

## Article

# Selecting Bacteria Candidates for the Bioaugmentation of Activated Sludge to Improve the Aerobic Treatment of Landfill Leachate

Justyna Michalska \*, Artur Piński , Joanna Żur  and Agnieszka Mrozik

Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia, Jagiellońska 28, 40-032 Katowice, Poland; apinski@us.edu.pl (A.P.); joanna.zur@us.edu.pl (J.Ż.); agnieszka.mrozik@us.edu.pl (A.M.)

\* Correspondence: jmichalska@us.edu.pl

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**Abstract:** In this study, a multifaceted approach for selecting the suitable candidates for bioaugmentation of activated sludge (AS) that supports leachate treatment was used. To determine the exploitation of 10 bacterial strains isolated from the various matrices for inoculating the AS contaminated with the Kalina pond leachate (KPL), their degradative potential was analyzed along with their aptitude to synthesize compounds improving remediation of pollutants in wastewater and ability to incorporate into the AS flocs. Based on their capability to degrade aromatic compounds (primarily catechol, phenol, and cresols) at a concentration of 1 mg/mL and survive in 12.5% of the KPL, *Pseudomonas putida* OR45a and *P. putida* KB3 can be considered to be the best candidates for bioaugmentation of the AS among all of the bacteria tested. Genomic analyses of these two strains revealed the presence of the genes encoding enzymes related to the metabolism of aromatic compounds. Additionally, both microorganisms exhibited a high hydrophobic propensity (above 50%) and an ability to produce biosurfactants as well as high resistance to ammonium (above 600 µg/mL) and heavy metals (especially chromium). These properties enable the exploitation of both bacterial strains in the bioremediation of the AS contaminated with the KPL.

**Keywords:** activated sludge; bacteria for bioaugmentation; catabolic traits; genomic sequencing; landfill leachate; selected features of bacteria

## 1. Introduction

Landfilling is currently the most common and the cheapest way to eliminate household, commercial, and industrial solid waste worldwide. However, a major drawback of this practice is the generation of heavily polluted leachate, which can contaminate the soil, groundwater, and surface water in the immediate vicinity of the landfill with hazardous substances [1,2]. Owing to the frequent discharge of the landfill leachate to the wastewater treatment plants (WWTPs), a variety of dynamically developing biological technologies in the world, including modern biological membrane reactors and bioelectrochemical systems, are implemented [3–6]. However, in the vast majority of WWTPs in Poland the landfill leachate treatment is still performed with the application of conventional activated sludge (AS) technology. Biological methods are considered to be a beneficial alternative to the physicochemical methods, which are the most cost intensive and have the inherent drawbacks due to the formation of toxic secondary compounds [3,7]. Implementing them for treating landfill leachate is extremely challenging, especially in the case of mature or old leachate due to its complex composition, fluctuations of dissolved organic carbon, and potential toxicity [8–10].

Although the successful treatment of landfill leachate by both conventional and granular AS has been reported in the literature [11–13], the number of reports that stress the negative impact of this

effluent on the sludge performance is definitely higher [14–19]. Because the AS process often cannot achieve an effective reduction of the chemical oxygen demand (COD) and total nitrogen in effluents contaminated with landfill leachate, this technology should be further improved in order to cope with the complex mixture of compounds as well as the fluctuations in the environmental factors associated with this type of wastewater.

The introduction of new metabolic functions into the AS by adding supplementary microorganisms, known as bioaugmentation, constitutes a promising approach for degrading recalcitrant compounds and may enable the performance of the AS process to be improved [20–22]. However, the usefulness of the microorganisms selected as candidates for the bioaugmentation of the AS is frequently limited by their low survival rate under the conditions that prevail in this ecosystem. The successful colonization of a bioreactor by inoculated bacteria is challenging as they are likely to encounter the following obstacles within the biological unit, i.e., environmental pressure due to protozoan grazing and/or competition with indigenous microorganisms as well as fluctuations of the abiotic factors such as temperature, pH, salinity, the hydrodynamic conditions, organic loading rate, and the availability of nutrients and oxygen, which causes them to die or to wash out of the system along with the effluent [23–26]. A versatile degradation capacity towards a variety of compounds is often reported as being a pivotal competence of potential candidates for bioaugmentation [27,28]. However, adding bacteria with the desirable catabolic capabilities into the AS does not always guarantee an enhanced biodegradation of the compounds in wastewater [29,30]. For this reason, the relative spatial and temporal abundance of inoculants and their ability to tolerate the conditions that prevail in the target environment is crucial to their survival.

To date, the majority of studies have only utilized one strain to inoculate the AS treating a synthetic wastewater contaminated with a single pollutant [31–33]. It is also worth stressing that the strains selected to bioaugment the AS in laboratory conditions often lose their ability to degrade contaminants after being inoculated into a bioreactor in a field experiment [34]. Taking this into account, the candidate bacteria for bioaugmentation should be carefully evaluated with respect to at least three criteria in order to confirm their predisposition to thrive in the conditions for which they were selected and that are designed to achieve an efficient performance [35]. They should be catabolically able to degrade the individual chemical components in a mixture of contaminants even in the presence of potentially inhibitory pollutants, persist and compete after their inoculation into a biosystem, and be compatible with autochthonous communities [34].

Utilizing microorganisms that have a proven degradative potential and survivability in the AS is crucial for successful bioaugmentation. In view of this, the aim of the present paper is to evaluate the selected bacterial strains in terms of their potential utilization in bioaugmentation of the AS exposed to the landfill leachate (KPL) originated from the bottom sediments of the Kalina pond in Świątchłowiec (Poland). In order to develop an effective bioaugmentation strategy, the catabolic abilities of microorganisms recently isolated from the environment as well as bacterial strains already known as degraders of various aromatic compounds were characterized. The procedure for selecting attractive candidates for inoculating the AS co-treated with the KPL along with municipal effluents, included the following specific goals: (1) screening the catabolic traits of selected bacterial strains, (2) determining the acute toxicity of the KPL for the tested bacteria, (3) evaluating the ability of the bacteria to survive and remain in the AS, (4) gauging the capability of the bacteria to produce substances that facilitate the degradation of the contaminants, (5) establishing the relationships between the factors being studied and selecting the most efficient candidates for bioaugmentation, and finally (6) exploring the whole genomes of the best candidates for the presence of the genes that are associated with the degradation of pollutants and other features that enable them to acclimatize in the AS.

## 2. Materials and Methods

### 2.1. Bacterial Strains

In this study, 10 bacterial strains were tested for their suitability for bioaugmentation of the AS. Three of them were purchased from the VTT Culture Collection in Espoo (Finland) and the Polish Collection of Microorganisms in the Ludwig Hirsfeld Institute of Immunology and Experimental Therapy in Wrocław (Poland) (PCM), while seven strains came from the Microbial Culture Collection in the Institute of Biology, Biotechnology, and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice (Poland) (IBBEP) (Table 1). The degradative potential of six strains: *Planococcus* sp. S5, *Stenotrophomonas maltophilia* KB2, *Pseudomonas putida* N6, *Pseudomonas* sp., *Pseudomonas putida* mt-2, and *Pseudomonas fluorescens* towards a variety of organic compounds has previously been reported in the literature [36–38], whereas four strains: *Pseudomonas putida* OR45a, *Pseudomonas putida* KB3, *Glutamicibacter soli* OR45b, and *Rhodococcus erythropolis* KB4 have only recently been isolated from the environment and are presented here for the first time.

**Table 1.** Microorganisms selected for bioaugmentation of the activated sludge (AS) in the combined treatment of municipal wastewater and landfill leachate.

Bacterial Strain	Site of Isolation	Selection Factor	Source
<i>Pseudomonas putida</i> OR45a	AS loaded with refinery wastewater, Jedlicze, Poland	Styrene	IBBEP
<i>Glutamicibacter soli</i> OR45b	AS loaded with refinery wastewater, Jedlicze, Poland	Styrene	IBBEP
<i>Planococcus</i> sp. S5	AS loaded with municipal wastewater, Miechowice Bytom, Poland	Sodium salicylate	IBBEP
<i>Stenotrophomonas maltophilia</i> KB2	AS loaded with municipal wastewater, Miechowice Bytom, Poland	Phenol	IBBEP
<i>Pseudomonas putida</i> N6	AS loaded with municipal wastewater from a furniture factory, Jasienica Poland	Phenol	IBBEP
<i>Pseudomonas putida</i> KB3	Uncontaminated, agricultural soil, Siewierz, Poland	Phenol + 4-chlorophenol Phenol + 2,4-dichlorophenol Phenol + 4-chlorophenol + 2,4-dichlorophenol	IBBEP
<i>Rhodococcus erythropolis</i> KB4	Uncontaminated, agricultural soil, Siewierz, Poland	Phenol + 4-chlorophenol Phenol + 2,4-dichlorophenol Phenol + 4-chlorophenol + 2,4-dichlorophenol	IBBEP
<i>Pseudomonas</i> sp. (VTT E-93486)	AS from a peat biofilter bed	Styrene	VTT
<i>Pseudomonas putida</i> mt-2 (VTT E-022150)	nd	nd	VTT
<i>Pseudomonas fluorescens</i> (PCM 2123)	nd	nd	PCM

IBBEP—Culture Collection in the Institute of Biology, Biotechnology, and Environmental Protection at the University of Silesia in Katowice (Poland), PCM—Culture Collection in the Ludwig Hirsfeld Institute of Immunology and Experimental Therapy in Wrocław (Poland), VTT—Culture Collection in Espoo (Finland), nd—data not available.

### 2.2. Isolating the Bacteria from the AS and Soil

The bacterial strains from the AS that was loaded with the refinery wastewater (Jedlicze, Poland) and the uncontaminated agricultural soil (Siewierz, Poland) were isolated using the classical enrichment technique [39] with a mineral salts medium (MSM) (Table S1) and 25 µg/mL of styrene, a combination of 100 µg/mL of phenol with 50 µg/mL of 4-chlorophenol or 10 µg/mL of 2,4-dichlorophenol, 50 µg/mL of 4-chlorophenol, or 10 µg/mL of 2,4-dichlorophenol as the selection factors, respectively.

### 2.3. Sample Collection

The landfill leachate (KPL) was obtained from the bottom sediments of the Kalina Pond in Świątchłowice (Upper Silesia, Poland), which is the site for the arbitrary disposal of industrial and municipal waste. The sediment samples were collected at the point at which the effluents from the waste dump entered the pond at a distance of 2 m from the shore and at a depth of 2 m in compliance with ISO 5667-13:2011 [40] and ISO 19458:2007P [41] in July 2017. The KPL was characterised by high concentrations of organic and inorganic pollutants and had an alkaline pH as well [19].

The activated sludge (AS) inoculum was obtained directly from the aeration tank of the wastewater treatment plant (WWTP) “Klimzowiec” (Chorzów, Poland).

### 2.4. Evaluating Catabolic Traits of the Bacterial Strains

The capability of bacterial strains to degrade phenol, 2-methylphenol, 3-methylphenol, 4-methylphenol, 4-aminophenol, catechol, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, and sodium benzoate at different concentration ranges was studied using Biolog<sup>®</sup> MT plates (Biolog, Inc., Hayward, CA, USA). The data were collected using Microlog 4.01 software. The reduction of colorless 2,3,5-triphenyltetrazolium chloride (TTC) to a purple 1,3,5-triphenyltetrazolium formazan (TPF), which was measured as the color development in the wells (WCD), indicated that the substrate had been used as the sole carbon and energy source by the tested bacteria, whereas no color development indicated that it had not been utilized. The metabolic versatility (MV) of each strain was expressed as the ratio of the number of substrates that were metabolized by the bacterial cells to all of the tested hydrocarbons. The capacity of the bacteria to degrade the aromatic compounds (AC-USE) in the Biolog<sup>®</sup> MT plates was calculated as follows (1):

$$AC - USE = \frac{WCD}{t_{cp}} \quad (1)$$

where:  $t_{cp}$ —time at which the value curve plateau of the WCD was reached.

### 2.5. Acute Toxicity of the KPL

The toxic effect of the KPL on selected bacteria that were grown in the synthetic wastewater (SW) contaminated with the leachate in a concentration range from 0% to 12.5% was determined according to Michalska et al. [19]. The initial optical density ( $OD_{600}$ ) of each bacterial strain was 0.1.

### 2.6. Evaluating the Auto-Aggregation and Co-Aggregation Ability of the Bacteria

The ability of the bacterial strains to auto-aggregate and co-aggregate with the indigenous microorganisms in the AS were determined by the modified method as described by Malik et al. [42]. Briefly, equal volumes of co-aggregating partners ( $OD_{660} = 1.0$ ) including the cell suspensions of each bacterial strain and the microorganisms released from the AS in phosphate buffered saline (PBS) (Table S1) were mixed together and incubated at  $23 \pm 2$  °C for 24 h with shaking at 130 rpm. Cell suspensions of pure bacterial strains were incubated under similar conditions in order to investigate their ability to auto-aggregate. Subsequently, the samples were allowed to settle and the  $OD_{660}$  of the supernatant was measured. The auto-aggregation (AAI) and co-aggregation (CAI) indices were calculated as follows (2):

$$AAI (CAI), \% = \frac{OD_{660i} - OD_{660s}}{OD_{660i}} \times 100\% \quad (2)$$

where:  $OD_{660i}$ —initial absorbance of the bacterial cell suspension,  $OD_{660s}$ —absorbance of the supernatant.

### 2.7. Determining the Flocculation Activity Index (FAI)

The flocculating activity of a bioflocculant produced by the bacterial strains was measured as the turbidity of a kaolin-clay suspension and determined in accordance with the methods of Kurane et al. [43] and Zhang et al. [44]. The flocculating activity index (FAI) was calculated according to the following Equation (3):

$$\text{FAI, \%} = \frac{A_{550\text{S}} - A_{550\text{C}}}{A_{550\text{S}}} \times 100\% \quad (3)$$

where:  $A_{550\text{S}}$ —absorbance of the clarifying solution after the bioflocculant was added,  $A_{550\text{C}}$ —absorbance of the clarifying solution after the fresh culture medium was added (control).

### 2.8. Measuring the Hydrophobicity of the Bacterial Cell Surface and the AS

The cell surface hydrophobicity (CSH) of the bacterial strains and the suspension of microorganisms released from the AS flocs was determined using a microbial adhesion to hydrocarbons (MATH) assay, according to the method described by Kos et al. [45]. For this purpose, microorganisms were grown in Luria–Bertani medium (LBB) (Difco Laboratories, Detroit, MI, USA) uncontaminated or polluted with 3.5% and 5.5% of the KPL at  $23 \pm 2^\circ\text{C}$  for 24 h. After incubation, the microbial cells were harvested by centrifugation at  $4^\circ\text{C}$  for 10 min at  $5000\times g$ , washed twice with sterile 0.85% NaCl and resuspended in it. The CSH was quantified by calculating the percentage of bacterial cells that were transferred to the *p*-xylene phase, according to the following Equation (4):

$$\text{CSH, \%} = \frac{1 - \text{OD}_{660\text{i}}}{\text{OD}_{660\text{a}}} \times 100\% \quad (4)$$

where:  $\text{OD}_{660\text{i}}$ —initial absorbance of the bacterial cell suspension,  $\text{OD}_{660\text{a}}$ —absorbance of the aqueous phase, which was measured after 20 min of the incubation of the bacteria with *p*-xylene.

### 2.9. Qualitatively Assessing the N-Acyl Homoserine Lactone Signal Molecules and Exopolysaccharide Production

The production of the N-acyl homoserine lactone signal molecules (N-AHLs) was evaluated qualitatively only for the gram-negative bacteria using the colorimetric method of Taghadosi et al. [46]. The presence of lactone compounds was indicated by a dark brown color, however, the appearance of a yellow color indicated a lack of N-AHLs in the sample.

The production of exopolysaccharides (EPS) was qualitatively assessed by cultivating the bacterial strains on Congo red agar (CRA) using the method modified by Freeman et al. [47]. The strains that formed reddish black colonies with a rough, dry and crystalline consistency were considered to be EPS-producers.

### 2.10. Detecting the Bacterial Motility and Chemotaxis

The swimming and swarming motilities of the bioaugmentation candidates were assayed on semi-solid agar plates according to Déziel et al. [48]. In order to determine the chemotactic response of the bacteria towards the KPL, the semi-solid agar plates were additionally supplemented with 3.5% and 5.5% of the KPL. Motility was assayed quantitatively by measuring the size of the turbid zone formed by bacterial cells migrating away from the inoculation point.

### 2.11. Qualitatively Screening the Biosurfactant Production and Determining the Emulsification Index

The bacterial strains were studied for the production of biosurfactants using the CTAB/methylene blue agar test [49]. A spot of bacterial culture, which had been grown overnight in an LBB medium contaminated with 5.5% of the KPL was placed on a modified mineral salts agar (MMSA) (Table S1) supplemented with 0.2 g/L of cetyltrimethylammonium bromide (CTAB) along with 0.005 g/L of methylene blue dye. After 48 h of incubation at  $23 \pm 2^\circ\text{C}$ , the appearance of a dark blue halo around the bacterial colonies indicated the presence of an extracellular glycolipid biosurfactant. The quality of the



biosurfactant was determined by measuring its emulsification capacity according to Pacwa-Płociniczak et al. [50]. The emulsification index ( $E_{24}$ ) was determined as follows (6):

$$E_{24}, \% = \frac{H_e}{H_m} \times 100\% \quad (5)$$

where:  $H_e$  is the height of the emulsion layer and  $H_m$  is the total height of the mixture.

#### 2.12. Qualitatively Assessing the Production of Siderophores

The production of siderophores by the tested strains was qualitatively assayed according to the protocol described by [51] using the blue indicator dye, chrome azurol S (CAS). The microorganisms that exhibited an orange halo after five days of incubation at  $23 \pm 2$  °C were considered to be positive for the production of siderophores.

#### 2.13. Determining the Non-Inhibitory Concentrations of Ions for the Bacteria

In order to estimate the resistance of the bacteria to heavy metals and ammonium, the non-inhibitory concentrations (NIC) of selected ions were determined. The assays were performed using 96-well microtiter plates. Briefly, a solution of  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cr}^{6+}$ , and  $\text{Pb}^{2+}$  derived from  $\text{Cu}(\text{NO}_3)_2$ ,  $\text{Cd}(\text{NO}_3)_2$ ,  $\text{ZnSO}_4$ ,  $\text{Ba}(\text{NO}_3)_2$ ,  $\text{K}_2\text{Cr}_2\text{O}_7$ , and  $\text{Pb}(\text{NO}_3)_2$  were introduced into the wells to obtain a final concentration of 300 µg/mL, whereas  $\text{NH}_4^+$  was introduced to obtain a final concentration of 2000 µg/mL. Subsequently, bacterial suspensions (optical density at 590 nm = 0.1) were inoculated into the wells contained the appropriate concentration of each ion. After 24 h of incubation in the dark at  $23 \pm 2$  °C, the absorbance in each well was measured at a wavelength of 590 nm using a Biolog<sup>®</sup> Microstation<sup>™</sup> (BIOLOG Inc., Hayward, CA, USA). The maximum concentration of ions that did not inhibit bacterial growth was considered to be the NIC value.

#### 2.14. Whole Genome Sequencing of *P. putida* OR45a and *P. putida* KB3

The whole genomes of *P. putida* OR45a and *P. putida* KB3, which had been selected as prospective candidates for bioaugmentation of the AS loaded with the KPL, were sequenced. The bacterial cells were maintained in a pure culture in an aerobic lysogeny broth at  $23 \pm 2$  °C prior to the DNA extraction using a GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit (Eurx, Gdańsk, Poland). The genome sequencing was performed with MicrobesNG [52] using the Illumina MiSeq platform with  $2 \times 250$ -bp paired-end reads. The results of the sequencing were put through a standard MicrobesNG analysis pipeline and deposited in the GenBank database under the following accession numbers: SPUU00000000.1 for *P. putida* OR45a and SPUT00000000.1 for *P. putida* KB3.

The functional annotation of both strains was performed using an eggNOG-mapper 1.0.3-35 with a one-to-one orthology restriction [53]. The genes that were assigned to multiple COG (clusters of orthologous groups) categories were counted as being present in each of these categories. The KO tags were mapped to the KEGG pathways using the Search Pathway tool from the collection of KEGG Mapper tools, which permit the genes involved in degrading xenobiotics to be visualized. The antibacterial biocide and metal resistance protein sequences were retrieved from the BacMet experimentally confirmed resistance genes database [54,55]. The proteome sequences of both strains were aligned with the BacMet database sequences using the BLAST algorithm and only the sequences with a coverage and identity more than 90% were considered to be relevant matches [56].

In order to generate a phylogenetic tree of the 13 high-quality bacterial genomes, the 31 single-copy housekeeping genes were retrieved from each genome using AmphoraNet [57]. The sequences of each individual marker gene were aligned using Geneious (version 11.1.2) (Biomatters Ltd., Auckland CBD, New Zealand) with the default parameters and the poorly aligned positions were removed using Gblocks (version 0.91b) (Integrated DNA Technologies, Iowa, IA, USA). Subsequently, the alignments were concatenated and a maximum likelihood phylogenetic tree was created in MEGA (version 7.0.26) (Pennsylvania State University, Mueller Laboratory, University Park, PA, USA). In order to assess

the support of the phylogenetic tree, a bootstrap test with 1000 replications was performed. Finally, the phylogenetic tree was visualized using the Interactive Tree of Life tool (iTOL) [58], which is available at [59].

### 2.15. Statistical Analysis

All of the data presented in this paper are expressed as the mean and standard deviation and were analysed using Microsoft Office Excel 2010, Statistica® 12.5 PL (StatSoft® Inc., Tulsa, OK, USA) and GraphPad Prism 5 software (Graphpad Software Inc., San Diego, CA, USA). For the matrix visualization and for the scrutiny of bioaugmentation potential of the tested bacteria, all of the data were first subjected to a min-max normalization according to the equation:

$$ND = \frac{RD_i - RD_{max}}{RD_{max} - RD_{min}} \quad (6)$$

where: ND (normalized data)—all of the variables that had scaled to the range (0, 1),  $RD_i$ —the initial raw data,  $RD_{min}$ —the minimal value of each parameter in a data set,  $RD_{max}$ —the maximal value of each parameter in a data set.

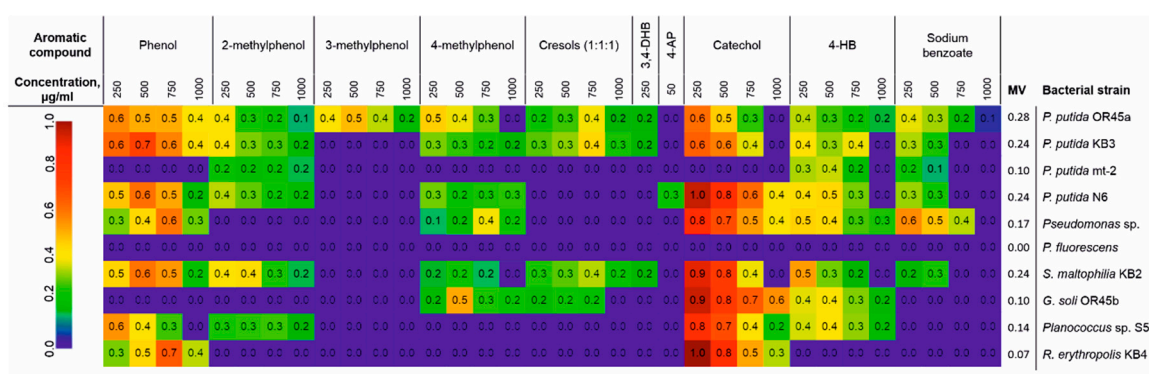
Qualitatively assayed characteristics of the tested bacteria were normalized as follows: the lack of bacterial ability to synthesize biosurfactants, siderophores, and exopolysaccharides was considered as 0, whereas the aptitude of bacteria to produce these compounds was considered as 1.

Any changes between the microorganisms were determined using a one-way analysis of variance (ANOVA, post-hoc test) and were followed by the separation of the treatments from the control as well as the microorganisms among them by applying the post-hoc LSD at confidence intervals of 95% ( $p < 0.05$ ). The values that are indicated by different lower case letters were statistically significant.

## 3. Results

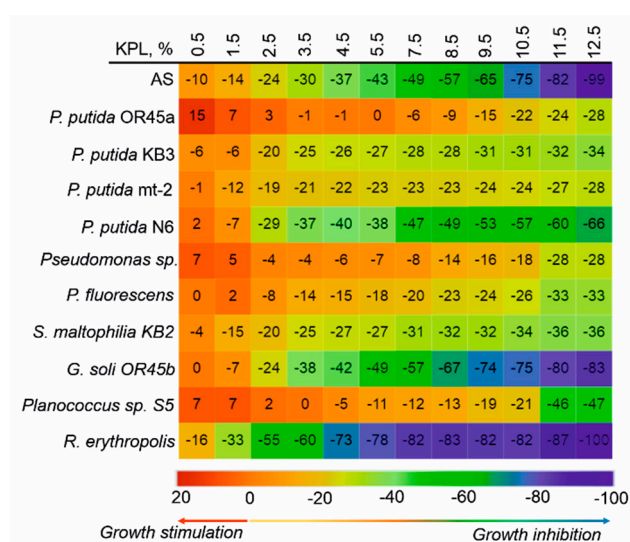
### 3.1. The Catabolic Potential of the Tested Bacteria and Their Ability to Grow in the Presence of the KPL

The degradative capacity of the potential candidates for bioaugmentation of the AS, which were analyzed using Biolog®<sup>MT</sup> plates, revealed that the metabolic versatility of the tested bacteria can be ordered as follows: *P. putida* OR45a > *P. putida* KB3 = *P. putida* N6 = *S. maltophilia* KB2 > *P. putida* sp. > *Planococcus* sp. S5 = *G. soli* OR45b = *P. putida* mt-2 > *R. erythropolis* KB4 > *P. fluorescens* (Figure 1). Of all of the tested strains, *P. putida* OR45a was characterized by the highest degree of metabolic versatility (0.28) and was the only strain able to degrade 3-methylphenol. In addition to this compound, it utilized seven other aromatic compounds as its sole carbon and energy source including phenol, 2-methylphenol, benzoic acid, and 4-hydroxybenzoic acid (up to 1000 µg/mL), 4-methylphenol and catechol (up to 750 µg/mL) as well as 3,4-dihydroxybenzoic acid (up to 250 µg/mL). *P. putida* KB3, *P. putida* N6, and *S. maltophilia* KB2, which had a metabolic versatility of 0.24, proved to be equally promising strains for degrading aromatic compounds. Conversely, *P. fluorescens* did not display any capacity for degrading the tested aromatic compounds.



**Figure 1.** Analysis of the metabolic versatility (MV) of the candidates for bioaugmentation using Biolog<sup>®</sup> MT plates. All of the data values were normalized and are presented as a color scale from navy blue (0) to red (1), which refer to inability and biodegradability of the tested aromatic compounds, respectively.

Previous studies have shown that exposing the AS to the KPL in wastewater at a concentration of 11.26% resulted in the complete inhibition of visible growth of microorganisms [20]. Taking this into account, in this study, a concentration range of 0.5% to 12.5% was used to investigate the ability of the bacterial strains to grow in the presence of the KPL in the effluent. The results are presented as the percentage of the stimulation or inhibition of bacterial growth (Figure 2). Although the increase in the KPL concentration in the SW was correlated with the increase of the effluent toxicity, the toxic effect varied in relation to the investigated microorganisms. It was found that at concentrations of 0.5 and 1.5%, the KPL in the SW stimulated the growth of *P. putida* OR45a, *P. putida* sp. and *Planococcus* sp. S5. By contrast, the addition of 1.5% of the KPL into the effluent resulted in the mortality of the other bacteria. Among the tested strains, *P. putida* OR45a proved to be the most resistant to the toxic effect of the KPL. The exposure of this bacterium to the KPL at a concentration of 12.5%, which was allocated to the 100% mortality of the AS microorganisms, merely resulted in the inhibition of the growth of *P. putida* OR45a by 28%. Generally, the toxicity of the KPL towards the tested bacteria can be ordered as follows: *P. putida* OR45a < *P. putida* sp. < *P. putida* mt-2 < *P. fluorescens* < *P. putida* KB3 < *P. putida* KB2 < *Planococcus* sp. S5 < *P. putida* N6 < *G. soli* OR45b < AS < *R. erythropolis* KB4.



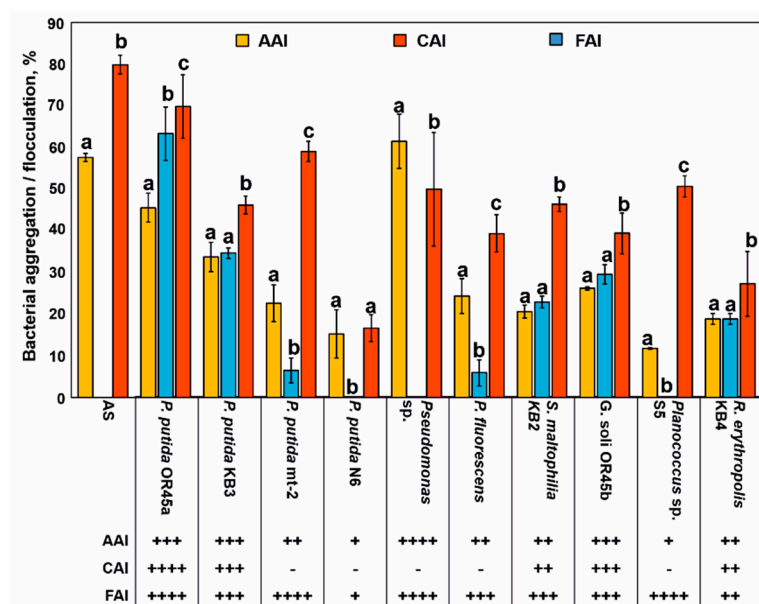
**Figure 2.** Effect of the different concentrations of the Kalina pond leachate (KPL) on the growth of the tested bacteria. The negative values indicate the inhibition of microbial growth whereas the positive values indicate its stimulation.



### 3.2. Ability of Bacterial Strains to Incorporate into the AS Structure

Aggregation and flocculation properties were important factors that promoted the survival of the inoculated strains in the AS. The results obtained in this study showed that although all of the tested bacteria were self-aggregating, *Pseudomonas* sp. had the strongest ability to form auto-aggregates (Figure 3). However, the strong auto-aggregation ability (AAI = 0.61%) of this bacterium did not support its co-aggregation with the microorganisms from the AS (CAI = 0%). Screening of the bacteria with co-aggregation ability revealed that only *P. putida* OR45a was able to co-aggregate with multiple species from the AS at a high co-aggregation rate of 63%. Moreover, this bacterium exhibited a good auto-aggregation (AAI = 46%). *P. putida* KB3 and *G. soli* OR45b can be categorized as both good auto- and co-aggregating strains, whereas *S. maltophilia* KB2, *R. erythropolis* KB4, *Planococcus* sp. S5, and *P. putida* N6 had a weak or very weak ability to aggregate.

An analysis of the bioflocculant-producing bacteria showed that eight of the evaluated strains including *P. putida* OR45a, *P. putida* mt-2, *Planococcus* sp. S5, *Pseudomonas* sp., *S. maltophilia* KB2, *P. putida* KB3, and *G. soli* OR45a exhibited promising flocculating activity of 70%, 59%, 51%, 50%, 46%, 46%, and 35%, respectively. Conversely, *R. erythropolis* KB4 and *P. putida* N6 were characterized as having a weak bioflocculant-producing ability (Figure 3).



**Figure 3.** Analysis of the ability of the tested strains to incorporate into the AS floc structure. AAI—auto-aggregation index, CAI—co-aggregation index, FAI—bio-flocculation index, (++++), strong ability, (++++) good ability, (++) weak ability, (+) very weak ability, (–) lack of ability.

The auto- and co-aggregation abilities of the investigated Gram-negative bacteria can also be enhanced by the production of N-AHLs and exopolysaccharides. It has been demonstrated that only *P. putida* N6, which exhibited very weak aggregation properties, can be regarded as a non-N-AHLs-producing strain. In turn, *P. putida* OR45a, *P. putida* KB3, *P. putida* mt-2, *P. fluorescens*, *Planococcus* sp. S5, and *S. maltophilia* KB2 were able to produce exopolysaccharides (Table 2).

**Table 2.** Production of N-AHLs and exopolysaccharides by the tested strains.

Characteristics	Capabilities Supporting Incorporation into the AS	
Bacterial Strain	N-AHLs	Exopolysaccharides
<i>P. putida</i> OR45a	+	+
<i>P. putida</i> KB3	+	+
<i>P. putida</i> mt-2 (VTT E-022150)	+	+
<i>P. putida</i> N6	—	—
<i>Pseudomonas</i> sp. (VTT E-93486)	+	—
<i>P. fluorescens</i> (PCM 2123)	+	+
<i>S. maltophilia</i> KB2	+	+
<i>G. soli</i> OR45b	nd	—
<i>Planococcus</i> sp. S5	nd	+
<i>R. erythropolis</i> KB4	nd	—

(+) production of N-AHLs/exopolysaccharides, (—) lack of production, nd—not determined.

### 3.3. Biosurfactant and Siderophore Production by the Bacterial Strains

All of the bacterial strains were also screened for a high biosurfactant-producing activity. The screening was performed against the qualitative (CTAB/methylene blue agar test) and semi-quantitative (emulsification index) parameters. Five bacterial strains were able to produce dark blue halos around the colonies, which indicated that they were biosurfactant-producers. The emulsification indices measured for these strains demonstrated that the *p*-xylene was emulsified by *P. putida* OR45a, *P. putida* KB3, *S. maltophilia* KB2, *P. putida* mt-2, and *P. fluorescens* to a level of 57%, 49%, 27%, 21%, and 16%, respectively (Table 3). Another metabolic capability of the tested bacteria, which can be harnessed for wastewater bioremediation, was the production of siderophores. Five of the microorganisms screened for the production of these compounds grew on CAS agar and changed its color from blue to orange with different intensities of the halo formed, which could be related to the concentration of siderophores (Table 3).

**Table 3.** Biosurfactant production, emulsification index, and siderophore production by the tested strains.

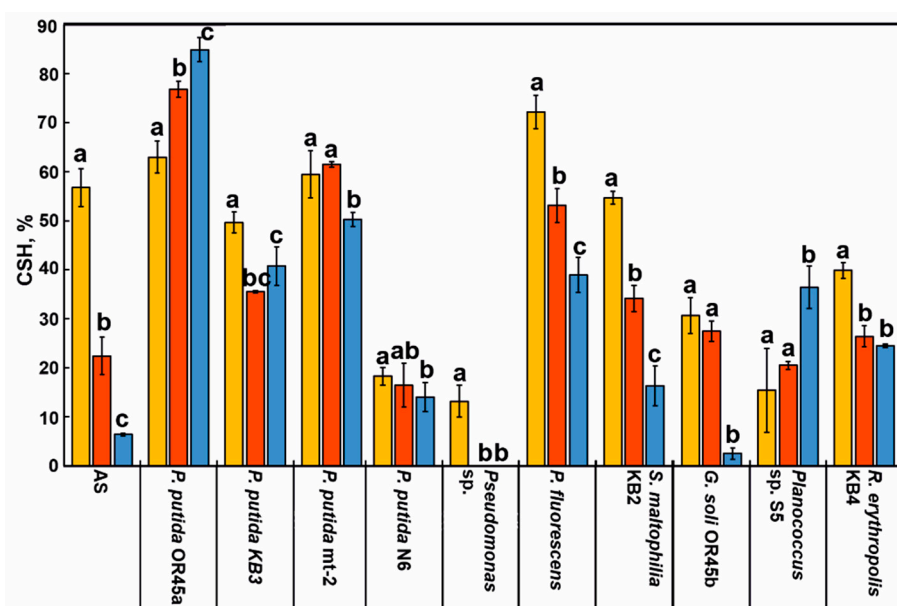
Characteristics	Capabilities Supporting the Degradation of Pollutants in Wastewater		
Bacterial Strain	Biosurfactant Production	Emulsification Index (E <sub>24</sub> ), %	Siderophore Production
<i>P. putida</i> OR45a	+	57 ± 8	+
<i>P. putida</i> KB3	+	49 ± 3	+
<i>P. putida</i> mt-2 (VTT E-022150)	+	27 ± 5	—
<i>P. putida</i> N6	—	nd	—
<i>Pseudomonas</i> sp. (VTT E-93486)	—	nd	+
<i>P. fluorescens</i> (PCM 2123)	+	16 ± 7	+
<i>S. maltophilia</i> KB2	+	21 ± 3	+
<i>G. soli</i> OR45b	—	nd	—
<i>Planococcus</i> sp. S5	—	nd	—
<i>R. erythropolis</i> KB4	—	nd	—

(+) capability of biosurfactant/siderophore production, (—) lack of capability, nd—not determined.

### 3.4. Cell Surface Hydrophobicity (CSH) of the Bacterial Strains and the AS

The CSH plays an indispensable role in the flocculation process and in the adhesion of bacteria to the flocs. The results of this study showed that the tested strains varied in the CSH from 13 to 72% (Figure 4). The cells of *P. fluorescens*, *P. putida* OR45a, *P. putida* mt-2, *S. maltophilia* KB2, and *P. putida* KB3 were highly hydrophobic (CSH ≥ 50%), while the cells of *G. soli* OR45b and *R. erythropolis* KB4 were characterized by a low hydrophobicity. By contrast, the cells of *P. putida* N6, *Planococcus* sp. S5, and *P. putida* sp. can be considered to be hydrophilic due to their low hydrophobicity values of 18%, 15%, and 13%, respectively.

In a parallel experiment, the influence of the KPL on the CSH of the tested bacterial strains and the overall hydrophobicity of the microorganisms that had been derived from the AS were examined. As is shown in Figure 4, the KPL affected the cell surface properties of the investigated microorganisms differentially. Incubating the AS-derived bacteria with 3.5% and 5.5% of the KPL decreased their overall hydrophobicity markedly from 57% to 22% and 6%, respectively. Cultivating *G. soli* OR45b, *P. fluorescens*, *S. maltophilia* KB2, and *Pseudomonas* sp. in the presence of 3.5% of the KPL resulted in a decrease of their CSH by 3%, 19%, 38%, and 100%, respectively. What is more, the CSH of *P. fluorescens*, *S. maltophilia* KB2, and *G. soli* OR45b was noted to decrease even more in response to the addition of 5.5% of the pollutant by 33%, 53%, and 93%. On the other hand, the KPL strongly promoted an increase in the hydrophobicity of *P. putida* OR45a from 63% to 85% and changed the cell character of *Planococcus* sp. S5 to being more hydrophobic.



**Figure 4.** Effect of the KPL on the cell surface hydrophobicity (CSH) of the tested bacterial strains and the overall hydrophobicity of microorganisms derived from the AS.

### 3.5. Bacterial Motility and Chemotaxis

Motility plays an important role in the microbial functions and processes that are connected with the survival of microorganisms in adverse conditions. Here, all of the tested strains were able to undertake both of the flagellum-mediated types of motility—swimming and swarming (Figure 5). However, among the tested microorganisms, *G. soli* OR45b and *P. putida* N6 were the strains with the weakest ability to move. The *P. putida* OR45a, *P. putida* mt-2, *Planococcus* sp. S5, and *R. erythropolis* KB4 strains had a stronger swimming tendency than *P. putida* KB3, *S. maltophilia* KB2, and *P. fluorescens*, which exhibited a strong swarming propensity.

The adaptation of the tested strains to the presence of the KPL was evaluated by analyzing their chemotactic responses. As Figure 5 shows, the swimming and swarming motility of the tested bacteria were markedly altered in the presence of the KPL. Addition of 3.5% and 5.5% of the KPL into the agar plates had a strong inhibitory effect on the swarming phenotype of almost all of the microorganisms that were investigated, except for *P. putida* mt-2 whose swarming ability was promoted by the presence of the KPL. Further observation revealed that *P. fluorescens*, *Pseudomonas* sp., *P. putida* KB3, *S. maltophilia* KB2, and *P. putida* OR45b completely lost their chemotactic swarming responses to the KPL. Interestingly, the KPL did not block the swimming motility of any of the bacterial strains. This study revealed that the KPL increased the swimming motility of *P. putida* OR45a, *G. soli* OR45b,

*Pseudomonas* sp., *P. putida* KB3, *P. fluorescens*, and *S. maltophilia* KB2 by 16%, 43%, 45%, 72%, 250%, and 288%, respectively.

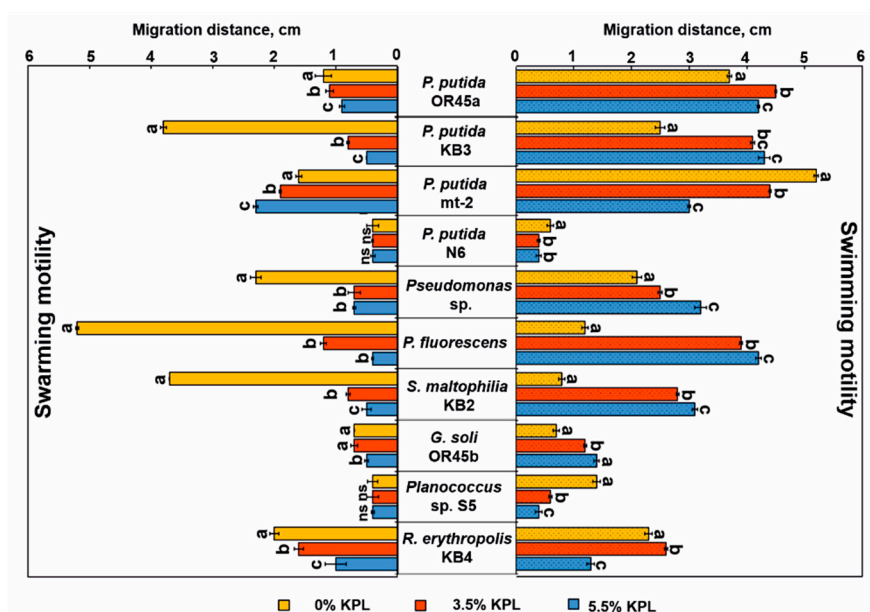
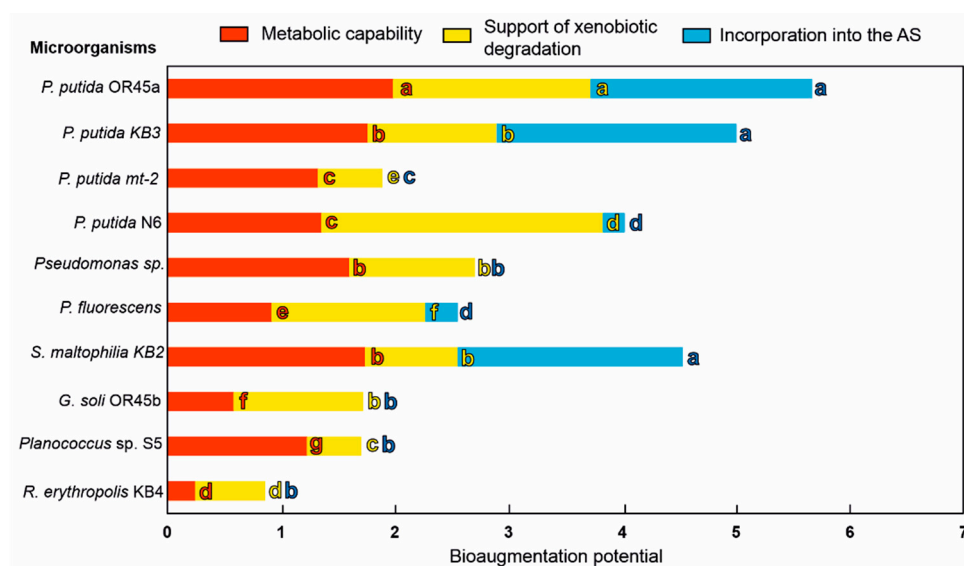


Figure 5. Motility behavior of the tested strains in response to the KPL.

### 3.6. Selecting the Most Promising Candidates for Bioaugmentation

The robust candidates for bioaugmentation of the AS were selected based on a multifaceted approach that included an analysis of their metabolic versatilities (degradative potential towards phenolic compounds, growth in the presence of the KPL), their ability to biodegrade contaminants (production of biosurfactants, siderophores, changes in the cell surface hydrophobicity, chemotaxis) and the features that supported their survival and incorporation into the AS flocs (auto-aggregation, co-aggregation, bioflocculation, exopolysaccharide production). An analysis of the crucial features of the bacteria to successfully bioaugment the AS revealed that *P. putida* OR45a, *P. putida* KB3, and *S. maltophilia* KB2 proved to be the most effective candidates for bioaugmentation of the AS in order to improve the aerobic treatment of the KPL (Figure 6). Although these bacteria had a similar metabolic capacity and ability to degrade xenobiotics, among them *P. putida* OR45a had the strongest metabolic potential and aptitude to incorporate into the AS. The results also indicated that *G. soli* OR545b, *R. erythropolis* KB4, *Planococcus* sp. S5, *P. putida* N6, and *Pseudomonas* sp. were not suitable for bioaugmentation due to the lack of their overall ability to enhance contaminant biodegradation and their low ability to survive in the sludge environment. Among the tested bacteria, *R. erythropolis* KB4 and *G. soli* OR45b had the lowest metabolic capacity. Nevertheless, these bacteria may be considered to be used as supportive agents during the construction of a microbial consortium because they had properties that enabled them to thrive in the AS. Finally, *P. putida* OR45a and *P. putida* KB3, which had the highest overall bioaugmentation capacity, were selected for the further analyses.



**Figure 6.** Comparison of the bioaugmentation potential of the tested bacterial strains.

### 3.7. Non-Inhibitory Concentration (NIC) of the Selected Ions for *P. putida* OR45a and *P. putida* KB3

Contamination of wastewater with organic compounds is often accompanied by pollution with heavy metals. Microorganisms with strong degradation capabilities that are also resistant to heavy metals are at the forefront of the candidates for bioaugmentation. Here, the resistance of *P. putida* OR45a and *P. putida* KB3 towards selected heavy metals (Zn, Cu, Cd, Ba, Cr, Pb), which are frequently present in sewage, were observed (Table 4). The trends in the toxicity of heavy metals for *P. putida* OR45a and *P. putida* KB3 were in the order of Cr > Cd > Zn > Cu > Pb > Ba and Cr > Pb > Cd > Zn > Cu > Ba, respectively.

**Table 4.** The non-inhibitory concentration (NIC) of the selected ions for *P. putida* OR45a and *P. putida* KB3.

Ions	NIC, µg/mL	
	<i>P. putida</i> OR45a	<i>P. putida</i> KB3
Zn <sup>2+</sup>	80.68 ± 2.50	81.15 ± 2.99
Cu <sup>2+</sup>	128.43 ± 19.90	104.15 ± 3.56
Cd <sup>2+</sup>	52.13 ± 2.48	59.97 ± 13.49
Ba <sup>2+</sup>	172.63 ± 2.94	224.65 ± 11.58
Cr <sup>6+</sup>	8.30 ± 0.59	11.30 ± 1.38
Pb <sup>2+</sup>	150.00 ± 1.29	50.00 ± 0.01
NH <sub>3</sub> -N	600.00 ± 52.30	900.00 ± 86.40

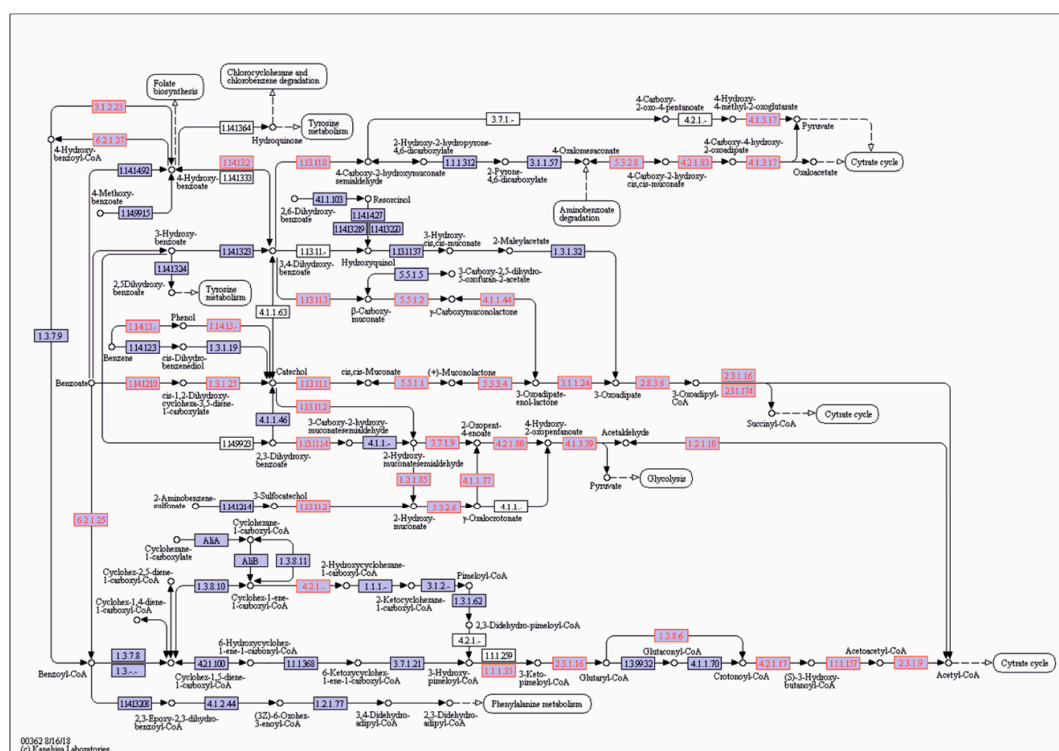
### 3.8. Genome-Guided Exploration of *P. putida* OR45a and *P. putida* KB3

The newly sequenced 5,993,405 bp and 5,772,482 bp genomes of *P. putida* OR45a and *P. putida* KB3 were assembled into 165 and 185 contigs with an average G + C content of 61.3% and 61.7%, respectively (Table S2). A total of 5433 protein encoding genes in *P. putida* OR45a and 5188 protein encoding genes in *P. putida* KB3 were classified into the appropriate clusters of orthologous groups (COG) categories in 21 functional categories (Table S3). More than 35% of all of the predicted genes in both genomes were assigned COG numbers that are associated with cellular metabolism. The largest fraction of the predicted genes was assigned as encoding proteins involved in amino acid transport and metabolism (E category) followed by genes encoding proteins involved in inorganic ion transport (P category) and genes encoding proteins involved in metabolism and energy production and conversion (C category). Specifically, the number of genes participating in cellular processing and signaling as well as information storage and processing were about 17% independent of the candidate for bioaugmentation. The protein

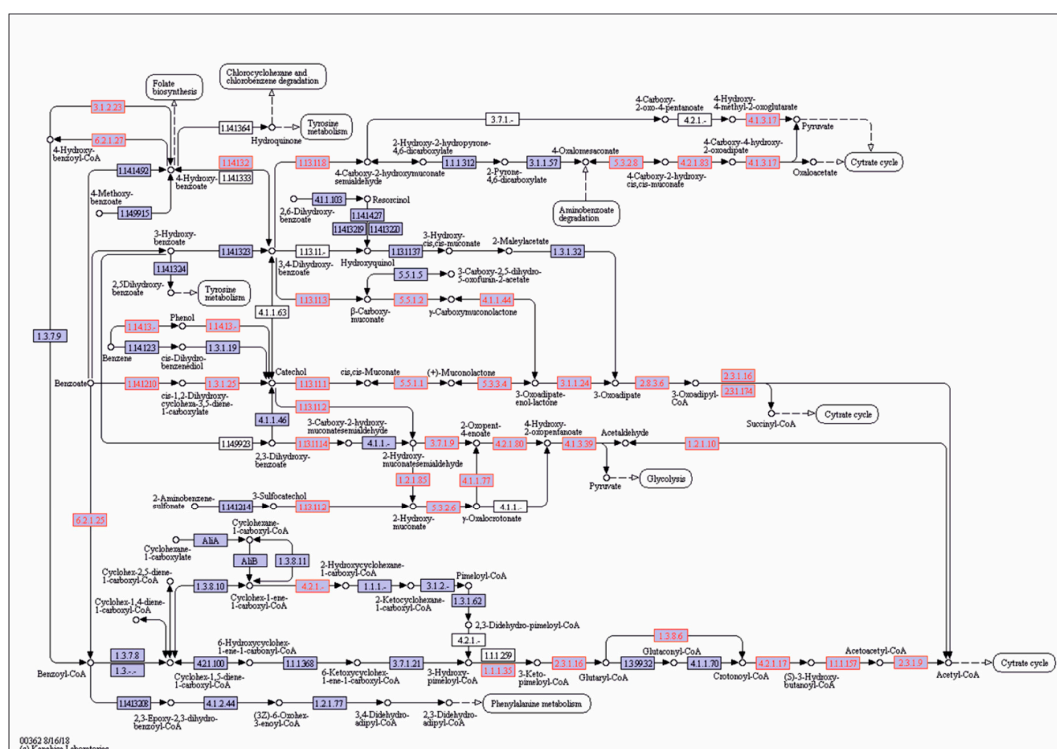


families for which the biological functions remain unknown constituted 18% and 19% of the genomes of *P. putida* OR45a and *P. putida* KB3, respectively.

A scrutiny of the genomes of *P. putida* OR45a and *P. putida* KB3 allowed the genes encoding enzymes of aromatic hydrocarbon degradation pathways to be identified. Locations of catabolic genes identified in these *Pseudomonas* strains in the general metabolic pathway of degradation of aromatic compounds by bacteria are illustrated in Figures 7 and 8, respectively. An exploration of both genomes revealed the presence of gene cluster *dmpKLMNOP*, which encodes multi-component phenol 2-monoxygenase, which is responsible for the cleavage pathway of (methyl)phenols (Table S4). Moreover, both strains contained the *dmpB*, *dmpC*, and *dmpD* genes, which encode catechol 2,3-dioxygenase, 2-hydroxymuconic semi-aldehyde dehydrogenase, and 2-hydroxymuconic semi-aldehyde hydrolase, respectively, involved in the meta-cleavage pathway of aromatic compounds. In addition, the *catA*, *catB*, and *catC* genes encoding enzymes that are implicated in the meta-cleavage pathway, were also identified in the genomes of the bacteria. It was inferred that tested bacteria harboured gene cluster *benABCD* encoding a three-component benzoate 1,2-dioxygenase and 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase, which are involved in the transformation of benzoate into catechol. Moreover, this region also harbored the *pcaDIJ* and *catABC* genes encoding the enzymes for the catechol branch of the  $\beta$ -ketoadipate pathway, which yields succinyl-CoA and acetyl-CoA, as well as the *pcaHGB* genes for the protocatechuate branch of the  $\beta$ -ketoadipate pathway, which enables the degradation of hydroxybenzoate. It is noteworthy that both bacterial strains may be able to degrade nitroalkane compounds due to the presence of the gene cluster *npd*, which encodes 2-nitropropane dioxygenase, in their genomes.



**Figure 7.** Locations of catabolic genes identified in *P. putida* OR45a (marked in pink) in the general microbial metabolic pathway for degradation of aromatic compounds.

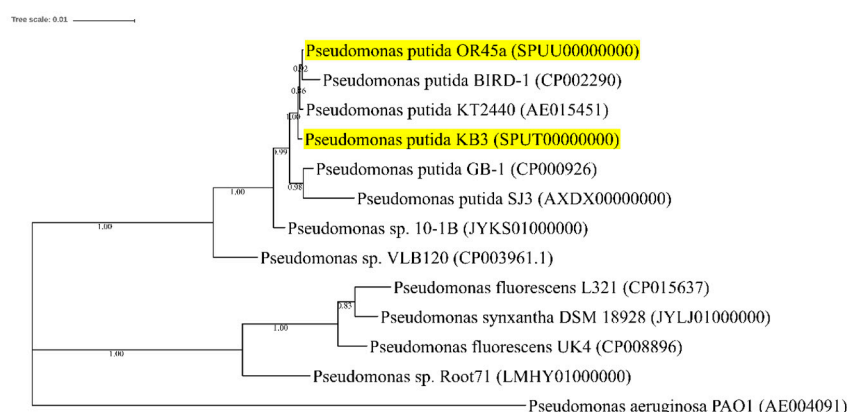


**Figure 8.** Locations of catabolic genes identified in *P. putida* KB3 (marked in pink) in the general microbial metabolic pathway for degradation of aromatic compounds.

The genes from the *P. putida* KB3 and *P. putida* OR45a genomes that are associated with biocide and metal resistance were mined using the BacMet database, which yielded 17 and 21 genes, respectively. The tolerance of *P. putida* KB3 to various solvents was proven by the presence of the *mepB* and *mepC* genes, which encode the inner and outer membrane multidrug/solvent efflux pump, respectively. The *mepBC* gene cluster contributes to the resistance to toluene, hexane, *p*-xylene as well as antibiotics such as ampicillin, penicillin G, erythromycin, novobiocin, and wycline. By contrast, no such gene cluster was found in *P. putida* OR45a. However, both of the tested strains were characterized by resistance to triclosan, *n*-hexane, and *p*-xylene due to the presence of the *mexE* and *mexF* genes, which encode the multidrug efflux RND membrane fusion protein and transporter, respectively. They also had the genes that are involved in the export of toluene, styrene, *m*-xylene, propylbenzene, and ethylbenzene. Moreover, the presence of the *oscA* and *chrR* genes in their genomes indicated a high degree of chromium(VI) resistance. It is also worth emphasizing that the genome of *P. putida* KB3 carried the *merAER* gene cluster, which is involved in mercury resistance.

The genomic-scale analysis of *P. putida* OR45a and *P. putida* KB3 revealed the presence of twelve genes (*algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, and *algA*) involved in the biosynthesis of alginate, which is a very common polysaccharide synthesized by *Pseudomonas* bacteria.

The phylogenetic relationships between *P. putida* OR45a and *P. putida* KB3 and other *Pseudomonas* strains, which were based on the concatenation of 31 single-copy genes were determined and are illustrated in Figure 9. The genome phylogeny with a 92% bootstrap support indicates that the *P. putida* OR45a and *P. putida* BIRD-1 strains [60] formed a monophyletic group or clade that also included, in a decreasing order of relevance, *P. putida* KT2440 [61] and *P. putida* KB3.



**Figure 9.** A maximum likelihood phylogenetic tree of the 13 *Pseudomonas* strains on the concatenation of 31 single-copy genes. *P. putida* OR45a and *P. putida* KB3 are indicated in yellow on the phylogenetic tree. The GenBank accession numbers are provided in the parentheses and the bootstrap support values are indicated on the nodes.

#### 4. Discussion

In this study, 10 bacterial strains isolated from different matrices were tested for the various criteria that they should meet in order to be bioaugmented into the AS polluted with the KPL to enhance the removal efficiency of COD and improve the sludge quality. The catabolic performance of the investigated microorganisms was analyzed in the presence of selected phenolic compounds that have commonly been found in both municipal and industrial leachates as the main components of many manufactured products [62,63]. The obtained results showed that *P. putida* OR45a, *P. putida* KB3, *P. putida* N6, and *S. maltophilia* KB3 exhibited a considerable capability to degrade phenolic compounds, including catechol, benzoate, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, phenol, 2-methylphenol, 4-methylphenol, and a mixture of cresol isomers. The microbial degradation of such compounds is possible due to the activity of iron-dependent oxygenases, which are responsible for opening the aromatic ring. The effective functioning of these enzymes can be improved by the secretion of siderophores, which increases the bioavailability of the iron ions ( $\text{Fe}^{3+}$ ) from sparingly soluble substrates and environments with a low concentration of this element for bacterial cells [64]. Here, three of the bioaugmentation candidates, *P. putida* OR45a, *P. putida* KB3, and *S. maltophilia* KB2, which had a high degradative potential towards aromatic compounds were found to successfully overcome the iron limitation by excreting siderophores. The literature data revealed that siderophores not only play an important role in iron scavenging but may also influence the swimming motility of bacteria as well as their resistance to oxidative and aromatic stress [65]. In our study, the relationship between siderophore production and the ability to withstand high concentrations of the KPL in wastewater was confirmed for two strains—*P. fluorescens* and *Pseudomonas* sp.

Although the application of bioaugmentation in sewage treatment plants can succeed in removing specific toxic compounds, this strategy may fail to enhance the removal efficiency of total COD from wastewater polluted with the landfill leachate because it constitutes a mixture of inorganic and organic contaminants [66]. Therefore, the successful exploitation of bacteria in the bioremediation of wastewater should be supported by selecting strong and adaptable strains. In this study, high concentrations of the KPL (12.5%) caused the complete inhibition of the growth of *G. soli* OR45b, *R. erythropolis*, and *P. putida* N6 as well as the AS microbial populations. In contrast, *P. putida* OR45a, *P. putida* KB3, and *S. maltophilia* KB2 could withstand high concentrations of the KPL, and therefore, they could be promising candidates to treat the leachate in the AS.

The self-aggregation and co-aggregation among genetically identical and distinct bacteria are factors that are believed to play a crucial role in forming activated sludge flocs, which act as a barrier to their mass transfer and reduce the penetration of toxic compounds into the inner cells in the

AS [42,66]. Therefore, screening bacteria with such capabilities may have important significance in bioaugmentation treatment [67]. An example could be the research of Jiang et al. [68], who reported that the bioaugmentation of the AS with *Propioniferax*-like PG-02, which was capable of degrading phenol and *Comamonas* sp. PB-08, which had a strong aggregation ability resulted in the acceleration of phenol degradation and an improvement of the aerobic granulation. A similar cooperation between bacteria that were used to bioaugment the AS loaded with tetrahydrofuran was observed by [69]. Moreover, studies by Adav et al. [70] and Di Gioia [71] indicated that inoculated bacteria promoted the degradation efficiency of other bacteria via their co-aggregation but not via the introduction of any additional degradative potential. Another critical factor for the success of bioaugmentation is the compatibility of inoculated bacteria with the indigenous microorganisms in the AS. In this study, good auto-aggregation propensities were confirmed for *P. putida* OR45a and *P. putida* KB3, which had a high potential to utilize phenol, and for *G. soli* OR45b and *Pseudomonas* sp., which had a poor phenol degradation capacity. Moreover, all of these strains manifested a good or even strong co-aggregation ability with the other microbial strains in the AS, which could mediate their integration into the sludge structure and thus improve the reactor efficiency.

An important feature for bacteria that are selected to bioaugment the AS is their ability to synthesize compounds referred as bioflocculants, which support cell aggregation and immobilization of the contaminants suspended in wastewater. The introduction of such microorganisms into the AS can accelerate sludge granulation and the formation of more stable flocs. This phenomenon was confirmed by Song et al. [72] who bioaugmented the AS with bacterial consortium composed of two bioflocculant-producing strains—*Devosia hwasunensis* and *Tetrasphaera elongata*. In another study, Guo et al. [73] showed that the bioflocculants synthesized by *Bacillus* sp. F2 and *Bacillus* sp. F6 contributed to the growth of the bacteria responsible for the cohesion of sludge flocs and improving the AS flocculation. In addition, bioflocculants can affect the removal of various contaminants in wastewater [74–76]. For example, a bioflocculant produced by *Bacillus salmalaya* 139SI in the treatment of the industrial wastewater resulted in a 99% removal of COD after treatment [77]. In other study, Zhang et al. [78] showed that the treatment of landfill leachate with wastewater using a microbial consortium composed of the bioflocculant-producing strains *Bacillus cereus* and *Pichia membranifaciens* enabled the removal of 73% of COD. In turn, Gomaa and El-Meihy [79] reported a great capacity of biosurfactant produced by *Citrobacter freundii* MG812314.1 to remove the heavy metals from wastewater. Our research revealed that two of the ten bioaugmentation candidates, namely *R. erythropolis* KB4 and *P. putida* N6, had weak bioflocculation properties. However, the other investigated microorganisms were characterized by good (39% to 49%) or strong (50% to 70%) flocculating capacities. This can suggest that the bioflocculation abilities of the bacterial strains presented in this study may support their resistance to the toxic compounds that are found in the KPL.

The capability of microorganisms to communicate with each other by sensing and responding to small signal molecules (auto-inductors), which is known as quorum sensing (QS), is very important during the selection of desirable bacteria for the AS bioaugmentation. The propensity of bacterial strains to secrete auto-inductors can support their intracellular communication with the autochthonous microorganisms in the AS and thus enable its effective settling. This phenomenon was studied by Wang et al. [80], who showed that the N-AHLs produced by *Acinetobacter* sp. TW, were responsible for protecting cells against the toxicity of industrial wastewater and enabled this bacterium to incorporate into the structure of the AS. Herein, among all of the tested gram-negative bacteria, only *P. putida* N6 did not produce N-AHLs, whereas the presence of this feature in other candidates confirmed their ability to incorporate into the AS flocs.

Another desirable feature of the bacteria that are used in the bioaugmentation strategy is their ability to produce exopolysaccharide substances (EPS). EPS are extremely important substances in mediating cell aggregation during the formation of activated sludge flocs, which determines the structure, charge and settleability of flocs [81]. The aggregation propensity of the bacteria inoculated into the AS not only mediates their communication with sludge autochthonous microorganisms but also

plays an important role in the bacterial defense against predators [82]. Furthermore, the hydrophobic regions and numerous anionic functional groups (e.g., carboxyl, phosphoryl, sulfhydryl, phenyl, and hydroxyl) present in the structure of EPS not only mediate the accumulation of nutrients but also bind the contaminants present in wastewater including heavy metals [83,84]. This was confirmed by Gupta and Thakur [85] who reported that the effectiveness of contaminant removal from wastewater inoculated with *Bacillus* sp. ISTVK1 was partially associated with its ability to produce a copious number of extracellular polymers. In this study, the ability of *P. putida* OR45a, *P. putida* KB3, *P. putida* mt-2, *P. fluorescens*, and *S. maltophilia* KB2 to produce EPS may support their resistance to the KPL in wastewater as well as help in the formation of larger and denser AS particles.

The formation and structure of the AS flocs are closely connected with the cell surface hydrophobicity (CSH). Previous research indicated that using microorganisms with a high CSH in the bioaugmentation of the AS accelerated the formation of sludge granules [70]. Moreover, it was found that along with an increase of microbial hydrophobicity, the cell–cell adhesion and aggregation also became stronger [86] and the removal of contaminants such as aromatic compounds, heavy metals, nitrogen, and phosphorus was accelerated [70]. In the present study, *P. putida* OR45a, *P. putida* KB3, *P. putida* mt-2, and *S. maltophilia* KB2 were categorized as the strains with the strongest hydrophobic properties (CSH above 50%). All of the tested bacteria had the ability to regulate CSH in order to adapt to the presence of the KPL in wastewater. However, for most of the strains, the reduction of their CSH was noted after addition of the KPL, which caused their membrane profile to become more hydrophilic. This phenomenon might be the result of diminishing the availability and permeability of bacterial cell wall to contaminants in the KPL in order to prevent pollutants from entering the cell [87]. However, two bacterial strains, *P. putida* OR45a and *Planococcus* sp. S5, significantly increased their CSH in the presence of the KPL, which may imply that they were capable of degrading the pollutants distributed in the KPL. Similarly, in the research of Wick et al. [88] the increase in cell hydrophobicity of *Mycobacterium frederiksbergense* after the addition of anthracene was attributed to its capability to utilize this compound as a carbon and energy source.

The hydrophobic character of the cells supports the uptake of the hydrophobic substrates from the environment without the production of surfactants, which predisposes their use in bioremediation technologies [89]. Because the microbial metabolism of xenobiotics could be impeded due to their hydrophobic character, the ability to synthesize biosurfactants is a desirable feature of potential candidates for bioaugmentation. Biosurfactants reduce both the surface and interfacial tension via emulsification and thus increase the availability of contaminants for microorganisms [86,87]. For example, the biodegradation efficiency of nonylphenol in wastewater significantly increased after bioaugmentation of the AS with biosurfactant-producing *Sphingomonas* sp. TTNP3 [31]. Similarly, Chen et al. [90] connected the increase in the removal of resin compounds from the effluent with the production of biosurfactants by *Gordonia* JW8 inoculated into the AS. In our study, *P. putida* OR45a, *P. putida* KB3, *P. putida* mt-2, *P. fluorescens*, and *S. maltophilia* KB2 were able to produce biosurfactants. However, the analysis of the potential biosurfactant producers based on their ability to emulsify hydrocarbons revealed that only *P. putida* OR45a and *P. putida* KB3 had a good emulsification activity (>45%).

It has been claimed that the ability of flagellated bacteria to move directly toward contaminants in wastewater plays an important role in the AS systems because it increases the bioavailability of the compounds for microorganisms. Moreover, this mechanism can support the competition of motile bacteria with the autochthonous microorganisms in the AS. Here, all of the tested bacteria exhibited swimming and swarming motility phenotypes. It was found that the presence of the KPL enhanced the chemotactic response of *R. erythropolis* KB4, *Planococcus* sp. S5, and *P. putida* N6, which resulted in a decrease in their swimming and swarming motility. The chemotaxis that was exhibited by *P. putida* OR45a, *P. putida* KB3, *S. maltophilia* KB2, *P. fluorescens*, *Pseudomonas* sp., and *G. soli* OR45b after the KPL had been added to the wastewater was primarily linked with the swimming motility and finally resulted in a decrease in the multicellular movement of the bacteria. Literature data indicated that this



phenomenon is common for many bacteria [91,92]. Interestingly, the KPL was found to increase the swarming motility of only *P. putida* mt-2.

Screening, which was based on phenotypes and biochemical tests, enabled the best two candidates *P. putida* OR45a and *P. putida* KB3 to be selected for the further bioaugmentation of the AS. They were characterized by the strongest metabolic capacity, the ability to support xenobiotic degradation and to incorporate into the AS. In order to get a deep insight into their genomes and identify the genes that belong to the metabolism of aromatic compounds, they were completely sequenced. An in silico genomic analysis revealed the presence of the genes encoding the oxygenase enzymes that are involved in the crucial steps in degrading aromatic compounds, i.e., hydroxylation and opening the aromatic ring (*hcA2* gene). It was found that *P. putida* OR45a and *P. putida* KB3 genomes harbored both the catechol 1,2-dioxygenase and catechol 2,3-dioxygenase genes (*catA* and *dmpB*), which are involved in degradation of catechol via the *ortho*- and *meta*-cleavage pathways, respectively. Moreover, the genomic data supported the results that had been obtained in the experimental tests, and thus confirmed the ability of *P. putida* OR45a and *P. putida* KB3 to biodegrade (methyl)phenols and benzoate efficiently. Because the degradation of aromatic compounds leads to the formation of reactive oxygen species such as  $H_2O_2$ ,  $OH^-$  and  $O_2^-$ , the presence of *oscA* gene, which encodes iron-dependent superoxide dismutase, can give these bacteria an advantage in surviving in unfavorable conditions. These results suggested that the bioaugmentation of the AS with *P. putida* OR45a and *P. putida* KB3 may be an auxiliary strategy to reduce the oxidative stress in the bioreactors exposed to various toxic compounds in effluents. Interestingly, both strains were able to biodegrade the toxic nitroalkane compounds due to the presence of gene cluster *npd* encoding 2-nitropropane dioxygenase, which belongs to the family of nitroalkane oxidizing enzymes and catalyze the oxidative denitrification of nitroalkanes to their corresponding carbonyl compounds and nitrites.

Literature data indicated that the embedment within an alginate matrix may be a suitable bioaugmentation approach to enhance the incorporation of inoculates into the AS flocs even in industrial-like wastewater treatment systems [93]. In this study, *P. putida* OR45a and *P. putida* KB3 were found to produce alginate. This exopolysaccharide is thought to have a protective function in a harsh environment in which microorganisms are continually subjected to oxidative stress [94]. Moreover, Lin et al. [95] reported that bacterial alginate greatly improved the formation and settleability of the aerobic granular bioparticles in the AS. In another study, Matz et al. [96] documented that the production of alginate by *P. aeruginosa* induced the formation of microcolonies that protected against protozoa.

Contamination of the leachate with phenolic compounds is often accompanied by heavy metals, organic solvents, antibiotics as well as ammonium ions. Therefore, the bacterial strains that will be used for bioaugmentation should be resistant to diverse toxic substances. The basic mechanisms of solvent tolerance in bacteria include the efflux pumps, which remove toxic solvents from bacterial cell membrane [97]. In both *Pseudomonas* candidates, a multidrug efflux pump of the resistance-nodulation-division family probably made a major contribution to the resistance to various solvents such as *n*-hexane, *p*-xylene, toluene, styrene, *m*-xylene, propylbenzene, and ethylbenzene. Moreover, *P. putida* KB3 was found to be resistant to antibiotics, including ampicillin, penicillin G, erythromycin, novobiocin, and tetracycline. From the application point of view, selected bacteria showed desirable traits to be used in the treatment of leachate-contaminated wastewater as they are simultaneously able to degrade aromatic hydrocarbons and thrive in the presence of pollutants that are known to limit the biodegradation of these compounds.

Here, *P. putida* OR45a and *P. putida* KB3 had a *mer* operon that confers a resistance to  $Hg^{2+}$ . However, the level of heavy-metal tolerance was higher in *P. putida* KB3 than in *P. putida* OR45a. The genome of *P. putida* KB3 harbored the *oscA* gene, which is involved in the sulfur starvation response in an ambient environment and is required to cope with the oxidative stress induced by chromium ions. Chromium is a heavy metal that is of commercial importance; thus, significant amounts of it are released into industrial wastewater. Unfortunately, this trace element was identified as an inhibitor of respiration and microbial growth in non-acclimated AS when was present at concentrations above

10 µg/mL [98]. In this study, the tolerance of *P. putida* OR45a and *P. putida* KB3 to 8.30 and 11.30 µg/µL of chromium was observed, respectively. Moreover, both of the *Pseudomonas* isolates exhibited a tolerance to a wide range of the heavy-metal ions (Cr, Ba, Cd, Pb, and Zn) that are commonly found in the KPL. Implementing the traditional AS technology for landfill leachate treatment is very challenging especially for mature and old leachates, which are characterized by high level of ammonia nitrogen (NH<sub>3</sub>-N) of 360 to 2150 µg/mL [99]. It is well known that highly toxic NH<sub>3</sub>-N significantly inhibits the growth and activity of the microorganisms in the AS systems and thus suppresses the biodegradation process [100]. Because of the aforementioned issues, it is necessary to remove ammonia nitrogen from wastewater. However, to date, there is scarce data about the successful bioaugmentation treatment of landfill leachate with high levels of NH<sub>3</sub>-N [101]. In this study, the results of the tolerance tests indicated that *P. putida* OR45a and *P. putida* KB3 could survive in the KPL when the ammonia nitrogen concentration was as high as 600 and 1000 µg/mL, respectively.

To sum up, the extensive methodology used in this work allowed accurate characterization of the different capacities of many bacterial strains and select *P. putida* OR45a and *P. putida* KB3 as the best candidates for bioaugmentation of the AS in a conventional wastewater treatment plant struggling with the problem of continuous exposure and management of complex wastewater with unknown composition, such as those contaminated with landfill leachate. Due to the high capacity of selected strains to degrade phenolic compounds and ability to tolerate heavy metal stress, it can be expected that their presence in bioreactors may improve the biodegradation of pollutants in contaminated wastewater. Our pilot scale study conducted in the sequencing batch reactor (SBR) revealed that the bioaugmentation of the AS polluted with the KPL with selected *Pseudomonas* strains succeeded in achieving of over 80% of the COD removal efficiency, while the reduction of organic contaminants in the non-inoculated SBR was about 40%. This can be explained by the activity of oxidative enzymes in bacteria cells, which can alleviate the potential oxidative stress in biological systems induced by high concentrations of toxic pollutants. More importantly, the ability of *P. putida* OR45a and *P. putida* KB3 to communicate among themselves as well as with other microorganisms from the AS may determine their survival in WWTP. Alternatively, bacterial biomass can be immobilized on various carriers for enhancing survivability and degradative potential of the cells. Although the limitations of bioaugmentation still exist, this strategy seems to be a beneficial solution for improving many aspects of the treatment of industrial and leachate polluted wastewater.

## 5. Conclusions and Future Prospects

Bioaugmentation of the AS is considered to be a promising decontamination approach in the context of the various strategies that are applied to quickly remove contaminants from wastewater. However, selecting robust bacterial strains for bioaugmentation should depend on our knowledge not only about their catabolic potential, but also about their ability to survive in the AS. To date, few studies have made a comprehensive analysis of the factors that determine the specific characteristics of candidates for bioaugmentation and how they contribute to the operation and maintenance of wastewater treatment systems. The present study underscores the importance of using a multifaceted approach for selecting promising candidates to enhance the treatment of wastewater contaminated with the KPL. Moreover, it demonstrated that the genome-centric approach may be useful to complement the biochemical tests to select the best strains for specific bioaugmentation. Here, based on all of the conducted tests regarding their ability to degrade aromatics compounds, their survival in the presence of the KPL, their resistance to heavy metals, and their potentially positive influence on the AS structure and settleability, two bacterial strains, namely *P. putida* OR45a and *P. putida* KB3, were selected as the best candidates for the bioremediation of KPL-contaminated wastewater. Currently, our research is focused on exploring whether using the selected *Pseudomonas* strains and their mixed co-culture can protect the structure and function of the AS microbial communities against the KPL and prevent operational problems in dedicated WWTP receiving the landfill leachate contaminated with aromatic compounds.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4441/12/1/140/s1>. Table S1: Composition of media used for characteristics of bioaugmentation candidates (section Materials and Methods), Table S2: General characteristics of genomes of *P. putida* OR45a and *P. putida* KB3, Table S3: A distribution of the genes associated with the clusters of orthologous groups (COG) categories in the genomes of *P. putida* OR45a and *P. putida* KB3, Table S4: Description of genes responsible for selected features of *P. putida* KB3 and *P. putida* OR45a.

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**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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