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Searching of Phenol-Degrading Bacteria in Raw Wastewater from Underground Coal Gasification Process as Suitable Candidates in Bioaugmentation Approach

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ABSTRACT

The aim of the conducted study was to isolate, identify and characterize suitable bacterial strains from UCG wastewater as potential candidates for the bioaugmentation approach. For this purpose, the straightforward cultivation procedure and unique biochemical selection were employed to gain insights into the specific properties of bacteria. From the 100 strains isolated from UCG wastewater, three (*Paenibacillus pasadensis* SAFN-007, *Peanibacillus humicus* Au34, and *Staphylococcus warneri* DK131) demonstrated the capacity to degrade phenol and specific biochemical properties. Phenol degradation reached more than 90% for the above-mentioned strains, while the average phenol removal rate for other selected strains was 82.9%, ranging from 66.1% to 90%. The bacterial strains belong to multi-enzyme producers and constitute a possible source of potential technologically important enzymes. Phenotypic microarray plates were used to characterise the metabolic properties of the strains. It was found that 74%, 67.4% and 94.2% of the carbon metabolites tested were utilised by *Paenibacillus pasadensis* SAFN-007, *Peanibacillus humicus* Au34 and *Staphylococcus warneri* DK131, respectively. Among C sources, the strains have the capability to metabolize some substrates appearing in phenol pathways, such as: N-acetyl-D-glucosamine, succinic acid, α-hydroxy-glutaric acid-γ-lactone, bromosuccinic acid, mono-methyl succinate, methyl-pyruvate, p-hydroxy-phenyl acetic acid, m-hydroxyphenylacetic acid, L-galactonic acid-γ-lactone, D-galactonic acid-γ-lactone, phenylethylamine. Bacteria show different levels of tolerance to pH and osmolality, and they can thrive in different habitats. Another characteristic of these strains is their high resistance to many antibiotics (multi-resistant bacteria). These properties allow the use of the isolated bacterial strains as good candidates for bioremediation of phenol-contaminated environments. The wastewater from the underground coal gasification process is an example of a good extreme environment for the isolation of unique bacteria with specific metabolic properties.

Keywords: post-processing wastewater, underground coal gasification (UCG) process, biodegradation, phenol, *Peanibacillus* sp., *Staphylococcus warneri*, phenotypic microarrays.

INTRODUCTION

One of the key sources of environmental pollution is industrial wastewater. The discharge of industrial wastewater containing the complex of various types of inorganic and organic pollutants into various water sources disrupts ecological sustainability and poses a risk to human health (Riggio et al., 2018; Vymazal, 2021). The wastewater from the underground coal gasification process (UCG process) and from the coke industry are examples of heavily

contaminated sources of environmental pollution. UCG is considered to be a clean green technology in which gas is obtained directly from a coal deposit in situ, without the need for coal extraction. Numerous organic and inorganic contaminants are formed during different process stages and after process termination. Then, the UCG process carries some environmental risks, mainly related to the raw post-processing wastewater. Underground gasification produces a higher total amount of polluted wastewater (Smolinski et al., 2012). The main contaminants in UCG wastewater are organic compounds, such as: BTEX, benzene, PAHs, phenols and inorganic substances, such as: heavy metals, ammonia and cyanides (Kapusta et al., 2011; Borgulat et al. 2022). The average values of some parameters in UCG wastewater, such as COD, BOD, TOC and phenol index were noted as very high, e.g.: 850 $mgO_2 \cdot L^{-1}$, 360 $mgO_2 \cdot L^{-1}$, 270 $mgC \cdot L^{-1}$, and 100 $mg \cdot L^{-1}$, respectively. In the case of BTEX, the concentration ranged from 600 to 1360 $\mu g \cdot L^{-1}$ and benzene was dominant. The average concentration of PAHs was 1300 $\mu g \cdot L^{-1}$ (from 190 to 2060 $\mu g \cdot L^{-1}$) (Wiatowski et al., 2023). UCG wastewater, despite its negative impact on the environment, can be considered as a source for the isolation of microorganisms with specific and useful catabolic properties.

The bioaugmentation strategy in environmental remediation technologies is one of the routes to apply suitable microbes. Bioaugmentation is known as the introduction of microbial strains or microbial consortia with new metabolic properties into the polluted environment. It is a promising but controversial approach used mainly for degrading recalcitrant compounds (Thompson et al., 2005). The basic idea of bioremediation is the multiplication of catabolically relevant microbes that are responsible for accelerating the efficiency of remediation processes. However, the success of bioaugmentation methods depends on the identification and isolation of suitable microbial strains as well as their subsequent survival and activity in the foreign environment. Thompson et al. (2005) discussed the bioaugmentation as one of the remediation strategies used in situ. Future prospects and ways to improve the selection process for isolating effective microbial strains for bioaugmentation were also discussed in this paper. The authors concluded that the initial selection of strains was based on a single key criterion, e.g. degradability, while the potential ability of the strains to replicate and be active at the sites of use was not considered. Then, the challenge of selecting the process of strains for bioaugmentation and the recent technical advances used in the selection methods of strains were discussed and summarized.

The bacteria considered for bioaugmentation should be able to degrade the respective pollutant even in the presence of potentially inhibitory pollutants, survive and compete in the environment after their inoculation and be compatible with autochthonous communities (Singer et al. 2005; Herrero & Stuckey, 2015). Bioaugmentation has been successfully used in the remediation of sites contaminated with aromatic compounds (Adams et al., 2015; Villaverde et al., 2019). The specific bioaugmentation research was carried out in different countries as described by Adams et al. (2015). The aim of the conducted study was to isolate, identify and characterize the selected bacterial strains from UCG wastewater as potential candidates for bioaugmentation approach in phenol-contaminated environments. For this purpose, the straightforward cultivation procedure and unique biochemical selection were employed to gain insights into the specific properties of bacteria. The isolates can be considered as a "tool" in the bioaugmentation approach for the remediation of phenol-polluted environments.

MATERIALS AND METHODS

Sampling of raw post-processing wastewater

The post-process wastewater samples were generated at the Central Mining Institute as a result of coal gasification experiments described by Wiatowski et al. (2023). The total amount of raw wastewater samples was collected in a plastic tank with a capacity of 1 m^3 (Mauser type) under sterile conditions. After mixing and decanting into smaller sterile containers, the raw wastewater samples were taken to the laboratory and used for microbiological analysis.

Isolation of bacteria from UCG post-processing water

Plating and serial dilution methods were used to isolate the culturable and aerobic bacteria. Aerobic bacteria were incubated on an SMA medium (Standard Methods Agar, BioMérieux) containing 100 mg cycloheximide/L at 30°C for 24–48 h. To obtain pure bacterial cultures by separating the

individual cells from each other, the individual bacterial colonies were transferred to Luria-Bertani solid medium with a pH of 7.0 containing 20.0 g·L^{-1} agar, 10.0 g·L^{-1} g·L asein peptone, 5.0 g·L^{-1} yeast extract and 5.0 g L^{-1} NaCl, using the strip plate technique. Then, the single bacterial isolates were transferred into a liquid LB medium, and the liquid cultures were incubated with constant shaking (100 rpm) at 30°C for 24 - 48 h. The strains are deposited at -80°C in a Luria-Bertani medium supplemented with 20% (v/v) glycerol in the Institute for Ecology of Industrial Areas (IETU) collection.

Identification of isolated bacterial strains

The genomic DNA from 100 bacteria was isolated with a Roche kit, according to the manufacturer's instructions. DNA concentration, yield and purity were assessed using absorbance ($A_{260/280}$, $A_{260/280}$), optical density and agarose gel electrophoresis. DNAs are stored at -20°C at IETU. Then, the DNA samples were sent to Genomed SA (Poland) for sequencing. The isolates were identified by 16S rRNA gene sequencing. The universal bacteria primers, 8F and 1492R were used to amplify region V3-V4 of 16S rRNA genes in accordance with the conditions established by Genomed SA (Poland). For each sequencing reaction, 3 µl of BigDye™ Terminator v 3.1 Ready Reaction Mix, 1 µl of BigDye™ Terminator v1.1 & v 3.1 5× sequencing buffer, 5 pmol of appropriate primer and 50–250 ng of DNA template were mixed in a final volume of 10 µl. Cycle sequencing was performed in 100 µl PCR tubes. Incubation at 96°C for 1 minute as the first denaturation step was followed by 25 cycles of 96°C for 10 seconds, 54°C for 5 seconds and 60°C for 4 minutes incubation. Prior to purification, the reaction mix was incubated at 4°C. The purified reaction products were separated by electrophoresis on the 3730XL DNA analyzer according to the producer (Thermofisher) instructions. The sequences were compared and analyzed in NCBI's BLAST program. Taxonomic identification was done by comparison with the National Centre for Biotechnology Information (NCBI) database using the BLASTN algorithm.

The 16S rRNA sequences of the strains, characterized by the greatest biodegradation of phenol, were deposited in the NCBI GenBank database with accession numbers (Table 1). The PhyML software (Approximate Likelihood-Ratio Test: SH-like) available at *phylogeny.fr* website was used to generate the phylogenetic tree (Dereeper et al., 2008). The Gblocks program was used to eliminate poorly aligned positions and divergent regions.

Biochemical characterization of bacteria

The following properties of bacterial strains were evaluated: phenol biodegradation on a laboratory scale, biosurfactant production, enzyme activities, and biochemical and metabolic properties by phenotypic microarrays (PMs plates, Biolog).

Phenol biodegradation

To evaluate phenol biodegradation, bacterial strains were grown aerobically in 100 mL liquid LB medium supplemented with various concentrations of phenol at 30°C with constant shaking (100 rpm). During a 10-day acclimatization period, phenol was continuously added to each culture every 48 hours. The degradation of phenol was measured after 24 hours in the liquid cultures to which a phenol concentration of 6.0 mM was added as the initial concentration. The amount of phenol in the culture was measured colorimetrically with p-nitroaniline.

Biosurfactant production

Bacterial cultures were grown in LB medium on an orbital shaker (100 rpm, 30°C, 24 hours) and then diluted with LB medium to achieve OD= 0.8 (λ= 600 nm). After 72 hours, the cultures were

Table 1. Phylogenetic affiliations of selected bacteria

Accesion number	NCBI affiliation	Similarity (%)	Phylum, class, order, family, genus
OQ996958	*Paenibacillus humicus* Au34 (MW534854.1)	99.6	Firmicutes, Bacilli, Paenibacillales, Paenibacillaceae, Paenibacillus
OQ996960	*Paenibacillus pasadensis* SAFN-007 (NR_042757.1)	100.0	Firmicutes, Bacilli, Paenibacillales Paenibacillaceae, Paenibacillus
OQ997235	*Staphyloccocus warneri* DK131 (MT642942.1)	100.0	Firmicutes, Bacilli, Staphylococcales, Staphylococcaceae, Staphylococcus

centrifuged (12,300 RCF, 10 min). The resulting supernatants were used to evaluate the production of biosurfactants. Indirect methods, e.g. blood agar, oil-spreading test and drop-collapse method as described by Płaza (2014) were used. The surface tension assessment was used as a direct method to evaluate biosurfactant production. The supernatant samples from bacterial cultures were measured for ST using a Du Nöuy ring with a tensiometer SIGMA T702 (Attension, Finland) according to the supplier`s instruction.

Measurements of each sample were taken three times at room temperature. Clean water was used to calibrate the device and as a reference measurement. The average of the measurements was given as the final SF result. The average values of surface tension were 72.01 ± 0.23 and 68.34 ± 0.86 for water and LB medium, respectively. An average standard deviation was calculated.

Enzymatic activities

First, the isolated bacterial strains were cultured on LB liquid medium (t = 72 h, T = 30°C, 90 rpm). The bacterial suspension was then applied to the previously prepared differentiation media. After incubation (incubation time varied depending on the medium used, T= 30°C), the ability of the selected strains to produce a specific group of enzymes was assessed (Table 2).

Phenotypic characterization by biolog phenotype microarrays (PMs)

An appropriate amount of liquid culture of the test strain (LB medium, T = 30°C, t = 48 h, 90 rpm) was added to the Biolog IF-0 a solution (5:1) diluted with water so that the final optical density (OD) of the obtained suspension of microorganisms was 42% (the turbidity of the suspension was determined with a turbidimeter). Subsequently, the previously prepared solutions according to the BIOLOG™ method (Biolog Inc., Hayward, USA) were added to the suspension so that a transmittance of 85% was obtained. The detailed methodology of the prepared solutions is described, among others, in the paper by Jałowiecki et al. (2020). Subsequently, the solutions together with the suspension of microorganisms were applied to PM plates using a multichannel pipette, 100 µl per well. The plates were incubated for 7 days in an OmniLog incubator at the same temperature as the culture (30°C). The growth of the microorganisms was read automatically every 30 minutes. Plates PM1-5 and PM9-PM13 were used in this study. The results are presented in the form of a circular chart (Fig. 2). The different parts of the diagram were marked according to the types of compounds present on the plates: (PM1-PM2) carbon sources, nitrogen sources (PM3), phosphorus and sulfur sources (PM4), nitrogen sources (PM5). The PM plates from 9 to 13 including tolerance/sensitivity to different osmolytes (PM9), pH conditions (PM10) and other chemicals i.e. antibiotics and chemicals (PM11-13) were studied.

RESULTS AND DISCUSSION

Around 100 bacterial strains were isolated and 100 DNA samples were sequenced. However, only 50% of the isolates were identified with a probability of 95–100%. The rest of the isolates were not identified because of the quality of the DNA samples or because they did not exist in the NCBI database. Among the identified bacteria, *Paenibacillus* and *Bacillus* species were dominant in UCG post-processing waters.

Table 2. Substrates used to assess enzymatic activity

Medium, incubation time, temperature	Enzymes activity	A positive result indicating the ability to produce the quoted enzymes
Urea Agar Base (UAB), 48 hrs., 30°C	Urease (ability to hydrolyse urea)	Change medium colour from pale yellow to violet
Nutrient agar with starch (1%), 48 hrs., 30°C	Amylase	A clear halo appeared around the colonies after the flooding the plate with 1% Lugol's liquid
Milk agar (SMA), 48 hrs., 300°C	Protease	Clear zones around the bacterial strains confirmed the proteolytic activity of the strains
Carboxymethyl cellulose agar (CMC), 7 days, 30°C	Celulase	Yellow color around the colonies after the flooding the plate with 1% Lugol's liquid
MHI medium (Li et al. (2011), 3 to 5 days, 30°C	Inulinase	A clear halo appeared around the colonies after the flooding the plate with 1% Lugol's liquid

The most numerous bacterial strains were selected for the biodegradation of phenol, e.g.: *Paenibacillus glucanotylicus* P69, *Bacillus altitudinis* VMFR48, *Bacillus nealsoni* LE3, *Bacillus pseudomycoides* S2015-2C, *Bacillus sp.* CMAA 1185, *Bacillus stratosphericus* TR4, *Margalitia shackletoni* LMG-18435, *Oceanobacillus picturae* B5, *Paenibacillus cineris* BB, *Paenibacillus favisporus* U3, *Paenibacillus humicus* Au34, *Paenibacillus humicus* Sp29, *Paenibacillus pasadensis* SAFN-007, *Paenibacillus cineris* cu1-7, *Peanibacillus cellulositrophicus* KACC 16577 and *Staphylococcus warneri* DK131. Figure 1 shows the phylogenetic tree of the above strains, which was constructed on the basis of 16S RNA sequences.

On the basis of their ability to survive the increasing concentration of phenol, *Pseudomonas humicus* Au34, *Paenibacillus pasadensis* SAFN-007 and *Staphylococcus warneri* DK131 were selected for further study. The selected strains were also characterized by the highest phenol degradation rate, amounting to more than 94% (Table 3). The average phenol degradation rate of the other selected strains was 82.9% and ranged from 66.1% to 90%. Many researchers examined and isolated phenol-degrading microorganisms. Microbes, such as *Pseudomonas* sp., *Alcaligenes* sp., *Ewingella americana, Bacillus* sp., *Acinetobacter* sp., *Streptomyces* sp., *Achromobacter* sp., *Corious versicolor*, *Ralstonia* sp., *Fusarium* sp., *Phanerocheate chrysosporium*, having the ability to decomposition various aromatic compounds including phenol, were used in several remediation technologies (Anhu et al., 2017; Tian et al., 2017; Alshabib & Onaizi, 2019; Filipowicz & Cieślinski, 2020; Shebl et al., 2022; Bibi et al., 2023). Screening bacteria with degradation capabilities is of great importance in the clean-up of contaminated environments using the bioaugmentation strategy. The properties and important role of bacteria of the genus *Paenibacillus* in sustainable farming and biotechnological processes in industry were described by Grade et al. (2016). The selected strains also belong to the multi-enzyme producers and represent a potential source of technologically important enzymes; for example, in the enzymatic pre-treatment of organic recalcitrant waste (biomasses), wastewater prior to its anaerobic/aerobic digestion or in the biodegradation of organic pollutants such as antibiotics, dyes and petroleum hydrocarbons. The enzymatic activity of the selected strains is listed in Table 3.

Enzymes are a crucial pillar of industrial biotechnology and can be used in a wide range of bioindustrial sectors, for example in the production of biofuels, washing detergents, food and feed production and in the preparation of bio-based chemicals, enhanced biological treatment

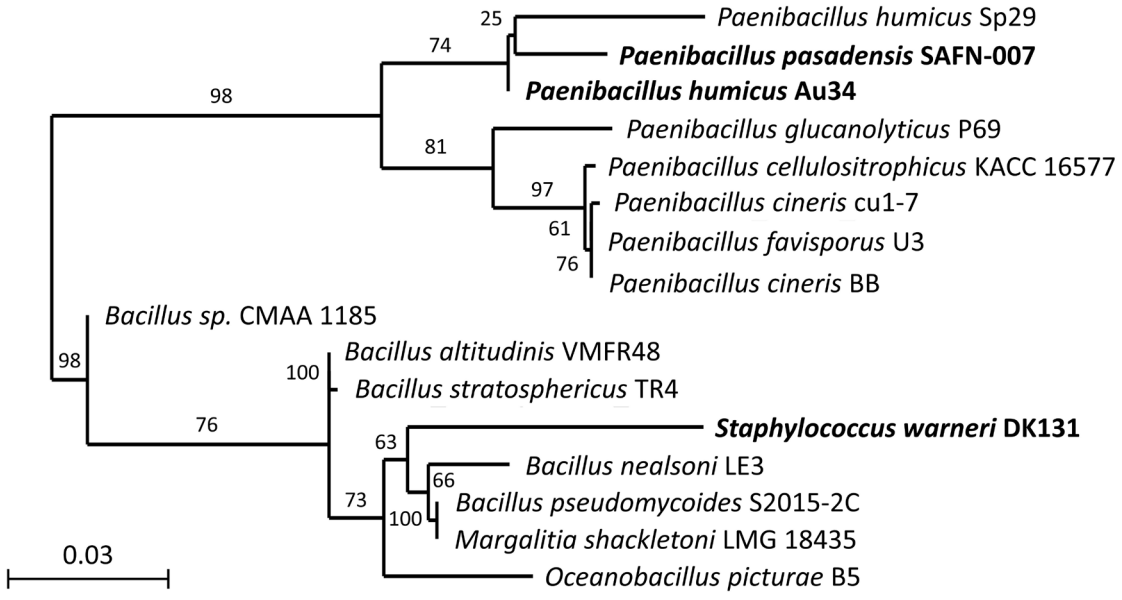

Figure 1. Maximum likelihood tree based on the 16S rRNA gene. The diagram shows, among other things, the relationships between the two selected *Paenibacillus* strains (*Paenibacillus pasadensis* SAFN-007 & *Paenibacillus humicus* Au34) and *Staphylococcus warneri* DK131 – strains isolated from UCG post-processing waters and characterised by the highest phenol biodegradation. The representation of the branches is the result of neighbour-joining (support values are given in %, number of bootstraps 500)

Table 3. Parameters indicating phenol biodegradation degree, biosurfactants production and enzymes activities

Measurements		*Paenibacillus humicus* Au34	*Paenibacillus pasadensis* SAFN-007	*Staphyloccocus warneri* DK131
Biosurfactants production	Surface tension (mN/m)[a]	52.14 ± 0.60	55.01 ± 0.59	52.25 ± 0.76
	Blood agar	-	-	-
	Oil spreading test	-	-	-
	Emulsification index (%)	0.0	0.0	0.0
Enzymatic activity[b]	Ureases	-	-	-
	Proteases	+	+	+
	Cellulases	+	+	+
	Inulinases	+	+	+
	Amylases	-	+	-
Phenol biodegradation Removal rate (%)[c]		93.3 ± 5.1	94.8 ± 6.2	92.8 ± 4.8

Note: [a] Control measurement (H_2O) – 56.73 mN/m, [b] Clearing zones: (–) lack of clearing zone, (+) clearing zone occurred, [c] Initial phenol concentration (C_o) in culture – 6.0 mM·L^{-1}

of biomass. The broad variability of enzyme applications is presented in 28 technological processes saving raw material energy and/or chemicals obtained by the implementation of enzymatic processing (Jałowiecki et al., 2017; Cabrera Blamey, 2018; Bhandari et al., 2021; Mesbah, 2022). Many authors support the hypothesis that enzyme technology is a promising means of moving toward cleaner industrial production and offers great potential for bioeconomy development. Industries are looking for new microbial strains in order to produce different enzymes to fulfil the current requirements of bio-based industry. The global enzyme market was valued at $5.8 billion in 2021 and is projected to reach $10.2 billion by 2031, growing by 6% from 2022 to 2031 (Mesbah, 2022). The discovery of new microbial enzymes through extensive and persistent

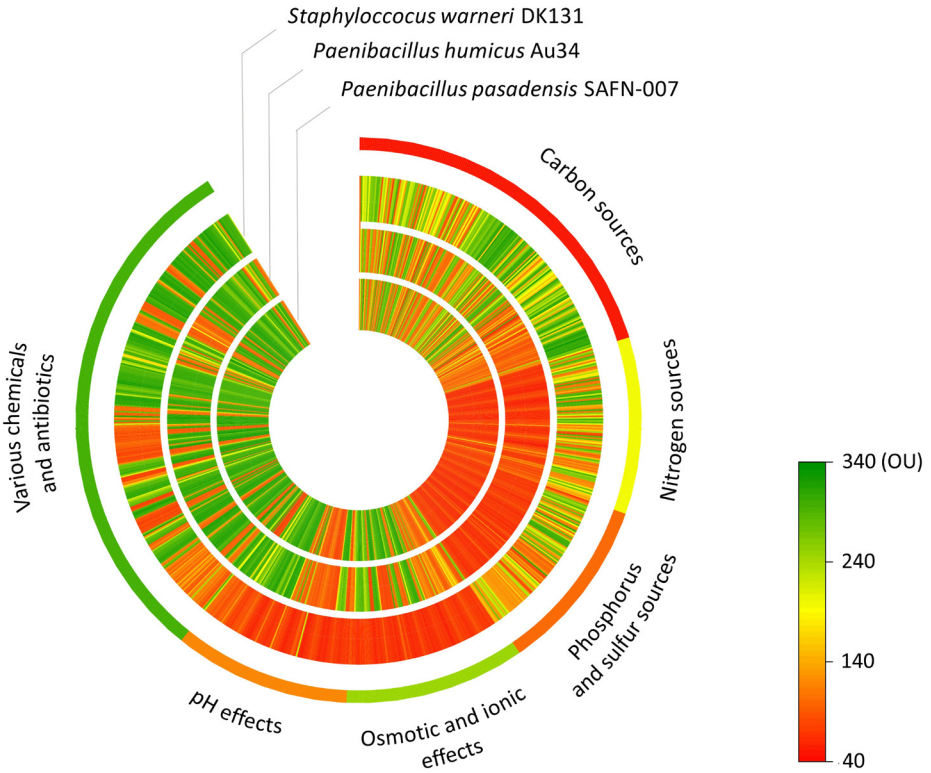

Figure 2. Changes in phenotypic diversity of investigated bacterial strains evaluated by PM plates

screening is an open and simple route for biosynthetic processes and, consequently, new ways of solving environmental problems are developed. The need for discovering new isolates with better enzymatic properties is now growing.

Biolog microplates were used to characterize the phenotypic properties of three strains that were the potential candidates for the bioaugmentation strategy. Figure 2 shows the metabolic profiles of the three strains using phenotype microarray plates. The results obtained showed that C-sources were the most used substrates. The following percentages of the carbon metabolites (PM1 and PM2): 74%, 67.4% and 94.2% were utilized by *Paenibacillus pasadensis* SAFN-007, *Peanibacillus humicus* Au34, and *Staphylococcus warneri* DK131, respectively. Among C sources, the strains have the capability to metabolize some substrates appearing in phenol pathways like N-acetyl-D-glucosamine, succinic acid, D-galactonic acid-γ-lactone, L-galactonic, acid-γ-lactone, α-hydroxy-glutaric, acid-γ-lactone, bromosuccinic acid, mono-methyl succinate, methyl-pyruvate, phenylethylamine m-hydroxy phenyl, acetic acid. *Staphylococcus warneri* DK131 metabolized 92% of 190 substrates belonging to nitrogen sources and phosphorus/sulfur sources. These substrates were metabolized with very low percentages, ranging from 2% to 4% by *Paenibacillus pasadensis* SAFN-007 and *Peanibacillus humicus* Au34. Bacteria have the ability to live in different habitats as well as show varying tolerance to the pH and osmolality of the environment. Therefore, the influence of pH and different osmolytes on the aerobic growth of bacteria was also assessed using 9-10 PM plates (data not presented). The strains tested grew in a wide pH range (4.5–9.5), especially in the presence of various amino acids. Growing *Paenibacillus pasadensis* SAFN-007 and *Peanibacillus humicus* Au34 cells were shown to cause progressive changes in pH

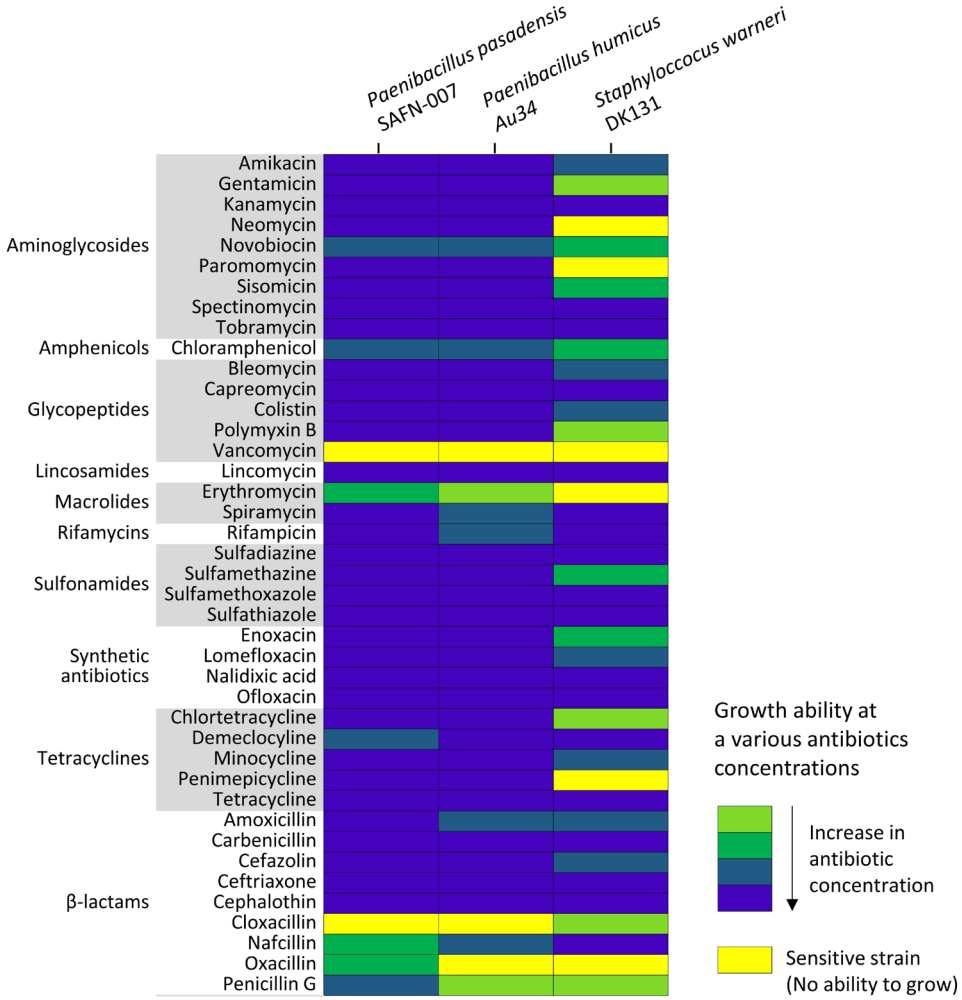

Figure 3. Heat map of antibiotic resistance of *Paenibacillus pasadensis* SAFN-007, *Paenibacillus humicus* Au34 and *Staphylococcus warneri* DK131 evaluated by microarrays plates (PM9-PM11)

variations as well as different osmolytes and their level. The behavior of both strains under the tested conditions was similar. Their growth curves are composed of the following phases: the lag phase, the exponential phase and the stationary phase. In the case of *Staphylococcus warneri* DK131, the growth curve showed a quite different pattern compared to *Peanibacillus* species. The growth curve was linear, at a constant level under varying conditions. The pH and osmolytes did not change the bacterial growth and the values of OmniLog units (OU) were in a similar range. These data could be useful for the selection and optimization of bacteria in many biotechnological applications such as the production of chemicals and in bioremediation technologies that predict the growth of bacteria in different ecological niches.

Using the PM11-PM13 plates, the three bacterial strains were tested for their sensitivity to 41 different antibiotics in four different concentrations (Figure 3). The producer of the PM plates (Biolog Inc.) does not specify the concentrations of the substances in its plates. The magnitude of the concentration increases in the four PM wells is also not known for the individual substances. The lack of information on the working range of the antibiotic concentrations makes it difficult to properly assess the sensitivity/resistance of the bacteria.The tested strains showed growth in the presence of examined antibiotics. In the case of *Paenibacillus* sp., both strains were resistant to most antibiotics belonging to various chemical classes in four concentrations. They showed full growth in all four wells for 30 from 41 antibiotics (73%) (Figure 3). In the case of vancomycin, erythromycin, cloxacillin, nafcillin, oxacillin and penicillin G no growth was observed. *Staphylococcus warneri* DK131 showed greater fluctuation in antibiotic resistance. It was sensitive to medium and high concentrations of antibiotics, showing tolerance to their lowest concentrations. Its growth was observed in all four concentrations of 18 antibiotics and amounted to 44%. The strains tested could be called multidrug-resistant bacteria, because they are resistant to several antibiotics from different chemical classes. The PM approach has been used previously as an additional tool to show variations in the antibiotic susceptibility of environmental bacteria (Jałowiecki et at., 2017). The literature discusses the use of microarrays as a tool to assess mutant phenotypes or to study phenotypic changes under different environmental stressors (Rodrigues et al., 2011; Greetham, 2014; Blumenstein et al., 2015; Dunkley et al., 2019). Moreover, the PM technology was used to identify the microorganisms and perform their characterization with selectable traits (Orro et al., 2015).

CONCLUSIONS

From the 100 strains isolated from UCG wastewater, three (*Paenibacillus pasadensis* SAFN-007, *Peanibacillus humicus* Au34, and *Staphylococcus warneri* DK131) demonstrated their capacity to degrade phenol. Their properties were evaluated by traditional microbiological methods and phenotype microarray plates. The obtained results show that *Paenibacillus pasadensis* SAFN-007, *Peanibacillus humicus* Au34, and *Staphylococcus warneri* DK131 can be used as suitable candidates in industrial and environmental biotechnology. UCG wastewater and other similar extreme environments are good sources for the isolation of specific microbes. The data obtained could be useful for the selection of bacteria in biotechnological applications as well as in bioremediation technologies by predicting the growth of bacteria in different ecological niches.

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