPAM degraders). *E. sibiricum* 1 exhibited the highest maximal growth as well as the highest exponential growth rate for pNIPAM and pHEMA. *E. sibiricum* 3 and 6 as well as *P. nitroguajacolicus* could also use both pNIPAM and pHEMA. Only *M. luteus* grew with pNIPAM and ESOA.

3.4. Substrates chemical alterations by promising isolates

Bacillus sp. 1 , *E. sibiricum* 1 and *A. koreensis* were selected to study the alteration of ESOA, pNIPAM and pHEMA, respectively, because of their high maximal growth coupled with a high exponential growth rate. The characterization of the chemical alterations was conducted through culture supernatant analyses using GPC, ^{1}H NMR and FTIR.

3.4.1 ESOA alterations by *Bacillus sp.* 1

The lack of growth in MM without any carbon source confirms that the observed growth with ESOA is due to the use of this molecule as carbon source. In contrast to the control with glucose exhibiting a lag phase, growth with ESOA starts immediately (Figure 3a). This phenomenon could rely either on a higher specialization toward hydrophobic substrates through hydrophobic cell surfaces or on the production of biosurfactants. ^{1}H NMR analyses were carried out on the supernatants of both abiotic control and bacteria-inoculated culture medium to detect the modification of ESOA and/or the production of new compounds. The abiotic control with ESOA but without bacteria also enabled to check that, despite its hydrophobic nature, ESOA did not totally sediment through centrifugation. Indeed, ^{1}H NMR ESOA characteristic signals could be observed in the control supernatant at 0.9-1.5 ppm (CH_2 and CH_3, fatty chain), 2.3 ppm (CH_2-C=O), 4.1-4.3 (CH_2, glycerol group), 3.5-5.5 ppm (CH_2-O, fatty chain), 5.25 ppm (CH, glycerol group), 5.9-6.4 ppm (acrylic group). The ESOA content in the culture supernatants was estimated by the CH_2-C=O signal intensity as compared to a standard (pyridine) added in the sample. An oil depletion of 83.1 % was recorded in the culture supernatant after incubation. This correlates well with the high maximal growth observed, in good agreement

with the hypothesis of a biodegradation of ESOA by *Bacillus sp.* 1. Acrylate groups could not be observed anymore on the ^{1}H NMR spectra after incubation of ESOA with the bacteria and CH_2 peaks of glycerol group dramatically decreased, excluding only adsorption phenomena. These results rather suggest a hydrolysis of the ESOA ester functions and consequently the production of esterase by the bacteria. Besides, a higher ratio of CH_2 and CH_3 groups to CH_2-C=O group was observed in the culture media spectra, which could not only be attributed to ESOA degradation, but may rely on the production of bacterial extracellular molecules such as biosurfactants (Figure 3b).

The FTIR spectrum of pure ESOA (Figure 3c) shows bands at 2900, 1724, 1627 and 1186 cm^{-1} due to CH stretching, C=O stretching of ester groups, C=C stretching of acrylic groups and C-O-C stretching of ester functions, respectively. The C=C stretching of acrylic groups (1627 cm^{-1}) and C-O-C stretching of ester functions (1186 cm^{-1}) was no more observed after incubation with *Bacillus sp.* 1 (Figure 3c). This result is in accordance with NMR data showing a decrease of the oil content and the disappearance of glycerol and acrylic groups in the presence of the strain. The hypothesis of ESOA hydrolysis by esterases could be confirmed by the presence of signals around 1750 cm^{-1} that can be attributed to a COOH group.

3.4.2 pNIPAM alterations by *Exiguobacterium sibiricum* 1

E. sibiricum 1 showed the most noteworthy growth kinetics with pNIPAM in term of both maximal absorbance and growth rate (Figure 4a). No growth was detected in MM, confirming that growth observed in other culture conditions relied on the presence of a carbon source only. *E. sibiricum* 1 could grow with pNIPAM over the 48 first hours, whereas growth decreased after 27 hours in the positive control containing glucose. The reason of this phenomenon remains to be investigated, but the absence of stationary phase could reflect the production of a toxic metabolite under this culture condition. GPC analyses were performed on freeze-dried culture supernatant in THF in order to evaluate polymer molecular weight modifications. Native pNIPAM has a molecular weight close to 1200 g/mol as determined by Refractive Index (RI) measurements using pMMA as standard. Size exclusion chromatography (SEC) chromatograms of pNIPAM after 4 days of incubation with *E. sibiricum* 1 showed a major peak at 17.4 min, which corresponds to a 470 g/mol molecular weight (Figure 4b). The shift of pNIPAM signal is compatible with an alteration of the polymer such as backbone modification or an amide bond hydrolysis.

^{1}H NMR was performed on the culture supernatant to confirm a possible alteration of the polymer (Figure 4c). ^{1}H NMR profile of culture supernatants after incubation with *E. sibiricum* 1 is very close to the abiotic control. The characteristic peaks of pNIPAM can be observed at 3.76 ppm, 1.94 ppm, 1.5 ppm, 1 ppm corresponding to -C-H of the isopropylamine group, -C-H in position alpha of the amide group, -C=O and -CH_2 groups of the carbon backbone, -CH_3 group of the isopropylamine, respectively. The signals at 2.6 ppm and 2.3 ppm were attributed to the CH_2 groups of the terminal function ($C_3H_5O_2S$). Terminal function of pNIPAM can be used as an internal standard to estimate the relative number of monomer units (noted “n”). ^{1}H NMR underlined a 18.3 ratio of the polymer backbone -CH_2- as compared to the terminal functions ($C_3H_5O_2S$). The n value dropped to 17.2 in the presence of *E. sibiricum* 1. So, the main backbone of pNIPAM seems to be degraded in smaller linear polymeric units. The same method was used to determine the isopropylamine unit number and the ratio between the monomer and isopropylamine unit numbers. A small decrease in this ratio was recorded in the presence of *E. sibiricum* 1. This observation suggests the partial hydrolysis of amide bonds, thus liberating isopropylamine in the culture medium, which may be used as a carbon source for bacterial growth. The low modifications detected by ^{1}H NMR spectra fits well with the low growth of the bacteria observed by OD_{600nm} monitoring.

The culture supernatant was also analyzed by the FTIR technique and compared to the native polymer (Figure 4d). Characteristic pNIPAM bands could be recorded in *E. sibiricum* 1 culture supernatants at 1628, 1558, 1460 and 1390 cm^{-1}, corresponding to amide bands I, II and III, respectively. This observation is in accordance with ^{1}H NMR spectra, which confirmed the presence of remaining pNIPAM in the culture supernatants. New bands around 1700 cm^{-1} and at 1080 cm^{-1} in *E. sibiricum* 1 culture supernatant suggests the appearance of carbonyl groups (C=O stretching) and

C-O or C-N groups of alcohols, lactones or amines. They may be related to polymer oxidation or to the production of new bacterial metabolites.

3.4.3 pHEMA alterations by *Arthrobacter koreensis*

The isolate identified as *A. koreensis* was selected to study the pHEMA alteration (Figure 5). No growth was observed in the negative MM control without substrate confirming that the growth observed in other culture conditions was due to the presence of an organic carbon source. The growth kinetics recorded with pHEMA was similar to the one obtained with glucose in the 10 first hours (Figure 5a). No lag phase was observed, and an exponential growth phase started after 45 min of incubation in both conditions with μ of 0.159 ±0.005 and 0.139 ±0.005 for glucose and pHEMA respectively. This exponential growth stopped earlier with pHEMA, after 6h of incubation, compared to 13h with glucose. The maximum $OD_{600\ nm}$ was 0.165 ±0.015 at 10 hours for pHEMA and then absorbance slightly decreased contrary to glucose for which maximal absorbance reached 0.348 ±0.010 after 45h. This profile could indicate a limitation in usable substrates or an accumulation of toxic metabolites with pHEMA.

A decrease in pHEMA molecular weight, from 21,800 g/mol in the abiotic control to 3150 g/mol in *A. koreensis* culture supernatant was determined by SEC analysis (Figure 5b).

No signal corresponding to native pHEMA could be recorded in *A. koreensis* culture supernatants through ^{1}H NMR analysis (Figure 5c). The presence of new peaks in the region of 7-8 ppm (characteristic of aromatic compounds), and 1-2 ppm (CH_2, CH_3) suggest that new metabolites related to the bacterial development did appear in the supernatants.

The FTIR spectrum (Figure 5d) obtained with the *A. koreensis* culture supernatant was very similar to the pure pHEMA ones with the presence of the characteristic bands at 2940 cm^{-1} and 2855 cm^{-1} (CH), 1702 cm^{-1} (C=O), 1452 cm^{-1}, 1365 cm^{-1}, 1154 cm^{-1} (C-O-O-R), 1071 cm^{-1} (C-OH).

4. Discussion

4.1. Ecology and biodegradation activities of the isolated species

Genera identified in this study are recurrently isolated from soil, water and both phyllosphere and rhizospheres of crops and ornamental plants. *E. sibiricum* have been isolated from different types of soils, including crop fields, permafrost or industrial contaminated soils, as well as from aquatic environments including marine sediments or industrial water effluent (Vishnivetskaya *et al.*, 2009). The isolates selection also includes four species of the *Bacillus* genus, among which one clearly identified as *Bacillus thuringiensis*. The 3 other strains could not be determined at the species level, but presented a 16S rRNA sequence similarity of 98 % with *B. pumilus*, *B. weihenstephanensis, B. simplex*, *B. cereus, B. licheniformis* and *B. subtilis*. All those strains have been previously isolated from very different types of soils such as cotton fields, lakeshores, mangrove (Anwar *et al.*, 2009; Lima de França *et al.*, 2015) and oil contaminated soils (Al-Sharidah *et al.*, 2000; Bezza and Chirwa, 2015; Calvo *et al.*, 2004; Maiti *et al.*, 2013; Kebria *et al.*, 2009). *B. pumilus* and *B. licheniformis* were also isolated from plant rhizospheres (Gutiérrez-Mañero *et al.*, 2001). The *Bacillus* strains identified in this study were recovered from all the sampled plants, underlying the presence of this genus in a diversity of habitats within plant phyllospheres. Rhodococci were also described in seawater, sediments, polluted soils, plant rhizosphere and plant surfaces (Larkin *et al.*, 2006). *Arthrobacter* is representative of soil bacteria (Lee *et al.*, 2003)**.** Among the genus Erwinia, *E. persicina* is a phytopathogenic strain with a broad host range (Zhang and Nan, 2014), and was found in the phyllosphere of rape in this study. The presence of several strains of *Bacillus*, *Arthrobacter*, *Pseudomonas* and *Micrococcus* with hydrocarbon degradation properties in the phyllosphere of a crop panel was also reported previously (Al-Awadhi *et al.*, 2012; Ilori *et al.*, 2006).

Degradation activities for recalcitrant and hydrophobic polymers have already been reported for some of the identified genera. *Exiguobacterium sp.* has been identified as a polystyrene (PS)-degrading microorganism present in the gut of mealworms, the larvae of *Tenebrio molitor* Linnaeus (Yang *et al.*, 2015). Besides, compounds entering the composition or used for the synthesis of plastic materials can be catabolized by *Arthrobacter* species. *Arthrobacter keyseri* was shown to catabolize phthalate *via* a plasmid-encoded pathway (Eaton, 2001). *Arthrobacter sp.* P1 could use methylamine as sole carbon source and degrade it *via* amine oxidase and facultative methylotrophic metabolisms

(Levering *et al.*, 1981). In addition, all the genera obtained through our screening were reported to possess degradative capacities for other types of recalcitrant molecules. This includes pesticides, alcohols, heterocyclic compounds, halogenated, sulfonated compounds and steroids (Larkin *et al.*, 2006; Tallur *et al.*, 2008; Laczi *et al.*, 2015; Kasana and Pandey, 2018) as well as oil components (Wright *et al.* 1993; Seo *et al.*, 2006; Kumar *et al.*, 2008; Mohanty and Mukherji, 2008; Binazadeh *et al.*, 2009; Bezza and Chirwa, 2015). However, none of these previous works has associated the identified species with the degradation of the substrates used in our study.

A critical aspect in hydrophobic compounds degradation such as ESOA resides in the access to the substrates. Interestingly, several of the identified genera were previously described either to be able to modify their cell surface hydrophobicity or to produce tensioactive molecules. The cell surface hydrophobicity modification was reported for *Exiguobacterium* (Mohanty, 2006; Mohanty and Mukherji, 2008). The production of tensioactive molecules was described for *Exiguobacterium*, *Bacillus* (Calvo *et al.*, 2004; Thavasi *et al.*, 2008; Cai et al., 2017), *Rhodococcus* (Bell *et al.*, 1998), *Arthrobacter* (Morikawa *et al.*, 1993), *M. luteus* (Tuleva *et al.*, 2009) or *Pseudomonas* (Kumar *et al.*, 2008; Noordman *et al.*, 2002).

The identification of our isolates is thus in great accordance with the largely furnished literature concerning polymers or hydrophobic molecules biodegradation, which tends to validate our isolation and screening strategy to target bacteria able to use this kind of molecules as substrates.

4.2. Relations between growth with selective substrates and phylogeny of the isolates

The analysis of $\Delta OD_{600\ nm}$ results in light of isolate identifications enabled to underline the selection of certain bacterial families. Several strains belonging to the *Rhodococcus* genus were shown to grow with pAM as substrate in our study. Bacteria of this genus were recurrently found in soils and could thus have already been exposed to pAM, which is used in agriculture as water absorbent to maintain soil humidity (Wenhua *et al.*, 2008). Bacillaceae were generally selected for positive $\Delta OD_{600\ nm}$ with ESOA and were less versatile. As mentioned previously, *Bacillus* can be found in diverse environments and have been described to be associated with the degradation process of a wide range of alkanes and hydrophobic compounds (Al-Sharidah *et al.,* 2000; Bezza and Chirwa; 2015, Calvo *et al.*, 2004; Maiti *et al.*, 2013: Kebria *et al.*, 2009). This suggests that species of this genus may possess specialized catabolic pathways to degrade waxes from the phyllosphere (Kunst and Samuels, 2003; Buschhaus *et al.*, 2007; Buschhaus and Jetter, 2012). Enterobacteriaceae and Pseudomonadaceae only contained one strain each and were more associated with ESOA, but these strains exhibited low $\Delta OD_{600\ nm}$ in the screening.

When considering growth capacities of isolates in view of stem size of the plant of origin, our results tend to show that bacteria from the phyllosphere of more aerial plants are more efficient in oily substrate utilization. This was mainly observed for the species from the *Bacillus* genus that appeared to preferentially utilize the triglyceride derivative ESOA. However most of the strains of interest were obtained from cabbage or sugar beet. Both of these plants are short-stem crops growing close to the soil surface, which is likely to enhance the isolation of soil-borne bacteria described as one of the main phylloplane microbial communities (Pieterse *et al.*, 2016). Notably *Exiguobacterium* strains were isolated from leaves of cabbage and sugar beet, and the *Arthrobacter* strain and the two Rhodococci came from sugar beet. These genera were isolated from soil in other studies as previously mentioned. These isolates appeared to be more polyvalent regarding substrates, which is correlated with the abundant literature about the capacity of these species to degrade a wide range of xenobiotics. Moreover, soils are environmental compartments with a high diversity of substrates and of microorganisms, which could explain a greater catabolic diversity resulting from the competition for substrates. Thus, in supplement to be a source of phylloplane microorganisms adapted to wax degradation, phyllosphere could be regarded as an environment naturally rich in hydrophobic and long chain compounds selecting strains of interest for xenobiotics biodegradation notably from the soil biodiversity.

Analyses of growth kinetics parameters enable to complete these interpretations. All the genera efficiently growing with ESOA as sole carbon source have been described to contain species

able to produce biosurfactants, notably lipopeptides for *Bacillus* species (Ben Ayed *et al.*, 2015; Bezza and Chirwa, 2015; Fooladi *et al.*, 2016; Lima de França *et al.*, 2015; Parthipan *et al.*, 2017; Yuan *et al.*, 2011). *E. sibiricum* 2 was the only *E. sibiricum* strain able to grow with ESOA and displayed the highest growth rate. Most of the strains growing with pHEMA are also identified as *E. sibiricum* in our study. *Exiguobacterium* bacteria have already been described to exhibit a wide range of catabolic activities such as the production of lipases and esterases (Vishnivetskaya *et al.*, 2009; Ali *et al.*, 2015; Kasana and Pandey, 2018), which could explain our results with ESOA and pHEMA. All strains selected for their growth ability with pNIPAM could grow with at least one other substrate, mainly pHEMA with notably 3 strains of *E. sibiricum* able to grow with the two polymers.

Growth on pAM and pAS observed in the first screening step for some isolates through OD variation measurements could not be reproduced for growth kinetics. The rare works reporting the biodegradation of pAM by isolated strains describe a degradation process occurring on time scales up to 14 days (Matsuoka *et al.*, 2002; Nakamiya and Kinoshita, 1995; Song *et al.*, 2017; Wen *et al.*, 2010). It may be possible that our growth kinetic studies over 4 days only may not be sufficient to obtain a visible effect. Besides, the pAM used in our study could also have a too high molecular weight (40,000 g/mol) limiting its biodegradation. Biodegradation generally decreases with an increased polymer molecular weight (Hayashi *et al.*, 1993; Hayashi *et al.*, 1994). No degradation has been reported for molecular weight over 4,000 g/mol and pAS used in our study was 5,000 g/mol. Moreover, Nyyssölä and Ahlgren (2019) recently reported that the pAS part would be more recalcitrant to bacterial degradation than the amide part during pAM biodegradation and confirmed that the biodegradation efficiency is notably linked to the polymer molecular weight.

4.3. Chemical alterations associated with bacterial growth

The chemical analyses confirm the isolation of a *Bacillus sp.* strain efficiently degrading ESOA. This biodegradation appears to involve esterase activities in correlation with the literature reporting esterases involvement in xenobiotics biodegradation by strains of this genus (Niazi *et al.* 2001; Shah *et al.*, 2016). The precise metabolic pathways and generated metabolites have now to be further investigated as well as the possibility of biosurfactants production suspected in our analyses.

Incubation of pNIPAM with *E. sibiricum* 1 led to a decrease of the polymer molecular weight, the appearance of low molecular mass compounds, ^{1}H NMR signals alterations and the appearance of C=O and C-O bands in IR spectra. These elements support the hypothesis of a polymer chain oxidation that more presumably lead to a polymer alteration through the cleavage of the main carbon chain. Such biodegradation potential of aliphatic and aromatic carbon structures by an *Exiguobacterium* strain through an oxidative pathway is in accordance with the PS-degrading capacities observed in this genus (Yang *et al.*, 2015). *E. sibiricum* could thus represent a strain of interest for acrylic polymers bioremediation through these specific oxidative pathways. Indeed, *E. sibiricum* strains isolated in our study also showed growth potential with pHEMA and triggered polymer amount as well as polymer weight decreases in the culture supernatants, as determined by SEC and ^{1}H NMR techniques. The appearance of new bands on the FTIR spectra is in accordance with these results, suggesting pHEMA alterations resulting from bacterial activity.

To our knowledge, this study is the first clear observation that *Arthrobacter koreensis* possesses a major degradation activity on a recalcitrant polymer. Mechanism of pHEMA alteration could rely on the hydrolysis of the ethylene glycol lateral chain. Indeed the biodegradation process of biopolymers such as polyhydroxyalcanoates (PHA), poly-β-hydroxybutyrate (PHB) and polylactic acid (PLA) is based on the hydrolysis of ester or amide bonds (Emadian *et al.*, 2017). However pHEMA alteration could also rely on the main long carbon chain oxidation with mechanisms closer to hydrocarbon biodegradation (Abbasian *et al.*, 2015). Interestingly, the presence of genes dedicated to the synthesis of plant growth promotors such as gibberellins, abscisic acid or strigolactones has been reported in *A. koreensis* genome (Manzanera *et al.*, 2015). All these metabolites are deeply related to molecules produced in isoprenoids or terpenoids metabolic pathways (Kuzma *et al.*, 1995; Das *et al.*, 2007). The presence of metabolic pathways dedicated to the synthesis of long chain isoprenoïds could be a possible line of explanation for the pHEMA degradation abilities of this strain either through reversible enzymatic reactions or through the direct use of partial degradation

products as precursors. In this respect, *A. koreensis* catabolic activities merit further investigations. Its versatile metabolic activities make it a very promising species for biotechnological, environmental and agricultural applications.

5. Conclusions

Plant phyllosphere was shown to be a suitable natural environment for the isolation of bacterial strains with catabolic activities enabling functional polymers biodegradation. Resulting alterations correspond to chemical structures oxidation and molecular weight decreases for ESOA, pNIPAM and pHEMA.

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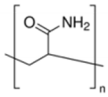
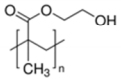
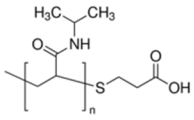
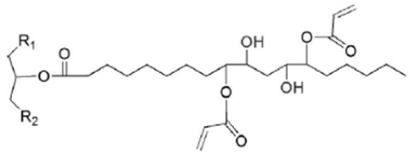
Table 1. Characteristics of selective substrates used in this study (information from the provider)

Substrate	Chemical structure	Mw g/mol	Concentration g/L
pAcrylamide		40,000	4
pAcrylate		5,100	5.84
pHEMA		20,000	3.6
pNIPAM		5,000	3.14
ESOA		990	2.6

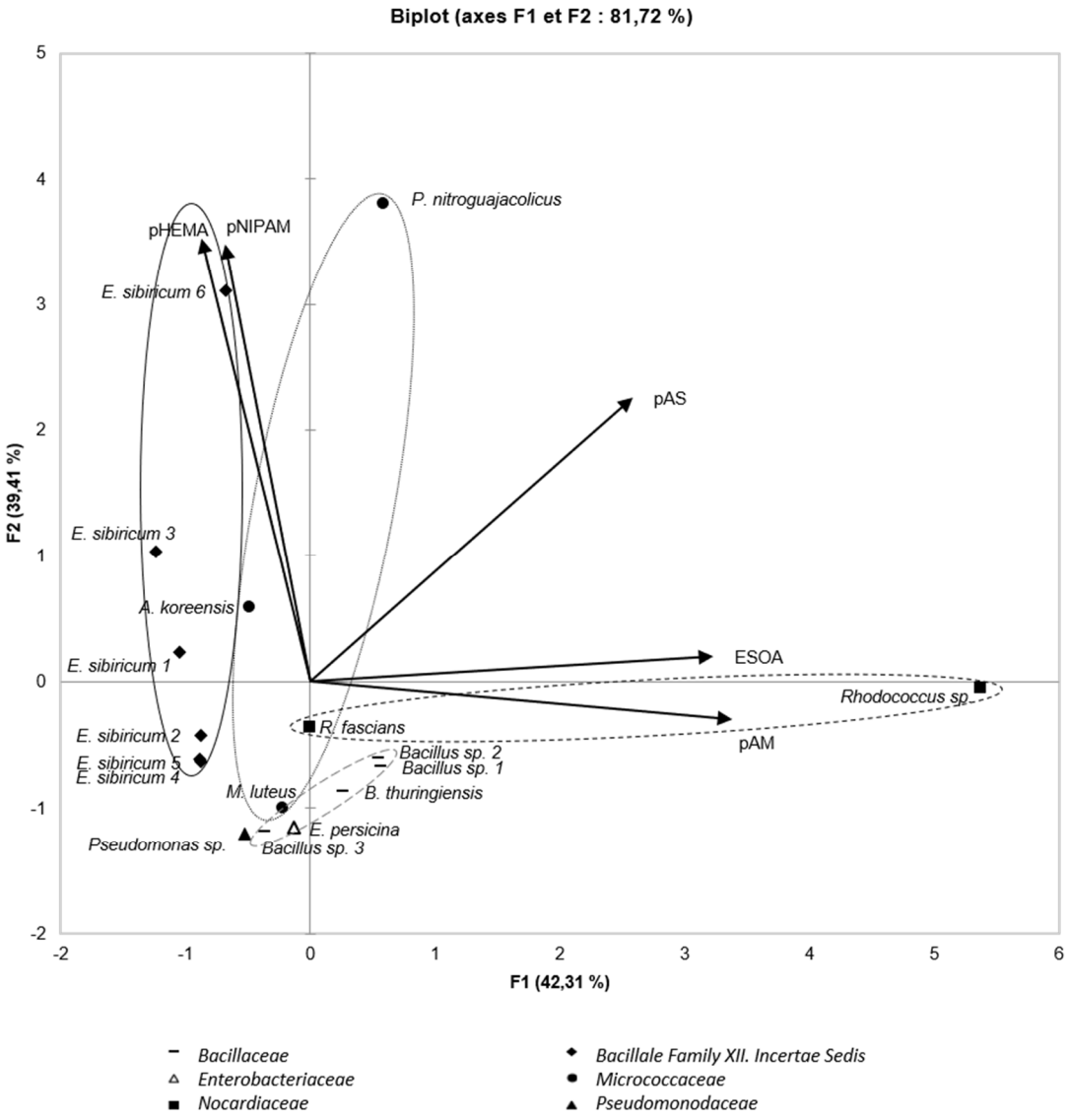


Figure 1. Principal Component Analysis (PCA) based on the ΔOD_{600nm} obtained after 7 days of incubation with each recalcitrant substrate for strains selected and identified after the qualitative screening. (81.7 % of the information is represented, ellipses underline pattern of bacterial families)

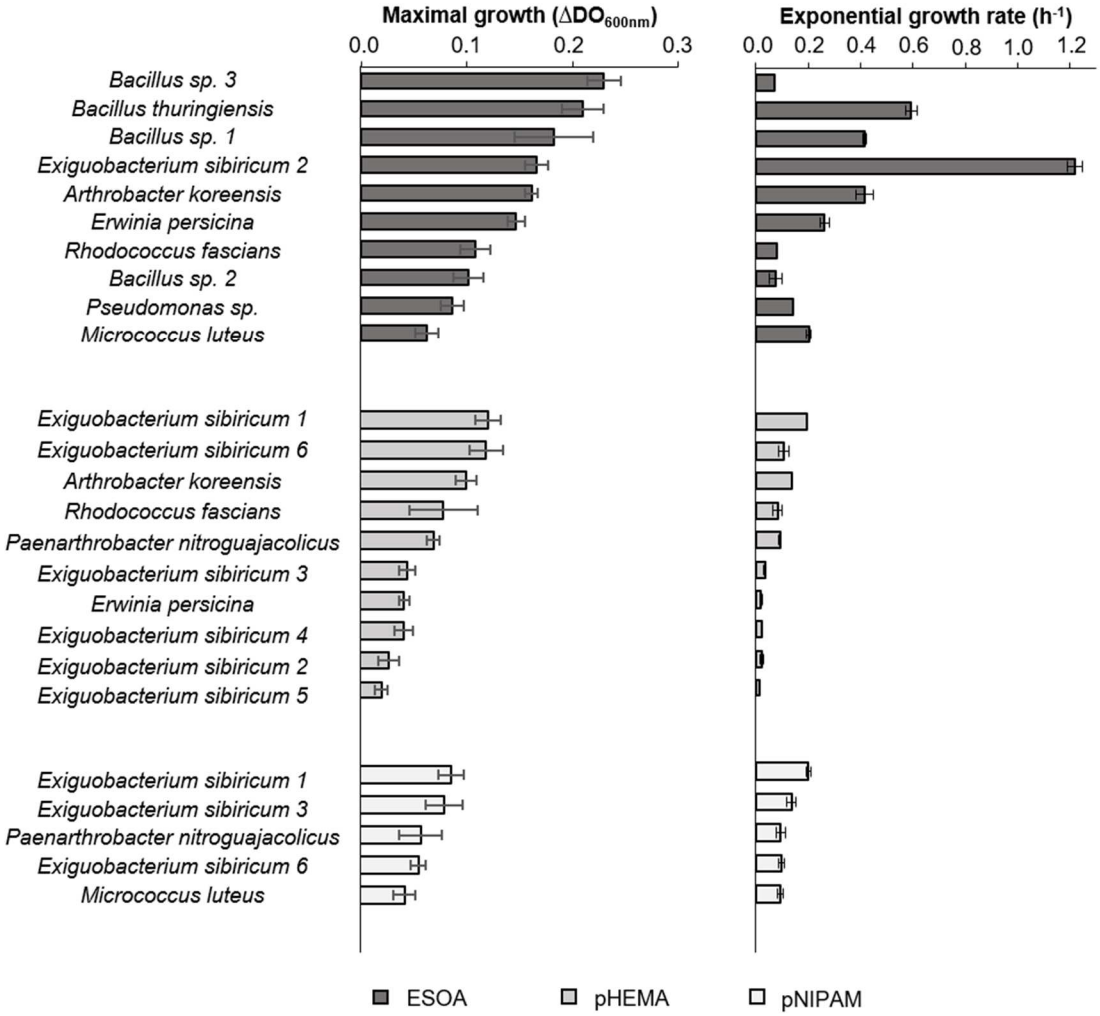


Figure 2. Maximal growth and exponential growth rate obtained on 4 days of growth kinetics of crop leaves isolated bacteria with ESOA, pHEMA, and pNIPAM as sole carbon source.

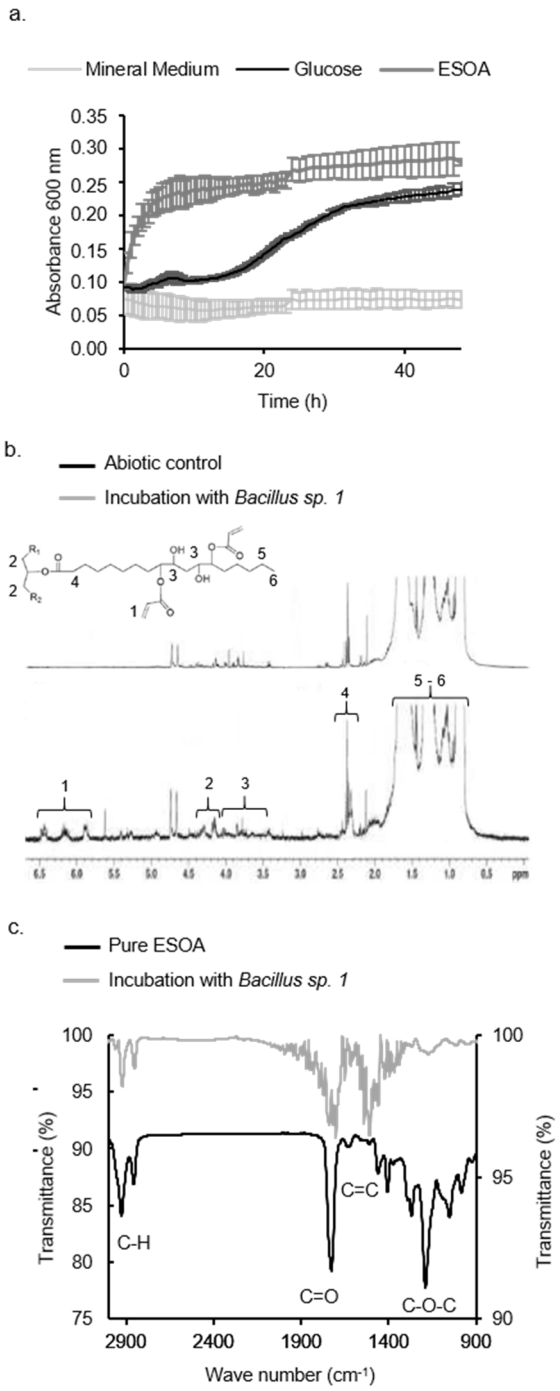


Figure 3. Analysis of *Bacillus sp.* 1 growth with ESOA as sole carbon source and characterization of alterations by comparing pure ESOA, abiotic control and the culture supernatants. a. Two first days of growth kinetic monitoring by 600 nm absorbance measurements, b. ^{1}H NMR spectra c. FTIR spectra

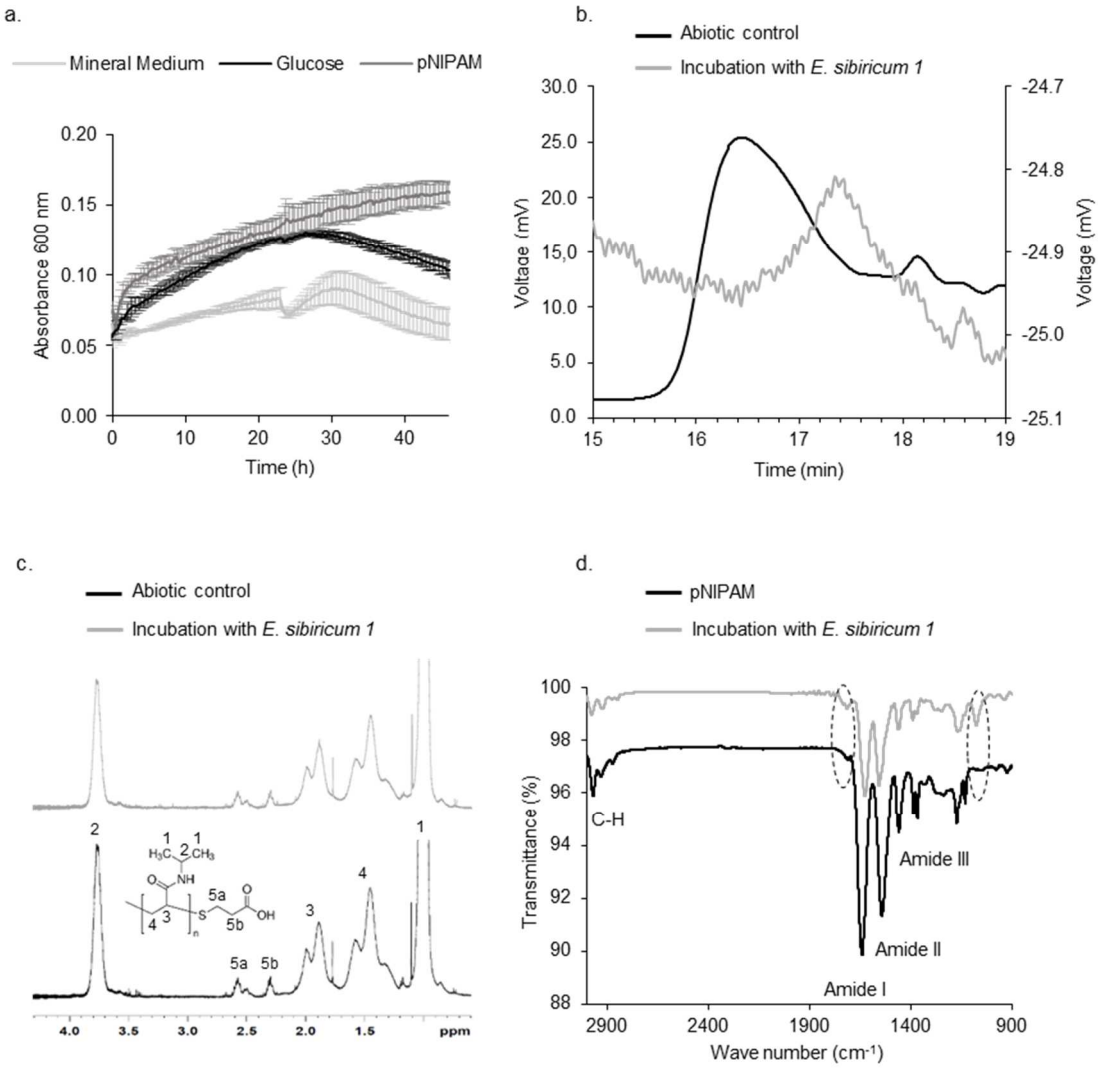


Figure 4. Analysis of *Exiguobacterium sibiricum* 1 growth with pNIPAM as sole carbon source and characterization of polymer alterations by comparing pure pNIPAM or abiotic control with culture supernatants. a. Two first days of growth kinetic monitoring by 600 nm absorbance measurements, b. SEC analysis of culture supernatants using RI as detector, c. ^{1}H NMR spectra, d. FTIR spectra

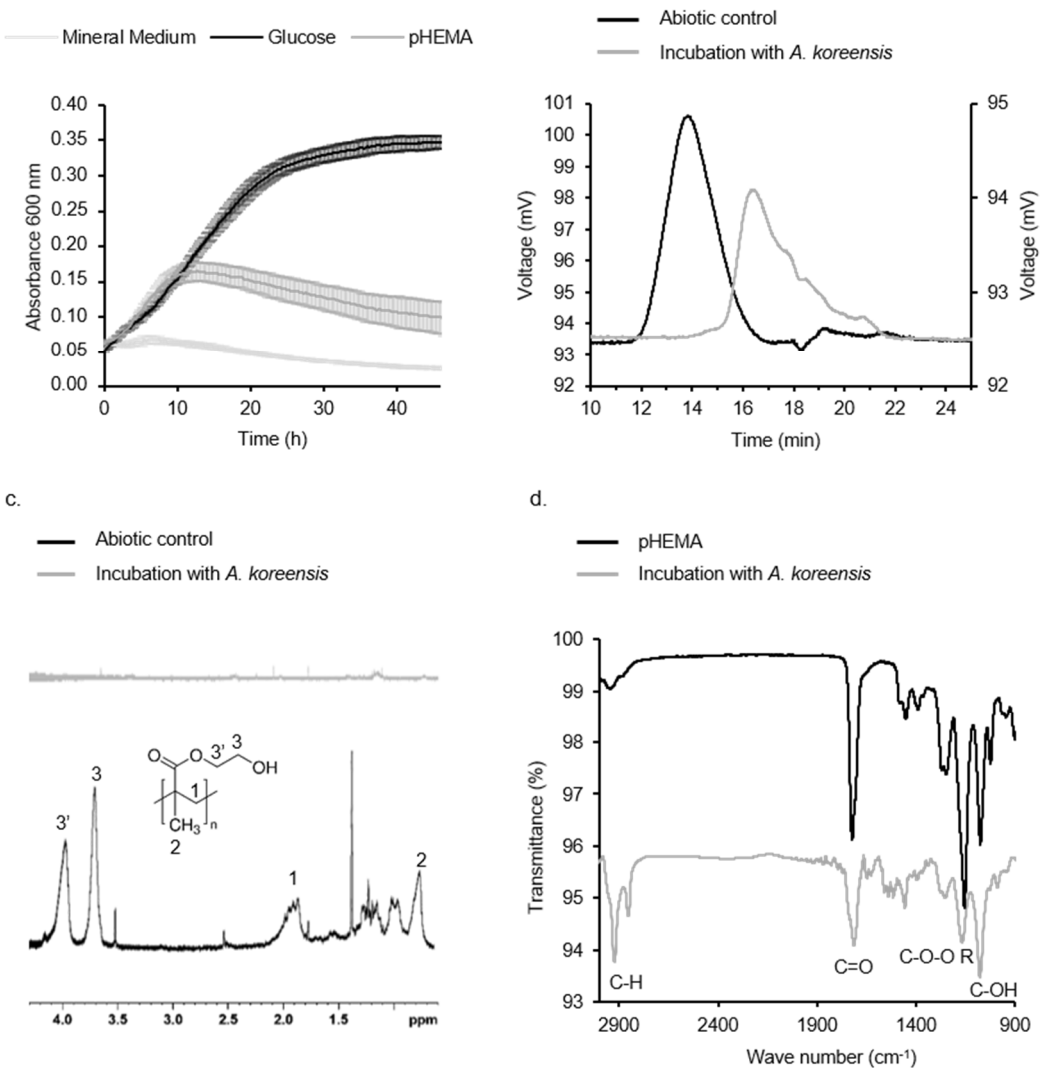


Figure 5. Analysis of *Arthrobacter koreensis* growth with pHEMA as sole carbon source and characterization of polymer alterations by comparing pure pHEMA or abiotic control with culture supernatants. a. Two first days of growth kinetic monitoring by 600 nm absorbance measurements. b. SEC analysis of culture supernatants using MALS as detector. c. ^{1}H NMR spectra d. FTIR spectra