Article

Bacterial Degradation of Ibuprofen: Insights into Metabolites, Enzymes, and Environmental Fate Biodegradation of Ibuprofen by *Achromobacter* Species

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Abstract: In recent years, pharmaceuticals have emerged as pollutants due to their incomplete degradation in sewage treatment plants and their ability to cause physiological problems in humans even at low doses. Understanding the environmental fate of pharmaceutical pollutants and the mechanisms involved in their degradation is crucial for developing strategies to mitigate their impact on ecosystems and human health. In this study, the degradation of pharmaceutical compound ibuprofen was achieved by employing two bacterial strains, *Achromobacter spanius* strain S11 and *Achromobacter piechaudii* S18, previously isolated from contaminated water. These strains were capable of degrading ibuprofen as their sole carbon source. The study aimed to identify intermediate metabolites, determine the enzymes involved, and detect specific genes related to ibuprofen degradation. Different concentrations of ibuprofen, temperatures, and pH levels were tested. Both *A. spanius* S11 and *A. piechaudii* S18 successfully degraded ibuprofen. *A. spanius* S11 showed a degradation efficiency of 91.18% after only 72 h and reached 95.7% after 144 h, while *A. piechaudii* S18 exhibited degradation efficiencies of 72.39% and 73.01% after three and seven days, respectively. The LC-MS technique was used to identify biodegradation metabolites produced by *A. spanius* S11. The results indicated that the first step was hydroxylation followed by oxidation via the combination of monooxygenases that catalyze the C-H hydroxylation and dehydrogenases. Furthermore, the detection of intermediate metabolites of trihydroxyibuprofen suggested that the biodegradation of ibuprofen by *A. spanius* S11 can occur through multiple mechanisms. The highest enzyme activities were recorded for catechol 1,2-dioxygenase, 4.230 $\pm$ 0.026 U/mg, followed by laccase, 2.001 $\pm$ 0.215 U/mg. This study demonstrates the potential of *Achromobacter* strains, particularly *A. spanius* (S11), in degrading ibuprofen. These findings provide insights into the ibuprofen degradation process, intermediate metabolites, and relevant genes.

Keywords: *Achromobacter* sp.; ibuprofen; biodegradation; pharmaceuticals; micropollutants

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1. Introduction

Water is a vital component of living systems; however, the purity of drinking water is steadily declining [1]. This decline can be attributed to the leakage of many pollutants into water sources as a result of human activities and the modern industrialization process. Emerging contaminants (ECs) are a broad category of chemical substances that have been found in the environment as a result of common human activities, such as household,

medical, agricultural, and industrial operations [2]. These pollutants encompass a variety of compounds, including pesticides, chemicals generated from different industries, pharmaceuticals and personal care products (PPCPs), artificial hormones, plastic waste, and pharmaceuticals [3–5]. PPCPs comprise many different pharmaceutical compounds including hormones, antibiotics, and non-steroidal anti-inflammatory drugs (NSAIDs), which are rapidly arising as organic contaminants [6]. The wastewater that is found in treatment plants consists of a combination of household waste, either of small- or medium-sized agricultural waste, industrial waste, wastewater from hospitals, and excretions from humans and animals [7,8].

One of the NSAID compounds is ibuprofen (IBU) [2-(4-isobutylphenyl-propionic acid)], with antipyretic, analgesic, and anti-inflammatory properties. It has been reported that ibuprofen, the third most consumed pharmaceutical worldwide, and its metabolites have been detected in water used for irrigation and municipal drinking water supplies [9]. The continuous presence of IBU in many environmental habitats such as soils and water bodies alongside its toxicity in non-target animals, especially to aquatic biota, raised increasing concern and led to the classification of IBU as an emergent pollutant [10].

Most of the employed techniques in wastewater treatment plants (WWTPs) cannot completely remove IBU; hence, it remains in the discharged effluent of these facilities [9]. Studies have reported the cytotoxic effects of IBU, practically, if shared with other pharmaceuticals, on human embryonic cells from the kidney [11]. IBU has been detected in the bile of wild fish living downstream of a WWTP [12], and it has been shown that prolonged exposure to IBU has adverse negative effects on aquatic organisms, practically, the reproduction process of zebrafish [13], planktonic crustaceans [14], and Japanese rice fish [15]. These results demonstrated the potential long-term ecological consequences of IBU on an aquatic environment. Due to population pressures leading to increased consumption, the toxicity and concentration of ibuprofen in WTPP and water bodies are escalating, raising concerns about its bioactive nature and potential hazardous impact on the environment. The largest producer of IBU in the world is China with a total annual production of about 13,000 tons, which represents more than a third of the global market of IBU [16]. The extensive consumption and widespread presence of ibuprofen highlight the need for effective methods to mitigate its environmental impact. Environmental concentrations of IBU have been found to range from low part-per-trillion to low part-per-billion levels [17,18]. It was reported that 15% of orally ingested doses of ibuprofen are excreted by humans unchanged, or as conjugates with glucuronide and thiol. The conjugates undergo further hydrolysis in the environment [19,20]. Recently, IBU can be eliminated efficiently in most modern wastewater treatment plants, with a removal rate of approximately 90%. However, traditional water treatment methods are ineffective in removing ibuprofen, causing it to become widespread in water bodies [21]. The combination of multiple drugs, each with distinct physicochemical characteristics and chemical structures, challenges their removal including IBU. This requires the creation of specific technologies for each, and makes it difficult for microbes or the environment to break them down [22]. Therefore, low-to-trace concentrations of pharmaceutical drugs like IBU continue to be detected in an aquatic environment. The environmental toxicity of ibuprofen on microbiota has been documented; IBU reduces the bacterial biomass of certain riverine biofilm communities when present alongside other pharmaceutical residues [23].

Microorganisms play a crucial role in the degradation process of different types of xenobiotic compounds, such as pesticides and pharmaceutical drugs, either metabolically or co-metabolically, through their strong array of efficient enzymatic processes [24,25]. Many factors determine the ecotoxicity of pharmaceuticals once they enter the ecosystem, depending on various processes such as biodegradation, photodegradation, hydrolysis, and sorption into suspended solids and bed sediments [24]. Photodegradation and microbe-mediated degradation methods have been effectively employed to remove toxicity of many pharmaceutical compounds from the environment. Among these, bacterial degradation is the most significant pathway for pharmaceutical dissipation [26]. The degradation rate is

influenced by factors such as temperature, pH, microbial richness, the presence of biosolids, and the lipophilicity of pharmaceuticals [27].

Some species of the genus *Achromobacter* were reported to have bioremediation potential of organic pollutants. Distinctive bioremediation properties of *Achromobacter* sp. have been reported for the degradation of phenolic pollutants [28], antibiotics [29], and other pharmaceutical drugs [30]. Therefore, this study was performed to explore the ability of two *Achromobacter* sp. for the efficient and complete degradation of IBU under optimized culture conditions. Furthermore, the catabolic degradation pathway for IBU was postulated for the first time based on the detected intermediate metabolites, detected relevant genes, and the activities of enzymes that participated in the degradation process with IBU being the only carbon source.

2. Materials and Methods

2.1. Chemicals and Bacterial Strains

The pure IBU was obtained from Al-Kahira Pharmaceuticals & Chemical Industries Company in Shubra (Cairo, Egypt). A minimal mineral salt (MMS) medium was prepared using the following ingredients: 33.9 g/L Na_2HPO_4, 15 g/L KH_2PO_4, 5 g/L NH_4Cl, 2.5 g/L NaCl, and 25 g/L $MgSO_4{\cdot}7H_2O$ (Laboratories Conda SA, Madrid, Spain). ABTS (2,2′-azino-bis (3-ethyl-benzothiazoline-6 sulfonic acid)), catechol 1, 2 dioxigenase, catechol 2, 3 dioxigenase, and pyrogallol were purchased from Sigma-Aldrich. All the chemicals were of the highest purity with an analytical grade. The soil bacterial strains used in this work are *Achromobacter spanius* strain S11 (OQ504475) and *Achromobacter piechaudii* S18 (OQ504476), which were identified in our previous study [30].

2.2. Biodegradation of IBU and Optimization of Achromobacter sp. Growth at Different Physicochemical Conditions

Both bacterial species were assessed for their growth ability at different concentrations of IBU including 10, 20, 30, and 40 mg/L as the only source of carbon and energy using a sterilized MMS medium. The flasks were prepared by mixing 99 mL of MMS with 1 ml of IBU stock solutions dissolved in distilled water. The IBU stock solutions were filtered through a Millipore syringe filter (0.22 µm) then added to a flask after autoclaving. Each flask was prepared with one of the selected concentrations of IBU. Four flasks were inoculated with an overnight culture of *A.spanius* strain S11, and the other four flasks were inoculated with *A. piechaudii* S18 besides one flask, which served as a control without an inoculum. *Achromobacter* sp. were enumerated by the viable plate counts technique utilizing LB agar media (pH 7.0, Laboratories Conda SA, Madrid, Spain), which were incubated at 37 °C aerobically for 24 h. The whole experiment was conducted three times under identical conditions, and the viable plate count was carried out in each flask at least three times to calculate the average reading. The number of bacterial colonies was expressed as CFU/mL and curves were plotted against incubation time. Similarly, the biodegradation of ibuprofen by each *Achromobacter* sp. was monitored at various temperatures (28, 37, and 40 °C) and different pH levels (4, 5, 6, 7, 8, and 9) following an incubation period ranging from 1 to 168 h using the same method previously described [30].

2.3. Quantification of Ibuprofen Biodegradation

High-performance liquid chromatography (HPLC) was employed to calculate the percentage of IBU degradation by measuring the residual IBU concentration, following a validated method described previously by Nguyen et al. [31]. Based on the previous experiment, samples were collected at two time points, three and seven days, from both cultures of *A. spanius* S11 and *A. piechaudii* S18 grown at 37 °C in MMS media supplemented with 10 mg IBU, pH 7. The samples were centrifuged at 8000× *g* for 10 min, and the cell-free supernatant was filtered (0.45 µm membrane filter; Millipore Corp., Billerica, MA, USA). A

sample volume of 100 µL was injected into the HPLC, and the detection limit was 10 µg/L. The percentage of IBP degradation was calculated using the equation

$$\text{degradation percentage} = (\text{Ci} - \text{Cf}) \times \frac{100}{\text{Cf}}$$

where Ci is the initial concentration of IBU and Cf is the concentration at the end of the experiment (supernatant being cell-free of the sample).

2.4. Metabolite Identification

The biotransformation of IBU by *A. spanius* S11 was monitored using an Ultra-Performance Liquid Chromatography with Electrospray Ionization Quadruple-Linear Ion Trap Tandem Mass Spectrometry (UPLC-ESI-MS/MS) instrument equipped with a C18 column (1.7 µm, 2.1 × 50 mm). The separation technique employed was reverse-phase chromatography and the mobile phase consisted of two solutions, A and B. A was acidified water containing 0.1% formic acid while B was acidified acetonitrile containing 0.1% formic acid. The elution was performed using a gradient of B (10 to 90 percent) over a period of 25 min. ESI-MS was performed in both negative and positive ion modes, with parameters such as cone voltage, capillary voltage, cone gas flow, source temperature, desolvation temperature, and desolvation gas flow that were optimized for the analysis. Mass spectra were detected in the ESI range of m/z 100–1000 atomic mass units, and retention time of IBU was recorded at 13.17 ± 0.20 min. Metabolites were identified based on their spectra and fragmentation patterns. Data processing and identification were performed utilizing Mass Lynx 4.1 software.

2.5. Enzyme Activities

A. spanius S11 was grown first in small flasks (50 mL) containing MMS broth supplemented with either 10 mg/L of ibuprofen or glucose as a control for 48 h. The enzymatic activities including laccase, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, and peroxidase were quantified as described before [30,32], from *A. spanius* S11 cell-free culture supernatants. In brief, the laccase activity was determined using a reaction mixture containing 2 mL of ABTS (10%) dissolved in a 100 mM acetate buffer (pH 4.9), and the activity was determined by measuring at 420 nm. Catechol 1,2-dioxygenase was assayed by quantifying the formation of cis, cis-muconic acid spectrophotometrically at 260 nm (ε_{260} = 16,800/M cm), while the activity of catechol 2,3-dioxygenase was determined by measuring the formation of 2-hydroxymuconic semialdehyde spectrophotometrically at 375 nm (ε_{375} = 36,000/M cm). The protocatechuate 4,5-dioxygenase activity was measured and quantified colormetrically by the formation of 2-hydroxy-4-carboxymuconic semialdehyde at 410 nm (ε_{410} = 9700/M cm). Peroxidase activities were measured based on the rate of the pyrogallol decomposition procedure. All these assays of enzymes were performed three times either at 37 °C or 30 °C, where the control was bacterial culture grown in the MMS supplemented with glucose instead of ibuprofen, and the reference blank contained the same contents but with inactivated enzymes (boiled). The specific enzyme activities' units were defined as a change in absorbance (unit/min/mg of protein).

2.6. Molecular Screening of Biodegradable Enzymes of Ibuprofen Using Polymerase Chain Reaction (PCR)

Monooxygenase and dioxygenase genes are the key enzymes in IBU degradation [19,20]. In order to screen for biodegradable enzymes of IBU, the polymerase chain reaction (PCR) was employed using specific primers for alkanal monooxygenase and phytanoyl-CoA dioxygenase genes. The primers were designed based on the full genome sequence of *A. spanius* strain NCTC13519 published in the GenBank using the NCBI PRIMER BLAST tool. The target partial sequence of the alkane monooxygenase gene was amplified using forward alkM-F 5′ GACAGGCCGATTGAATTCCC 3′ and reverse alkM-R 5′ GCGATG-GAAAATGCAGCAAC 3′ primers, while Dioxy-F 5′ GGGAACGCTTTGAAGAGGAC 3′

and Dioxy-R5′ CTCAGATCGCGTTGCAAGAA were used for phytanoyl-CoA dioxygenase genes. Pure DNA was extracted from each bacterial strain using a DNA extraction kit (Wizard SV Genomic DNA, Promega, Madison, WI, USA). PCR was performed with an Applied Biosystems 9700 thermal cycler (PE Life Sciences, New York, NY, USA) programmed as the following: 5 min initial denaturation at 94 °C, followed by 30 cycles (94 °C denaturation for 30 s, 52 °C annealing for 30 s, 72 °C extension for 40 s), and a final extension at 72 °C for 10 min. In total, 10 µL of PCR products from each *Achromobacter* species were analyzed on agarose gel (1% *w*/*v*) stained with ethidium bromide (0.5 mg/mL) and then visualized under ultraviolet light. The expected amplicon sizes for alkanal monooxygenase and phytanoyl-CoA dioxygenase genes were 550 and 500 bp, respectively. The DNA ladder (100 pb Gene Ruler, GeneDirex Taipei, Taiwan) was used as a DNA size marker. The purified amplicons of the alkane monooxygenase gene fragment (550 bp) were sequenced using the alkM-F and alkM-R primers by Macrogen (Seoul, Republic of Korea) and a contig sequence was created and then deposited in the NCBI nucleotide sequence databases. The obtained contig was aligned with references' sequence with high similarity to other *A. spanius* strains in GenBank by using the NCBI BLAST server. The phylogenetic relationship was constructed using the neighbor-joining method using DNASTAR software (version 7, DNASTAR, Inc., Madison, WI, USA), with 1000 bootstrap replications.

2.7. Statistical Analysis

All results are represented in figures or tables as the mean value ± standard deviation (SD) of three replicates. The statistical differences between different treatments were identified using one-way ANOVA programming followed by Tukey's HSD test (Minitab 18) and are represented by different letters ($p \leq 0.05$).

3. Results

3.1. Optimum Growth Parameters for Ibuprofen-Degrading Bacteria

The tested *Achromobacter* species used in this work, *Achromobacter spanius* strain S11 and *Achromobacter piechaudii* S18, had the ability to grow on MMS plates containing IBU as a sole carbon and energy source. To optimize their growth conditions and subsequently IBU degradation, different growth parameters were tested individually in the MMS medium supplemented with IBU as a sole carbon source. The results revealed that both bacterial species can grow and utilize IBU at all tested concentrations (10, 20, 30, and 40 mg/L) with incubation time. The growth of the *A. spanius* S11 bacterium at different concentrations of IBU was affected by increasing the tested concentration of IBU, and the CFU counts were increased up to 30 mg/L. However, at a higher concentration (40 mg/L), a considerable decrease in growth was observed compared to low concentrations of IBU, 10, 20, and 30 mg/mL. The maximum growth was recorded after 48 h of incubation at 30 mg/L (Figure 1a). A similar trend was observed for *A. piechaudii* S18, which showed maximum growth at 30 mg/L after 48 h (Figure 1b). Therefore, the concentration of 30 mg/L was further tested to optimize the growth at different temperatures and pH.

The effect of incubation temperature influences the ability of bacterial growth and consequently the degradation of IBU. The results revealed that 37 °C is the best temperature for growth of both bacteria after 48 h of incubation and the decrease in the temperature to 28 °C decreases the growth of both bacterial species, S11 and S18 (Figure 2a,b).

In addition, at 43 °C, the growth of bacteria was unfavorable for both *Achromobacter* species (Figure 2a,b). The effect of pH on the growth and biodegradation of IBU (30 mg/L) at 37 °C was further tested at different pH, ranging from 5 to 9. The results in Figure 3a,b show that the neutral pH was favored for the growth and biodegradation of IBU for both species. The deviation from pH 7 led to a considerable decrease in growth of *A. spanius* S11, whereas slight changes were observed in the *A. piechaudii* S18 growth after 48 h of incubation.

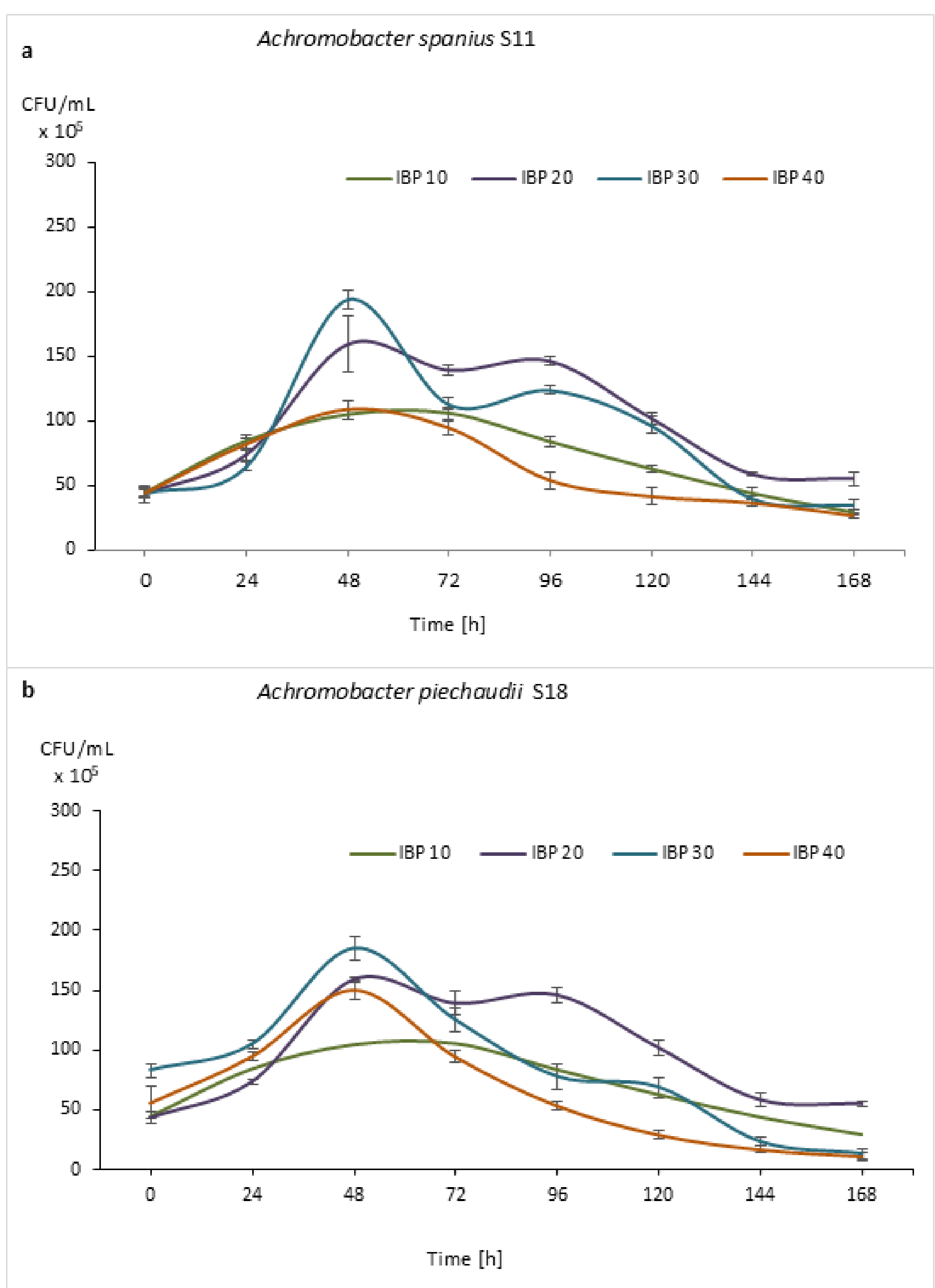

Figure 1. The growth of *Achromobacter spanius* strain S11 (**a**) and *Achromobacter piechaudii* S18 (**b**) as monitored by the viable count method (CFU/mL) after different time intervals (1–168 h) of bacterial growth in minimal mineral salt (MMS) media supplemented with different concentrations of ibuprofen (10, 20, 30, and 40 mg/L) as a sole carbon source.

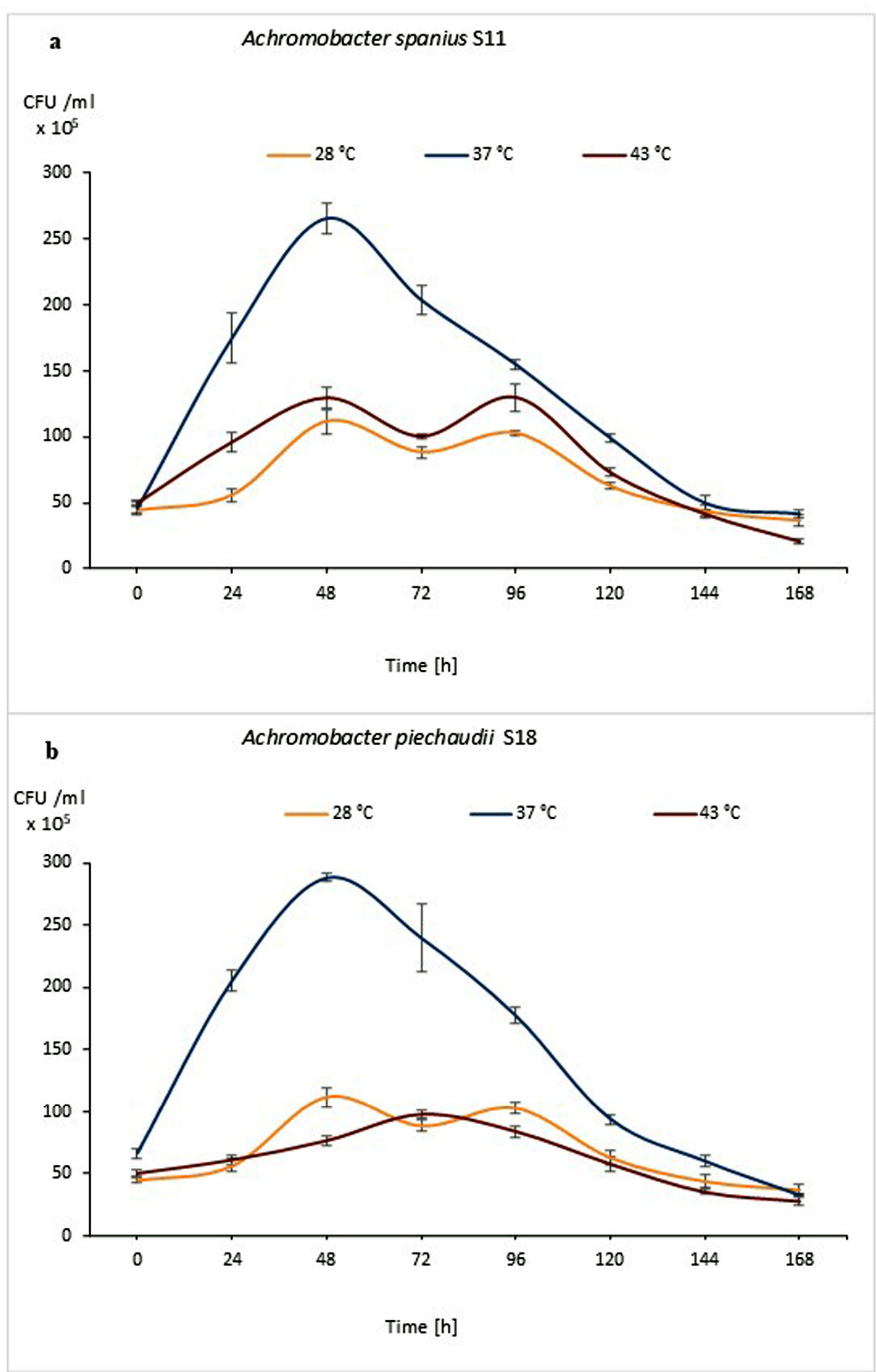

Figure 2. The effect of temperature (28, 37, and 43 °C) on the growth of *Achromobacter spanius* strain S11 (**a**) and *Achromobacter piechaudii* S18 (**b**) as monitored by the viable count method (CFU/mL) after different time intervals (1–168 h) in minimal mineral salt (MMS) media supplemented with 30 mg/L IBU as the sole carbon source. The curve was drawn by mean values ± SD of three independent replicates.

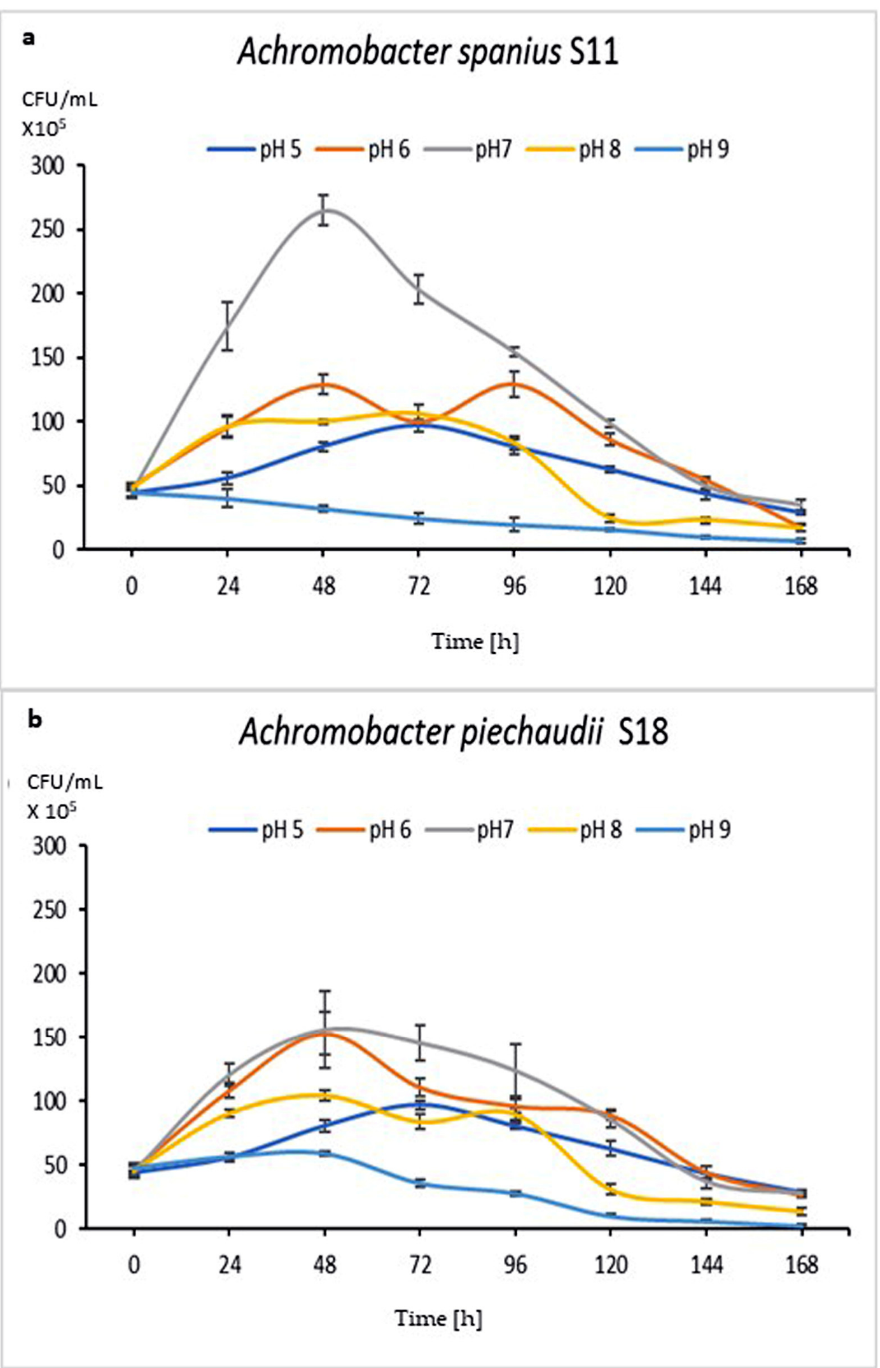

Figure 3. The effect of different pH values (5, 6, 7, 8, and 9) on the growth (CFU/mL) of *Achromobacter spanius* strain S11 (**a**) and *Achromobacter piechaudii* S18 (**b**) as monitored by the viable count method (CFU/mL) after different time intervals (1–168 h) with an initial ibuprofen concentration of 30 mg/L as the sole carbon source in the minimal mineral salt (MMS) medium. The curve was drawn by mean values ± SD of three independent replicates.

3.2. Efficiency of IBU Biodegradation by Achromobacter Species

Based on the obtained data for the optimal conditions of bacterial growth in MMS media supplemented with IBU as a sole carbon source, the efficacy of biodegradation was monitored using an HPLC analysis. The results revealed specific peaks of candidate intermediate IBU products, indicating the utilization of IBU by *A. spanius* S11 as a carbon and energy source (Figure 4).

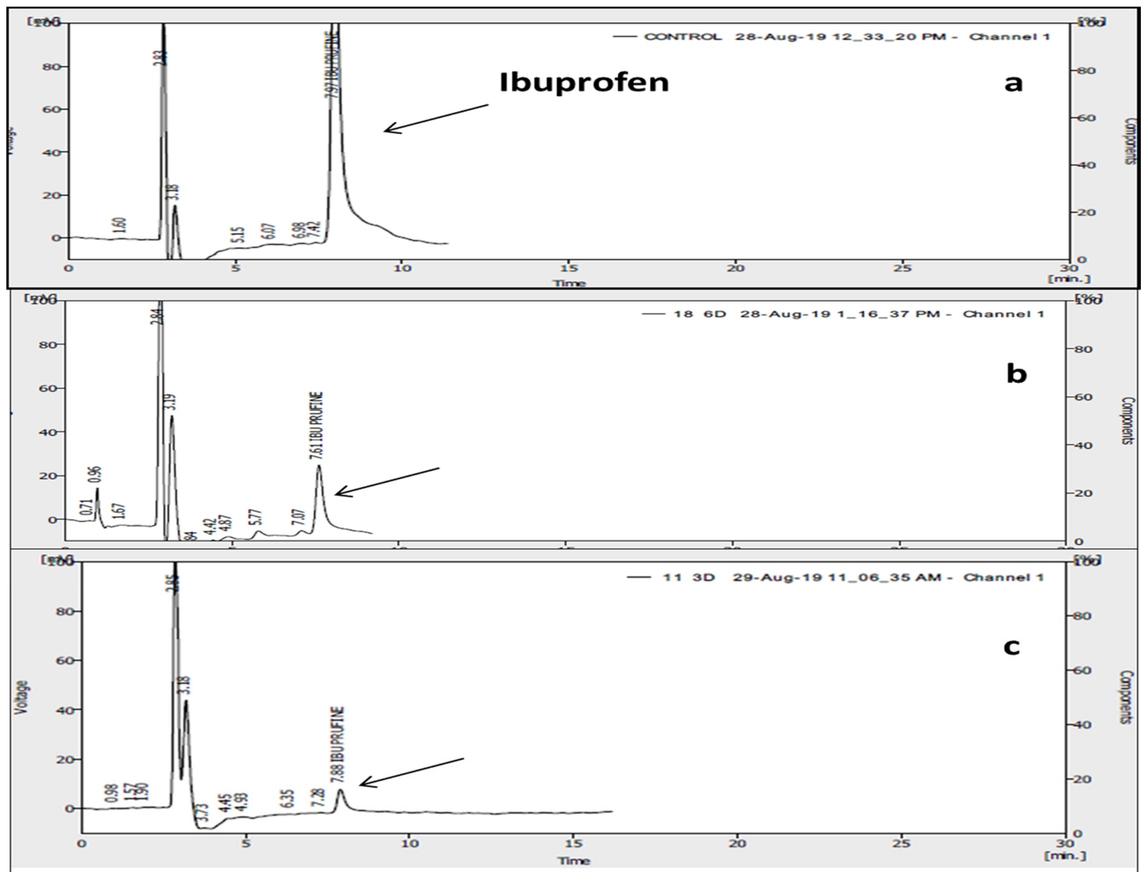

Figure 4. The LC-MS chromatogram product ion profile for the biodegradation of IBU in the minimal mineral medium with an initial concentration of ibuprofen of 40 mg/L. (**a**) The standard without bacterial inoculation, (**b**) medium inoculated by *A. piechaudii* S18, and (**c**) medium inoculated by *A. Spanius* S11 after 144 h of incubation. The black arrow indicates the characteristic peak of IBU.

Table 1 summarizes the percentage of the degradation of IBU; a remarkable high percentage of degradation was recorded for the *A. spanius* S11 after only 72 h of incubation, 91.18%. A prolonged incubation period to 144 h slightly increased the degradation to 95.4%. On the other hand, *A. piechaudii* S18 showed significant lower degradation rates than *A. spanius* S11 and recorded 72.39% and 73.01% after 72 h and 144 h of incubation, respectively. The bacteria-free control recorded almost no change after the same period of incubation under the same experiment condition.

Table 1. The Ibuprofen degradation percentages in MMS after incubation at 37 °C for 72 and 144 h.

Isolates	Degradation of Ibuprofen Percentage *	
	72 h	144 h
S11	91.18 ± 1.19 ^{c}	95.40 ± 0.84 ^{c}
S18	72.39 ± 1.55 ^{b}	73.01 ± 0.89 ^{b}
Control	01. 02 ± 0.28 ^{a}	01.15 ± 0.47 ^{a}

* The initial ibuprofen concentration in the MMS medium was 40 mg/L. The percentages of degradation are calculated as means of three independent replicates ± SD. The different lowercase letters (a, b, c) beside the value of means indicate a significant difference at the probability level of 0.05 within the same time point, as analyzed by Tukey's test.

The biotransformation process of IBU was most effective when the cells of *A. spanius* were pre-grown in an LB medium for two days and collected during the exponential growth phase. The complete biotransformation of IBU was achieved after 144 h of incubation at concentrations of 10, 20, 30, and 40 mg/L. The average rate of IBU bioconversion was

0.66 ± 1.44 mg/h, where maximum values were reached after 72 h at an average rate of 1.26 ± 3.25 mg/h. Based on the previous degradation results, *A. spanius* S11 was further characterized because it was more efficient than *A. piechaudii* S18 for IBU degradation. To determine and identify the intermediate biodegradation metabolites, the LC-MS technique was carried out for the metabolite's identification.

3.3. Postulation of IBU Biotransformation Pathway by A. spanius S11

The results of the LC-MS product ion profile (Figure 4) represent the main 14 metabolites detected through the experimental degradation metabolites as compared to the parent (IBU, m/z 205.211). The chemical structures of these products were elucidated from their mass patterns (Table 2). Based on these structures, the biodegradation mechanism could be anticipated (Figure 5). These results demonstrated that IBU biotransformation was achieved by hydroxylation, leading to the formation of a monohydroxylated product with a mass of 221.117 m/z, confirming the molecular composition of a $C_{13}H_{18}O_3$ fragmentation pattern of the monohydroxylated product detected by the LC-MS/MS and corresponding to 1-hydroxyibuprofen (2-[4-(1-hydroxy-2-methylpropyl) phenyl] propanoic acid) (metabolite 2) or 2-hydroxyibuprofen (2-[4-(2-hydroxy-2-methylpropyl) phenyl] propanoic acid) (metabolite 3), and the subsequent formation of a 1,2-Dihydroxyibuprofen derivative with 237.048 m/z, confirming the molecular composition of $C_{13}H_{18}O_4$ (metabolite 4). Consequently, another intermediate metabolite with m/z 281.0655 and molecular formula $C_{13}H_{14}O_7$ corresponds to 4-(2-formyl-1,1-dihydroxy-4-oxobutyl) phenyl] (hydroxy)acetic acid (metabolite 5).

Table 2. Summary of compounds detected by LC-MS/MS with retention times, molecular composition, chemical structure, and characteristic ions detected by (+) ESI-MS during IBU biodegradation by *A. spanius* S11.

	Compound Name	RT (Min)	Molecular Composition	M.Wt.	[M-H] + Measured	Chemical Structure
1	Ibuprofen	13.17	$C_{13}H_{18}O_2$	206.2808	205.2113	OH, O
2	1-hydroxyibuprofen	11.58	$C_{13}H_{18}O_3$	222.2802	221.1386	CH3, CH3, H3C, OH, O, OH
3	2-hydroxyibuprofen 2-[4-(2-hydroxy-2-methylpropyl)phenyl] propionic acid	11.58	$C_{13}H_{18}O_3$	222.2802	221.1386	CH3, HO, CH3, H3C, O, OH
4	1,2-Dihydroxyibuprofen 2-[4-(1,2-dihydroxy-2-methylpropyl) phenyl] propionic acid	19.44	$C_{13}H_{18}O_4$	238.2796	237.0482	CH3, HO, CH3, H3C, OH, O, OH
5	4-(2-formyl-1,1-dihydroxy-4-oxobutyl)phenyl] (hydroxy)acetic acid	22.42	$C_{13}H_{14}O_7$	282	281	OH, CHO, OH, OHC, OH, OH, O

Table 2. *Cont.*

	Compound Name	RT (Min)	Molecular Composition	M.Wt.	[M-H] + Measured	Chemical Structure
6	Trihydroxyibuprofen 2-[4-(1,2-dihydroxy-2-methylpropyl)-3-hydroxyphenyl] propanoic acid	23.17	$C_{13}H_{18}O_5$	254.279	253.2053	
7	2-ethyl-5-methylbenzene-1,4-diol	26.84	$C_9H_{12}O_2$	152.19	151.075	
8	(5-ethyl-4-hydroxy-2-methylphenyl)oxidanyl	23.25	$C_9H_{11}O_2$	151.182	150.06	
9	(5-ethyl-4-hydroxy-2-methylphenyl)oxidanyl	23.46	$C_8H_9O_2^+$	137.155	136.05	
10	(4-ethenylcyclohexa-1,3-dien-1-yl) methylium	27.45	$C_9H_9^+$	117.167	116.89	
11	(3,4-dihydroxyphenyl) acetic acid		$C_8H_8O_4$	168.146	167.033	
12	benzene-1,2,4-triol	22.69	$C_6H_6O_3$	126.11	125.896	
13	(2E,4Z)-3-hydroxyhexa-2,4-dienedioic acid	17.9	$C_6H_6O_5$	158.108	157.1706	
14	2-methylpentanoic acid	23.63	$C_6H_{12}O_2$	116	115.08	
15	(2S)-2,3-dihydroxy butanedioic acid	28.69	$C_4H_6O_6$	150.09	149.95	

Figure 5. Proposed pathway for ibuprofen biodegradation by *A. spanius* S11.

The presence of metabolite 6 with m/z 253.138 confirming the molecular composition of $C_{13}H_{18}O_5$ corresponds to trihydroxy ibuprofen (2-[4-(1,2-dihydroxy-2-methylpropyl)-3-hydroxyphenyl] propanoic acid). In addition, metabolite 7 with m/z at 151 and a molecular formula of $C_9H_{12}O_2$ corresponds to 2-ethyl-5-methylbenzene-1,4-diolindicating. Moreover, five intermediates were identified as metabolites of trihydroxyibuprofen: the metabolite with m/z 167.0338 and molecular formula $C_8H_8O_4$ corresponds to (3,4-dihydroxyphenyl) acetic acid (metabolite 11), the metabolite at m/z 125.9603 and with molecular formula $C_6H_6O_3$ corresponds to 2-hydroxy–1,4 hydroquinol (metabolite 12), and the metabolite with m/z 157.01315 and molecular formula $C_6H_6O_5$ corresponds to 3-hydroxycis, cis-muconic acid (metabolite 13).

The above products were detected in the first 72 h of *A. spanius* S11 incubation with IBU in the MMS medium. However, after prolonged incubation (144 h), two other major metabolites spectroscopically similar to 2-methylpentanoic acid (metabolite 14) and (2S)-2,3-dihydroxybutanedioic acid (metabolite 15) were detected. Taken together, the biodegradation of IBU can be performed by *A. spanius* S11 by more than one mechanism (Figure 5).

3.4. Specific Activity of Enzymes Involved in Ibuprofen Degradation

The proposed degradation pathway of IBU by the *Achromobacter spanius* strain S11 as well as the detected genes established the involvement of different enzyme activities including monooxygenase, dioxygenase, peroxidase, and laccase. To confirm the production of these expected enzymes in the biodegradation pathway, the activities of selected enzymes including laccase, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, catechol 4,5-dioxygenase, and peroxidase were assessed in the liquid culture of *A. spanius* S11. Therefore, the enzymatic activities of above-mentioned enzymes were measured both in MMS supplemented with IBU and in the control medium supplemented with glucose instead of IBU as a carbon source, while monooxygenase was not determined in this study. The results demonstrated that the activity of the catechol 1,2-dioxygenase enzyme recorded the highest specific activity, 4.230 ± 0.026 U/mg protein, in the presence of IBU followed by the enzyme laccase, 2.001 ± 0.215 U/mg protein (Table 3). These enzymatic activities were induced only in the cells grown in the medium supplemented with IBU compared to control culture supplemented with glucose under the same conditions.

Table 3. Activities of some ibuprofen degradation-related enzymes (U/mg protein) for *A. spanius* strain S11, in the presence of either ibuprofen or glucose as a control.

Specific Enzyme Activity (U/mg Protein)		
Enzyme	**Control ***	**MMS+ Ibuprofen ****
Laccase	0.098 ± 0.013	2.001 ± 0.215
Catechol 1,2-Dioxygenase	0.655 ± 0.064	4.230 ± 0.026
Catechol 2,3-Dioxygenase	0.002 ± 0.006	0.016 ± 0.004
Catechol 4,5-Dioxygenase	0.000 ± 0.00	0.000 ± 0.00
Peroxidase	0.0184 ± 0.022	0.255 ± 0.059

* The minimal mineral salt (MMS) medium supplemented with 10 mg/L glucose was used as the control. ** The MMS medium with 10 mg/L ibuprofen was the sole carbon source.

However, the specific activity of peroxidase achieved 0.255 ± 0.059 U/mg protein and almost no activities were recorded for either catechol 2,3-dioxygenase or catechol 4,5-dioxygenase (Table 3).

3.5. Detection of Genes Involved in Biodegradation of Ibuprofen by Achromobacter spanius Strain S11

In order to detect possible genes involved in the IBU biodegradation, specific primers targeting a partial sequence of the alkane monooxygenase and phytanoyl-CoA dioxygenase genes was designed based on sequences published from the *A. spanius* strain NCTC13519 genome. The results revealed that both genes were amplified by PCR and amplicons were detected at the expected size of 550 for alkanal monooxygenase and 500 for phytanoyl-CoA dioxygenase (Figure 6).

To confirm the identity of the formed PCR products, a sequencing analysis was performed on a 550 bp fragment of the alkane monooxygenase gene. The sequences were compared to other nucleotide sequences in GenBank using the NCBI BLAST program, which revealed the high similarity to other alkane monooxygenase genes from different *Achromobacter* species including *A. spanius* strain UQ283 (99.9% similarity), *A. spanius* strain NCTC13519 (95.85% similarity), and *A. spanius* strain DSM 23806 (95.85% similarity). The top five homologous hits were selected for neighbor joining and multiple alignments using Clustal W. The alignment results were used to construct a phylogenetic tree. The nucleotide sequence data were deposited in the NCBI nucleotide sequence databases (Figure 7).

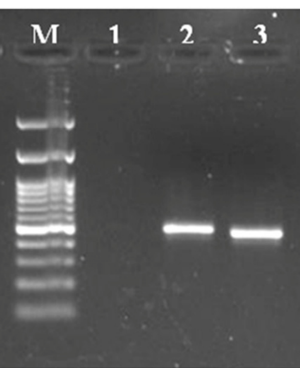

Figure 6. Detection of alkanal monooxygenase and phytanoyl-CoA dioxygenase genes in genomic DNA of *Achromobacter spanius* S11 by PCR. Lanes: M, 100 bp ladder; 1, non-template control; 2, alkanal monooxygenase gene fragment; and 3, phytanoyl-CoA dioxygenase gene fragment.

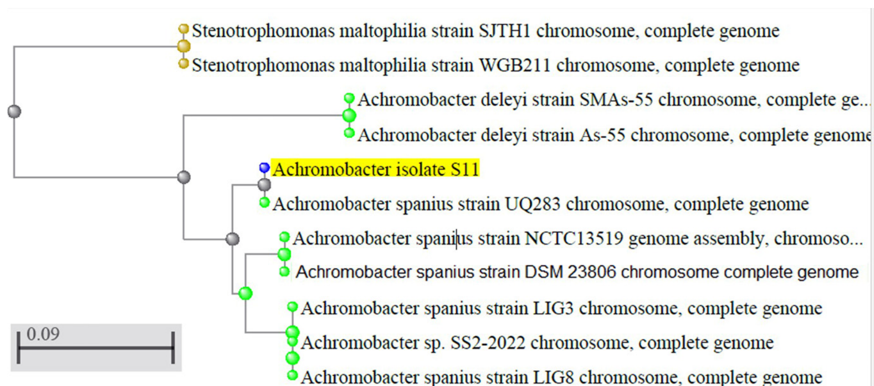

Figure 7. The neighbor-joining phylogenetic tree for the alkanal monooxygenase gene sequence of the best ibuprofen-degrading strain, *A. spanius* strain S11, with the top ten aligned sequences in GenBank. The tree was generated by Clustal W and the bacterium, *A. spanius* S11, is highlighted in yellow.

4. Discussion

Pharmaceuticals play a crucial role in public health; however, their extensive usage, unsafe removal in the environment, and contribution as micropollutants are pressing concerns for the improvement of degradation processes. The management of pharmaceutical contaminants, including used and expired products, personal home-care items, and over-the-counter medications, has emerged as a significant global health challenge [33]. IBU is a widely used pain-relieving drug in both human and veterinary medicine, listed in the WHO Essential Drug List, and is known for its anti-inflammatory, antipyretic, and analgesic effects. It is employed in the treatment of conditions such as osteoarthritis, gout, pericarditis, and cancer [10,34]. The production volume of IBU is estimated to be in the thousands of tons per year [16]. IBU primarily enters the environment through wastewater due to its extensive use, stable molecular structure, incomplete breakdown in the human body, and improper disposal practices of unused and expired IBU [10,35]. In this study, the tested bacteria, *A. spanius* and *A. piechaudii*, were found to thrive in the presence of IBU as the sole carbon and energy source, providing evidence of their high capabilities of the use of IBU in their metabolic processes. *A. spanius* exhibited efficient degradation, even at high concentrations of up to 40 mg/L of IBU. It was observed that both bacterial species required a relatively extended lag phase with increasing IBU concentration, and their cell count reached a stationary phase after seven days of incubation. Previous studies have also investigated the biodegradation of IBU using different bacterial isolates such as *Nocardia* sp. NRRL 5646 [36], *Sphingomonas* sp. Ibu-2, and *Variovorax* sp. Ibu-1 at IBU concentrations ranging from 50 to 1000 mg/L, with degradation rates ranging from approximately

70 mg/L/day to 870 mg/L/day [37]. Another study examined a relatively lower IBU concentration (10 µg/L) under varying biomass concentrations (~50–1000 mg/L) and reported degradation rates ranging from approximately 0.001 to 0.06 mg/L/day [38]. Furthermore, strain B1(2015b) was capable of degrading 20 mg/L of ibuprofen within six days, but it only degraded 46.56% of 25 mg/L of ibuprofen over 20 days [20]. These studies also highlighted the ability of different bacterial species to degrade ibuprofen at high concentrations. In the present study, *A. spanius* S11 and *A. piechaudii* S18 were found to degrade IBU. After 72 h of incubation, *A. spanius* S11 degraded IBU by 91.18%, while *A. piechaudii* S18 degraded it by 72.39%. After 144 h of incubation, *A. piechaudii* S18 achieved 73.01% degradation. In this consideration, we conducted a basic examination utilizing a run of databases and survey in the literature related to the biodegradation of IBU and *Achromobacter* species. This deficiency of exploration underscores the peculiarity and potential value of examining *Achromobacter's* capacity for IBU biodegradation, considering the known bacterium efficient enzymatic activity and applicability in xenobiotic degradation [30]. Therefore, there is a gap in knowledge of *Achromobacter's* metabolic pathways involved in IBU degradation. This gap highlights a critical opportunity for future exploration to clarify the nuclear components and specific genetic components that empower IBU biodegradation.

Many pharmaceutical pollutants, including IBU, exhibit acute toxicity, with their toxicity assessed through short-term EC_{50} values (ranging from 10 to 100 mg/L). The cytotoxic and genotoxic effects of these analgesics are evaluated after prolonged exposure. Prolonged exposure is primarily associated with imbalances in the redox state of a cell [39]. According to Van't Hoff's principle, the chemical reaction speed increases by 2–4 fold for a 10 °C rise in temperature [40]. Nonetheless, the effect of temperature on the processes of degradation in biological systems is more complex due to the relationship between cell membrane function and temperature. Excessively high temperatures can cause the denaturation of membrane-bound proteins, while excessively low temperatures can increase the viscosity of membrane phospholipids, leading to membrane rigidity and hindered membrane transport [41]. The concentration of IBU typically found in the Nile River in Cairo, Egypt, is 0.002 mg. To overcome the limitations of HPLC in determining the categories of substances, LC-MS was utilized to identify possible intermediate products based on m/z values and previous reports.

The LC-MS analysis resulted in the identification of 14 main metabolites during the experimental degradation of IBU compared to the parent compound (IBU, m/z 205.211). The chemical structures of these products were elucidated based on their mass patterns (Table 1). These structures provide insight into the anticipated biodegradation mechanism (Figure 5). The results indicated that the first step in the IBU biotransformation was hydroxylation, confirmed by the formation of monohydroxylated products with a mass of m/z 221.117 (metabolite 2 or metabolite 3) and a derivative of metabolites 2 and 3 with a mass of m/z 237.048 and a molecular composition of $C_{13}H_{18}O_4$ (metabolite 4), indicating a possibility that cytochromp-450 monooxygenase plays a key role in the first step of IBU biotransformation. Under toxic environmental conditions, the hydroxylation of xenobiotic compounds is a typical first reaction during the degradation process of IBU in both bacteria as well as eukaryotes [19,20]. The formation of metabolite 5, which verifies the double oxidation of 1,2-dihydroxyibuprofen to the corresponding aldehydes, was possible via the combination of NADH- or NADPH-dependent monooxygenases that catalyzed the C-H hydroxylation with dehydrogenases that are $NAD(P)^+$-dependent to facilitate the conversion of the formed intermediate alcohol into the carbonyl product [30]. The CH_3 group's selective oxidation to their corresponding aldehydes poses a challenge in bacterial cell biotransformation, as it involve factors beyond mere enzymatic activities [42]. This is attributed to the activity of endogenous aldehyde dehydrogenases, which catalyze the undesired overoxidation [43].

Another scenario that could be suggested due to the presence of metabolite 6 was the detection of metabolite 7, which may be a substrate for acyl-CoA synthase. Activity of this enzyme was also observed during ibuprofen degradation by *Sphingomonas* Ibu-2 [37]. Fur-

thermore, the involvement of this enzyme in ibuprofen decomposition was demonstrated in a study performed by *Patulibacter* sp. strain I 11 [44]. Indeed, 2-(4-hydroxyphenyl-) propionic acid was suggested to be the substrate for acyl-CoA synthase/thiolase during the pathway degradation of ibuprofen by *Bacillus thuringiensis* B1 [44]. The hydroxyl derivative was exposed to the subtracting of an electron and H^+ from the hydroxyl phenolic group by peroxidases, generating a phenoxy radical. The formation of phenoxy radicals followed by the sequential formation of carbonium ions' metabolite 8 to 10 can be generated by both of the enzymes laccase and peroxidase [30,45,46]. In addition, intermediate metabolites of trihydroxyibuprofen that were detected, the metabolite with m/z 167.0338 (metabolite 11), the metabolite with m/z 125.9603 (metabolite 12), and the metabolite with m/z 157.01315 (metabolite 13), strongly supported this scenario. These findings indicate that the biodegradation of IBU by *A. spanius* S11 can occur through multiple mechanisms.

On the other hand, the results of enzymatic activities of *A. spanius* S11 reflect the induction effect of the IBU for the enzyme production of catechol 1,2-dioxygenase and laccase enzymes. The enhanced degrading activities of *A. spanius* S11 are in line with the capabilities of different *Achromobacter* strains for the production of monooxygenase, dioxygenase, laccase, and peroxidase enzymes that facilitate the biodegradation of different types of xenobiotic compounds such as *Achromobacter* sp. HZ01, *Achromobacter* sp. DMS1, and *Achromobacter xylosoxidans* DN002 for fluoranthene degradation [16], and *Achromobacter denitrificans* PR1 during the degradation process of antibiotic sulfonamides as the only carbon and nitrogen source [29]. Indeed, we previously reported the involvement of these enzymes in the biodegradation of another pharmaceutical compound, diclofenac, by *A. spanius* and *A. piechaudii* [30]. The presence of genes encoding for laccases, in the genome of *Achromobacter denitrificans* strain EPI24, confirmed the bioremediation potential of *Achromobacter* species under versatile and efficient conditions [47]. Moreover, the detection of monooxygenase and dioxygenase in the *A. spanius* and *A. piechaudii* suggested high enzymatic capabilities of *Achromobacter* spp. in the biodegradation process.

The study highlights the potential of these bacterial strains to degrade IBU, shedding light on the biodegradation pathways involved. The LC-MS analysis provided valuable insights into the intermediate products formed during the degradation process, contributing to our understanding of the transformation of IBU in the environment. Further research and analyses are necessary to fully elucidate the complete degradation pathways and the ecological implications of IBU degradation by these bacterial strains.

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