Thesis

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Enrichment, cultivation and genomic analysis of pharmaceutical-residues (ibuprofen, diclofenac) degrading bacteria

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TABLE OF CONTENTS

1. INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) encompasses all substances that are used for medical care, cosmetics, antiseptics and health improvement. Among them non steroidal anti-inflammatory drugs (NSAIDs) are one of the most common drug as it can be given over the counter as well as by prescription. Consumption of PPCPs have increased exponentially over the last decades although they have caused an improvement in the life expectancy in humans as well as in livestock animals by treatment of deadly diseases such HIV, diabetes, cancer and also pain reliefs which consequently reduce the stress. However their continuous release either via urination or disposal of unused one into sewage system, they find their way into soil and groundwater and other aquatic environments. Furthermore, advances in analytical technology have made their detection possible even in very small quantity (ng L^{-1}). Since those pharmaceutical products, such as diclofenac and ibuprofen have been produced to be bioactive in a tiny concentration, therefore they can affect negatively in non-target species as well.

Although the current method of wastewater treatment can eliminate large percentage of PPCPs but not completely, for that reason it is advisable to discover new ways of elimination. Microorganisms such us bacteria have shown to be very effective in degrading organic compounds from the environment. In the present study we attempted to isolate bacterial strains from groundwater sediment source which has been contaminated with BTEX compounds, assuming that some of the strains have adapted to use them as carbon. To achieve this goal we have applied classical microbiology methods (e.g. enrichment cultivation, pure strains isolation) additionally molecular biology methods (e.g. T-RFLP analysis, Sanger sequencing, Illumina 16S rDNA amplicon sequencing) and chemical method (HPLC technique) also. Enrichments were performed with either diclofenac or ibuprofen as a sole carbon.

2. LITERATURE REVIEW

2.1. Pharmaceuticals studied

Since the fate of most of pharmaceuticals in the environment is not known, the number of drugs that could have been included in our research was fairly high. The research focused on two pharmaceuticals, each of which has been found in the environment. The compounds are: diclofenac and ibuprofen.

2.1.1. Diclofenac

Diclofenac is a phenylacetic acid derivative (2-[2,6- dichloranilino]phenylacetic acid) which belongs to a non-steroidal anti-inflammatory drug. First introduced in Japan in 1974 for is indicated for osteoarthritis (OA), rheumatoid arthritis (RA), ankylosing spondylitis, and mild to moderate pain (Gan 2010). It is taken by mouth or rectally in a suppository, used by injection, or applied to the skin. Similar to other NSAID its mechanism of action involve inhibition of cyclogenase (COX1,COX2) which is involved in conversion of arachidonic acid to prostaglandin H2, which is expressed in inflammation (Gan 2010). Diclofenac is completely absorbed via gastrointestinal tract (Gi), when taken orally only 60% reaches blood circulation unmodified. Metabolism of diclofenac can be divided into two pathways: oxidation which leads to formation of hydoxyl metobolite such as 4'-hydroxy diclofenac, 3'-hydroxy diclofenac, 3'-hydroxy-4'methoxy diclofenac, 4',5'-dihydroxy diclofenac. The other pathway is conjugation, which forms diclofenac glucuronide (Tang, 2003). Diclofenac is mainly eliminated via metabolism. Of the total dose, 60-70% is eliminated in the urine and 30% is eliminated in the feces (*Diclofenac Compound*, 2023.).

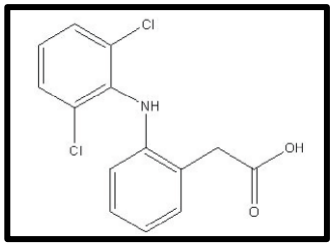

Figure 1. Structure of diclofenac (B. Yilmaz et al., 2015)

2.1.2. Ibuprofen

Ibuprofen is a propionic acid derivate and nonsteroidal anti-inflammatory drug (NSAID) with anti-inflammatory, analgesic, and antipyretic effects. First introduced in United Kingdom in 1969. Same as the other NSAIDs it binds and chelates to Cyclogeneses' (COX 1,2) and prevents formation of prostaglandins and thromboxane to reduce inflammation and platelet aggregation. (Rainsford 2009). Ibuprofen completely absorbed after oral intake and 99% of the drug binds to plasma proteins. Pharmacokinetic studies have revealed that about 66% of the drug is eliminated in the urine whereas about 34% is excreted in the feces. Four oxidative metabolites of ibuprofen, including 1-hydroxyibuprofen, 2-hydroxyibuprofen, 3-hydroxy-ibuprofen, and carboxyibuprofen, have been found in human urine and plasma samples after oral ibuprofen administration. Oxidative metabolism is the primary pathway for the biotransformation of ibuprofen (IBU). The parent drug and its metabolites are discovered to be conjugated with glucuronic acid in humans (Magiera & Gülmez 2014).

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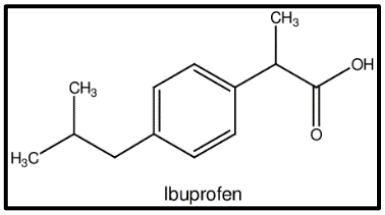

Figure 2. Structure of ibuprofen (Abualhasan et al., 2015)

2.2. Occurrence of diclofenac and ibuprofen in the environment

Non steroidal anti-inflammatory drugs (NSAID) such as diclofenac and ibuprofen groups of drugs are becoming contaminants of emerging concern. They can be given prescribed or over the counter. There are many routes that NAISDs can release to the environment. Effluents from wastewater treatment plants (WWTPs) are recognized as the main source (Villa et al. 2020). Hospitals come in second place as the main source of pharmaceuticals in the environment. As they are linked sewage system plants and these systems are not designed to remove micropollutants as it is a case for pharmaceuticals. In the third position comes animal husbandry sector particularly in developing countries where environment related threshold values are not

very frequent (aus der Beek et al. 2016). They can be found in variety of environments –surface water, ground water, drinking water, sediment and accumulated in the aquatic organism's body.

2.2.1. Surface water

Surface water refers to water body that locates above the ground including lakes, streams, reservoirs, rivers and oceans, and NSAIDs are considered as emerging pollutants in those environments. Based on study of Chaves et al. (2022), which have gathered published articles between year 2014-2022 shows that NSAIDs are the most frequently detected group of pharmaceuticals in surface water. The highest diclofenac concentration was found in Asia, which was 8500 ng L^{-1} (in Pakistan) (Scheurell et al. 2009) followed by African continent (8174 ng L^{-1} in South Africa (Agunbiade & Moodley, 2016b)) and in the European continent (4806 ng L^{-1} (Palma et al., 2020)), which also reported to have high risk to aquatic organisms (algae, crustaceans, and shells).

Antipyretic drugs are commonly used for pain relief in Africa, particularly among people who work in high-energy, manual labor and among lower-income earners, as is the case in most suburban, informal settlements. South America had concentrations of up to 1540 ng L^{-1} in Colombia (Pemberthy et al. 2020), and Asia had concentrations of up to 1390 ng L^{-1} in Saudi Arabia (Picó et al. 2020). The highest concentration reported in North America was 40 ng L^{-1} in Canada (Comtois-Marotte et al. 2017a).

Ibuprofen had a 51% detection rate in the studies with concentrations of up to 84 600 ng L^{-1} in the African continent (in South Africa) (Matongo et al. 2015a) and 57 600 ng L^{-1} in the European continent (in Spain) (Sadutto et al. 2021), where it had been shown to pose a high environmental risk to algae and crustaceans. Ibuprofen concentrations in surface water may be high due to its ease of access and thus use, as it can be purchased over the counter in large quantities. The highest ibuprofen concentration in the Asian continent was 2407 ng L^{-1} in Saudi Arabia (Picó et al. 2020), while in South America highest rate was detected in Argentina which was 2143 ng L^{-1}(Comtois-Marotte et al. 2017a) In North America it was 1670 ng L^{-1} in Canada (Comtois-Marotte et al. 2017b). In Danube river maximum detected concentration was 115 ng/ L^{-1} (Kondor et al. 2020).

2.2.2. Sediment

The NSAIDs (ibuprofen, diclofenac, acetaminophen, naproxen and ketoprofen) were one of the most commonly detected PPCPs group in sediments (Chaves et al. 2022).

Ibuprofen had a 46% detection rate and concentrations five times higher in Africa (up to 659 ng g^{-1} in South Africa) (Matongo et al. 2015b) than in North America (up to 150 ng g^{-1} in the USA)(Deere et al. 2020) and Asia (132 ng g^{-1} in India) (Chakraborty et al. 2019). Concentrations of up to 100 ng g^{-1} in South America, Mexico and 31 ng g^{-1} in Europe, in Spain concentrations were quantified in dry weight in each continent. (Carmona et al., 2017; Díaz & Peña-Alvarez, 2017).

Diclofenac was detected in 38% of the studies and presented the highest concentrations in the African and Asian continents, up to 309 ng g^{-1} in South Africa 54 and 109 ng g^{-1} in China (Agunbiade & Moodley, 2016b; Huber et al., 2016).. The concentrations of diclofenac were quantified above15 ng g^{-1} in Europe, South and North America (in Spain, in Brazil and in Greenland) (Díaz & Peña-Alvarez, 2017; Beretta et al., 2014; Huber et al., 2016).

2.3. Ecotoxicology

In human NSAIDs may have adverse effect on stomach, cardiovascular, kidney and liver as well as other effect on skin and pulmonary system (Ghlichloo & Gerriets 2022). For that reason even small concentrations 1.0 µg L^{-1} can be dangerous to non-target organisms. Diclofenac have shown to be extremely toxic to *Gyps* vultures it causes abnormal deposition of uric acid soft tissues, renal necrosis, and death after few days of exposure (Rattner et al. 2008). Based on (Gröner et al. 2017) findings, which has indicated that diclofenac can cause histopathological changes in nile tilapia *Oreochromis niloticus* gills by causing epithelial cells lifting as well as hyperplasia followed by increased size of the chloride cells (hypertrophy). It can affect sexual differentiation and gametogenesis by causing estrogen disruption potential. LH luteinizing hormone level reduced with the elevation in diclofenac dose. In another study, which was done by Guiloski et al. (2017) has discovered reduction in antioxidant enzymatic activitity such as catalyze, superoxide dismutase in the liver and lowering of dopamine (DA/DOPAC) level as well as reduction in lipid peroxidation level in South Amercian cat fish (*Rhamdia quelen*) after being exposed to a fresh water containing diclofenac (0.2 µg L^{-1}, 2 µg L^{-1}, 20 µg L^{-1}). In Zebrafish

(*Danio rerio*) ibuprofen has been shown to cause disruption in hypothalamus-pituitary –gonad (HPG) axis by upregulating of genes which involves in male hormones production and down-regulating female hormones and also diclofenac and Ibuprofen can be neurotoxic and affect the larval motion (Ji et al. 2013; Xia et al. 2017). Other aquatic organisms have negatively affected by these compounds. For instance, Baltic sea Mussels (*Mytilus edulis trossulus*), which was revealed to have lower byssus strength and byssus threads' abundance also, additionally was detected less scope of growth, when they have been exposed to these two compounds (Ericson et al. 2010).

2.4. Methods for detection and removal of pharmaceutical compounds

There are several techniques used to detect NSAIDS compounds such as Liquid Chromatography-Mass Spectrometry (LC-MS)(Chen et al. 2011), Gas Chromatography-Mass Spectrometry (GC-MS) (Kovacs et al. 2021) and High-Performance Liquid Chromatography (HPLC)(Aguilar-Arteaga et al. 2010). The methods typically involve extraction of the NSAIDs from the environmental sample, followed by separation using gas chromatography, and detection and quantification using mass spectrometry.

Many pharmaceuticals are environmentally persistent, because stability is often considered to be a beneficial property to humans. Their constant inflow into the environment is another factor that contributes to persistence. Molecules with aromatic rings linked to chloro, nitro, and fluoro functionalities have greater persistence. Due to electron removal, which keeps the aromatic ring stable, these structures withstand oxidation (Khetan & Collins 2007) as it's a case for diclofenac and ibuprofen. In most countries sewage water treatment plant are not designed for removal of NSAIDS. They have been detected in the effluents of this systems. The most common ways of removal of pharmaceuticals are through waste water treatment plants (WWTPs) and constructed wetlands.

2.4.1. Wastewater treatment plants

Wastewater treatment process typically involves sedimentation, anaerobic digestion, and tertiary treatment. Sedimentation allows particles to settle out of the suspension, while anaerobic digestion reduces sludge using anaerobic microorganisms. Tertiary treatment removes excess

phosphorus and nitrogen to improve water quality. NSAIDs, such as diclofenac, are not easily removed by adsorption onto activated sludge due to their polar and hydrophilic properties (Zorita et al. 2009). Diclofenac has low removal efficiency in most studies due to its degradation, deconjugation of metabolites, low water solubility, and inability to adsorb onto sludge (Kermia et al. 2016). The removal of NSAIDs occurs mainly during the biological treatment process, and disinfection by UV or chlorination contributes to diclofenac removal (Sari et al. 2014).

2.4.2. Constructed wetland

Constructed wetlands (CWs) are wastewater treatment systems that use natural processes to remove wastewater constituents, and they are economical and require less operation and maintenance effort than other decentralized treatment technologies. CWs are capable of removing nonsteroidal anti-inflammatory drugs (NSAIDs) through bio- and photo-degradation, sorption, plant uptake, and sedimentation (Kadlec & Wallace 2008). The removal efficiency of NSAIDs in CWs varies depending on several factors, including the concentration of the influent and temperature (Ávila et al. 2010). Aquatic plants have been found to play a significant role in the removal of diclofenac from CWs through phytoremediation. The removal efficiency of NSAIDs also depends on the plant species used (Zhang et al. 2012).

2.5. Biodegradation

Microbial biodegradation of NSAIDS have gained huge interest of researchers around the world due to their incredible degradation potential and production of less toxic materials as well as cost effectiveness compared to other treatments methods such as ozonation and photolysis which have shown to be tremendously costly due to high operational and energy cost (Fischer et al. 2015), which makes it very challenging for developing countries to afford it. The breakdown of NSAIDs begins with the molecule's oxidation. Biodegradation refers to the breakdown of organic substances into smaller compounds via bacteria, fungi, and other organisms (Adamek et al. 2016). Biological oxidizers of NSAIDs include bacteria, fungi, microbial consortiums, algae and higher plants. The vast majority of studies are devoted to the processes of NSAID biodegradation using bacteria and fungi. Biodegradation of pharmaceutically active compounds (PhACs) in wastewater is influenced by a number of variables, including the structure and chemical characteristics of PhACs, the type of microorganisms utilized, pH, redox potential,

temperature, the type and effectiveness of the microorganisms used, and PhACs concentration. The solubility of PhACs plays a key role in their biodegradation in WWTPs. The retention of hydrophobic PhACs in the sludge due to their adsorption on suspended solids always gives time for microbial breakdown (Tiwari et al. 2017).

2.5.1. Biodegradation of ibuprofen and diclofenac by fungi

Several studies have described fungal species capability to biotransformation and biodegrade ibuprofen, mostly white-rot fungi (*Trametes versicolor*, *Irpex lacteus*, *Ganoderma lucidum* and *Phanerochaete chrysosporium)* additionally *Penicillium chrysogenum* and *Aspergillus niger* (Gonda et al. 2016; Marco-Urrea et al. 2009). Marco-Urrea et al. (2009) investigated the ability of *Trametes versicolor*, a white rot fungus, to degrade ibuprofen (IBU). The identification of putative IBU metabolites was achieved through Nuclear Magnetic Resonance (NMR). Three metabolites were identified as 2-hydroxy ibuprofen, 1-hydroxy ibuprofen, and 1,2-dihydroxy ibuprofen. The metabolism of IBU by *T. versicol*or leads to the oxidation of its isopropyl chain. The propionic acid chain remains the same. Two main metabolites, 2-hydroxy ibuprofen and 1-hydroxy ibuprofen, were detected in the first phase of the biodegradation process. In contrast, 1,2-dihydroxy ibuprofen was observed as the main metabolite in the second phase of the biodegradation process (after seven days of the experiment), with none of the formers detected. The study provides insight into the potential of *T. versicolor* in the bioremediation of pharmaceuticals and their metabolites in wastewater treatment. Manganese peroxidase and cytochrome P450 monooxygenases are involved in the first step of IBU degradation (Marco-Urrea et al. 2009).

Diclofenac is characterized by one of the most persistent pharmaceutical compound and highly detected in the environment. However, there some microbial species which have been revealed to degrade diclofenac in high percentage such as *Phanerochaete chrysosporium, Aspergillus niger*, *Trametes versicolor, Phanerochaete sordida YK-624*. Both parent drug and breakdown metabolites (4′-OH and 5′-OH diclofenac) found in fungal cultures disappeared within 24 hours. This is consistent with the reduced ecotoxicity measured by the Microtox bioassay. According to the data obtained using the cytochrome P450 inhibitor 1-aminobenzotriazole. Beside these two metabolites 4′,5′-dihydroxydiclofenac (4′,5′-diOH-DCF) was also detected when *Phanerochaete sordida YK-624* was used. The cytochrome P450 system appears to play a significant role in the

first phase of diclofenac breakdown. Although purified laccase can also catalyze the conversion of diclofenac to 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol, it does not appear to be the enzyme mechanism responsible for diclofenac degradation in *T. versicolor* pellets (Aracagök, 2018; Hata et al. 2010; Marco-Urrea et al. 2010). Desipite high degradation activity of fungi there are some drawback of utilizing fungi in waste water treatment plant. As the pH of wastewater is around 7, while fungi thrive at a slightly lower pH of 4.5. Further problems could be: the requirement for an external energy substrate; the possibility of bioreactor fouling; the nature of the seed (spore) material; and the production of mycotoxins (Tyumina et al. 2020).

2.5.2. Biodegradation ibuprofen and diclofenac using bacterial species

Biodegradation by bacteria has become a hot topic among researchers compared to Fungi. Bacteria can use wider range of substrates as a carbon and energy source, which makes them to utilize wider range of organic compounds. They are more toxic resistant, which enables them to survive harsh conditions.

A number of bacteria shown to breakdown ibuprofen either to smaller subunits or to undetectable amounts. Such as *Sphingomonas* sp. Ibu-2, *Variovorax* sp. Ibu-1, *Sphingobium yanoikuyae*, and *Pseudoxanthomonas* sp. DIN-3. *Stenotrophomonas humi* DIC_5 and *Rhizobium daejeonense* IBU_18 c, *Patulibacter medicamentivorans* (Balciunas et al. 2020; Lu et al. 2019a; Marchlewicz et al. 2017; Murdoch & Hay 2013, 2015; Pápai et al. 2023; Salgado et al. 2020). This process of degradation occurs through the IBU biodegradation pathway, which is initiated by the action of an enzyme, ibuprofen-CoA ligase, followed by the ibuprofen-CoA-1,2-dioxygenase. This pathway leads to the formation of the metabolite 2-phenylpropanoic acid and isobutylbenzene that undergo enzymatic biodegradation and catechol formation before ring cleavage. The presence of the CoA ligase is significant in the transformation of the IBU molecule, and it also has an effect on the esterification of the carboxylic acids. The process of degradation is initiated by the up-regulation of the Rieske (2Fe-2S) iron-sulphur domain protein, which is an oxidoreductase that acts on the CH-CH group of donors with nicotinamide adenine dinucleotide NAD+ (oxidized) or nicotinamide adenine dinucleotide phosphate (NADP+) as an electron acceptor. This transformation results in the formation of the metabolites 2,3-dihydroxysuccinic acid, 2-hydroxysuccinic acid (malic acid), and propionic acid. The study of the IBU biodegradation pathway by *P. medicamentivorans* suggests that this bacterium has a

potential application in the bioremediation of IBU-contaminated environments (Murdoch & Hay 2013, 2015; Salgado et al. 2020).

In case of diclofenac fewer bacterial species were able to completely degrade as sole carbon source or even as a secondary substrate. However, more and more bacteria have been discovered lately to degrade diclofenac into less toxic metabolites such as *Pseudomonas moorei* KB4, *Klebsiella* sp. KSC, *Labrys portucalensis* F11, *Pseudoxanthomonas* sp. DIN-3, *Rhodococcus ruber*, *Staphylococcus haemolyticus*, *Raoultella* sp. DD4 *(*(Domaradzka et al. 2016; Ivshina et al. 2019; Lu et al. 2019b; Moreira et al. 2018; Murshid & Dhakshinamoorthy 2019; Stylianou et al. 2018; Żur et al. 2020). 4′-OH-DCF and DCF-lactam were the key metabolites detected in both mono-substrate and co-metabolic culture and also up-regulation of genes encoding degredative monooxygenase, dihydroxylating dioxygenases, cleavage dioxygenases or cytochrome P-450 during *Pseudomonas moorei* KB4 application (Żur et al. 2020) The main degradation pathways of diclofenac appear to involve hydroxylation, decarboxylation and partly dechlorination of the compound, detachment of acetate group, and cleavage of the non-chlorinated aromatic ring in *Klebsiella* sp. KSC (Stylianou et al. 2018). There was a significant change in size and shape of the cells as well as cell aggregation, increased hydrophobicity in the cells exposed to the diclofenac in *Rhodococcus ruber* beside (4′-OH-DCF, 5′-OH-DCF) benzoquinone imine-type compounds were also detected (Ivshina et al. 2019) complete degradation of DCF was observed in case of *Labrys portucalensis* F11 (Moreira et al. 2018).

3. MATERIALS AND METHODS

3.1. Sample collection

Samples were collected from groundwater from a site that was contaminated with petroleum hydrocarbons for decades due to the leakage in one of the gasoline tanks in Siklós, a town located in Baranya county, in Hungary (45′51″25.8°N, 18′17″32.3°E) (Táncsics et al. 2013) during September 2021. Several studies have been conducted on this site for bacterial biodegradation of BTEX (benzene, toluene, ethylbenzene, and xylenes) compounds, which achieved a significant degradation rate (Banerjee et al. 2022; Bedics et al. 2022; Táncsics et al. 2020). Both BTEX and investigated pharmaceutical compounds possess aromatic ring for this study we were expecting that some bacterial strains, which were adapted to live underground on BTEX contaminated environment would be able to degrade pharmaceuticals.

3.2. Selective enrichment

Selective enrichment was carried out in 300 mL Erlenmeyer flasks containing mineral salts solution supplemented with vitamins with addition of either diclofenac (DCF) or ibuprofen (IBU) as a sole carbon and energy source. The medium was prepared with 100 mL each of solutions A and B, 1 mL of solution C, and 800 mL of sterile MQ water. Solution A: 5 g $MgSO_4 \cdot 7H_2O$ and 1 g $CaCl_2 \cdot H_2O$ L^{-1} (filtered). Solution B: 11.1 g Na_2HPO_4, 2.5 g KH_2PO_4 and 10 g NH_4NO_3 L^{-1} (autoclaved). Solution C: 10 g $FeSO_4 \cdot 7H_2O$, 0.64 g $Na_2EDTA \cdot 3H_2O$, 0.1 g $ZnCl_2$, 0.015 g H_3BO_3, 0.175 g $CoCl_2 \cdot 6H_2O$, 0.15 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g $MnCl_2 \cdot 4H_2O$ and 0.01 g $NiCl_2 \cdot 6H2O$ (Fahy et al. 2006) with addition of 1 mg of B1 vitamin and 20 µg from B12 vitamin and 15 µg from biotin. 100 mL from the medium was transferred to 300 mL Erlenmeyer flasks then 1.0 mL of inoculum. Followed by the addition of 100 mg L^{-1} DCF and IBU and then the flasks were sealed with cotton and the experiment was done in three parallel enrichment for each pharmaceutical compounds. Enrichment cultures were incubated at 28 °C on a rotary shaker (120 rpm) for 3 months in total (Certomat® Bs-1, Sartorius Stedim Biotech GmbH., Germany). After each month 10 mL of enrichment culture medium were transferred to fresh enrichment medium. The remaining volume of the enrichment was preserved for further experiments such as DNA isolations, T-RFLP analysis and 16S rDNA amplicon sequencing.

3.3. Isolation of pure strains

Isolation of bacterial strains was carried out by transferring 1.0 mL from 3-month-old medium of both DCF and IBU enrichments to 15 mL falcon tube containing 9 mL of physiological salt solution (0.9 % NaCl). Then the samples were serially diluted (10^{-1} … 10^{-8}). 100 µL from each dilution were spread onto R2A media agar plates (0.5 g proteose peptone, 0.5 g casamino acids, 0.5 g yeast extract, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g dipotassium-phosphate, 0.05 g MgSO4 ·7H2O, 0.3 g sodium pyruvate) and labeled with diluted value. After that, agar plates were incubated at 28°C. Pure bacterial colonies showing different shapes and forms were isolated from dilution (10^{-4} , 10^{-5} , 10^{-6}) and inoculated onto new R2A agar plates using a streak plating technique. Sealed with parafilm and incubated at 28°C for 72h. Purified strain were maintained on R2A agar plates at 4 °C and stored long-term at − 80 °C in a glycerol-R2A solution (30% v/v). Next steps of identification will be described later.

3.4. Identification of bacterial community

For identification of bacterial community grown in the enrichment media community DNA was isolated and sent for genomic analysis by 16S rDNA amplicon sequencing using Illumina MiSeq platform and also fragment analysis through utilizing T-RFLP technique which will be described later in methodology part.

3.5. Isolation of DNA

In the first step of DNA isolation 50 mL from the enrichment culture was centrifuged at 2360 g for 10 min using a Rotanta 460 R centrifuge (Hettich, Germany), and the community DNA was extracted from the pellet. The genomic DNA of isolates was extracted by using the DNeasy® UltraClean® Microbial DNA isolation Kit (Qiagen, Germany) following the instructions of the manufacturer. And then DNA concentration was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The presence of DNA then was confirmed via the gel electrophoresis technique. 1 % agarose gel was prepared by adding 80mL of 10X TBE buffer (10.8 g Tris base, 10.5 g Boric acid, 0.93 g EDTA (disodium salt) with 0.8 g from agar powder, then 3 µL of ethidium bromide were added to the solution (intercalates between DNA bases to make them visible under the UV light). The flask then heated in a microwave, and agar was completely dissolved. The solution was cooled down to about 50-

60°C, then poured into a casting tray. The comb was inserted into the casting tray wells were created, and the gel was let to solidify in about 20-30 minutes at room temperature. 3 µL of gene ruler (Thermo Scientific GeneRuler™ DNA Ladder Mix, 0.5 µL μL^{-1}, Thermo Fisher Scientific™ Inc., USA) were added in first well. After that 2 µL of 5X Loading Dye with 3 µL of DNA samples were mixed on the parafilm tape and loaded into the wells. The samples were run at 120 V for 20 minutes, then the gel was placed in the Syngene™ Ingenius LHR device to verify the success of the isolation under UV light. This procedure was repeated after each month for the period of three months.

The same procedure was performed for DNA isolation from purified bacterial strains except the first step were the strains suspended in PowerBead Solution.

3.6. Polymerase chain reaction (PCR)

A technique that was invented by Kary Mullis in 1983 (Mullis, 1990) the technique is mainly used to amplify certain fragments of nucleic acids into thousands of copies. It includes three main steps: denaturation, annealing and extension. During denaturation, the double-stranded DNA is heated to usually 94-96°C to separate the strands. During annealing, the temperature is lowered to 55- 65°C allowing the primers to bind to the complementary single-stranded DNA. Finally, during extension the temperature risen again to 72°C in order for the DNA polymerase mainly Taq polymerase to add nucleotides to the 3' end of the primers. These three steps are typically repeated for 25-40 cycles, each cycle doubling the amount of DNA in the reaction. The resulting product is a large amount of DNA that is identical to the target sequence.

PCR components and reaction

4 µL BSA (Bovine Serum Albumin, 250 ng mL^{-1}), 28.75 µL Milli-Q ultra-pure water, 5 µL Taq buffer (Thermo Scientific 10X DreamTaq™ Green Buffer included: 20 mM $MgCl_2$, Thermo Fisher Scientific™ Inc., USA), 10 µL dNTPs (0.2-0.2 mM, consisting of all four types of deoxynucleotides triphosphate), 0.5 µL 27F forward primer, 0.5 µL 1492R reverse primer, 0.25 µL Taq polymerase (Thermo Scientific™ DreamTaq™ DNA Polymerase, 5 U μL^{-1}; Thermo Fisher Scientific Inc., USA), 1 µL of template, resulting in a final volume of 50 µL per sample.

Reaction profile:

95 °C 3:00		1X
94 °C 0:30	}	
56 °C 0:30	}	32X
72 °C 1:10	}	
72 °C 10:00		1x

In this PCR reaction, 16S rRNA region was amplified using a forward primer 27F (5'AGAGTTTGATCCTGGCTCAG-3') and a reverse primer 1492R (5'-GGTTACCTTGTTACGACT-3') (Lane 1991). For both T-RFLP analysis and Sanger sequencing the only difference was that in the case of T-RFLP 27F was labeled with a 5'-end fluorophore (VIC). The amplification took place in the Applied Biosystems™ ProFlex™ PCR device. The final product of the reaction was checked by agarose gel electrophoresis to confirm the relative length and purity of the sequences.

16S rDNA region was targeted for bacterial identification because it conserved enough to be present in almost all bacteria and archaea, but also contains variable regions that can be used to distinguish between different taxa and also it has big databases (NCBI, EZBIOCLOUD) where investigated strains can be compared to the already identified one.

3.7. T-RFLP of genes encoding 16S rDNA

T-RFLP is a molecular fragment analysis technique that is used to study a community's microbial diversity (Liu et al. 1997). It mainly involves the amplification of 16S rRNA region as were described before in chapter in 3.6. The technique implies PCR in which one of the two primers is fluorescently labeled which limits the analysis of terminal fragments of the digestion. After that amplified product will be digested using restriction enzymes which were utilized for the first time in 1971 (Danna & Nathans 1971). They are produced by bacteria and are used as a defense mechanism against invading viruses by cutting their DNA. The restriction enzyme recognizes the palindromic sequence and cuts between 4-8 base pairs. And then, by using gel electrophoresis size of the generated fragments can be relatively measured. After that using an automated DNA sequencer, the fluorescently labeled terminal restriction fragment is precisely quantified.

The gained electropherograms have provided information about diversity of bacterial community (the numbers of peaks) and from the peaks' heights (areas) can be concluded semiquantitative information about OTU's (Marsh 1999).

3.7.1. Steps of analysis

3.7.1.1. Preparatory steps

DNA isolation from the enrichments, PCR reaction and gel electrophoresis were conducted, which were described in chapters 3.5. and 3.6. as preparatory steps.

3.7.1.2. PCR digestion

PCR-products were digested using a mixture containing 0.3 µL restriction enzyme (*Alu*I ; (AG↓CT)) (Thermo Scientific™, 10 U µL, Thermo Fisher Scientific Inc., USA), 2 µL buffer solution (10X Tango™ buffer, with BSA, Thermo Fisher Scientific Inc., USA), 7.7 µL Milli-Q water and 10 µL from PCR product. The final concentration was 20 µL, which was incubated for one hour at 37 °C.

3.7.1.3. Precipitation step

After the digestion step digested PCR product needs to be precipitated in this step 80 µL from the mixture of 62.5 µL 95% ethanol, 14.5 µL Milli-Q water, 3 µL Na-acetate was added to the digested product and incubated in room temperature for 10 minutes. Then the samples were centrifuged (4600 rpm) for 20 minutes at 4 °C. Next samples were discarded, and 180 µL from 70% ethanol was added to the samples. Followed by 20 minutes centrifugation (4600 rpm) and discarding the samples as the previous step. After that the tube were placed upside-down and centrifuged (900 rpm) for one minute. Finally, 20 µL of Milli-Q water was added to the samples.

3.7.1.4. Fragment analysis

After the precipitation 18 µL from Hi-Di™ and 0.4 µL from GeneScan™ 1200 LIZ™ size standard were added to the samples. The former was used to resuspend samples before electrokinetic injection in capillary electrophoresis system while the latter was added as gene ruler is designed for sizing DNA fragments in the 20-1200 nucleotides range. After that, the samples were kept at 4 °C overnight. Fragment analysis was carried out in Applied Biosystems®

3500 Genetic Analyzer. The results were analyzed using GeneMapper™ 4.0. software (Applied Biosystems, USA). Terminal restriction fragment (T-RF) peaks with a peak height below 50 relative fluorescence units or with a peak abundance contribution below 1% were excluded from further analysis.

3.8. 16S rDNA amplicon sequencing

Bacterial diversity for all six enrichment samples (IBU1, IBU2, IBU3, DIC1, DIC2, DIC3) was examined by Illumina 16S amplicon sequencing. The variable V3 and V4 regions of the 16S rRNA gene were amplified with the universal primer pair SD (S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21) with Illumina adapter overhanging nucleotides (Klindworth et al. 2013). The steps of DNA isolation, PCR reaction and gel electrophoresis was similar as mentioned in chapters 3.5. and 3.6.

PCR reaction for amplicon sequencing contained 1.0 μL of DNA, 0.5 μL from both forward and reverse primers, 12.5 μL of 2X KAPA HiFi HotStart Ready Mix (KAPABiosystems, London, UK), and 10.5 μL of Milli-Q water.

Reaction profile:

95 °C 3:00
95 °C 0:30
55 °C 0:30 } 25x
72 °C 0:30

PCR products were checked by agarose gel electrophoresis. Paired-end fragment reads were generated on an Illumina MiSeq sequencer using MiSeq Reagent Kit v3 (600-cycle) by Eurofins BIOMI Ltd. (Gödöllő, Hungary). The raw sequencing data were analysed according to Banerjee et al. (2022). Read numbers for all six enrichment samples were above 58 723. Trimmed sequences were processed using mothur v 1.41.1. (Schloss et al. 2009) as recommended by the MiSeq SOP page (https://www.mothur.org/wiki/MiSeq_SOP) (Kozich et al. 2013). The sequence assortment based on the alignment with the SILVA 132 SSURef NR99 database (Yilmaz et al. 2014). The ‘split.abund’ command was used to cut off singleton reads (Kunin et al. 2010). Chimera detection was fulfilled with the mothur’s uchime command (Edgar et al. 2011). As

proposed for prokaryotic species delimitation, the conventional 97% similarity criteria was employed to identify operational taxonomical units (OTUs) (Tindall et al. 2010).

3.8.1. Bray-Curtis similarity

The ‘subsample.shared’ command was used to gain abundances’ datas about the OTU’s. To visualize them we have used Paleontological Statistics (PAST 2.0) software (Hammer et al. 2001). With the gained output file (.xls) we have done cluster analysis by Bray-Curtis method (Bray & Curtis 1957). The Bray-Curtis similarity index compares the relative abundances of a community across two locations. A value of 1 indicates total similarity, if the value is 0 that indicates total dissimilarity among enrichments (TerpConnect). By this method we have got information the direction and the parallelism of enrichment processes.

3.9. Sanger sequencing

A molecular biology technique which was invented by Fred Sanger in 1977 (Sanger et al. 1977). It is also called chain termination method because of addition of called dideoxyribonucleotides (ddNTPs), which are missing the 3′ hydroxyl group that is required for 5’ to 3’ extension of a DNA polynucleotide chain. The sequencing process involves four main parts PCR amplification and sequencing of DNA, capillary electrophoresis, and data analysis. A mixture of primer, DNA polymerase, normal dNTPs, fluorescently labeled ddNTPs, and DNA template is transferred into a PCR machine which is connected to the capillary electrophoresis filled with gel polymer. When the ddNTPs are randomly incorporated and terminated the chain strands of each possible length are produced. The extension products are subsequently resolved to single-nucleotide size variations by electrophoresis. Each color identifies one of the terminating ddNTPs.

3.9.1. Steps of the test

3.9.1.1. Preparatory steps

DNA isolation from the isolates, PCR reaction and gel electrophoresis were conducted which were described in chapters 3.5. and 3.6. as a preparatory steps.

3.9.1.2. PCR cleaning

PCR cleaning was performed as next phase of preparation by using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH& Co. KG, Germany) according to the protocol supplied by the manufacturer. Primers, DNA polymerase, unused nucleotides, buffers, and dyes were washed away from the PCR product. Spin columns containing a silica matrix to which DNA was selectively bound. Then washed off from the silica matrix by using an elution buffer. After that purity of the samples was visualized by gel electrophoresis.

3.9.1.3. Sequencing PCR

After PCR-clean up samples were ready for sequencing in order to sequence longer region of the 16s rDNA three primers 27F, 338F (5'-ACTCCTACGGGGGGCAGCAG-3'), and 803F (5'-ATTAGATACCCTGGTAG-3') were used.
Reaction components: 0.75 µL buffer (BigDye® Terminator 5X Sequencing, Applied Biosystems, UK); 0.5 µL BigDye® (Applied Biosystems, UK); 0.25 µL 27F/338F/803F primer; 3 µL of Milli-Q water; 0.5 µL of template, so the final volume is 5 µL per sample.

Reaction profile:

96 °C 0:10
50 °C 0:05 } 28x
60 °C 4:00

3.9.1.4. DNA precipitation

PCR products resulted from sequencing PCR were subjected to precipitation step, which was similar to precipitation step described in chapter 3.7.1.3. except that in final step 20 µL Hi-Di™ were added to the samples instead of Milli-Q water. Sequencing reaction was carried out with the Applied Biosystems® 3500 Genetic Analyzer, as in the case of the T-RFLP analysis.

3.9.1.5. Result Analysis

Around 1500 bp long nucleotide sequence was obtained in case of each bacterial strain, which reflect the whole length of 16S rDNA region. Each of the three used primers was designed to generate around 700 bp long fragments. Mega X software was used to trim and align generated

16S region. EzBioCloud database homepage (EzBioCloud.net | Search about Bacteria or Archaea) 16S-based ID function was used to identify bacterial isolates (Yoon et al. 2017).

3.10. The resazurin screening assay

The resazurin screening assay is a common method used to screen for bacterial growth or activity in a sample. Resazurin is a blue dye that is reduced to a pink, fluorescent dye, resorufin in the presence of metabolically active bacteria (Sarker et al. 2007). To assess the growth and activities of the bacterial strains on the individual pharmaceutical compounds as a sole carbon source. The screening was conducted in a 300 mL Erlenmeyer flask triplicate for each strain. 100 mL from Bushnell-Haas mineral medium (0.002 g $CaCl_2$, 0.02 g $MgSO_4$, 1.0 g NH_4NO_3, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 0.005 g $FeCl_3 \cdot 6H_2$ L^{-1} H_2O with pH=7) supplemented with resazurin redox indicator (1 mg l^{-1}). Then 10 mg L^{-1}, either DIC, IBU was added to the media. Next, 100 µL from bacterial suspension (OD_{600}=1.00) obtained in physiological salt was added to the solution. Triplicates of biotic control which did not contain pharmaceuticals and abiotic control without the addition of bacteria were also prepared. After that, all flasks were incubated for a period of two weeks in a rotary shaker incubator at 145 rpm and 27 °C. The activity of the tested isolates was detected spectrophotometrically using (GENESYS™ 10 UV/Vis Spectrophotometer, Thermo Fisher Scientific, USA) by measuring the absorbance of the test solutions at 610 nm (absorbance of the resazurin indicator, A610) according to Benedek et al. (2022).

3.11. HPLC measurement

To confirm the capability of bacterial isolates to degrade ibuprofen and diclofenac HPLC technique (High Performance Liquid Chromatography) was used. HPLC is a liquid-phase physical separation technique in which a sample is separated into its constituent components by distribution between the mobile phase and a stationary phase. A chromatogram is generated by an online detector that monitors the concentration of each separated component in the column effluent (Ornaf & Dong 2005). Biodegradation tests were conducted according to Benedek et al. (2022) in triplicates in 100 mL of mineral salts medium, and 1.5 mg L^{-1} of either ibuprofen or diclofenac was added to the media as a sole carbon source. Subsequently, co-metabolic biodegradation tests were also conducted, easily assimilable carbon sources such as yeast extract (0.3 g L^{-1}), or glucose (3 g L^{-1}) were added to the test solutions mentioned above next to the 1.5

mg L^{-1} of pharmaceutical compounds. The test solutions were inoculated with 100 µL of bacterial cell suspensions (OD_{600} = 1) obtained in a physiological saline solution. Abiotic controls, containing all the above-mentioned except the bacterium were also set up. Test runs were incubated for the period of three weeks on a rotary shaker at 145 rpm and 28 °C. The concentration of the pharmaceuticals was measured at zero time and three weeks later. Prior to injection into the HPLC instrument the aqueous samples were filtered by passing through Labfil nylon syringe filters (0.22 µm). A Chromaster Hitachi instrument consisting of a Model 5110 pump, a Model 5210 autosampler, and a Model 5430 Diode-array detector (DAD) was used. The separation and data processing were operated by an EZChom Alite software. The separation of the pharmaceutical compounds was performed on Ascentis C18, 150 × 0.46 mm column with isocratic elution. HPLC set up shown in Table 1 .The DAD was operated at a wavelength range between 190 and 400 nm. For the quantitative determination a calibration curve for each compound was done between concentration and absorbance at maximum wavelengths (ibuprofen 196 nm and diclofenac 202 nm). The compounds were identified based on a comparison of retention time and spectral characteristics with those of standard solution.

Time [min]	**KH_2PO_4[%]**	**Acetonitrile [%]**	**Flow rate [mL/min]**
4	52	48	0,75
8	40	60	0,75
15	40	60	0,75
18	52	48	0,75
20	**52**	**48**	**0,75**

Table 1. Set up of HPLC method

4. RESULTS AND DISCUSSION

4.1. Enrichment of bacteria with capability of degrading pharmaceuticals

The below figure represents the diclofenac and ibuprofen flasks after three months of enrichment. In the case of ibuprofen, the enrichment flasks that start becoming turbid may indicate bacterial growth and activity. While diclofenac flasks showed to be less turbid and remained relatively the same throughout the period of enrichment.

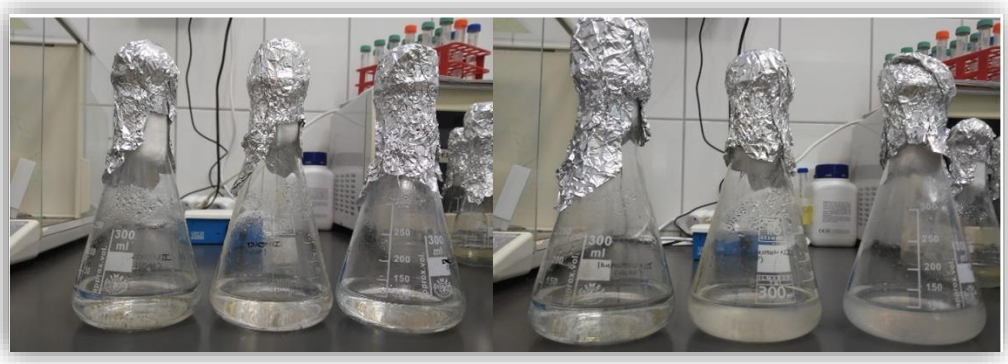

Figure 3. Diclofenac (left) and ibuprofen (right) enrichment's flasks at the 30. days

4.2. Bacterial pure strains isolation

Eight pure bacterial strains were isolated from the enrichment cultures, after that all the strains were maintained and transferred to new media every two weeks. As we described before (in chapter 3.9.1.5.) we have identified our pure strains. The aligned sequences that we have gained would be about 1280-1440 bp long partial 16S rDNA sequences.

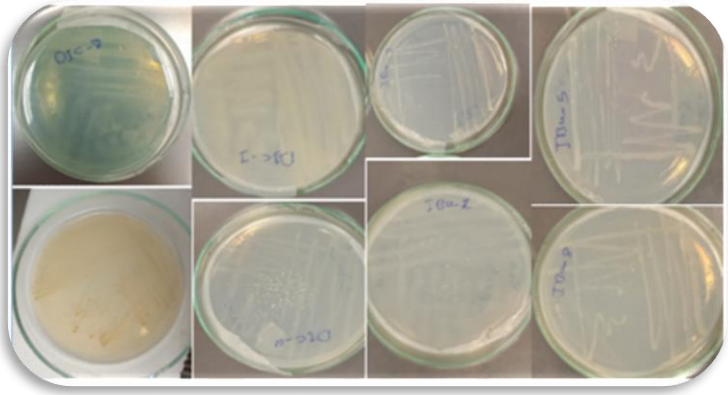

Figure 4. Isolated bacterial strains (in order: IBU-3, DIC-3, IBU-6, IBU-5, KM-8, DIC-4, IBU-2, IBU-8)

4.2.1. Description of bacterial strains

4.2.1.1. *Rhizobium petrolearium* IBU-8

Gram-negative aerobic bacterium belongs to the family *Rhizobiaceae.* Motile with polar or lateral flagella, non-endospore-forming rods (0.5–1.0×1.0–2.5 μm). Colonies are circular. The pH range for growth is 6.0–10.0 and the optimum pH is 7.0–8.0. The range temperature for growth is 10–40 °C and the optimum temperature is 30 °C. Catalase- and oxidase-positive. GC-content is 62.2 mol%. NaCl concentrations up to 3% (w/v) are tolerated, but the optimal concentration for growth is 1% (w/v) it has been discovered in oil-contaminated soil (Zhang et al. 2012). The strain SL-1 have shown to use polycyclic aromatic hydrocarbons as its only source of carbon and energy, including naphthalene, fluorene, anthracene, pyrene, 1-naphthol, gentisic acid, protocatechuate, and 1-hydroxy-2-naphthoic acid. The strain could completely eliminate phenanthrene (PHE) without co-metabolism or the use of surfactants. Due to its high PHE degradation efficiency, metabolic adaptability and stress resistance, it is a promising candidate for bioremediation of PAH-contaminted sites (Huang et al. 2016).

4.2.1.2. *Ancylobacter lacus* IBU-6

The species is gram-negative, non-spore-forming, nonmotile, has pear-shaped cells (1.0–1.5 × 2.0–2.2 μm). A facultative methylotrophic bacteria, which is oxidase-, catalase- and urease-positive. Optimal growth at 25–29°C, pH 7.0–7.5 and 0.5 vol % CH_3OH, in the presence of 0.05–1.5% NaCl. Highly resistant to a wide range of antibiotics. Utilizes ammonium salts and nitrates as nitrogen sources. The DNA G + C content is 67.5 mol %. It has been mainly detected in the

aquatic environment (Chemodurova et al. 2020). Previously Pápai et al. (2023) could isolate from IBU containing enrichment a strain (*Ancylobacter* sp. nov. IBU_2), which belongs to the Ancylobacter genus, but according to the literature its biodegradation capacity are not known so far.

4.2.1.3. *Pseudomonas aeruginosa* IBU-3

A gram-negative, motile, rod-shaped bacterium. It exhibits oxidase and catalase activity. Some of the strains can resist extreme conditions in a temperature as high as 50 °C and in an environment with a pH of 11 (Dharshini et al. 2021). It can be found in a wide range of environments such the body of animals, aquatic environments, and soil (*Https://Microbeatlas.Org*, 2023). *Pseudomonas aeruginosa* has been shown to utilize and degrade crude oil and it has reduced the viscosity of the crude oil (Varjani & Upasani 2016). It has also been reported that degradative capabilities of *P. aeruginosa* on surfactant namely sodium dodecyl sulphate (SDS) is highly significant (Ambily & Jisha 2012). Another study revealed that could produce some extracellular-enzyme capable of degrading aflatoxin compounds (Xu et al. 2023). Additionally the strain PCN-2 was able to degrade sulfamethoxazole (a type of antibiotic) and were identified seven metabolites during the experiment also (Zhao et al. 2023).

4.2.1.4. *Pseudomonas furukawaii* IBU-2

Pseudomonas furukawaii is a Gram-stain-negative, aerobic, motile, straight rod, 1.5–2.0 μm long and 0.7–0.9 μm wide species. The DNA G+C content is 65.5 mol% of chromosome and 60.5 mol% of a plasmid. It has been discovered in soil environments (Kimura et al. 2018). It has been reported that the degradation capability of *Pseudomonas furukawaii* in the crude oil is relatively high, its capability has been increased when combined with extracellular polymeric substances, which are produced by microbial biofilm (Vandana & Das 2021).

4.2.1.5. *Starkeya novella* IBU-5

The species is Gram-negative, non-motile, non-spore forming, rod, coccoidal shape, aerobe, and requires biotin for growth on most substrates. The GC content of the DNA is 67±3–68±4 mol%, a facultative sulfur- oxidizing chemolithoautotroph bacterium (Kelly et al., 2000). It can be found mainly in aquatic and soil environments (https://Microbeatlas.Org/, 2023-a). *S. novella*

has shown to degrade organophosphorus pesticides (OPs) (Dichlorvos, chlorofos, Methyl-parathion and fenitrothion, monocrotophos) in a very high range especially when supplemented with carbon and nitrogen sources (Sun et al. 2016). Bessa et al. (2017) have isolated and described the strain *Starkeya* sp. C11 was able to biodegrade 30% of 10 mg L^{-1} of carbamazepine, which is an antiepileptic drug.

4.2.1.6. *Azospirillum thiophilum* DIC-4

It is a Gram-negative, spiral, curved rods, 1.1–2.0 mm wide and 3.6–7.0 mm long, motile and aerobic. It can grow at a temperature of 40 °C. It has been discovered in the aquatic environment in sulfur spring. It can also grow on a wide range of organic substrates (pyruvate, succinate, asparagine, aspartate, phenylalanine, cysteine, lysine, and methionine). The DNA G+C content of the type strain is 67 mol%. It exhibits thiosulfate: ferricyanide oxidoreductase activity which makes it capable of using a thiosulfate electron donor for energy metabolism under microaerobic conditions (Lavrinenko et al. 2010). A study which was done by Alexandrino et al. (2020) has revealed that *Azospirillum thiophilum* along with some other bacterial species were able to degrade two highly persistent fluorinated fungicides (epoxiconazole and fludioxonil).

4.2.1.7. *Sphingomonas asaccharolytica* KM-8

Gram-negative, non-sporing, straight rods, motile with the single polar flagellum, colonies are lemon-yellow colour. G +C content of DNA is 62-64 mol% (Yabuuchi et al. 1990). Most of the *Sphingomonas* species are found in aquatic environment (*Https://Microbeatlas.Org/*, 2023-b). The main difference between *Sphingomonas* and other bacterial families is the exhibition sphingoglycolipid, 2-hydroxy fatty acids and the lack of 3-hydroxy fatty acids (Busse et al. 1999). It has been reported that members of genus were able to degrade and utilize some polycyclic aromatic hydrocarbons (e. g. pyrene, 1-nitropyrene) as a source of energy. Except for chrysene which was co-metabolically utilized (Ye et al. 1996). One of the members of S*phingomonas* genus namely S*phingomonas* sp. Ibu-2 was able to use ibuprofen as a sole source of carbon and energy (Murdoch & Hay 2005).

4.2.1.8. *Pseudomonas stutzeri* DIC-3

Pseudomonas stutzeri is a Gram-negative bacterium, cells are rod-shaped, motile, and aerobic. Colonies are adherent, have a characteristic wrinkled appearance, and are reddish brown. G+C content of their genomic DNA lies between 60 and 66 mol%. It can resist temperature up to 42 °C. The strain is also known as a denitrifier. It has been isolated from straw, manure, and canal water (Lalucat et al. 2006). *P.stutzeri* has shown the ability to grow on a wide range of organic compounds. The strain is capable of oxidizing and utilizing *o*-xylene, 2,3- and 3,4-dimethylphenol as a sole carbon and energy source (Baggi et al. 1987). The complete decolorization of procion red dye has been reported while subjected to the *P.stutzeri.* The degradative capability of the species on pharmaceuticals such as alprazolam has also appeared to be relatively high (Shahriarinour et al. 2021). Pápai et al. (2023) have successfully isolated from their pharmaceuticals containing enrichment of a *P. stutzeri* strain namely *Pseudomonas stutzeri*_Dic_1. At the case of DIC-enrichments the *Pseudomonas* genus was the most dominant bacteria according to Illumina 16S rDNA sequencing's results, and the case of ibuprofen and carbamazepine enrichments the *Pseudomonas* genus was ranked second/third places based on relative abundance's datas, but they have not mentioned any biodegradation capacity of this strain.

Designation of the isolates	Nearest cultured neighbour based on 16S rRNA gene	Phylogenetic affiliation	16S rDNA sequence homology (%)	16S rRNA gene sequence accession number
IBU-1,-3; DIC-7,-8,-9	*Pseudomonas aeruginosa* JCM 5962(T)	Gammaproteobacteria	99.51	BAMA01000316
IBU-2	*Pseudomonas furukawaii* KF707(T)	Gammaproteobacteria	99.58	AJMR01000229
KM-8	*Sphingomonas asaccharolytica* NBRC 15499(T)	Alphaproteobacteria	98.05	BCYU01000001
IBU-5,-7,-12	*Starkeya novella* DSM 506(T)	Alphaproteobacteria	98.48	CP002026
IBU-6,-9,-10,-11	*Ancylobacter lacus* F30L(T)	Alphaproteobacteria	98.53	MK931436
IBU-8	*Rhizobium petrolearium* SL-1(T)	Alphaproteobacteria	99.45	EU556969
DIC-1,-2,-3,	*Pseudomonas stutzeri* ATCC 17588(T)	Gammaproteobacteria	99.16	CP002881
DIC-4	*Azospirillum thiophilum* DSM 21654(T)	Alphaproteobacteria	99.64	LAEL01000007

Table 2. Isolated strains from three months old selective enrichment samples

4.3. Microbial community analysis by T-RFLP

On the T-RFLP electropherograms the x-axis shows the base pair lengths associated with the peaks/cleavage sites and the y-axis shows the intensity of the signal of the fluorophore-labelled and cleaved fragments, so that the area under the peaks is proportional to the amount of these fragments. By *in silico* method we have determined expected cleavage sites of our pure strains and tried to identify them on the gained electropherograms.

Microbial community in ibuprofen containing enrichment tends to be more diverse and also parallel to one another, the T-RFLP profile generated by *AluI* digestion showed 4-5 major T-RFs (Figure 4.). The T-RFs between 50 bp and 300 bp were detected. T-RF of about 71 bp corresponded to *Pseudomonas* genera, and of 205 bp corresponded to *Azospirillum* genus while the one of 247 bp corresponded to *Rhizobium* genus. The other T-RFs could not be assigned to a cluster or group.

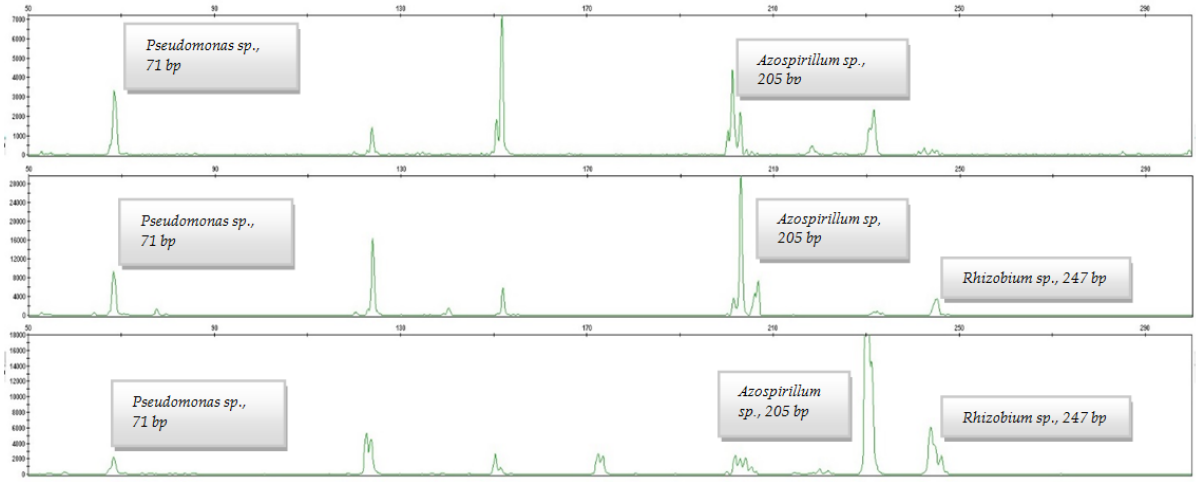

Figure 5. Electropherograms of T-RFLP analysis, 16s rDNA was digested by *Alu*I (IBU-1,2,3 enrichments)

The microbial community in the case of diclofenac degrading enrichments have showed lower diversity comparing with the IBU enrichments, but seems to be also parallel. Only one OTU could be identified, which is corresponded to *Pseudomonas* genera (at 71 bp).

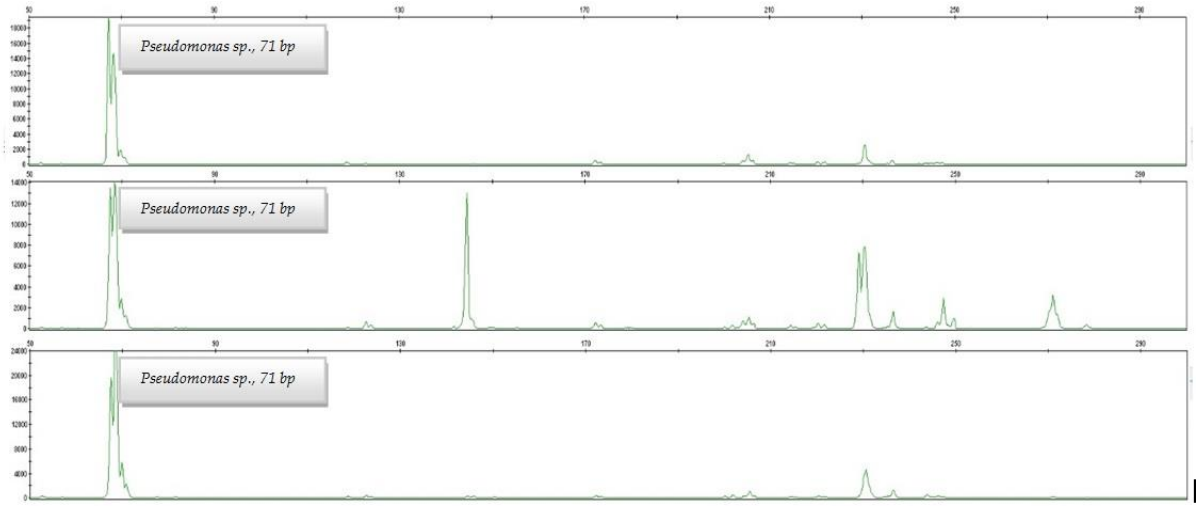

Figure 6. Electropherograms of T-RFLP analysis, 16s rDNA was digested by *Alu*I (DIC-1,2,3 enrichments)

4.4. Bacterial community composition based on Illumina 16S rRNA gene amplicon sequencing

In the ibuprofen-containing enrichments the two most dominant classes were Alphaproteobacteria (54.9%) and Gammaproteobacteria (38.5%), followed by Actinobacteria class, which represents 3.7%. The two other classes were Bacteroidia and Gemmatimonadetes, but they represent less than 1% of the total bacterial community in the enrichments. Regarding the genera, the distribution in the microbial community according to the relative abundances' values was as follows: *Pseudomonas* (36.5%), *Hyphomicrobium* (22.9%), *Azospirillum* (13.4%), *Rhizobium* (7.1%), *Bradyrhizobium* (2.9%), *Nocardioides (*2.1%) and *Sphingomonas (*2%).

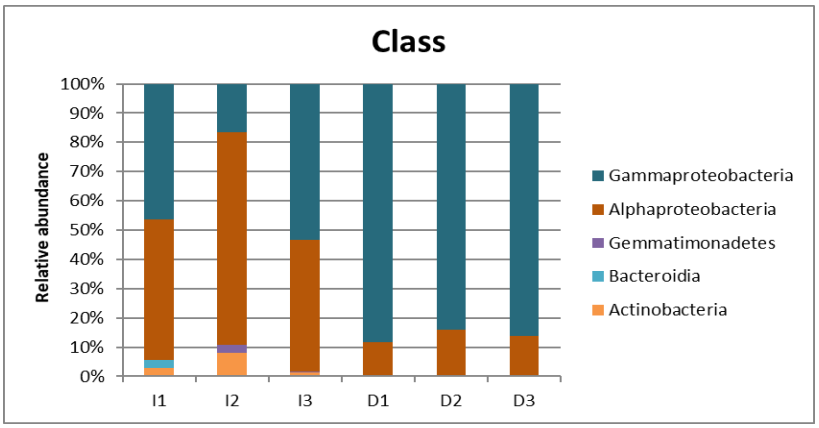

Figure 7. Taxonomic composition of the bacterial community found in (IBU 1-2-3 and DIC 1-2-3) enrichments after three months of selective enrichment in the level of class with a percentage relative abundance ≥ 1% in at least one of the samples.

In the case of diclofenac degrading enrichments, the most dominant class was by far the Gammaproteobacteria (85.5%) with a high abundance of the genus *Pseudomonas* (73.1%) and co-dominant community members were *Pseudoxanthomonas (*3%) and *Solimonas* (3%). The second most abundant class was Alphaproteobacteria (13.7%) represented mainly by the genera *Hypomicorbium* (3.4%), *Bradyrhizobium* (3.3%), and *Azospirillum (*2.8%).

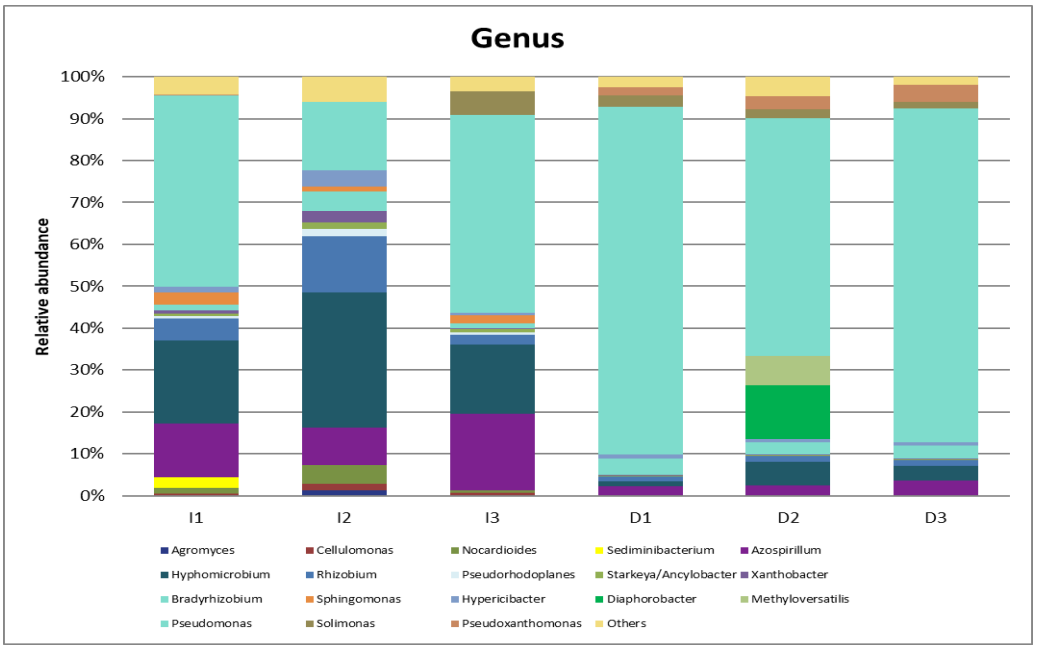

Figure 8. Taxonomic composition of the bacterial community found in (IBU1-2-3 and DIC 1-2-3) enrichments after three months of selective enrichment in genus level with a percentage relative abundance ≥ 1% in at least one of the samples.

In the species level three strains, namely *Pseudomonas stutzeri* DIC-3, *Pseudomonas aeruginosa* IBU-3 and *Azospirillum thiophilum* DIC-4 were successfully isolated from the diclofenac containing enrichments. *Pseudomonas stutzeri* (OTU 1) was found to be the most abundant species with an average of 69.2%. However, this value increased to 81.5% in the DIC-1 enrichment. The relative abundance value of OTU 2, which contains *Pseudomonas aeruginosa* species, was 3.5%. It should be noted that *Pseudomonas furukawaii* IBU-2 belongs to this OTU also, but could only be isolated from IBU degrading enrichments. *Azospirillum thiophilum* DIC-4 species belonging to OTU 4 was also isolated with a relative abundance value of 2.7%. Therefore, four dominant or co-dominant strains were isolated from the top 6 OTU's.

In the case of IBU degrading enrichments, six strains were successfully isolated, including *Pseudomonas aeruginosa* IBU-3, *Pseudomonas furukawaii* IBU-2, *Sphingomonas* sp. KM-8., *Starkeya* sp. IBU-5, *Ancylobacter* sp. IBU-6, and *Rhizobium petrolearium* IBU-8. The highest relative abundance value of 33.2% among the enrichments was found in the case of OTU 2, which contains *Pseudomonas aeruginosa* and *Pseudomonas furukawaii* species. OTU 4, which is related to *Azospirillum thiophilum* DIC-4, had a relative abundance value of 13.3% on average,

but it could only be isolated from DIC enrichments. *Rhizobium petrolearium* IBU-8 belonging to OTU 5 had a relative abundance value of 5.6%. Additionally, OTU 1 showed a value of 3.2%, but *Pseudomonas stutzeri* DIC-3 could be isolated from the DIC enrichments. The relative abundance values of the other three strains, *Sphingomonas* sp. KM-8 (belonging to OTU 14), *Starkeya* sp. *IBU-5* and *Ancylobacter* sp. IBU-6 (both belonging to OTU 15) were between 1% and 2%. Therefore, five dominant strains were isolated and identified from the top 6 OTUs, and three co-dominant strains were isolated from the top 15 OTU's.

	Isolated strains	Average relative abundance of IBU-degrading enrichment	Average relative abundance of DIC-degrading enrichment
Otu0001	*Pseudomonas stutzeri* DIC-3	3,2%	69,2%
Otu0002	*Pseudomonas aeruginosa* IBU-3, *Pseudomonas furukawaii* IBU-2	33,2%	3,5%
Otu0004	*Azospirillum thiophilum* DIC-4	13,3%	2,7%
Otu0005	*Rhizobium petrolearium* IBU-8	5,6%	0,6%
Otu0014	*Sphingomonas* sp. KM-8	1,6%	0,0%
Otu0015	*Ancylobacter* sp. IBU-6, *Starkeya* sp. IBU-5	1,0%	0,2%

Table 3: Relative abundance of the isolated strains after three months of selective enrichment

4.4.1. Bray-Curtis similarity

Dendrogram was generated after using Brays-Curtis cluster analysis methods based on obtained relative abundancy of the enrichment samples from 16S rRNA gene amplicon sequencing results. Two enrichment samples were considered parallel, when similarity index was above 60%. It was clear-cut to identify the IBU from DIC enrichments as the degree of similarity between them was below 20%. Concerning the DIC enrichments the similarity between the replicates was relatively high, the similarity between DIC-1 and DIC-2 was 90%, however, DIC-3 showed 75% with the other two replicates, for that reason the three replicates were considered parallel to one another. Regarding the IBU enrichment, the similarity between IBU-1 and IBU-3 was 85%, but this index decreased to 55%, when compared with IBU-2, which is a median value of the similarity index. So they were also considered to be parallel.

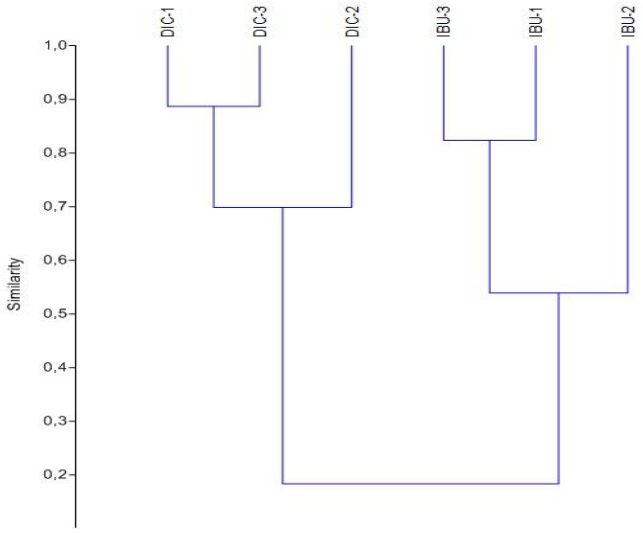

Figure 9. Dendrogram based on the Bray Curtis similarity method

4.5. Resazurin screening assay

Figure 10. Resazurin screening experiment after two weeks of incubation

After 5-6 days of the experiment, there was an apparent change in colour of the biotic control as well as testing samples, which can be viewed as a clear indicator of microbial activity in those flasks.

4.5.1. Potential ibuprofen degrading isolates

Name of strains	Species group representative isolates	Resazurin Standard Deviation Value	Resazurin sample measurement after 2 weeks
KM-8	*Sphingomonas asaccharolytica*	0.140 ± 0.014	0.097 ± 0.005
IBU-2	*Pseudomonas furukawaii*	0.140 ± 0.014	0.048 ± 0.021
IBU-3	*Pseudomonas aeruginosa*	0.140 ± 0.014	0.119 ± 0.019
IBU-5	*Starkeya novella*	0.140 ± 0.014	0.101 ± 0.008
IBU-8	*Rhizobium petrolearium*	0.140 ± 0.014	0.041 ± 0.002
IBU-6	*Ancylobacter lacus*	0.140 ± 0.014	0.120 ± 0.010
DIC-3	*Pseudomonas stutzeri*	0.140 ± 0.014	0.060 ± 0.030
DIC-4	*Azospirillum thiophilum*	0.140 ± 0.014	0.130 ± 0.008

Table 4. Absorbance values at the case of ibuprofen at tested strains at 610 nm after two weeks of incubation

The extent of the effect varies among the bacterial strains tested. The test samples, which contain both the bacterial strains and ibuprofen, have higher or similar absorbance values compared to the biotic control, indicating reduced bacterial growth in the presence of ibuprofen. For example in the case of DIC-4 or IBU-3 strains, suggesting that they are more sensitive to the inhibitory effect of ibuprofen. However, IBU-8, IBU-2 and DIC-3 had relatively lower absorbance values in the test samples compared to other strains, indicating that they are more resistant to the inhibitory effect of ibuprofen, and have become a promising candidate for further experiments.

The abiotic control, which contains ibuprofen and resazurin without any bacterial strains, has the highest absorbance value among all samples, indicating that the presence of bacteria is necessary for the reduction of resazurin and consequent reduction in absorbance values.

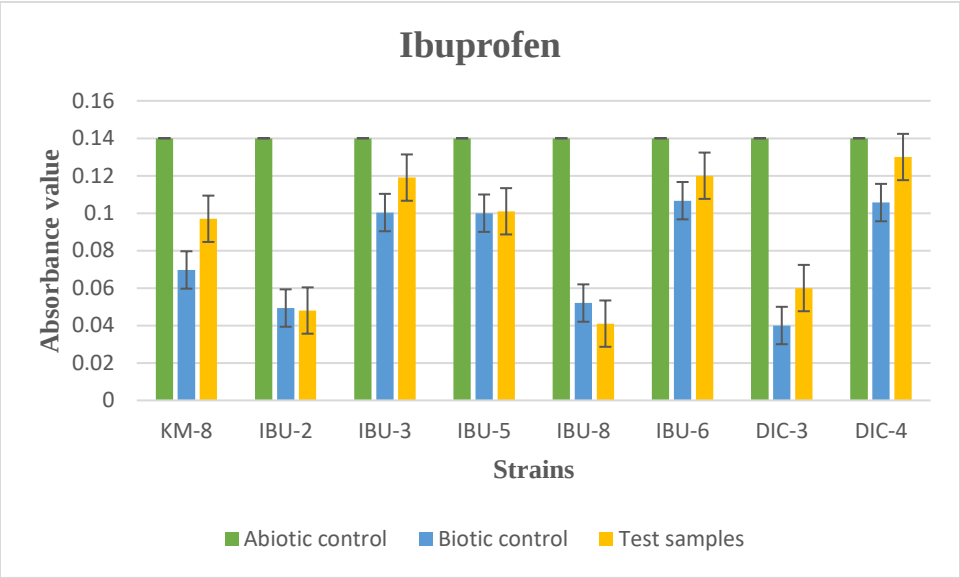

Figure 11: Absorbance values of the resazurin test solutions at 610 nm after two weeks of incubation

4.5.2. Potential diclofenac degrading isolates

Name of strains	Species group representative isolates	Resazurin Standard Deviation Value	Resazurin sample measurment after 2 weeks
KM-8	*Sphingomonas asaccharolytica*	0.140 ± 0.014	0.094 ± 0.001
IBU-2	*Pseudomonas furukawaii*	0.140 ± 0.014	0.048 ± 0.021
IBU-3	*Pseudomonas aeruginosa*	0.140 ± 0.014	0.116 ± 0.020
IBU-5	*Starkeya novella*	0.140 ± 0.014	0.097 ± 0.005
IBU-8	*Rhizobium petrolearium*	0.140 ± 0.014	0.051 ± 0.014
IBU-6	*Ancylobacter lacus*	0.140 ± 0.014	0.140 ± 0.017
DIC-3	*Pseudomonas stutzeri*	0.140 ± 0.014	0.063 ± 0.032
DIC-4	*Azospirillum thiophilum*	0.140 ± 0.014	0.113 ± 0.021

Table 5. Absorbance values at the case of diclofenac at tested strains at 610 nm after two weeks of incubation

In the case of diclofenac the test samples showed lower absorbance values compared to the biotic control, indicating that the presence of diclofenac had a neutral or positive impact on the growth of the bacterial strains. However, the degree of effect varied between the different strains tested. For example, strain IBU-2 showed a fair resistance to the diclofenac, with a test sample absorbance value of only 0.048 compared to the biotic control value of 0.049. On the other hand, strains IBU-6 and IBU-3 showed greater sensitivity, with test sample absorbance values higher than their respective biotic control values.

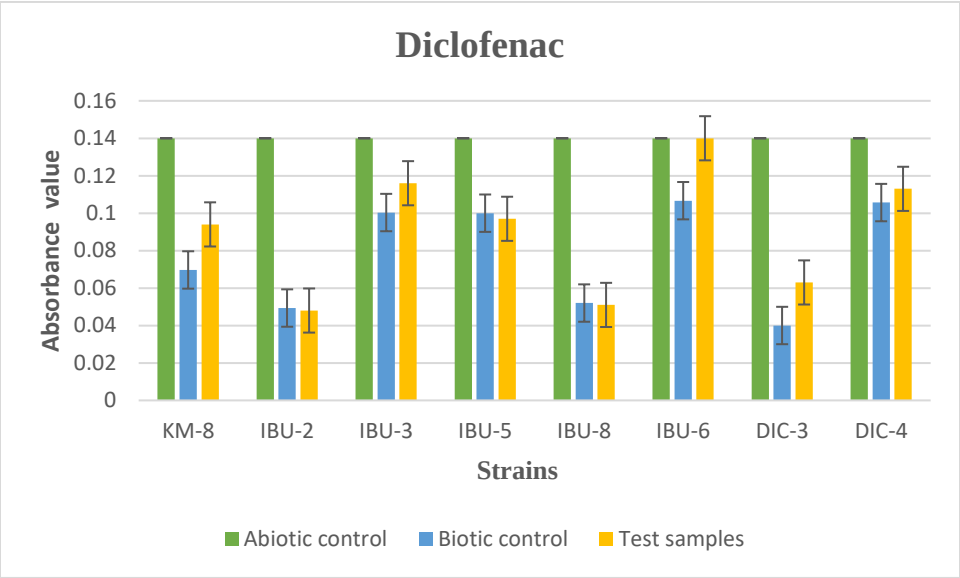

Figure 12: Absorbance values of the resazurin test solutions at 610 nm after two weeks of incubation

4.6. Biodegradation analysis using HPLC

4.6.1. Testing pharmaceutical biodegradation capacity of strains in a metabolic way using the HPLC technique

As was described before in chapter 3.11. (in 100 mL of mineral salts medium was added 1.5 mg L^{-1} of either ibuprofen or diclofenac, inoculated with 100 µL of suspensions) the experiment was performed. Based on the resazurin screening assay results four bacterial strains (*Sphingomonas* sp. KM-8, *Pseudomonas furukawaii* IBU-2, *Rhizobium petrolearium* IBU-8 and *Pseudomonas stutzeri* DIC-3) were chosen. Those strains showed higher metabolic activities in the presence of ibuprofen or diclofenac. Contrary to our expectations after three weeks, our strains have not shown detectable biodegradation activity in the case of ibuprofen or diclofenac either.

4.6.2. Testing pharmaceutical biodegradation capacity of strains in a co-metabolic way using the HPLC technique

The experiment was performed as we have described before in chapter 3.11., plus the addition of easily assimilable carbon sources also in this case (yeast extract and glucose). During the experiment huge amount of biomass was formed in the test flasks as seen below Figure 13.

Figure 13. The flasks of the tested strains in the second day (KM-8+ibuprofen; IBU-2+diclofenac; IBU-8+diclofenac and DIC-3+diclofenac)

For ibuprofen, the control showed no significant change in the concentration of the drug over three weeks. As in the literature written, "the abiotic degradation has a negligible role in its transformation" (Barra Caracciolo et al. 2015).

		c_{T0} [mgL^{-1}]	c_{T3} [mgL^{-1}]	Degradation rate
Ibupofen	Control	1.50	1.50	0.0%
	Pseudomonas furukawaii IBU-2	1.48	1.40	5.4%
	Rhizobium petrolearium IBU-8	1.48	1.34	9.4%
	Pseudomonas stutzeri DIC-3	1.49	1.49	0.0%
	Sphingomonas sp. KM-8	1.52	0.75	50.8%
Diclofenac	Control	1.50	1.50	0.0%
	Pseudomonas furukawaii IBU-2	1.38	1.02	26.2%
	Rhizobium petrolearium IBU-8	1.35	0.90	33.0%
	Pseudomonas stutzeri DIC-3	1.48	0.98	33.5%
	Sphingomonas sp. KM-8	1.48	1.38	6.5%

Table 6. Biodegradation percentage of bacterial isolates after 3 weeks of incubation

Among the bacterial strains tested, *Sphingomonas* sp. KM-8 as expected showed the highest degradation rate of 50.8%, reducing the concentration of ibuprofen from 1.52 mg L^{-1} to 0.75 mg L^{-1} .Members of *Sphingomonas* genus have great degradation capability, which was described in detail (in chapter 4.2.1.7). *Pseudomonas furukawaii* IBU-2 and *Rhizobium petrolearium* IBU-8 also showed some ability to degrade ibuprofen, with degradation rates of 5.4% and 9.4%, respectively. *Pseudomonas stutzeri* DIC-3 showed negligible degradation of ibuprofen.

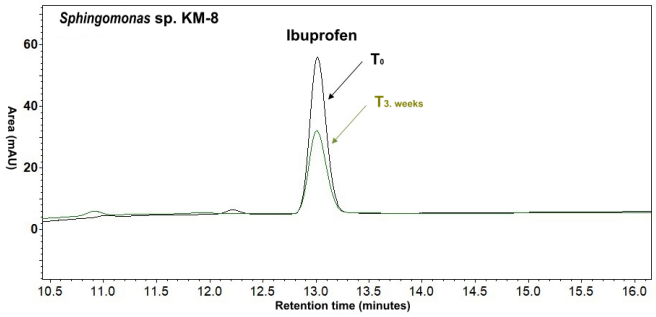

Figure 14. Biodegradation pattern of IBU induced by isolates (*Sphingomonas* sp. KM-8)

For diclofenac, the control also showed no significant change in the concentration of the drug over three weeks. Among the bacterial strains tested, *Rhizobium petrolearium* IBU-8 and *Pseudomonas stutzeri* DIC-3 showed the highest degradation rates of 33.0% and 33.5%, respectively, reducing the concentration of diclofenac from 1.35 mg L^{-1} and 1.48 mg L^{-1} to 0.90 mg L^{-1} and 0.98 mg L^{-1}, respectively. *Pseudomonas furukawaii* IBU-2 also showed some ability to degrade diclofenac, with degradation rates of 26.2%. Members of the genus *Pseudomonas* have been described in the breakdown of several xenobiotics e.g. carbamazepine-degrading *Pseudomonas* sp. CBZ-4 (Li et. al 2013). The information regarding biotransformation capabilities has been described in scientific literature, which is mentioned in chapter 4.2.1.1. In the case of *Sphingomonas* sp. KM-8 6.5% reduction rate was detect.

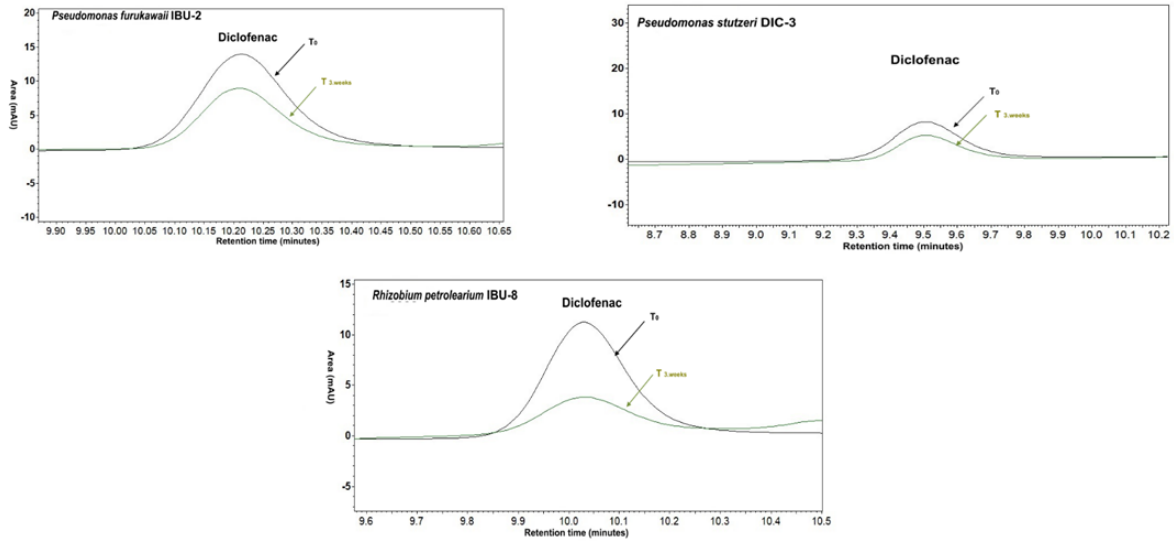

Figure 15: Biodegradation pattern of DCF induced by isolates (*Pseudomonas furukawaii* IBU-2, *Pseudomonas stutzeri* DIC-3 and *Rhizobium petrolearium* IBU-8*)*

5. CONCLUSION

The capacity of eight bacterial strains isolated from BTEX contaminated groundwater to degrade pharmaceuticals IBU, DCF was examined in this work. The results of 16s rDNA amplicon showed that the ibuprofen containing enrichment had a higher microbial diversity, with Alphaproteobacteria and Gammaproteobacteria as the dominant classes, while Gammaproteobacteria was the most dominant class in diclofenac containing enrichment. *Pseudomonas* was the most abundant genus in both enrichments, but the distribution of other genera was different. *Pseudomonas stutzeri* DIC-3, *Pseudomonas aeruginosa* IBU-3 and *Azospirillum thiophilum* DIC-4 were the dominant species in diclofenac containing enrichments, while *Pseudomonas aeruginosa* IBU-3, *Pseudomonas furukawaii* IBU-2, *Pseudomonas stutzeri* DIC-3 were dominant species, and *Sphingomonas* sp. KM-8, *Starkeya* sp IBU-5, *Ancylobacter* sp. IBU-6, and *Rhizobium petrolearium* IBU-8 were the co-dominant species in ibuprofen containing enrichments.

Based on resazurin pre-testing and scientific literature four promising strains namely *Sphingomonas sp.* KM-8, *Pseudomonas furukawaii* IBU-2, *Rhizobium petrolearium* IBU-8, *and Pseudomonas stutzeri* DIC-3 were chosen for performing degradation experiment. Those strains showed higher metabolic activities in the presence of ibuprofen or diclofenac, indicating their ability to utilize them as carbon source. However, the effect of ibuprofen and diclofenac on the growth of the bacterial strains varied, with some strains showing higher resistance to the inhibitory effects of the drugs than others.

The HPLC analysis confirmed that *Sphingomonas* sp. KM-8 was the most effective strain in degrading ibuprofen, reducing its concentration by 50.8% over three weeks. *Rhizobium petrolearium* IBU-8 and *Pseudomonas stutzeri* DIC-3 showed the highest degradation rates for diclofenac, reducing its concentration by 33.0% and 33.5%, respectively.

It is important to note that the bacterial strains were only able to degrade ibuprofen and diclofenac in the presence of an additional carbon source, such as yeast extract and glucose, indicating that these drugs cannot be used as sole carbon sources for bacterial growth. Another worth mentioning point is that during the experiment large amount of biomass was formed, which may have affected the results of the experiment. Therefore, further experiments are needed to

confirm that the reduction of the pharmaceuticals rate was not due to the adsorption to the biomass.

Overall, the study provides valuable insights into the potential of bacterial strains in bioremediation of pharmaceutical pollutants in the environment, and further research can explore the optimization of the conditions for their efficient degradation.

6. SUMMARY

The presence of pharmaceutical residues in the environment poses a serious threat to aquatic life as well as other non-target organisms. The fate of many of these pharmaceuticals in the environment has not been thoroughly investigated. Diclofenac and ibuprofen are among these pharmaceuticals, which are widely consumed around the globe with significant bioactivity that are considered to be persistent pollutants. The biodegradation of these compounds has been studied in this project in order to assess the fate of these pharmaceuticals in the environment. An easily degradable carbon source was added in these biodegradation experiments to optimize co-metabolism as a removal mechanism. In the first experiment for evaluation of microbial biodegradation capabilities resazurin screening assay was conducted as a preliminary test, which is widely used to assess the microbial activity and metabolism. Eight bacteria which were isolated from selectively enriched samples were used to determine if the selected drugs were biodegradable, and also to identify the level of activity which was measured according to their absorbance value. It was demonstrated that biodegradation occurred for both studied compounds with diclofenac showing more resistant to the biodegradation.

It appeared the bacteria *Sphingomonas* sp. KM-8 showed a particular ability to degrade 50.8% of ibuprofen. Regarding diclofenac degradation, *Rhizobium petrolearium* IBU-8 and *Pseudomonas stutzeri* DIC-3 showed the highest degradation rates of 33.0% and 33.5%, respectively, which were all confirmed by HPLC analysis.

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