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Biodegradation of ibuprofen and fluoxetine by bacterial strains isolated from environmental samples and identification of candidate catabolic genes

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Msc. In Biotechnology

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Declaration of Authorship

I declare that I am the author of this work, which is original and unpublished. The sources consulted have been duly cited in the text and included in the list of references.

AYLEEN D. VARGAS VILLAGÓMEZ

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Ayleen V.

Abstract

Pharmaceuticals are commonly found in surface waters due to high global demand. Conventional wastewater treatment plants don't remove them completely being a major source of these compounds, which pose a risk to the environment and human health despite their low concentrations. The objective of the present investigation was to isolate bacteria from environmental and wastewater samples from the Algarve area potentially exposed to recalcitrant contaminants (aromatic compounds), capable of degrading pharmaceuticals. Ibuprofen (IBU) and fluoxetine (FLX), two of the most common drugs found in effluents worldwide, were the targets. Twenty bacterial strains were isolated using enrichment cultures. However, only three strains (TIBU2.1, LOI1.1 and LOI1.2) showed the ability to degrade IBU, and two (LOFLX1.1 and LOFLX1.3) FLX. The drug concentration was monitored by HPLC, as well as the presence of IBU metabolites eventually formed during biodegradation. In addition, two bacterial strains (*Mycolicibacterium aubagnense* HPB1.1 and *Micrococcus yunnanensis* TJTP4), previously isolated for other pharmaceuticals, were investigated in this work. *Klebsiella pneumoniae* TIBU2.1 and *M. yunnanensis* TJTP4 exhibited complete degradation of IBU after 15 and 14 days, respectively, while *M. aubagnense* HPB1.1 was also able to degrade 60.2% ± 0.4 of IBU after 21 days. Additionally, the complete genomes of these three bacterial strains were sequenced to conduct a preliminary analysis of candidate genes involved in the degradation pathway of IBU. Catabolic enzymes reported in databases and literature for IBU biodegradation were used to search for similar proteins translated by the obtained genome sequences. These *in silico* analyses on the bacterial genomes showed similarities with most of the reported proteins. This work provides useful genetic information for the development of bioaugmentation techniques and reports new bacteria for bioremediation processes transforming IBU into less toxic metabolic compounds.

Keywords: Bacterial isolation; IBU; FLX; Bioaugmentation; Degrading genes.

Resumo

Os produtos farmacêuticos constituem um mercado em crescimento, altamente procurado ano após ano, o que faz com que estes compostos sejam continuamente detetados nos efluentes e nas águas superficiais. Apesar desses compostos poderem chegar ao ambiente através de diferentes fontes, as estações de tratamento de águas residuais (ETARs) são incapazes de os eliminar completamente, sendo por isso a maior fonte de contaminação ambiental destes poluentes. Foi demonstrado que, apesar das baixas concentrações de descarga presentes nas águas residuais (de ng L^{-1} a µg L^{-1}), a sua presença nos ecossistemas representa um risco para o meio ambiente e para a saúde humana. A utilização de processos de bioaumentação e bioestimulação apresentam-se como técnicas promissoras para a eliminação desses contaminantes. Para alcançar esse objetivo é fundamental isolar bactérias que possuam a capacidade de degradação dessas substâncias. Além disso, o entendimento da via de degradação e dos genes envolvidos pode ser útil no desenvolvimento de ferramentas a ser utilizadas na bioaumentação de bactérias, ou bioaumentação genética por transferência horizontal de genes de enzimas catabólicas em plasmídeos. Assim, o objetivo principal deste trabalho, a fim de melhorar os processos de tratamento de águas residuais, foi isolar estirpes bacterianas com a capacidade de degradar o anti-inflamatório monocíclico não esteroidal ibuprofeno (IBU) e o antidepressivo fluoxetina (FLX), dois medicamentos comumente encontrados nos efluentes de águas residuais que acabam no mar. Estas bactérias foram isoladas de amostras de sedimentos ambientais de diferentes pontos da região do Algarve, potencialmente afetados por contaminantes recalcitrantes como a gasolina e óleos lubrificantes e também de águas residuais de lagar de azeite – águas ruças - e seus sedimentos, uma vez que estes contêm compostos com aneis aromáticos, tal como o IBU e a FLX. Para selecionar bactérias potencialmente degradadoras foram feitos enriquecimentos em solução aquosa com meio mineral salino (MSM) suplementado com altas concentrações de IBU e FLX (100 mg L^{-1}), a partir dos quais foram isoladas em placa um total de 20 estirpes bacterianas (11 para IBU e 9 para FLX) com diferenças morfológicas entre elas. Dessas apenas 3 (TIBU2.1, LOI1.1 e LOI1.2) foram capazes de degradar IBU e 2 (LOFLX1.1 e LOFLX1.3) FLX, em solução aquosa. Para além dos novos isolamentos obtidos neste trabalho, também foi avaliado o potencial de degradação de duas bactérias previamente isoladas para outros fármacos no

laboratório de Tecnologias Ambientais do CCMAR onde decorreu o trabalho: *Mycolicibacterium aubagnense* HPB1.1 e *Micrococcus yunannensis* TJPT4. Nos ensaios de biodegradação, as amostras e os produtos metabólicos formados foram analisados por HPLC. A estirpe isolada TIBU2.1, identificada como *Klebsiella pneumoniae* por sequenciação do gene 16S rRNA, apresentou a maior taxa de degradação após 15 dias (100% de 5 mg L^{-1} inicial), sendo descrita pela primeira vez como degradadora de IBU. Seguido pelas estirpes LOI1.1e LOI1.2 identificadas como *Klebsiella variicola* e *Pseudomonas aeruginosa*, alcançando uma degradação de IBU de 60% e 65% (5 mg L^{-1} inicial) após 13 dias. Das estirpes testadas com vista à degradação de FLX, a maior taxa de degradação alcançada foi pela estirpe LOFLX1.3 (29% de 5 mg L^{-1} inicial). Sendo uma taxa de degradação relativamente baixa, decidiu-se não prosseguir com os demais ensaios com FLX. Das bactérias previamente isoladas para outros fármacos e recentemente testadas neste trabalho (*M. yunannensis* TJPT4 e *M. aubagnense* HPB1.1), ambas mostraram capacidade de degradação de IBU, no caso da estirpe TJPT4 uma degradação completa de IBU foi atingida e 60% de 5 mg L^{-1} inicial após 21 dias no caso da estirpe HPB1.1. Para melhor compreensão da cinética de crescimento, útil para otimizar a preparação de inóculos para futuros ensaios de degradação, estudou-se a cinética de crescimento das bactérias selecionadas. Com o objetivo de usar as informações genéticas em estudos futuros para o desenvolvimento de técnicas de bioaumentação, enviou-se ADN genómico de três isolados (TIBU2.1, TJPT4 e HPB1.1) selecionados para o sequenciamento do genoma completo. Estes isolados apresentaram os melhores resultados nos estudos de biodegradação. TIBU2.1 e TJPT4 revelaram um único grande contig cromossómico, enquanto HPB1.1 apresentou 1 contig cromossómico e 5 contigs de tamanhos condizentes com plasmídeos. Cada bactéria foi comparada em busca de homologias genéticas com as sequências das enzimas dos genes codificadores da via de degradação de IBU mais completa encontrada até o momento. Foi realizada uma análise preliminar *in silico* das enzimas e dos genes envolvidos na via de degradação do IBU. Isso fez-se através do alinhamento de 15 enzimas catabólicas de referência descritas na literatura com proteínas traduzidas dos genomas sequenciados das bactérias degradadoras selecionadas. A via metabólica de degradação da estirpe *Rhizorhabdus wittichii* MPO218 foi utilizada como referência, uma vez que é a via geneticamente mais completa descrita até à data. Dessas descobertas, cabe destacar que a bactéria TPJP4 apresentou similaridades acima de 25% apenas para 6 enzimas

enquanto as HPB1.1 e TIBU2.1 apresentaram percentagens de similaridade acima de 25% para a maioria das enzimas estudadas. Nestas duas bactérias verifica-se entre 25% e 56% similaridade com proteínas do cluster de genes (*ipfNSQT*) relacionados com a transformação do ácido 2-hidroxi-5-isobutil-hexa-2,4-dienóico em produtos finais que são facilmente metabolizados.

Em particular, a bactéria HPB1.1 apresentou semelhanças com praticamente todos as proteínas comparadas, confirmando uma relação entre essa via de degradação e a de referência. Por outro lado, a TIBU2.1 apresentou proteínas codificadas pelo cluster de genes *ipfABHI* relacionados com o início da via catabólica onde se forma dihidrodiol, e proteínas codificadas pelo cluster de genes *ipfNSQT* relacionados com o fim dessa via onde é produzido ácido 2-hidroxi-5-isobutil-hexa-2,4-dienóico, mas não apresentou proteínas codificados pelos genes *ipfDE* da parte intermediaria onde se formam o isobutilcatecol e o propionil-CoA. Isto sugere que a TIBU2.1 segue rotas alternativas na parte intermediária. Em conclusão, este trabalho permitiu isolar com sucesso a bactéria degradadora de IBU *K. pneumoniae* TIBU2.1, e confirmar também a atividade degradadora de IBU da bactéria *M. aubagnense* HPB1.1, ambas descritas pela primeira vez neste estudo com essa capacidade. No caso da FLX, mais estudos devem ser realizados para isolar bactérias capazes de degradar de forma mais eficiente esse fármaco.

Palavras-chave: Isolamento bacteriano; IBU; FLX; Bioaumentação; Genes de degradação.

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List of Abbreviations

°C	Degree Celsius
16S rRNA	16 Svedberg ribosomal ribonucleic acid
Blast	Basic local alignment search tool
bp	Base pairs
CAS #	Chemical abstracts service number
CFU	Colony forming units
CFU mL^{-1}	Colony forming units per milliliter
DNA	Deoxyribonucelic acid
DT_{50}	Time required to reach 50% degradation
DT_{90}	Time required to reach 90% degradation
EDTA	Ethylenediaminetetraacetic acid
EU	Europe union
F	Primer foward
FLX	Fluoxetine
FLX Hydro	Fluoxetine hydrochloride
FOMC	First-order multi-compartment mode
GC	Guanine-Cytosine content
gDNA	Genomic DNA
HPLC	High performance liquid chromatography
IBU	Ibuprofen
IUPAC	International union of pure and applied chemistry
k	Degradation constant
LB	Luria broth medium
LC-MS	Liquid chromatography mass spectrometry
MB	Marine broth medium
MSM	Mineral salt medium
NCBI	National center for biotechnology information
nm	Nanometer
NSAIDs	Non-steroidal anti-inflammatory drugs
OD_{600}	Optical density at 600 nm
PCR	Polymerase chain reaction
PES	Polyethersulfone
R	Primer reverse
RAST	Rapid annotation using subsystem technology
SFO	Single first-order kinetics
SSRI	Selective serotonin reuptake inhibitor
TAE	Tris-acetate-EDTA
td	Duplication time
t_R	Retention time
WWTP	Waste water treatment plant
X^2	Chi-square test
ʎ	Wavelength

α	Shape parameter
β	Location parameter
μ	Specific growth rate

1. Introduction

1.1 Pharmaceutical compounds

Pharmaceuticals such as anti-inflammatory drugs, analgesics and antidepressants generate trillions of dollars worldwide. By the year 2025, it is expected that pharmaceutical industry will generate approximately 1.6 trillion dollars (Figure 1.1). With the over-the-counter sale of some of these compounds, new diseases, increasing and aging population, the demand for pharmaceutical compounds has inevitably risen in recent years. Despite bringing countless benefits to society, these ubiquitous drugs represent an ecological risk when they are not completely eliminated from wastewater and make their way into aquatic environments. There are now regarded as emerging water pollutants. Of particular concern are widely used non-prescription drugs like the non-steroidal anti-inflammatory (NSAID) ibuprofen (IBU) and common antidepressants such as fluoxetine (FLX), which have been frequently detected in surface waters, groundwater and oceans worldwide (Figure 1.2). These pharmaceuticals have been found at concentrations that may adversely impact aquatic life (Luo et al., 2014; Martins et al., 2018; Mezzelani et al., 2018b; Shi et al., 2020; González Peña et al., 2021; Silva et al., 2021).

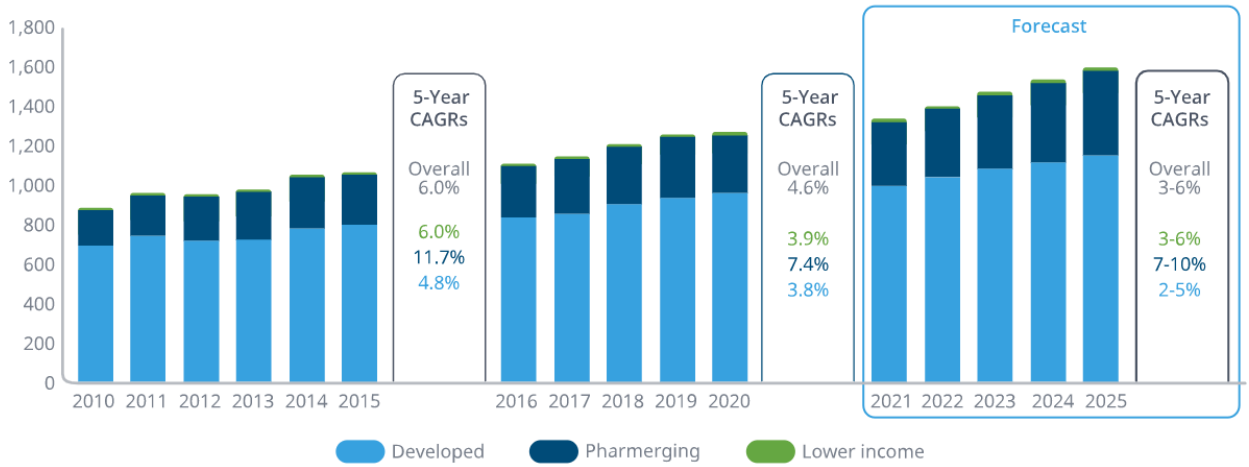


Figure 1.1 Global pharmaceutical market in trillions dollars from 2010 to 2025 (IQVIA, 2021).

Given the potential long-term harm to non-target organisms, and their classification as emerging pollutants, it is imperative to adopt sustainable measures for their comprehensive elimination. Considering their extensive usage, persistence in the environment, potential chronic toxicity to non-target organisms, and their classification as emerging pollutants for the ecological impacts of NSAIDs and antidepressants, appropriate measures are required for their complete elimination.

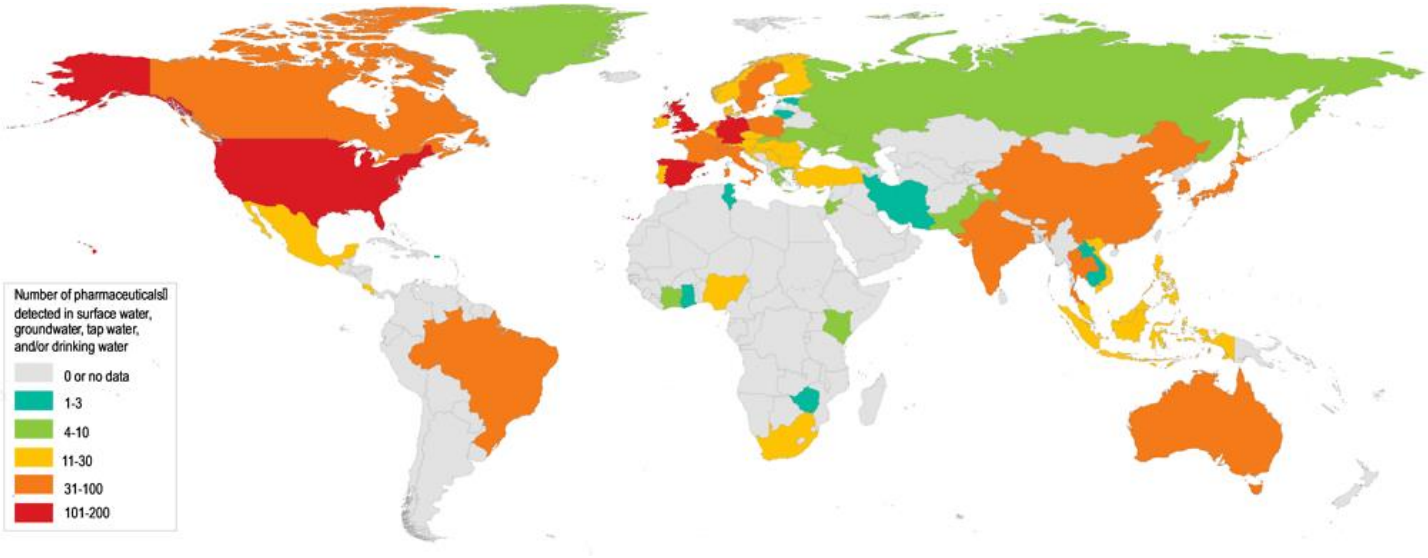


Figure 1.2. Global pharmaceutical detection in surface water, groundwater or drinking water (OECD, 2019).

1.2 Pharmaceuticals of interest

The presence of pharmaceuticals in wastewater, treated water effluents, surface water and groundwater is a concern. A wide range of these compounds has been identified in these aquatic systems (Pereira et al., 2020; Reyes et al., 2021). Drug concentrations in wastewater depend on several factors, including their usage in each country, climatic conditions, per capita water consumption, sewage systems, population size and density, the percentage of removal by water treatment processes, and their persistence in the environment (Tran et al., 2018). Seasonal variations also impact the concentration of pharmaceuticals in the influents and effluents of WWTPs, including the dilution rate, the influence of temperature on microbial activity, and changes in hydraulic retention time (Sun et al., 2014).

The NSAID drugs, such as IBU, are among the most abundant pharmaceuticals in effluents and surface waters worldwide, with concentrations ranging from ng L^{-1} to µg L^{-1} (Tran et al., 2018; Hodkovicova et al., 2022). On the other hand, antidepressants, such as FLX, are highly consumed drugs, and their usage increased even more after the COVID-19 pandemic, resulting in continuous finding of these compounds in effluents at concentrations ranging from ng L^{-1} to µg L^{-1} (Weinberger & Klaper, 2014; Yan et al., 2023). A study carried out in Portugal (Silva et al., 2021) revealed that in the Faro region (south Portugal), the second most frequently detected compound in urban wastewater influent was IBU with a concentration of 13,3 µg L^{-1}, while the antidepressant FLX was detected with a median concentration of 11 ng L^{-1}.

1.5.1 Ibuprofen

IBU ($C_{13}H_{18}O_2$) is a poorly water-soluble propionic acid derivative, that contains an aromatic ring in its structure (Figure 1.3). It is a non-stereoidal monocyclic anti-inflammatory compound and has become one of the most widely used drugs worldwide to relieve muscle and neurological pain, treat fever, headache and menstrual discomfort (Marchlewicz et al., 2016; National Center for Biotechnology Information, 2022). IBU undergoes biotransformation by microorganisms, human or animal metabolism results in two main metabolites: carboxybuprofen and 2-hydroxybuprofen (Figure 1.3). Other minor metabolites, such as conjugates or hydroxylated derivatives such as 1-hydroxyibuprofen or 3-hydroxyibuprofen can also be present (Ferrando et al., 2012; Larsson et al., 2014; Aguilar et al., 2022). Even though IBU and its metabolite compounds are removed during the activated sludge process in WWTPs with an efficiency of 90% to 99%, they continue to be found in WWTP effluents, surface water and in non-target organisms due to their high usage and its continuous release in the environment (Ferrando et al., 2012; Larsson et al., 2014).

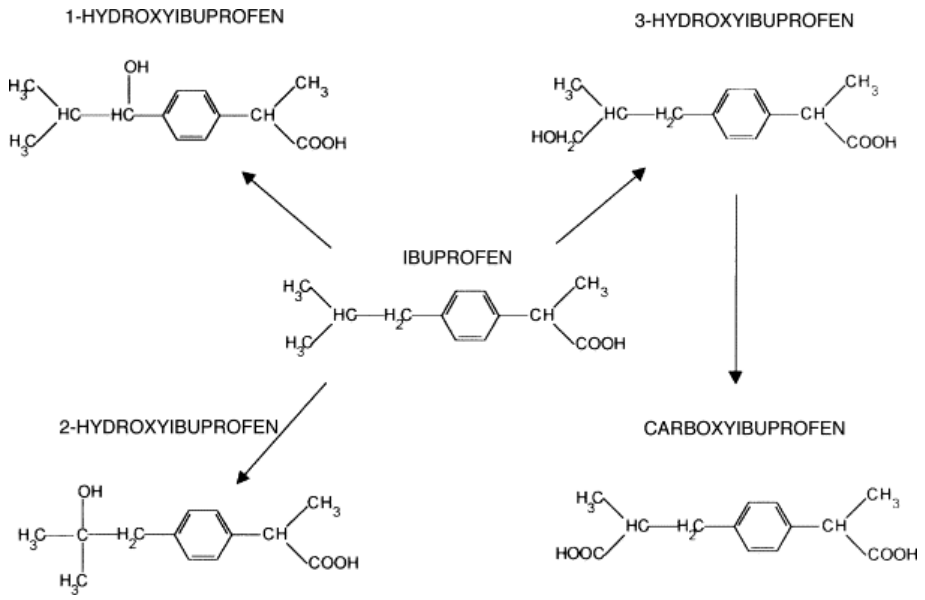
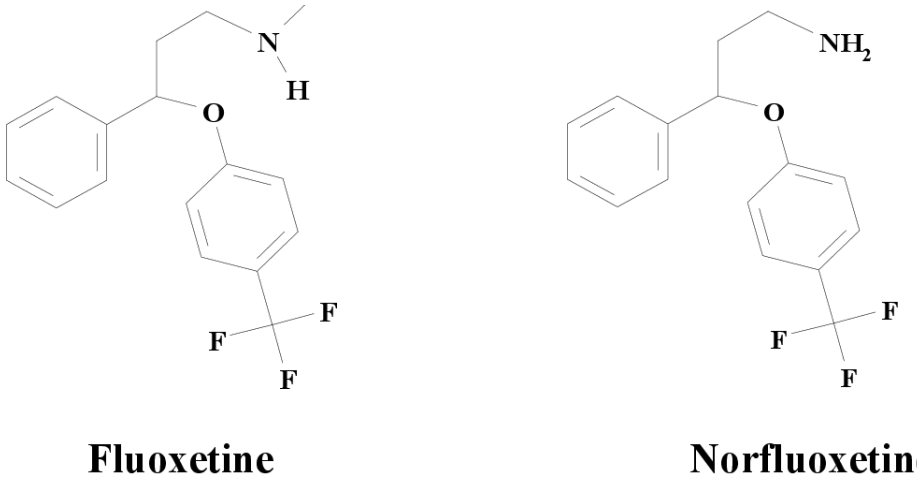


Figure 1.3. Chemical structure of IBU and their metabolites (De Oliveira et al., 2005).

1.5.2 Fluoxetine

FLX ($C_{17}H_{18}F_3NO$) a selective serotonin reuptake inhibitor (SSRI) is an antidepressant drugs widely prescribed for depressive and anxiety disorders worldwide (Runfola et al., 2018). Norfluoxetine (Figure 1.4) is the main metabolite in the metabolic pathway of FLX detected in surface waters (Whitlock et al., 2019; Över et al., 2020; Deodhar et al., 2021). Studies have shown that FLX and its metabolite are recalcitrant contaminants, due to their structure and properties, resisting various removal methods including the basic treatments of WWTPs (Ferrando et al., 2012; Khan & Murphy, 2021). Their persistence in the environment makes FLX an ecotoxicological concern when it ends up in the effluents of sewage plants and subsequently in surface waters (Moreira et al., 2015; Khan & Murphy, 2021).



Fluoxetine **Norfluoxetine**

Figure 1.4. Chemical structure of FLX and their metabolite norfluoxetine (Lewis et al., 2007).

1.3 Sources of pharmaceutical contamination to the environment

Pharmaceutical compounds are ubiquitously detected in the environment due to their continuous introduction through multiple pathways, including production and manufacturing waste streams, hospitals, veterinarians, industrial and agricultural discharge, due to human and animal consumption. These compounds are excreted in the form of metabolites or initial compounds via urine or feces into municipal wastewater or the environment. Even waste-water treatment plants (WWTPs) with conventional treatments (primary and secondary), are not designed to eliminate pharmaceuticals, thereby allowing the release of the initial compounds or by-products into the environment (Mezzelani et al., 2018b; OECD, 2019; Sharma et al., 2019). Figure 1.5 illustrates the primary pathways through which pharmaceutical compounds and their metabolites are dispersed into the environment. The majority of these residues end up in wastewater treatment plants, where, they are not entirely removed, they are subsequently released into the environment. This leads to their accumulation and the consequent emergence of detrimental on aquatic ecosystems. Furthermore, when these contaminants enter the food chain, they pose a risk to human health. Therefore, it is necessary to regulate the discharge and spread of these compounds, as well as to implement additional treatment methods in WWTPs to reduce the dispersion of these contaminants into the environment.

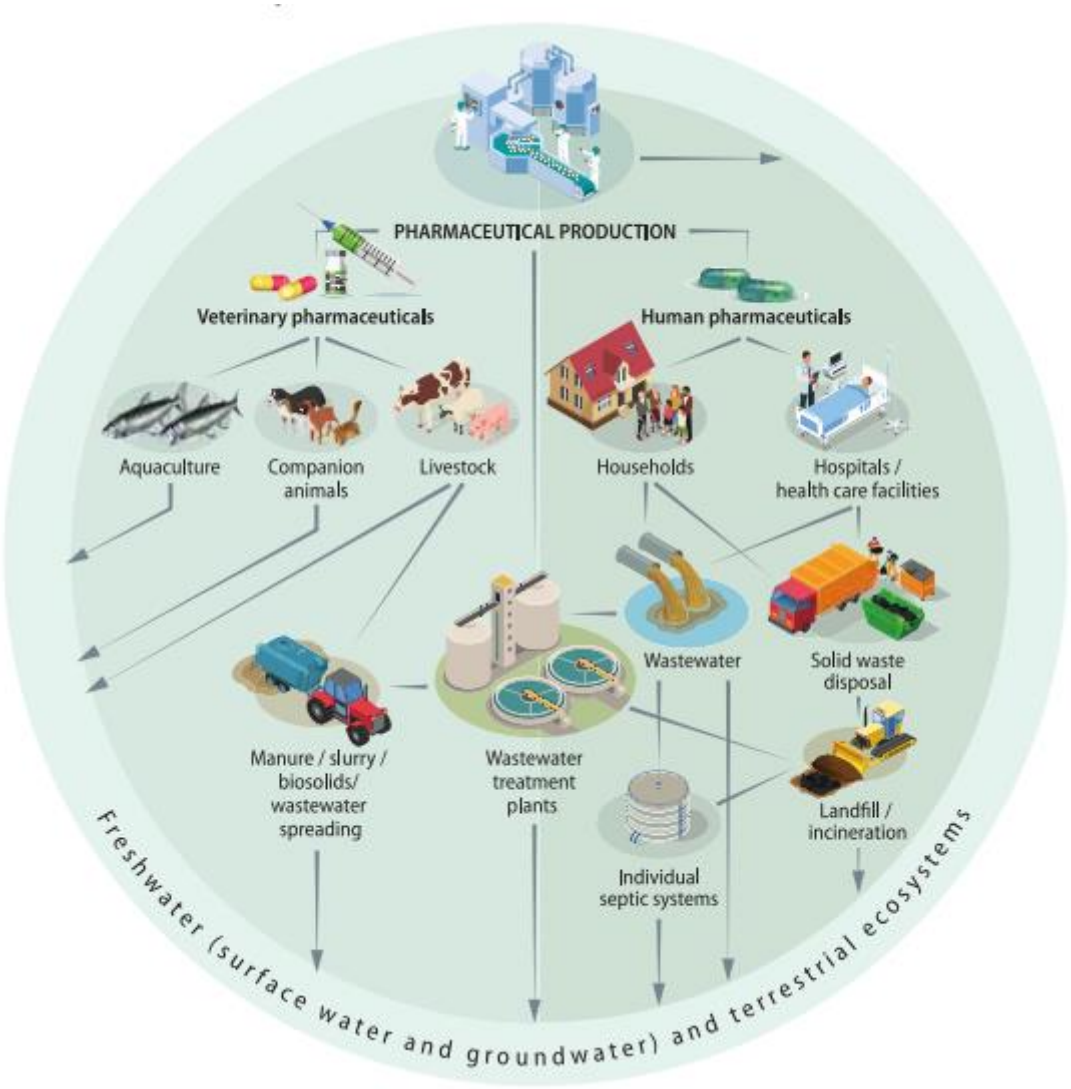


Figure 1.5. Major pathways of release of pharmaceuticals compounds into the environment (OECD, 2019).

1.4 Environmental legislation on pharmaceutical products in the European Union

Although pharmaceutical compounds are considered as emerging contaminants of concern and are consistently detected in wastewater, as indicated by the European Union's Environmental Quality Standards Directive 2013/39/EU (which is still in effect), there are currently no legal requirements for treating effluents contaminated with pharmaceutical compounds such as IBU and FLX. This is due to the assumption that low concentrations found in drinking water do not pose a threat to human health. Consequently, these measures are not considered a priority (WHO, 2017).

Several studies have evidenced how chronic exposure to low concentrations of these compounds can result in long-term adverse effects on the environment and human health (Schultz et al., 2011; aus der Beek et al., 2016; Mezzelani et al., 2016; Mathias

et al., 2018; Mezzelani et al., 2018a; OECD, 2019). However, the pharmaceutical reform legislation of April 2023 (Document 52023DC0190), which is based on the Strategic Approach to Pharmaceutical in the Environment, derived from Directive 2013/39/EU, now mandates that all pharmaceutical companies in the European Union (EU) conduct an environmental risk assessment throughout the entire life-cycle of marketed medicinal products, from production to disposal. Directive 2013/39/EU maintains a watch list to which substances are periodically added based on the collected information. In the current EU proposal (Document 52022PC0540) to amend the latest directive (Decision 2022/1307/EU), the addition of 23 individual substances to the list of priority substances for surface waters is proposed, and among these substances, IBU is included. The inclusion of IBU in the priority list signifies a positive step toward increased monitoring and control of these contaminants.

1.5 Environmental fate of pharmaceuticals

The ecotoxicological implications of these emerging contaminants represent a relatively new and constantly evolving research field (OECD, 2019; Samal et al., 2022). The continuous discharge of these compounds, even at low concentrations ranging, from ng L^{-1} to µg L^{-1}, they can affect the environment and the health of aquatic and terrestrial organisms, leading to adverse effects. This is because pharmaceuticals are designed to be biologically active at very low doses (Zhang et al., 2013; Mezzelani et al., 2018b). Their accumulation in the environment can directly or indirectly affect human health through the ingestion of contaminated food or drinking water, and the health consequences of this prolonged exposure are not yet fully understood (Patneedi & Durga, 2015; Koopaei & Abdollahi, 2017; Keerthanan et al., 2021). Their presence can also lead to the spread of genes and organisms resistant to antibiotics or drugs (Shi et al., 2020), as well as causing fundamental behavioral changes for survival and reproduction in aquatic and terrestrial organisms (Brodin et al., 2014; Patel et al., 2019; González Peña et al., 2021).

Several authors have studied the exposure of organisms to different drugs such as antidepressants and NSAIDs, like FLX and IBU. In the case of IBU, it has been shown that in the mussel *Mytilus galloprovincialis*, it can bioaccumulate, induce genotoxic effects, modulate lipid metabolism, alter immunological parameters, and affect cellular turnover when exposed for 60 days at a concentration of 2,5 µg L^{-1} (Mezzelani et al., 2018a). Another study, demostrated alterations in embryonic development and

teratogenic effects in oocytes and embryos of common carp *Cyprinus carpio* when were exposed to concentrations between 1,5 μg L^{-1} and 11,5 μg L^{-1} (Gutiérrez et al., 2020). Furthermore, after 42 days of exposure to 2 μg kg^{-1} and 200 μg kg^{-1} of IBU in the freshwater trout *Oncorhynchus mykiss*, it induced oxidative stress, disruptions in organism homeostasis, potentiated inflammation, and caused gut dysbiosis (Hodkovicova et al., 2022). In the presence of FLX at concentrations ranging from 5 to 40 ng L^{-1} , oxidative stress was induced in *Danio rerio* (zebrafish) embryos, affecting their normal development and leading to their death (Orozco et al., 2022). FLX also reduced predatory behavior in *Carassius carassius* by decreasing reaction distance and feeding rate when exposed for 4 weeks to 360 ng L^{-1} of FLX (Grzesiuk et al., 2023). Additionally, at concentrations of 61 and 352 ng L^{-1}, FLX altered anxiety-related behaviors in *Gambusia holbrooki* in a non-monotonic, sex-specific manner after 28 days of exposure (Martin et al., 2019).

These studies demonstrate that at environmentally relevant concentrations, IBU and FLX have effects on the behavior, physiology, survival, and cellular and genetic material of aquatic wildlife. An important aspect to consider are the toxicological risks associated with the metabolites of these drugs. For example, norfluoxetine, the main active metabolite of FLX, has a similar level of toxicity to FLX and can accumulate in tissues, posing a greater challenge for elimination than FLX itself (Yan et al., 2023). Similarly, toxic intermediate compounds are generated during IBU metabolism (Zheng et al., 2011; Rácz et al., 2012). Therefore, it is crucial to implement and employ removal methods for IBU and FLX in wastewater effluents to avoid the discharge of both the parent compounds and their metabolites into the environment. Further studies are required to fully comprehend the impacts of these emerging contaminants and their metabolites on ecosystems and to develop effective mitigation strategies.

1.6 Bioremediation

The toxicity and persistence of IBU and FLX in the environment, threaten water quality and ecological health. Since these compounds continuously enter the environment through the effluents of wastewater treatment plants, the development of improved wastewater treatment solutions is an important step to reduce the environmental impacts. Innovative, cost-effective, and sustainable approaches are essential to ensure the complete removal of these pharmaceuticals and their

metabolites from wastewater, which will help to prevent the discharge of these compounds into the environment.

Bioremediation techniques have gained attention for the removal of pharmaceutical compounds in WWTPs, primarily due to their ability to produce non-toxic byproducts alongside to their impressive removal capacity (Show et al., 2022). These techniques employ biological systems to degrade toxic compounds. Certain microorganisms such as bacteria, algae and fungi possess the metabolic capacity to transforms complex chemicals into simpler ones (metabolites) or achieve complete degradation, mineralizing the drug to carbon dioxide and water (Nyirenda et al., 2020). It is essential to utilize advanced instrumental methods to identify the metabolic products resulting from degradation (Kosjek et al., 2007). Liquid Chromatography Mass spectrometry (LC-MS) or High-Performance Liquid Chromatography (HPLC) are widely used in the detection of biodegradation of pharmaceutical compounds (Sharma et al., 2019; Khan & Murphy, 2021; Chen et al., 2022a). Cultures enriched with the target contaminants as a carbon source are used to obtain adapted microorganisms with the ability to degrade these compounds (Park & Oh, 2020a, 2020b). Despite the time and complexity required to isolate a microorganism or microbial consortium with the ability to degrade environmentally harmful pollutants like pharmaceutical compounds, biodegradation is one of the most commonly used strategies because its cost-effectiveness, and environmental friendly (Aguilar et al., 2022), especially when compared to other methods that generate harmful by-products requiring treatment and having high operating costs, such as nanofiltration membranes (Maryam et al., 2020), electrochemical techniques (Zhang et al., 2021; Nabgan et al., 2022), bioaccumulation using microalgae (Xiong et al., 2017), among others.

1.6.1 Bioaugmentation and Biostimulation

Although the conventional activated sludge method may not be effective in removing biodegradable organic compounds (Liang et al., 2021), some studies have already indicated that bioaugmentation and biostimulation can enhance the degradation of various compounds, including pharmaceuticals. They have demonstrated their effectiveness in the removal of aromatic hydrocarbons such as pharmaceutical compounds (e.g., IBU and FLX) (Balciunas et al., 2020; Park & Oh, 2020b; Raimondo et al., 2020; Dalecka et al., 2021; Yaman et al., 2021; Fang et al., 2022).

Bioaugmentation and biostimulation have been described as potential strategies to facilitate biodegradation in wastewater treatment. Bioaugmentation involves the introduction of selected microbial inoculants into a biological matrix to enhance degradation rates by strengthening endogenous microbiota (Tyagi et al., 2011; Rodríguez et al., 2014). Biostimulation entails the addition of limiting nutrients (nitrogen, phosphorus, oxygen, or electron donors) to stimulate the activity of endogenous or exogenous microorganisms (Herrero & Stuckey, 2015; Goswami et al., 2018). When combined, both strategies increase the degradative potential of the native microorganisms. However, the effectiveness of these processes can be constrained by several critical factors, such as oxygen and nutrient limitations, the adaptability of the introduced microorganisms to the environment, and microbial competition. Factors like pH, temperature, and organic matter content also exert influence on these strategies (Tyagi et al., 2011; Masy et al., 2016; Goswami et al., 2018; Jiménez et al., 2019) .

Despite the challenges, research indicates that bioaugmentation and biostimulation are capable of removing contaminants. For instance, Muter et al. (2017) demonstrated that bioaugmentation, in conjunction with biostimulation stimulates the nitrification process and degradation of pharmaceuticals detected in wastewater. In another study, bioaugmentation with fungi in biopile systems effectively removed pharmaceuticals from wastewater (Llorens et al., 2018). An alternative approach to overcome the limiting factors of bioaugmentation with microorganisms is bioaugmentation with plasmids or genes encoding specific catabolic pathways. With the aim to stimulate horizontal genetic transfer between exogenous donor cells and the native microbial community, thereby enhancing their capacity to degrade emerging contaminants such as the pharmaceuticals IBU and FLX (Morales et al., 2023). Several articles have reported the application of this potential strategy (Ikuma & Gunsch, 2012; Garbisu et al., 2017; Aguilar et al., 2021; Varner et al., 2022).

1.6.2 Fluoxetine and Ibuprofen biodegradation

Several studies have described isolated bacterial strains or microbial consortia that efficiently remove pharmaceuticals through biodegradation (De Gusseme et al., 2011; Zhang et al., 2013; Aissaoui et al., 2017; Yang et al., 2021). For instance, Palma & Costa, (2021) isolated a microbial consortium from the anaerobic sludge of Faro East WWTPs with the ability to remove up to 66% of FLX from an initial concentration of

50 mg L^{-1} after 31 days. In another study, a heterotrophic consortium demonstrated the ability to biodegrade FLX up to 85% with an initial concentration of 10 µg L^{-1} in 24 days (Velázquez & Nacheva, 2017). Moreira et al. (2014), showed how the bacterium *Labrys portucalensis* was able to degrade a concentration of 2 µM of FLX after 30 days through enantioselective biodegradation. In the case of IBU, several studies demonstrate the degradative potential of bacteria, consortia, and fungi (Marco et al., 2009; Marchlewicz et al., 2017a; Marchlewicz et al., 2017b; Rutere et al., 2020). For example, a bacterial community composed of *Comamonas aquatica* and *Bacillus sp.* was able to degrade 100 mg L^{-1} of ibuprofen in 33 h (Fortunato et al., 2016). The article by Xu et al. (2018) describes how the IBU-degrading bacterium *Serratia marcescens,* isolated from activated sludge, was able to increase IBU removal by up to 44% when applied in a pilot system of aerated biological filter. Another study shows that *Bacillus siamensis*, a bacterium isolated from untreated wastewater, was able to degrade a concentration of 20 mg L^{-1} IBU in 24 h (Chopra & Kumar, 2022). The isolation of bacteria capable of biodegrading pharmaceutical contaminants displays great potential for the development of complementary strategies to conventional treatments in WWTPs, aimed at improving the removal of these compounds, such as bacterial bioaugmentation. To achieve this, it is essential to better understand the pathways and mechanisms involved in the transformation of IBU and FLX.

1.6.1.1 Biodegradation pathways and pharmaceuticals degrading genes

Despite IBU and FLX being recalcitrant pharmaceutical compounds in the environment due to their chemical structure, characterized by an aromatic ring formed by six carbon atoms bonded by π bonds, this ring imparts stability to them and makes them naturally resistant to degradation (Jan & Cruz, 2023). This explains the difficulties they encounter in biodegrading in the environment or in WWTPs. However, there are microorganisms capable of using these compounds as a source of carbon and transforming them into more biodegradable and less toxic compounds. The metabolic pathways for these compounds can vary depending on the microorganism, for this reason, these pathways are continuously under study. In the case of IBU, several studies have described various metabolic pathways (Murdoch & Hay, 2005, 2013, 2015; Marchlewicz et al., 2017a; Salgado et al., 2020). Figure 1.6 provides a summary of the main metabolic pathways.

Hydroxylation enzymes are crucial during the degradation of IBU, especially in the initial degradation steps. Whether it's through direct hydroxylation on its side chains, the aromatic ring, or even binding to coenzyme A (CoA) (Murdoch & Hay, 2013, 2015; Marchlewicz et al., 2017a). The first complete degradation pathway of IBU in the presence of *Sphingomonas sp.* strain Ibu-2, was reported by Murdoch & Hay, (2013). In this pathway, the initial step begins with the enzyme CoA ligase encoded by the gene *ipfF*, where a coenzyme A (CoA) binds to IBU to form ibuprofen-CoA, followed by hydroxylation by a dioxygenase enzyme encoded by *ipfABHI* and then deacetylated, encoded by *ipfDE* to produce p-isobutylcatechol and propionyl CoA (Aulestia et al., 2022). Another degradation pathway described by Marchlewicz et al. (2017a) was followed by the bacterium *Bacillus thuringiensis* B1. In this pathway, the first step involves the hydroxylation of IBU to form either 2-hydroxyibuprofen or 1-hydroxyibuprofen, which is carried out by an aliphatic monooxygenase enzyme. These compounds are then transformed into 2-(4-hydroxyphenyl)-propionic acid, followed by acyl-CoA synthase activity forming 1,4-hydroquinone. Subsequently, it is converted into 2-hydroxy-1,4-quinol through the hydroxylation action of the hydroquinone monooxygenase enzyme. Finally, the enzyme hydroxyquinol 1,2-dioxygenase performs an intradiol cleavage, producing 3-hydroxy cis,cis-muconic acid.

In degradation pathway of the bacterium *Variovorax* Ibu-1, IBU is converted into trihydroxyibuprofen through hydroxylation of the aromatic ring, leading to the destabilization of IBU by meta cleavage pathway (Murdoch & Hay, 2015). Aulestia et al. (2022) expanded upon the metabolic pathway originally described by Murdoch & Hay, (2013), wherein they included the lower part of the IBU degradation pathway, encompassing the enzymes and encoding genes (*ipfPONSQT*). For this reason, in this study, it was decided to utilize this degradation route as a reference point for identifying potential catabolic genes and enzymes associated with IBU biodegradation.

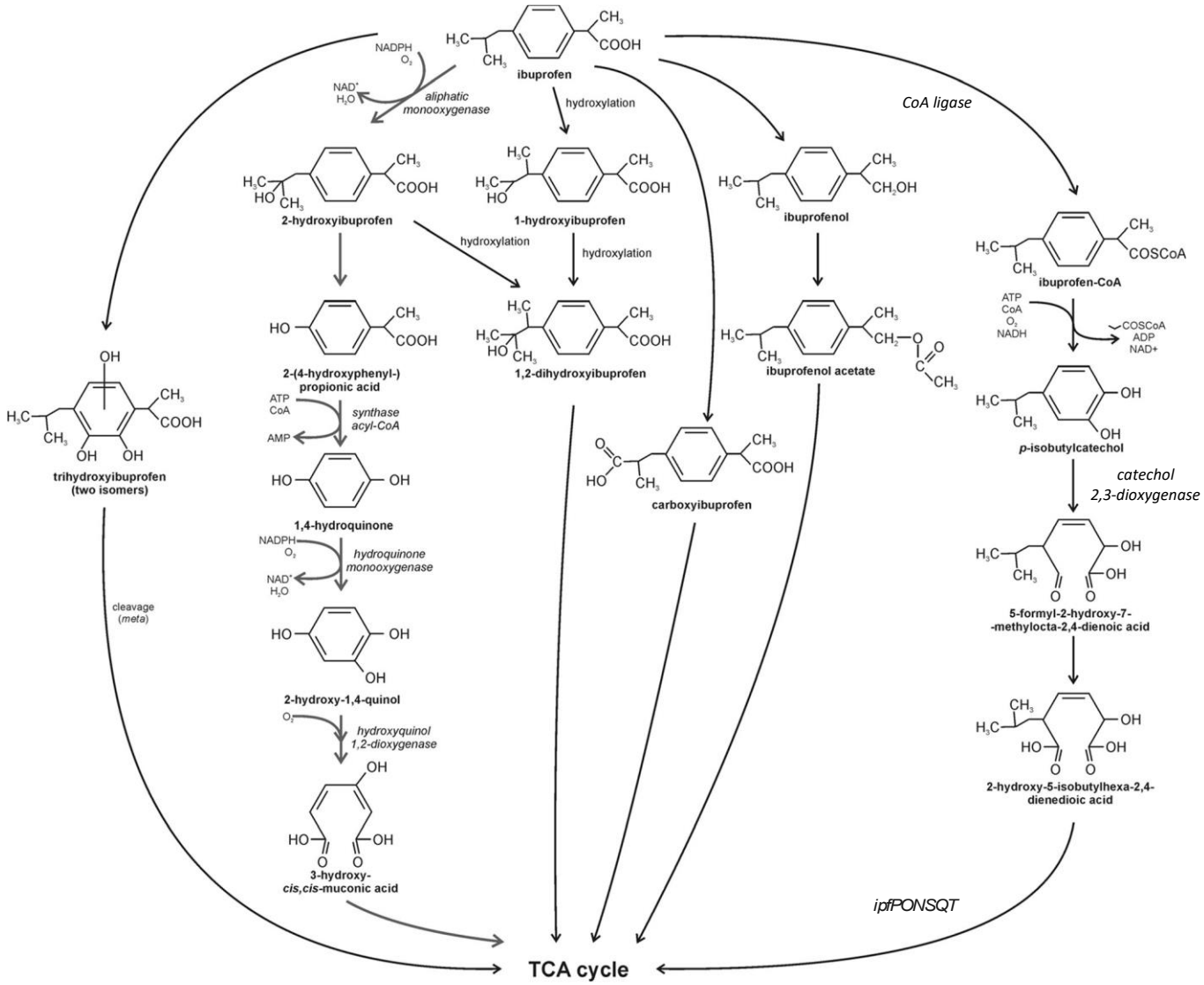


Figure 1.6. Summary of IBU biodegradation pathways, adapted from Żur et al. (2018).

On the other hand, several studies have described FLX degradation pathways using various methods (Patel et al., 2019; Chen et al., 2022b; Lin et al., 2022). However, the degradation pathway described by Khan & Murphy, (2021) represents the first instance in which a group of common environmental bacteria has been shown to transform this compound (Figure 1.7). Initially, the ether bond of FLX is hydrolyzed and converted into 4-(trifluoromethyl)phenol (TFMP) and 3-(methylamino)-1-phenylpropan-1-ol. Both compounds were found to be degraded by bacteria, although TFMP proved to be more persistent and accumulated in the environment. Continuing

along the degradation pathway, this article suggests that TFMP is transformed into 4-(trifluoromethyl)catechol through a meta-cleavage pathway, resulting in the production of trifluoroacetic acid, a recalcitrant product, and fluoride ions, which are photosensitive. This article also performed an *in silico* comparison of the fundamental *btf* cluster in the degradation pathway of benzotrifluoride and isopropylbenzene, where only the *BtfC* gene coding for 2,3-dioxygenase showed similarities with the sequences of the FLX-degrading bacteria of this work.

This pathway may serve as a baseline for future works; however, further complementary studies are needed to expand our understanding of FLX degradation pathways, identify intermediate products, and determine the genes responsible for catalyzing these processes. These tools can be used as supplementary measures in wastewater treatments, contributing to the removal of these emerging compounds.

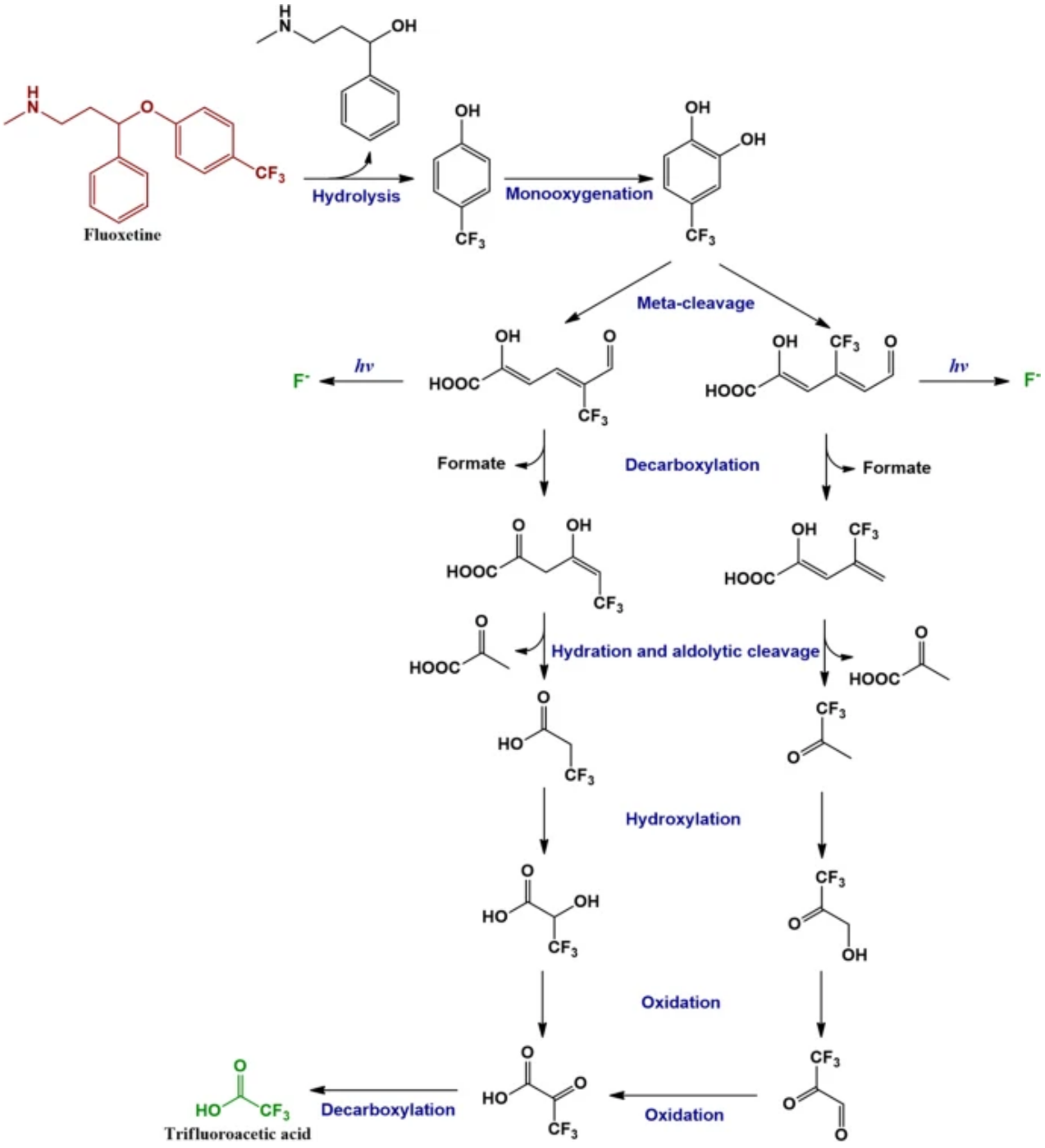


Figure 1.7. FLX biodegradation pathway described by (Khan & Murphy, 2021).

2. Objectives

The main objective of this study is to improve the quality of environmental water contaminated with IBU and FLX. To achieve this goal, the feasibility of developing an effective remediation strategy to enhance the removal of wastewater treatments has been assessed. This strategy involves using bacterial strains isolated from environmental sediments affected by recalcitrant pollutants, which can degrade IBU and FLX and their metabolites as the sole source of carbon and energy. The pharmaceutical compounds under study have been selected because they are among the most frequently detected drugs in wastewater The specified objectives and the work plan are the following:

2.1. Selecting potential degrading bacteria through enrichment cultures in the presence of IBU and FLX, using environmental sediment samples from the Algarve region affected by recalcitrant pollutants such as fuels.

2.2. Isolation of bacterial strains obtained by enrichment culture and selection of pure cultures with the ability to degrade IBU and FLX in plates supplemented with the drugs.

2.3. Confirming the degradative capacity of the isolated bacteria in an aqueous solution and monitoring biodegradation by HPLC detection.

2.4. Taxonomic identification of the degrading bacteria by sequencing of the 16S rRNA gene.

2.5. Sequencing of the whole-genome of the most relevant degrading bacteria, to identify the genes involved in the biodegradation pathway.

2.6. Comparing the whole-genome sequences of the selected strains with proteins codified by genes from IBU-degrading bacteria retrieved from databases and literature with the aim of gaining insight into the mechanism of IBU biodegradation.

3. Materials and Methods

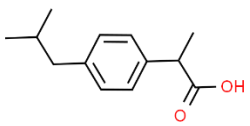
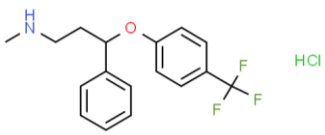
3.1 Materials

3.1.1 Chemicals and Sample Collection

The study drugs IBU (2-(4-Isobutylphenyl) propanoic acid, > 98% purity) and FLX Hydrochloride (N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine, > 90% purity) were provided by Sigma-Aldrich (Algés, Portugal). Some physico-chemical properties of pharmaceutical compounds are presented in Table 3.1.

Environmental samples used in this work to obtain drug-degrading microorganisms were collected in August and September of 2022 from different points located in the Algarve area: 1. Tavira, a marine sample located close to a boat fuel stand, 2. Santa Luzia was sampled from an isolated enclosure near the harbor with gasoline sediment, 3. Fuseta, a marine sample collected in a maritime port next to the municipal market, and 4. Santa Catarina da Fonte do Bispo, an olive mill wastewater and sediment. Samples were stored in plastic bottles at room temperature until the enrichment culture was performed.

Table 3.1. Physico-chemical properties of the pharmaceutical compounds used in this work (Kim et al., 2023).

Properties	**Pharmaceuticals**	
	IBU	**FLX Hydro**
Molecular formula	$C_{13}H_{18}O_2$	$C_{17}H_{19}ClF_3NO$
IUPAC name	2-(4-Isobutylphenyl) propanoic acid	N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine
CAS #	15687-27-1	56296-78-7
Water solubility	21 mg L^{-1}	14 mg $^{L-}$
Molecular weight	206,28	34,8
Structure		

3.1.2 Bacterial strains

Degrading bacteria were isolated using enrichment cultures. To assess the biodegradation ability of IBU and FLX, in addition to the bacteria isolated in this work, two strains previously isolated in the environmental technologies laboratory (ECOREACH group) of the Center for Marine Sciences (CCMAR) in the University of Algarve, where this work was carried out, were used: *Micrococcus yunnanensis* strain TJPT4 isolated from consortia recovered from the marine organism *Filograna implexa* that was able to degrade paracetamol, fluoxetine and 17α-Ethinylestradiol (Palma et al., 2022), and *Mycolicibacterium aubagnense* strain HPB1.1 acquired during the PROBIOMA project from Poderosa mines with the ability to degrade paracetamol and hydroquinone (Ismail, 2022). For future studies, the isolated bacteria were stored in cryovials at -20°C and -80°C in the presence of 40% glycerol.

3.1.3 Culture medium

Luria Broth (LB Broth) medium (Nzytech, Portugal) used for the cultivation of bacterial strains, is composed of the following components (diluted per liter): Tryptone 10g, yeast extract 5g, and NaCl 10g, with pH adjusted to 7. Marine Broth (MB) medium (PanReac, Spain) was utilized to grow *M. yunnanensis* TJPT4, it is composed of the following components (g L^{-1}): H_3BO_3 0,022, NH_4NO_3 0,0016, $CaCl_2$ 1,8, $SrCl_2$ 0,034, yeast extract 1, $FeC_6H_4O_7$ 0,1, $MgCl_2$ 8.8, bacteriological peptone 5, KBr 0,08, KCl 0,55, NaCl 19,4, NaF 0,0024, $NaHCO_3$ 0,16, Na_2HPO_4 0,008, Na_2SiO_3 0,004 and Na_2SO_4 3,24. At a pH between 7,6 to 0,2. Finally, mineral salt medium (MSM) described by Zhang et al., (2013) used to conduct the enrichment culture and biodegration assays. The modified MSM composition per liter of deionized water is as follows: 500 mg KH_2PO_4, 500 mg K_2HPO_4, 1000 mg $(NH_4)_2HPO_4$, 10 mg NaCl, 200 mg $MgCl_2$-$6H_2O$, 20 mg $CaCl_2$, 0,339 mg $MnSO_4$, 0,428 mg $ZnSO_4$, 0,347 mg $(NH_4)_6Mo_7O_{24}$-$4H_2O$), 0,4 mg $CoCl_2$-$6H_2O$ and 15 mg EDTA. With pH between 7,2 to 7,4. After autoclaving at 121 °C for 20 min 10 mg L^{-1} of sterile $MgSO_4$ was added.

3.1.4 Ibuprofen and fluoxetine enrichment cultures and isolation of degrading bacterial strains

IBU and FLX degrading-bacteria were obtained using enrichment cultures from environmental sediment samples and olive mill wastewater sediment. In 250mL Erlenmeyer flask, 6g of each individual sample were mixed with 60mL of MSM and

100 mg L^{-1} of drug, IBU or FLX as the sole carbon and energy source. Cultures were incubated with orbital shaking at room temperature. A total of three successive enrichments were performed by re-inoculating 10% (v/v) of the volume of the previous culture, at 6 and 21 days.

After first and second enrichment, 100 µL of the culture were plated on MSM agar medium supplemented with 50 mg L^{-1} of FLX or 20 mg L^{-1} of IBU. The grown bacterial colonies were then plated with LB medium to distinguish morphological differences until isolation was achieved. Isolated bacteria were labeled with codes according to isolation place and contaminant.

3.2 Inoculum preparation for biodegradation experiments

For bacterial growth, a colony was incubated in sterile flasks with 25g L^{-1} of LB medium, in the case of *M. aubagnense* HPB1.1 10 mL.L^{-1} of 40% Glycerol was added to the LB medium. 40g L^{-1} of MB medium was used exclusively for the growth of *M. yunnanensis* TJPT4 (Palma et al., 2022). The bacterial cultures were incubated overnight at 30 °C on an orbital shaker at 160 rpm.

Bacterial cultures were centrifuged (4000 rpm, 10 mins). The total amount of pellet was washed with MSM to ensure that no traces of LB or MB medium remained. Bacterial growth was monitored by the optical density (OD) at 600nm at the beginning of the assay in a spectrophotometer Hach Lange DR 2800. Each flask contained the bacterial inoculum (OD_{600} = 1).

3.3 Biodegradation experiments in solution

Biodegradation assays were carried out by inoculating 250 mL sterile Erlenmeyer flasks in triplicate with 50 mL of MSM medium supplemented with IBU or FLX as the sole carbon source at an initial concentration of 5 mg L^{-1}. Flasks without bacterial inoculation were used as a negative control to observe potential abiotic degradation. Inoculated flasks (OD_{600} = 1) were agitated at 160 rpm and 30 °C. For analysis, 2 mL of the samples were centrifuged, and the supernatant was filtered using 0,22 µm PES sterile syringe filters (VWR, China). To ensure that the drug did not remain on the filter, 1 mL was discarded. The filtrated samples were stored at 4 °C in 1,5 mL vials until HPLC analysis. The degradation percentage was obtained by considering the

negative control and the residual concentration at each time point during the assay, using the following equation:

$$\textbf{Degradation}\ \% = 100 - \left(\frac{Residual\ concentration \times 100}{Negative\ control}\right)$$

3.4 Pharmaceuticals analytical methods

The concentration of IBU and FLX in the samples was quantified using an Ultra High Performance Liquid Chromatography Nexera system (SHIMADZU), equipped with a XBridgeTM C18 5µm, 4.6 x 250 mm column (Waters, USA). For the IBU samples, the mobile phase composition was Methanol:Water (80:20 v/v), while for the FLX samples it was Acetonitrile:Water (60:40 v/v), both adjusted at pH ~ 3 with orthophosphoric acid 85%, using an isocratic method. The flow rate was set at 1.3 mL min^{-1}, with an injection volume of 200 µL. The total run time was 6,5 mins for IBU and 5 mins for FLX. Two metabolites of IBU: carboxyibuprofen and 2-hydroxyibuprofen, were analyzed using the same method as IBU. The detection wavelength (ʎ) was 204 nm for IBU and 190 nm for FLX, for the IBU metabolites it was 224nm. The sample retention time for the samples were approximately 4,89 mins for IBU and 3,98 mins for FLX, for the IBU metabolites was 3,33 and 3,27 mins, respectively. The concentration of IBU and FLX in the samples was determined by calculating the slope of the calibrations curves, which were prepared using five concentrations from 5 to 1 mg L^{-1} in volumetric flasks. The calibration curves were prepared from the stock solution in MSM with 5 mg L^{-1} for both IBU and FLX, which were used for the biodegradation tests. Mili-Q water was used as the diluent.

3.5 Biodegradation Kinetics Modelling

The biodegradation curves of IBU and FLX were analyzed and fitted to the best kinetic model using an Excel spreadsheet developed by the FOCUS working group (FOCUS, 2006), that provides a guideline for calculating degradation kinetic rates of environmental samples. The Solver tool and a statistical package from Microsoft, was utilized for this purpose.

The biodegradation curves were fitted to the most appropriate model using the parameters recommended by the FOCUS group and employing the least squares method (FOCUS, 2006). Two kinetic models were employed: Single first-order

kinetics (SFO) and First-Order Multi-Compartment mode (FOMC). The equations used for each model were as follows:

$$\text{(SFO):} \quad M = M_0 e^{-kt} \;\; ; \;\; DT_{50} = {Ln2}/{k} \;\; ; \;\; DT_{90} = {Ln10}/{k}$$

$$\text{(FOMC):} \quad M = {M_0}/{\left({t}/{\beta} + 1\right)^{\alpha}} \;\; ; \;\; DT_{50} = \beta\left(2^{1/\alpha} - 1\right) \;\; ; \;\; DT_{90} = \beta\left(10^{1/\alpha} - 1\right)$$

In the equations, M and M_0 represents the concentration of IBU or FLX (mg L^{-1}) at time t and the initial concentration, respectively. The rate constant of degradation is denoted k (day^{-1}). In the FOMC model, β represents the location parameter, and α is the shape parameter, which is determined by the coefficient of variation of the k values. The DT_{50} and DT_{90} reflects the time required by the bacteria to degrade 50% and 90%, respectively, of the initial contaminating drug. The Chi-square (X^2) test is used as a tool to compare the goodness of fit of kinetic models by assessing the deviations between observed and calculated values. A suitable model should pass the significance level equal to or less than 5%.

3.6 16S rRNA sequencing and taxonomic classification

To characterize the degradative bacterial strains, DNA extraction was carried out from each bacterium culture in LB liquid medium using the Nzytech microbial gDNA isolation kit (Nzytech, Portugal). The DNA concentration was quantified by an ultratrace UV spectrophotometer Nanodrop 1000 (Thermo Scientific). To amplify the 16S rRNA gene, PCR was performed with 0,5 µL of the universal prokaryotic primers: Primer forward, 8F (5′- AGA GTT TGATCC TGG CTC AG -3′) (Weisburg et al., 1991) and Primer reverse, 1492R (5′- GGT TAC CTT GTTACG ACT T-3′) (Lane, 1991). A total of 10 µL of Supreme NZYTaq 2x Green Master Mix (Nzytech, Portugal), 7 µL of H_2O Milli-Q and 2 µL of DNA at a concentration of 5 - 50 ng μL^{-1} were used in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA). The amplification was performed according to the following program: an initial denaturation at 95°C for 5 min followed by 35 cycles of: 94°C for 30s, 57°C for 30s, 72°C for 90s and a final step at 72°C for 7 min.

The amplified PCR product was analyzed by electrophoresis on a 1% agarose gel in 1x TAE buffer (AMRESCO, Solon, USA). Standard Ladder VII (NZYTech, Portugal) was used as a marker. To stain the DNA, 2,5 µL (50 µL L^{-1}) of SYBR Safe Premium

(Invitrogen, USA) was added to the gels containing the samples which were run at 50V (5V per cm gel length). Subsequently, the amplified PCR product was sequenced by the sanger method (Sanger et al., 1977) in both directions using the Genetic Analyzer Model 3130xl capillary electrophoresis sequencing system (Applied Biosystems, Foster City, USA). For sequence editing and assembly, the BioEdit 7.2 software (BioEdit Sequence Alignment Editor) was utilized. The taxonomic classification was performed by comparing the sequences with the NCBI (National Center for Biotechnology Information) database using Blastn program (Zhang et al., 2000; Morgulis et al., 2008). Figure 3.1 represents a graphical abstract of the methodologies employed in this work.

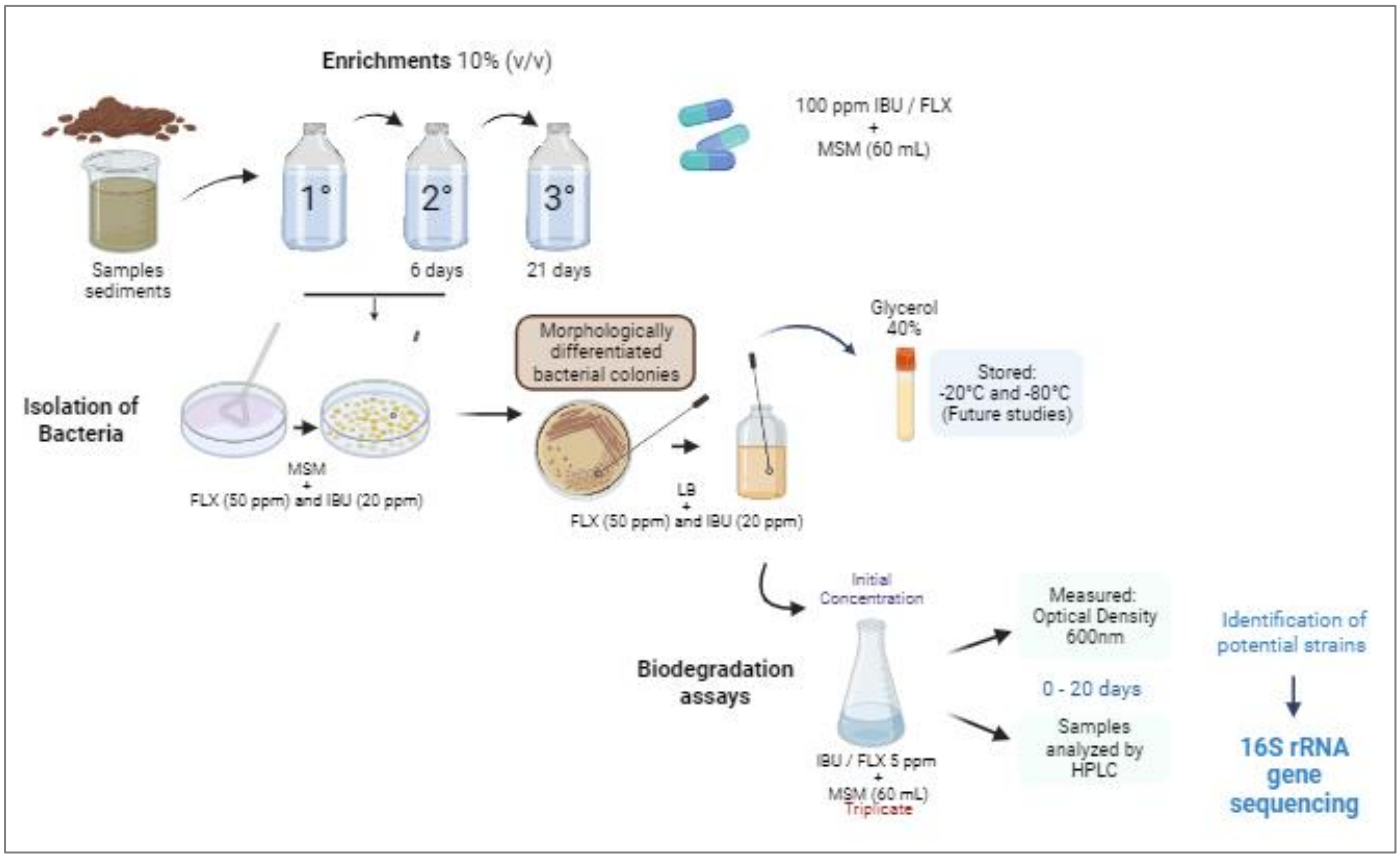


Figure 3.1. Graphical representation of the isolation process of ibuprofen (IBU) and fluoxetine (FLX) degrading bacterial strains.

3.7 Bacteria growth curves and enumeration of colonies

For bacteria that exhibited a percentage of biodegradation higher than 30%: *Klebsiella pneumoniae* TIBU2.1, *Klebsiella variicola* LOI1.1, *Pseudomona aeruginosa* LOI1.2 and *M. yunannensis* TJPT4 bacterial growth was monitored, except for the *M. aubagnense* HPB1.1 since the curve performed in a previous work was used. The pre-inoculum of the bacteria, obtained from an overnight culture, was used to inoculate

100 mL in a sterile 250 mL Erlenmeyer of LB medium, and *M. yunannensis* TJPT4 was inoculated in MB medium. Bacterial growth was monitored by measuring the optical density at 600nm (OD_{600nm}) every 2 h until 68h. The initial OD of the cultures was set to 0,1 with agitation at 125 rpm and 30 °C. To calculate the colony forming units (CFU), serial dilutions were performed in LB medium from 10^{-4} to 10^{-11}. Two drops of 20 µL each from each dilution were plated on divided plates containing LB and MB medium, respectively as shown in figure 3.2. After 48h, the number of CFU mL^{-1} was determined. The specific growth rate (µ) was calculated using the slope of at semilogarithmic curve during the exponential phase, and the duplication time (td) was calculated with the equation $td = Ln\ 2/\mu$, where the natural logarithmic of two is divided by the specific growth rate.

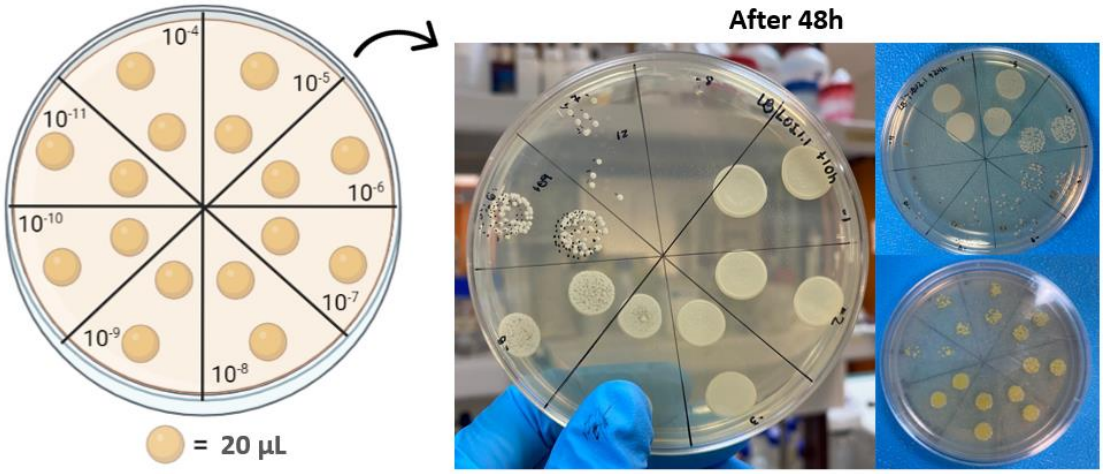


Figure 3.2. Scheme of serial dilutions in plates.

3.8 Genome sequencing and gene annotation

The whole genome of three IBU degradative bacterial strains were sequenced: *K. pneumoniae* TIBU2.1, *M. aubagnense* HPB1.1 and *M. yunannensis* TJPT4. This sequencing aimed to obtain valuable genetic information regarding the genes involved in the IBU biodegradation pathway. Genomic DNA was extracted using a microbial gDNA isolation kit, and it was sent to sequence to Integrated Microbiome Resource company (IMR, Canada) https://imr.bio. The sequencing method used was PacBio Sequel 2, which enables the sequencing of long amplicons or whole genomes up to 240 - 320Gb. For genomic annotation, the Rapid Annotation using Subsystem Technology (RAST) server (Aziz et al., 2008) a bioinformatics tool, was employed. Candidate catabolic genes were identified by comparing the sequences with reference

sequences obtained from a literature search on genes associated with IBU biodegradation. Similarities were analyzed using the NCBI tblastn database program by selecting the mode of aligning sequences (Zhang et al., 2000; Morgulis et al., 2008). The results were then compared with the genomic annotation data obtained from the RAST server.

4. Results and Discussion

4.1 Isolated bacteria by enrichment cultures

Environmental sediment samples were selected to carry out the enrichment cultures because they have been in contact with gasoline (aromatic hydrocarbons) for a long period of time, thus having a structure with some similarity to those of the pharmaceuticals under study, and, therefore, the endogenous microbiota of the sediments could be adapted to their presence (Parajuli et al., 2017), improving the ability of its microbiota to degrade IBU and FLX. Several studies have mentioned that high concentrations in enrichment cultures facilitate the proliferation of degrading strains (Park & Oh, 2020b; Vargas et al., 2023). Therefore, a concentration of 100 mg L^{-1} was selected to isolate strains with the capability to degrade IBU or FLX (Figure 4.1). As explained un the methods section, an aliquot of the first and second enrichment was inoculated on MSM plates containing IBU and FLX and then transferred to plates with LB medium where the bacteria were isolated based on their morphological differences. A total of 20 bacteria were isolated: 11 corresponding to IBU and 9 to FLX (Table 4.1). As shown in Table 4.1, all the bacteria isolated from Tavira, Santa Luzia and Fuseta come from the second enrichment, while the bacteria from the olive sludge were isolated from the first enrichment.

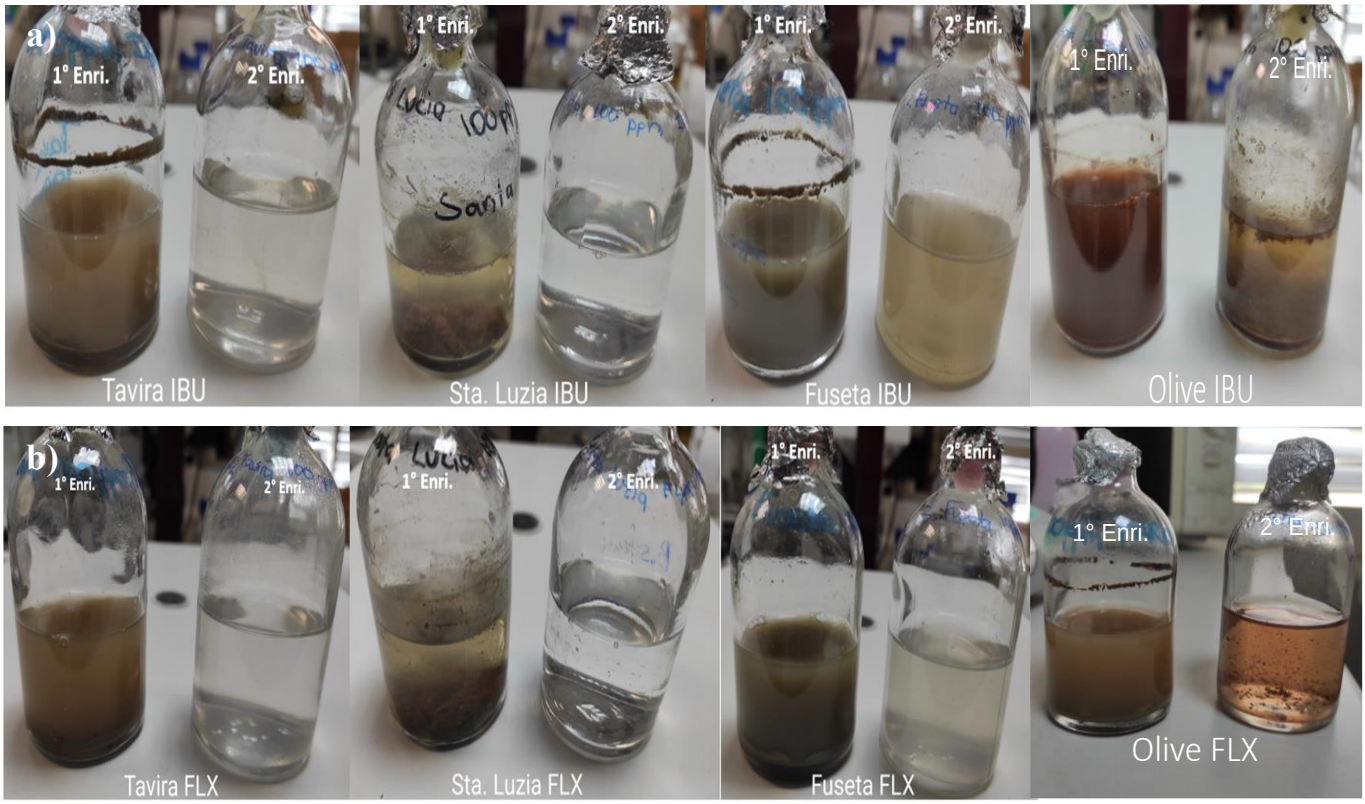


Figure 4.1. First and second enrichment cultures from environmental sediment sample of Tavira, Santa Luzia, Fuseta and Olive mill wastewater sediment in the presence of: a) IBU and b) FLX.

Table 4.1. Table of isolated bacteria with the respective codes

Origin location	Ibuprofen	Fluoxetine
Tavira	TIBU2.1	TFLX2.1
	TIBU2.2	
	TIBU2.3	
	TIBU2.4	
	TIBU2.5	
Sta. Luzia	STIBU2.1	STFLX2.1
	STIBU2.2	STFLX2.2
	STIBU2.3	
Fuseta	FIBU2.1	FFLX2.1
		FFLX2.2
		FFLX2.3
Olive sludge	LOI1.1	LOFLX1.1
	LOI1.2	LOFLX1.2
		LOFLX1.3

4.2 Potential biodegradation of IBU and FLX by the isolated bacteria in an aqueous solution

After the growth of bacteria on MSM plates using IBU or FLX as the sole carbon source, it was decided to proceed with biodegradation assays in an aqueous solution with all the isolated bacteria (Table 4.1) in triplicates including the negative control (without inoculum). In all biodegradation assays, the negative control remained stable, indicating the absence of abiotic factors influencing in biodegradation process. Of the 20 isolated bacteria, only 5 were capable of degrading the studied drugs: TIBU2.1, LOI1.1, LOI1.2, LOFLX1.1 and LOFLX1.3. This fact could be explained due to in liquid media, the carbon source (drug) is more readily accessible to bacteria, whereas in agar plates, it is limited (Bonnet et al., 2020), highlighting the potential disparity between degradation on solid agar plates and in aqueous solution.

Based on the results obtained, it was decided to represent the biodegradation curves only for the bacteria that showed a degradation percentage higher than 15% for the IBU and FLX bacterial isolates (Figures 4.2 to 4.7). These corresponded to the following bacteria: in the case of IBU, TIBU2.1, LOI1.1, LOI1.2, *M. aubagnense* HPB1.1 and *M. yunannensis* TJPT4, and for FLX, LOFLX1.1 and LOFLX1.3. IBU and FLX biodegradation kinetic parameters determined from the kinetic models are shown in Table 4.2. Only strain LOFLX1.3 followed the FOMC kinetic model, while the rest of the bacteria followed the SFO model. According HPLC analysis, TIBU2.1 achieved a complete degradation of the initial IBU concentration (5 mg L^{-1}) after 15 days (Figure 4.3.), coinciding with the lowest DT_{50} (5 days) and the highest degradation rate constant (k) of 0,153. LOI1.2 and LOI1.1 were able to degrade 65,47% ± 1,1 and 59,48% ± 0,1, respectively, of the initial IBU concentration and a longer time was also necessary to reach 50% of the biodegradation (DT50 = 13 and 12 days, for LOI1.2 and LOI1.1, respectively) (Figure 4.4. and 4.5, Table 4.2). Among the bacteria previously isolated, *M. yunannensis* TJPT4 exhibited the highest degradation percentage of 100% ± 0,1 in 14 days with the lowest DT_{50} (4 days). This result was expected considering the prior report by Sharma et al., (2019), which highlighted the bacterium's degradative potential. Through optimized growth conditions, it was able to degrade up to 90.87% of the IBU. Finally, *M. aubagnense* HPB1.1, isolated from a mine sample, displayed the 50% of IBU degradation after 19 days and 60,15% ± 0,4 of IBU degraded at the end of the assay (21 days) (Figure 4.7.,

Table 4.2). It is worth emphasizing that it is the first time that *Mycolicibacterium sp.* is reported as a degrader of IBU. Not only that, but it is also the first time it is been reported as a pharmaceutical degrader. However, articles about *Mycolicibacterium* genus bacteria report their degradative potential for compounds like pyrene (Yang et al., 2021) and fuel oxygenates (Zsilinszky et al., 2022).

In the FLX biodegradation tests, the LOFLX1.3 strain displayed the highest degradation percentage (29,02% ± 0,1) in comparison with the LOFLX1.1 strain (18,59% ± 0,3). However, it also exhibited the longest DT_{50} (more than 3 years), possibly due to a rapid initial degradation followed by slower degradation over the course of 14 days (Figure 4.2).

Table 4.2. Kinetic parameters calculated from IBU and FLX biodegradation in aqueous solution inoculated with TIBU2.1, LOI1.1, LOI1.2, *M. aubagnense* HPB1.1, *M. yunannensis* TJPT4, LOFLX1.1 and LOFLX1.3.

Drug	Strain	Model Kinetic	k (day^{-1})	α (day^{-1})	β (day^{-1})	DT_{50} (days)	DT_{90} (days)	X^2	Extent degraded (%)
IBU	TIBU2.1	SFO	0,153	-	-	4,54	15,07	12,08	100,00
	LOI1.1	SFO	0,059	-	-	11,66	38,74	1,84	59,48
	LOI1.2	SFO	0,053	-	-	13,11	43,56	13,95	65,47
	HPB1.1	SFO	0,036	-	-	19,16	63,66	7,21	60,15
	TJPT4	SFO	0,177	-	-	3,92	13,01	33,24	100,00
FLX	LOFLX1.1	SFO	0,018	-	-	39,26	130,43	4,09	18,59
	LOFLX1.3	FOMC	-	0,051	0,018	12837,71	4,942E+17	1,02	29,02

* X^2 calculated values < X^2 corresponding to the tabulated value ($p < 0.05$).

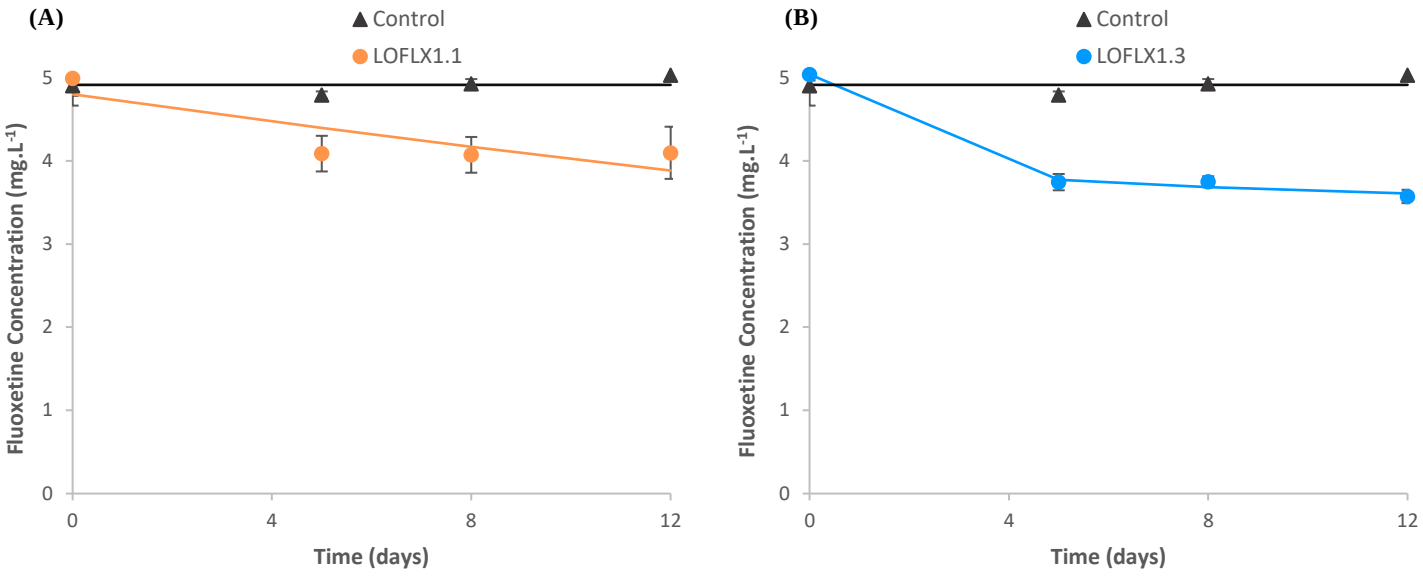


Figure 4.2. FLX concentration (mg L^{-1}) as a function of time (days) in the presence of strains (●) in A) LOFLX1.1 and B) LOFLX1.3, and the negative control (▲). The error bars represent the average deviation for three replicates. When error bars are not visible, it means they are smaller than the data point markers. Solid lines show model fitting to the experimental results (symbols).

In parallel to the studies of IBU biodegradation, the behavior of various metabolites derived from IBU has been studied. To identify these metabolites, two IBU metabolites, carboxyibuprofen and 2-hydroxyibuprofen, were analyzed by HPLC, with retention times of 3,33 and 3,27 mins, respectively. However, the detected metabolites did not correspond to the analyzed metabolites, leading to their designation as "unknown metabolite". For the identification of these metabolites, analyses could be conducted using widely employed methods for metabolite detection such as nuclear magnetic resonance (NMR), HPLC-Mass Spectrometry (HPLC-MS), or gas chromatography mass spectrometry (GC-MS) (Kamal et al., 2022).

Bacterium TIBU2.1 presented two unknown metabolites, one detected at 3.06 mins and the other at 4,22 mins at 224nm, as shown in Figure 4.3. Both metabolites were initially detected on day 7 of the degradation assay when 57% of IBU had already degraded. The areas of both metabolites begin to increase as the parent compound (IBU) is degraded, however, after 12 days the unknown metabolite detected at 4,22 min began to be degraded by the study degrading bacteria (Figure 4.3A). By contrast, the metabolite detected at 3,06 mins continued to increase by day 15, when 100% of IBU had been degraded (Figure 4.3B). This could indicate a possible transformation

of unknown metabolite 4,22 min into unknown metabolite 3,06 min. On the other hand, the bacterium LOI1.1 also exhibited two unknown metabolites, with retention times very similar to those detected in TIBU2.1, therefore could be the same compounds, this can also be confirmed by comparing the absorption spectrum between both strains (*Annex 1*), where the similarities between them can be observed. The first metabolite was detected at 3,09 mins, and the other at 4,26 mins at 224nm (Figure 4.4). After 8 days and 41% of IBU degraded, both metabolites were identified for the first time. The area under both metabolite peaks followed an exponential trend, reaching their maximum point at day 13, when IBU degradation had reached 59%.

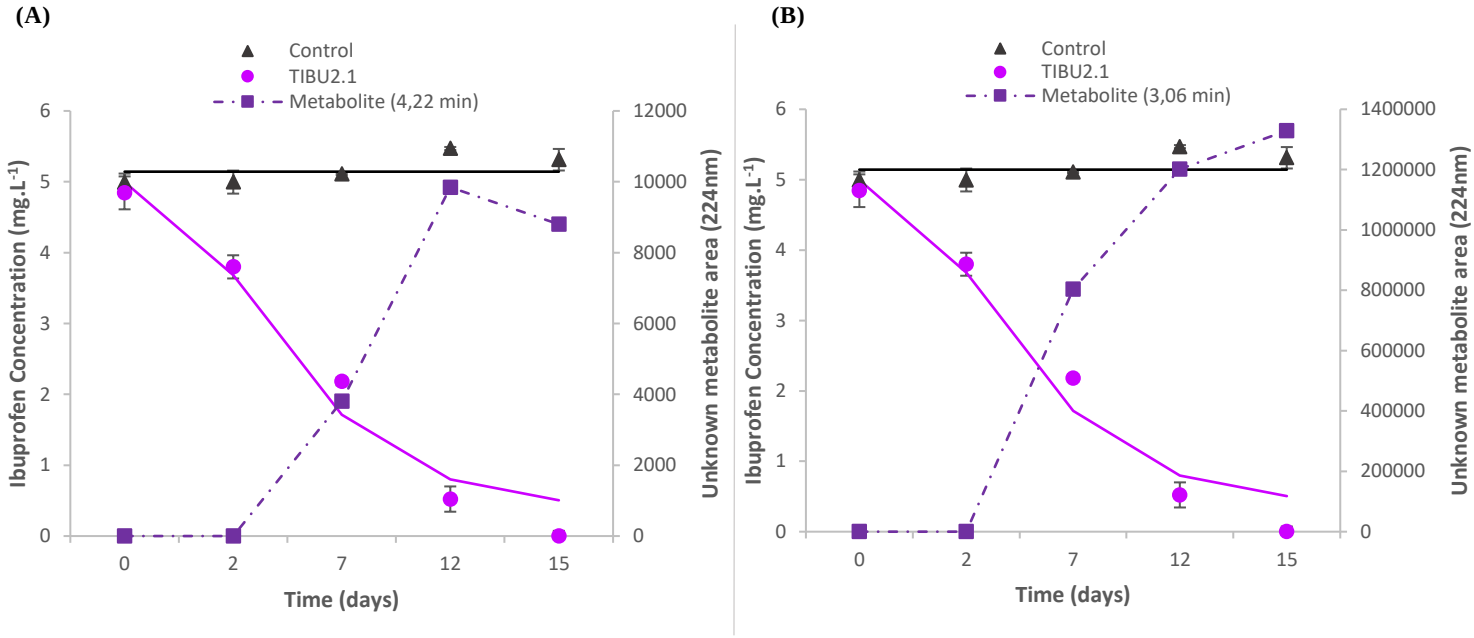


Figure 4.3. IBU concentration (mg L^{-1}) as a function of time (days) in the presence of TIBU2.1 strain (●) and the negative control (▲). On the secondary axis is represented the area at 224nm (■) of the unknown metabolites detected by HPLC, in A) the metabolite detected at 4,22 mins and in B) the metabolite detected at 3,06 mins. The error bars represent the average deviation for three replicates. When error bars are not visible, it means they are smaller than the data point markers. Solid lines show model fitting to the experimental results (symbols).

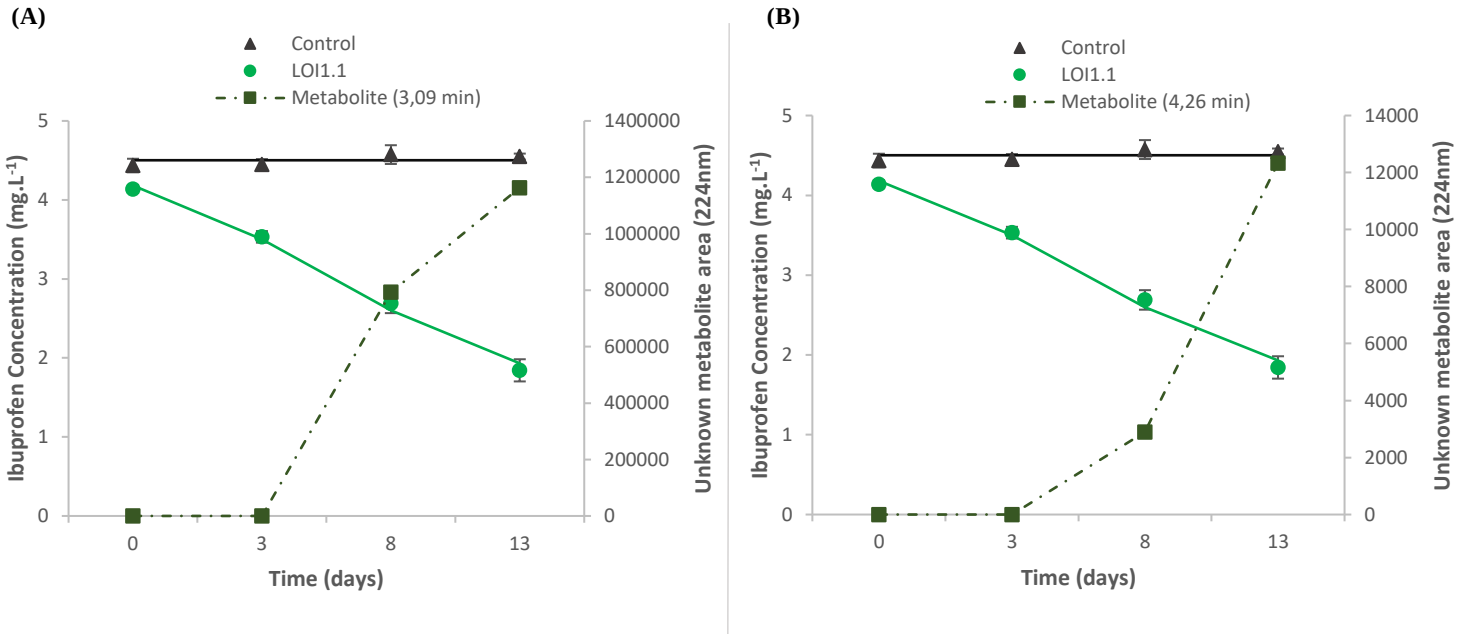


Figure 4.4. IBU concentration (mg L^{-1}) as a function of time (days) in the presence of LOI1.1 strain (●) and the negative control (▲). On the secondary axis is represented the area at 224nm (■) of the unknown metabolites detected by HPLC, in A) the metabolite detected at 3,09 mins and in B) the metabolite detected at 4,26 mins. The error bars represent the average deviation for three replicates. When error bars are not visible, it means they are smaller than the data point markers. Solid lines show model fitting to the experimental results (symbols).

For LOI1.2 and *M. aubagnense* HPB1.1 (Figure 4.5 and 4.6), only one metabolite was detected for each bacterium, both at similar retention times 4,35 and 4,39 mins, respectively. However, when comparing the absorption spectrum between the two metabolites, differences were observed (*Annex 1*). Both metabolites were first identified on day 3, with 4% and 33% of IBU degraded, respectively. In the case of LOI1.2, the metabolite area emerged immediately alongside IBU degradation and remained exponentially steady until reaching its peak on day 13 (65% IBU degraded). Meanwhile, in the HPB1.1 bacterium, the metabolite had already reached its peak on day 3, and then, it began to decrease along with IBU, suggesting simultaneous degradation of both compounds.

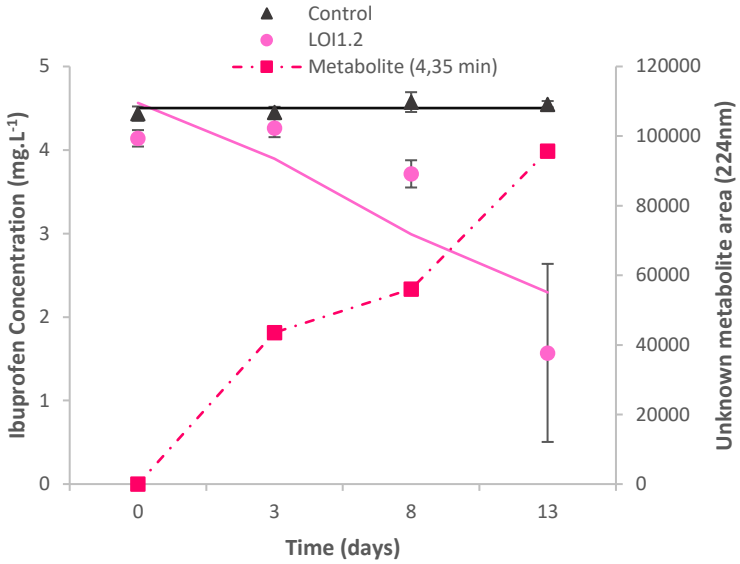


Figure 4.5. IBU concentration (mg L^{-1}) as a function of time (days) in the presence of LOI1.2 strain (●) and the negative control (▲). On the secondary axis is represented the area at 224nm (■) of the unknown metabolite detected by HPLC, at 4,35 mins. The error bars represent the average deviation for three replicates. When error bars are not visible, it means they are smaller than the data point markers. Solid lines show model fitting to the experimental results (symbols).

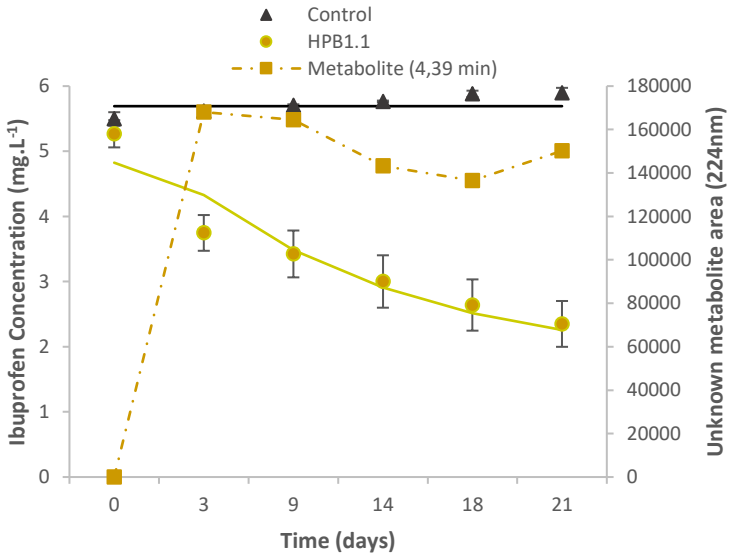


Figure 4.6. IBU concentration (mg L^{-1}) as a function of time (days) in the presence of *M. aubagnense* HPB1.1 strain (●) and the negative control (▲). On the secondary axis is represented the area at 224nm (■) of the unknown metabolite detected by HPLC, at 4.39 mins. The error bars represent the average deviation for three replicates. When error bars are not visible, it means they are smaller than the data point markers. Solid lines show model fitting to the experimental results (symbols).

Finally, two metabolites were detected in the treatment with *M. yunannensis* TJPT4 strain (Figure 4.7), one at 5,21 and another at 3,13 mins. Considering the retention time, the metabolite that appears at 3,13 mins could be the same as in the case of the bacteria TIBU2.1 and LOI1.1, which could be confirmed by the similarities in the absorption spectrum when comparing them. This suggests the potential existence of a relationship among them, possibly even being the same metabolite, and the likelihood of being closely involved in the IBU catabolic pathway. The metabolite detected at 3,13 mins began to be identified starting from day 9 when 94% of IBU had been degraded, and it remained constant until the end of the test. On the other hand, the second metabolite (t_R = 5,21 mins) began to be detected after the complete degradation of IBU (day 14), gradually increasing until reaching its peak on day 21. This implies that the detected compound corresponds to a different compound that emerges once IBU disappears.

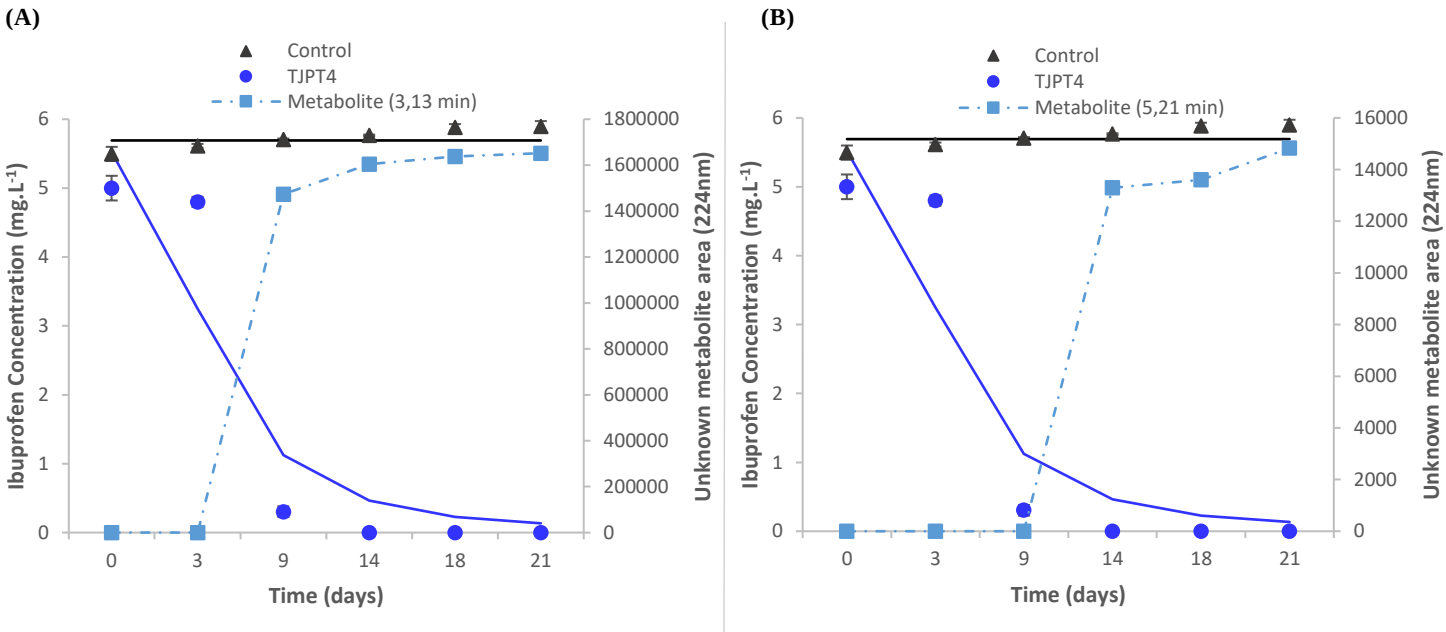


Figure 4.7. IBU concentration (mg L^{-1}) as a function of time (days) in the presence of *M. yunannensis* TJPT4 strain (●) and the negative control (▲). On the secondary axis is represented the area at 224nm (■) of the unknown metabolites detected by HPLC, in A) the metabolite detected at 3,13 mins and in B) the metabolite detected at 5.21 mins. The error bars represent the average deviation for three replicates. When error bars are not visible, it means they are smaller than the data point markers. Solid lines show model fitting to the experimental results (symbols).

4.3 Taxonomic classification of selected IBU degrading bacteria by 16S rRNA gene sequencing

After the biodegradation test of IBU and FLX, it was decided to identify only those bacteria that showed a high potential to degrade the study drugs. The 16S rRNA gene of TIBU2.1, LOI1.1 and LOI1.2 was amplified by PCR. The quality of the PCR results was analyzed using agarose gel electrophoresis (Figure 4.8), where all bacteria exhibited an expected band at 1500 bp (Hassan et al., 2015), except for the Blank control. This fact indicates that the PCR mix was not contaminated and that the samples can be sent for sequencing.

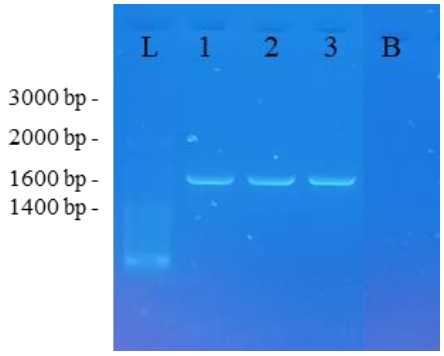


Figure 4.8. PCR products of 16S rRNA genes on 1% agarose gel with 50 µL L^{-1} SYBER Safe imaged after electrophoresis at 100V in 1x TAE buffer at room temperature. L = Standard Ladder VII; 1 – TIBU2.1; 2 – LOI1.1; 3 – LOI1.2; B - Blank.

The Sanger sequencing method was used to sequence the samples. The sequenced data were edited to remove low-quality regions from the chromatograms. Once edited, both forward and reverse sequences were assembled using BioEdit 7.2 software. The sequences in FASTA format (*Annex 2*) were then used to search for phylogenetic similarities using the BLASTn database at NCBI. Among the database results, the species with the highest percentage of identity was selected, with a minimum requirement of 98,7% (Yarza et al., 2014). The results presented in Table 4.3 indicate that two of the isolates belong to the Klebsiella genus, while the remaining one belongs to the Pseudomonas genus. All three isolates exceed the required minimum threshold: TIBU2.1 shows an identity match of 99,71% with *Klebsiella pneumoniae*, LOI1.1 exhibited an identity of 99,46% with *Klebsiella variicola*, and LOI1.2 corresponds to *Pseudomonas aeruginosa* with an identity match of 99,86%.

Table 4.3. Results of the phylogenetic analysis of the isolated bacterial strains capable of IBU degradation.

Isolate	Scientific name	Query cover	E-value	Per Ident.	Accession number
TIBU2.1	*Klebsiella pneumoniae*	100%	0.0	99.71%	NR_037084.1
LOI1.1	*Klebsiella variicola*	100%	0.0	99.46%	NR_025635.1
LOI1.2	*Pseudomonas aeruginosa*	100%	0.0	99.86%	NR_117678.1

To date, the capacity for IBU degradation by bacteria of the *Klebsiella* genus, especially *Klebsiella pneumoniae*, has not been previously reported in the scientific literature, highlighting the importance of this study in the bioremediation field. Despite *K. pneumoniae* is known to be a pathogenic opportunistic bacterium (Venezia et al., 2017), its potential in biotechnology has also been demonstrated. For example, it has been used in the production of chemical compounds such as 1,3-propanediol and 2,3-butanediol through fermentation (Oh et al., 2018; Mitrea & Vodnar, 2019) as well as in the bioremediation of organic compounds like phenols (Mahgoub et al., 2023), antibiotics such as tetracycline (Yin et al., 2020), and NSAID drugs like diclofenac, it achieved a degradation of 79% in 72 h from an initial concentration of 10 mg L^{-1} (Sharma et al., 2021).

Other species of the *Klebsiella* genus have demonstrated their ability to degrade toxic compounds such as nitrobenzene (Ren et al., 2015) and drugs like diclofenac, degrading 70 mg L^{-1} in less than 72 h (Stylianou et al., 2018). *Klebsiella variicola*, a nitrogen-fixing bacterium, genetically very similar to *K. pneumoniae* (Rosenblueth et al., 2004), has been used in the removal of various chemical substances, such as phenols (Mahgoub et al., 2023), heavy metals (Das & Osborne, 2017), herbicides (Zhang et al., 2019), and dyes (Eslami et al., 2019). Moreover, some articles mention its contribution to plant development (Kusale et al., 2021; Wang et al., 2022). However, to date, no scientific articles have described the capacity of *K. variicola* to degrade drugs.

Pseudomonas aeruginosa is a bacterium widely described in the literature for its degradative capacity of various organic chemical compounds, such as pyrene (Ma et al., 2013), carbazole, phenols (Mahgoub et al., 2023), and drugs like paracetamol (Hu

et al., 2013), or the NSAID drug flurbiprofen (Liu et al., 2023), achieving a degradation of 400 mg L^{-1} in 96 h. A recent study shows how the biosurfactant capacity of this bacterium can be used to remove IBU (Jayalatha & Devatha, 2023).

Among the three identified bacteria, *K. pneumoniae* showed the highest degradation percentage, making it the most promising candidate for future studies and potential applications in bioremediation. However, further research is necessary to optimize the IBU degradation in wastewater using this bacterium in a controlled manner to mitigate the risks associated with its pathogenicity, especially if considering its use in bioaugmentation. Several articles (Izquierdo et al., 2003; Guo et al., 2010; Jung et al., 2013) have suggested that through genetic modifications, it is possible to transform this strain into a non-pathogenic variant by eliminating or deactivating virulence genes, which could serve as a starting point for further projects.

4.4 Growth curves of the selected degrading bacteria

The degrading bacteria were grown in LB media to study the growth kinetics, except in the case of *M. yunannensis* TJPT4 which was inoculated in MB medium, under agitation at 125 rpm and 30 °C. Growth was monitored over 68 h by measuring OD_{600nm}. Simultaneously, colony-forming units (CFU mL^{-1}) were determined using serial dilutions (Figure 3.2). Data from a previous study were utilized for the growth curve of the bacterium *M. aubagnense* HPB1.1 (Ismail, 2022). The results were represented in logarithmic graphs, comparing CFU mL^{-1} and OD_{600} values over time, for each bacterium (Figures 4.9 to 4.13).

Table 4.4 presents the growth rates (μ) and duplication times (td), calculated during the exponential phase for the five degrading bacteria. The bacterium *K. pneumoniae* TIBU2.1 presented the highest growth rate μ of 0,60 h^{-1}, corresponding to a doubling time td of 1,15 h. On the other hand, *M. aubagnense* HPB1.1 showed the lowest μ of 0,12 h^{-1} and a td of 5,99 h. *K. variicola* LOI1.1, *P. aeruginosa* LOI1.2 and *M. yunannensis* TJPT4 bacteria showed intermediate growth rates between 0,41 and 0,56 h^{-1} with doubling times from 1,23 to 1,69 h (Table 4.4). These findings highlight differences in the growth kinetics among the isolated degrading bacteria.

Table 4.4. Kinetics growth rate and duplication time of isolated degrading bacteria

Isolated bacteria	***K. pneumoniae* TIBU2.1**	***K. variicola* LOI1.1**	***P. aeruginosa* LOI1.2**	***M. yunannensis* TJPT4**	***M. aubagnense* HPB1.1**
Growth rate μ (h^{-1})	0,60	0,56	0,42	0,41	0,12
Duplication time td (h)	1,15	1,23	1,66	1,69	5,99

The bacterial strains *K. pneumoniae* TIBU2.1 and *K. variicola* LOI1.1, belonging to the *Klebsiella* genus, show exponential growth (Figures 4.9 and 4.10), with no lag phase, reaching the stationary phase within 6 h of starting the assay. The exponential phase, when the bacteria reach their maximum growth and productivity, extends from h 0 to 6 h, reaching a maximum value of $1,4x10^9$ CFU mL^{-1} at an OD_{600} of 2,12 and $1,9x10^9$ CFU mL^{-1} with an OD_{600} of 1,54, respectively. Subsequently, the value of OD_{600} remained, indicating the beginning of the stationary phase.

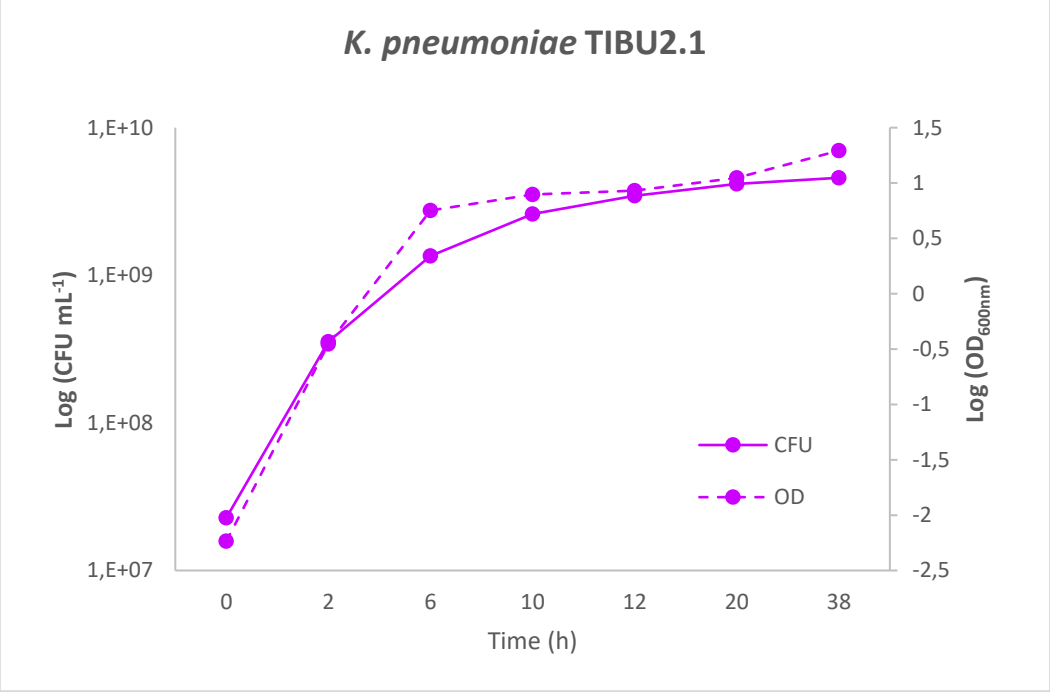


Figure 4.9. Growth curve of *K.pneumoniae* TIBU2.1.

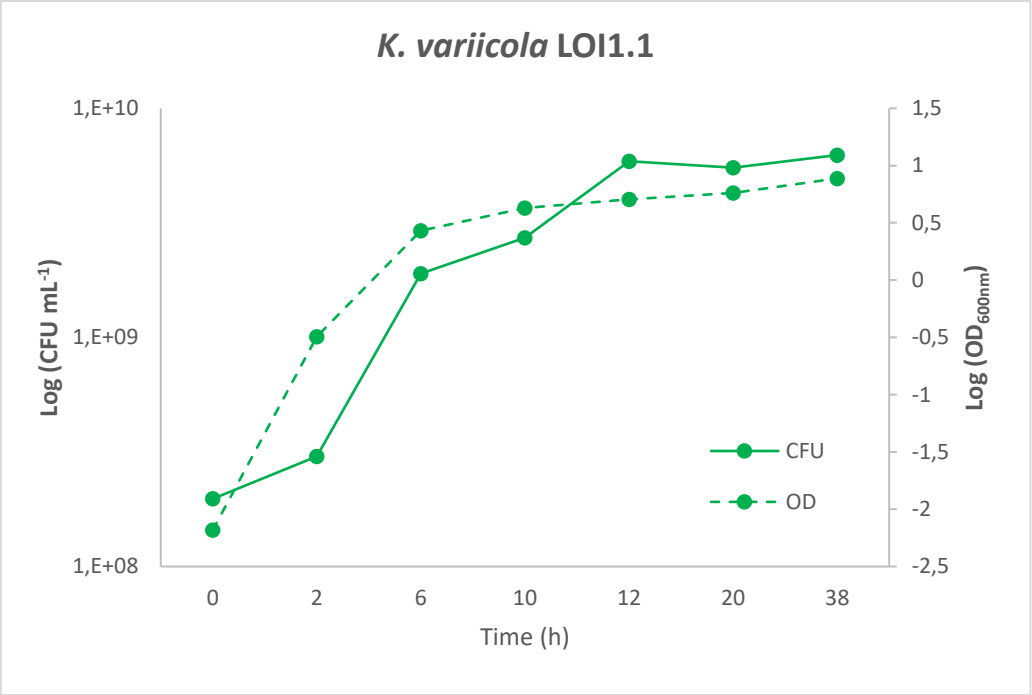


Figure 4.10. Growth curve of *K. variicola* LOI1.1.

On the other hand, the bacteria: *P. aeruginosa* LOI1.2 and *M. yunannensis* TJPT4 (Figures 4.11 and 4.12), also showed no lag phase, where the exponential phase occurs between 0 and 6 h, until reaching the stationary phase. At the maximum growth point during the exponential phase, 2,43x10^8 CFU mL^{-1} was obtained at the OD_{600} of 1,12 and 1,1 x10^7 CFU mL^{-1} at the OD_{600} of 1,02, respectively. Meanwhile, the bacterium *M. aubagnense* HPB1.1, presented a longer exponential phase (between 0 and 16 h) (Figure 4.13), reaching a maximum value of 80 CFU mL^{-1} at OD_{600} 0,48. These results provide useful baseline information for optimizing inoculum preparation during the exponential phase before conducting future degradation assays with these bacterial strains.

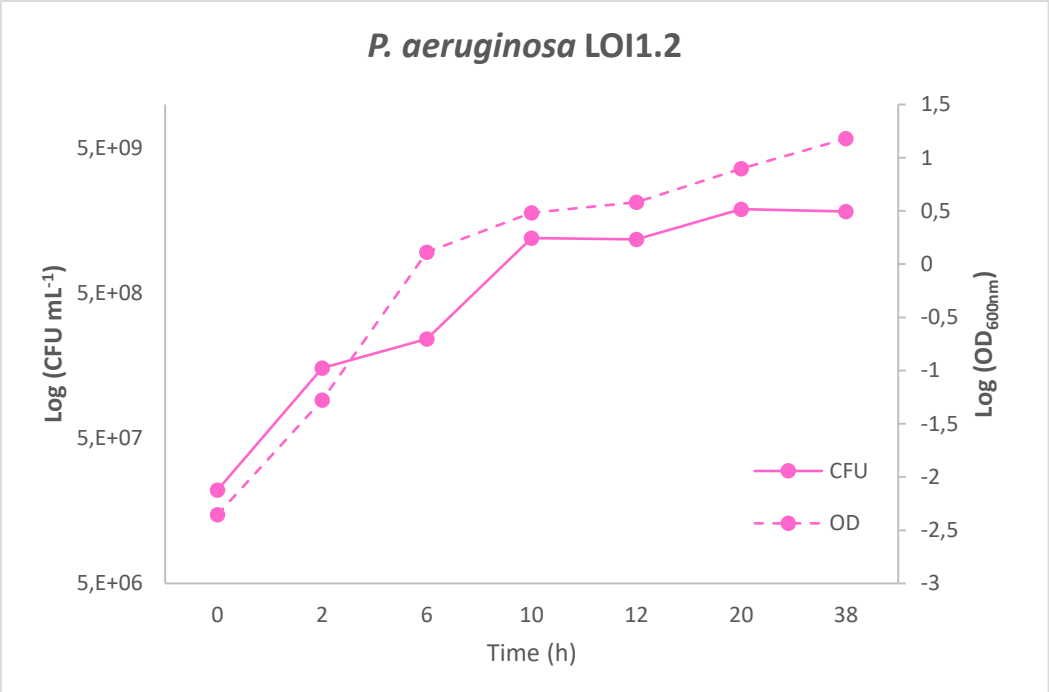


Figure 4.11. Growth curve of *P. aeruginosa* LOI1.2.

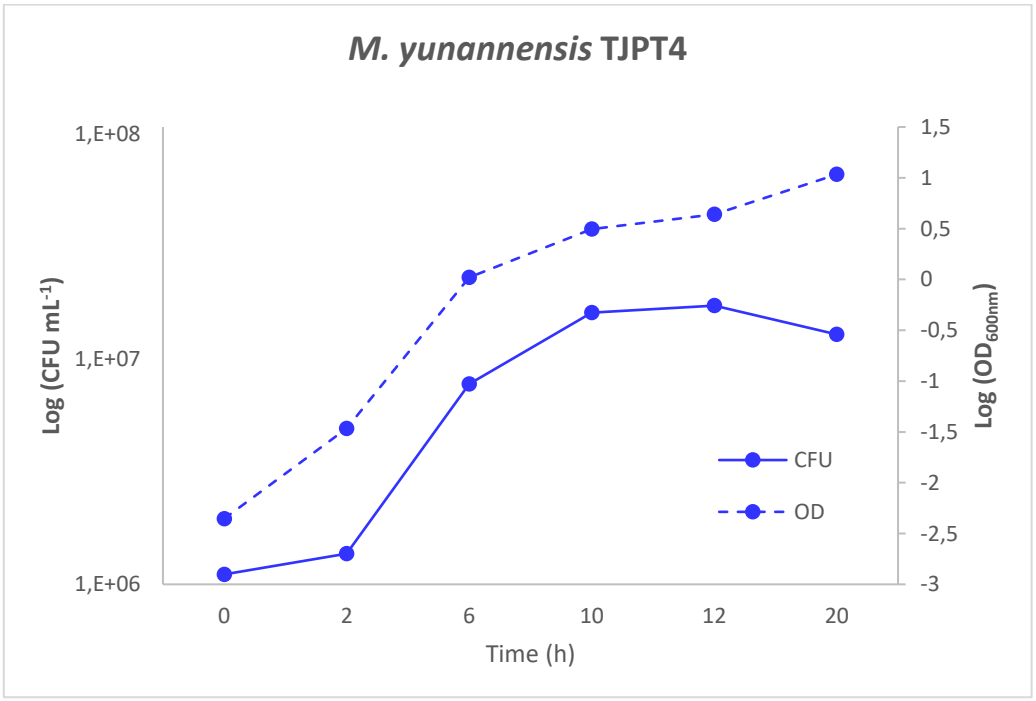


Figure 4.12. Growth curve of *M. yunannensis* TJPT4.

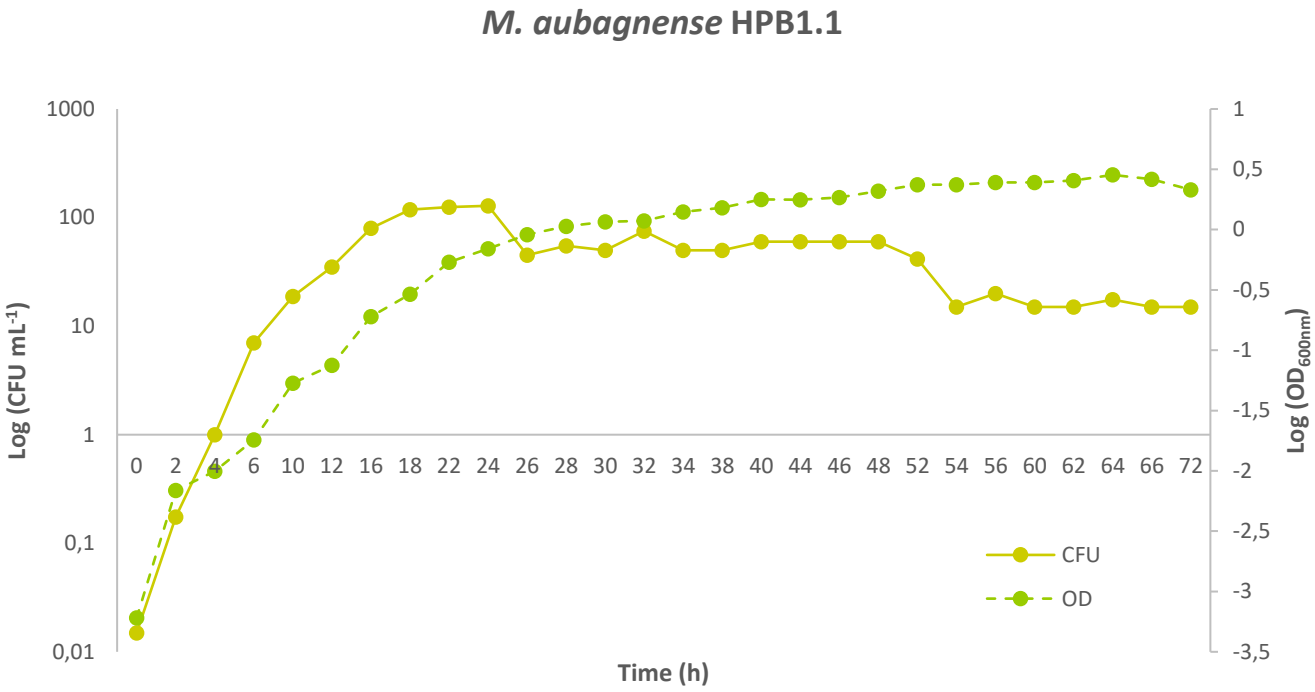


Figure 4.13. Growth curve of *M. aubagnense* HPB1.1 adapted from Ismail, (2022).

4.5 *In silico* prediction of candidate genes involved in the IBU degradation pathway

The genomes of three IBU-degrading bacteria were sequenced using the PacBio Sequel method to identify candidate genes involved in the IBU degradation pathway. The assembled genomes of the bacteria, *K. pneumoniae* TIBU2.1, and *M. yunnanensis* TJPT4 consist of a single contig, corresponding to a circular chromosome with total sizes of 5,188,999 bp and 2,432,733 bp, and Guanine-Cytosine (GC) content of 57,6%, and 73,1%, respectively. They have 4,991 and 2,260 annotated genes, respectively. On the other hand, the assembled genome of the bacterium *M. aubagnense* HPB1.1 is deposited in the NCBI Genbank (accession numbers: CP122994 - CP12299), and exhibits a total size of 6,224,392 bp, composed of 6 contigs: one circular chromosome (CP122994) with a size of 5,734,470 bp, three circular plasmids (CP122995, CP122998, CP122999) of 168,657 bp, 23,691 bp, and 16,144 bp and two linear sequences probably derived from unclosed circular plasmids (CP122996, CP122997) of 181,565 bp and 99,865 bp, respectively. The HPB1.1 genome has a GC content of 66,4% and is annotated with 5838 genes.

A total of 58 genes involved in the metabolism of aromatic compounds were identified in the genome of TIBU2.1 (Figure 4.14), 7 in TJPT4 and 50 in HPB1.1 (Figures 4.15 and 4.16). These genes are classified into 4 subcategories: (1) peripheral pathways for catabolism of aromatic compounds, (2) anaerobic degradation of aromatic compounds, (3) metabolism of central aromatic intermediates, and (4) metabolism of aromatic compounds - no subcategory.

The peripheral category (1) genes are related to the degradation of quinate, benzoate, p-Hydroxybenzoate, salicylate ester, and biphenyl. The bacterium TIBU2.1 has 11 genes, TJPT4 has only 1 gene and HPB1.1 has 15 genes in this category. On the other hand, in the anaerobic category (2), only the bacterium TIBU2.1 has 3 genes belonging to the hydroxyaromatic decarboxylase family. Meanwhile, in the central intermediates category (3), TIBU2.1 has 39 genes, TJPT4 and HPB1.1 have 6 and 34 genes, respectively. These genes are involved in central metabolism, such as catechol and protocatechuate branch of the beta-ketoadipate pathway, central meta-cleavage and homogentisate pathway, 4-hydroxyphenylacetic acid catabolic pathway, and salicylate and gentisate catabolism. Finally, in the last category (4), the TJPT4 bacterium does not present any gene, while TIBU2.1 has 5 genes related to aromatic amin catabolism and the HPB1.1 bacterium shows 1 gene involved in gentisate degradation. Despite the potential variations in catabolic pathways across species, a few of the previously mentioned genes have been linked to the pathway involved in the degradation of IBU. Enzymes such as ibuprofen-CoA ligase, aliphatic monooxygenases, hydroxylation enzymes, and dioxygenases have been closely associated with the IBU metabolic pathway (Murdoch & Hay, 2005; Marchlewicz et al., 2017a).

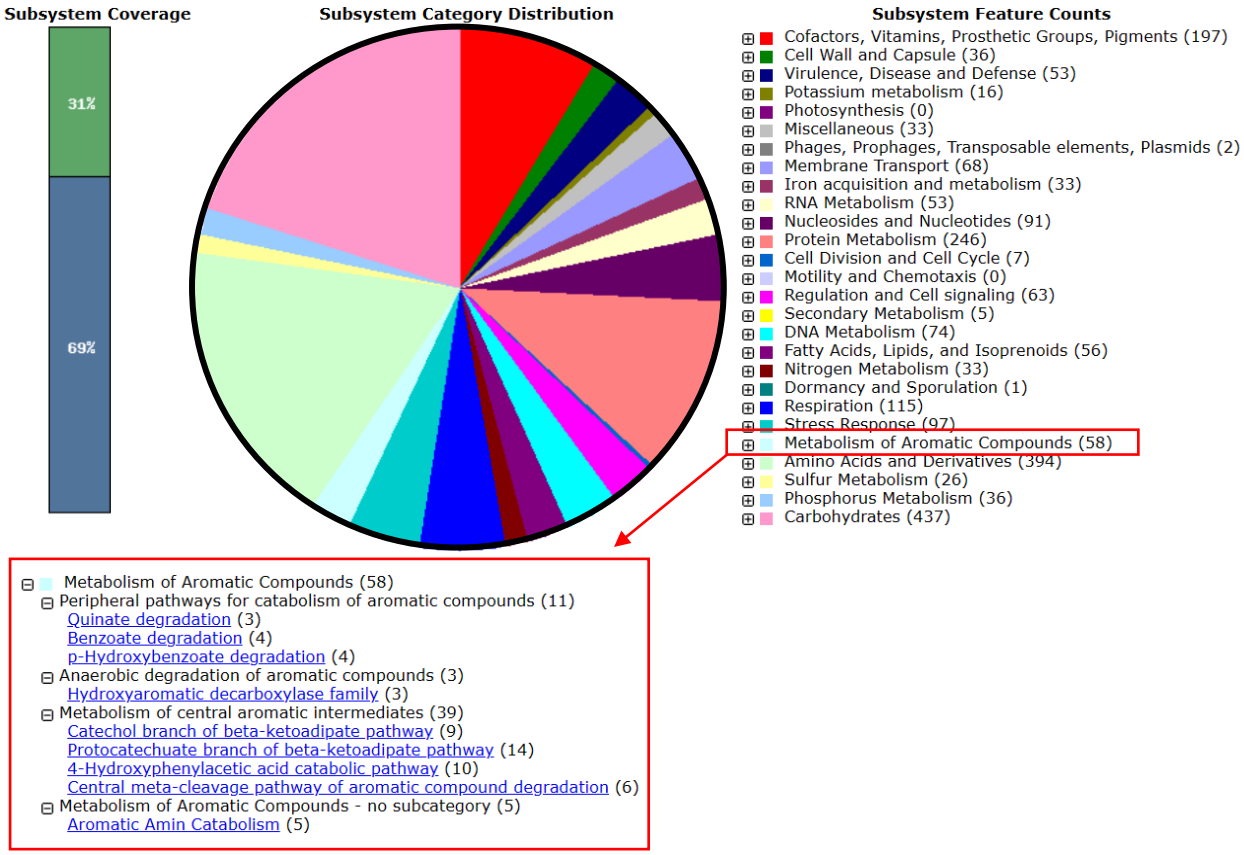


Figure 4.14. Subsystem category distribution of major protein-coding genes of *K. pneumoniae* TIBU2.1 as annotated by the RAST annotation server. The bar chart shows the subsystem coverage in percentage (the blue bar corresponds to the percentage of proteins included). The pie chart shows the percentage distribution of the 27 most abundant subsystem categories.

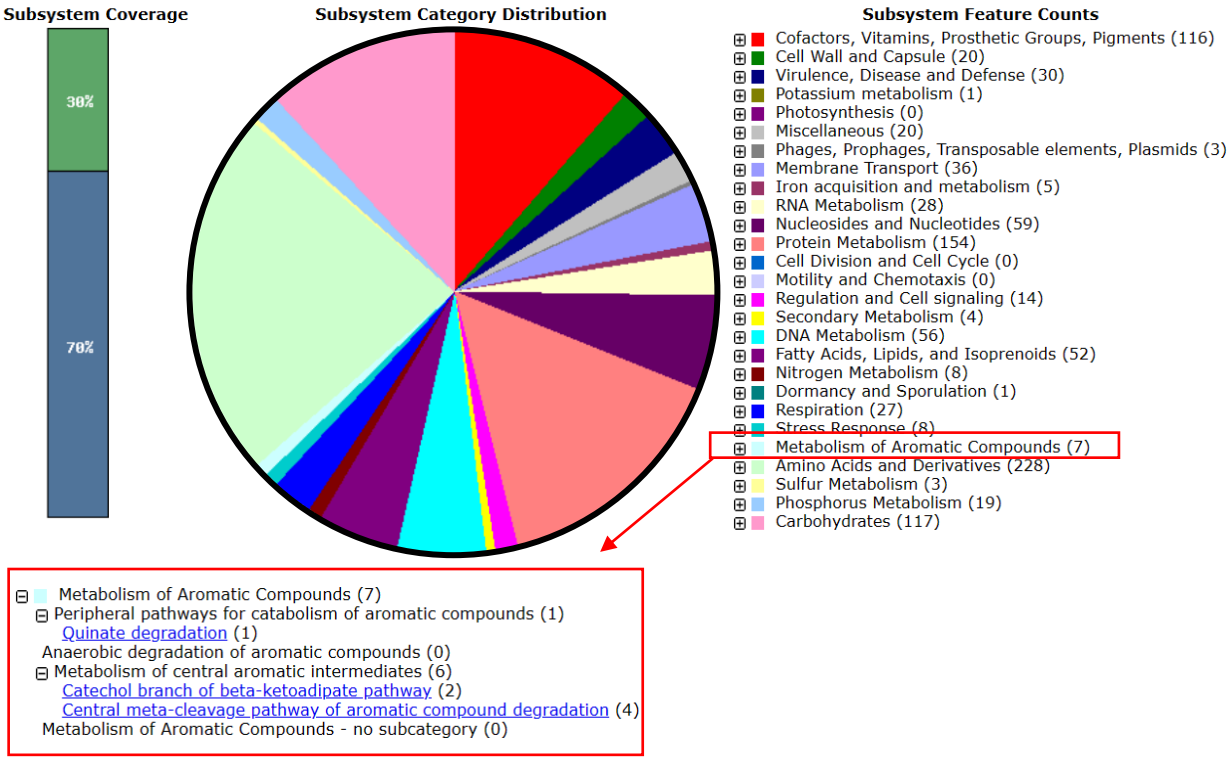


Figure 4.15. Subsystem category distribution of major protein-coding genes of *M. yunannensis* TJPT4 as annotated by the RAST annotation server. The bar chart shows the subsystem coverage in percentage (the blue bar corresponds to the percentage of proteins included). The pie chart shows the percentage distribution of the 27 most abundant subsystem categories.

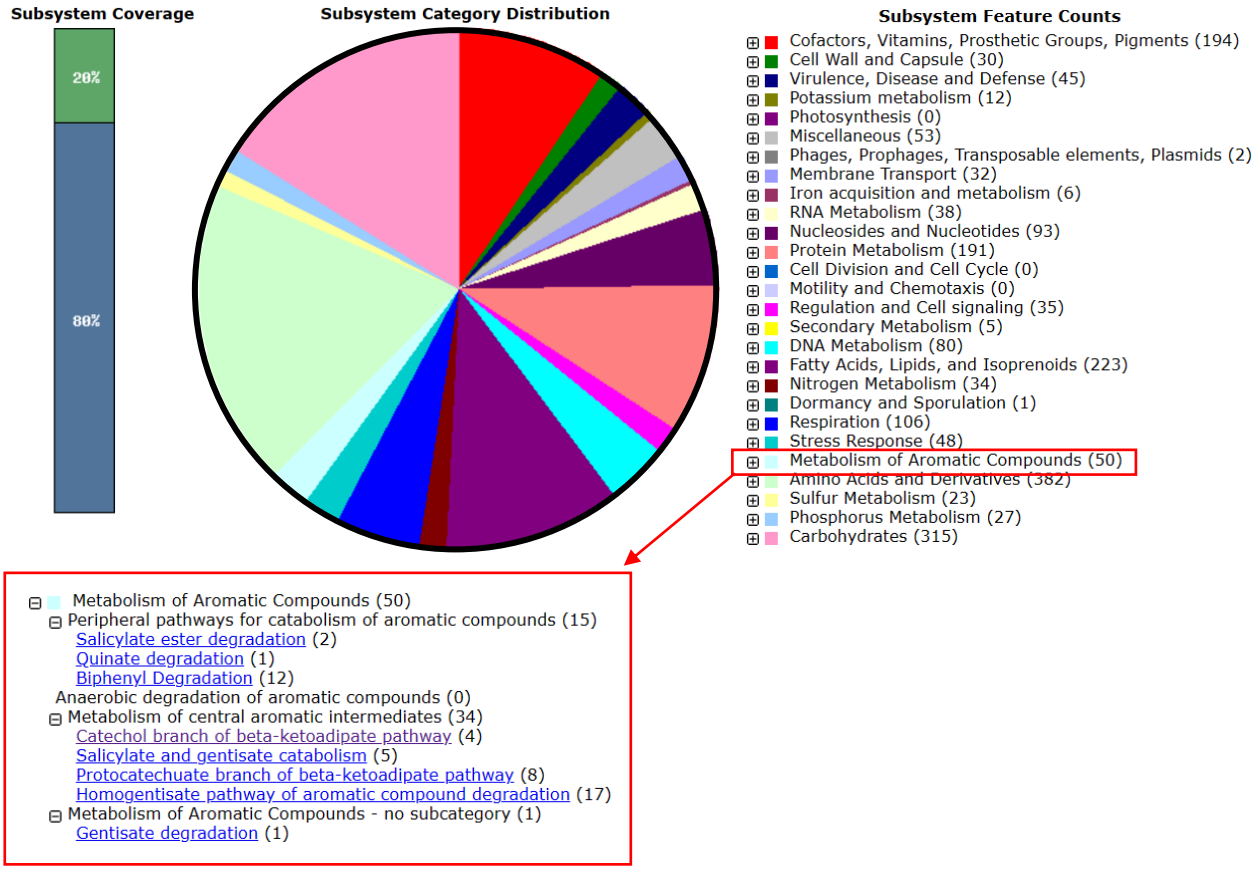


Figure 4.16. Subsystem category distribution of major protein-coding genes of *M. aubagnense* HPB1.1 as annotated by the RAST annotation server. The bar chart shows the subsystem coverage in percentage (the blue bar corresponds to the percentage of proteins included). The pie chart shows the percentage distribution of the 27 most abundant subsystem categories.

To identify potential enzymes involved in the IBU biodegradation pathway, an *in-silico* analysis was conducted to predict the genes associated with IBU biodegradation within the genomes of three distinct bacterial strains, namely, TIBU2.1, TJPT4, and HPB1.1. The sequences were compared to search for genetic homologies with the proteins described in the IBU degradation pathway (Figure 4.17) as reported by Aulestia et al. (2022). The results, based on the percentage of similarity, were obtained by individually aligning the reference proteins with the genome of each bacteria using the tblastn database program from NCBI, these results were shown in Table 4.5. This table presents the percentages of identity and coverage, along with a comparison of the protein functions. It should be noted that in the case of the HPB1.1 bacterium, despite being composed of 6 DNA molecules, similarities were only found with the CP122994 chromosome and the CP122995 plasmid (designated as C and P in Table

4.5, respectively). Regarding the TIBU2.1 and TJPT4 strains, both chromosomes were denoted as C in Table 4.5.

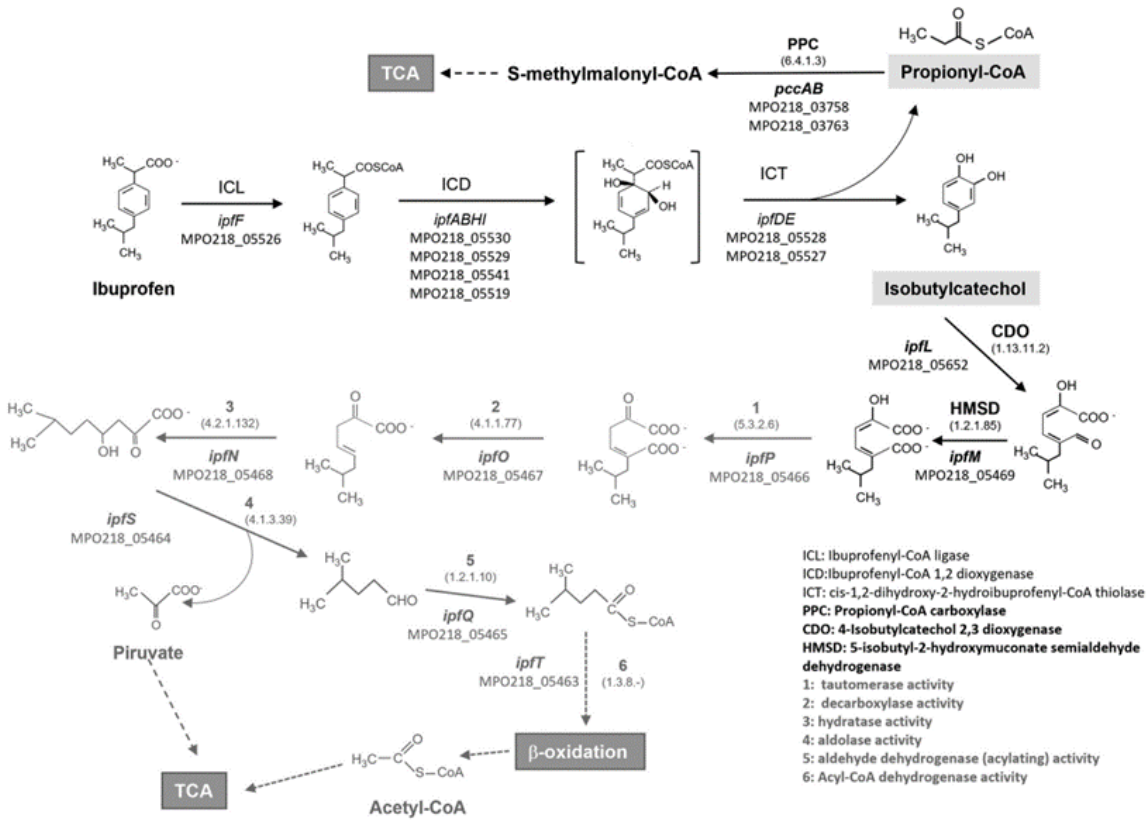


Figure 4.17. Metabolic pathway for IBU degradation in *Rhizorhabdus wittichii* MPO218 from Aulestia et al. (2022).

The percentage of identity between homologous proteins from different bacterial species can vary depending on the evolutionary distance between them. Despite the absence of an exact threshold for the percentage of identity required to infer functional similarity among homologous proteins from different bacteria, research suggests that identity percentages > 40% indicate high conservation of biochemical function among homologs, while below 30%, functional variation becomes significant. Sequences with lower identity percentages indicate significant evolutionary divergence, and protein structure becomes essential for understanding the molecular basis of functional differences (Todd et al., 2001; Pearson, 2014).

In the IBU biodegradation pathway described by Aulestia et al. (2022), the first step is catalyzed by the enzyme ibuprofenyl-CoA ligase (*ipf*F), which is responsible for binding the IBU to coenzyme A, facilitating degradation. The AMP-binding protein (Accession number: WP_208634566.1) was compared with the genome sequences of

the three degrading bacteria TIBU2.1, TJPT4, and HPB1.1. The percentage of identity among them was 25%, 28%, and 32%, respectively for TIBU2.1, TJPT4, and HPB1.1 strain (Table 4.5), with coverage ranging from 98% to 99%. Similarities in the case of the bacteria HPB1.1, were only found in the chromosome. Interestingly, the three bacteria share the same functional annotation: Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3). This suggests that this enzyme is likely responsible for the initial step in the IBU biodegradation. The evolutionary differences between these species and the bacterium *Sphingomonadaceae* may explain why they possess an identical protein function despite having a < 40% identity.

In the second step of IBU degradation, it is known that a dioxygenase system, encoded by the *ipfABHI* genes, acts to form the dihydrodiol. This group of proteins was individually compared, consisting of the aromatic ring-hydroxylating dioxygenase subunit Alpha (Accession number: WP_208634570.1) from the bacterium *Rhizorhabdus wittichii*, followed by the aromatic-ring-hydroxylating dioxygenase subunit beta (Accession number: WP_208634569.1), FAD-dependent oxidoreductase (Accession number: WP_226870539.1), and non-heme iron oxygenase ferredoxin (Accession number: WP_185210326.1) from the *Sphingomonadaceae* bacterium. The bacterium TIBU2.1 exhibited similarity with the entire group of proteins, showing identity percentages of 30%, 31%, 28%, and 29%, for *ipfA, B, H* and *I*, respectively. Despite the identity % when compared to the reference proteins indicating significant evolutionary differences in the function of these proteins, the functional annotation reveals them to be dioxygenase subunit alpha and beta, reductase large subunit, and oxygenase, respectively. As they belong to the same enzyme group involved in the second step of the IBU degradation catabolic pathway, structural analysis of the protein or catalytic residue could be crucial for ruling out or integrating these proteins in the IBU metabolic pathway. On the other hand, the bacterium TJPT4 only showed similarity (identity percentage < 30% in both) with two proteins from the aforementioned protein group, which correspond in the genomic annotation of the strain to ferredoxin reductase (encoded by *ipfH*) and dioxygenase ferredoxin subunit (encoded by *ipfI*). Lastly, the bacterium HPB1.1 exhibited similarities with the entire protein group on the chromosome contig (identity percentage 45%, 44%, 32%, and 34%, respectively), suggesting the presence of dioxygenases responsible for adding two hydroxyl groups (OH). Meanwhile, the plasmid contig showed similarities only

with the dioxygenase alpha subunit (encoded by *ipfA*) and the FAD-dependent oxidoreductase (encoded by *ipfH*) (coverage 69% and 83%, identity 32% and 31%, respectively).

The next step in the IBU biodegradation pathway involves the formation of the main central intermediate, isobutylcatechol, and propionyl-CoA through the action of the thiolase enzyme encoded by the *ipfDE* genes (Aguilar et al., 2021; Aulestia et al., 2021). The presence of the proteins encoded by the *ipfDE* genes in the TIBU2.1, TJPT4 and HPB1.1 genomes was analyzed. The alignment of sterol carrier protein and acyl-CoA transferase domain revealed a protein with 36 and 38% identity, respectivelyfor HPB1.1; however, no similarities were found for TIBU2.1 and TJPT4 strain. Appropriate analysis as molecular studies could help to determine whether these encode for the *ipfDE* genes.

The further step in IBU degradation is the opening of the aromatic ring of 4-isobutylcatechol, which occurs through a reaction catalyzed by the enzyme catechol 2,3-dioxygenase (Accession number: WP_208634508.1), encoded by the *ipfL* gene. This is followed by the action of a semialdehyde dehydrogenase (Accession number: WP_208634595.1), encoded by the *ipfM* gene, to form 2-hydroxy-5-isobutylhexa-2,4-dienoic acid. Both genes were first described by (Aulestia et al., 2022). Interestingly, TJPT4 strain was the only one to exhibit similarities with both genes. In the case of semialdehyde dehydrogenase (encoded by *ipfL*), showed 27% identity to annotated catechol 2,3-dioxygenase, suggesting the presence of this gene in the genome of the strain. Several articles mention the importance of catechol 2,3-dioxygenase in the degradation pathways of aromatic compounds by microorganisms, as it catalyzes the incorporation of dioxygen into catechol, facilitating ring cleavage and enabling the degradation of various aromatic contaminants (Kita et al., 1999; Hakim et al., 2015). On the other hand, TJPT4 showed a 40% identity with the enzyme encoded by the *ipfM* gene, sharing annotation with the TIBU2.1 strain (39% identity), with the enzyme 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (EC 1.2.1.60). Meanwhile, the HPB1.1 bacterium displayed similarities on its chromosome with 36% identity with aldehyde dehydrogenase (EC 1.2.1.3). As mentioned earlier, molecular analyses would help determine their relationship with the IBU biodegradation pathway.

After analyzing the primary potential candidate genes involved in the IBU degradation metabolic pathway, it is worth noting that an alignment was also performed with the enzymes encoded by the *ipfO, ipfN, ipfS, ipfQ,* and *ipfT* genes (Table 4.5), responsible for encoding enzymes with decarboxylase, hydratase, aldolase, aldehyde dehydrogenase (acylating) and acyl-CoA dehydrogenase activity, respectively. These proteins are associated with the final steps of IBU degradation, responsible for metabolizing 2-hydroxy-5-isobutylhexa-2,4-dienoic acid into end products. These end products can be readily metabolized by the tricarboxylic acid (TCA) cycle and beta-oxidation. No similarities were found with any bacteria between the tautomerase enzyme encoded by the *ipfP* gene. However, similarities were found between the bacteria TIBU2.1 (36, 40, 48, 55 and 28 % identity, respectively) and HPB1. 1 (36, 43, 48, 48, 56 and 37 % identity, respectively) with the enzymes encoded by the *ipfO, N, S, Q* and *T* genes, it is worth mentioning that only in the acyl-CoA dehydrogenase (*ipfT*) protein, the plasmid of HPB1.1 showed similarities at a 24% identity. On the other hand, TJPT4 showed similarities with the decarboxylase (*ipfO*), hydratase (*ipfN*) and acyl-CoA dehydrogenase (*ipfT*), with 36%, 40% and 31% identity, respectively. It is important to highlight that almost all the percentages of identity with this group of proteins vary between 30% and 40%, suggesting the presence of the genes that encoded the fundamental proteins for the transformation of IBU into small metabolic products that are less toxic and easier to degrade (Murdoch & Hay, 2013; Salgado et al., 2020).

Summarizing, the genomic study showed that the HPB1.1 bacterial strain presents practically all the proteins encoded by the degradation genes described in the literature, for this reason, it could be confirmed that it follows the degradation pathway described by Aulestia et al. (2022). However, the TIBU2.1 strain contains in its genome the proteins of the degradation genes involved at the beginning (*ipfABHI*) and at the end (*ipfNSTQT*) of the pathway proposed by Aulestia et al. (2022). This indicates that in the intermediate part of the pathway encoded by *ipfDE* genes where isobutylcatechol and propionyl-CoA are formed, *K. pneumoniae* TIBU2.1 could follow other alternative routes that have not been described in the current literature. Nevertheless, the catabolic pathway of the TJPT4 strain requires further exploration, as it deviates from the established pattern described in the literature.

Table 4.5. Information about the alignment of protein known to degrade IBU and proteins annotated in the genome of *K. pneumoniae* TIBU2.1, *M. yanunnensis* TJPT4 and *M. aubagnense* HPB1.1.

Reference Protein Data				Alignment Data				Annotated Protein Data	
Gene name	**Protein (Accession number)**	**Reference bacterium**	**Function NCBI**	**Bacteria strain**	**Coverage (%)**	**Identity (%)**	**E-value**	**Plasmidium/ Cromosome**	**Function annotation**
ipfF	WP_208634566.1	*Sphingomonadaceae*	AMP-binding protein	TIBU2.1	98	25	$7e^{-39}$	C	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)
				TJPT4	99	28	$9e^{-41}$	C	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)
				HPB1.1	99	32	$2e^{-56}$	C	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)
					-	-	-	-	-
ipfA	WP_208634570.1	*Rhizorhabdus wittichii*	aromatic ring-hydroxylating dioxygenase subunit alpha	TIBU2.1	89	30	$1e^{-46}$	C	Benzoate 1,2-dioxygenase alpha subunit (EC 1.14.12.10)
				TJPT4	-	-	-	-	-
				HPB1.1	98	45	$1e^{-111}$	C	putative dioxygenase hydroxylase component
					69	32	$2e^{-33}$	P	Phthalate 3,4-dioxygenase alpha subunit
ipfB	WP_208634569.1	*Sphingomonadaceae*	aromatic-ring-hydroxylating dioxygenase subunit beta	TIBU2.1	66	31	$6e^{-09}$	C	Benzoate 1,2-dioxygenase beta subunit (EC 1.14.12.10)
				TJPT4	-	-	-	-	-
				HPB1.1	90	44	$6e^{-36}$	C	Benzoate 1,2-dioxygenase beta subunit (EC 1.14.12.10)
					-	-	-	-	-
ipfH	WP_226870539.1	*Sphingomonadaceae*	FAD-dependent oxidoreductase	TIBU2.1	63	28	$5e^{-09}$	C	Nitrite reductase [NAD(P)H] large subunit (EC 1.7.1.4)
				TJPT4	92	27	$3e^{-26}$	C	Ferredoxin reductase

				HPB1.1	91	32	$5e^{-47}$	C	Anthranilate dioxygenase reductase
					83	31	$2e^{-35}$	P	Ferredoxin reductase
ipfI	WP_185210326.1	*Sphingomonadaceae*	non-heme iron oxygenase ferredoxin	TIBU2.1	69	29	$2e^{-05}$	C	Vanillate O-demethylase oxygenase subunit (EC 1.14.13.82)
				TJPT4	93	29	$2e^{-09}$	C	3-phenylpropionate dioxygenase ferredoxin subunit
				HPB1.1	90	34	$2e^{-08}$	C	Pyruvate dehydrogenase (quinone) (EC 1.2.5.1)
					-	-	-	-	-
ipfD	WP_226949243.1	*Sphingomonadaceae*	hypothetical protein	TIBU2.1	-	-	-	-	-
				TJPT4	-	-	-	-	-
				HPB1.1	72	36	$5e^{-32}$	C	Sterol carrier protein IgrF
					-	-	-	-	-
ipfE	WP_208634567.1	*Sphingomonadaceae*	OB-fold domain-containing protein	TIBU2.1	-	-	-	-	-
				TJPT4	-	-	-	-	-
				HPB1.1	51	38	$4e^{-08}$	C	Acyl-CoA transferase domain of IgrD / Nucleic-acid-binding domain of IgrD
					-	-	-	-	-
ipfL	WP_208634508.1	*Sphingomonadaceae*	catechol 2,3-dioxygenase	TIBU2.1	-	-	-	-	-
				TJPT4	96	27	$3e^{-20}$	C	Catechol 2,3-dioxygenase (EC 1.13.11.2)
				HPB1.1	-	-	-	-	-
					-	-	-	-	-
ipfM	WP_208634595.1	*Sphingomonadaceae*	2-hydroxymuconic semialdehyde dehydrogenase	TIBU2.1	100	39	$1e^{-106}$	C	5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (EC 1.2.1.60)
				TJPT4	99	40	$2e^{-105}$	C	5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (EC 1.2.1.60)
				HPB1.1	100	36	$2e^{-86}$	C	Aldehyde dehydrogenase (EC 1.2.1.3)

					-	-	-	-	-
ipfP	WP_208634520.1	*Sphingomonadaceae*	tautomerase family protein	TIBU2.1	-	-	-	-	-
				TJPT4	-	-	-	-	-
				HPB1.1	-	-	-	-	-
					-	-	-	-	-
ipfO	WP_208634521.1	*Sphingomonadaceae*	2-oxo-3-hexenedioate decarboxylase	TIBU2.1	97	36	$2e^{-43}$	C	2-oxo-hepta-3-ene-1,7-dioic acid hydratase (EC 4.2.-.-)
				TJPT4	87	36	$6e^{-46}$	C	2-oxo-hepta-3-ene-1,7-dioic acid hydratase (EC 4.2.-.-)
				HPB1.1	97	36	$2e^{-37}$	C	2-oxopent-4-dienoate hydratase
					-	-	-	-	-
ipfN	WP_208634522.1	*Sphingomonadaceae*	fumarylacetoacetate hydrolase family protein	TIBU2.1	98	40	$4e^{-56}$	C	2-keto-4-pentenoate hydratase (EC 4.2.1.80)
				TJPT4	95	40	$2e^{-51}$	C	2-oxo-hepta-3-ene-1,7-dioic acid hydratase (EC 4.2.-.-)
				HPB1.1	97	43	$4e^{-51}$	C	2-oxopent-4-dienoate hydratase
					-	-	-	-	-
ipfS	WP_208634518.1	*Sphingomonadaceae*	4-hydroxy-2-oxovalerate aldolase	TIBU2.1	95	48	$1e^{-102}$	C	4-hydroxy-2-oxovalerate aldolase (EC 4.1.3.39)
				TJPT4	-	-	-	-	-
				HPB1.1	95	48	$2e^{-94}$	C	4-hydroxy-2-oxovalerate aldolase (EC 4.1.3.39)
					-	-	-	-	-
ipfQ	WP_208634519.1	*Sphingomonadaceae*	acetaldehyde dehydrogenase (acetylating)	TIBU2.1	94	55	$7e^{-86}$	C	Acetaldehyde dehydrogenase, acetylating, (EC 1.2.1.10) in gene cluster for degradation of phenols, cresols, catechol
				TJPT4	-	-	-	-	-
				HPB1.1	95	56	$2e^{-98}$	C	Acetaldehyde dehydrogenase, acetylating, (EC 1.2.1.10) in gene cluster for degradation of phenols, cresols, catechol
					-	-	-	-	-

ipfT	WP_208634517.1	*Sphingomonadaceae*	acyl-CoA dehydrogenase	TIBU2.1	49	28	$5e^{-18}$	C	Alkylation response protein AidB, acyl-CoA dehydrogenase family
				TJPT4	61	31	$1e^{-26}$	C	Branched-chain acyl-CoA dehydrogenase (EC 1.3.99.12)
				HPB1.1	87	37	$2e^{-71}$	C	3-methylmercaptopropionyl-CoA dehydrogenase (DmdC)
					63	24	$7e^{-13}$	P	Acyl-CoA dehydrogenase

(-) Not detected.

5. Conclusion

In conclusion, the use of enrichment cultures in this work successfully enabled the isolation on plates, of twenty differentiated bacterial strains from polluted environmental sediment. Among these, three bacteria (TIBU2.1, LOI1.1, and LOI1.2) isolated from the potentially contaminated sediments from Tavira region and olive mill wastewaters exhibited the ability to degrade between 59% and 100% of the emerging contaminant IBU at an initial concentration of 5 mg L^{-1} in aqueous solution. For FLX, LOFLX1.3 strain achieved only 29% degradation in 15 days, this being the maximum percentage degraded, in the following trials FLX was not considered, so complementary studies are necessary to find degrading bacteria with the capacity to degrade FLX efficiently. It was also included *M. yunannensis* TJPT4 and *M. aubagnense* HPB1.1, strains previously isolated to degrade other pharmaceuticals, which have also demonstrated the capability to degrade IBU in this work. HPLC analysis revealed the presence of unknown metabolites, which when compared to the main metabolites of IBU, carboxyibuprofen and 2-hydroxyibuprofen, no similarities to either were found. This could serve as starting point for future research, involving specific techniques such as LC-MS, to aid in the identification of these compounds and gain a better understanding of the products resulting from the transformation of IBU by these bacteria. Sequencing of the 16S rRNA gene allowed the taxonomic identification of IBU-degrading bacteria (TIBU2.1, LOI1.1, and LOI1.2), which were found to belong to the genera *Klebsiella* and *Pseudomonas*. Among the isolated strains, TIBU2.1, identified as *K. pneumoniae*, exhibited the highest IBU degradation capacity and was selected for complete genome sequencing, along with *M. aubagnense* HPB1.1 and *M. yunannensis* TJPT4. The genetic information from this work serves as a basis for future studies aimed at the development of bioaugmentation techniques. By aligning 15 enzymes reported to be involved in the IBU catabolic pathway with proteins translated from the sequenced genomes of the three selected bacteria, candidate catabolic genes for these degrading bacteria were identified. *M. aubagnense* HPB1.1 showed similarities to almost all proteins compared, followed by *K. pneumoniae* TIBU2.1 with similarities to proteins related to dihydrodiol formation and 2-hydroxy-5-isobutyl-hexa-2,4-dienoic acid transformation. Both strains were identified for the first time as IBU degrading bacteria in this work. The presence of such genes probably encoding enzymes able to break the aromatic ring of IBU

reinforces the possibility of using the selected strains to transform this drug into less persistent compounds and opens the way for further studies to confirm the functions of those encoded proteins.

6. Future Perspectives

Genomic information is a valuable tool for future applications in the bioremediation of wastewater containing these emerging contaminants. For instance, the presence of genes encoding catabolic enzymes in isolated bacteria can serve as a foundation for designing optimized synthetic microbial consortia for the bioremediation of pharmaceuticals in contaminated water and soil. Moreover, the enzyme information can be the starting point for the development of other strategies such as the direct addition of purified or partially purified degrading enzymes to contaminated waters or soils. Furthermore, in the context of bioaugmentation, the knowledge of genes and enzymes involved in the IBU degradation pathways can facilitate the development of strategies where these genes are introduced into native bacterial strains using plasmid vectors that transport the degrading genes. This approach can enhance the degradative capacity of microbial communities within wastewater treatment plants, expediting the removal of these emerging contaminants to improve the quality of surface waters. The next step will be the use of these bacteria in bioaugmentation assays for the degradation of IBU in bioreactors with granular sludge.

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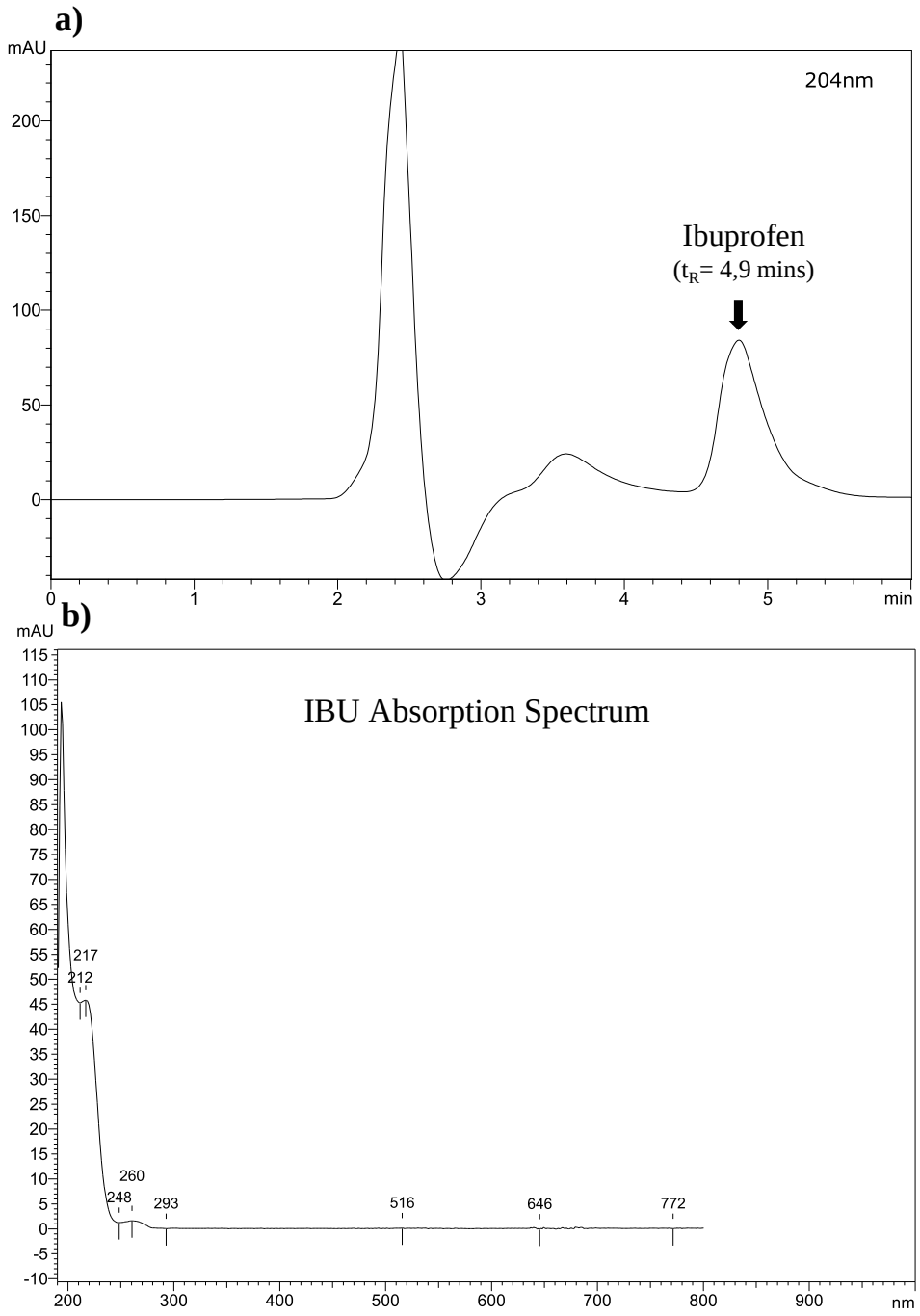
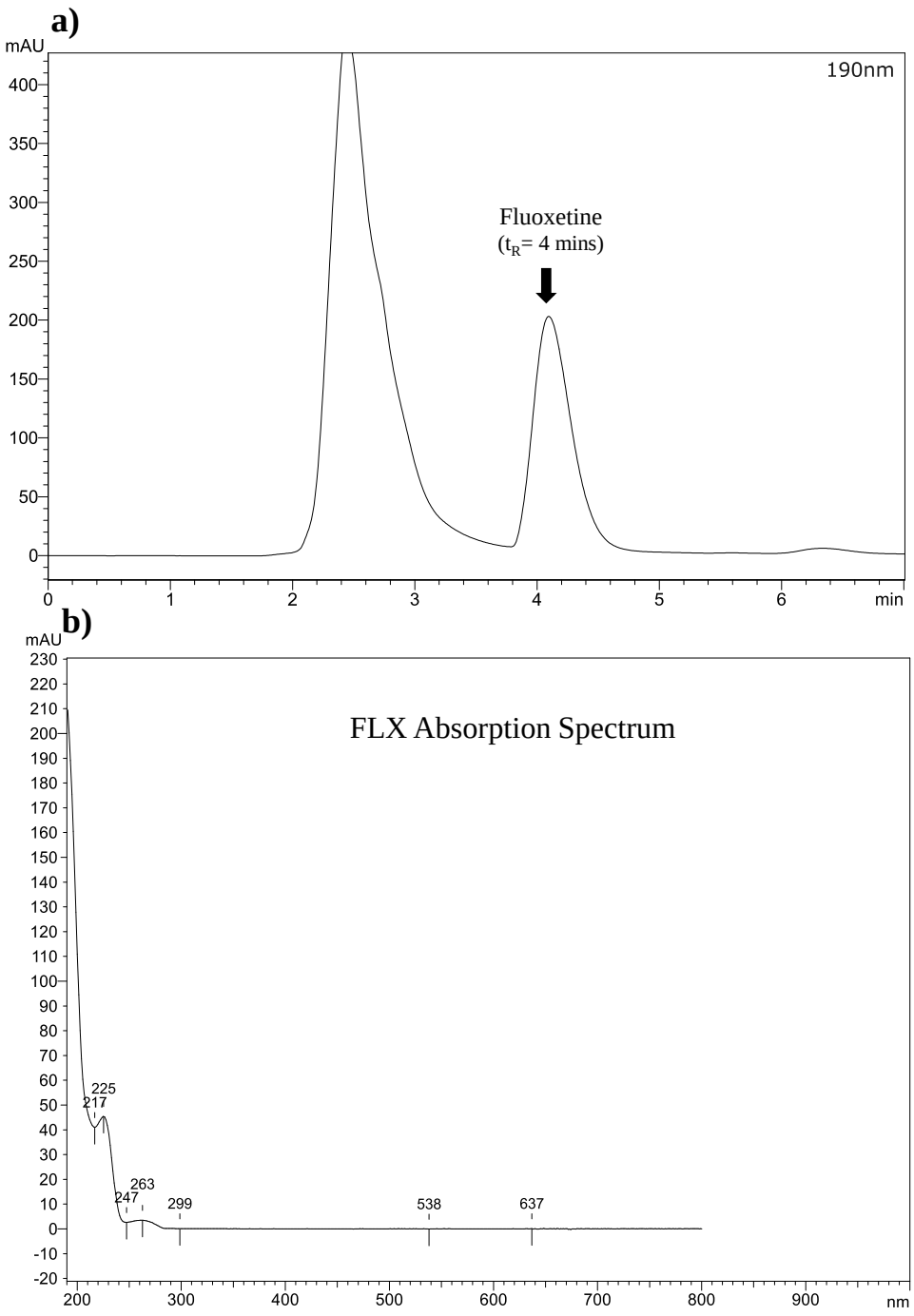
8. Annexes

Annex 1: Chromatograms and absorption spectrum detected by HPLC.

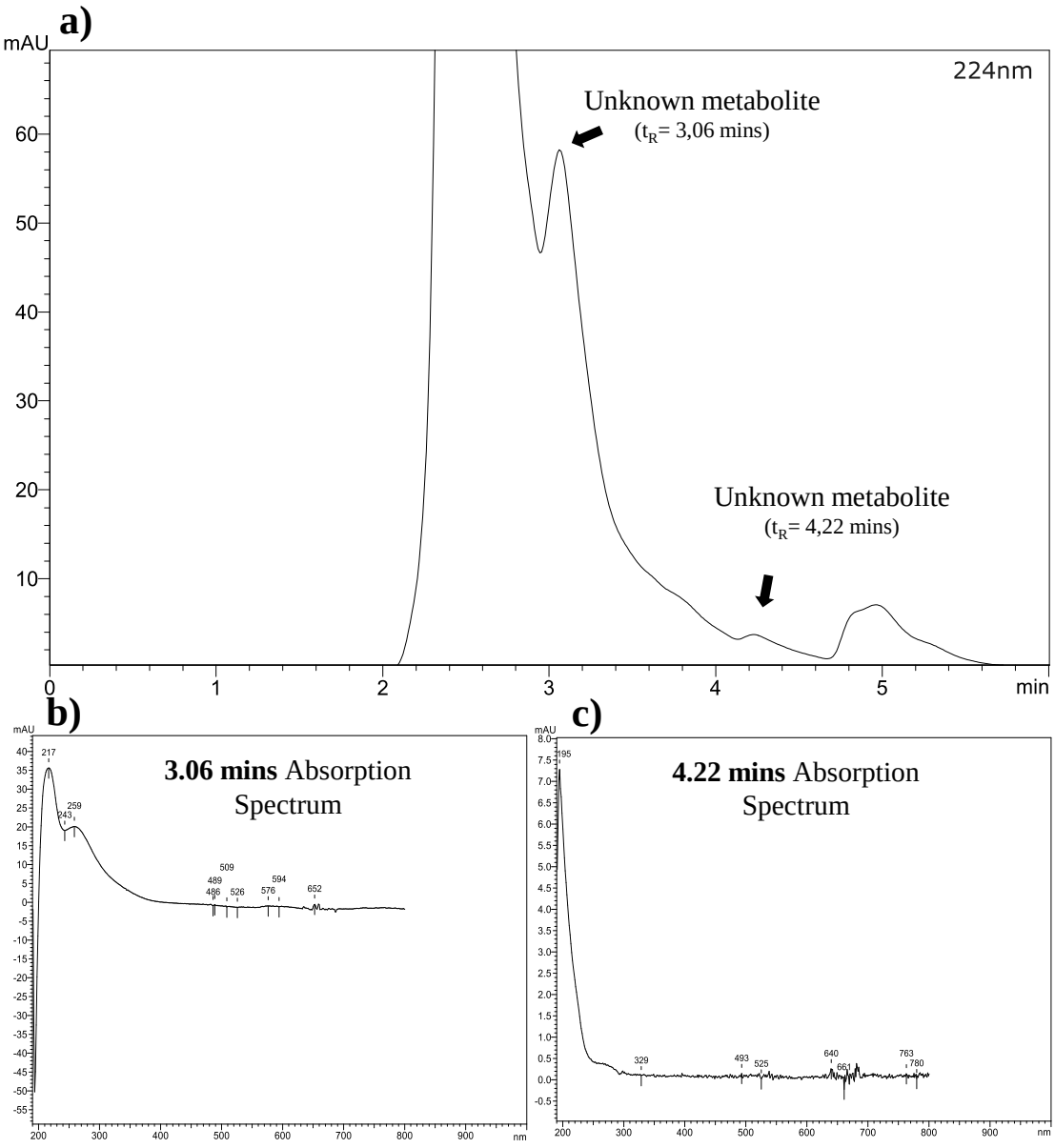
IBU: a) Chromatogram of IBU detected at 204nm and b) absorption spectrum by HPLC.



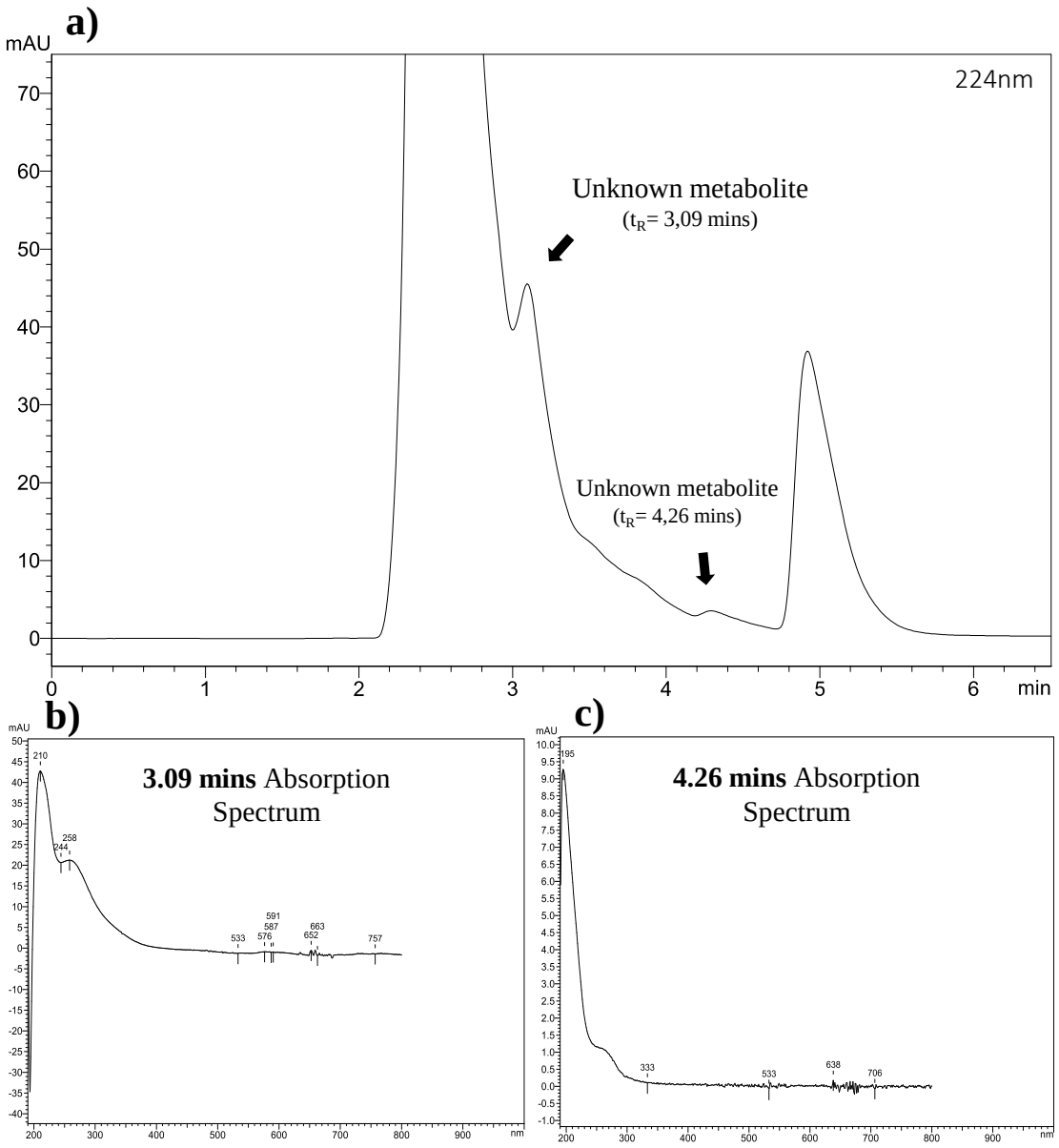
IBU: a) Chromatogram of FLX detected at 190nm and b) absorption spectrum by HPLC.



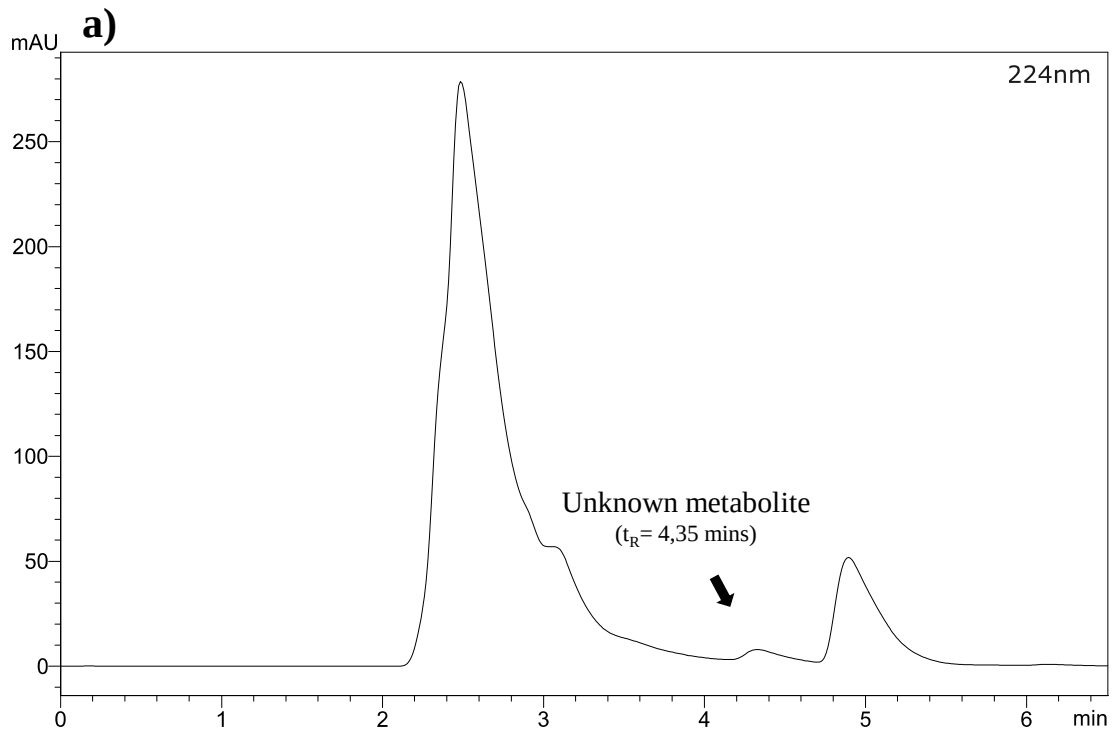
TIBU2.1 strain: UBU solubilized in the aqueous Mineral Salt Medium (MSM) analyzed by HPLC in the presence of *K. pneumoniae* TIBU2.1. a) Chromatogram of unknown metabolites detected at 224nm, b) absorption spectrum of 3,06 mins metabolite, and c) absorption spectrum of 4,22 mins metabolite.

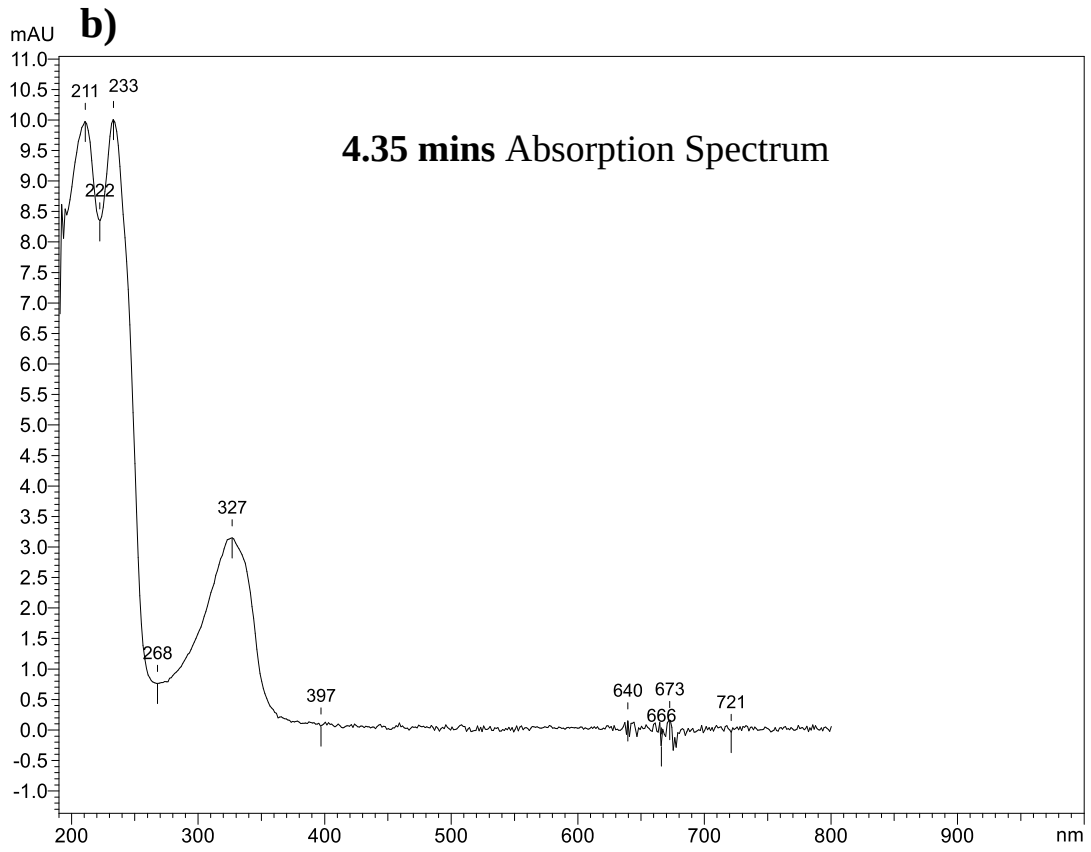


LOI1.1 strain: UBU solubilized in the aqueous Mineral Salt Medium (MSM) analyzed by HPLC in the presence of *K. variicola* LOI1.1. a) Chromatogram of unknown metabolites detected at 224nm, b) absorption spectrum of 3,09 mins metabolite, and c) absorption spectrum of 4,26 mins metabolite.

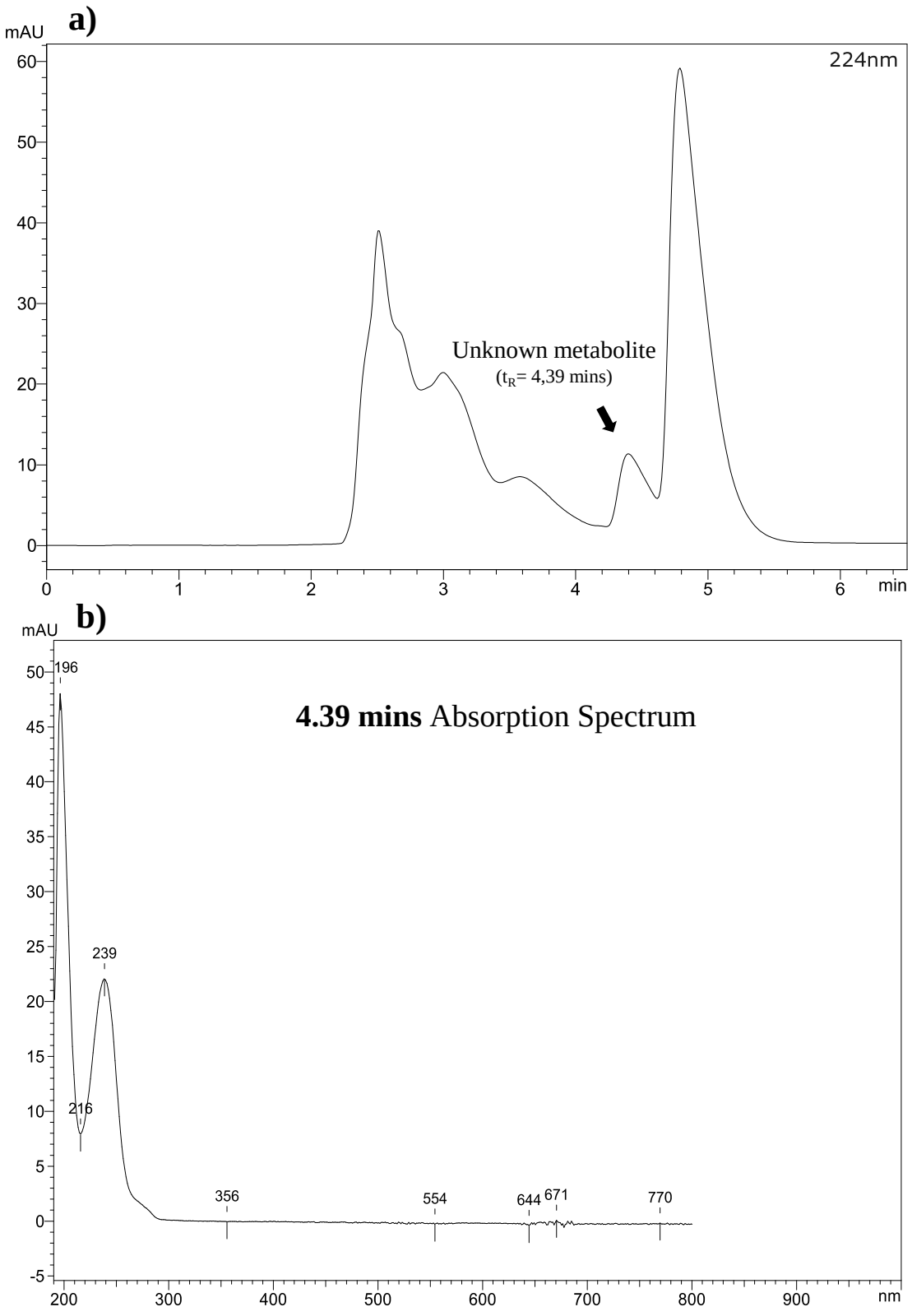


LOI1.2 strain: IBU solubilized in the aqueous Mineral Salt Medium (MSM) analyzed by HPLC in the presence of *P. aeruginosa* LOI1.2. a) Chromatogram of unknown metabolite detected at 224nm and b) absorption spectrum of 4,35 mins metabolite.

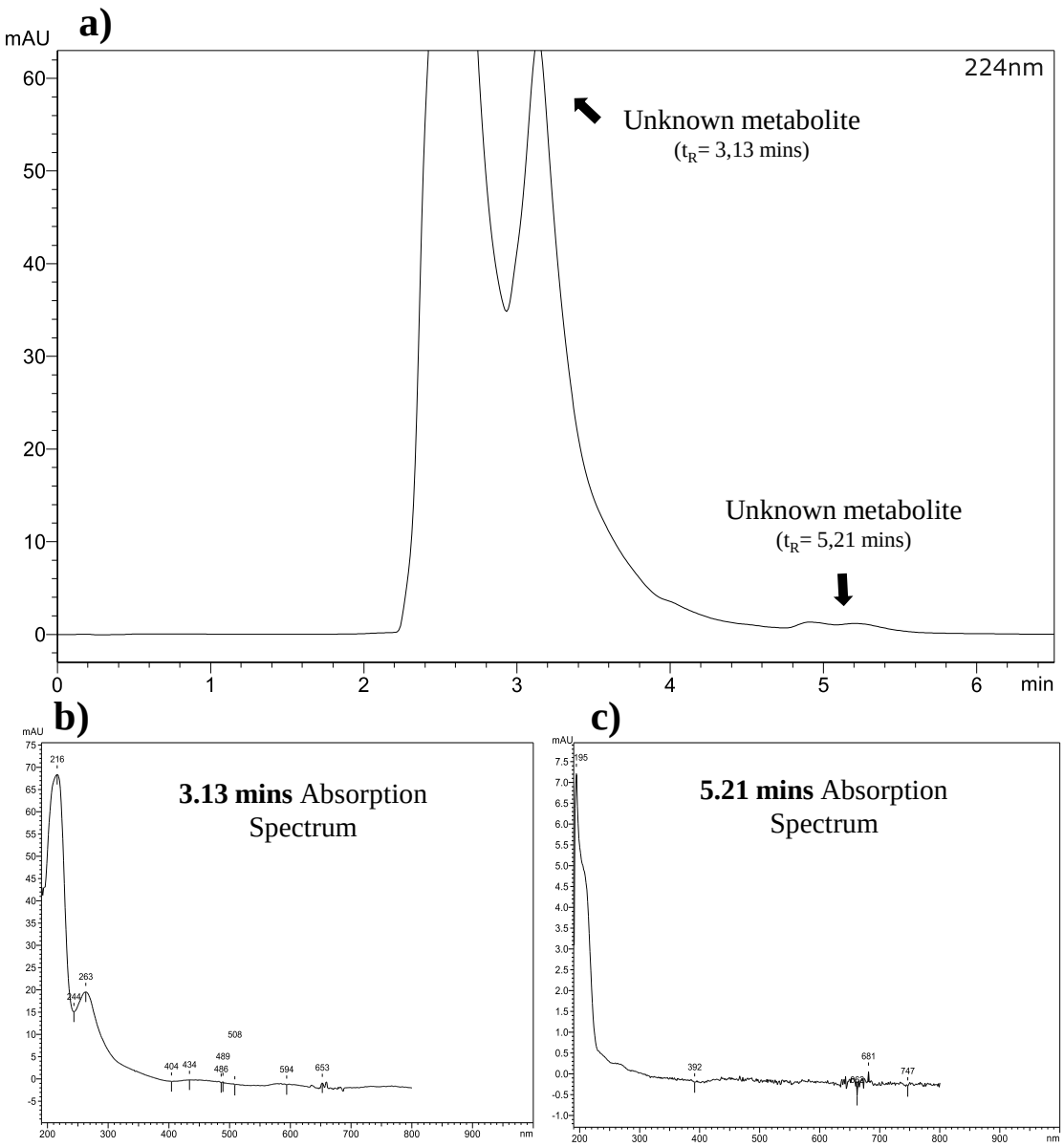




HPB1.1 strain: IBU solubilized in the aqueous Mineral Salt Medium (MSM) analyzed by HPLC in the presence of *M. aubagnense* HPB1.1. a) Chromatogram of unknown metabolite detected at 224nm and b) absorption spectrum of 4,39 mins metabolite.



TJPT4 strain: IBU solubilized in the aqueous Mineral Salt Medium (MSM) analysed by HPLC in the presence of *M. yunannensis* TJPT4. a) Chromatogram of unknown metabolites detected at 224nm, b) absorption spectrum of 3,13 mins metabolite and c) absorption spectrum of 5,21 mins metabolite.



Annex 2: **16S rRNA gene sequeces** in FASTA format of IBU degrading bacteria.

K. pneumoniae **TIBU2.1**

>Contig-TIBU2.1

CTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAA
CGTATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGC
AGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCT
TTGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGACGTCAT
CCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAAC
AAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGAC
AGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGCACCAAACCATCTCTGCTAAGTTCTGTGG
ATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTG
CGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAA
CGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAATCGACATCGTTTACGGCGT
GACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTC
CAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGA
ATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTTCCCAGGTTGAGCCCG
GGGATTTCACATCCGACTTGACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAAC
GCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTA
ACGTCAATCGACAAGGTTATTAACCTTATCGCCTTCCTCCCCGCTGAAAGTGCTTTACAACCCG
AAGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCA
CTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGA
CCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTAGCTAATCCCATCTGGGCA
CATCTGATGGCATGAGGCCCGAAGGTCCCCCACTTTGGTCTTGCGACATTATGCGGTATTAGCT
ACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCCGCCG
CTCGTCACCCGAGAGCAAGCTCTCTGTGCTACCGCTCGACTTGCATGTGTTAG

K. variicola **LOI1.1**

>Contig-LOI1.1

CACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGA
GTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGC
ATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGG
GATTAGCTGGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGAT
GACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGGAGGCAAAACAGTGGGGA
ATAGATACACAATGGTAGTCGCAAGCCTGTAAACGATGCCGATTTGCAGCGTTGTGCCCTTGA
AGAATGGCCTTCCGGATGCTAAAGCACTTAAATCGACCGCCTGGGGAGGAAGGCCCGCAAGG
TTAATAACCTCACCAATTGAAGTTGACCCGCAGAAGAAGCACCGCACTAACTCCGTGCCAGCA
TGCCGCGGTTAATACGGAGGGTGCAACGCGTTAATCGGAACCTTACCTGGGCGTAACAGCGCA
CAGAAGGCGGTCTGTCCAGAGATCGGATGTGAAATCCCTTCGGGCTCAACCTGGGAGACAGCA
TTCGAAACTGGCATGGCTAGAGTCTTGTCAGAGCGGGTTGTAGAAATCCAGGTGTAGCGGTGA
AATCCCGCAGAGATCTGGAGCGCAACCCTTATCCGGTGTTGCCAAAGGCGGCCAGGCCGGGA
ACTCAAAGGACTGACTGCTCAGTGATAAACTGGAGGAAGCGTGGGGATGACGTCAAGTCATC
ATGGACCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTC
GCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCC
ATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGG
GAGGGCGCTTACCACTT

P. aeruginosa **LOI1.1**

>Contig-LOI1.2

TGCGGTTAGACTAGCTACTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAA
GGCCCGGGAACGTATTCACCGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCA
GTCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCT
TGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGA

CTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCCGAG
GTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGA
AAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTC
CACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGC
GGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAACGGCTAGTCGACATC
GTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTG
TCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACC
GCTACACAGGAAATTCCACCACCCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCC
AGGTTGAGCCCGGGGATTTCACATCCAACTTGCTGAACCACCTACGCGCGCTTTACGCCCAGT
AATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCT
TATTCTGTTGGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGT
GCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGT
CCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGA
TCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACTAGCTAAT
CCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAGAAACCCCACTTTCTCCCTCAGGACGTA
TGCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGATTCCTAGGCATTACT
CACCCGTCCGCCGCTGAATCCAGGAGCAAGCTCCCTTCATCCGCTCGAC