INVESTIGATION OF THE PHENOL-DEGRADING ABILITY AND METABOLIC PATHWAYS OF BACTERIA ISOLATED FROM LANDFILL LEACHATE

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Phenol is an aromatic compound which can be found in many industrial wastewaters such as those of petrochemical, pharmaceutical industries and plastic manufacturing. Due to the toxic properties of phenols, outflow of the phenol-containing wastewaters to the environment must be prevented. Therefore, a number of methods can be used of which microbial degradation processes are environmentally friendly and cost-effective solutions. In this study, the biodegradation capacity of bacterial strains was analyzed at 100 mg/L initial phenol concentration. Among the strains studied Acinetobacter sp. CFII-97, Acinetobacter sp. CFII-99A, Arthrobacter crystallopoietes CFII-104 and Arthrobacter crystallopoietes CFII-112 were able to degrade the phenol in 8, 12, 28 and 32 hours respectively. The other strains showed no growth and no phenol degradation during the experiment. Acinetobacter sp. CFII-97 and Acinetobacter sp. CFII-99A degraded phenol by the meta-cleavage pathway, whereas the Arthrobacter crystallopoietes CFII-104 and Arthrobacter crystallopoietes CFII-112 strains by the ortho-cleavage pathway.

Keywords: phenol, landfill leachate, phenol biodegradation, phenol biodegradation pathway, phenolic wastewater treatment

1. Introduction

Phenol is an aromatic white-to-colorless crystalline (acicular) compound with a characteristic odor (sweet tarry), hygroscopic and becomes red when contacts with air. The phenol molecule contains one hydroxyl group (–OH) attached to a phenyl ring (C_6H_5–) [1,2]. The solution of phenol is weakly acidic, it has a little tendency to lose H^+ cation from the hydroxyl group to form water-soluble

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phenoxide anion $C_6H_5O^-$ [3]. Phenol and phenolic compounds can occur naturally in the environment, for example in coal tar and are formed when plant organic material decomposes. Since phenol is soluble in water (8.28 g/100 mL at 20°C), in the environment it can spread far from the source point [4].

Phenolic compounds can be also produced artificially by different industries such as resin production, pesticide production, plastic manufacturing, petroleum processing, pharmaceutical production and so on.

Furthermore, all industries that use or produce phenol may be responsible for the phenol emissions [3,5]. Thus, wastewaters derived from these industries may contain high concentration of phenols (refinery wastewater had approximately 1 g/L phenol) [6] and the insufficient treatment of these wastewaters may be a significant anthropogenic phenol source in the environment, contaminating the surface waters bodies, groundwater bases, and the soil.

According to the available studies, phenol can be present in surface waters in significant amounts, e.g., one of the highest concentration detected was 2.11 mg/L in Isebo River, Nigeria [7]. This fact is causing a major concern, due to its harmful effects on living organisms. Phenol has bactericide, fungicide and algaecide properties, it was used for disinfection, and acts as a protoplasmic poison and denatures proteins. The values of median lethal concentration (LC_{50}) are commonly used to characterize the toxicity of a chemical compound to a living organism. For example, the mrigal carp (*Cirrhinus mrigala*) was one of the most sensitive freshwater organism with a 96 h LC_{50} of 1.55 mg/L phenol. Among the seawater organisms, the opossum shrimp (*Archaeomysis kokuboi*) is one of the most sensitive with a 96 h LC_{50} of 0.26 mg/L phenol [8].

Since phenol quickly penetrates the skin, acute ingestion, inhalation, and dermal exposure can cause irritation to the skin, eyes, and respiratory tract in humans. Also, phenol provokes mutagenesis and carcinogenesis in humans and other organisms [9]. Due to these adverse properties of phenols, it is essential to remove them from wastewater in order to prevent their toxic effect in the environment [10].

Many methods have been developed in order to eliminate phenols from wastewaters. These methods can be divided into destructive and non-destructive methods [11]. The non-destructive methods do not degrade the phenol, just move it to another medium. Some examples of non-destructive procedures: steam distillation, liquid-liquid extraction, adsorption, membrane separation. In contrast, the destructive methods allow the degradation of phenol. Some examples for destructive procedures: chemical oxidation (ozone, chlorine, chlorine dioxide, chloramines, ferrate [Fe(VI)], and permanganate [Mn(VII)] are the most commonly applied reagents in oxidative wastewater treatment), electrochemical oxidation, advanced oxidation processes (UV/H_2O_2 treatment, Fenton reagent, wet air oxidation), biological treatment and enzymatic treatment [12,13].

The biological treatment is based on the microbial catabolism of compounds. As shown in Fig. 1, microbial degradation is an alternative method to remove the toxic compounds from soil or wastewater, thus, bacteria that are able to degrade the phenol can be used for phenolic wastewater treatment.

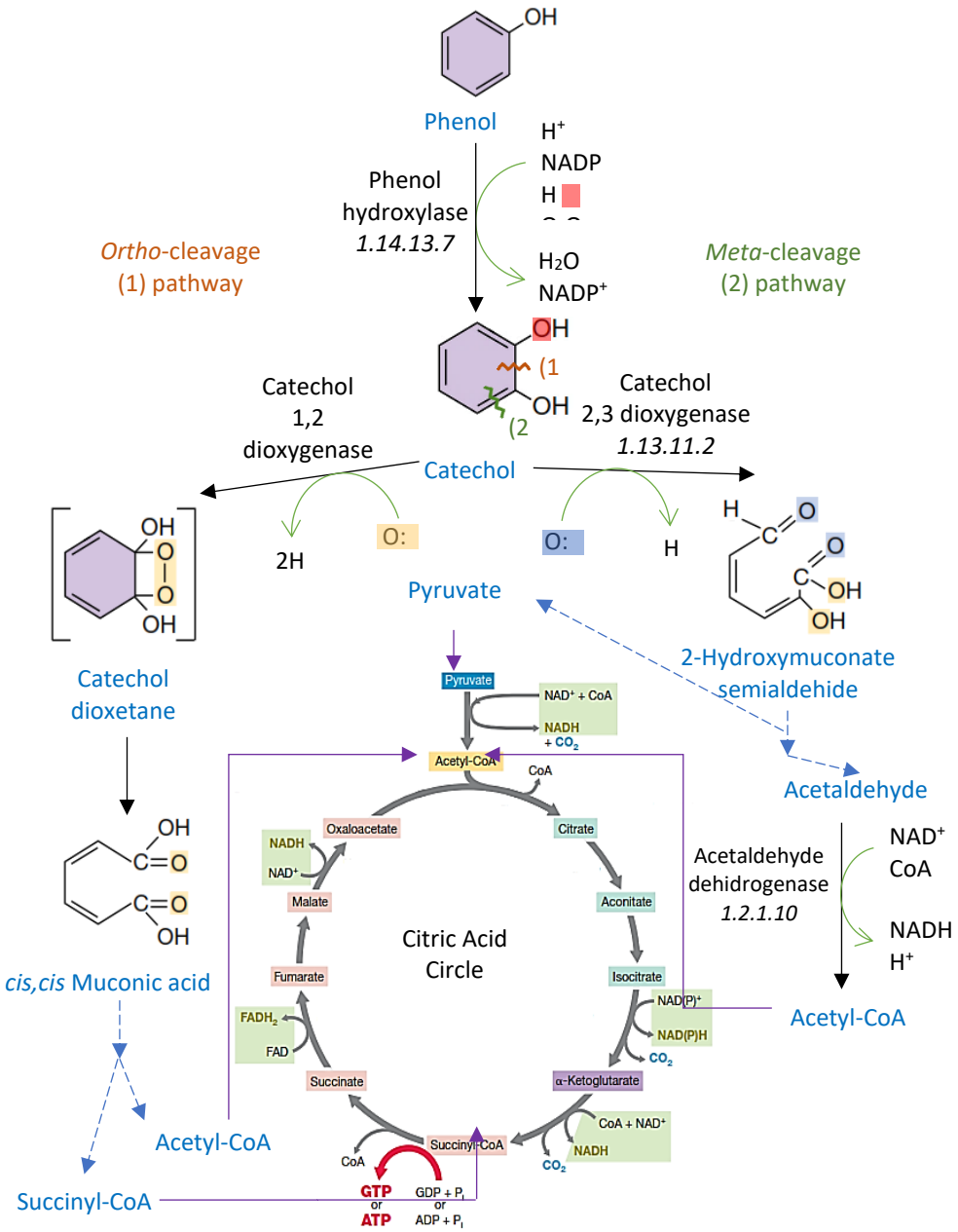

Fig. 1. Aerobic phenol biodegradation pathways (black arrows: consecutive products, blue dashed arrows: intermediate products, purple arrows: phenol degradation end-product pathways) [15,16]

Microbial wastewater treatment is considered a cost-effective, environmentally friendly and society accepted method. In order to remove the phenol from contaminated media via enzymatic treatment we need to identify the enzymes which are involved in phenol degradation. In bacteria, the first step in aerobic phenol catabolism is the conversion of phenol to catechol. This step is catalyzed by phenol hydroxylase which oxidizes phenol by forming catechol. In the next step the catechol can be cleaved either between the hydroxyl groups (*ortho*-cleavage) or neighboring to one of the hydroxyl groups (*meta*-cleavage). In the case of *ortho*-cleavage *cis,cis*-muconic acid is formed by catechol 1,2-dioxygenase. In the other pathway 2-hydroxymuconate semialdehyde is formed by catechol 2,3-dioxygenase. After some additional enzymatic steps in the case of *ortho*-cleavage pathway acetyl-CoA and succinyl-CoA are formed. In the case of *meta*-cleavage pathway pyruvate and acetaldehyde are formed. These compounds finally enter the citric acid cycle, which provide the cells energy and carbon source (Fig. 1) [14].

3. Materials and methods

3.1. Sampling

The samples were collected from a landfill leachate-treating bioreactor situated in Cekend-plateau, Harghita country, Romania. The activated sludge samples were taken from a depth of 40 cm from the water surface, then stored in sterile bottles at 4°C (Fig. 2).

Fig. 2. Geographical location of the sampling site and a snapshot of the bioreactor

3.2. Phenol-degrading bacteria enrichment

In order to enrich the phenol-degrading bacteria, 5 mL activated sludge sample was added to 95 mL mineral salt medium (MP) proposed by Watanabe et al. (1998) [17]. The composition of the MP medium was as follows: 2.75 g K_2HPO_4, 2.25 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.1 g NaCl, 0.02 g $FeCl_3 \cdot 6H_2O$, 0.01 g $CaCl_2$, 1 L distilled water (pH 6.8 to 7.0). In order to mimic the environmental conditions for microbes the MP was modified to leachate's pH, thus the pH was adjusted to 8.0±0.05 and the modified medium was named as Cekend Mineral Salt Medium (C-MSM) which was used throughout the experiments. The C-MSM medium was supplemented with 100 mg/L phenol as sole carbon and energy source. Then the flasks were incubated with shaking (GFL, Burgwedel, Germany) at 150 rpm at 28°C for one week. This procedure was repeated twice, 5 mL from the first enrichment culture was transferred aseptically to a new sterile flask containing 95 mL C-MSM medium supplemented with 500 and then 750 mg/L phenol. After incubation, the isolation of the bacterial strains was carried out from the last enrichment culture.

3.3. Isolation of the bacterial strains

Isolation of the strains was carried out by the spread-plate method using Reasoner's 2A (R2A) medium (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) medium 830, DSMZ GmbH, Germany) with incubation at room temperature, following the general microbiological laboratory practice [18]. The composition of the R2A medium was as follows: 0.5 g yeast extract, 0.5 g proteose peptone, 0.5 g casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g Na-pyruvate, 0.3 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 15 g agar, 1 L distilled water (pH 7.2). Colonies with different morphology were selected for isolation. After purification, strains were subsequently maintained on R2A medium and stored in a fridge at 4°C.

3.4. Identification of the bacterial strains

In order to identify the strains taxonomically, the total genomic DNA was extracted by AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Republic of Korea) following the protocol given by the manufacturer. The 16S rRNA gene (16S rDNA) was amplified by polymerase chain reaction (PCR) using primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') as described by Máthé et al. (2014) [19]. Sequencing of the isolated bacterial strains was performed by the LGC Genomics GmbH (Germany). The 16S rDNA sequences were processed using the MEGA X software [20]. The taxonomic identification of the strains was carried out by comparing the obtained sequences to the type strain sequences using the EzBioCloud online database [21].

3.5. Examination the phenol-degrading ability of the strains

Strains were inoculated onto R2A agar slopes and incubated at room temperature for 24 hours. From the cultures were prepared 5 mL cell suspension with 0.5 optical density using C-MSM medium and a Biolog turbidimeter (Biolog Inc. USA). This cell suspension was used for inoculating the Erlenmeyer flasks containing 95 mL C-MSM medium and phenol in 100 mg/L concentration. In the beginning, the solid phenol was thawed and sterilized by using 0.2 μm syringe filter (Whatman, Buckinghamshire, UK) and stored in sterile Eppendorf tubes. Prior to the experiment, the sterile phenol was carefully thawed again, and the required quantity (93.5 μL) was pipetted quickly to the liquid medium. In addition, an uninoculated medium was also prepared as a control to be able to check the possible abiotic phenol elimination and the undesirable microbial contamination. After preparing the flasks, they were sampled (1.5 mL) at the initial time point and then sampled every 4 hours for 48 hours. The flasks were incubated with shaking at 150 rpm and 28ºC (GFL, Burgwedel, Germany). The optical density was determined at 590 nm using a spectrophotometer (Hach DR6000, Colorado, USA), and the samples were frozen until processing (stored at –22ºC). After defrosting, the samples were centrifuged (Beckman Coulter, Indianapolis, USA) at 10000 rpm, 10 min., at 4°C in order that the cells do not affect the measurement of phenol concentration, and then the phenol content of the sample was determined [22].

3.6. Phenol measurement procedure

In order to determine the phenol concentration of the samples, the 4-aminoantipyrine-based colorimetric standard method was used. According to the protocol, the following steps were carried out: 10 mL diluted sample + 0.5 mL NH_4OH/NH_4Cl buffer (1 L buffer = 570 mL NH_4OH (25%) + 67.5 g NH_4Cl + 430 mL distilled water) + 0.2 mL 4-aminoantipyrine (2% w/V) + 0.2 mL potassium hexacyanoferrate (III) (8% w/V). After 15 minutes reaction time, absorbance was measured at 510 nm. Standard solutions were prepared and used for making a calibration curve. The calibration curve has proved to be linear until 10 mg/L phenol concentration, so more concentrated samples were diluted with distilled water [23].

3.7. Determination the phenol biodegradation metabolic pathway

The metabolic pathways of the phenol degrading strains were determined by the spray-plate method. In order to stimulate the phenol degrading enzyme production of the strains, the R2A agar was supplemented with 100 mg phenol/1 L culture media. The sterile thawed phenol was pipetted to the medium after autoclaving (121°C for 20 min.) and then the phenol containing culture medium was poured into Petri dishes. A suspension (OD_{600nm} = 0.5) of the strains to be tested was prepared, from which 5 μL cell suspension was inoculated onto R2A agar plates with point inoculation. As a negative control the *Escherichia coli* K-12 strain

was used. After cell growth, the plates were sprayed with 0.1% ethereal solution of 3-methylcatechol.

After 12 h of incubation at room temperature, a yellowish color will appear surrounding the colonies if the *meta*-cleavage pathway is active. Otherwise, if the surrounding of the colonies is transparent the *orto*-cleavage pathway is active [24].

4. Results

4.1. Taxonomic identification

In this work 14 bacterial strains were isolated from the last enrichment culture (750 mg/L phenol) and identified taxonomically by 16S rRNA sequence analysis. The 16S rRNA gene sequences showed that these strains belonged to different genera, including *Acinetobacter, Arthrobacter, Microbacterium, Citricoccus, Kocuria, Corynebacterium* and *Georgenia.*

The identification showed that these bacteria belonged to phyla Proteobacteria and Actinobacteria. From the Actinobacteria phylum we found 6 strains which belonged to genus *Georgenia* and showed relatively low similarity to *Georgenia subflava* (~97%). According to Tindall et al. (2010) strains with near 97% nucleotide similarity may represent new species [25].

For the phenol degradation studies, an additional strain was added to these strains which was isolated from the same landfill leachate-treating bioreactor and was the first new bacterial genus described from Transylvania (Romania). This strain was named as *Quisquiliibacterium transsilvanicum* CGI-09 and described by Felföldi et al. (2017) [26].

4.2. Phenol degradation

In total, the phenol-degrading ability of 15 bacterial strains were investigated. According to our results, 4 strains were able to degrade phenol under the conditions used. The phenol degradation profiles are shown in Fig. 3. and Fig. 4. Fig. 3. also shows the phenol-degrading profile of *Quisquiliibacterium transsilvanicum* CGI-09 strain, which was not able to degrade phenol. The profile of the control and non-phenol-degrading strains was similar to the profile of *Quisquiliibacterium transsilvanicum* CGI-09, so their data are not shown.

Our measurements showed that only strains belonging to genera *Acinetobacter* and *Arthrobacter* were able to degrade phenol. Strains *Acinetobacter* sp. CFII-97 and *Acinetobacter* sp. CFII-99A were able to degrade 100 mg/L phenol in 8 and 12 hours, respectively. In the case of *Acinetobacter* sp. CFII-99A the phenol has depleted earlier than 12 h, as shown in Fig. 4 but the depletion is not clearly visible because of the 4-hour sampling period (phenol is just detectable in the 8^{th} hour sample = 2.8 mg/L). Furthermore, strains *Arthrobacter crystallopoietes* CFII-104 and *Arthrobacter crystallopoietes* CFII-112 were able to degrade the phenol in 28 and 32 hours, respectively. Adav et al. (2007) reported an

Acinetobacter strain able to degrade 100 mg/L phenol in 9 hours [27]. Margesin et al. (2004) studied an *Arthrobacter* strain which was able to degrade 200 mg/L phenol within 48 hours [28].

4.3. Metabolic pathway determination

From the 4 phenol-degrading strains, 2 belonged to genus *Acinetobacter* and 2 belonged to genus *Arthrobacter*. Strains *Acinetobacter* sp. CFII-97 and *Acinetobacter* sp. CFII-99A showed a yellowish color surrounding the colonies, which indicated the catechol 2,3 dioxygenase production, thus *meta*-cleavage pathway was active. In the case of *Arthrobacter crystallopoietes* CFII-104 and *Arthrobacter crystallopoietes* CFII-112, no yellowish coloration was observed, thus the colorless product suggested the catechol 1,2 dioxygenase activity, so the *ortho*-cleavage pathway was active [29].

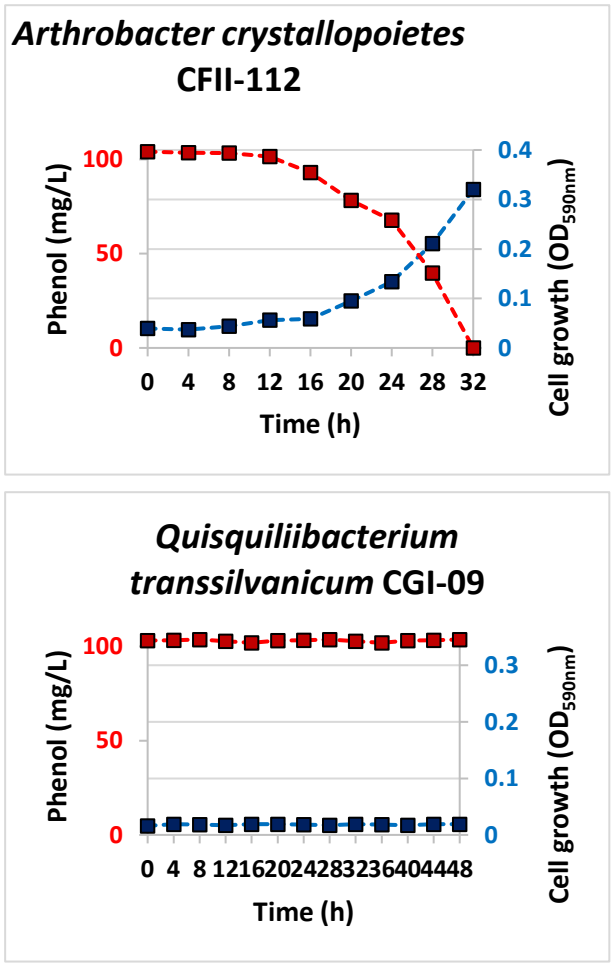

Fig. 3. Phenol biodegradation of strains *Arthrobacter crystallopoietes* CFII-112 and *Quisquiliibacterium transsilvanicum* CGI-09. The measurements were performed in duplicate.

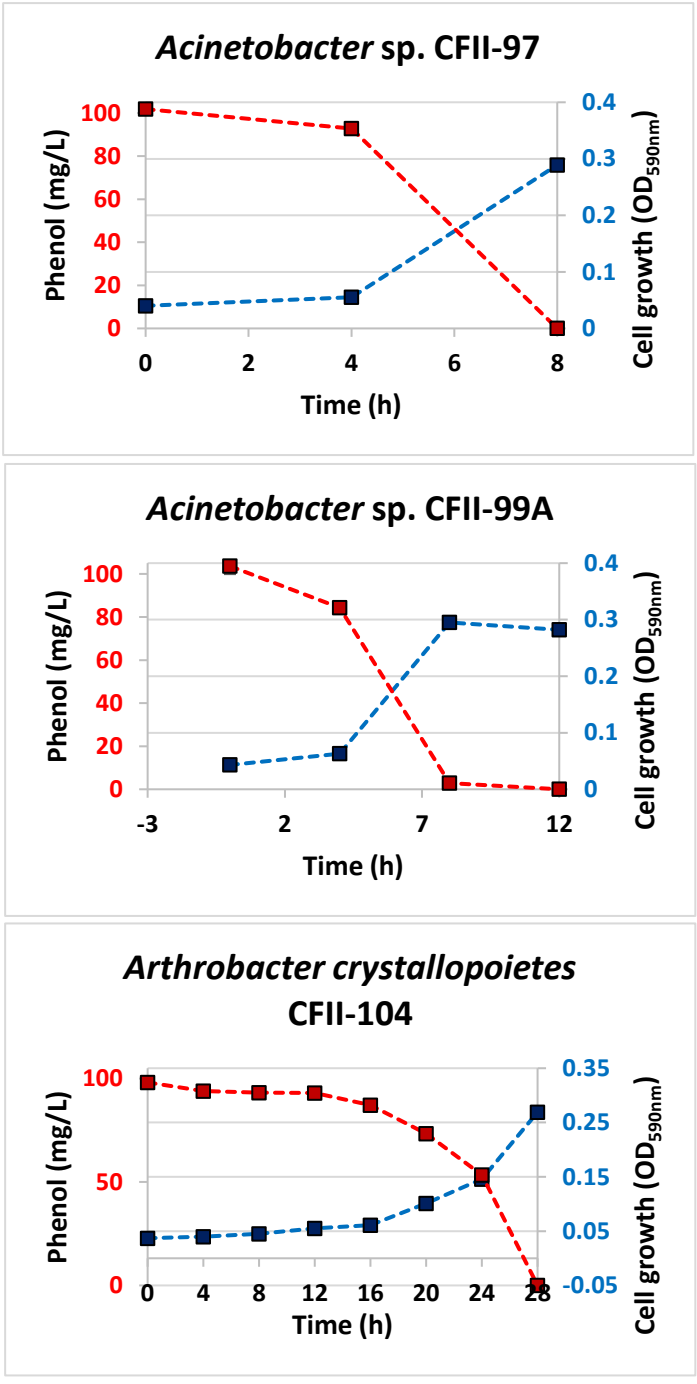

Fig. 4. Phenol biodegradation of strains *Acinetobacter* sp. CFII-97, *Acinetobacter* sp. CFII-99A and *Arthrobacter crystallopoietes* CFII-104. The measurements were performed in duplicate.

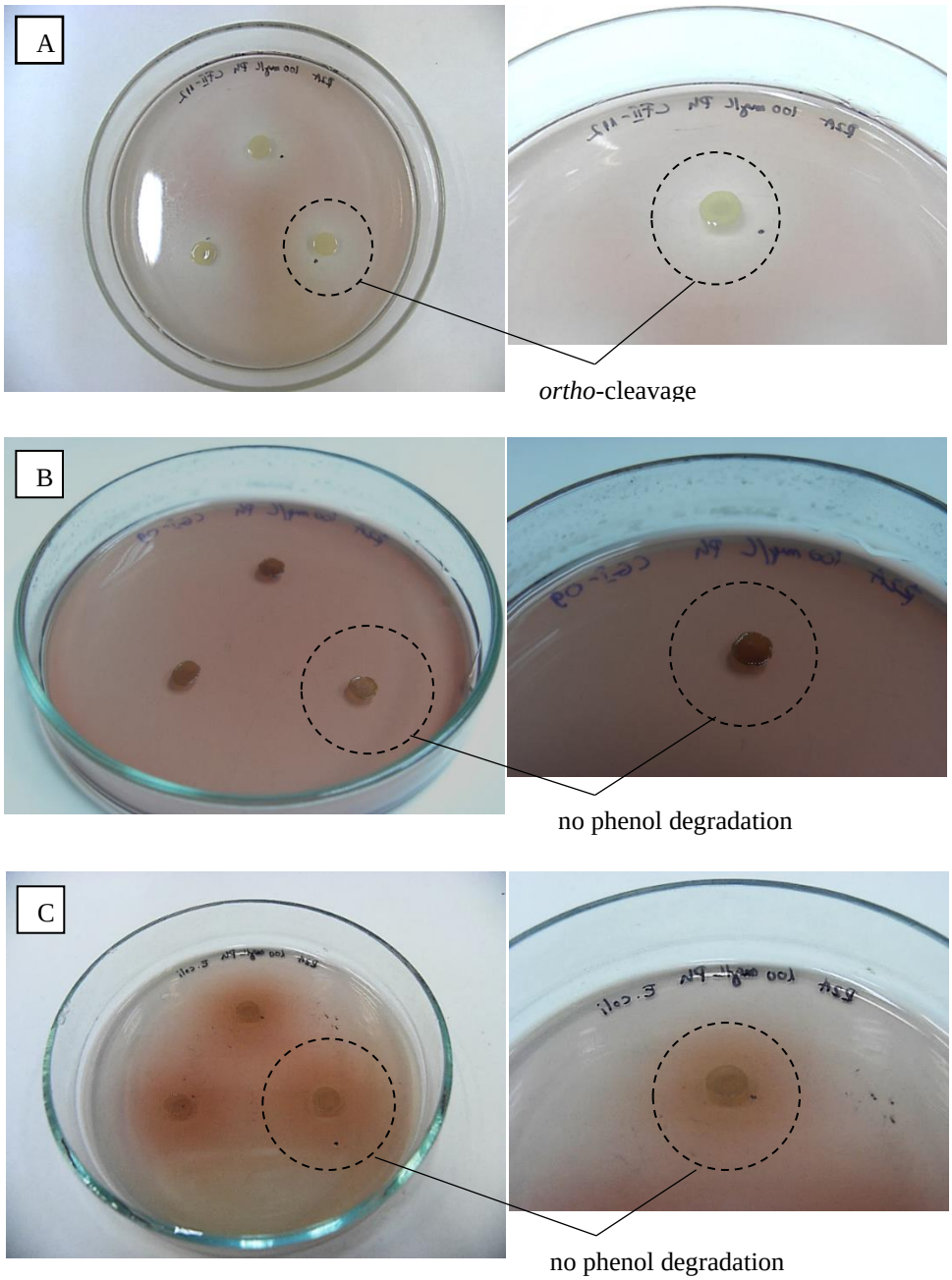

Fig. 5. Determination the metabolic pathways by the spray-plate method, A: *Acinetobacter* sp. CFII-97, B: *Acinetobacter* sp. CFII-99A, C: *Arthrobacter crystallopoietes* CFII-104

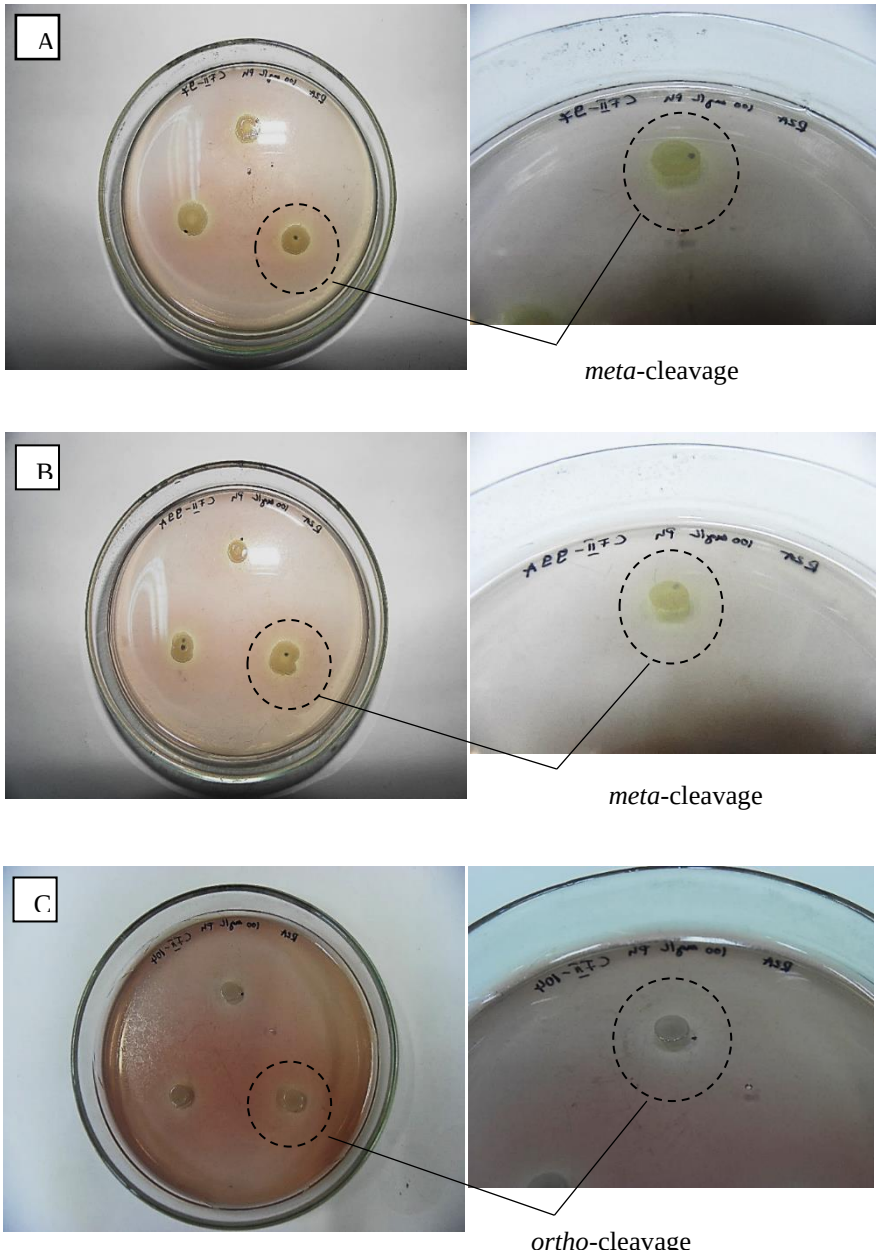

Fig. 6. Determination the metabolic pathways by the spray-plate method, A: *Arthrobacter crystallopoietes* CFII-112, B: *Quisquiliibacterium transsilvanicum* CGI-09, C: *Escherichia coli* K-12.

Ahmad et al. (2016) published first that strain *Acinetobacter* sp. AQ5NOL 1 had its phenol degradation activity via the *meta*-cleavage pathway [30].

Arora and Sharma (2015) studied an *Arthrobacter* sp. strain and in that case also the *ortho*-cleavage pathway was observed as in the case of our *Arthrobacter* strains [30]. Margesin et al. (2004) also got this result examining another *Arthrobacter* sp. strain [29,31].

5. Conclusions

In this work were examined the phenol degradation ability of 15 bacterial strains isolated from landfill leachate-treating bioreactor. From the 15 bacterial strains, 4 strains proved to be able to use phenol while 11 were unable to use phenol as sole carbon and energy source. Despite the fact that 11 bacterial strains did not degrade phenol, we found several bacterial strains that may represent new species for science, such as the previously described isolate *Quisquiliibacterium transsilvanicum* CGI-09 strain.

The strains *Acinetobacter* sp. CFII-97 and *Acinetobacter* sp. CFII-99A were able to degrade 100 mg/L initial phenol concentration in 8 and 12 hours respectively. The *Arthrobacter crystallopoietes* CFII-104 and *Arthrobacter crystallopoietes* CFII-112 strains consumed phenol within 28 and 32 hours respectively, and shows a longer adaptation time and a more delayed degradation compared to the *Acinetobacter* strains presented in this study. Based on the data available in the literature the obtained results are promising since rapid phenol removal was achieved with small amounts of bacteria (initial $OD_{590} \approx 0.04$).

The *Acinetobacter* strains degraded phenol via *meta*-cleavage pathway, while the *Arthrobacter* strains via *ortho*-cleavage pathway. Since *Acinetobacter* strains degraded phenol faster than *Arthrobacter* strains, in our case the *meta*-cleavage pathway proved to be faster than the *ortho*-cleavage pathway. Further kinetic studies are needed to determine the biokinetic parameters of the strains.

Strains belonging to genera *Acinetobacter* and *Arthrobacter* isolated in this study, either individually or in a consortium, can serve as a potential bioaugmentation material for the bioaugmentation of bioreactors treating phenolic wastewaters.

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