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Wydział Nauk Przyrodniczych

Instytut Biologii, Biotechnologii i Ochrony Środowiska

ANNA DZIONEK

Praca doktorska

Wpływ immobilizacji komórek bakteryjnych na biodegradację naproksenu

Promotor:
dr hab. Urszula Guzik, prof. UŚ
Uniwersytet Śląski w Katowicach

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Spis treści

I. Autoreferat rozprawy

I.1. Wprowadzenie

W ostatnich latach, coraz więcej uwagi poświęcane jest obecności niesteroidowych leków przeciwzapalnych (NLPZ) w środowisku naturalnym. Pomimo, że są wykrywane w stężeniach nie przekraczających 1 μg/L, ich chroniczne oddziaływanie na ekosystemy może zaburzać ich prawidłowe funkcjonowanie. Do grupy trudnodegradowalnych NLPZ należy naproksen (kwas 2-(6-metoksynaftalen-2-ylo)propanowy), który ze względu na działanie przeciwbólowe oraz przeciwzapalne jest powszechnie stosowany na całym świecie. Jednakże, lek ten nie jest całkowicie metabolizowany w organizmie ludzkim [Wojcieszyńska i in. 2016; Domaradzka i in. 2015; Grenni i in. 2013].

Ze względu na złożoną strukturę naproksenu, jego utylizacja w oczyszczalniach ścieków nie zachodzi z dużą wydajnością. Ponadto lek ten wykazuje wrażliwość na światło UV. Warto podkreślić, że ostatnią fazą oczyszczania ścieków jest ich sterylizacja z wykorzystaniem lamp UV. Skutkuje to uwolnieniem do środowiska naturalnego nie tylko naproksenu, ale również produktów jego fototransformacji. Badania wykazały, że produkty fotodegradacji naproksenu są bardziej toksyczne dla organizmów żywych niż sam lek [Isidori i in. 2005]. Zasadnym jest zatem poszukiwanie i opracowywanie biologicznych metod utylizacji naproksenu.

Dotychczas opisano tylko kilka szczepów mikroorganizmów posiadających systemy enzymatyczne umożliwiające degradację naproksenu. Spośród szczepów z kolekcji Zespołu Biochemii i Genetyki Mikroorganizmów jedynie *Bacillus thuringiensis* B1(2015b) rozkłada całkowicie naproksen w stężeniu 6 mg/L w ciągu 30 dni, w obecności dodatkowego źródła węgla [Marchlewicz i in. 2016]. Szczep *Planococcus* sp. S5 taką samą dawkę leku degraduje po upływie 38 dni [Dzionek i in. 2018]. Ze względu na potencjał degradacyjny tych szczepów mogą one znaleźć zastosowanie w oczyszczaniu ścieków.

Bioaugmentacja systemów bioremediacyjnych szczepami zdolnymi do rozkładu konkretnych zanieczyszczeń ma swoje zalety. Największą korzyścią jest usunięcie zanieczyszczeń, których nawet niewielkie stężenie może wykazywać toksyczny wpływ na autochtoniczną mikroflorę systemu bioremediacyjnego. Jednakże, aby biodegradacja zanieczyszczenia była efektywna przez dłuższy okres czasu, wprowadzony szczep powinien wykazywać zdolność do kolonizacji systemu i namnażania się w nim. Sukces bioaugmentacji jest uzależniony od wielu czynników. Najważniejszym z nich są predyspozycje wprowadzanych mikroorganizmów oraz sposób ich wprowadzenia do systemu. Wynika to z faktu, że systemy bioremediacyjne, w szczególności w oczyszczalniach ścieków, stanowią złożone i kompleksowe społeczności mikroorganizmów, które ukształtowały się

w wyniku szeregu oddziaływań międzygatunkowych i środowiskowych. Zatem wprowadzenie obcego szczepu do takiego środowiska może skutkować jego szybkim wyparciem z systemu [Xu i in. 2010; Ma i in. 2009].

Aby zwiększyć przeżywalność mikroorganizmów, stosuje się ich immobilizację, która dodatkowo obniża koszty procesów bioremediacyjnych, jednocześnie zwiększając ich efektywność. Najpopularniejszą metodą immobilizacji stosowaną w bioremediacji jest adsorpcja na powierzchni, ponieważ skutkuje wprowadzeniem do systemu ukształtowanego biofilmu, który wykazuje niższą wrażliwość na metabolity innych mikroorganizmów oraz wahania parametrów środowiskowych [Martins i in. 2013]. Zdolność do wytwarzania biofilmu na powierzchni porowatych materiałów jest kluczową cechą warunkującą efektywną immobilizację. Ponadto, poprzez dobór optymalnych warunków immobilizacji, a także odpowiedniego nośnika, możliwe jest zwiększenie efektywności wytwarzania biofilmu. Jednak unieruchomienie mikroorganizmów w biofilmie może wiązać się również z ograniczeniem dyfuzji oraz osłabieniem ich możliwości katalitycznych [Xu i in, 2010]. Wynika to ze zróżnicowanych profili ekspresji genów komórek znajdujących się w biofilmie [Flemming i in. 2016].

Ciekawym zagadnieniem są także interakcje bakterii z powierzchnią nośnika. Wykazano bowiem, że rozpoczęcie procesu adhezji jest zróżnicowane w zależności od rodzaju materiału i skutkuje utworzeniem przez ten sam szczep bakteryjny biofilmu o różnej strukturze i właściwościach [Dalton i in. 1994]. Interakcje bakteria-nośnik są zależne od oddziaływań elektrostatycznych, hydrofobowych, topografii powierzchni, a także od składu chemicznego materiału, w tym od obecności i rodzaju grup funkcyjnych [Tuson i in. 2013]. Przedstawiona złożoność procesu immobilizacji może skutkować znamiennym wpływem na zdolność, kinetykę, a także szlak biodegradacji zanieczyszczeń przez szczepy bakteryjne. Charakterystyka metod immobilizacji, ze szczególnym uwzględnieniem adsorpcji na powierzchni oraz przykładów ich zastosowania w bioremediacji, została opisana w publikacji przeglądowej **Dzionek i in. [2016]**, zamieszczonej w **rozdziale II.1** niniejszej rozprawy doktorskiej.

I.2. Cel pracy doktorskiej

Ze względu na ograniczoną wiedzę na temat degradacji niesteroidowych leków przeciwzapalnych przez immobilizowane mikroorganizmy, konieczne jest zbadanie wpływu unieruchamiania szczepów zdolnych do degradacji leków na kinetykę ich rozkładu. W związku z powyższym, w pracy podjęto próbę immobilizacji szczepów bakteryjnych wykazujących zwiększoną zdolność do degradacji naproksenu. Dokonano również analizy wpływu unieruchamiania na kinetykę rozkładu leku zarówno w warunkach monokulturowych, jak i w obecności autochtonicznej mikroflory systemu bioremediacyjnego.

Głównymi celami badawczymi pracy doktorskiej były:

- Charakterystyka metaboliczna biofilmu;
- Optymalizacja warunków immobilizacji;
- Charakterystyka nośnika oraz unieruchomionych mikroorganizmów;
- Opracowanie modelu rozkładu naproksenu przez immobilizowane szczepy bakteryjne oraz określenie wpływu immobilizacji na ten proces;
- Określenie wpływu wprowadzenia immobilizowanych mikroorganizmów do złoża biologicznego na rozkład naproksenu.

I.3. Materiały i metody

W celu przygotowania inokulum do immobilizacji, sporządzono zawiesiny dwóch szczepów bakteryjnych należących do kolekcji Zespołu Biochemii i Genetyki Mikroorganizmów. Szczep *Bacillus thuringiensis* B1(2015b) wytrząsano w bulionie odżywczym lub pożywce HCT o składzie: 5 g/L tryptonu, 2 g/L hydrolizatu kazeiny, 3 g/L glukozy, 6,8 g/L KH_2PO_4, 0,12 g/L $MgSO_4$ x $7H_2O$, 0,0022 g/L $MnSO_4$ x $4H_2O$, 0,014 g/L $ZnSO_4$ x $7H_2O$, 0,02 g/L $Fe_2(SO_4)_3$ oraz 0,18 g/L $CaC1_2$ x $4H_2O$ przez 24 godziny w 30°C [Lecadet i in. 1980]. Szczep *Planococcus* sp. S5 hodowano w bulionie odżywczym przez 72 godziny w 30°C. Następnie, namnożone hodowle zwirowano (5000*g*, 4ºC, 15 min) i zawieszono w jałowej minimalnej pożywce mineralnej (MSM) o składzie: 3,78 g/L Na_2HPO_4 x $12H_2O$, 0,5 g/L KH_2PO_4, 5 g/L NH_4Cl, 0,2 g/L $MgSO_4$ x $7H_2O$ oraz 0,01 g/L ekstraktu drożdżowego [Greń i in. 2009].

Jako nośniki, w immobilizacji metodą adsorpcji na powierzchni, zastosowano piankę poliuretanową (PUR; Instapak®, Charlotte, NY, USA) oraz gąbkę Loofah (York, Bolechowo, Poland). Przygotowanie pianki poliuretanowej do immobilizacji obejmowało uformowanie z niej sześcianów o wymiarach 1 x 1 x 1 cm, ważących 10 ± 5 mg. Gąbki Loofah poddawano procesowi suszenia w eksykatorze w celu ustalenia suchej masy oraz uformowano z nich prostopadłościany o wadze 150 mg. Uzyskane nośniki kolejno dwukrotnie płukano w wodzie destylowanej oraz sterylizowano (121ºC, 1,2 atm, 20 min).

Immobilizacja komórek B1(2015b) metodą adsorpcji na powierzchni na piance poliuretanowej przeprowadzona była w następujący sposób: 0,1 g PUR inkubowano w 100 ml zawiesiny komórek B1(2015b) o gęstości optycznej 0,8 (OD_{600}) w 30°C. Po 72 godzinach nośnik zawieszono w 0,9% NaCl, zwirowano (500 rpm, 4ºC, 2 min) i zawieszono w 0,9% NaCl. W celu dokonania charakterystyki metabolicznej biofilmu oraz znajdujących się w nim komórek bakteryjnych, przeprowadzono optymalizację i walidację metody pomiaru aktywności niespecyficznych esteraz z zastosowaniem dioctanu fluoresceiny, bez naruszania struktury biofilmu. Aby tego dokonać, zmodyfikowano metodę opracowaną przez Liang i in. [2009]. Modyfikacja polegała na pominięciu etapu odrywania komórek od nośnika. Analizie poddawano cały nośnik wraz z nienaruszonym biofilmem. Wykonano testy adsorpcji fluoresceiny przez nośnik oraz autohydrolizy dioctanu fluoresceiny. Przeprowadzono optymalizację parametrów reakcji, takich jak sposób aplikacji dioctanu fluoresceiny, prędkość wytrząsania, pH buforu fosforanowego oraz czas inkubacji. W celu ustalenia czułości metody, analizę aktywności niespecyficznych esteraz immobilizowanych bakterii na nośniku przeprowadzono po inkubacji przez 24, 48 oraz 72 godzin w pożywce bez źródła węgla. Ponadto w celu oceny zmian strukturalnych biofilmu przeprowadzono w skaningowym mikroskopie elektronowym (SEM) obserwację

biofilmu poddanego procesowi głodzenia. Aby dokonać walidacji metody, uzyskane wyniki testów czułości porównano z wynikami pomiaru zużycia tlenu (OUR, ang. *oxygen uptake rate*) przez immobilizowane bakterie wraz z nośnikiem zgodnie z procedurą opracowaną przez De Beer i in. [1994] oraz Amon i in. [1996]. Szczegółowy opis modyfikacji metody analizy aktywności mikroorganizmów z zastosowaniem dioctanu fluoresceiny i warunków analiz znajduje się w pracy **Dzionek i in. [2018a]** zamieszczonej w **rozdziale II.2** niniejszej rozprawy doktorskiej.

W kolejnym etapie badań, przeprowadzono proces immobilizacji szczepów *Planococcus* sp. S5 oraz *Bacillus thuringiensis* B1(2015b) na gąbce Loofah. Aby uzyskać najwyższą wydajność immobilizacji oraz biofilm o największej aktywności metabolicznej, dokonano optymalizacji warunków immobilizacji, która obejmowała parametry takie jak: wiek hodowli, pożywka, suplementacja glukozą, chlorkiem sodu lub solami metali, początkowa gęstość optyczna inokulum, czas i temperatura inkubacji oraz pH pożywki, a także prędkość wytrząsania. Wybór optymalnych warunków procesu immobilizacji dokonywano na podstawie pomiaru masy uzyskanego biofilmu oraz opracowanej analizy aktywności niespecyficznych esteraz z zastosowaniem dioctanu fluoresceiny, bez naruszania struktury biofilmu. Zoptymalizowana procedura immobilizacji komórek S5 przedstawiała się następująco: 0,75 g gąbki Loofah inkubowano w 100 ml zawiesiny komórek S5 o gęstości optycznej 1,2 (OD_{600}) w pożywce MSM o pH 7,2, suplementowanej glukozą (0,5 g/L), NaCl (10 g/L) oraz $MnSO_4$ (0,01 g/L) i wytrząsanej przy prędkości 90 rpm przez 72 godziny w 30°C. Natomiast zoptymalizowany protokół immobilizacji komórek B1(2015b) był następujący: 0,75 g gąbki Loofah inkubowano w 100 ml zawiesiny komórek B1(2015b) o gęstości optycznej 0,2 (OD_{600}) w pożywce HCT o pH 8, suplementowanej glukozą (0,5 g/L) oraz $MnSO_4$ (1 g/L) i wytrząsanej przy prędkości 110 rpm przez 48 godzin w 20°C. W kolejnym kroku gąbki Loofah z immobilizowanymi komórkami S5 lub B1(2015b) płukano dwukrotnie 0,9% roztworem NaCl i wykorzystywano w dalszych badaniach [Dzionek i in. 2018b; 2020].

Aby opracować modele rozkładu naproksenu przez immobilizowane szczepy bakteryjne, wykonano testy kometabolicznej biodegradacji naproksenu. W tym celu przygotowano układy zawierające immobilizowane komórki szczepu S5 oraz pożywkę MSM suplementowaną naproksenem w stężeniu 6, 9, 12 lub 15 mg/L. Co 3 dni, układy suplementowane były glukozą w stężeniu 0,5 g/L. Aby określić wpływ immobilizacji na degradację leku, przygotowano układy zawierające zawiesiny komórek S5 oraz naproksen w analizowanych stężeniach. Ponadto przygotowano układ zawierający immobilizowane komórki S5 oraz pożywkę MSM, której suplementacja naproksenem w stężeniu 6 mg/L wykonywana była każdorazowo po całkowitym rozkładzie podanej dawki [Dzionek i in. 2018b].

Stężenie naproksenu określano z użyciem wysokosprawnej chromatografii cieczowej w odwróconym układzie faz (HPLC) [Wojcieszyńska i in. 2014].

W trakcie badań poddano analizie aktywność enzymów zaangażowanych w rozkład naproksenu. W tym celu przygotowano hodowle immobilizowanych na gąbce Loofah komórek szczepu S5 (15-dniowe) oraz nieimmobilizowanych zawiesin komórek S5 (15 oraz 35-dniowe) na podłożu MSM suplementowanym glukozą (0,5 g/L) oraz naproksenem (6 mg/L). Komórki oddzielono od medium poprzez wirowanie (4500*g*, 4ºC, 15 min), a w celu oderwania ich od nośnika dodatkowo zastosowano worteksowanie. Otrzymany osad komórek bakteryjnych zawieszono w 50 mM buforze fosforanowym o pH 7,0, poddano procesowi sonikacji (6 x 15 sek.) oraz ponownie zwirowano (9000*g*, 4ºC, 30 min.).

Aktywność *O*-demetylazy wyznaczono poprzez analizę ubytku substratu, kwasu wanilinowego przy λ = 260 nm. Mieszanina reakcyjna (1 ml) obejmowała 100 mM buforu TRIS-HCl, 500 µM kwasu wanilinowego, 1 mM kwasu tetrahydrofoliowego oraz ekstrakt enzymatyczny. Reakcję przeprowadzano w warunkach beztlenowych przez 10 min. w 30ºC. Po zatrzymaniu reakcji metanolem, mieszaninę poddano analizie HPLC [Abe i in. 2005]. Aktywność monooksygenaz aromatycznych określono spektrofotometrycznie poprzez śledzenie szybkości utleniania NADH przy długości fali λ = 340 nm (ε = 6220 M^{-1} cm^{-1}). Mieszanina reakcyjna zawierała 50 mM buforu fosforanowego, 200 µM NADH oraz 8,8 µM FAD, wybrane związki aromatyczne w stężeniu 1 mM (fenol, naproksen), a także ekstrakt enzymatyczny [Wojcieszyńska i in. 2011]. Aktywności dioksygenazy naftalenowej mierzono spektrofotometrycznie przez monitorowanie powstawania produktu reakcji - *cis,cis*-dihydrodiolu przy długości fali λ = 262 nm (ε = 8230 M^{-1} cm^{-1}), w obecności 50 mM wysyconego naftalenem buforu fosforanowego Na-K [Cidaria i in. 1994]. Dla oznaczenia aktywności dioksygenazy 1,2-gentyzynianowej kontrolowano pojawianie się produktu reakcji - maleilopirogronianu przy długości fali λ = 330 nm (ε = 10800 M^{-1} cm^{-1}) w obecności 0,33 mM gentyzyny rozpuszczonej w 0,1 M buforze fosforanowym [Feng i in. 1999]. Aktywność dioksygenazy 1,2-salicylowej określono poprzez spektrofotometryczne oznaczenie powstawania produktu reakcji - kwasu 2-oksohepta-3,5-dienodiowego przy λ = 283 nm (ε = 13600 M^{-1} cm^{-1}). Mieszanina reakcyjna zawierała 20 µM buforu TRIS-HCl oraz 0,1 µM salicylanu [Hintner i in. 2001].

Aby określić wpływ immobilizacji mikroorganizmów na rozkład naproksenu w systemie bioremediacyjnym skonstruowano złoża biologiczne. Każde z nich składało się z czterech ruchomych podjednostek o wymiarach 400 x 100 mm (łącznie 1600 x 100 mm), aby możliwe było wprowadzenie immobilizowanych na gąbce Loofah bakterii B1(2015b) na różnych wysokościach systemu. Złoża biologiczne augmentowano autochtoniczną mikroflorą pochodzącą z komory

przepływowej osadnika Imhoff'a w Krupskim Młynie – Ziętek, w celu ukształtowania błony biologicznej. Po 21 dniach do złóż wprowadzono immobilizowane komórki B1(2015b) oraz syntetyczne ścieki o następującym składzie: 0,317 g/L CH_3COONH_4, 0,04 g/L NH_4Cl, 0,024 g/L K_2HPO_4, 0,008 g/L KH_2PO_4, 0,1 g/L $CaCO_3$, 0,2 g/L $MgSO_4$ x $7H_2O$, 0,04 g/L NaCl, 0,005 g/L $FeSO_4$ x $7H_2O$, 0,5 g/L glukozy oraz 1 mg/L naproksenu w zamkniętym obiegu. Co 3 dni syntetyczne ścieki suplementowano glukozą oraz makro- i mikroelementami. W celu oceny wydajności pracy złóż biologicznych wykonywano pomiar chemicznego zapotrzebowania na tlen (ChZT) metodą z dichromianem potasu [Dzionek i in. 2020].

W trakcie badań określono również wpływ wprowadzenia do złoża biologicznego immobilizowanych na gąbce Loofah komórek B1(2015b) na autochtoniczną mikroflorę systemu bioremediacyjnego. W tym celu, przed i po procesie biodegradacji naproksenu, z próbek błony biologicznej oraz gąbek Loofah wyizolowano DNA oraz amplifikowano metodą PCR region V3-V5 bakteryjnego genu 16S rRNA oraz region ITS1/ITS2 eukariotycznego genu rRNA. Dokonano rozdziału uzyskanych fragmentów DNA poprzez elektroforezę w gradiencie czynnika denaturującego (DGGE) oraz poddano sekwencjonowaniu [Płocinniczak i in. 2013; Anderson i in. 2003]. Na podstawie analizy intensywności prążków DNA na żelach DGGE wyliczono indeks Shannon'a-Wiener'a (*H*') obrazujący różnorodność mikrobiologiczną populacji [Xia i in. 2008].

W trakcie badań dokonano również obserwacji struktury gąbki Loofah oraz biofilmu z zastosowaniem skaningowej mikroskopii elektronowej. W tym celu fragmenty nośnika, bez lub z immobilizowanymi bakteriami B1(2015b) lub S5, poddano utrwaleniu w 3% glutaraldehydzie przez 24 godziny oraz 1% czterotlenku osmu przez 3 godziny. Kolejno, dokonano odwodnienia próbek poprzez szereg alkoholowy (30, 50, 70, 80, 90, 95 oraz 100% etanol) oraz poddano liofilizacji. Uzyskane próbki następnie pokryto złotem i analizowano w mikroskopie skaningowym JSM-7100F TTL LV [Dzionek i in. 2018b].

I.4. Wyniki i dyskusja

Obecnie najczęściej stosowane metody oceny wydajności immobilizacji zakładają wysiew oderwanych z biofilmu mikroorganizmów i szacowanie jednostek tworzących kolonie (CFU), bądź oznaczanie suchej masy biofilmu. Jednakże żadna z tych metod, nie przedstawia stanu fizjologicznego unieruchomionych komórek. Badania aktywności enzymatycznej immobilizowanych mikroorganizmów opierają się na ich uwolnieniu z macierzy, co skutkuje uzyskaniem niepełnej aktywności całego biofilmu. W ramach badań, podjęto próbę modyfikacji istniejącej metody oceny stanu fizjologicznego immobilizowanych komórek w biofilmie bazującej na hydrolizie dioctanu fluoresceiny (FDA) [Dzionek i in 2018a; Liang i in. 2009]. Modyfikacja polegała na ominięciu etapu odrywania biofilmu od nośnika i wykonanie testu na nienaruszonym biofilmie wraz z nośnikiem.

Badania nad modyfikacją testu przeprowadzono na immobilizowanych na piance poliuretanowej (PUR) komórkach szczepu *Bacillus thuringiensis* B1(2015b). Analiza wstępna wykazała, że w badanym zakresie stężenia fluoresceiny (0,5–5 μg/mL), jej adsorpcja przez PUR nie przekraczała 9%, jednakże zależała od początkowego stężenia. Z tego względu, aby test przedstawiał prawidłową aktywność niespecyficznych esteraz, należy przeprowadzić analizę adsorpcji fluoresceiny na zastosowanym nośniku. Istotnym czynnikiem wpływającym na powtarzalność testu, okazał się być sposób aplikacji FDA. Wynika to ze złożonej i heterogennej struktury biofilmu, co utrudnia dyfuzję FDA do wnętrza biofilmu [Flemming i in. 2016]. Zatem najniższy współczynnik zmienności wynoszący 7% uzyskano w próbach, w których FDA wstrzykiwane było bezpośrednio w nośnik. Przeprowadzona optymalizacja warunków testu wykazała, że najwyższe wartości całkowitej aktywności metabolicznej (TEA) uzyskano podczas wytrząsania próbek w buforze fosforanowym o pH w zakresie 7,4-7,6 przez 1 godzinę. Istotną obserwacją był brak akumulacji fluoresceiny w biofilmie po 1,5 godz. inkubacji.

W celu określenia czułości testu, dokonano oceny wpływu obniżenia metabolizmu pod wpływem głodzenia na proces immobilizacji. W pierwszej dobie eksperymentu obserwowano najwyższą wartość TEA (360 ± 24 μg/g suchej masy na godz.), co świadczy o zużywaniu przez immobilizowane komórki nagromadzonych zapasów energetycznych. Wraz z wydłużaniem czasu inkubacji, po 72 godzinach, TEA obniżyła się dwukrotnie (170 ± 7 μg/g suchej masy na godz.) ze względu na wyczerpanie substancji zapasowych i ograniczenie aktywności metabolicznej. Uzyskane wyniki były zgodne z obserwacjami Gengenbacher i in. [2010] oraz Voelker i in. [1995], którzy zaobserwowali wyraźny spadek produkcji ATP przez szczepy *Bacillus subtilis* i *Mycobacterium tuberculosis*, w wyniku pozbawienia związków odżywczych. Porównując wartości TEA uzyskane przez unieruchomione komórki B1(2015b) oraz przez wolne komórki

inkubowane w tych samych warunkach zaobserwowano, że immobilizacja ogranicza niekorzystny wpływ głodzenia na komórki. Po 48 godzinach inkubacji w medium pozbawionym źródeł węgla, wartość TEA wolnych komórek B1(2015b) wynosiła 161 ± 17 μg/g suchej masy na godz. i utrzymywała się na takim poziomie aż do końca eksperymentu. Można zatem wnioskować, że minimalny endogenny metabolizm szczepu *Bacillus thuringiensis* B1(2015b) wynosi 161-170 μg/g suchej masy na godz. Obserwacja biofilmu dokonana z zastosowaniem skaningowej mikroskopii elektronowej (SEM) wykazała pozytywny wpływ głodzenia na proces tworzenia biofilmu. Wynika to z faktu, że niedobór substancji odżywczych indukuję sporulację, która promuje proces produkcji egzopolisacharydów u szczepów blisko spokrewnionych z B1(2015b), takich jak *Bacillus subtilis* i *Bacillus cereus* [Mielich-Süss i in. 2015; Sonenshein, 2000].

Uzyskane wyniki TEA metodą z dioctanem fluoresceiny podczas głodzenia porównano z wynikami pomiaru zużycia tlenu. Zaobserwowano bardzo wysoką korelację trendu spadku TEA oraz OUR podczas eksperymentu, co świadczy o wiarygodności opracowanej metody. Szczegółowe informacje dotyczące walidacji metody oceny stanu fizjologicznego immobilizowanych komórek bakteryjnych bez naruszania struktury biofilmu, a także wpływu głodzenia na unieruchomione na piance poliuretanowej komórki szczepu *Bacillus thuringiensis* B1(2015b) zaprezentowano w pracy **Dzionek i in. [2018a]** zamieszczonej w **rozdziale II.2** niniejszej pracy.

Badania nad wpływem immobilizacji szczepów *Bacillus thuringiensis* B1(2015b) oraz *Planococcus* sp. S5 na gąbce Loofah na ich zdolności degradacji naproksenu poprzedzono optymalizacją procesu unieruchamiania, w celu uzyskania biofilmu charakteryzującego się najwyższą wartością TEA.

W trakcie optymalizacji immobilizacji szczepu *Planococcus* sp. S5 wykazano efektywniejszą indukcję wytwarzania biofilmu w obecności glukozy. Götz [2002] wykazał, że blisko spokrewnione z rodzajem *Planococcus* szczepy *Staphylococcus aureus* oraz *Staphylococcus epidermidis* wymagały suplementacji glukozą podczas immobilizacji, która indukowała syntezę adhezyn biorących udział w początkowej adsorpcji komórek do powierzchni nośnika. Cechą charakterystyczną szczepów z rodzaju *Planococcus* jest zwiększona tolerancja na stres osmotyczny. Cecha ta była również widoczna u komórek szczepu S5, których biofilm osiągał wyższe wartości TEA w medium dodatkowo suplementowanym NaCl w stężeniu 19 g/L. Fenomen ten wynika z przystosowań halofilów do wyższego zasolenia. Szczepy halofilne pod wpływem stresu osmotycznego produkują większe ilości egzopolisacharydów, których dodatkową funkcją jest zwiększanie pojemności wodnej biofilmu [Quarashi i in. 2012]. Procedura immobilizacji szczepu *Planococcus* sp. S5 na gąbce Loofah skutkowała najwyższymi wartościami TEA podczas 72 godzinnej inkubacji w minimalnej

pożywce mineralnej (MSM) o pH 7,2 suplementowanej glukozą (0,5 g/L), NaCl (19 g/L) oraz $MnSO_4$ (0,05 g/L), wytrząsanej przy 90 rpm w 30°C oraz przy wysokiej koncentracji komórek (OD_{600} = 1,2). Uzyskany tą procedurą biofilm na powierzchni jednej gąbki Loofah charakteryzuję się TEA wynoszącą 1250,26 ± 87,61 μg/g suchej masy na godz.

Immobilizacja szczepu *Bacillus thuringiensis* B1(2015b) na gąbce Loofah wykazała odmienne mechanizmy indukcji biofilmu. Proces unieruchamiania był najwydajniejszy w pH 8, w pożywce HTC bogatej w źródła węgla oraz sole mineralne, dodatkowo suplementowanej glukozą. Wytłumaczeniem tego zjawiska jest neutralizacja ujemnego ładunku ściany komórkowej oraz powierzchni nośnika poprzez wysoką siłę jonową bogatej pożywki HTC [Palmer i in. 2007]. Ograniczyło to oddziaływania elektrostatyczne i tym samym umożliwiło adhezję komórek B1(2015b) do gąbki Loofah. Ze względu na fakt, że mangan stymuluje sporulację i tym samym produkcję biofilmu u szczepów z rodzaju *Bacillus*, sprawdzono jak dodatkowa suplementacja manganem wpłynie na aktywność enzymatyczną biofilmu szczepu B1(2015b). Badania wykazały, że mangan w stężeniu 0,33 mM znacząco wpłynął na zwiększenie TEA uzyskanego biofilmu. Warto zaznaczyć, że Morikawa i in. [2006] zaobserwował, że mangan w stężeniu nawet 1 M stymulował produkcję biofilmu przez szczep *Bacillus subtilis* B1. Podsumowując, immobilizacja szczepu *Bacillus thuringiensis* B1(2015b) na gąbce Loofah przebiegała najwydajniej na podłożu HTC o pH 8, suplementowanej glukozą (0,5 g/L) oraz $MnSO_4$ (1 g/L), wytrząsanej przy 110 rpm w 20°C przez 48 godziny przy niskiej koncentracji komórek (OD_{600} = 0,2). Biofilm szczepu B1(2015b) na powierzchni gąbki Loofah charakteryzował się wartością TEA wynoszącą 790,14 ± 40,60 μg/g suchej masy na godz. Szczegółowe informacje dotyczące procesów optymalizacji immobilizacji szczepów *Planococcus* sp. S5 oraz *Bacillus thuringiensis* B1(2015b) na gąbce Loofah zaprezentowano w pracach **Dzionek i in. [2018b]** oraz **Dzionek i in. [2020]** zamieszczonych odpowiednio w **rozdziałach II.3** i **II.4** niniejszej pracy.

W kolejnym etapie badań przeprowadzono testy biodegradacji naproksenu przez immobilizowane na gąbce Loofah szczepy *Planococcus* sp. S5 oraz *Bacillus thuringiensis* B1(2015b).

Analiza modelu rozkładu leku przez szczep S5 została przeprowadzona w warunkach monokulturowych z zastosowaniem różnych stężeń naproksenu (6, 9, 12 oraz 15 mg/L), zarówno przez komórki unieruchomione na gąbce Loofah, jak i przez zawiesiny komórek. Zaobserwowano, że wolne komórki S5 były zdolne do całkowitego rozkładu leku w stężeniu 6, 9 oraz 12 mg/L w odpowiednio 38, 44 oraz 62 dni. Biodegradacja najwyższego analizowanego stężenia leku (15 mg/L) zakończyła się po rozkładzie 29% leku w czasie trwania eksperymentu. Wyniki te udowodniły, że naproksen w stężeniu wyższym niż 12 mg/L wykazuje

działanie hamujące na wolne komórki szczepu *Planococcus* sp. S5. Analiza krzywej rozkładu leku przez szczep S5 wykazała wolniejszy przebieg degradacji w początkowej fazie inkubacji, który był niezależny od początkowego stężenia leku. W tej fazie wolne komórki S5 rozkładały 6,3 ± 3,4 μg naproksenu na godzinę. Po 29 dniach zaobserwowano fazę szybszej degradacji, podczas której komórki S5 były zdolne do rozkładu 12,0 ± 4,5 μg leku w ciągu godziny.

Immobilizowane na gąbce Loofah komórki S5, były zdolne do całkowitej degradacji naproksenu w analizowanych dawkach w ciągu 17, 32, 43 oraz 53 dni. Zatem dzięki immobilizacji, czas rozkładu naproksenu przez szczep S5 został niemal dwukrotnie skrócony. Wykazano również niższą wrażliwość unieruchomionych komórek S5 na lek, co skutkowało całkowitym rozkładem najwyższej analizowanej dawki leku. Analiza szybkości rozkładu w poszczególnych dniach inkubacji wykazała, że tempo rozkładu leku było stałe, niezależne od dnia inkubacji, a także od początkowego stężenia naproksenu. Co ciekawe, prędkość ta wynosiła 12,1 ± 4 μg leku na godzinę i nie różniła się od tempa rozkładu naproksenu przez wolne komórki S5 w fazie szybszej degradacji leku. Wynika to z faktu, że immobilizowane komórki wykazują cechy charakterystyczne hodowli w fazie stacjonarnej [Flemming i in. 2016], a to właśnie w tej fazie wolne komórki S5 degradowały lek najwydajniej. Brak fazy adaptacyjnej immobilizowanych komórek S5 wskazuje na ich dobrą adaptację do rozkładu leku, nawet w najwyższym stężeniu. Biofilm charakteryzuje się dobrymi właściwościami sorpcyjnymi, a także ograniczoną dyfuzją. Dzięki tym cechom, komórki w biofilmie mają mniejszy kontakt z zanieczyszczeniem, który jest transportowany do komórek w stałym, wolniejszym tempie. W efekcie, immobilizowane komórki są mniej narażone na ich szkodliwe oddziaływanie, i tym samym, mogą degradować ich wyższe stężenia [Rahman i in. 2006].

W trakcie badań sprawdzono również przebieg wielokrotnych cykli degradacji naproksenu w stężeniu 6 mg/L przez immobilizowane na gąbce Loofah komórki *Planococcus* sp. S5. Całkowity rozkład naproksenu był obserwowalny w trzech cyklach. Pierwsza i druga dawka leku została zdegradowana po odpowiednio 17 i 15 dniach inkubacji, w trzecim cyklu zauważono natomiast wolniejszy przebieg rozkładu, który trwał 21 dni. W czwartym cyklu, tylko 27% leku uległo biodegradacji. Tempo rozkładu leku w ciągu pierwszych trzech cykli było podobne i wynosiło odpowiednio 14,8 ± 3,0, 16,0 ± 6,9 oraz 11,4 ± 3,9 μg na godzinę. Jednakże immobilizowane na gąbce Loofah komórki szczepu *Planococcus* sp. S5 wykazywały pełną zdolność degradacyjną przez 55 dni, co sugeruje, że immobilizacja wydłuża aktywność katalityczną komórek, co może być efektem zwiększenia stabilności błon komórkowych [Rahman i in. 2006]. Dokonano także analizy w skaningowym mikroskopie elektronowym (SEM) zmian struktury biofilmu szczepu S5 na powierzchni gąbki Loofah, w wyniku ekspozycji

na kolejne dawki leku. Zaobserwowano znaczący wzrost wydzielanych egzopolisacharydów, co skutkowało powiększeniem bariery ochronnej przez szczep S5. Mechanizm ten jest odpowiedzią na obecność toksycznych czynników i dodatkowo zmniejsza wrażliwość komórek na ten czynnik [Zhou i in. 2013].

Istotną częścią badań było określenie wpływu immobilizacji komórek *Planococcus* sp. S5 na aktywność enzymów zaangażowanych w rozkład naproksenu. Zgodnie z pracą Domaradzka i in. [2015], dokonano analizy aktywności takich enzymów jak *O*-demetylaza, monooksygenazy aromatyczne, dioksygenaza naftalenowa, dioksygenaza 1,2-gentyzynianowa oraz dioksygenaza 1,2-salicylowa. Aktywność analizowanych enzymów zarówno w ekstraktach wolnych, jak i immobilizowanych komórek szczepu S5 świadczy o braku zmian w szlaku biodegradacji naproksenu w wyniku unieruchomienia. Znaczące zmiany zaobserwowano natomiast w wartościach tych aktywności. Zgodnie z oczekiwaniami, aktywność analizowanych enzymów wolnych komórek S5 była niższa w fazie wolniejszego rozkładu (15 dzień degradacji). W tym samym czasie, aktywność większości badanych enzymów z komórek immobilizowanych była dwukrotnie wyższa. Zaobserwowano także znacznie wyższą aktywność monooksygenaz aromatycznych, po zastosowaniu naproksenu zamiast fenolu jako substratu reakcji. Jednakże ze względu na specyfikę metodologii wyznaczania aktywności aromatycznych monooksygenaz (oznaczanie redukcji NAD), niewykluczonym jest, że więcej enzymów należących do klasy oksydoreduktaz jest zaangażowanych w degradację naproksenu przez szczep *Planococcus* sp. S5. Zwiększona aktywność dioksygenazy 1,2-gentyzynianowej komórek immobilizowanych może świadczyć o zwiększonym udziale w rozkładzie leku w stosunku do dioksygenazy 1,2-salicylowej. Ze względu na zbliżone tempo biodegradacji naproksenu przez wolne komórki S5 w szybszej fazie rozkładu leku oraz komórki immobilizowane, zakładano, że aktywność analizowanych enzymów będzie również zbliżona. Jednakże aktywność komórek immobilizowanych w 15 dniu degradacji leku była znacznie wyższa niż komórek wolnych w 35 dniu, w wyniku czego obserwowano istotne przyspieszenie czasu degradacji leku. Szczegółowe informacje dotyczące przebiegu degradacji naproksenu przez szczep *Planococcus* sp. S5 w analizowanych stężeniach oraz w wielokrotnych cyklach, a także wpływu immobilizacji na aktywność enzymów zaangażowanych w rozkład naproksenu zaprezentowano w pracy **Dzionek i in. [2018b]** zamieszczonej w **rozdziale II.3** niniejszej pracy.

Badania nad przebiegiem degradacji naproksenu przez immobilizowany na gąbce Loofah szczep *Bacillus thuringiensis* B1(2015b) prowadzono w złożu biologicznym, zawierającym ukształtowaną autochtoniczną mikroflorę pochodzącą z komory przepływowej osadnika Imhoff'a w Krupskim Młynie – Ziętek. Ponadto sprawdzono przebieg degradacji leku przez immobilizowane komórki B1(2015b)

umieszczone w złożu biologicznym bez autochtonicznej mikroflory. Po 15 dniach inkubacji, immobilizowane komórki B1(2015b) w systemie bez autochtonicznej mikroflory były zdolne do degradacji 70% leku. Na podstawie przeprowadzonych badań wstępnych ustalono, że autochtoniczna mikroflora złoża biologicznego nie wykazywała zdolności do degradacji naproksenu. Po wprowadzeniu do złoża biologicznego immobilizowanych na gąbce Loofah komórek B1(2015b) zaobserwowano prawie 90% rozkład leku po 15 dniach inkubacji. Otrzymane wyniki wskazują na synergistyczne oddziaływania pomiędzy wprowadzonymi immobilizowanymi komórkami szczepu *Bacillus thuringiensis* B1(2015b) a autochtoniczną mikroflorą złoża biologicznego podczas rozkładu naproksenu. Tego typu interakcje w systemach bioremediacyjnych nie są do końca poznane. Mogą one być efektem zwiększania biodostępności zanieczyszczeń poprzez produkcję biosurfaktantów, wykorzystywaniem metabolitów, które nie mogą być dalej transformowane, wymianą czynników wzrostowych, bądź indukcją agregacji. Przykład synergii obserwowanej w glebie przedstawił Byss i in. [2008]. Zaobserwował on, że w wyniku inokulacji gleby szczepem *Pleurotus ostreatus* znacząco wzrosła liczba bakterii Gram-dodatnich i tym samym, wzrosła wydajność biodegradacji wielopierścieniowych węglowodorów aromatycznych przez te bakterie (WWA). Ponadto obecność beztlenowych bakterii z rodzaju *Clostidium* w dolnych partiach złoża biologicznego mogła przyczynić się do przyspieszenia rozkładu naproksenu, ze względu na posiadanie enzymów demetylujących oraz degradujących kwas weratrowy, bądź katechol [Mechichi i in. 2005].

W trakcie trwania eksperymentu monitorowano również wydajność pracy złoża biologicznego. Analiza chemicznego zapotrzebowania na tlen (ChZT) wykazała znacznie wydajniejszą pracę złoża biologicznego z immobilizowanymi komórkami B1(2015b) oraz autochtoniczną mikroflorą (82,65 ± 1,91% redukcji ChZT w ciągu 3 dni) niż złoża biologicznego zawierającego wyłącznie immobilizowane komórki B1(2015b) (68,85 ± 0,074% redukcji ChZT w ciągu 3 dni). Jednakże, immobilizowane komórki B1(2015b), były zdolne do rozkładu znaczącej części materii organicznej z syntetycznych ścieków, co pokazuje ich potencjał w bioremediacji.

Obserwacja w skaningowym mikroskopie elektronowym powierzchni gąbki Loofah wraz z immobilizowanymi komórkami B1(2015b) po 15 dniach przebywania w złożu biologicznym wykazała, że została ona niemalże całkowicie zasiedlona autochtoniczną mikroflorą. Uformowany biofilm charakteryzował się znaczną ilością zewnątrzkomórkowych wydzielin i wykazywał zróżnicowanie w zależności od wysokości złoża, z której został pobrany. W dolnej i środkowej części złoża obserwowano głównie biofilm bakteryjny, natomiast w górnej części przeważały strzępki grzybowe. Ze względu na to, że komórki szczepu B1(2015b) nie były widoczne na powierzchni gąbek Loofah, przeprowadzono analizę

bakteryjnych regionów V3-V5 genu 16S rRNA z zastosowaniem elektroforezy w gradiencie czynnika denaturującego (DGGE), aby potwierdzić obecność komórek B1(2015b) w złożu biologicznym oraz ustalić zmiany jakościowe mikroflory bakteryjnej. Dodatkowo przeprowadzono analizę jakościową szczepów grzybowych poprzez analizę regionów ITS1/ITS2 genu 18S rRNA. Badania wykazały znaczące zmiany jakościowe społeczności autochtonicznej mikroflory bakteryjnej po 15 dniach ekspozycji na naproksen. Skutkowało to obniżeniem indeksu Shannon'a-Wiener'a o niemal połowę. Szczepy grzybowe natomiast wykazały mniejszą wrażliwość na naproksen, co w efekcie nie wpłynęło na zmianę indeksu bioróżnorodności. Badania Grenni i in. [2014] wykazały, że nawet 3-godzinna ekspozycja na naproksen w stężeniu 100 µg/L powodowała znaczący spadek żywych komórek mikroorganizmów rzeki Tyber. Naproksen w stężeniu 10 µM spowodował także zahamowanie produkcji azotanów (III) przez bakterie nitryfikacyjne *Nitrosomonas europaea*, będące fundamentalnym szczepem systemów bioremediacyjnych w oczyszczalniach ścieków [Wang i in. 2011].

Obecność sekwencji genu 16S rRNA immobilizowanego szczepu B1(2015b), zarówno na gąbce Loofah, jak i wypełnieniu złoża, na różnych wysokościach złoża biologicznego, potwierdziła obecność szczepu i namnażanie się w systemie po zakończonym procesie degradacji naproksenu. Zaobserwowano również znaczące zmiany jakościowe społeczności bakteryjnych i grzybowych w wyniku wprowadzenia immobilizowanych na gąbce Loofah komórek szczepu B1(2015b). Obserwowano wzrost bioróżnorodności, zmiany grup dominujących, a także wzrost szczepów wrażliwych na naproksen. Uzyskane wyniki wskazują jednoznacznie na zagrożenie wynikające z ekspozycji na naproksen dla autochtonicznej mikroflory systemów bioremediacyjnych. Pokazują również, że szybka degradacja leku, skutkuje przywróceniem równowagi systemu oraz ogranicza eliminację cennych szczepów bakteryjnych autochtonicznej mikroflory systemu bioremediacyjnego. Szczegółowe informacje dotyczące degradacji naproksenu w złożu biologicznym przez immobilizowane na gąbce Loofah komórki *Bacillus thuringiensis* B1(2015b) oraz analizy DGGE zaprezentowano w pracy **Dzionek i in. [2020]** zamieszczonej w **rozdziale II.4** niniejszej pracy.

I.5. Literatura

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II. Publikacje wchodzące w skład rozprawy

II.1.

Dzionek A., Wojcieszyńska D., Guzik U.: Natural carriers in bioremediation: A review. Electronic Journal of Biotechnology, 2016, 19, 28-3

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Review

Natural carriers in bioremediation: A review

Anna Dzionek, Danuta Wojcieszyńska, Urszula Guzik *

Department of Biochemistry, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Jagiellońska 28, 40-032 Katowice, Poland

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ABSTRACT

Bioremediation of contaminated groundwater or soil is currently the cheapest and the least harmful method of removing xenobiotics from the environment. Immobilization of microorganisms capable of degrading specific contaminants significantly promotes bioremediation processes, reduces their costs, and also allows for the multiple use of biocatalysts. Among the developed methods of immobilization, adsorption on the surface is the most common method in bioremediation, due to the simplicity of the procedure and its non-toxicity. The choice of carrier is an essential element for successful bioremediation. It is also important to consider the type of process (*in situ* or *ex situ*), type of pollution, and properties of immobilized microorganisms. For these reasons, the article summarizes recent scientific reports about the use of natural carriers in bioremediation, including efficiency, the impact of the carrier on microorganisms and contamination, and the nature of the conducted research.

Contents

* Corresponding author.
E-mail address: urszula.guzik@us.edu.pl (U. Guzik).
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1. Introduction

The twentieth century went down in history as a period of extremely dynamic civilizational and technological development. Industrialization, wars, and intensive use of large-scale heavy metals and synthetic xenobiotics led to many environmental problems [1,2].

The contamination of the environment by petroleum products, pharmaceutical compounds, chloro- and nitrophenols and their derivatives, polycyclic aromatic hydrocarbons, organic dyes, pesticides and heavy metals is a serious problem [3,4,5,6,7,8,9]. These pollutants enter the environment by different ways. For example, one of the major consequences of the armed conflict between Iraq and Kuwait was the release into the environment millions of barrels of crude oil. After the war ended, scientists began numerous studies aimed at the removal of oil from the contaminated environment. Other sources of crude oil in ecosystems are accidental oil spills. One of the biggest marine disasters took place in Mexico in 2010, and it resulted in the spewing out of about 2.8 million barrels of crude oil from the British Petroleum (BP) oil rig Deepwater Horizon into the sea [10,11].

Pesticides are other serious pollutants present in soils. USEPA reported that in 2007, global consumption of pesticides for agricultural purposes was 2.36 million tonnes [12]. These compounds, used in bulk for long periods of time in a limited area, lead to serious disorders in indigenous microflora and humans, because pesticides are also toxic to non-target organisms [12,13,14]. Moreover, many metabolites of pesticide biodegradation are also toxic and constitute priority pollutants. For example, the major metabolites of parathion and 2,4-dichloropenoxy acetic acid biodegradation are *p*-nitrophenol and 2,4-dichlorophenol, respectively [9,15,16,17,18].

It has been reported that many microorganisms are able to biodegrade different pollutants [4,5,7,8,19,20]. However, the biodegradation rate depends on the physiological state of the microorganisms, which are sensitive to variable environmental factors. It is known that immobilization improves microorganisms' resistance to unfavourable environmental impacts [6,8].

The main purpose of this review is to present and discuss the latest reports about the natural carriers in the processes of bioremediation by immobilized cells. In the article immobilization methods for bioremediation are also presented.

2. Bioremediation methods

In 1930 Tausz and Donath [21] presented the idea of using microorganism to clean soil contaminated with petroleum derivatives, giving rise to biodegradation processes. Today, bioremediation is a commonly used method to restore the natural and useful values of contaminated sites by microorganism able to degrade, transform, or chelate various toxic compounds [22]. Microorganisms can break down organic pollutants by using them as a source of carbon and energy, or by cometabolism. Heavy metals cannot be degraded or destroyed biologically and undergo transformation from one oxidative state or organic complex to another. It changes their water solubility and decreases their toxicity [22,23].

Bioremediation is eco-friendly, non-invasive, cheaper than conventional methods, and it is a permanent solution that can end with degradation or transformation of environmental contaminants into harmless or less toxic forms [23,24,25,26]. Soil bioremediation can be carried out at the place of contamination (*in situ*), or in a specially prepared place (*ex situ*). *In situ* technology is used when there is no possibility to transfer polluted soil, for example when contamination affects an extensive area [26,27,28].

There are three basic methods of *in situ* bioremediation with microorganisms: natural attenuation, biostimulation, and bioaugmentation [24,29,30].

Natural attenuation is connected with the degradation activities of indigenous microorganisms. This method avoids damaging the habitat, allows ecosystem revert to its original condition and enables detoxification of toxic compounds [24,31].

Removal of contaminations by the natural attenuation takes a long time because degrading microorganisms in soil represent only about 10% of the total population. The increase of bioremediation efficiency *in situ* may be realized in the bioaugmentation process, in which the specific degraders are introduced into the soil [30,31]. This method is applied when the indigenous microflora are unable to break down pollutants, or when the population of microorganisms capable of degrading contaminants is not sufficiently large. To make the process of bioaugmentation successful, microorganisms introduced into the polluted environment as a free or immobilized inoculum should be able to degrade specific contamination and survive in a foreign and unfriendly habitat, be genetically stable and viable, and move through the pores in the soil. Microorganisms can be previously isolated from the contaminated soil and propagated, or their functional ability can be enhanced in the laboratory. Nonindigenous strains or genetically modified microorganisms (GMM) can also be incorporated into the remediated soil [31,32,33,34]. However, the result of bioaugmentation depends on the interaction between exogenous and indigenous populations of microorganisms because of the competition, mainly for nutrients [31].

To accelerate *in situ* bioremediation processes, biostimulation is used in order to modify the physical and chemical parameters of the soil. For this purpose, compounds such as nutrients (*e.g.* biogas slurry, manure, spent mushroom compost, rice straw and corncob) or electron acceptors (phosphorus, nitrogen, oxygen, carbon) are introduced into the soil [29,30,32,35].

Because *in situ* processes are out of hand it is difficult to predict the effect of remediation of contaminated sites [28]. *Ex situ* methods allow more efficient removal of pollutants, by controlling the physico-chemical parameters, resulting in a shortening of the total time of reclamation. These advantages outweigh *ex situ* methods' disadvantages such as additional cost and risk connected with the possibility of dispersion of the contamination during transport. During the *ex situ* processes contaminated medium is excavated or extracted and moved to the process location. Liquids can be clean in constructed wetlands while semi-solid or solid wastes in slurry bioreactors. Solid contaminations are biodegraded through land farming, composting and biopiles [26,28,36,37].

Constructed wetlands are used with success in the treatment of wastewater derived from domestic, industrial or agricultural sources [38]. They require the competition of microbes (bioremediation) and plant (phytoremediation). Microorganisms degrade or sorb organic substance present in the water undergoing treatment. Plants are used to remove, transfer or stabilize contaminants through metabolism, accumulation, phytoextraction or immobilization at interface of roots and soil [37]. Bioremediation processes in slurry bioreactors can be performed under aerobic or anaerobic conditions [28]. These systems utilize naturally occurring microorganisms or strains possessing specific metabolic capabilities to transform toxic compounds [27]. Slurry bioreactors are one of the best applied technologies used in the bioremediation of contaminated soils because they undergo under controlled operating conditions. It allows for the enhancement of microorganisms activity [27,39,40].

Landfarming is one of the most widely used soil bioremediation technologies. In this technology, excavated contaminated soils are spread out in a thin layer on the ground surface. Aerobic microbial activity within the soil is stimulated through the aeration and addition of minerals, nutrients and moisture [41,42]. Landfarming is a relatively simple technology however it is inexpensive and effective for easily biodegradable contaminants only at low concentration [28,37,41,42,43]. Composting is a controlled biological process that treats of agricultural and municipal solid wastes and sewage sludge using microorganisms under thermophilic and aerobic conditions [28,37]. Through composting, it is possible to reduce the volume of residues in landfills.

Biodegradation of solid contaminants takes place mainly as a result of oxidation and hydrolysis. The optimum temperature for growth of microorganisms engaged in composting is in the range of 40 to 70°C. The risk of contamination by pathogens is small, because most of them are inactivated at 70°C. A key factor during composting is microbial accessibility to the pollutants and the characteristics of the amending agents. This method is eco-friendly, has simple protocols, allows the control of large volumes of waste and ends with the total mineralization of pollutants [26,44,45]. Composting has been applied to bioremediation of soils contaminated with petroleum hydrocarbons, solvents, chlorophenols, pesticides, herbicides, polycyclic aromatic hydrocarbons and nitro-aromatic derivatives [28,37,46]. More advanced systems of composting are biopiles that are more expensive but enable more effective control of the process and its higher efficiency [28]. It is possible as the aerated composted piles are equipped with the dissolved oxygen, moisture and nutrient control systems and the proper aeration is forced by vacuum or injection system. This technology has been used for remediation of petroleum-contaminated soil [28,37,46].

3. Immobilization methods

In recent times, bioremediation processes more and more often employ immobilization methods. Immobilization is defined as limiting the mobility of the microbial cells or their enzymes with a simultaneous preservation of their viability and catalytic functions [47,48,49,50,51]. This process may use the natural ability of microorganisms to form biofilms on the surface of various materials, which is commonly observed in the environment. Immobilization significantly reduces costs of bioremediation processes and improves their efficiency. This method brings many benefits to bioremediation, such as higher efficiency of pollutant degradation, multiple use of biocatalysts, reduced costs — the stage of cell filtration is eliminated, ensuring a stable microenvironment for cells/enzymes, a reduced risk of genetic mutations, ensured resistance to shear forces present in bioreactors, increased resistance of biocatalysts to adverse environmental conditions and heavy metals, increased biocatalyst survival during storage, and increased tolerance to high pollutant concentrations [1,47,51,52].

There are five main techniques of immobilization: adsorption, binding on a surface (electrostatic or covalent), flocculation (natural or artificial), entrapment, and encapsulation (Fig. 1). Flocculation does not require carriers, and therefore will not be discussed [1,47].

3.1. Adsorption

Immobilization of microbial cells and enzymes by adsorption takes place through their physical interaction with the surface of water-insoluble carriers. This method, commonly used in bioremediation processes, is quick, simple, eco-friendly and cost-effective. Adsorption on a carrier surface is achieved by the formation of weak bonds. For that reason there is a high probability of cells leaking from the carrier into the environment, and this method is not used for GMM immobilization [1,53,54].

3.2. Binding on a surface

Electrostatic binding on a surface is very similar to physical adsorption, but the probability of microorganisms leaking is lower. This method requires washing the surface of the carrier with a buffer solution to obtain a hydrophilic surface that can attract the negatively charged cells or enzymes [55,56].

The procedure for immobilization is different in the case of covalent binding, because it requires the presence of a binding agent. Immobilization can be performed only on chemically activated carriers enriched with amide, ether and carbamate bonds. This

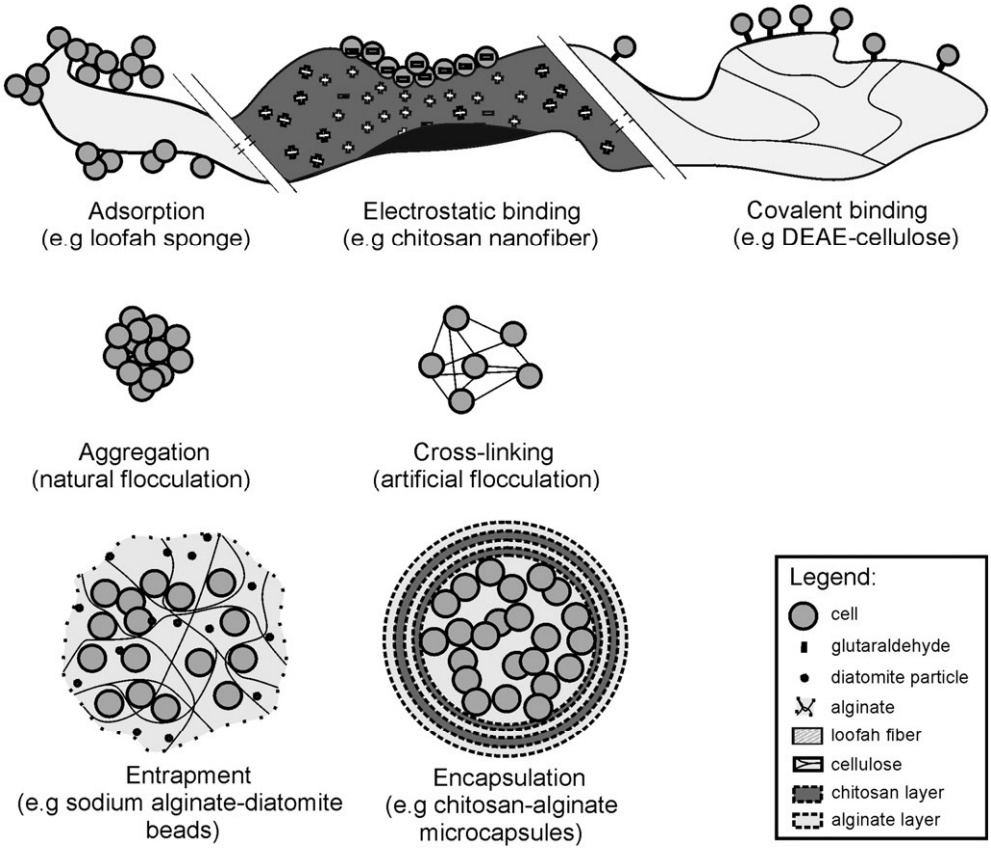

Fig. 1. Methods of immobilization [12,129,130,131,132].

method is mainly used for the immobilization of enzymes, because binding agents are often toxic to cells, and for that reason microbial viability and activity are lowered. The advantage of the covalent bonds is that they are strong enough to prevent the leaking of molecules into the environment [1,48,57,58].

3.3. Entrapment in porous matrix

Entrapment of microorganisms is well-known and widely used in bioremediation. After the entrapment, microbial cells are able to move only within a carrier. This prevents the cells from leaking into the environment but may limit the exchange of nutrients and metabolites. Microorganisms entrapped in the heterogeneous carrier are physiologically diverse. The cells located near the surface have high metabolic activity in contrast to starved cells in the interior of the carrier [1,47,51,59]. Entrapment is a rapid, nontoxic, inexpensive and versatile method [8,51]. Entrapped microorganisms are protected against environmental factors. The most important parameter in entrapment of microorganisms is the ratio of the size of the pores of the carrier to the size of the cells. In a situation where the pores are larger than the immobilized cells, they leak into the environment [1,47,51,59].

3.4. Encapsulation

Encapsulation is very similar to the entrapment, but in this case immobilized particles are separated from the external environment with a semi-permeable membrane. The biggest advantage of this method is the significant protection of biological material against the adverse conditions of the external environment. However, due to the limited permeability of the used membrane and the probability of its damage by growing cells, encapsulation is rarely used in *ex situ* bioremediation [1,47,60].

4. Support materials

Not every material is suitable for immobilization. A good carrier should be insoluble, non-toxic, both for the immobilized material and the environment, easily accessible, inexpensive, stable and suitable for regeneration. The immobilization process should be simple and harmless. Another important aspect is that different immobilization methods require carriers with specific properties. For example, carriers used for adsorption or binding on the surface should have a high porosity to ensure that the contact area of the immobilized material and the carrier is as large as possible [1,61,62]. The nature of performed bioremediation processes also has an impact on the choice of the carrier. Carriers used in bioaugmentation ought to be readily biodegradable. In wastewater treatment processes the carrier should have a high mechanical resistance because it may be exposed to different kinds of physical forces [1,63].

Carriers are classified as organic and inorganic or natural or synthetic. Natural organic carriers have many functional groups which stabilize biocatalysts. This class of carriers includes: alginate, κ-carrageenan, chitosan, sawdust, straw, charcoal, plant fibres, corncob, bagasse, rice, husks of sunflower seeds, diatomite and mycelium [64,65,66,67,68,69,70,71,72,73,74,75,76]. These supports are hydrophilic, biodegradable, biocompatible, and inexpensive because they are mostly waste from the food industry. However, the possibility of their application in bioremediation processes is limited because of low resistance to biodegradation, sensitivity to organic solvents, and stability in a narrow pH range [1,52,74,77].

Synthetic organic carriers have numerous functional groups with diversified characters. This class includes polypropylene, polyvinyl chloride, polystyrene, polyurethane foam, polyacrylonitrite and polyvinyl alcohol [78,79,80,81,82,83,84,85]. Their advantage is the possibility to regulate their structure at the macromolecular level — the selection of the proper molecular weight, the spatial structure and the manner and arrangement order of each active functional group in the chain. Moreover, during synthesis, the porosity, pore diameter, polarity and hydrophobicity of the carrier may be controlled. Furthermore, synthetic supports can be formed into various shapes (tubes, membranes, coatings, carriers of various shapes from spherical to oval), and they are easily available and relatively inexpensive [1,86,87].

Inorganic carriers (natural and synthetic) have a high chemical, physical and biological resistance. They are represented by magnetite, volcanic rocks, vermiculite, porous glass, silica-based materials, ceramics and nanoparticles [88,89,90,91,92]. A significant disadvantage of these carriers is the presence of a small number of functional groups, which prevents sufficient bonding of the biocatalyst. For that reason they are used in the formation of hybrid carriers, combining natural polymers and synthetic nanoparticles [47,88,93].

5. Immobilization in bioremediation

Higher biodegradation efficiency observed after the use of immobilized microorganisms in comparison to free ones caused the increase interest in their application in bioremediation processes [94,95]. It is assumed that carrier protects and hinders the spread of organic pollutants and in this way reduces the surface contaminants concentration on the immobilized microorganisms. Moreover, changes in microenvironment after immobilization may lead to changes in cell morphology, physiology and metabolic activity [96,97]. Wastes from the food industry are very good and inexpensive candidates for carriers [52,98,99,100,101]. Some researchers have also started to explore inorganic adsorbents, such as expanded perlite or tezontle [99,102,103]. Table 1 presents a list of carriers used in bioremediation processes.

5.1. Plant fibres

The most often applied vegetable fibre in immobilization is a sponge derived from *Luffa cylindrica* or *Luffa aegyptiaca*. These plants grow in tropical and subtropical climates. The loofah sponge shows important advantages required for immobilization processes: high porosity (85–95%) with simultaneous low density (0.018–0.05 g/cm^3). The sponge is composed of fibre networks that form an open and free space for the exchange of matter [2,104].

The first usage of the loofah sponge was reported in 2003. Microalgal-luffa sponge immobilized discs were applied in nickel biosorption processes. It has been shown that loofah sponge restricts the leaking of the immobilized biomass into the environment. It is an extremely stable carrier and can be regenerated at least 7 times [2]. Mazmanci et al. [105] reported that the loofah sponge was a source of carbon and energy for white rot fungi, and therefore should not be used for their immobilization (long-term bioremediation). On the other hand, it provides an excellent support for *in situ* or short-term bioremediation (without a source of carbon and energy) with these fungi.

5.2. Sugarcane bagasse

Sugarcane bagasse, derived from the extrusion of a plant *Saccharum officinarum*, is widely used for the production of ethanol, and is an excellent biosorbent. Bagasse is rich in carbohydrates, mainly lignin and cellulose. The spatial structure of bagasse is formed by parallel-arranged fibres and micropores (0.5–5 μm). It is an ideal place to attachment bacteria and fungal hyphae. Another advantage of this carrier is its mechanical strength. After centrifugation at 1500 rpm no disintegration or microorganism leaking into the medium were observed [100,106].

Mohammadi and Nasernejad [72] demonstrated that immobilization of *Phanerochaete chrysosporium* on sugarcane bagasse significantly

Table 1
Natural carriers used in bioremediation.

Carrier	Removed pollution	Immobilized microorganisms	Efficiency of bioremediation	References
Plant fibres (Loofah sp.)	Aromatic hydrocarbons	*Bacillus cereus*	Unimmobilized — 74% Immobilized — 79%	[122]
	Phenol	*Trametes versicolor*	Unimmobilized — 39% Immobilized — 87%	[96]
	Methyl parathion	Bacterial consortium	Unimmobilized — 55% Immobilized — 98%	[12]
	Carbendazim (pesticide)	Bacterial consortium	Unimmobilized — 12% Immobilized — 95%	[70]
	Ni	*Chlorella sorokiniana*	Unimmobilized — 64% Immobilized — 88%	[2]
Baggase	Tetradecane	*A. venetianus*	Unimmobilized — 22.3% Immobilized — 76.8%	[106]
	Anthracene	*P. chrysosporium*	Unimmobilized — 43% Immobilized — 82%	[72]
	Mesotrione (herbicide)	*Bacillus pumilus* HZ-2	Unimmobilized — 48% Immobilized — 75%	[99]
	Chromium	*Acinetobacter haemolyticus*	Unimmobilized — 38% Immobilized — 92%	[123]
Sawdust	Petroleum oil	*Arthrobacter* sp.	Unimmobilized — 18% Immobilized — 36%	[67]
	Crude oil hydrocarbon	Bacterial consortium	Unimmobilized — 79.37% Immobilized — 95.9%	[101]
	Chromium	*A. haemolyticus*	Unimmobilized — 80% Immobilized — 99.8%	[124]
Corncob	*p*-Nitrophenol	*Arthrobacter protophormiae* RKJ100	Unimmobilized — 39% Immobilized — 79%	[99]
	Carbofuran	*B. cepacia* PCL2	Unimmobilized — 67.69% Immobilized — 96.97%	[125]
	Hexadecane	*Pseudomonas* sp.	Unimmobilized — ~33% Immobilized — ~56%	[52]
	Chlorophenols	Bacterial consortium	Unimmobilized — 87% Immobilized — 89.7%	[77]
Expanded perlite	Methyl *tert*-butyl ether	Soil consortium	Unimmobilized — 22% Immobilized — 50%	[110]
	Hexadecane	*Aspergillus niger*	Unimmobilized — 81% Immobilized — 96%	[126]
	Styrene	*P. aeruginosa*	Immobilized — 90%	[111]
Tezontle	Sulfonated azo dyes (Acid Orange 7, Acid Red 8)	Bacterial consortium	Abiotic test — 16.8 mg/(L * 24 h) Immobilized — 80 mg/(L * 24 h)	[127]
	Propanil (herbicide)	Bacterial consortium	Immobilized — 36.78 mg/(L * 24 h)	[128]
	Methyl paration	Bacterial consortium	Abiotic test — 9% Immobilized — 58%	[102]
		E. coli RAZEK	Unimmobilized — 49% Immobilized — 95%	[114]
	DDT (pesticide)	*P. fluorescens*	Unimmobilized — 55% Immobilized — 99%	[113]
Coco-peat	Oil	Bacterial consortium	Unimmobilized — 51.2% Immobilized — 86.6%	[115]
Husks of sunflower seeds	Crude oil	*Rhodococcus* sp. QBTo	Unimmobilized — 28% Immobilized — 66.1%	[74]
Cotton fibres	*n*-Heptadecane	*Acinetobacter* sp. HC8-3S	Unimmobilized — 82% Immobilized — 96%	[82]

increased the production and activity of manganese peroxidase during the biodegradation of anthracene. After the immobilization of *Acinetobacter venetianus* on this carrier, a higher rate of tetradecane degradation was observed. This was probably connected with binding of the contaminant on the hydrophobic surface of the carrier, and in consequence the easier access of microorganisms to hydrocarbon [82]. Increased efficiency of phenol degradation by immobilized *Candida tropicalis* PHB5 was also observed [100]. The microorganisms immobilized on the bagasse are suitable for bioremediation in bioreactors because they remain active for up to 8 bioremediation cycles [100,106].

5.3. Sawdust

One of the most common agro-wastes is sawdust, which has been successfully used for the immobilization of bacterial cells. *Arthrobacter* sp. immobilized on sawdust did not lose their enzymatic activity after 6 weeks of storage (at 25°C and 45°C) and was still able to degrade similar quantities of crude oil [67]. Sawdust possesses a labyrinthine structure providing very high surface area for cellular attachment. High hydrophilicity of this carrier may hamper the adsorption of oil-degrading microorganisms on the carrier. However, this difficulty may be overcome by non-toxic hydrophobic coating of sawdust [107]. Hazaimeh et al. [101] during studies on degradation of oil by a bacterial immobilized consortium, demonstrated that immobilization significantly increased the production of biosurfactants by bacteria. This was to increase the solubility, and thus the bioavailability of hydrophobic hydrocarbons.

5.4. Corncob

Materials derived from agro-industrial residues (AIR), such as corncobs, offer many advantages over synthetic matrices. Corncobs are robust, porous and perforated. This increases the attachment area for organisms and allows their growth without limiting diffusion. Corncobs have a high water holding capacity, improve soil structure

and oxygen diffusion, are readily available in maize processing plants, and their usage in the processes of bioremediation provides an alternative method of AIR disposal [12,52,98].

The first study of corncobs used as a carrier in bioremediation [108] showed that they are a good carrier for the bioaugmentation of soil contaminated with oily-sludge. It was also noted that after the introduction of immobilized bacteria, the degree of oxygenation of the lower layers of the soil had increased as a result of the creation of air pockets by corncobs. Plangklang et al. [109] showed that *Burkholderia cepacia* PCL3 bacteria grow very well on the surface of the corncob, and thereby leaked into the medium due to the lack of space on the carrier. Rivelli et al. [52] observed the increased degradative activity after immobilization of bacteria on corncob powder. Additionally, this carrier stabilized soil and improved the oxygen diffusion and the water-mass transfer [52].

5.5. Expanded perlite

Volcanic rocks are known for their sorption and mechanical properties. They are widely used in construction, filters and hydroponics. One of these rocks is perlite which is excavated worldwide. Because crude perlite has a relatively high density and small surface area, it is subjected to heat treatment, resulting in a significant extension and the forming of air bubbles inside. Expanded perlite obtained in this way has a low density (0.032–0.4 g/cm^3), high porosity, and high surface area [89,110].

For the first time, expanded perlite was used for the bioremediation studies by Paca et al. [111]. It was shown that biofilter consisted of perlite particles with immobilized cells of *Pseudomonas aeruginosa* was more effective in styrene biodegradation [111]. Emtiazi et al. [112] demonstrated that the transformed cells of *Escherichia coli* immobilized on perlite were more genetically stable than in other carriers, and they were able to produce biosurfactants, which increased the solubility of petroleum hydrocarbons, and therefore the degree of their biodegradation.

5.6. Tezontle

Tezontle is a volcanic rock tested recently as a carrier in bioremediation. This rock is commonly used in Mexico as a building material, and has a characteristic reddish colour (due to the presence of iron ions). The surface is highly porous and perforated, which provides a good place for biofilm formation by microorganisms [102].

Santacruz et al. [113] demonstrated that *Pseudomonas fluorescens* immobilized on a tezontle biofilter is able to degrade DDT up to 999.8 mg/L per day and 2,4-dichlorophenoxyacetic acid up to 2634 mg/L per day. Yáñez-Ocampo et al. [103] observed biodegradation of a methyl-parathion and tetrachlorvinphos mixture by a consortium of bacteria immobilized on tezontle. They showed a decrease in the optical density of bacteria after 13 d of the experiment, whereas the death of free cells occurred after 6 d. In addition, the immobilized cells did not require supplementation with glucose during pollutant breakdown. This demonstrates that after immobilization the new environment is more friendly for bacteria which are able to degrade greater amounts of insecticides [103]. Similar results were obtained by Abdel-Razek et al. [114] during research on methyl-parathion biodegradation by transformed *E. coli* RAZEK immobilized on tezontle.

5.7. Other carriers

Recently, increasing interest is observed in the usage of coco-peat, husks of sunflower seeds and cotton fibres as carriers in bioremediation. These carriers have not gained popularity yet, but so far studies have shown their promising possibilities in bioremediation [74,82,115].

Nunal et al. [115], during the biodegradation of heavy-oil by a bacterial consortium immobilized on coco-peat, showed that the carrier, because of its porous and perforated surface, is a good place to create a stable biofilm. Moreover, they observed that the immobilized bacteria, after 60 d of the experiment, degraded 86.6% of the heavy-oil, while the free cells decomposed only 51.2% of it. After 90 d of storage, bacteria immobilized on the coco-peat had a greater survival rate than those encapsulated in sodium alginate. This makes coco-peat an excellent candidate carrier in bioaugmentation [115].

Bioremediation of crude oil by *Rhodococcus* spp. QBTo immobilized on sunflower seed husks, also shown that immobilization improves the survival and enzymatic activity of microorganisms. After 120 d of storage at 10°C the bacterial survival rate was about 76%, and therefore sunflower seed husks are an appropriate carrier for the bioaugmentation of contaminated soils [74].

Lin et al. [82] noted that the negative charge and the presence of hydroxyl and carboxylic acid groups make cotton fibres a good carrier for immobilization of microorganisms. It was shown that *Acinetobacter* sp. HC8-3S degraded more than 70% of the crude oil with 90 g/L NaCl, whereas free cells degraded about only 15% under the same conditions. This opens up the possibility of inexpensive bioremediation in areas of high salinity by immobilized microorganisms. The authors showed that the adsorption properties of cotton fibres allow the use of this carrier for the biodegradation of floating oil from oil spills [82].

5.8. Pros and cons of natural and synthetic carriers

Application of immobilized cell systems in bioremediation indicates several advantages over the usage of free microorganisms: prolonged activity, stability of biocatalyst, feasibility of continuous processing, increased tolerance to high toxic compounds concentration, easier recovery, possibility of regeneration and reuse of biocatalyst, reduction of microbial contamination risk and ability to use smaller bioreactors with simplified process [1,94,99,116]. Because each support has its own requirements in terms of the microorganisms used and the degraded compounds, the support selection is a key step which influences the success of bioremediation process [61,94].

The main feature of the carriers is mechanical resistance, which allows to the recovery, regeneration and reuse of biocatalyst in bioremediation processes [94,116,117]. This feature is typical for sawdust, wood chips, shavings, loofah sponge and polyvinyl alcohol beads, polyurethane foam, among from natural and synthetic carriers, respectively [94,96,97,117,118]. In bioremediation processes very important is the use of low-price and easy accessible carriers because only than they may be applied on the large scale. This condition fulfils plant residue, polyvinyl alcohol beads, polyurethane foam, different ceramics, and natural polymers such as agarose, κ-carrageenan, alginate, agar, and chitosan [52,62,97,103,107]. However, most of the natural polymers are non-mechanically resistant. One of the most often described natural carriers is alginate. It is cheap, biocompatible, non-toxic and easy to use [51,48,49,97,119]. Unfortunately, it cannot be used in continuous conditions because of the problems with gel degradation and low physical strength resulting in the leakage of immobilized microorganisms from the matrix [61,120].

Equally important carriers potentially useful in bioremediation have to meet other requirements of good matrices: non-toxicity and insolubility. These features characterize both natural (chitosan, loofah sponge, corncob, sawdust, tezontle, sugarcane bagasse, wood chips) and synthetic (polyvinyl alcohol, polyurethane, polypropylene, polystyrene) carriers [36,52,81,94,95,100,101,103,107,116,117,118,121].

It is possible to find among both natural and synthetic carriers almost ideal one, which may be used with success in bioremediation. However, the predominance in the usage of natural carriers is connected with their biodegradability, renewability and availability in nature. Moreover, many of natural carriers are agro-waste that may be further used in biotechnological processes. The immobilization of microorganisms on natural carriers is environmentally friendly

because it causes less disposal problems that may occur for synthetic ones [52,99,100,101,107].

6. Conclusions

Interest in organic carriers, which are wastes from the agricultural and food industries, increases continuously, because they are very good material for immobilization. All of them have many functional groups, which positively affect the degree of colonization by microorganisms. Moreover, volcanic rocks (expanded perlite and tezontle) are also known as carriers which have good sorption properties and high mechanical resistance.

Carriers such as the loofah sponge and corncob have been used with success in bioremediation *in situ*, and the former has also shown the greatest support for pesticide biodegradation. In *ex situ* bioremediation the best results have been obtained using carriers such as bagasse, sawdust, expanded perlite and tezontle. Coco-peat, sunflower seed husks, cotton fibres and porous glass seem to be promising materials for immobilization, although their application requires further studies.

Conflict of interest

The authors declare no conflict of interest.

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II.2.

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Fluorescein Diacetate Hydrolysis Using the Whole Biofilm as a Sensitive Tool to Evaluate the Physiological State of Immobilized Bacterial Cells

Anna Dzionek [1], Jolanta Dzik [2], Danuta Wojcieszyńska [1,*] and Urszula Guzik [1]

[1] Department of Biochemistry, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Jagiellońska 28, 40-032 Katowice, Poland; adzionek@us.edu.pl (A.D.); urszula.guzik@us.edu.pl (U.G.)

[2] Institute of Technology and Mechatronics, University of Silesia in Katowice, Żytnia 12, 41-200 Sosnowiec, Poland; jolanta.dzik@us.edu.pl

* Correspondence: danuta.wojcieszynska@us.edu.pl; Tel.: +32-2009-567

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Abstract: Due to the increasing interest and the use of immobilized biocatalysts in bioremediation studies, there is a need for the development of an assay for quick and reliable measurements of their overall enzymatic activity. Fluorescein diacetate (FDA) hydrolysis is a widely used assay for measuring total enzymatic activity (TEA) in various environmental samples or in monoculture researches. However, standard FDA assays for TEA measurements in immobilized samples include performing an assay on cells detached from the carrier. This causes an error, because it is not possible to release all cells from the carrier without affecting their metabolic activity. In this study, we developed and optimized a procedure for TEA quantification in the whole biofilm formed on the carrier without disturbing it. The optimized method involves pre-incubation of immobilized carrier in phosphate buffer (pH 7.6) on the orbital shaker for 15 min, slow injection of FDA directly into the middle of the immobilized carrier, and incubation on the orbital shaker (130 rpm, 30 °C) for 1 h. Biofilm dry mass was obtained by comparing the dried weight of the immobilized carrier with that of the unimmobilized carrier. The improved protocol provides a simple, quick, and more reliable quantification of TEA during the development of immobilized biocatalysts compared to the original method.

Keywords: immobilization; fluorescein diacetate; polyurethane foam; biofilm; total enzymatic activity

1. Introduction

Increasing technological and civilization progress resulted in the level of anthropogenic pollution (e.g. pesticides, heavy metals, pharmaceuticals, dyes) in the natural environment increasing significantly in recent years. However, scientific progress made it possible to cheaply and effectively reduce the amount of these pollutants in the environment through bioremediation. This process is based on microorganisms equipped with systems of enzymes that allow them to obtain carbon and energy from xenobiotics [1–3].

An important attribute of stable bioremediation systems is their well-shaped microflora. For that reason, introduction of new microorganisms into the bioremediation systems very often ends, however, with their quick removal by the microflora present in the system. One of the common methods used to increase the chance of survival upon introducing microorganisms into the new system is their immobilization. In addition, immobilized biocatalysts bring certain advantages into bioremediation studies, such as reducing costs, ensuring a stable microenvironment for cells and their enzymes, and increasing the efficiency and resistance of biocatalysts to adverse environmental conditions and

high pollutant concentration. Immobilized biocatalysts were extensively examined in the treatment of wastewaters contaminated with various pollutants, and their potential is promising [2,4–7].

Among various immobilization techniques, particular attention in bioremediation studies is paid to the ability of some bacterial strains to form biofilms on various materials. This technique is simple, fast, cheap, and non-toxic for cells and the environment. One of the most important advantages of this method, considering bioremediation systems, is also the spread of the introduced cells within the system, caused by the detachment of external parts of the biofilm in one of its growth phases. The necessary condition, in this technique, to receive a stable and efficient immobilized biocatalyst, is the development of a biofilm strongly attached to the surface of the carrier [4,8–10]. To obtain this kind of biofilm, it is necessary to optimize conditions of the immobilization process for each strain and the carrier [4,11].

Currently, the most commonly method used to determine the efficiency of immobilization is the plate method which relies on plating and subsequent counting of colony-forming units (CFUs) released from the carriers [12] or determination of dry weight of the immobilized biomass [13]. However, none of the above methods determine the physiological state of immobilized cells, which is significantly affected by the quality of the formed biofilm. An indirect method allowing determination of immobilization efficiency is to conduct pollution degradation tests for which an immobilized biocatalyst was developed [14]. However, with multifactor optimization, determining the immobilization efficiency using this method is problematic, especially in the case of hardly biodegradable pollutants that are decomposed over a long period of time. In such cases, enzymatic determination of the metabolic activity of microbial cells may be the solution.

Fluorescein diacetate (3′,6′-diacetyl-fluorescein; FDA) is a prefluorophore, which can be hydrolyzed by a wide spectrum of non-specific extracellular enzymes and membrane-bound enzymes like proteases, lipases, and esterases. Fluorescein, which is a product of hydrolysis, has a yellow-green color and is characterized by strong light absorption at 490 nm. For this reason, the concentration of fluorescein after enzymatic reactions can be easily measured spectrophotometrically. Moreover, measurements of enzymatic activity using FDA hydrolysis correlate with other parameters, such as biomass, ATP content, oxygen consumption, or optical density, and therefore, are often expressed as the total enzymatic activity (TEA) [15–17].

Despite its simplicity, determination of enzymatic activity of immobilized bacterial cells with FDA was presented so far in only one study [18]. The method proposed by Liang et al. [18] assumes the determination of FDA-hydrolyzing enzyme activity of cells that are detached from the carrier. A measurement of enzymatic activity performed in this way carries an error for two very important reasons. Firstly, it is impossible to detach the entire biofilm from the carrier in a non-toxic way because of the biofilm binding strength [19]. On the other hand, bacterial cells at different depths of the biofilm are characterized by different enzymatic activities [11,20]. Therefore, depending on the biofilm binding strength, its various layers with different enzymatic activities can be released and assumed as a total activity. In this study, we made an attempt to apply an appropriate modification to this method to eliminate the mentioned errors. The most important modification was to skip the step of cell removal from the biofilm and to conduct the FDA assay on the entire biofilm with the carrier. To achieve a reliable and reproductive assay, tests were started by determining the ability of carrier to adsorb the product of FDA hydrolysis. We also examined the influence of shaking, and determined which of the substrate application methods resulted in the highest FDA hydrolysis efficiency and the lowest coefficient of variation. Due to the fact that the repeatability and sensitivity of methods based on enzymatic activity depend on the operational conditions [21,22], the optimization of conditions such as pH and incubation time was performed. As a result, a sensitive and reproducible method was developed to determine the total enzymatic activity (TEA) of the entire biofilm formed on the carrier without disturbing it. Using this method, it is possible to determine the efficiency of immobilization during the optimization of its conditions quickly and precisely.

2. Results and Discussion

2.1. Fluorescein Adsorption by Polyurethane Foam (PUR)

After the decision to carry out the enzymatic assay on the biofilm along with the carrier, particular attention should be paid to the possible interaction of the reaction product with the carrier. Fluorescein, without ionic functional groups (e.g., COO^-), is characterized by very limited solubility in water. As the ionization increases, the interaction of the dye with oppositely charged functional groups of the carrier will also increase due to ion exchange. For this reason, the sorption of fluorescent dyes depends on both the pH and the functional groups of the carrier. Due to the presence of two negatively charged groups, and the absence of positive charges, fluorescein is much better adsorbed by positively charged surfaces than by negative ones [23–25].

In this study, the immobilization of the naproxen- and ibuprofen-degrading bacterium *Bacillus thuringiensis* B1 (2015b) [26] was conducted on PUR as a carrier. This is one of the most commonly used materials for microorganism immobilization, and it is characterized by good mechanical strength, non-toxicity, large surface area, and low price [8,27]. It was also shown that polyurethane foam, due to the presence of neutral carbamate groups, is a good sorbent of hydrophobic compounds [8,28]. Therefore, since fluorescein exhibits hydrophobic characteristics [24], its adsorption by polyurethane foam was investigated.

Sterile PUR cubes were incubated for 1 h with fluorescein formed during the hydrolysis of fluorescein diacetate to test the adsorption capacity of PUR. Conducted tests showed that, in the analyzed range of fluorescein concentrations (0.5–5 µg/mL), its adsorption by PUR did not exceed 9% of the dye, but the adsorption value depended on the initial concentration of fluorescein (Figure 1). The average value of adsorbed fluorescein in concentrations below 2.5 µg/mL was equal to 3.8 ± 1.6%, which was a statistically insignificant result (*t*-test; $p \geq 0.05$). However, when the initial fluorescein concentration was higher than 2.5 µg/mL, 7.7 ± 1.12% of dye adsorption was observed. Due to the fact that this result was a statistically significant difference (*t*-test, $p \leq 0.05$), in this study, when the obtained concentration was in the range of 2.5–5 µg/mL, the adsorption of fluorescein by PUR was included in the final concentration.

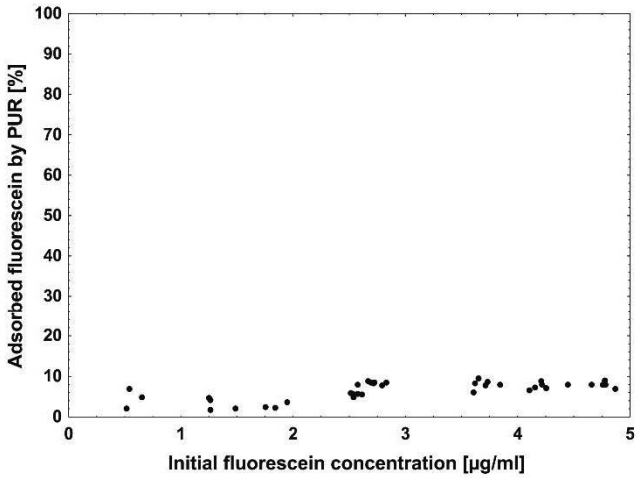

Figure 1. Fluorescein adsorption by sterile polyurethane foam (PUR) cubes depending on the initial concentration of the dye after 1 h of incubation.

Adsorption of fluorescein by materials used as carriers for immobilization is not yet extensively described. A good sorbent material, zeolite, was shown to adsorb 17% of the dye during overnight incubation [23]. However, the material which did not adsorb fluorescein, due to the negative charge of its surface, was silica gel [24].

The adsorption capacity of the carrier can be one of the most important factors which significantly affects the reliability of the FDA assay. For this reason, the adsorption test should be performed for each carrier at the beginning of the optimization of immobilization.

2.2. Fluorescein Diacetate Application and Impact of Shaking

Depending on bacterial strain, the physiological condition of the cells, and the environmental conditions, biofilms can be flat or consist of numerous water channels and extensive structures. They may contain a small number of cells and a rich matrix, or be very densely packed with cells. The structure of the biofilm and the condition of the cells at different depths differ significantly. However, the transport of water, metabolites, or nutrients in any type of biofilm is conducted in the same way. Mass transfer in the biofilm follows the principles of diffusion (in the biofilm matrix) and advection (in the water channels). Because mass transport in the biofilm is limited in its deeper layers, due to the slower diffusion through the matrix, a chemical gradient is created that affects the physiological state of cells at different heights of the biofilm [4,11,20,29]. For that reason, an examination of the physiological state of the biofilm should concern each of its layers. However, this causes technical complications that must be investigated to correctly perform the enzymatic assay and obtain reliable results.

In order to check whether the method of application of fluorescein diacetate would affect the reproducibility and efficiency of its hydrolysis, FDA was applied to the buffer solution or injected directly into the immobilized PUR cube and incubated for 1 h. Depending on the site of FDA application, a different hydrolysis efficiency and coefficient of variation was observed (Table 1). The most reproducible and efficient result was obtained when the substrate was applied directly into the center of immobilized PUR cubes (262 $\pm$ 18 μg/g dry mass per h). Addition of FDA to the phosphate buffer caused a large discrepancy in the obtained results (210 $\pm$ 48 μg/g dry mass per h).

Table 1. Reproducibility of the method for determining fluorescein diacetate (FDA) hydrolytic activity depending on the method of FDA application. TEA—total enzymatic activity; SD—standard deviation; CV—coefficient of variation.

Location of Application	Biofilm Dry Mass (g)	Fluorescein Concentration (μg/mL)	TEA (μg/g dry mass per h)	Mean	SD	CV (%)
Solution	0.0082–0.0089	1.35–2.30	157–267	210	48	23
Carrier	0.0084–0.0086	2.02–2.40	238–283	262	18	7

The immobilization of bacterial cells on polyurethane foam often results in the formation of a very abundant biofilm, both on its surface and inside the pores. As a result, a high cell density can be obtained in a small volume of the carrier, but also with limited mass transfer to the internal parts of the carrier [20,30]. For that reason, the application of FDA to the buffer solution could cause the adsorption of the FDA to only occur due to a biofilm located on the outer parts of the PUR. Therefore, different amounts of substrate could penetrate into the PUR interior, causing divergences. Nevertheless, it should also be taken into account that the release of fluorescein from the biofilm, especially from the internal parts of the carrier, may be slower due to the limited mass transfer and electrostatic repulsion with amino acids present in the biofilm matrix [31]. In order to achieve results with the smallest error, the final procedure assumes injecting the FDA directly into immobilized carriers placed in a phosphate buffer.

To evaluate the impact of agitation on the efficiency and reproducibility of FDA assay with immobilized B1 (2015b) cells on PUR, hydrolytic activity was measured after 1 h in static conditions, and upon subjection to a rotation rate of 130 rpm (Table 2). Under static conditions, a higher concentration of fluorescein (275 μg/g dry mass per h) was observed in comparison to assays conducted with shaking (249 μg/g dry mass per h). However, this result was the least reproducible as confirmed

by the obtained coefficient of variation (46%). Results obtained during assays shaken at 130 rpm proved to be the most reproducible with the smallest coefficient of variation (8%).

Table 2. Impact of shaking on the reproducibility of FDA assay.

Agitation	Dry Biofilm Mass (g)	Fluorescein Concentration (μg/mL)	TEA (μg/g dry mass per h)	Mean	SD	CV (%)
With	0.0079–0.0086	1.36–3.89	164–469	275	126	46
Without	0.0081–0.0089	1.92–2.32	226–273	249	21	8

In enzymatic assays, proper mixing is necessary to ensure sufficient substrate contact with enzyme active sites. However, excessive shaking, due to the shear forces, can deactivate the enzymes and reduce the efficiency of enzymatic reactions [32]. On the other hand, in static incubation, FDA will be rapidly hydrolyzed near the biofilm, while the rest of the FDA may not be transferred to the biofilm surface and matrix [17]. However, it was shown that shaking at below 200 rpm does not damage the enzymes and provides the best efficiency of enzymatic reactions in soils [17,33]. For that reason, in the final method, samples were incubated with shaking at 130 rpm.

2.3. pH Optimization

One of the crucial factors influencing enzyme activity is the pH of the assay mixture. Therefore, each enzyme is characterized by a specific pH value at which it works most efficiently. At the optimal pH, the active site of the enzyme is properly spatially shaped. This behavior is related to the proper protonation of amino acids included in the active site. However, due to the fact that FDA hydrolysis is carried out by many different enzymes, determining the optimum for the reaction involves determining the optimum of the enzyme group. It should also be noted that one of the FDA hydrolysis products is acetic acid; therefore, it is necessary to perform the assay in a buffer with an appropriate buffering capacity [17,34]. The temperature of the assay mixture also affects it pH value. Thus, to best assess the physiological state of the analyzed bacterial cells, the assay was carried out at the optimal temperature for their growth (30 °C).

In order to select the optimal pH of phosphate buffer, the hydrolysis of fluorescein diacetate in pH-buffering solutions ranging from 6.8 to 7.6 was examined.

Conducted assays showed significant differences in FDA hydrolysis at different pH levels (Figure 2). Incubation of the immobilized B1 (2015b) strain with FDA in the buffer with the lowest pH (6.8) resulted in the smallest amount of released fluorescein in 1.5 h (54 ± 6 μg/g dry mass per h). As the pH of the buffer increased, hydrolytic activity also increased. Maximum FDA hydrolysis was observed at pH 7.4–7.6 (138 ± 7 μg/g dry mass per h to 128 ± 5 μg/g dry mass per h). According to Guilbault and Kramer [35], FDA-hydrolyzing enzymes exhibit the highest activity at a pH from 7 to 8. However, most researchers use pH 7.6, which is very beneficial [22], mainly because of the fact that abiotic FDA hydrolysis is statistically significant at higher pH values. For this reason, pH above 7.6 was not examined during evaluation.

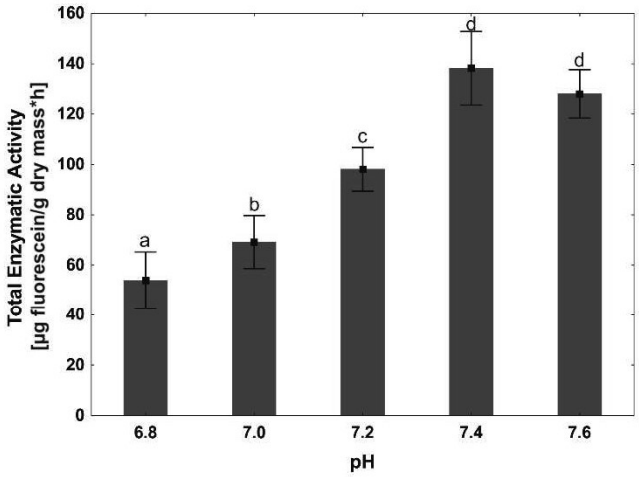

Figure 2. Effect of pH on the enzymatic hydrolysis of fluorescein diacetate (FDA) by *Bacillus thuringiensis* B1 (2015b) cells immobilized onto PUR. Error bars were obtained based on the standard deviation. Statistically significant differences are marked with letters (post hoc, $p \leq 0.05$).

Depending on the type of the carrier and its ionization, pH of the environment may influence abiotic degradation of FDA [17]. In this study, abiotic and spontaneous FDA hydrolysis in the presence of PUR in the analyzed pH range was not statistically significant (data not shown).

The lack of background in quantification of microbial enzymatic activity is undoubtedly an advantage. However, not every carrier will affect the abiotic FDA degradation; therefore, the above test should be performed in phosphate buffer (pH 7.6) before the FDA assay with immobilized cells.

2.4. Incubation Time

The biofilm matrix is a complex mixture of many compounds such as polymers, proteins, polysaccharides, and nucleic acids. Other important components of the biofilm matrix are also cellular elements, including enzymes. They may come from autolysed cells or may be secreted by viable cells to facilitate degradation of macromolecular substances adsorbed by extracellular polymeric substances (EPS) [11,20]. Frølund et al. [36] also demonstrated the presence of enzymes responsible for the hydrolysis of FDA in the biofilm matrix. They observed much greater enzymatic activity per cell in activated sludge flocs than in sludge cultures. Jørgensen et al. [37] also noted that they may be responsible for 20–30% of FDA hydrolysis reactions from samples. However, due to the anionic nature of the biofilm, accumulation of negatively charged fluorescein in the biofilm matrix after 1.5 h of incubation was not statistically significant (data not shown).

In this study, we investigated the temporal variation of fluorescein release from B1 (2015b) cells immobilized onto PUR during 1.5 h of incubation. A linear relationship was observed throughout all analyzed times of incubation with the maximum amount of released fluorescein after 1.5 h of incubation (128 $\pm$ 5 µg/g dry mass per h, Figure 3). This result show that FDA hydrolysis was not limited by substrate concentration over the analyzed period of time. Due to the fact that the assay was conducted at a favorable temperature for bacterial cell proliferation (30 °C) [38], it was suggested that a long-term incubation could lead to a result that would not reflect the enzymatic activity of the original sample [17,22,39]. Adam and Duncan [39] also pointed out that it is more important to estimate the hydrolytic potential of the samples than to obtain the highest concentration of fluorescein; therefore, they recommend that incubation last not longer than one hour. On the other hand, Green et al. [22] recommended that incubation last longer than 2 h for soil samples, thereby allowing better differentiation of the results.

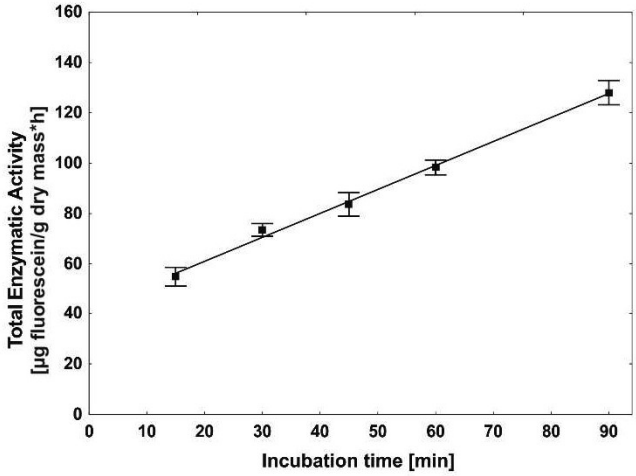

Figure 3. Fluorescein release over time during FDA hydrolysis by immobilized B1 (2015b) cells onto PUR. Error bars were obtained based on the standard deviation.

In the analyzed period of time, errors associated with the growth of microorganisms were eliminated. However, to allow a longer differentiation of samples, an incubation time of 1 h was chosen for the final procedure.

2.5. Sensitivity Assay—Carbon Starvation

The incubation time during immobilization is an extremely important parameter which determines the formation of a stable and strong biofilm, which, after reaching maturity, will be fully resistant to adverse environmental conditions and will be able to degrade higher concentrations of impurities. However, to produce biofilms, bacterial cells must be metabolically active. One of the basic factors affecting metabolic activity is the availability of easily assimilable carbon sources. During a shortage of carbon sources, bacterial cells will reduce their size, and very often, their shape as well (they become more round). If, however, nutrition level drops to a minimum, the response to these conditions involves limiting endogenous metabolism to such a level that they will not be able to reproduce, but will remain active [40–42]. Under these conditions, the vegetative bacterial cells can survive, depending on the strain, from a few to even 100 days (e.g., *Arthrobacter crystallopoietes*) [43].

In order to determine sensitivity of the optimized method, it was observed how the total enzymatic activity (TEA) of bacterial cells in the developing biofilm decreased to the point of minimal endogenous metabolism under starvation during the immobilization process. Seventy-two hours of incubation without a carbon source in the medium resulted in a gradual decrease in TEA (Table 3). After 24 h of incubation, when immobilized B1 (2015b) cells were using accumulated sources of energy, the highest enzymatic activity (360 $\pm$ 24 µg/g dry mass per h) was observed. Along with the progressing starvation, after 72 h, a nearly twofold reduction in mean TEA was observed (170 $\pm$ 7 µg/g dry mass per h), which indicates the exhaustion of energy reserves and restriction of metabolic activity. The obtained results agree with those obtained by Gengenbacher et al. [44] and Voelker et al. [45], in which a significant decrease in the amount of ATP was demonstrated in nutrient-starved *Bacillus subtilis* and *Mycobacterium tuberculosis*, which indicates a reduction of metabolic activity. It should be also noted that the obtained fluorescein concentration after the analyzed period of time does not differ despite increasing biofilm mass (Table 3). This result can be caused by continuous EPS production by bacterial cells without progressing colonization of the carrier. However, monitoring of the changes in the optical density (OD_{600}) of the medium during immobilization reveal that, with progressing incubation, more cells migrated from the medium. After 24, 48, and 72 h, reductions in the initial OD_{600} value were observed to be 39.5 $\pm$ 0.9, 48.8 $\pm$ 3.5, and 54.8 $\pm$ 9.1%, respectively. It was observed also that, despite the increasing amount of EPS during incubation, it did not exceed 13–15% of the

biofilm mass (Table 3). These results clearly indicate that the drop in TEA was caused by the decreasing activity of newly colonizing bacterial cells, instead of the increasing amount of EPS.

Table 3. Impact of carbon starvation on the TEA of the immobilized *Bacillus thuringiensis* B1 (2015b) strain during the immobilization process. EPS—extracellular polymeric substances.

Incubation time (h)	Biofilm dry mass (g)	Dry EPS mass(g)	Fluorescein concentration (μg/ml)	TEA (μg/g dry mass per h)	Mean	SD	CV (%)
24	0.0034–0.0049	0.0005–0.0007	1.21–1.66	326–383	360	24	7
48	0.0064–0.0078	0.0008–0.0010	1.63–2.45	255–325	287	28	10
72	0.0080–0.0091	0.0010–0.0013	1.43–1.50	166–180	170	7	4

In comparison to the TEA values obtained from planktonic B1 (2015b) cells present in the medium, cells immobilized in the biofilm were characterized by better resistance to starvation. After 48 h of incubation, unimmobilized B1 (2015b) cells showed the lowest TEA value (161 ± 17 μg/g dry mass per h), which was maintained until the end of the analyzed period of time. This result shows that the TEA in the range of 160–170 μg/g dry mass per h indicated that the B1 cells (2015b) were limited to endogenous metabolism.

It was noticed that starvation of *Bacillus thuringiensis* B1 (2015b) cells promoted their immobilization on polyurethane foam. To observe the progress of immobilization, SEM micrographs were prepared after 24, 48, and 72 h of incubation of polyurethane foam with *Bacillus thuringiensis* B1 (2015b) cells (Figure 4).

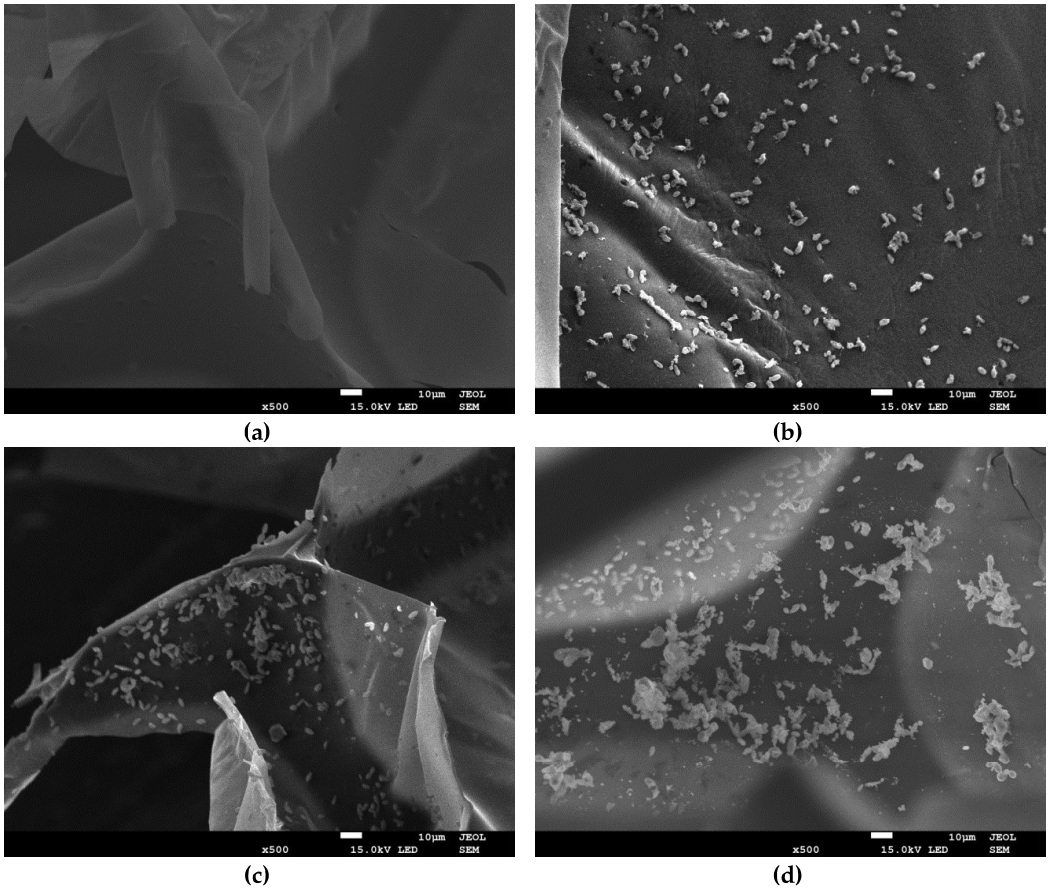

Figure 4. SEM micrographs of biofilm formation by the B1 (2015b) strain onto PUR cubes during starvation after 24 h (**b**), 48 h (**c**), and 72 h (**d**) of incubation. The surface of the unimmobilized control PUR cube is shown in (**a**).

As can be seen in Figure 4b, the colonization of polyurethane foam by B1 (2015b) cells was already evident after 24 h of immobilization. Adsorption of bacterial cells onto the surface of polyurethane foam was observed, which indicates the start of the biofilm formation process. Over time, the bacterial cells began forming microcolonies and secreting extracellular polymeric substances (EPS; Figure 4c). After 72 h, accumulated cells and extracellular matrices in the form of connected aggregates were observed on the PUR surface (Figure 4d). As is known, the limitation of nutrients such as carbon, nitrogen, or phosphorus in the medium is an inducer of sporulation in *Bacillus subtilis* and *Bacillus cereus* cells. The transcription factor *Spo0A* is activated, which, apart from participating in the production of spores, also promotes the formation of biofilm by induction of EPS production. This is one of the defense mechanisms of this genus during the absence of nutrients [46,47]. Due to the fact that the *Spo0A* gene was found in the genome of *Bacillus thuringiensis* [48,49], the mechanism of biofilm induction during starvation in *B. thuringiensis* B1 (2015b) used in this study could be similar. As a result, the decrease in total enzymatic activity of immobilized B1 (2015b) cells was caused by spending energy reserves on EPS synthesis and biofilm formation.

During estimation of the immobilization efficiency, only by determining the dry mass of immobilized bacterial cells could 72 h of incubation be considered optimal. However, after examination of the enzymatic activity, it is shown that the bacterial cells were weakened. The biodegradation tests in this case could be significantly prolonged due to the time when the biofilm would be regenerating. If, however, contamination indicates toxic effects on the strain, it could even lead to their death. With the developed method, it is possible to examine the physiological state of the biofilm formed on the carrier, thereby optimizing the immobilization process, allowing one to obtain a biofilm with the highest enzymatic activity.

2.6. Comparision of the Modified FDA Method with Oxygen Consumption

Oxygen is the key substrate conditioning the metabolism of aerobic organisms. It is necessary for ATP synthesis, and therefore, for the growth, proliferation, and synthesis of various cellular elements. However, due to its poor solubility in water, cultures of aerobic microorganisms must be constantly mixed to ensure its transition from the gas phase. For this reason, oxygen availability in the medium can be a growth-limiting factor for cell cultures [50]. Because of the unique mass transfer properties of biofilms, they are able to adsorb oxygen even at low concentrations. As a result, biofilms ensure, in the top layers, a constant amount of oxygen depending on the cell's oxygen demand, which, in turn, results from the physiological state of bacterial cells [51]. Recent studies showed that differences in oxygen concentration in the horizontal direction at the same depth of aerobic biofilms are statistically insignificant; however, like the previously mentioned nutrients, they are significant in the vertical direction [52,53].

The oxygen uptake rate (OUR), due to its good correlation with metabolic activity, provides valuable information on the physiological state of microbial cells. Due to its relatively simple calculation, OUR is often used to characterize activated sludge [54], production processes [55], and bioremediation [56,57].

To test whether the optimized method would present the same relationships as those used to assess the physiological state of microbial cells, the oxygen uptake rate (OUR) was measured during the starvation assay described above. Table 4 summarizes the obtained values of OUR and TEA after 24, 48, and 72 h of incubation of B1 (2015b) cells with PUR in a medium without carbon sources. The trend in oxygen uptake rate showed a very good correlation with the decrease in total enzymatic activity. After 72 h, immobilized cells showed a nearly twofold reduction in OUR (70 ± 4 μg/g dry mass per h) compared to values obtained after 24 h of incubation (176 ± 13 μg/g dry mass per h). A similar decrease in OUR value in a trickling filter biofilm was also observed by Cox et al. [41] in the absence of toluene, which was the only carbon source in the experiment.

Table 4. Comparison of TEA and oxygen uptake rate (OUR) during carbon starvation of immobilized B1 (2015b) cells during the immobilization process. Data are presented as means ± standard deviation of three replicates.

Incubation time (h)	TEA (μg/g dry mass per h)	OUR (μg O_2/g dry mass per h)
24	360 ± 24	176 ± 13
48	287 ± 28	120 ± 9
72	170 ± 7	70 ± 4

A comparison of the proposed and optimized method in this study for evaluating the physiological state of immobilized cells in biofilms and the method based on the determination of oxygen uptake requires a consideration of the advantages and disadvantages of each technique. OUR is well-established method that allows an indirect estimation of metabolic activity. Its biggest advantage is the duration of the measurement, because results can be obtained after 10 minutes. On the other hand, this method requires experience, due to the fact that, depending on the method of flask sealing and oxygen removal from the gas phase, the result may be burdened with various errors caused by the transfer of oxygen from the air. The determination of the physiological state by direct analysis of the activity of non-specific enzymes responsible for organic matter degradation proposed herein does not require any technical steps that may disturb the final results.

To conclude, in this study, a modification of the FDA assay was optimized in a way which allows results which are reproducible and have a low coefficient of variation. The result also implies the diversity of activities resulting from the heterogeneity of the biofilm. However, due to the possible fluorescein adsorption by the carrier, it is necessary to carry out adsorption tests. With the proposed method, it is possible to monitor changes in the physiological state of the biofilm formed on the carrier through optimization of the immobilization process. By conducting the optimization in this way, the development of an immobilized biocatalyst was possible with the highest enzymatic activity, and thus, with the highest biodegradation capacity or resistance to harsh environmental conditions.

3. Materials and Methods

3.1. Materials

Polyurethane foam (PUR) used in this study is a commonly used material to protect packages during transport (Instapak®, Charlotte, NY, USA). The carrier was trimmed into 1 × 1 × 1 cm cubes with a weight of 10 ± 5 mg, and was washed two times with distilled water to remove impurities, before being autoclaved (121 °C, 1.2 atm, 20 min). All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Bacterial Strains and Growth Conditions

Bacterial strain *Bacillus thuringiensis* B1 (2015b) isolated from the soil of the chemical factory "Organika-Azot" in Jaworzno (Poland) was used for immobilization [26]. Strain B1 (2015b) was grown in the nutrient broth (BBL) at 30 °C on a rotary shaker at 130 rpm for 24 h. After cultivation, cells were harvested by centrifugation (5000 rpm, 15 min), washed twice with a sterile mineral salt medium according to Greń et al. [58], and re-suspended in the same medium. A bacterial suspension at a final concentration corresponding to an optical density (OD_{600}) of 0.8 was used for immobilization.

3.3. Immobilization Procedure

Each Erlenmeyer flask (250 mL) containing sterile carrier material (0.1 g) was inoculated with the bacterial cell suspension (100 mL). The mineral salt medium [58] in which the immobilization process was conducted did not contain any carbon sources. The immobilization process was carried out on the orbital shaker (130 rpm) at 30 °C for 72 h. After incubation, the medium was removed

and immobilized PURs were suspended in NaCl solution (0.9%), centrifuged at 500 rpm for 2 min to remove unbound microorganisms, rinsed with 0.9% NaCl, and used for further analysis.

3.4. Standard Method of Non-Specific Esterase Activity with FDA Assay

The physiological state of the bacterial cells was determined via measurements of non-specific esterase activity with fluorescein diacetate (FDA) as a substrate. The original method [18] includes detaching microorganisms from the carriers by shaking (5 g) in 100 mL of distilled water (200 rpm, 30 min). In the next step, 2 mL of the microorganism suspension was added to 8 mL of phosphate buffer (pH 7.0) and incubated for 15 min with shaking at 30 °C. After pre-incubation, 0.1 mL of FDA stock solution (4.8 mM, dissolved in acetone) was added to each sample and incubated for 2 h. Fluorescein concentration was measured spectrophotometrically (Genesys 20, Thermo Fisher Scientific, Inc., Rochester, NY, USA) at 490 nm and was calculated on the basis of a standard curve.

3.5. Abiotic Controls for FDA Assay

To examine fluorescein adsorption by PUR, sterile carrier cubes (one cube per assay) were placed in solutions with different concentrations (0.5–5 μg/mL) of sterile fluorescein suspended in phosphate buffer (pH 7.0) and incubated in the dark on the orbital shaker (130 rpm, 30 °C). After 1 h of incubation, absorbance (λ = 490 nm) was measured. Additionally, control samples were prepared in the case of FDA autohydrolysis and the natural coloration of the sample with and without sterile carriers.

3.6. Optimization Procedure

The main aim of the optimization procedure was to skip the step of detachment of microorganisms from the carrier in such a way to allow testing of the enzymatic activity of the entire biofilm formed on the carrier without disturbing it. For the best reproducibility, the impacts of substrate application method (FDA added to the liquid or into the carrier) and agitation (with or without) were examined. In order to maximize the activity of non-specific esterases, optimizations of the pH (6.8–7.6) and of the incubation time of immobilized strain B1 (2015b) (15–90 min) with FDA solution were also performed.

3.7. Modified Method of Non-Specific Esterase Activity with FDA Assay

The final methodology is defined as follows: an immobilized PUR cube was placed into 8 mL of phosphate buffer (pH 7.6) and incubated for 15 min on the orbital shaker. In the next step, 0.1 mL of FDA solution in acetone (4.8 mM) was slowly injected directly into the middle of the carrier and incubated on the orbital shaker (130 rpm, 30 °C) for 1 h. Fluorescein concentration was measured as described in Section 3.4.

3.8. Sensitivity Assay—Carbon Starvation

To determine the sensitivity of the method, the impact of carbon starvation on the metabolic response of bacterial cells and the immobilization process was monitored and expressed as total enzymatic activity (TEA). In this test, bacterial cells were immobilized onto PUR as described in Section 3.3 with the incubation time varied to 24, 48, or 72 h. After incubation, the FDA hydrolysis potential of the immobilized bacterial cells was examined. The biofilm's dry mass was calculated by comparing the dried weight of the immobilized carrier (dried at 105 °C for 2 h and stored in a desiccator) with that of the unimmobilized carriers incubated and dried under the same conditions. TEA was expressed in μg of fluorescein obtained from 1 g of biofilm dry mass for 1 h [59]. TEA values for unimmobilized cells of B1 (2015b) were obtained in the same way as for immobilized cells, except that 2 mL of the culture was added to the phosphate buffer (pH 7.6) and, after 1 h of incubation with FDA, bacterial cells were collected through filtration on 0.2-μm Nuclepore filters [15,17,59]. Migration of the bacterial cells from the medium was determined using spectrophotometry (OD_{600}; Genesys 20, Thermo Fisher Scientific, Inc., Rochester, NY, USA). EPS extraction from the immobilized PUR cubes

was conducted according to the protocol proposed by Subramanian et al. [60] with some modifications. The PUR cube after 24, 48, or 72 h of incubation was transferred from the medium into 20 mL of distilled water, centrifuged (500 rpm for 2 min) to remove unbound microorganisms, and re-suspended in the same volume of Milli-Q water (Burlington, MA, USA). In the next step, the sample was ultrasonically treated three times for 15 s with a time interval of 10 s, and centrifuged (without carrier, 14000 rpm for 20 min at 4 °C). The collected supernatant containing EPS was precipitated with 2.2 volumes of absolute chilled ethanol through incubation of the mixture at 20 °C for 1 h, and was separated by centrifugation at 6000 rpm for 15 min at 4 °C. The dry EPS mass was obtained by drying the pellet at room temperature and overnight storage in the desiccator.

3.9. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to illustrate biofilm formation onto a carrier during starvation. For this purpose, immobilized carrier cubes were fixed in 3% glutaraldehyde and 1% osmium tetroxide, dehydrated with ethanol (30, 50, 70, 80, 90, 95, and 100%, each for 10 min), dried by lyophilization, covered with gold, and observed with a high-resolution electron microscope JSM-7100F TTL LV (JEOL, Tokio, Japan).

3.10. Oxygen Consumption

Oxygen uptake rate (OUR) was determined using an Elmetron multiparameter equipped with a Clark electrode. One immobilized PUR cube was introduced into a flask containing 15 mL of oxygen-saturated phosphate buffer (pH 7.6, 20 °C). To minimize the measurement error, the vessels were placed on a magnetic stirrer and sealed. The decrease in oxygen concentration was registered every 30 sec for 10 min. Oxygen uptake rate was calculated from the slope of a linear regression line through the obtained results and expressed as OUR (μg of consumed O_2 by 1 g of biofilm dry mass during 1 h) [54,61].

3.11. Statistical Analysis

All experiments were performed in at least three replicates. The values of the efficiency of immobilization and enzyme activities were analyzed by one-way ANOVA ($p \leq 0.05$ was considered significant) using the STATISTICA 12 PL software package (StatSoft Inc., Kraków, Poland). A post hoc test was applied to assay the differences between the treatments. To express the repeatability and precision of conducted assays, the coefficient of variation (CV) was calculated as the quotient of the standard deviation and the mean of the obtained TEA from each flask.

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II.3.

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Immobilization of *Planococcus* sp. S5 Strain on the Loofah Sponge and Its Application in Naproxen Removal

Anna Dzionek [1], Danuta Wojcieszyńska [1], Katarzyna Hupert-Kocurek [1], Małgorzata Adamczyk-Habrajska [2] and Urszula Guzik [1,*]

[1] Department of Biochemistry, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Jagiellońska 28, 40-032 Katowice, Poland; adzionek@us.edu.pl (A.D.); danuta.wojcieszynska@us.edu.pl (D.W.); katarzyna.hupert-kocurek@us.edu.pl (K.H.-K.)

[2] Institute of Technology and Mechatronics, University of Silesia in Katowice, Żytnia 12, 41-200 Sosnowiec, Poland; malgorzata.adamczyk-habrajska@us.edu.pl

* Correspondence: urszula.guzik@us.edu.pl; Tel.: +32-200-9567

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Abstract: *Planococcus* sp. S5, a Gram-positive bacterium isolated from the activated sludge is known to degrade naproxen in the presence of an additional carbon source. Due to the possible toxicity of naproxen and intermediates of its degradation, the whole cells of S5 strain were immobilized onto loofah sponge. The immobilized cells degraded 6, 9, 12 or 15 mg/L of naproxen faster than the free cells. *Planococcus* sp. cells immobilized onto the loofah sponge were able to degrade naproxen efficiently for 55 days without significant damage and disintegration of the carrier. Analysis of the activity of enzymes involved in naproxen degradation showed that stabilization of S5 cells in exopolysaccharide (EPS) resulted in a significant increase of their activity. Changes in the structure of biofilm formed on the loofah sponge cubes during degradation of naproxen were observed. Developed biocatalyst system showed high resistance to naproxen and its intermediates and degraded higher concentrations of the drug in comparison to the free cells.

Keywords: whole-cell immobilization; loofah sponge; *Planococcus* sp. S5; naproxen

1. Introduction

In recent years more attention has been paid to the presence of various medicines in the natural environment. One of them is naproxen (2-(6-methoxy-2-naphthyl)propionic acid) which belongs to the group of polycyclic Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). This drug is not metabolized in human body and the sewage treatment plants are not adapted to its utilization. Therefore, naproxen has been releasing into the natural environment in an unchanged form for over 40 years. Due to its continuous accumulation in the environment, naproxen is now one of the most frequently detected drug in surface and drinking water (concentration in the range 0.01–2.6 μg/L) [1–3].

The most efficient methods for naproxen removal from the environment are based on the physicochemical processes. However, the biggest disadvantage of these methods is generation of products with greater toxicity than the drug itself and formation of free radicals that directly damage biological structures [3–6]. The use of microorganisms in naproxen utilization brings many benefits. Bioremediation is based on the capabilities of selected microorganisms for accumulation, transformation, detoxification or degradation of pollutants. This technology is environmentally friendly, cheap and effective [7–9].

During application of microbial cells in bioremediation processes, extremely important is their immobilization. It allows increasing microbial degradation capacity, extends viability and catalytic

activity of cells introduced into bioremediation systems as well as increases the chances of microbial cells survival and adaptation to changing environment. In immobilization process, microbial cells are trapped in polymeric gels (entrapment, encapsulation) or on the surface of various carriers (adsorption, electrostatic or covalent binding on the surface). However, the main disadvantages of immobilization are: limitation of diffusion, leakage of cells into the medium or lowering their catalytic functions depending on the binding compounds used. Recently, immobilization based on the natural ability of some microorganisms to biofilm formation on the surface of various carriers is gaining more attention. Through the existence of many microenvironments in the biofilm, cells are less vulnerable to changing environmental conditions. Created in this way stable matrix is also characterized by a high degree of heterogeneity in which microbial cells are protected against anti-bacterial agents and bacteriophages and are able to degrade contaminants at higher concentrations [8,10,11].

From an economic point of view, the carrier and the procedure for immobilization of microorganisms should be cheap. Therefore, natural and organic carriers are widely used in bioremediation processes [8,12]. These biodegradable and biocompatible supports are characterized by hydrophilic surface on which many functional groups are located. Among these natural carriers, particular attention is paid to the group of lignocellulolistic plant materials. One of them is loofah sponge derived from the dry mature fruit of *Luffa aegyptiaca*, grown in most subtropical and tropical regions. This sponge is composed of an open network of fibres that form the skeleton of the fruit. The main advantages of this material are its high porosity, low price, non-toxicity, simple application and operation technique and high mechanical resistance [13–15].

The present work is a continuation of studies on bacterial degradation of NSAIDs. Our previous study showed the ability of Gram-positive *Planococcus* sp. S5 to catalyse naproxen degradation under cometabolic conditions [2]. The aim of this study was to investigate the effect of immobilization of *Planococcus* sp. S5 cells on the loofah sponge on degradation processes. The degradation capacity of the developed biocatalyst at various concentrations of naproxen and its reusability were examined. To investigate how immobilization affected degradation activity of S5 strain, the activity of enzymes involved in naproxen utilization was examined. Additionally, visualization of biofilm formed on the surface of the carrier and its changes during drug degradation was performed. This is the first report about degradation of naproxen by immobilized bacterial cells. Moreover, the results of these studies enable evaluation of potential application of tested strain in bioremediation systems.

2. Results and Discussion

2.1. Immobilization of Planococcus sp. S5 on Loofah Sponge

The natural ability of some microorganisms to colonize surface of porous materials is a key feature for efficient immobilization. At the beginning, process of cells attachment to the surface is reversible and cells may be easily removed from the carrier by washing. In the second phase, cells which synthesize extracellular polymeric substances bind to the surface of the carrier with such a force that more invasive process is needed to remove them. Taking into consideration stability of the constructed biocatalyst, it is desirable that the formed biofilm should be strongly bound to the carrier. This feature depends on the species of the microorganism and the type of the surface. Additionally, selection of proper conditions of immobilization can improve the quality of biofilm [16–18]. To verify the quality of biofilm formed by S5 strain, which corresponds to its catalytic functions, its physiological state was evaluated by determination of its ability to hydrolyse fluorescein diacetate by non-specific esterases produced by alive bacterial cells (total enzymatic activity). This assay was chosen due to its simplicity, short incubation time (1 h) and possibility of spectrophotometric determination of the data. What is more important, the test used is correlated with other bacterial indicators such as an amount of biomass or adenosine triphosphate (ATP) and the oxygen consumption [19,20].

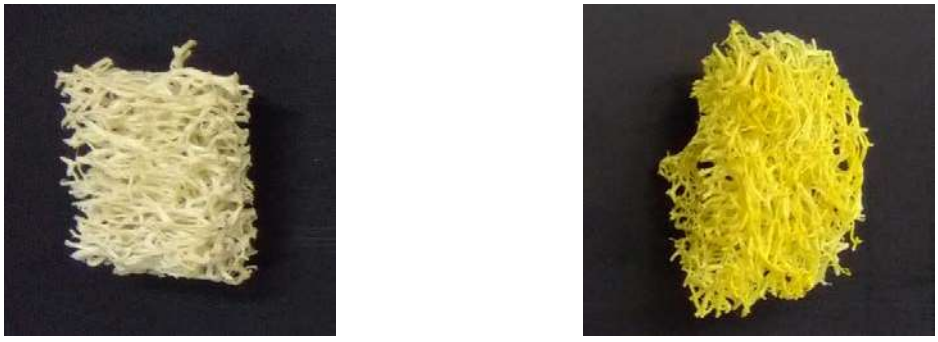
Selection of the optimal conditions for immobilization of the whole bacterial cells by their adsorption on the surface is an extremely important in designing of biocatalyst systems. Due to

the diversity of bacteria, optimization should be carried out for each immobilized strain. Strain *Planococcus* sp. S5, which was isolated from the activated sludge from a sewage treatment plant in Bytom Miechowice (Poland), was able to degrade aromatic compounds like salicylate, benzoate, hydroxybenzoate and dihydroxybenzoate and phenol [21,22]. However, this is the first report regarding its immobilization and one of a few on the ability of biofilm formation by bacteria from *Planococcus* genus [23].

Procedure of *Planococcus* sp. S5 immobilization on the loofah sponge through its adsorption on the surface was developed by optimizing each parameter to obtain the highest amount and enzymatic activity of immobilized microorganisms. Immobilization of S5 cells on the loofah sponge was the most effective in mineral salts medium (pH 7.2), in the presence of glucose and manganese salt, during bacteria incubation with shaking (90 rpm) at 30 °C. The effect of the growth phase on immobilization process was also observed. The best results were obtained for a culture at stationary phase. Immobilization of S5 strain was also more efficient during the osmotic stress caused by a higher concentration of NaCl (19 g/L) and at high number of cells in the medium (initial OD_{600} equal to 1.2) (Figure S1).

It has been proven that limitation of carbon source in the medium stimulates biofilm formation by *Bacillus subtilis* by activating the Spo0A transcription factor [24]. This mechanism enables survival and proliferation of bacteria during nutrient deficiency at which growth in the form of planktonic cells is impossible. On the other hand, *Staphylococcus aureus* and *Staphylococcus epidermidis* form biofilms only when glucose is present in the medium because it is necessary for the synthesis of adhesins [25]. Therefore, since S5 strain is not spores producing bacterium [21], efficient immobilization in the presence of glucose probably resulted from synthesis of adhesins which are involved in attachment of cells to the carrier. Bacteria of genus *Planococcus* are known to be moderately halophilic [26–28]. Recent research [23] shows that osmotic stress favours the formation of biofilm by *Planococcus rifietoensis* by increasing production of exopolysaccharides (EPS), which additionally improves water holding capacity at higher salt concentrations.

The developed method of S5 strain immobilization on the loofah sponge resulted in 0.0191 ± 0.0022 g of *Planococcus* sp. S5 cells (dry mass) immobilized on each loofah cube, able to hydrolysis of 23.88 ± 1.06 μg of fluorescein diacetate during 1 h (total enzymatic activity) (Figure 1). Results for fluorescein diacetate hydrolysis and fluorescein adsorption by unimmobilized loofah cubes were not statistically significant.



(**a**) (**b**)

Figure 1. Loofah sponges cubes after total enzymatic activity assay; (**a**) unimmobilized carrier (**b**) loofah cubes after incubation with *Planococcus* sp. S5 cells.

2.2. Naproxen Biodegradation

2.2.1. Biodegradation of Different Concentration of Naproxen

Naproxen (2-(6-methoxy-2-naphthyl)propionic acid)) belongs to the propionic acid derivatives family. Due to the presence of two aromatic rings, naproxen biodegradation is more difficult than monocyclic NSAIDs. Only a few strains (mainly white-rot fungi) are known to possess enzymatic systems which enable them to acquire carbon and energy from naproxen [29,30]. Biodegradation of naproxen by bacterial strains is a process that is being explored. However, none of the known bacterial strains—*Planococcus* sp. S5 [2], *Bacillus thuringiensis* B1(2015b) [31], *Stenotrophomonas maltophilia* KB2 [32], *Pseudomonas* sp. CE21 [33]—are able to use naproxen as the sole carbon source. First study about naproxen biodegradation by strain *Planococcus* sp. S5 [2] revealed that the addition of glucose which was readily available source of carbon and energy resulted with complete biodegradation of the drug. However, due to the negative effect of naproxen on the total enzymatic activity of free S5 cells, the degradation of the drug at a concentration of 6 mg/L lasted 38 days (data not published). For this reason it was decided to carry out the immobilization process.

Loofah sponge due to the relatively large surface area and chemical composition (cellulose, hemicellulose, lignin) shows good sorption properties. However, making the loofah sponge capable of sorption of hydrophobic substances (like naproxen) requires subjecting it to a cooking process with NaOH to increase its hydrophobicity and to create available vacant sites [34,35]. For this reason, the loofah sponge in this study has not been subjected to a cooking process with NaOH to limit its sorption capacity. Another important factor that needed to be consider in biodegradation studies with immobilized cells by adsorption of the surface is accumulation of xenobiotics in biofilm. This phenomenon is related to the sorption properties of exopolysaccharides (EPS). While the sorption of positively-charged compounds by EPS is more efficient due to anionic nature of biofilm, sorption of anionic organic molecules is limited due to electrostatic repulsion [36].

There was no changes observed in drug concentration during incubation with sterile non-immobilized carriers, which confirms lack of the naproxen adsorption capacities by prepared in that way loofah sponges. Naproxen was also not detected in the biofilm formed onto the loofah sponges by *Planococcus* sp. S5. Obtained results demonstrated that loss of the drug from the medium during biodegradation experiments was caused only by immobilized cells of *Planococcus* sp. S5.

To investigate whether the immobilization onto the loofah sponges affected the degradation capacity of *Planococcus* sp. S5, biodegradation of various concentrations of naproxen (6, 9, 12 or 15 mg/L) by immobilized cells with respect to non-immobilized cells was tested (Figures 2 and 3).

Observation of the efficiency of the naproxen cometabolic biodegradation conducted by free cells of *Planococcus* sp. S5 showed that they were able to degrade naproxen in concentration 6, 9 and 12 mg/L respectively in 38, 44 and 62 days. Biodegradation of the highest tested concentration of the drug (15 mg/L) stopped after biodegradation of 29% of naproxen (Figure 2a). Obtained results shows that the free cells of S5 strain were able to complete cometabolic naproxen biodegradation at a concentration up to 12 mg/L. Higher drug concentrations showed inhibitory effects on free cells of the S5 strain. Immobilization of *Planococcus* sp. S5 cells onto the loofah sponges resulted in a significant acceleration of the naproxen cometabolic biodegradation. It was observed a complete biodegradation of 6, 9, 12 and 15 mg/L of naproxen respectively after 17, 32, 43 and 53 days (Figure 2b).

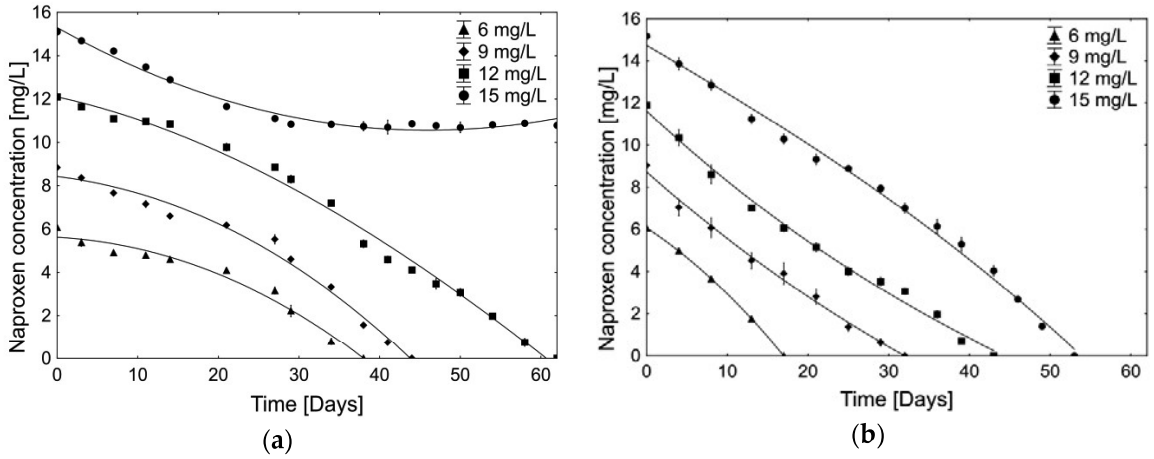


Figure 2. Cometabolic degradation of 6, 9, 12 and 15 mg/L naproxen by (**a**) free cells of *Planococcus* sp. S5; (**b**) cells of *Planococcus* sp. S5 immobilized onto the loofah sponge. Data presented as mean ± standard deviation of three replicates.

In order to evaluate changes in the course of the biodegradation process, the rate of naproxen biodegradation was calculated (Tables 1 and 2). Biodegradation rates by free cells of S5 were statistically different (Test T, $p \geq 0.05$) than by immobilized cells. During the biodegradation of naproxen by free cells, in the initial phase the slower drug degradation was observed, independently of the drug concentration (average 6.3 ± 3.4 μg/h). After 29 days twice faster biodegradation of the drug (12.0 ± 4.5 μg/h) was observed that lasted until the end of biodegradation (Table 1). Designation of the naproxen biodegradation rates by immobilized cells of S5 strain revealed its almost linear and constant course (12.1 ± 4 μg/h) (Table 2). Interestingly, there was no significant difference between naproxen biodegradation rates by the immobilized cells and by free cells during faster drug degradation phase (Test T, $p \geq 0.05$). This situation was most likely caused by the fact that cells in the biofilm exhibit characteristics of the stationary phase (altered genetic expression profile and slower growth) [37]. Confirmation of this hypothesis is the fact that the phase of faster naproxen degradation by free cells of *Planococcus* sp. S5 occurred when cells entry into the stationary phase (data not published).

Table 1. Naproxen degradation rates by free cells of *Planococcus* sp. S5. Data presented as a mean ± standard deviation of three replicates.

Period [Day–Day]	Average Naproxen Degradation Rate [μg/h]			
	6 mg/L	9 mg/L	12 mg/L	15 mg/L
0–3	9.8 ± 2.2	6.6 ± 0.6	6.4 ± 1.1	5.7 ± 0.9
3–7	4.8 ± 2.2	7.3 ± 1.7	5.8 ± 1.0	5.0 ± 1.7
7–11	1.3 ± 0.5	5.3 ± 2.5	1.2 ± 0.5	7.6 ± 0.2
11–14	2.6 ± 1.4	7.7 ± 1.5	1.7 ± 2.6	8.1 ± 1.9
14–21	3.0 ± 0.4	2.5 ± 0.4	6.4 ± 1.5	7.4 ± 0.1
21–27	6.5 ± 1.4	4.5 ± 1.7	6.4 ± 0.5	3.8 ± 0.6
27–29	19.6 ± 3.3	19.2 ± 6.0	11.5 ± 4.6	5.5 ± 1.2
29–34	11.9 ± 0.9	10.6 ± 1.4	9.2 ± 1.5	0.1 ± 1.9
34–38	8.2 ± 1.6	18.4 ± 1.3	19.6 ± 0.6	1.0 ± 3.2
38–41		11.4 ± 0.5	10.1 ± 0.7	0.6 ± 7.3
41–44		10.3 ± 0.6	6.7 ± 1.1	−2.2 ± 4.9
44–47			8.9 ± 5.1	1.3 ± 3.8
47–50			5.5 ± 4.0	1.2 ± 4.0
50–54			11.4 ± 0.9	−1.3 ± 3.7
54–58			12.8 ± 1.1	−0.7 ± 2.4
58–62			7.8 ± 2.0	1.0 ± 1.9

Table 2. Average naproxen degradation rates by immobilized cells of *Planococcus* sp. S5. Data presented as a mean ± standard deviation of three replicates.

Period [Day–Day]	Average Naproxen Degradation Rate [μg/h]			
	6 mg/L	9 mg/L	12 mg/L	15 mg/L
0–4	11.2 ± 1.2	20.7 ± 8.6	16.1 ± 6.7	13.9 ± 0.6
4–8	13.9 ± 0.9	10.1 ± 1.4	18.0 ± 14.6	10.5 ± 2.5
8–13	15.8 ± 1.1	12.9 ± 1.8	13.2 ± 5.4	13.4 ± 4.2
13–17	18.2 ± 1.7	6.4 ± 2.4	10.1 ± 1.2	10.0 ± 1.4
17–21		11.6 ± 3.0	9.4 ± 0.9	10.0 ± 1.0
21–25		15.0 ± 5.8	12.0 ± 4.8	4.6 ± 4.2
25–29		7.6 ± 3.9	5.1 ± 0.6	9.7 ± 5.6
29–32		8.7 ± 4.2	6.4 ± 2.9	13.1 ± 5.6
32–36			11.5 ± 1.7	10.8 ± 6.0
36–39			17.4 ± 4.8	9.4 ± 4.3
39–43			7.3 ± 2.4	13.0 ± 2.5
43–46				19.0 ± 2.8
46–49				17.7 ± 0.6
49–53				14.6 ± 2.2

It is known that one of the most important parameters that can significantly affect the biodegradation process is initial substrate concentration. Analysis of the patterns and correlations between the time of incubation, naproxen concentration and biodegradation rate shows that free cells of *Planococcus* sp. S5 were capable of the fastest biodegradation of naproxen at the lowest dose. With increasing drug concentration the rate of naproxen biodegradation was decreased (Figure 3a). Obtained results indicated that during decomposition of naproxen, accumulation of metabolites occurred, which negatively affect free cells of S5 strain and caused increasing difficulties with its total degradation. Therefore, biodegradation of the highest dose of naproxen (15 mg/L) ended with 29% efficiency most likely due to the critical level of inhibitory or toxic metabolites. Recently, more attention has been focused on the antibacterial activity of certain NSAIDs or their derivatives [38,39]. Although the mechanisms of this process are not known, one study found that vedaprofen, bromfenac and carprofen—by binding to polymerase α subunit—inhibit the proliferation of *E. coli*, *A. baylyi*, *S. aureus* and *B. subtilis* cells [39]. Inhibitory effect of naproxen on the ammonia oxidizing bacterium (AOB) *Nitrosomonas europaea* was observed by Wang et al. [40]. They revealed that naproxen at concentration of 10 μM significantly inhibits nitrile production by AOB by affecting membrane integrity of the cells, while exposure on the drug in concentration of 1 μM did not influences AOB cells. To reveal possibility of antibacterial activity of naproxen or its intermediates on *Planococcus* S5 cells more research should be attempted.

No correlation between analysed variables was observed for the immobilized cells. Degradation proceeded with the same trend regardless of the initial naproxen concentration (Figure 3b). Obtained results indicated good adaptation of immobilized S5 cells to the presence of higher concentrations of naproxen. Lack of the lag phase and concentration-independent drug biodegradation course suggests that formed biofilm reduced the sensitivity of the cells to naproxen and its intermediates. Due to the good sorption properties and the limited diffusion in the extracellular biofilm matrix, immobilized microorganisms have limited contact with xenobiotics, which are transported to the cells at a constant, slower rate. This mechanism allows the immobilized cells to biodegrade higher concentrations of impurities, without causing toxic effects [41]. Thus, the immobilized cells of *Planococcus* sp. S5 onto the loofah sponge have a promising potential to use them for the bioremediation of naproxen-contaminated sites. In addition, lack of the toxic effect of naproxen and its metabolites on immobilized cells allowed them to utilize higher concentrations of the drug compared to the free cells.

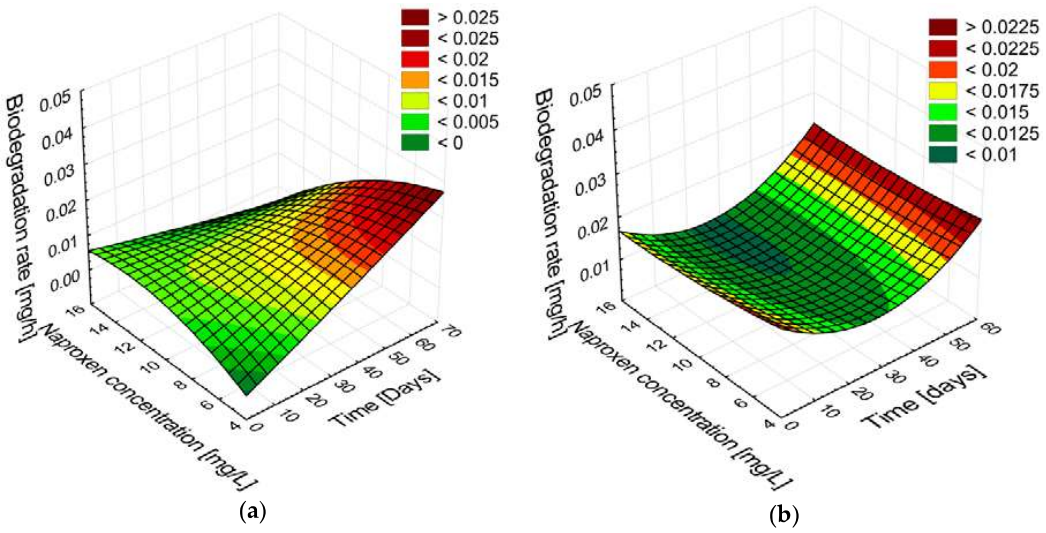


Figure 3. Naproxen (6, 9, 12 and 15 mg/L) biodegradation rate by (**a**) free cells of *Planococcus* sp. S5; and (**b**) immobilized on loofah sponges cells of *Planococcus* sp. S5; presented as patterns and the relationships among the time of incubation, naproxen concentration and biodegradation rate.

2.2.2. Stability and Degradation Capacity of the Developed Whole-Cell Biocatalyst System

The stability during long-term operations is a crucial factor for practical application of immobilized biocatalysts. To test reusability of *Planococcus* sp. S5 cells immobilized on the loofah sponges, the efficiency of naproxen biodegradation in subsequent cycles has been determined. In the presence of fresh sterile mineral salts medium in each cycle, immobilized cells were able to complete drug degradation in 3 repetitions (Figure 4). The first and the second dose of naproxen were utilized within respectively 17 and 15 days. In the next cycle it was observed deceleration of the degradation efficiency (21 days) while in the 4th cycle, the biodegradation ended after decomposition of 27% of the drug. However, cells of *Planococcus* sp. S5 immobilized on loofah sponges demonstrated the ability to efficient naproxen biodegradation for 55 days. After the end of the experiment, no significant damage and disintegration of the loofah sponges was observed, which confirms its high mechanical resistance [14].

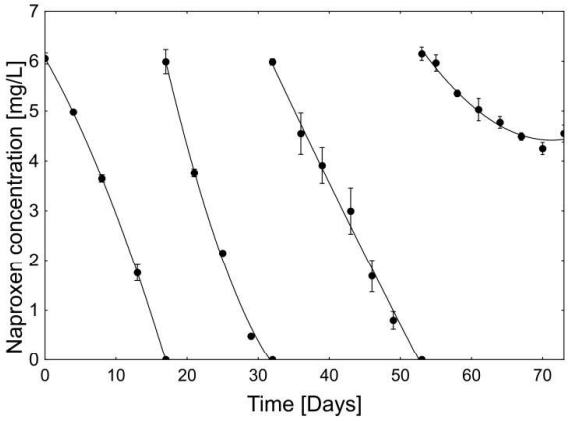


Figure 4. Cycles of 6 mg/L naproxen degradation by immobilized *Planococcus* sp. S5 on the loofah sponge. Data presented as a mean ± standard deviation of three replicates.

Evaluation of the biodegradation rate during each cycle shows not significant differences in the first 3 cycles of naproxen biodegradation amounting 14.8 ± 3.0, 16.0 ± 6.9 and 11.4 ± 3.9 µg/h respectively (Table 3, Figure 5). Only during the first cycle it was observed increasing rate of

drug degradation. The second and third cycle, however, were characterized by slowing down biodegradation rate, while the last one proceeded unstable (Table 3). It is suggested that one of the reasons why immobilization extends the catalytic activity of cells is to ensure the stability of cell membranes and adequate permeability [41].

Table 3. Average biodegradation rate of 6 mg/L naproxen by immobilized cells of *Planococcus* sp. S5 in each cycle. Data presented as a mean ± standard deviation of three replicates. Different letters (a, b, c) indicate a statistically significant difference between biodegradation rates during subsequent cycles ($p \geq 0.05$).

Period [Day–Day]	Average Naproxen Degradation Rate [µg/h]			
	I Cycle	II Cycle	III Cycle	IV Cycle
0–4	11.2 ± 1.2 ^{a}			
4–8	13.9 ± 0.9 ^{b}			
8–13	15.8 ± 1.1 ^{b}			
13–17	18.2 ± 1.7 ^{c}			
17–21		23.2 ± 1.8 ^{a}		
21–25		16.8 ± 0.7 ^{b}		
25–29		17.4 ± 0.2 ^{b}		
29–32		6.7 ± 0.1 ^{c}		
32–36			15.0 ± 3.8 ^{a}	
36–39			8.9 ± 1.4 ^{b}	
39–43			9.6 ± 1.2 ^{b}	
43–46			18.2 ± 2.0 ^{a}	
46–49			12.3 ± 2.4 ^{ab}	
49–53			8.3 ± 1.8 ^{b}	
53–55				3.8 ± 3.5 ^{a}
55–58				8.5 ± 3.2 ^{b}
58–61				6.8 ± 0.1 ^{ab}
61–64				3.5 ± 1.7 ^{a}
64–67				3.9 ± 1.0 ^{a}
67–70				3.3 ± 1.9 ^{a}
70–73				−4.2 ± 2.5 ^{c}

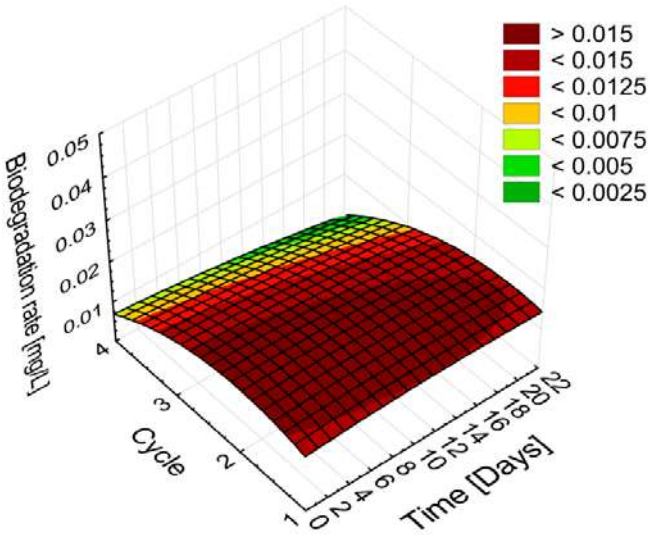


Figure 5. Naproxen (6 mg/L) biodegradation rate during subsequent cycles by immobilized on loofah sponges cells of *Planococcus* sp. S5 presented as patterns and the relationships among the time of incubation, cycle and biodegradation rate.

The ability of immobilized cells of *Planococcus* sp. S5 to carry out the biodegradation of naproxen in several cycles indicates the possibility of their use in bioremediation. However, to study their behaviour in such systems it is necessary to conduct pilot studies.

2.3. The Influence of Immobilization on Enzymes Activity

The process of biodegradation of naproxen by white rot fungi is much better understood than by bacteria [29,42–44]. One of the proposed mechanisms demonstrates naproxen demethylation carried out by cytochrome P-450 [32]. The reaction product, 6-desmethylnaproxen appeared during biodegradation of naproxen by the *Planococcus* sp. S5 strain. Domaradzka et al. [2] showed that some enzymes involved in the degradation of polycyclic aromatic hydrocarbons were active during cometabolic (with glucose) biodegradation of naproxen by free cells of *Planococcus* sp. S5. The activity of phenol monooxygenase, naphthalene dioxygenase, hydroxyquinol 1,2-dioxygenase and gentisate 1,2-dioxygenase was demonstrated.

A typical degradation pathway of aromatic compounds by bacteria is the initial hydroxylation followed by the ring cleavage [45]. The first step in degradation of naphthalene (the derivative of which is naproxen) is the hydroxylation of C1 and C2 catalysed by naphthalene dioxygenase [46]. It was suggested that this enzyme is responsible for hydroxylation of C7 and C8 of naproxen. Moreover, on the base of the activity of phenol monooxygenase additional hydroxylation was proposed. Formed trihydroxylated derivative probably is cleaved by hydroxyquinol 1,2-dioxygenase [2]. One of the most important steps in catabolism of naphthalene is its conversion to salicylate, which bacterial pathway has been well described. The most important part of the catabolism of salicylate is direct ring cleavage by salicylate 1,2-dioxygenase or gentisate 1,2-dioxygenase (in case salicylate transformation to gentisate by monooxygenase). The salicylate transformation products are next introduced into the tricarboxylic acid cycle [47–49].

Because the immobilization of bacterial cells may change the xenobiotic degradation pathway, the activity of enzymes that are involved in naproxen degradation were examined. This phenomenon is related to the change in the gene expression profile that occurs during the formation of the biofilm and can be related to the type of carrier surface [50,51].

The activities of *O*-demethylase, aromatic monooxygenase (with phenol or naproxen as a substrate), naphthalene dioxygenase, gentisate 1,2-dioxygenase and salicylate 1,2-dioxygenase were compared. As is shown in Table 4, the activity of all analysed enzymes was observed, both in free and immobilized cells. Obtained results showed that the naproxen biodegradation pathway in immobilized S5 cells probably did not change markedly. Significant changes, however, were observed in the values of activity (Table 4). As expected, during the slower degradation phase of naproxen (15th day), free S5 cells were characterized by the lowest activity of enzymes associated with drug degradation. At the same time, the enzymatic activity of immobilized cells was about 2 times higher in the case of *O*-demethylase, aromatic monooxygenase (with phenol as a substrate), naphthalene dioxygenase and salicylate 1,2-dioxygenase. Interesting increase in the activity of aromatic monooxygenase (with naproxen as a substrate, 10 times higher) and gentisate 1,2-dioxygenase (4 times higher) was observed in the immobilized cells. However, due to the methodology for determining the activity of aromatic monooxygenase (determination of reduced nicotinamide adenine dinucleotide - NADH oxidation), it is not excluded that more of the enzymes belonging to the class of oxidoreductases are involved in the degradation of naproxen by immobilized cells of *Planococcus* sp. S5. A higher gentisate 1,2-dioxygenase activity may have been caused by the increase of its participation (in relation to salicylate 1,2-dioxygenase) in the drug biodegradation due to immobilization.

Due to the non-statistically different biodegradation rate of naproxen by free cells in the phase of faster drug degradation and immobilized cells, it was suspected that the enzymatic activity of these systems would also not be different. However, according to the analysis (Table 4), the activity of all analysed enzymes was higher in the immobilized cells. Higher activity of enzymes associated with the biodegradation of naproxen in immobilized cells confirms that the sensitivity to the drug of cells in the

developed biocatalyst was reduced. For that reason, the significant acceleration of its biodegradation was observed.

Table 4. Specific activity of enzymes involved in naproxen degradation under cometabolic conditions by immobilized and non-immobilized *Planococcus* sp. S5. Different letters (a, b, c) indicate a statistically significant difference between activity of enzymes from free and immobilized cells ($p \geq 0.05$).

Enzyme	Specific Enzyme Activity (U/mg protein)		
	Free Cells		Immobilized Cells 15th Day
	15th Day	35th Day	
O-demethylase	412.84 ± 48.53 [a]	737.16 ± 55.81 [b]	1051.84 ± 65.57 [c]
Aromatic monooxygenase (Phe)	13.06 ± 0.83 [a]	27.14 ± 2.40 [b]	31.55 ± 1.18 [c]
Aromatic monooxygenase (Npx)	14.78 ± 1.28 [a]	65.17 ± 3.59 [b]	123.71 ± 12.39 [c]
Naphthalene dioxygenase	8.16 ± 0.82 [a]	10.71 ± 2.23 [a]	15.73 ± 1.80 [b]
Gentisate 1,2-dioxygenase	52.95 ± 2.90 [a]	122.26 ± 9.44 [b]	203.03 ± 18.55 [c]
Salicylate 1,2-dioxygenase	388.26 ± 11.12 [a]	520.38 ± 24.60 [b]	714.87 ± 71.58 [c]

2.4. Changes in Biofilm Formed Onto the Loofah Sponge during Naproxen Degradation

Visualization of bacterial biofilm in high resolution using SEM (Scanning Electron Microscopy) is one of the best methods to determine the biofilm structure. Preparation of samples for analysis by SEM involves the fixation of their structures (using glutaraldehyde and osmium tetraoxide), dewatering, drying and covering with a conductive layer. However, due to the fact that the structure of EPS (which stability is dependent on the presence of water) often collapses during dewatering and drying, identifying certain structures in biofilm sometimes is problematic [52]. The procedure of sample preparation very often leads to the creation of artefacts. One of them, especially at higher magnifications, is the appearance of EPS as fibres or granularity and not as a gel structure surrounding the cells [53]. However, the advantage of this visualization method is the possibility of determining the distribution of microorganisms and changes in the biofilm structure caused by specific factors. Considering the identical preparation of samples for analysis, demonstration of changes in the biofilm structure in relation to the state of biofilm before exposure to a specific factor reduces the probability of incorrect image analysis.

Loofah sponge which was used as a carrier for immobilization, observed in SEM revealed its multidirectional highly fibrous network and porous surface with a small protuberances (Figure 6a) which was a suitable place for the attachment of microorganisms.

After immobilization of *Planococcus* sp. S5 cells through the adsorption on the surface (lasting 72 h), accumulation of cells covered with an extracellular matrix (Figure 6b) was observed. Flat biofilm formed on the loofah sponges had the form of irregular rods of different lengths (Figure 6b) and appeared in aggregates.

When the first and the second dose of naproxen (6 mg/L) have been degraded (respectively 17th and 36th day of incubation), immobilized loofah sponges were observed in SEM to reveal changes in biofilm structure during naproxen biodegradation. After complete degradation of the first dose of naproxen, a more diversified and intense colonization of the carrier was observed (Figure 6c). There was present formation of the connections between aggregates (Figure 6c) and new fibrous extracellular matrix-like structures. When the second dose of naproxen was degraded, surface of loofah sponges was completely covered by biofilm formed by *Planococcus* sp. S5 (Figure 6d). A decrease in the amount of aggregates in relation to the newly formed structures was observed.

In the presence of toxic substances bacteria trigger different protective mechanisms. One of them is the secretion of large amounts of EPS, which will create a protective layer for cells. At the same time, by forming clusters, the cells reduce the contact surface with the toxin-containing environment [54]. Due to the problematic utilization of naproxen by free S5 cells (especially in the first phase of degradation), increased tolerance of immobilized cells appears to be the result of the accumulation of large amounts of extracellular substances in the biofilm. The same defence mechanism was observed by Ma et al. [55] against to immobilized bacteria from activated sludge during biodegradation of phenol. They showed that with the increase in the amount of toxic phenol nol photodegradation products, the number of microbial cells in the biofilm decreased. On the other hand, the amount of secreted EPS was increasing, which resulted with a lack of differences in the phenol biodegradation way.

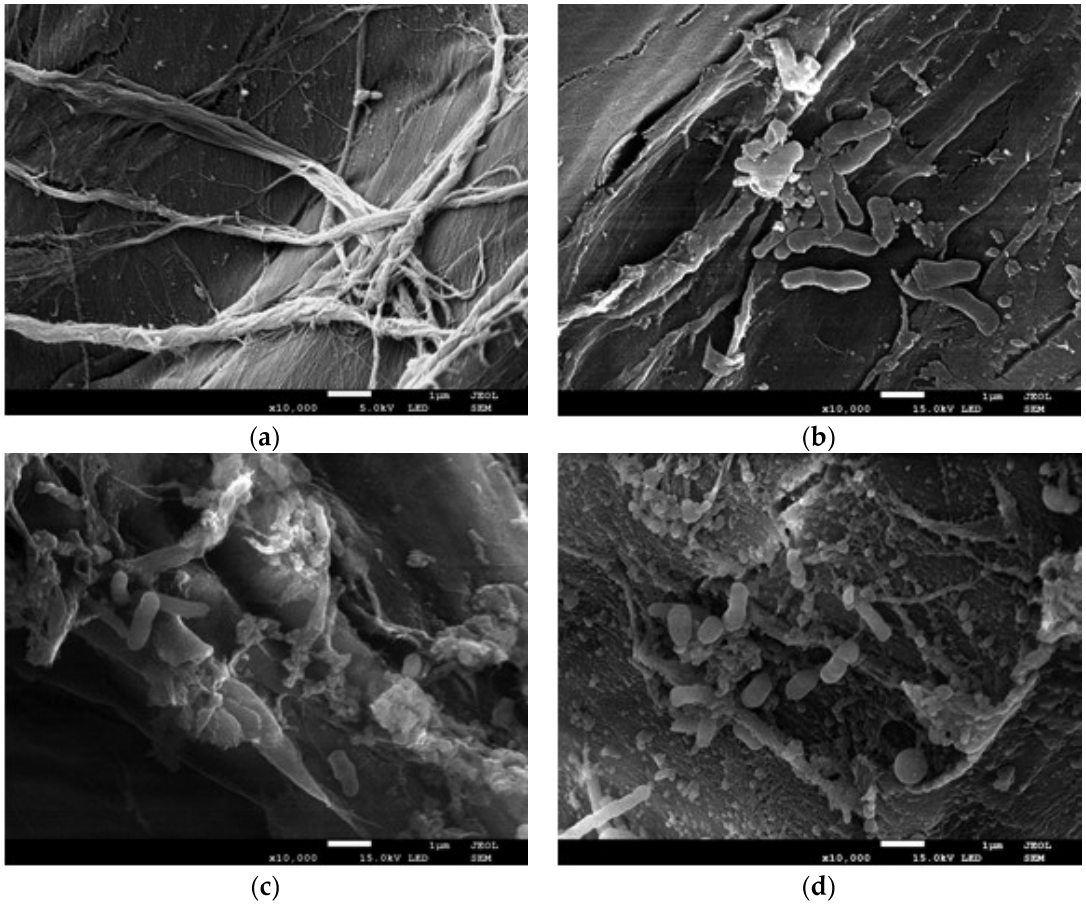


Figure 6. Scanning electron microscopy (SEM) micrographs of unimmobilized loofah sponge (**a**), biofilm formed by *Planococcus* sp. S5 cells onto loofah sponge before naproxen degradation (**b**), after decomposition of the first dose of naproxen (6 mg/L) (**c**) and the second dose of the drug (6 mg/L) (**d**).

3. Materials and Methods

3.1. Bacterial Cultures Cultivation

Isolated from activated sludge Gram-positive strain *Planococcus* sp. S5 described by Łabużek et al. [21] was used. Proliferation of S5 cells was carried out in the nutrient broth (BBL) at 30 °C on a rotary shaker at 130 rpm. After 72 h of incubation bacterial cultures were centrifuged (5000 rpm, 15 min), washed twice with mineral salts medium [56] and resuspended in the same medium. Prepared bacterial suspensions were used as an inoculum for immobilization and control non-immobilized cells experiments.

3.2. Carrier Preparation for Immobilization

The first step in the preparation of the loofah sponges (York, Bolechowo, Poland) for immobilization was drying them in a desiccator to establish constant weight and cutting out fragments weighing 0.15 g. Obtained cubes were washed according to Iqbal et al. [14] and sterilized (121 °C, 1.2 atm) two times at an interval of 24 h.

3.3. Immobilization Procedure

Cells of S5 strain were immobilized through the adsorption on the surface of loofah sponge. Immobilization was conducted in 250 mL Erlenmeyer flasks, which contained 0.75 g of the carrier and 100 mL of the mineral salts medium (pH 7.2) with *Planococcus* sp. S5 cells (optical density at 600 nm equal to 1.2; Genesys 20, Thermo Scientific). Medium was supplemented with glucose (0.5 g/L), NaCl (10 g/L) and $MnSO_4$ (0.01 g/L). Flasks were incubated with shaking (90 rpm), at temperature of 30 °C. After 72 h of incubation, loofah sponges with immobilized bacteria were rinsed with aqueous solution of NaCl (0.9%) to remove unbound microorganisms and used for biodegradation experiments.

3.4. Characterization of Immobilized Loofah Sponges

Dry mass of the immobilized bacteria was obtained by comparing the dried weight of immobilized carrier (105 °C, 2 h and stored in a desiccator) with unimmobilized carriers incubated and dried under the same conditions. Enzymatic activity of biofilm formed onto loofah sponge was measured as follow (modified method proposed by Jiang et al. [19]): immobilized carrier (1 cube) was added to 8 mL of phosphate buffer (pH 7.6) and incubated for 15 min on the orbital shaker (130 rpm, 30 °C). After pre-incubation, 0.1 mL of FDA (Sigma-Aldrich, St. Louis, MO, USA) (4.8 mmol L^{-1}) was slowly injected directly into middle of the carrier and incubated in the dark on the orbital shaker (130 rpm, 30 °C) for 1 h. Fluorescence intensity in the liquid was measured spectrophotometrically at 490 nm (Genesys 20, Thermo Scientific, Waltham, MA, USA). Concentration of fluorescein was calculated on the basis of a standard curve.

3.5. Biodegradation Experiments

Naproxen decomposition was conducted in 500 mL Erlenmeyer flask containing 250 mL of the mineral salts medium [56] and 10 pieces of the loofa sponge colonized by bacteria. Each flash was supplement with naproxen (Sigma-Aldrich, USA) to obtain a final concentration of 6, 9, 12 or 15 mg/L and at every 3 days with glucose (0.5 g/L, POCH, Gliwice, Poland) and incubated with shaking (130 rpm) at 30 °C. The control cultures contained non-immobilized cells of *Planococcus* sp. S5 were also prepared. For estimation of naproxen accumulation in the biofilm the drug extraction with modified Huerta et al. protocol was performed [36]. Loofah sponges with immobilized bacteria were cut into small pieces and placed in 15 mL falcon tube with 10 mL of mixture of citric buffer (pH 4) and acetonitrile (1:1, *v/v*, Sigma-Aldrich, USA). Mixtures were subsequently sonicated 3 times for 10 min and centrifuged (15,000 rpm, 20 min). Obtained supernatants were analysed by HPLC and naproxen concentrations were calculated based on a standard curve prepared with extraction mixture. Determination of naproxen adsorption on the carrier was conducted by incubation sterile loofah sponges (1.5 g) with 250 mL of MSM medium supplemented with naproxen (6 mg/L) and glucose (0.5 g/L) in 500 mL Erlenmeyer flasks. Medium samples were taken every 24 h for 7 days and analysed by HPLC to determine naproxen concentration.

3.6. Determination of Naproxen Concentration

Decomposition of naproxen was monitored by HPLC (Merck HITACHI, Darmstadt, Germany) equipped with a LiChromospher® RP-18 column (4 × 250 mm), liChroCART® 250-4 Nucleosil 5 C18 and a DAD detector (Merck HITACHI). Medium samples from each flask were taken at 3 days period and centrifuged (14,000 rpm, 20 min). Naproxen identification and quantification in the supernatant

was done by following the protocol proposed by Wojcieszyńska et al. [32]. The mobile phase consisted acetonitrile and 1% acetic acid (50:50 v/v) with a flow rate of 1 mL/min and column temperature was 23 °C. The detection wavelength was set at 260 nm. Naproxen was identified by way of comparison of HPLC retention time (2.41 min) and ultraviolet-visible spectra with those of the external standards. To determine the abiotic degradation of naproxen, uninoculated controls were prepared.

3.7. Enzyme Assay

After 15 days of incubation (additionally free cells after 35 days), free and immobilized cells of *Planococcus* sp. S5 were separated from the medium by centrifugation (4500× g for 15 min at 4 °C). The release of immobilized cells from the carrier was carried out by vortexing. The obtained pellet was washed with 50 mM phosphate buffer (pH 7.0), disrupted by sonication (6 times for 15 s) and centrifuged at 9000× g for 30 min at 4 °C. Obtained crude extract was used for the measurement of enzyme activities.

The activity of *O*-demethylase was determined by measuring the loss of vanillic acid (λ = 260 nm) with the use of the HPLC method [57]. In order to determine monooxygenase activity (with phenol or naproxen as a substrate), NADH oxidation (ε_{340} = 6220/M cm) was measured spectrophotometrically [58]. The naphthalene dioxygenase, gentisate 1,2-dioxygenase and salicylate 1,2-dioxygenase activity was measured spectrophotometrically by the formation of *cis*,*cis*-dihydrodiol (ε_{262} = 8230/M cm) [46], maleylpyruvate (ε_{330} = 10,800/M cm) [49] and 2-oxohepta-3,5-dienedioic acid (ε_{283} = 13,600/M cm) [48], respectively. Protein concentration was determined using the Bradford method [59]. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of product per minute.

3.8. Scanning Electron Microscopy

To observe the structure of biofilm formed onto the loofah sponges and its changes during naproxen biodegradation, samples for Scanning Electron Microscopy were prepared. For this examination, unimmobilized and immobilized loofah sponges before and after naproxen biodegradation were collected from the medium and prepared as follows: fixation in 3% glutaraldehyde (24 h), in 1% osmium tetroxide (3 h), dehydration with ethanol (30, 50, 70, 80, 90, 95 and 100%, each for 10 min), drying by lyophilisation and covering with gold. Samples were observed in high-resolution electron microscope JSM-7100F TTL LV (JEOL, Tokio, Japan).

3.9. Statistical Analysis

All experiments were performed in at least three replicates. The values of the efficiency of naproxen biodegradation and enzymes activities were analysed by STATISTICA 12 PL software package. Statistically significant differences and similarities have been demonstrated by the *t*-test or the Least Significant Differences (LSD) test ($p \geq 0.05$).

4. Conclusions

Ability of some bacterial strains to form a biofilm on the surface of various materials is a key element for efficient immobilization process. By optimizing each parameter of the immobilization procedure, a biocatalyst that is characterized by increased naproxen biodegradation capacities has been developed. Immobilized cells of *Planococcus* sp. S5 strain on the loofah sponge, compared to the free cells, were able to faster biodegradation of naproxen added at higher doses. Additionally, due to the maintenance of full catalytic activity for 3 cycles (55 days), immobilized onto the loofah sponge S5 cells, show promising potential in their application in bioremediation systems. Analysis of the effect of immobilization on the activity of enzymes associated with naproxen biodegradation showed that it caused a significant increase in the activity of all examined enzymes. The significant increase in the efficiency of the naproxen biodegradation by immobilized S5 cells was most probably caused by the synthesis of large amounts of EPS, which by sorption and limitation of the substrates diffusion increased the tolerance of the strain to the drug.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/8/5/176/s1, Figure S1: Effect of various environmental and physiological factors on the efficiency of *Planococcus* sp. S5 cells immobilization onto the loofah sponge.

Author Contributions: A.D. conceived, designed and performed the experiment, analysed the data and wrote the paper; D.W. analysed the data and wrote the paper; K.H.-K. corrected manuscript, M.A.-H. contribution in analysis of SEM micrographs, U.G. designed the experiment, analysed the data and wrote the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

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catalysts

MDPI

Supplementary Material

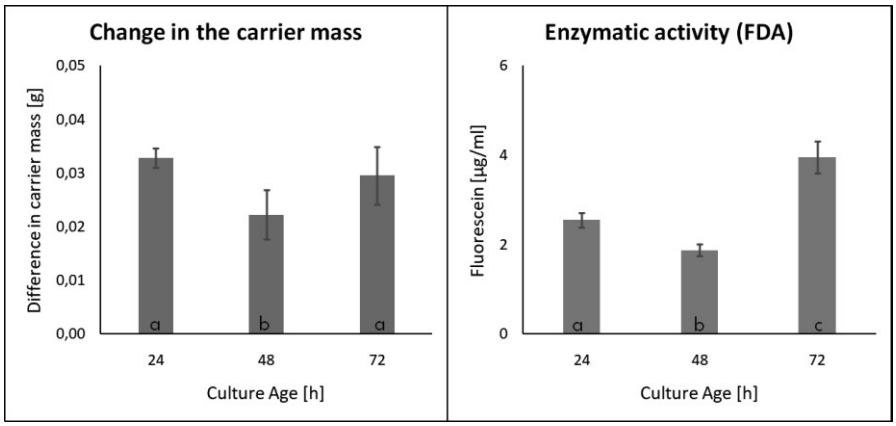
Immobilization of *Planococcus* sp. S5 cells on Luffa sponge and its application in naproxen removal.

Anna Dzionek , Danuta Wojcieszyńska and Urszula Guzik*

University of Silesia, Faculty of Biology and Environment Protection, Department of Biochemistry, Jagiellońska 28, 40-032 Katowice

* Correspondence: urszula.guzik@us.edu.pl; Tel.: +32-2009-567

Contents: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b
a
24
48
72
Culture Age [h]
Enzymatic activity (FDA)
Fluorescein [µg/ml]
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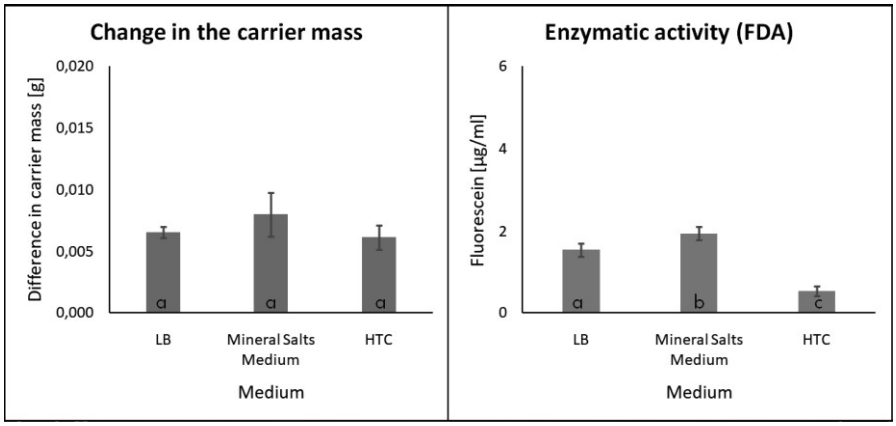
4
2
0
a
b
c
24
48
72
Culture Age [h]

(a)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HTC
Medium
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b
c
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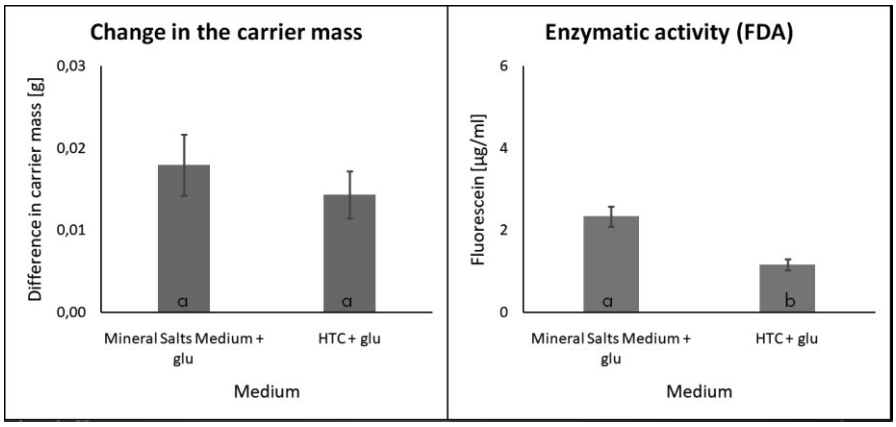
Mineral Salts Medium
HTC
Medium

(b)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Medium
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0
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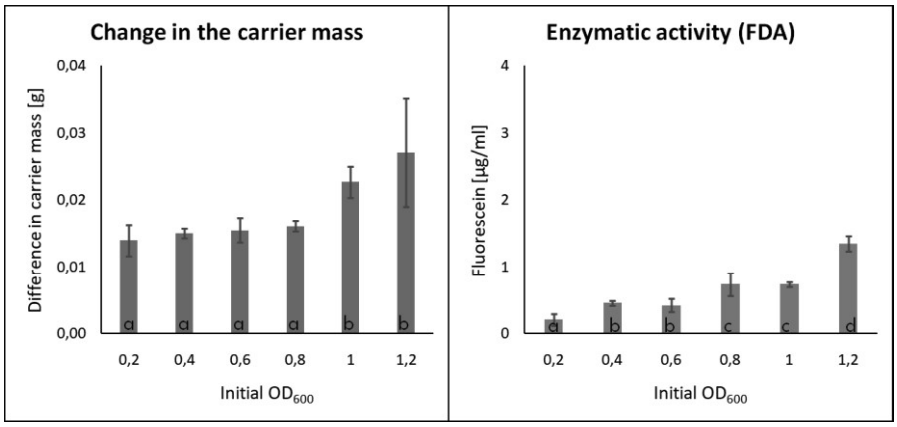
b
Mineral Salts Medium + glu
HTC + glu
Medium

(c)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0,6
0,8
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1,2
Initial OD600
Enzymatic activity (FDA)
Fluorescein [µg/ml]
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3
2
1
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c
c
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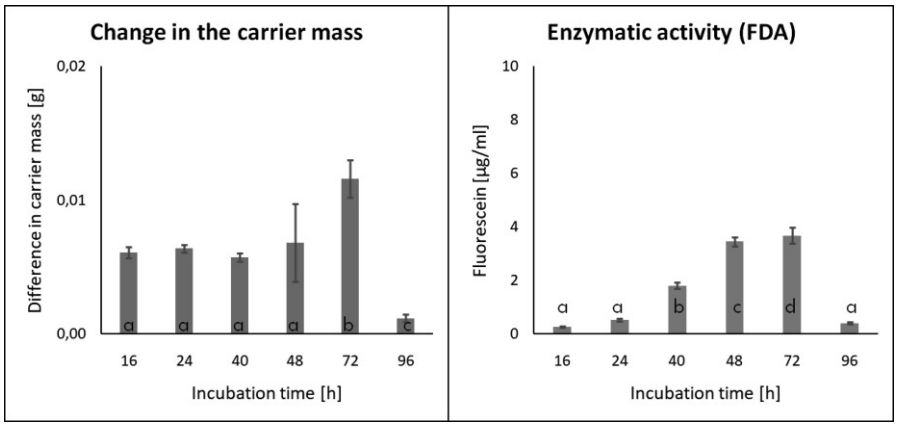
1
1,2
Initial OD600

(d)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Fluorescein [µg/ml]
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8
6
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0
a a b c d a
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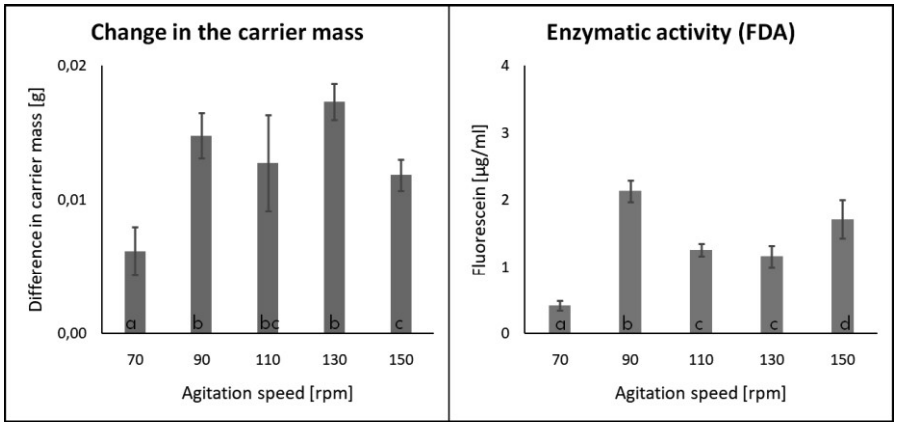
Incubation time [h]

(e)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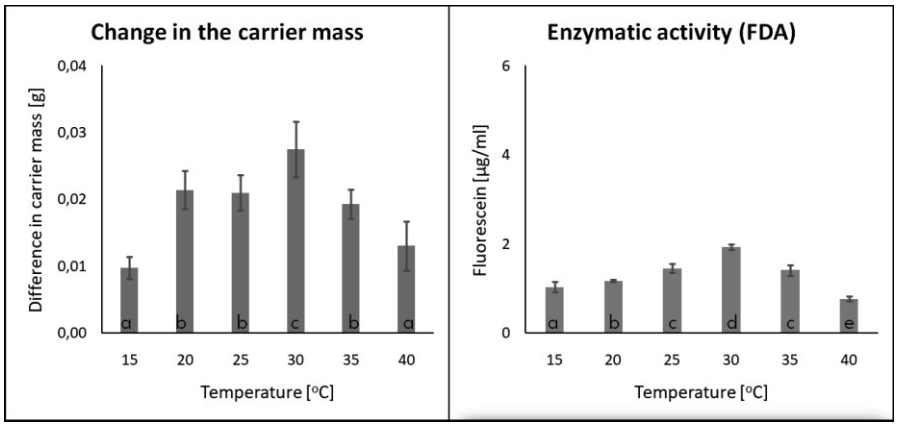
Agitation speed [rpm]
Enzymatic activity (FDA)
Fluorescein [µg/ml]
4
3
2
1
0
a b c c d
70 90 110 130 150
Agitation speed [rpm]

(f)


Change in the carrier mass
Difference in carrier mass [g]
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0,03
0,02
0,01
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a b b c b a
15 20 25 30 35 40
Temperature [°C]
Enzymatic activity (FDA)
Fluorescein [µg/ml]
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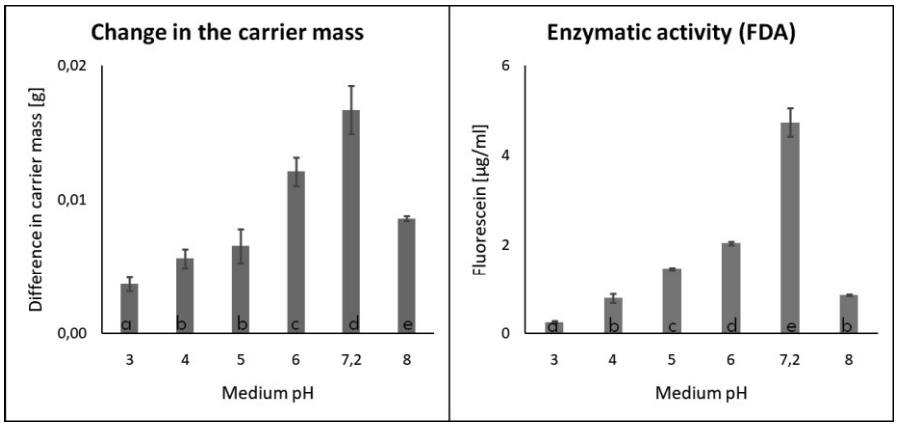
4
2
0
a b c d c e
15 20 25 30 35 40
Temperature [°C]

(g)


Change in the carrier mass
Difference in carrier mass [g]
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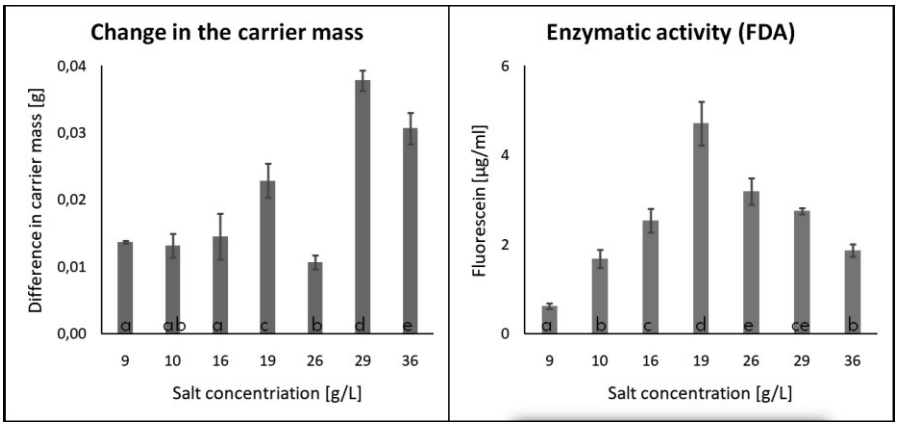
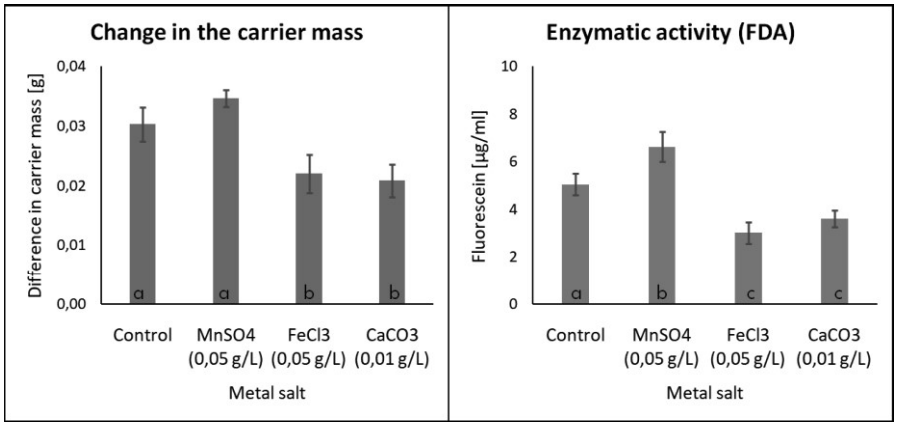
0,01
0,00
a b b c d e
3 4 5 6 7,2 8
Medium pH
Enzymatic activity (FDA)
Fluorescein [µg/ml]
6
4
2
0
a b c d e b
3 4 5 6 7,2 8
Medium pH

(h)



(i)



(j)

Figure S1: Effect of different variants of environmental and physiological factors on the efficiency of the immobilization of cells *Planococcus* sp. S5 Luffa sponge:
(**a**) Age of the culture taken for immobilization (24, 48 or 72 h);
(**b**) Immobilization medium (Lysogeny Broth (LB), Mineral Salts Medium or HTC (medium rich in both carbon and ion sources)
(**c**) Addition of a carbon source (Mineral Salts Medium + 0.5 g/L glucose or HTC + 0.5 g/L glucose)
(**d**) Initial Optical Density of the culture (0.2, 0.4, 0.6, 0.8, 1.0 or 1.2)
(**e**) Incubation time of the carrier with bacterial culture (16, 24, 40, 48, or 72 h)
(**f**) Agitation speed during immobilization (70, 90, 110, 130 or 150 rpm)
(**g**) Temperature of the process (15, 20, 25, 30, 35 or 40 °C)
(**h**) Immobilization medium pH (3, 4, 5, 6, 7.2 or 8)
(**i**) Salt concentration in immobilization medium (9, 10, 16, 19, 26, 29 or 36 g/L)
(**j**) Addition of a metal ions (manganese, iron or calcium)
All experiments were performed in at least five replicates. The values of the efficiency of immobilization and enzyme activities were analyzed by one-way ANOVA ($p<0.05$) using STATISTICA 10 PL software package. A post-hoc test or T-test were applied to assay the differences between the treatments (differences are marked with subsequent letters).

II.4.

Dzionek A., Wojcieszyńska D., Adamczyk-Habrajska M., Guzik U.: Enhanced Degradation of Naproxen by Immobilization of *Bacillus thuringiensis* B1 (2015b) on Loofah Sponge. Molecules, 2020, 25, 872

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Enhanced Degradation of Naproxen by Immobilization of *Bacillus thuringiensis* B1(2015b) on Loofah Sponge

Anna Dzionek [1], Danuta Wojcieszyńska [1], Małgorzata Adamczyk-Habrajska [2] and Urszula Guzik [1,*]

[1] Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Science, University of Silesia in Katowice, Jagiellońska 28, 40-032 Katowice, Poland; adzionek@us.edu.pl (A.D.); danuta.wojcieszynska@us.edu.pl (D.W.)

[2] Institute of Materials Engineering, Faculty of Science and Technology, University of Silesia in Katowice, 75 Pułku Piechoty 1a, 41–500 Chorzów, Poland; malgorzata.adamczyk-habrajska@us.edu.pl

* Correspondence: urszula.guzik@us.edu.pl; Tel.: +32-2009-567

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Abstract: The naproxen-degrading bacterium *Bacillus thuringiensis* B1(2015b) was immobilised onto loofah sponge and introduced into lab-scale trickling filters. The trickling filters constructed for this study additionally contained stabilised microflora from a functioning wastewater treatment plant to assess the behavior of introduced immobilized biocatalyst in a fully functioning bioremediation system. The immobilised cells degraded naproxen (1 mg/L) faster in the presence of autochthonous microflora than in a monoculture trickling filter. There was also abundant colonization of the loofah sponges by the microorganisms from the system. Analysis of the influence of an acute, short-term naproxen exposure on the indigenous community revealed a significant drop in its diversity and qualitative composition. Bioaugmentation was also not neutral to the microflora. Introducing a new microorganism and increasing the removal of the pollutant caused changes in the microbial community structure and species composition. The incorporation of the immobilised B1(2015b) was successful and the introduced strain colonized the basic carrier in the trickling filter after the complete biodegradation of the naproxen. As a result, the bioremediation system could potentially be used to biodegrade naproxen in the future.

Keywords: bacteria; immobilization; loofah sponge; naproxen; trickling filter; wastewater treatment

1. Introduction

Pharmaceuticals and personal care products are nowadays being detected in surface water and groundwater more and more frequently. Although their concentration typically does not exceed 1 $\mu g \times L^{-1}$, chronic exposure on non-target organisms can have significant consequences. One of the drugs that can affect entire ecosystems is naproxen (2-(6-methoxy-2-naphthyl)propionic acid), which is a polycyclic non-steroidal anti-inflammatory drug (NSAID). This drug is not metabolized by humans and wastewater treatment plants do not have strains that are capable of degrading naproxen with high efficiency. Additionally, it is one of the drugs that is easily photolyzed. It has been proven that the naproxen phototransformation products are often more toxic than the drug itself [1]. It should also be noted that in the last phase of water purification in wastewater treatment plants sterilization using UV light is performed. Hence, not only is naproxen released into the environment, but also the products of its phototransformation.

Among the various bioremediation systems that are used in wastewater treatment plants, particular attention has been paid to those that are based on immobilised microorganisms. Naturally occurring

biofilm and immobilization ensure the retention and accumulation of the biomass, which decreases the filtration costs. One of the systems that is based on this attached growth is a trickling filter. These systems are characterized by a simpler design and a smaller environmental footprint due to their lower installation and operational costs compared to other wastewater treatment systems [2]. As was mentioned above, most wastewater treatment plants are not adapted to remove NSAIDs. One possible solution might be bioaugmenting the existing bioremediation systems.

Introducing strains that are capable of utilising specific pollutants in bioremediation systems has many advantages as well as a few disadvantages. The biggest benefit is associated with removing the pollutants, which despite the use of low concentrations may have a toxic effect on autochthonous microflora. If bioaugmentation is successful and the introduced strains are not removed from the system, the biodegradation of the contaminants will remain constant over a long period of time. However, in order to make this possible, after the introduction, the strains must show a degradative activity, colonize the system and be able to propagate in it, which does not always occur. The success of bioaugmentation is determined by many factors. Not only can the selection of the strains with the appropriate features be crucial, but also the way that they are introduced into a complex community. In addition to preparing a sufficiently high biomass concentration, it is also important to conduct an acclimatization pre-run. Such a process should be performed in a separate system that is run under the same conditions but only with the strain that was selected for the bioaugmentation so that it can adapt to the conditions that prevail in the system [3]. To increase the chance of the survival of the introduced strains, they can also be introduced in an immobilised form. This method is fast, inexpensive and simple and does not require any acclimatization or specialized equipment. The most commonly used method for immobilization for bioaugmentation is adsorption on the surface because it results with a shaped biofilm that is introduced into the system. Additionally, immobilization provides a barrier that protects the introduced cells from other microorganisms and the substances that they excrete as well as from toxic shock or environmental fluctuations [4]. However, bioaugmentation can also have negative consequences. One of these is the significant changes in the composition of the autochthonous microflora that result from the competition and inhibition of the new strains. One consequence may be a significant drop in the effectiveness of the system [5]. For that reason, an experiment that examines the interactions between the immobilised strains that are introduced into wastewater treatment systems and the microbial communities that are present in these systems as well as the influence of these communities on the degradation capacity should be performed.

In our work, we present the process of developing an immobilised biocatalyst which was constructed to bioaugmentation of the bioremediation system and to investigate its naproxen degradation capabilities in such a system. A Gram-positive *Bacillus thuringiensis* B1(2015b), which is able to degrade naproxen under cometabolic conditions, was selected to be the introduced strain [6]. The cells were immobilised on the loofah sponge through adsorption on the surface and were introduced into a trickling filter that contained stable microbial communities from the wastewater treatment plant. To determine the influence of the autochthonous microflora that was present in the bioremediation system on the efficiency of naproxen biodegradation by the immobilised B1(2015b), the removal of the drug in the bioaugmented trickling filter and in a system without autochthonous microflora was monitored. We present a visualization of the biofilm that was created on the loofah sponges and its colonization after its introduction into the trickling filter. Additionally, the impact of an acute, short-term naproxen exposure and bioaugmentation on the qualitative composition of the autochthonous microflora was also evaluated. This is the first report about wastewater treatment system bioaugmentation with immobilised cells that are capable of degrading naproxen that includes its impact on the autochthonous microflora.

2. Results

2.1. Immobilization of Bacillus thuringiensis B1(2015b) on Loofah Sponge

The immobilization process in this study was the most efficient in a HCT medium (pH 8) in the presence of glucose with shaking (110 rpm) at 20 °C for 48 h. A HCT medium is very rich in both carbon sources and metal ions and therefore it was used to form biofilm by the *Bacillus thuringiensis* genus [7]. Analysis of the initial amount of the cells shows that the smallest number of cells (OD_{600} equal to 0.2) resulted in the highest mass of the biofilm and the enzymatic activity. It was observed that the age of a culture that is used for immobilization has a significant influence on the metabolic activity of a biofilm. The lowest enzyme activity was demonstrated by a 24 h old biofilm. The highest activity was observed in 48 h and 72 h old cultures. That is why a 48 h old culture of B1(2015b) was selected for the immobilization process in this study. Interesting results we obtained during the analysis of the medium pH and addition of the different metal salts. A significant increase in the enzymatic activity of the biofilm was noted when incubation was conducted in pH 8.0 and with supplementation with manganese (Figure S1, Supplementary Materials).

After the optimization of each immobilization parameter immobilized biocatalyst contained 28 ± 3.5 mg of dry biofilm mass per loofah cube, which hydrolyzed fluorescein diacetate (FDA) to 19.07 ± 1.06 μg/mL of fluorescein in 1 h (Table 1). FDA abiotic hydrolysis and fluorescein adsorption by the loofah cubes that were not immobilised was not statistically significant.

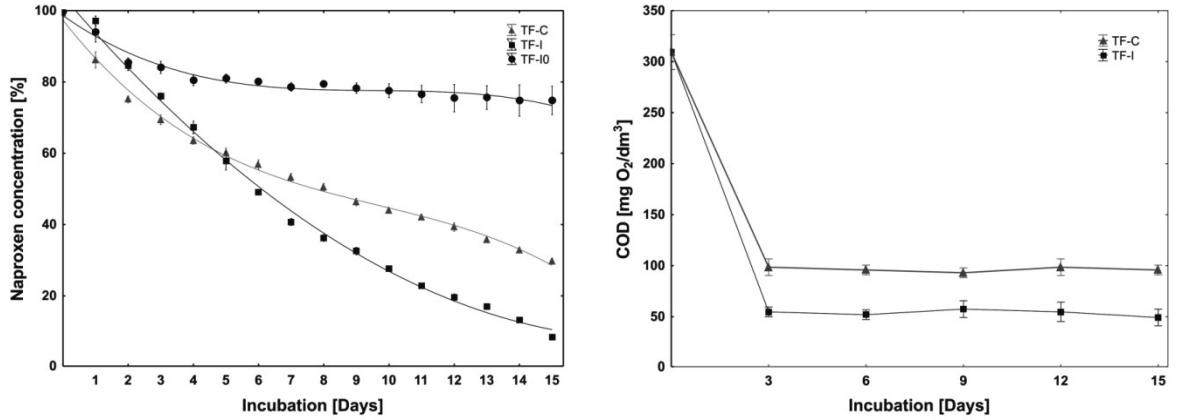
Table 1. Comparison of the *B. thuringiensis* B1(2015b) biofilm mass that was created on loofah sponge and its metabolic activity before and after the optimization of the immobilization process.

Development Stage	Dry Biofilm Mass [mg]	Fluorescein Concentration [μg·mL^{-1}]	Total Enzymatic Activity [μg·g Dry Mass^{-1}·h^{-1}]
Before optimization	8.3 ± 0.9	4.56 ± 0.48	532.77 ± 39.09
After optimization	28 ± 3.5	19.07 ± 1.06	709.14 ± 40.60

2.2. Naproxen Biodegradation in the Trickling Filters

Trickling filters TF-I and TF-C were designed to recreate the conditions that prevail in wastewater treatment plants. The only distinguishing parameter was maintaining a constant, room temperature. The flow rate was adjusted to 0.0066 m^3/h in order to prevent the wastewater from spraying through packing material too strongly or quickly. Simultaneously, this enables the time of contact between wastewater and microorganisms to be established, which is expressed as the hydraulic retention time (HRT), which was set for six hours.

In the TF-I0 system, which only contained the lightweight expanded clay aggregate (LECA) and the microflora from the Imhoff tank, there was an almost 20% loss of the drug (Figure 1a) in the first four days, which was caused by its adsorption by the LECA and not because of biodegradation. Over the following days, naproxen concentration was constant. In the TF-C control system that contained both the LECA and immobilised B1(2015b) cells on the loofah sponges, 70% of the drug was removed. In the TF-I tricking filter with introduced B1(2015b) cells on the loofah sponges and the microflora from the Imhoff tank, almost 90% of the naproxen was degraded. These results indicate a synergistic interaction between introduced immobilized biocatalyst and autochthonous microflora on naproxen biodegradation.



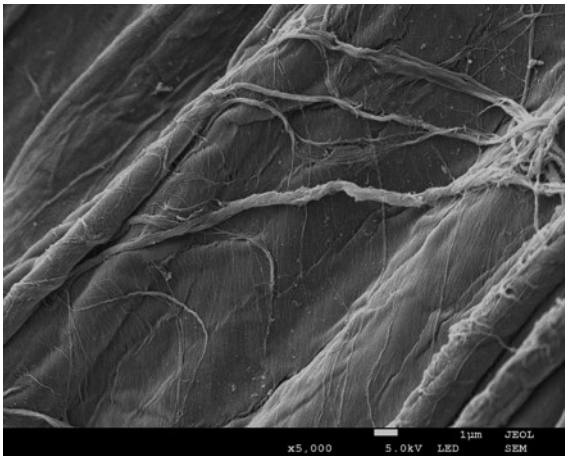
(**a**) (**b**)

Figure 1. The trickling filters (TF) performance: (**a**) naproxen (1 mg/L) biodegradation: TF-C (Δ), TF-I (□) and TF-I0 (o); (**b**) removal of the COD in TF-C (Δ), TF-I (□).Data are presented as the mean ± the standard deviation of three replicates.

At the same time that the naproxen biodegradation was being monitored in the trickling filters, the level of chemical oxygen demand (COD) was also determined. Samples for this analysis were taken from the collection tanks before the supplementation with the nutrients and glucose. After the stabilization processes, the decrease in the COD in TF-C and TF-I remained at a constant level (Figure 1b). More efficient decomposition of the organics from the synthetic wastewater was observed in TF-I (82.65 ± 1.01%) compared to TF-C (68.85 ± 0.074%). This was caused by the addition of microorganisms into TF-I that were specialized for wastewater treatment. The obtained results indicated that despite the relatively short time period (21 days), the stabilization processes were performed successfully and a fully functioning biofilm in the TF was developed.

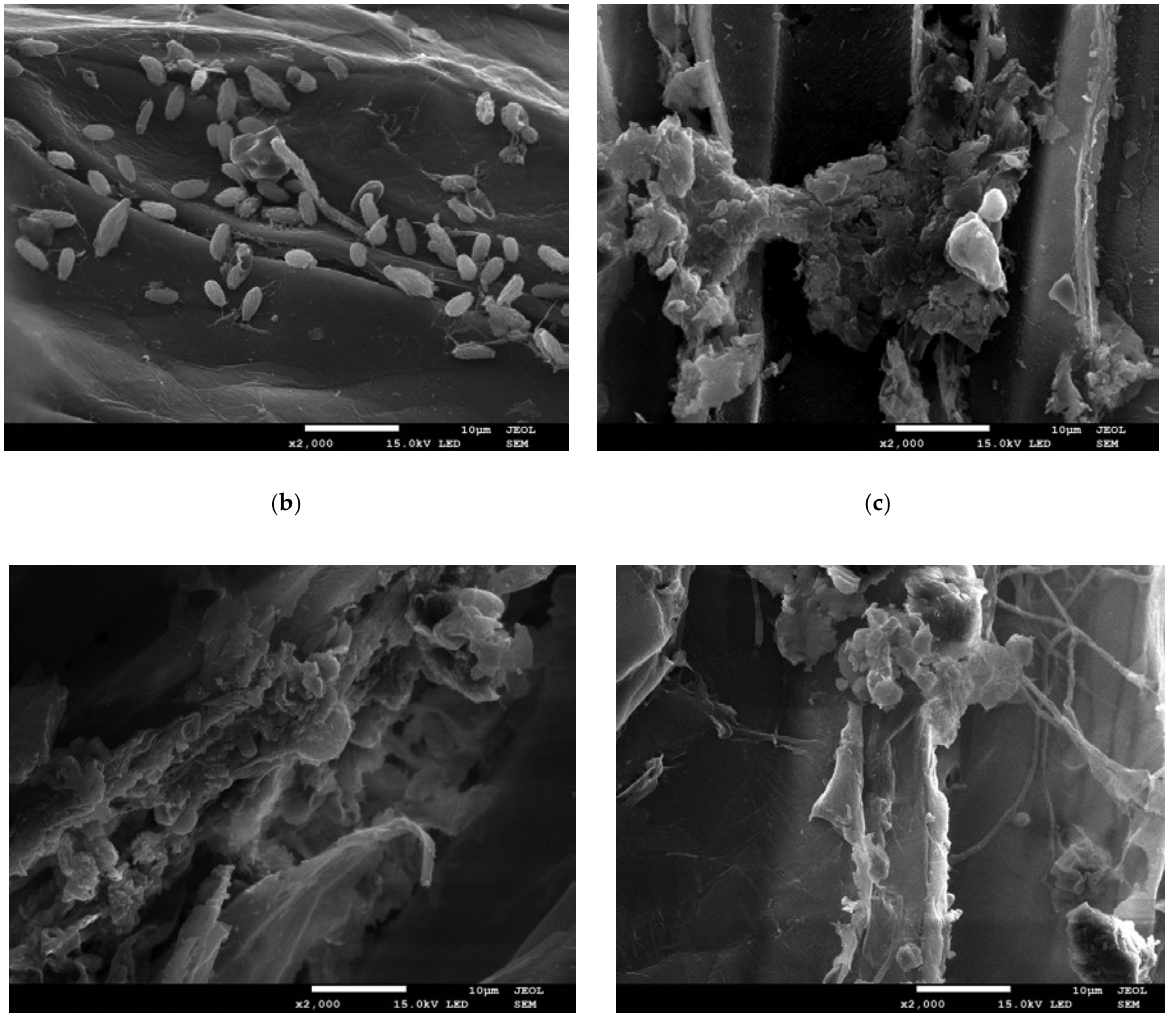
2.3. Colonization of the Loofah Sponges

Observation of the loofah sponges using scanning electron microscope (SEM) showed their highly porous structure, which was a suitable site for the attachment of the *Bacillus thuringiensis* B1(2015b) cells (Figure 2a). At the beginning of the experiment, immobilised B1(2015b) cells were clearly visible on the surface of the loofah sponges (Figure 2b).



(**a**)

Figure 2. *Cont.*



(**b**) (**c**)

(**d**) (**e**)

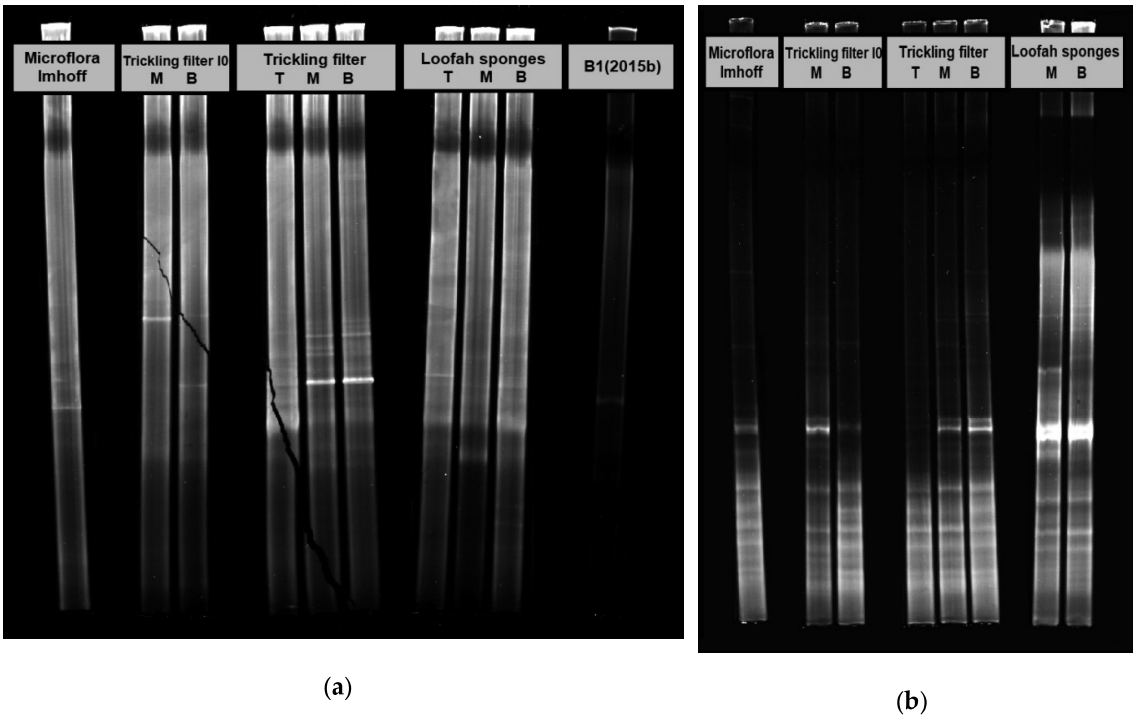
Figure 2. Scanning electron microscopy (SEM) micrographs of the uncolonized loofah sponges (**a**), that were colonized by *Bacillus thuringiensis* B1(2015b) (**b**), microflora from the Imhoff tank at the bottom (**c**), in the middle (**d**) and at the top (**e**) of the trickling filter.

After 15 days, the loofah sponges that remained at the top, in the middle and at the bottom of the trickling filter were almost completely covered by the microflora from the Imhoff tank flow chamber (Figure 2c–e). The biofilm that had formed on the surface of the sponges was characterized by a large amount of extracellular matrix, and therefore detecting individual cells was very difficult. There were differences in the formation of the biofilm depending on the site in the trickling filter into which the immobilised strain was incorporated. We observed that bacterial biofilm was formed in the lower and the middle parts of the trickling filters, while fungal hyphae were dominant in the upper parts (Figure 2c–e).

2.4. Phylogenetic Characterization of the TF Microbial Population

In this study, we analyzed the qualitative changes in both the bacterial (V3-V5 regions of the 16S rRNA gene) and fungal populations (ITS1/2 regions of the 18S rRNA gene) after acute, short-term exposure to naproxen (1 mg/L). As can be seen in Figure 3a, the untreated microflora from the Imhoff tank flow chamber was formulated by different bacterial strains with the *Pseudomonas* species being the dominant group. After 15 days of microflora exposure to naproxen, there were significant changes in the qualitative composition and a decrease in the diversity index (Table 2). The dominant groups of

microorganisms that had been observed before the exposure to naproxen had probably the highest sensitivity to the drug, except for the strains that belong to the *Clostridium* sp. Interestingly, the presence of the drug caused a significant growth of the aerobic bacterium *Chryseolinea* sp. (Basic Local Alignment Search Tool (BLAST) similarity 100%), which to date has been found in soils in China and Korea and in Europe only in Germany. Although this genus is characteristic for uncontaminated forest soils, its tolerance to polycyclic aromatic hydrocarbons has not yet been studied. However, because this strain was no longer observed in the system after the bioaugmentation, it could be limited by other strains.



(**a**) (**b**)

Figure 3. The denaturing gradient gel electrophoresis (DGGE) profiles that were obtained at different stages of the experiment using the amplified (**a**) bacterial V3-V5 regions of the 16S rRNA gene and (**b**) the fungal ITS1/2 regions of the 18S rRNA gene. The lines that are marked as Microflora Imhoff contain the sequences that were obtained from the middle part of TF-I0 before the exposure to naproxen; Trickling filter I0 after the exposure to naproxen; Trickling filter and loofah sponges after the complete biodegradation of naproxen; B1(2015b) contains the sequences that were obtained from pure cultures of B1(2015b) cells. The letters T M B represent the sampling sites: T—top, M—middle and B—bottom of the TF.

Table 2. Changes in the Shannon-Wiener index (*H'*) of the bacterial (16S rDNA) and fungal (18S rDNA) communities corresponding to the denaturing gradient gel electrophoresis (DGGE) profiles that were obtained after the treatments. Columns marked as Microflora Imhoff contain the *H'* values that were calculated from the middle part of TF-I0 before the exposure to naproxen; Trickling filter I0 after the exposure to naproxen; Trickling filter and the loofah sponges after the complete biodegradation of naproxen. The letters T M B represent the sampling sites: T—top, M—middle and B—bottom of the TF.

	Microflora Imhoff	**Trickling Filter I0**		**Trickling Filter**			**Loofah Sponges**		
		M	**B**	**T**	**M**	**B**	**T**	**M**	**B**
16S rDNA	2.164	1.039	1.386	2.025	2.307	2.253	1.886	1.791	2.342
18S rDNA	2.686	2.761	2.043	2.564	2.780	2.718	-	2.800	2.841

The fungal strains demonstrated a greater tolerance to naproxen than the bacteria. Changes in their composition, dominant groups and diversity index were not significant after their exposure to

naproxen, except in the upper part of the TF, which contained a smaller amount of the 18S rDNA sequences (Figure 3b; Table 2).

Among the identified species, it was possible to distinguish fungi that belong to the genera *Trichosporon* sp. and *Vanrija* sp. The vast majority of the fungal DNA sequences (9 of 13) that were obtained were not similar to the sequences in the NCBI library.

By analyzing the 16S rDNA sequence from the B1(2015b) cells and comparing them with the sequenced genetic material from the TF and loofah sponges, it was confirmed that the bioaugmentation had been performed successfully. The immobilised B1(2015b) cells on the loofah sponges that remained in the trickling filter after 15 days were still present not only on the loofah sponges, but also in the core of the trickling filters (BLAST similarity 99%) (Figure 3a).

Therefore, after the biodegradation of naproxen, the influence of the immobilised B1(2015b) cells on the composition of the microflora from the Imhoff tank flow chamber in the trickling filter was analyzed. A comparison of the bacterial composition in the TF after exposure to naproxen (TF-I0) and after bioaugmentation shows a significant increase in biodiversity as a result of the introduction of the immobilised B1(2015b) cells (Figure 3a; Table 2). Changes were also observed in the dominant groups (*Clostridium* sp. and *Pseudomonas* sp. new domination) as well as in the growth of the bacteria that were present prior to the drug exposure and were sensitive to it (e.g., *Pseudomonas* sp.). Additionally, strains that were below the detection level before bioaugmentation were also present, probably as a result of the changes in the community composition. Analysis of the qualitative composition of the bacterial population that was introduced onto the loofah sponges indicated that the carrier was a good site for colonization. This was confirmed by the presence of strains from the LECA on the surface of the carrier.

The lack of significant changes in the fungal community in this study indicates that bioaugmentation did not affect the composition of autochthonous strains or their diversity on the basic carrier (LECA) in the trickling filter (Figure 3b; Table 2). However, significant changes were observed in the number of fungal strains that colonized abundantly lignocellulosic sponges.

3. Discussion

3.1. Immobilization of Bacillus thuringiensis B1(2015b) on Loofah Sponge

The ability of bacteria to form a biofilm on the surface of various materials can provide many advantages for cells such as a higher level of resistance to the toxic compounds in the environment. In bioremediation, a biofilm matrix additionally ensures a better chance for the adaptation and survival of bacteria in a new environment with autochthonic microflora. Because the efficiency of bioremediation using a trickling filter depends on the quality of the biofilm that is formed, its parameters for immobilization were optimized by adsorption on loofah sponge. Among the various accessible materials that are used for immobilization, it is desirable that carriers should be biodegradable and biocompatible when being used for bioremediation. At the same time, carriers have to also be characterized by a high level of porosity, mechanical resistance and a low price. Loofah sponges, which are composed of cellulose, hemicellulose and lignin, are an eco-friendly material for bacterial cell immobilizations due to their high mechanical resistance and high porosity [8,9].

The *Bacillus thuringiensis* B1(2015b) that was used in this study was isolated from the soil of the chemical factory "Organika-Azot" in Jaworzno, Poland. This Gram-positive strain is able to degrade various aromatic compounds such as phenol, vanillic acid, protocatechuic acid, benzoic acid or 4-hydroxybenzoic acid as well as some non-steroidal anti-inflammatory drugs such as naproxen and ibuprofen [6,10]. Because of these valuable features, this strain was immobilised on the loofah sponge.

One of the major factors that influences attachment is the surface charge of bacterial cells, which depends on the positively or negatively charged molecules that are predominant on the surface of the bacterial cell wall. Most bacteria have a negatively charged surface (negative zeta potential) at physiological pH (pH 7). However, the bacteria cell wall zeta potential is strongly determined by the

ionic strength of the surrounding medium and pH value [11]. It has been shown that *Bacillus cereus* spores exhibit a higher efficiency of attachment to hydrophobized, negatively charged glass when the medium has the same pH as the isoelectric point of their cell wall (pH 3) [12]. Due to the fact that loofah sponge is composed of polysaccharides, its charge at pH equal to or higher than 7 is negative. For that reason, the same charge of the cell surface and the carrier complicates the initial cell adhesion to the carrier surface. High ionic strength facilitates the neutralization of the cell wall charge when the cell and carrier surfaces have the same charge and in this way, it reduces their electrostatic repulsion [11].

It is known that adding manganese significantly increases the amount of the biofilm of *Bacillus* species [13]. Manganese belongs to the group of essential metals that regulate the activity of many enzymes by functioning as a catalytic cofactor. One of these enzymes is phosphoglycerate phosphomutase (EC 5.4.2.1), which plays a crucial role during the initiation of sporulation. This enzyme is responsible for the accumulation of 3-phosphoglyceric acid (3-PGA) in spores and its utilisation during germination [14]. On the other hand, other studies have indicated that manganese is complexed with Spo0F (a signal mediator), which is involved in the sporulation phosphorelay. In this mechanism, the phosphate is transferred from histidine kinase to Spo0F and later to Spo0B (the phosphotransferase). The final step is the transition of the phosphate to Spo0A, which is a transcription factor that initiates spore or biofilm formation [13,15]. Simultaneously, an increase in the amount of phosphorylated Spo0A results in unblocking the sinI transcription. SinI subsequently represses the activity of SinR and thereby prevents the blocking of the *eps* and *tapA* operons, which are involved in exopolysaccharide (EPS) and TasA protein (one of the matrix components) synthesis, respectively [16]. Despite the fact that a HCT medium already contained manganese (0.015 mM), during the optimization of the immobilization conditions, the addition of 0.33 mM of manganese significantly improved the quality of biofilm that was formed by the B1(2015b) cells. It is noteworthy that Morikawa et al. [17] showed that even at a concentration of 1000 mM, manganese stimulated biofilm formation by the *Bacillus subtilis* B1 strain.

3.2. Naproxen Biodegradation in the Trickling Filters

Because of its polycyclic structure, naproxen belongs to the group of hard-to-biodegrade xenobiotics. However, naproxen removal has been observed in monoculture conditions by only a few bacterial strains [4,6]. Moreover, Górny et al. [10] proposed a naproxen biodegradation pathway by *Bacillus thuringiensis* B1(2015b). According to their studies, the first stage comprises demethylation by tetrahydrofolate-dependent *O*-demethylase, which was observed so far only during fungal biotransformation. In the next step, *O*-desmethylnaproxen probably undergoes hydroxylation to 7-hydroxy-*O*-desmethylnaproxen. Due to the fact that naproxen is naphthalene derivative, it might be degraded like naphthalene however, only 2-formyl-5-hydroxyphenylacetic acid was detected. The last observed intermediate was salicylic acid which is transformed into maleyl puryvate, 2-oxo-3,5-heptadienedioic acid or *cis*,*cis*-muconic acid. These results indicated that strain *Bacillus thuringiensis* B1(2015b) is able to degrade naproxen into the tricarboxylic acid cycle intermediates. The ability of strain B1(2015b) to naproxen mineralization has made it a promising strain for the complete removal of this drug from wastewater.

On the other hand, only a few studies have been done with mixed microflora under non-sterile conditions. Due to the overgrowth of autochthonous bacteria and the inhibition of the growth of the microorganisms that are able to degrade these types of contaminants, the removal efficiency of naproxen was significantly lower than in monoculture conditions [18]. Because of the growing problem of drugs in the environment, there is a need to develop bioremediation methods adapted to their removal.

One of the key elements of a trickling filter is the carrier on which the biofilm is created. To date, various packing materials, such as plastics, stones [19], polyurethane foam [20] or zeolite have been used [21]. However, by selecting the correct packing material, it is possible to improve the effectiveness of the performance of a trickling filter. In this study, we selected lightweight expanded clay aggregate (LECA) (particle size 10–20 mm) due to its high porosity, natural origin, low cost and lack of toxicity.

Additionally, studies on the usability of LECA in bioremediation have revealed its high sorption properties for heavy metals and certain pollutants [22].

In order to create a fully functioning trickling filter, there was a need to implement its by biological active microflora. The TF-I and TF-I0 systems that were constructed for this study were inoculated with microflora from the Imhoff tank flow chamber from the wastewater treatment plant of Krupski Młyn-Ziętek (Poland), which was built almost 60 years ago and has never been modernized or otherwise augmented since that time.

The TF-I0 system was constructed in order to examine if the autochthonous microflora from the Imhoff tank flow chamber was able to biodegrade naproxen. The conducted study revealed that the initial drug loss was caused by LECA adsorption and not as an effect of biodegradation. It was confirmed in an additional control system, which contained only LECA and synthetic wastewater contaminated with naproxen (results not shown). This result shows that even wastewater treatment system with almost 60 years of history do not have microflora able to degrade or remove naproxen. Fungi primarily have the ability to degrade non-steroidal inflammatory drugs, which also implies that they are less sensitive to these compounds. This is related to the presence of more advanced enzyme complexes and detoxification pathways than those that are observed in bacterial strains [23]. We could suppose that the presence of fungi in bioremediation systems will provide a higher possibility of xenobiotic degradation for drugs such as NSAIDs. However, fungi only constituted a small percentage of the entire microflora of the trickling filters and activated sludge.

After introducing the immobilized cells of *Bacillus thuringiensis* B1(2015b) into the trickling filters, they were able to degrade naproxen in the presence of the autochthonous microflora and beyond their optimal growth conditions. Moreover, we observed a positive influence of the synergism between the introduced B1(2015b) strain and the autochthonous microflora on naproxen biodegradation. Although this type of ecological interactions occurs often in bioremediation systems, their mechanisms of action are not well known. It is assumed that this might be caused by increasing the bioavailability of a contaminant through the production of surfactants, a metabolic association of intermediates that cannot be further degraded, an exchange of growth factors or enhanced aggregation. For example, Byss et al. [24] observed that the inoculation with *Pleurotus ostreatus* of soil that had been contaminated with PAH resulted in significant stimulation of the growth of G+ bacteria and simultaneously more efficient bioremediation. In our study, we suspected that synergisms could be caused by the exchange of growth factors and the interaction of the Imhoff population and the B1(2015b) strain. Additionally, the presence of the anaerobic bacterial communities in the lower part of the TF (e.g., *Clostridium* sp.), could accelerate the naproxen biodegradation, due to the ability to demethylation or degradation of aromatic compounds like veratrate or catechol [25]. However, the B1(2015b) cells were able to degrade naproxen.

A further major factor that affects the performance of attached-growth bioreactors is the selection of an appropriate carbon to nitrogen (C:N) ratio in synthetic wastewater, which is associated with ensuring adequate amounts of carbon compounds, which are electron donors in the denitrification process. Under carbon-deficit conditions (C:N 3:1), nitrification and denitrification rates may not be in equilibrium and as a result, there is a lower performance of the bioreactors [2]. In our study, we prepared synthetic wastewater with a C:N ratio of 10:1, which according to Xia et al. [2], provides the best efficiency for the reduction of the COD in biofilm-based bioreactors. Additionally, immobilized cells of B1(2015b) introduced into the TF without autochthonous microflora were able to degrade most of the organic carbon from the synthetic wastewater. However, the addition of the specialized microflora from the wastewater treatment plant resulted in the higher removal of the organic carbon.

3.3. Colonization of the Loofah Sponges

In response to deficiencies of organic and mineral compounds, temperature and pH fluctuations, the presence of heavy metals as well as the substances that are secreted by other organisms, microorganisms began to aggregate into extensive communities in the form of biofilm. This solution

ensured the most critical advantages in the context of the colonization of aquatic environments. Biofilm forms various structures and water channels, which provide mechanical stability, mass transport, and functional heterogeneity. However, the key element is the biofilm matrix, whose main component is a highly hydrated EPS. Because of its sorption properties, biofilm accumulates nutrients as well as the exopolysaccharides, lipids, nucleic acids and enzymes that are secreted by cells while it also limits the direct contact of cells with toxins [4,8,26]. In natural habitats, biofilm is composed of multiple, highly selected strains of microorganisms. This microbiological diversification ensures metabolic cooperation and genetic exchanges among the populations in the biofilm [26]. For example, a synergistic effect occurred during the testing of the sodium dodecyl sulfate (SDS) resistance of *Pseudomonas aeruginosa*, *P. protegens* and *Klebsiella pneumoniae* strains. In monospecies biofilms, strains had different levels of resistance, whereas in a consortium, the more resistant species protected the rest of the community [27]. In bioremediation systems consortium we can also observe a more abundant biofilm matrix, in which the microorganisms are exposed to higher concentrations of potentially toxic compounds and required a protective barrier [3].

A characteristic feature of trickling filters is the diversity of conditions depending on the height. This also results in a different variety of microorganisms at different levels which is connected with the occurrence of two regions—the upper part of the aerobic region and a progressive lower anaerobic region due to the lack of additional aeration [21].

3.4. Phylogenetic Characterization of the TF Microbial Population

In bioremediation systems, autochthonous microflora operates in the form of cooperating communities. However, the presence of factors such as pollution or the addition or depletion of nutrients can significantly affect the diversity of microbial communities. The impact of pollutants on diversity depends on its type and the duration of the exposure. It was demonstrated that naproxen can negatively affect non-target organisms and entire ecosystems [28]. To date, there have only been a few studies on the effects of naproxen on microbial communities. Grenni et al. [28] observed that after 3 h of naproxen exposure (100 μg/L), the number of live cells of the microorganisms from the Tiber River decreased drastically. At a concentration of 10 μM, naproxen also inhibits nitrite production by the ammonia-oxidizing bacterium (AOB) *Nitrosomonas europaea,* which is a fundamental member of the microflora in wastewater treatment systems [29].

However, little is known about how naproxen affects the compositions of the microorganisms communities in bioremediation systems that are not adapted to degrade NSAIDs. In this study, we observed significant changes in the bacterial community which resulted in a drastic reduction in their biodiversity. This result shows how much risk naproxen can pose to bacteria in wastewater treatment plant if it enters in large quantities.

It should be also stressed that bioaugmentation is not indifferent to the autochthonous microflora in a bioremediation system. This effect can be positive or negative. The presence of new strains can change the composition of the microbial community through competition or inhibition. In the case of immobilization, the carrier onto which a strain is introduced should also be considered to be an influencing factor.

A carrier that can be a carbon and energy source for a specific group of microorganisms should be introduced carefully. A too large amount can cause these species to be too dominant, which could disrupt the performance of the entire system. In this study, we analyzed the influence of bioaugmentation over a short period of time and after 15 days, the beginning of overgrowth was observed. Further analyses should be performed to assess the long-term impact of the introduction of cellulosic materials into the communities in wastewater treatment systems.

4. Materials and Methods

4.1. Immobilization Optimisation and Procedure

In order to develop an immobilisation procedure that will results in the most abundant biofilm, ten immobilization parameters for optimization were accessed. These included the type of growth medium used: nutrient broth, mineral salts medium, HCT medium and its supplementation with glucose (0.5g/L). Additionally, we also assess the age of the culture that was harvested for immobilization (24, 48 or 72 h) and initial culture optical density (0.2, 0.4, 0.6, 0.8, or 1.0). The influence of the agitation speed (70, 90, 110, 130 or 150 rpm), incubation time of the bacterial culture with carrier (16, 24, 48 or 72 h), temperature (15, 20, 25, 30, or 35 °C), medium pH (3, 4, 5, 6, 7.2 or 8), salt concentration (7, 8, 14, 17, 24 or 34 g/L) and additional supplementation with metal salts (manganese, iron or calcium) was studied.

The final procedure used after immobilization was as follows: the bacterial strain *Bacillus thuringiensis* B1(2015b), which was cultivated according to Marchlewicz et al. [6] and re-suspended in the HCT medium, was used for the immobilization [7]. The optical density value (OD_{600}) of the bacterial suspension that was prepared for the immobilization was equal to 0.2. Loofah sponges, which were prepared according to Dzionek et al. [4], were used as the carrier for the immobilization of the B1(2015b) cells. For the immobilization, each Erlenmeyer flask (1000 mL) that contained sterile carrier material (7.5 g) was inoculated with the bacterial cell suspension (600 mL). The HCT medium (pH 8) in which the immobilization process was conducted was additionally supplemented with glucose (0.5 g/L) and manganese sulphate (1 g/L). The flasks were incubated with shaking (110 rpm) at 20 °C for 48 h. After incubation, loofah sponges with the immobilised B1(2015b) cells were rinsed with NaCl (0.9%) in order to remove any unbound microorganisms and were then used for bioremediation experiments.

4.2. Configuration and Operational Conditions of the Trickling Filters (TFs)

Three lab-scale TFs were constructed for this study, namely TF-I, TF-C, and TF-I0. Each TF was composed of four filter units. One filter unit consisted of polyvinyl chloride (PVC) pipe (H × W = (400 × 100 mm) × 4), which was protected from the bottom by steel mesh and filled with the biofilm carriers. The total volume of each TF was 0.015 m^3 and the filling was 46% of the reactor volume. As a base, biofilm carriers were selected lightweight expanded clay aggregate (LECA), which constituted 70% of each filter unit filling volume. The remaining 30% of the filling comprised the loofah sponges with immobilized bacteria (Figure 4).

As the nutrient and carbon sources, synthetic wastewater (15 L) was continuously circulated into the trickling filters by a peristaltic pump with a flow rate of approximately 0.0066 m^3/h. The synthetic wastewater was based on the one that was proposed by Kosjek et al. [30] with some modifications. The composition per 1 L was as follows: 0.317 g CH_3COONH_4; 0.04 g NH_4Cl; 0.024 g K_2HPO_4; 0.008 g KH_2PO_4; 0.1 g $CaCO_3$; 0.2 g $MgSO_4 \times 7H_2O$; 0.04 g NaCl; 0.005 g $FeSO_4 \cdot \times 7H_2O$ and 0.6 g glucose. A nutrient and glucose stock solution were added to the collection tanks every three days. The addition of calcium carbonate prevented the excessive acidification of the wastewater, whose pH was adjusted to 7.6 and was inspected every three days. The temperature in the trickling filters was maintained within a range of 21–23 °C. The hydraulic residence time (HRT) was determined using a draining test and is expressed as the ratio of the volume of the liquid in the trickling filter to the volumetric flow rate [31]. The chemical oxygen demand (COD) was analyzed using the potassium dichromate method according to the standard procedures [32].

Trickling filter TF-I was inoculated with biomass that had been taken from the Imhoff tank flow chamber in the wastewater treatment plant of Krupski Młyn-Ziętek (Poland). To colonize the trickling filter, a mixture of biomass and synthetic wastewater was continuously circulated through TF-I. When the biofilm on the surface of the LECA reached a thickness of 2–3 mm, the stabilisation of TF-I was considered to be complete (21 days) and loofah sponges with the immobilized bacteria were added to each filtration unit. The first control trickling filter (TF-C) contained only loofah sponges with

immobilized bacteria and the LECA. The second control trickling filter (TF-IO) contained only stabilised biomass (2–3 mm thick biofilm) from the Imhoff tank flow chamber and LECA.

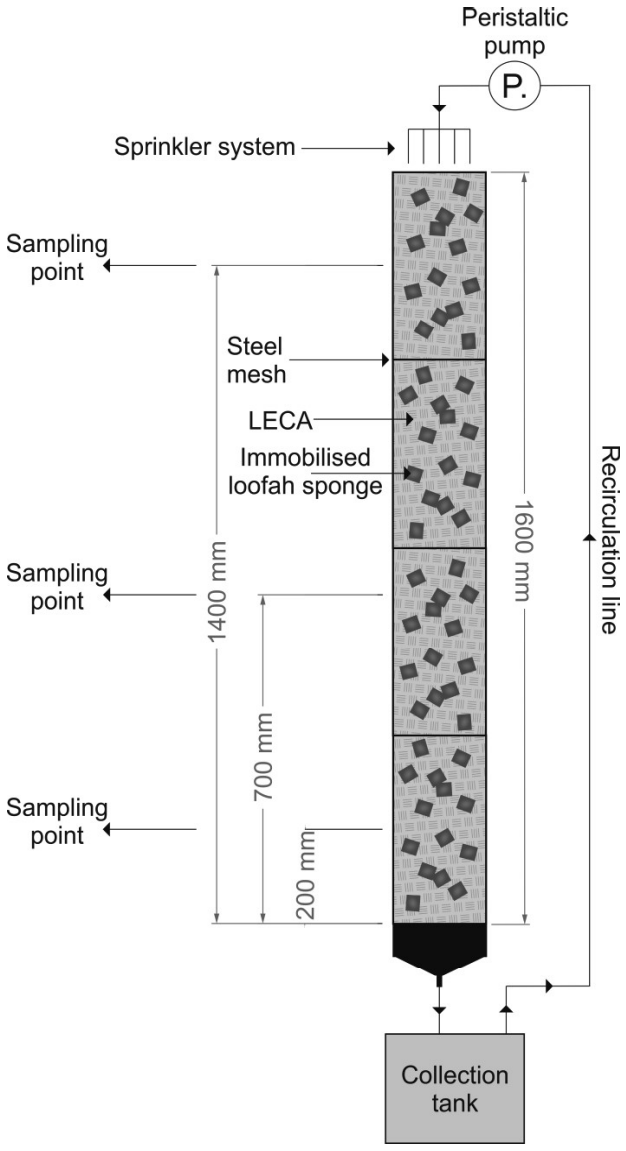


Figure 4. Experimental set-up of the trickling filters.

4.3. Naproxen Biodegradation Experiments

Fresh synthetic wastewater (15 L) that had been supplemented with naproxen (1 mg/L) was added to each trickling filter and circulated for the next 15 days. All of the experiments were conducted in a closed circuit. To determine the naproxen concentration, synthetic wastewater samples were taken from the collection tanks every 24 h for 15 days and analyzed using HPLC equipped with a LiChromospher® RP-18 column (4 × 250 mm), liChroCART® 250-4 Nucleosil 5 C18 and a DAD detector (Merck HITACHI, Darmstadt, Germany). As a mobile phase was used acetonitrile and 1% acetic acid (50:50 *v/v*) at a flow rate of 1 mL × min^{-1}. Naproxen was detected in the supernatant at wavelength 260 nm. Identification and quantification by comparison the HPLC retention times and UV-visible spectra with the external standards were conducted [23].

Uninoculated, additional controls (contained only LECA or synthetic wastewater supplemented with naproxen) were also prepared in order to determine adsorption or abiotic degradation of the drug.

To investigate the microbial population on the LECA and loofah sponges, the carriers were taken from three sampling points at depths of 20, 700 and 1400 mm (Figure S1, Supplementary Materials). To examine the effect of naproxen on the microflora from the Imhoff tank flow chamber (TF-I0), the LECA samples were taken before the naproxen was added and after 15 days of runoff. In order to investigate the colonization of the loofah sponges by the microflora from the Imhoff tank flow chamber, samples of the loofah sponges were taken after the complete biodegradation of naproxen (TF-I).

4.4. Biofilm Analysis Using Scanning Electron Microscopy

The biofilm structure on the loofah sponges was observed before and after naproxen biodegradation in trickling filter TF-I using Scanning Electron Microscopy. Samples were prepared according to Dzionek et al. [4] and were observed with a JSM-7100F TTL LV high-resolution electron microscope (JEOL, Tokyo, Japan).

4.5. Phylogenetic Characterization of the Microbial Population in the TFs

Changes in the microbial population on the carriers were determined using the denaturing gradient gel electrophoresis (DGGE) method. In order to obtain the DNA samples, the biofilm from the carrier samples was removed by shaking and rinsing with a NaCl solution (0.9%), centrifuged (14,000 rpm, 20 min) and re-suspended in the same solution. DNA was immediately extracted from various materials using a Genomic DNA isolation kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions.

The PCR amplification of the bacterial V3-V5 region of 16S rRNA gene (about 570 bp) was performed using the universal primers MF341-GC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCCG CCT ACG GGA GGC AGC AG-3′) and MR907 (5′–CCG TCA ATT CMT TTG AGT TT-3′) [33]. In order to obtain the sequences of the highly conserved regions of ITS1 and ITS2 from the fungal rRNA gene (500–800 bp), the primers ITS1F (5′–CTT GGT CAT TTA GAG GAA GTAA-3′) and ITS4 (5′–TCC TCC GCT TAT TGA TAT GC-3′) were used [34]. Each 25 µL PCR reaction contained 1 µL of extracted DNA, a 1 × PCR buffer, a 10 mM dNTP mixture, 10 mM of forward and reverse primers, 0.2 mg/L BSA and 1.25 U Pfu DNA Polymerase. Amplification was carried out with the cycling conditions for bacterial 16S rDNA according to Płociniczak et al. [33] and Anderson et al. [34] for the fungal ITS. The PCR products were examined with a 1.5% agarose gel in order to isolate the DNA fragments of the required length and to reamplify them.

DGGE was performed with the D-code System (Bio-Rad, Hercules, CA, USA) according to Płociniczak et al. [33]. The PCR products were loaded directly onto 6% (for 16S rRNA gene) or 8% (for ITS regions) polyacrylamide gels in a 1 × TAE buffer. The gels were prepared with a denaturing gradient in the range of 30–60% (for the 16S rRNA gene) or 18–58% (for the ITS regions). The electrophoresis was first run at 180 V for 30 min and then at 80 V for 17 h at 60 °C. The gels were subsequently stained with SYBR Gold (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The strong bands were cut out and diluted in 25 µL sterile water overnight, reamplified with the primers described above (except for primer MF341-GC, which was used without a GC clamp) and sequenced. The nucleotide sequences that were obtained were compared with known sequences in GenBank using the BLAST program (https://blast.ncbi.nlm.nih.gov).

The intensity of the individual DGGE bands was evaluated using ImageJ software and was scored as absent (value 0) or present on a scale of 1 to 4 in order to generate a data set. The diversity of a microbial population was determined using the Shannon Wiener index (*H*′) according to Xia et al. [2].

4.6. Statistical Analysis

All of the experiments were performed in at least three replicates. The values of the efficiency of naproxen biodegradation and the values of the physico-chemical parameters that were obtained were analyzed using the STATISTICA 12 PL software package (StatSoft, Tulsa, OK, USA). Statistically

significant differences and similarities were determined using the t-test or the Least Significant Differences (LSD) test ($p \geq 0.05$).

5. Conclusions

The immobilised *Bacillus thuringiensis* B1(2015b) introduced onto loofah sponges were successfully incorporated into the trickling filters. Synergistic influence of the autochthonous microflora on the naproxen biodegradation that was performed by the immobilised B1(2015b) cells was revealed. This short-term analysis revealed the possible effects of introducing a larger quantity of naproxen into a wastewater treatment plant. One of them can be a large decrease in the microbial diversity.

Supplementary Materials: The following are available online: Figure S1: Influence of different variants of environmental and physiological factors on the immobilisation efficiency of *Bacillus thuringiensis* B1(2015b) cells on loofah sponges.

Author Contributions: Conceptualization, A.D., and U.G.; Methodology, A.D., D.W. and U.G.; Validation, A.D., D.W. and U.G.; Formal Analysis, A.D.; Investigation, A.D. and M.A.-H.; Resources, A.D. and U.G.; Data Curation, A.D.; Writing—Original Draft Preparation, A.D.; Writing—Review & Editing, D.W. and U.G.; Visualization, A.D. and M.A.-H.; Supervision, D.W. and U.G.; Project Administration, U.G.; Funding Acquisition, U.G. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Sample Availability: Not Available.

Supplementary Material

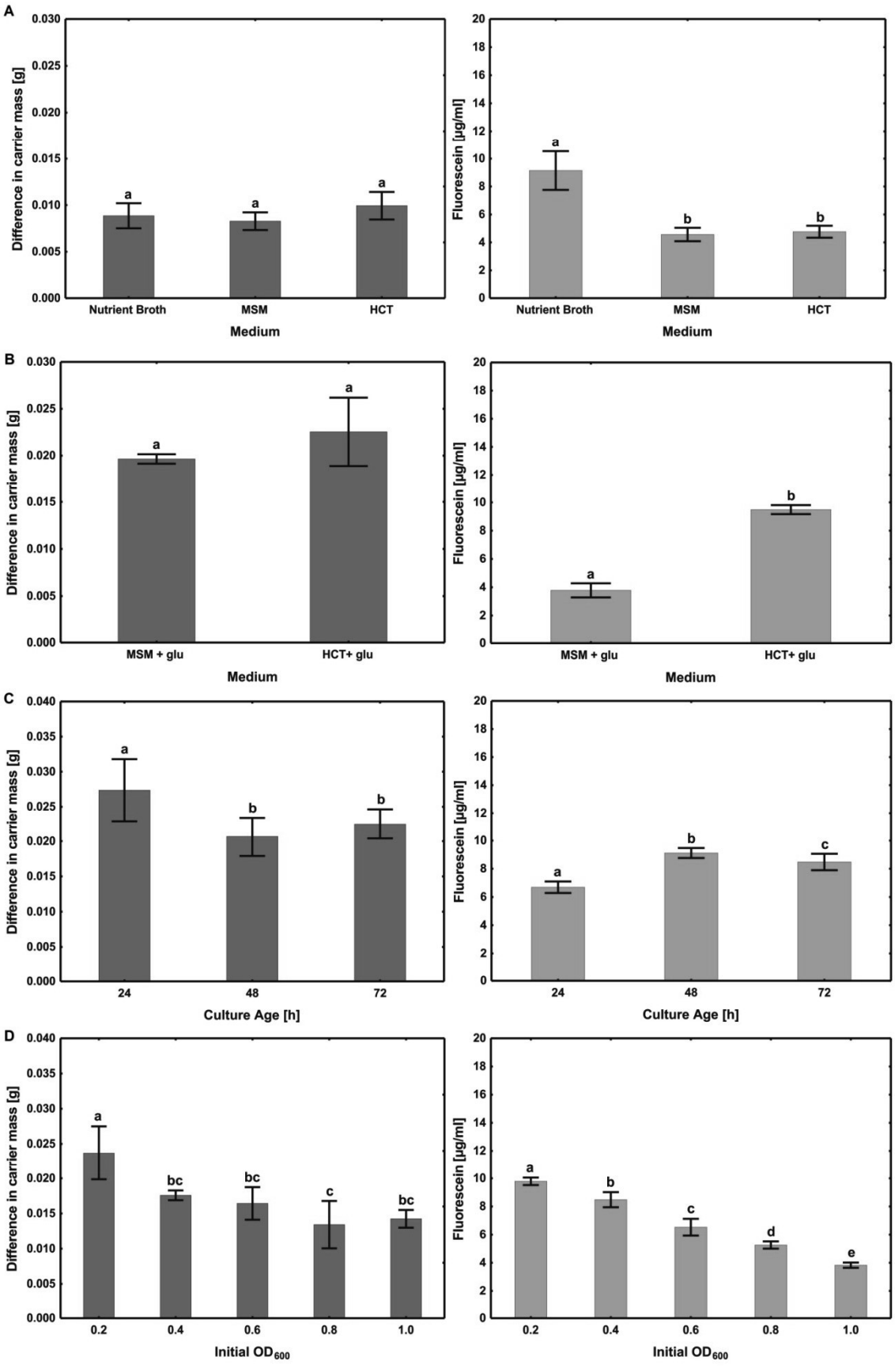
Enhanced degradation of naproxen by immobilization of *Bacillus thuringiensis* B1(2015b) on loofah sponge

Anna Dzionek[1], Danuta Wojcieszyńska[1], Małgorzata Adamczyk-Habrajska[2] and Urszula Guzik[1]*

[1] University of Silesia in Katowice, Faculty of Natural Science, Institute of Biology, Biotechnology and Environmental Protection, Jagiellońska 28, 40-032 Katowice, Poland
[2] University of Silesia in Katowice, Faculty of Science and Technology, Institute of Materials Engineering, 75 Pułku Piechoty 1a, 41–500 Chorzów, Poland

* Correspondence to: urszula.guzik@us.edu.pl; Tel.: +32-2009-567

Contents: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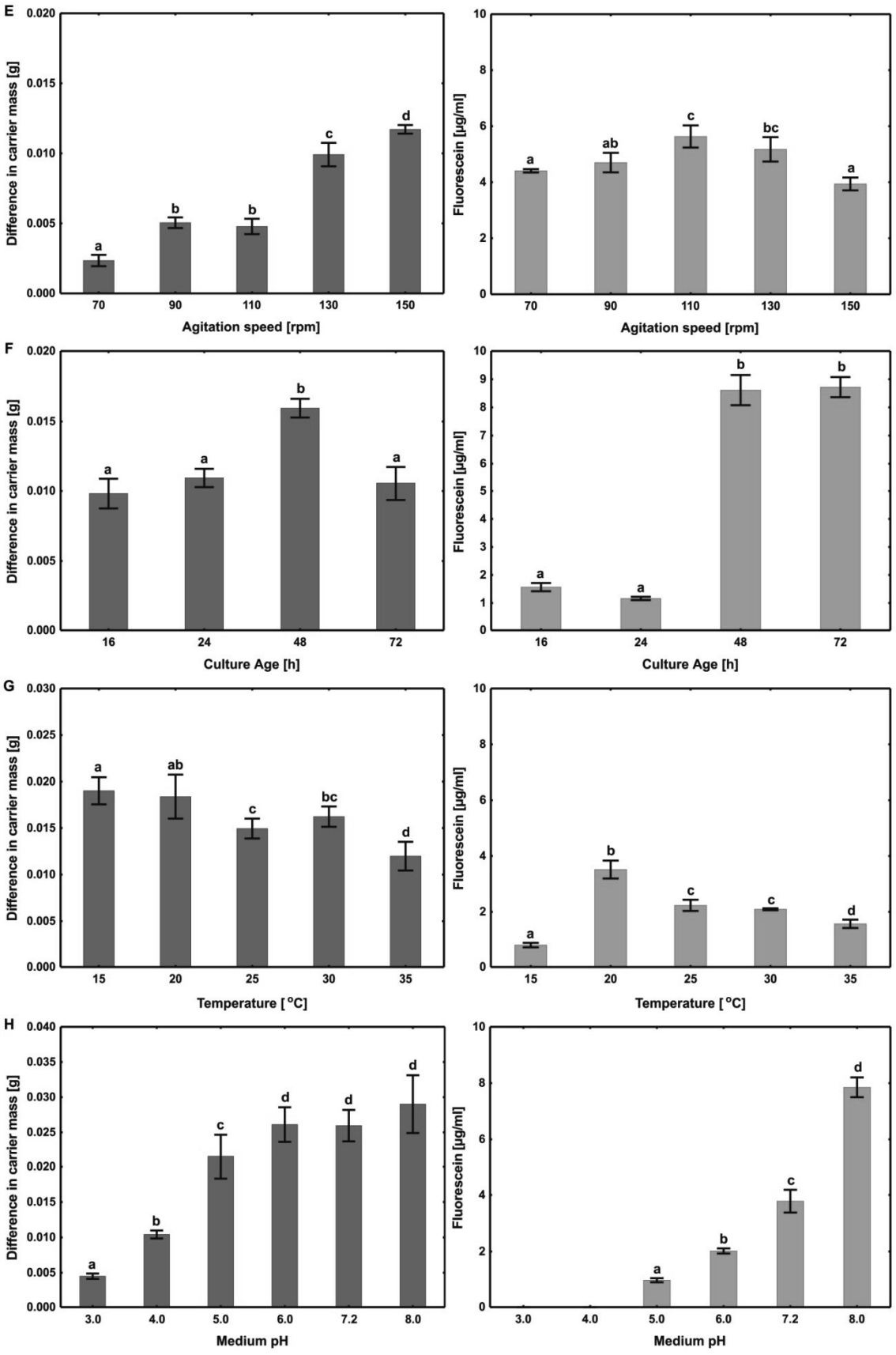
0.8
1.0
Initial OD600


E
Difference in carrier mass [g]
0.020
0.015
0.010
0.005
0.000
a
b
b
c
d
70
90
110
130
150
Agitation speed [rpm]
Fluorescein [µg/ml]
10
8
6
4
2
0
a
ab
c
bc
a
70
90
110
130
150
Agitation speed [rpm]
F
Difference in carrier mass [g]
0.020
0.015
0.010
0.005
0.000
a
a
b
a
16
24
48
72
Culture Age [h]
Fluorescein [µg/ml]
10
9
8
7
6
5
4
3
2
1
0
a
a
b
b
16
24
48
72
Culture Age [h]
G
Difference in carrier mass [g]
0.030
0.025
0.020
0.015
0.010
0.005
0.000
a
ab
c
bc
d
15
20
25
30
35
Temperature [°C]
Fluorescein [µg/ml]
10
8
6
4
2
0
a
b
c
c
d
15
20
25
30
35
Temperature [°C]
H
Difference in carrier mass [g]
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
a
b
c
d
d
d
3.0
4.0
5.0
6.0
7.2
8.0
Medium pH
Fluorescein [µg/ml]
10
8
6
4
2
0
a
b
c
d
3.0
4.0
5.0
6.0
7.2
8.0
Medium pH

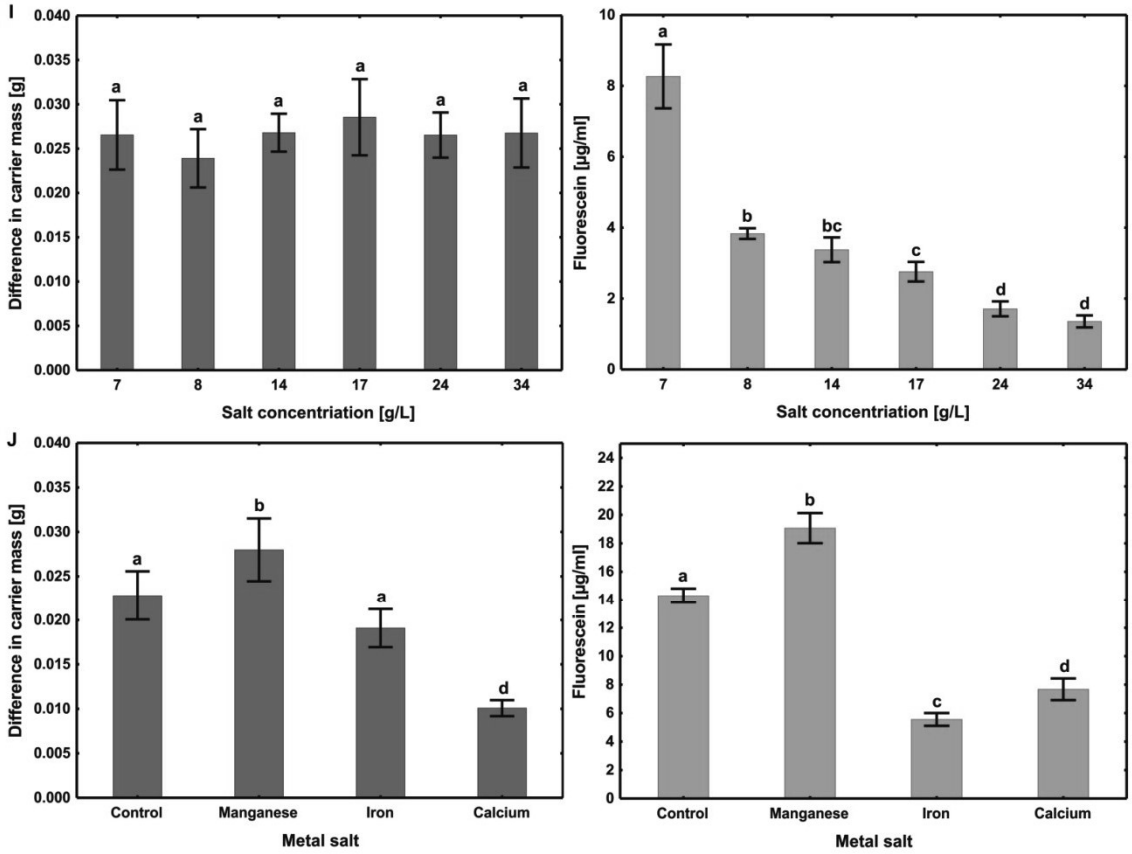


Figure S1: Influence of environmental and physiological factors on the immobilisation efficiency of *Bacillus thuringingiensis* B1(2015b) cells on loofah sponges:

(**a**) Immobilisation medium (Nutrient Broth, Mineral Salts Medium or HCT (medium rich in both carbon and ion sources));
(**b**) Additional carbon source (Mineral Salts Medium + 0.5 g/L glucose or HTC + 0.5 g/L glucose);
(**c**) Age of the culture that was harvested for immobilisation (24, 48 or 72 h);
(**d**) Initial culture Optical Density (0.2, 0.4, 0.6, 0.8, or 1.0);
(**e**) Agitation speed (70, 90, 110, 130 or 150 rpm);
(**f**) Incubation time of the bacterial culture with a carrier (16, 24, 48 or 72 h);
(**g**) Temperature (15, 20, 25, 30, or 35^{o}C);
(**h**) Immobilisation medium pH (3, 4, 5, 6, 7.2 or 8);
(**i**) Salt concentration in the immobilisation medium (7, 8, 14, 17, 24 or 34 g/L);
(**j**) Additional metal ions (manganese, iron or calcium).
All experiments were performed in at least five replicates. The values of the efficiency of immobilisation and enzyme activities were analyzed using a one-way ANOVA ($p \geq 0.05$) using STATISTICA 10 PL software package. A post-hoc test or T-test was applied to assess the differences between the treatments (differences are marked with successive letters a, b, c, d or e).

III. Podsumowanie

1. Dokonano modyfikacji i walidacji testu pozwalającego na ocenę stanu fizjologicznego immobilizowanych komórek w biofilmie, bazującego na hydrolizie dioctanu fluoresceiny.
2. Na podstawie wyników optymalizacji procesu immobilizacji szczepów *Planococcus* sp. S5 oraz *Bacillus thuringiensis* B1(2015b) na gąbce Loofah poznano mechanizmy indukcji biofilmu przez te szczepy.
3. W wyniku immobilizacji na gąbce Loofah komórek *Planococcus* sp. S5 zmniejszono wrażliwość szczepu na naproksen. W efekcie rozkład leku został niemal dwukrotnie przyspieszony, a także możliwa była degradacja najwyższego analizowanego stężenia naproksenu, który na wolne komórki S5 wykazywał działanie hamujące.
4. Immobilizowany na gąbce Loofah szczep *Planococcus* sp. S5 wykazywał pełną aktywność degradacyjną przez 55 dni, w ciągu których zdegradowane zostały 3 dawki naproksenu.
5. Nie zaobserwowano zmian szlaku degradacji naproksenu przez szczep *Planococcus* sp. S5 w wyniku immobilizacji. Obserwowano natomiast znaczące zmiany w wartościach aktywności następujących enzymów zaangażowanych w rozkład naproksenu: *O*-demetylazy, monooksygenazy aromatycznej, dioksygenazy naftalenowej, dioksygenazy 1,2-gentyzynianowej oraz dioksygenazy 1,2-salicylowej.
6. Bioaugmentacja złoża biologicznego immobilizowanymi na gąbce Loofah komórkami *Bacillus thuringiensis* B1(2015b) skutkowała synergistycznymi oddziaływaniami pomiędzy wprowadzonymi komórkami B1(2015b) a autochtoniczną mikroflorą. Spowodowało to znaczące przyspieszenie rozkładu naproksenu w złożu biologicznym.
7. Ekspozycja autochtonicznej mikroflory złoża biologicznego na naproksen skutkowała znaczącym zubożeniem bioróżnorodności społeczności bakteryjnych obecnych w złożu.

IV. Streszczenie

Obecnie pojawia się coraz więcej doniesień o obecności farmaceutyków, w tym niesteroidowych leków przeciwzapalnych, w środowisku naturalnym. Jednym z nich jest naproksen, który nie ulega całkowitej biodegradacji w organizmie człowieka. Dodatkowo, oczyszczalnie ścieków nie są przystosowane do jego utylizacji. W ostatnich latach wyizolowano i opisano szczepy bakteryjne, charakteryzujące się zwiększonymi możliwościami degradacyjnymi naproksenu. Celem pracy doktorskiej była immobilizacja szczepów bakteryjnych zdolnych do degradacji naproksenu. Dokonano charakterystyki zastosowanego nośnika oraz utworzonych na jego powierzchni biofilmów bakteryjnych. Ustalono wpływ unieruchamiania na przebieg rozkładu leku w warunkach monokulturowych, a także w obecności autochtonicznej mikroflory złoża biologicznego. Ponadto zbadano zmiany aktywności enzymów zaangażowanych w rozkład naproksenu w wyniku immobilizacji.

Badania nad wpływem immobilizacji na biodegradację naproksenu rozpoczęto przeprowadzeniem optymalizacji procesu unieruchamiania. Jednakże, aby poprawnie ocenić stan fizjologiczny immobilizowanych bakterii w biofilmie, zmodyfikowano metodę bazującą na hydrolizie dioctanu fluoresceiny. Wprowadzona modyfikacja polegała na ominięciu odrywania biofilmu od nośnika i przeprowadzenie testu na nienaruszonym biofilmie wraz z nośnikiem. Opracowana procedura zakłada wytrząsanie próbek w buforze fosforanowym o pH w zakresie 7,4-7,6 przez 1 godzinę, a wynik wyrażano jako całkowitą aktywność metaboliczną (TEA). Analiza czułości testu, w trakcie której mierzono zmiany TEA w wyniku głodzenia pozwoliła wyznaczyć minimalny endogenny metabolizm szczepu *Bacillus thuringiensis* B1(2015b), wynoszący 161-170 μg/g suchej masy na godz. Zaobserwowano również, że niedobór substancji odżywczych indukuje na powierzchni pianki poliuretanowej proces tworzenia biofilmu przez komórki szczepu B1(2015b).

W wyniku optymalizacji unieruchamiania szczepu *Planococcus* sp. S5 na gąbce Loofah zaobserwowano, że najwyższe wartości TEA (1250,26 ± 87,61 μg/g suchej masy na godz.) osiągano podczas 72-godzinnej inkubacji w minimalnej pożywce mineralnej (MSM) o pH 7,2 dodatkowo suplementowanej glukozą, NaCl oraz $MnSO_4$, wytrząsanej przy 90 rpm w 30°C oraz przy wysokiej koncentracji komórek. Szczep *Bacillus thuringiensis* B1(2015b) immobilizowany na gąbce Loofah wykazywał największe wartości TEA (790,14 ± 40,60 μg/g suchej masy na godz.) po 48-godzinnej inkubacji w podłożu HTC o pH 8, suplementowanej glukozą oraz $MnSO_4$, wytrząsanej przy 110 rpm w 20°C przy niskiej koncentracji komórek.

Analiza rozkładu naproksenu przez szczep *Planococcus* sp. S5 wykazała działanie hamujące leku w stężeniu wyższym niż 12 mg/L na wolne komórki tego

szczepu. Zaobserwowano, że wolne komórki S5 były zdolne do całkowitego rozkładu leku w stężeniu 6, 9 oraz 12 mg/L w odpowiednio 38, 44 oraz 62 dni. Rozkład leku przebiegał dwufazowo. Pierwsza faza, trwająca 29 dni charakteryzowała się wolniejszym rozkładem naproksenu. Podczas drugiej fazy obserwowano dwukrotnie szybszy rozkład leku. Immobilizowane na gąbce Loofah komórki S5 były zdolne do całkowitej degradacji leku we wszystkich analizowanych stężeniach, a tempo rozkładu było stałe, niezależne od dnia inkubacji oraz zbliżone do szybkości degradacji w trakcie II fazy przeprowadzanej przez komórki wolne. Badania nad przebiegiem wielokrotnych cykli degradacji naproksenu w najniższym analizowanym stężeniu wykazały, że w wyniku immobilizacji, komórki *Planococcus* sp. S5 zachowały pełną zdolność degradacyjną przez 55 dni, rozkładając w tym czasie 3 dawki leku. W trakcie rozkładu leku, komórki S5 wydzielały znaczne ilości egzopolisacharydów w celu zwiększenia bariery ochronnej przed naproksenem. Badania nad wpływem immobilizacji szczepu *Planococcus* sp. S5 na aktywność enzymów zaangażowanych w rozkład naproksenu wykazały, że immobilizacja nie spowodowała zmiany szlaku jego degradacji. Zaobserwowano natomiast znaczące zmiany w wartościach tych aktywności. Wykazano, że aktywność enzymatyczna w I fazie rozkładu leku przez wolne komórki S5 była znacznie niższa niż w fazie szybszego rozkładu. Pomimo zbliżonego tempa degradacji leku przez wolne komórki S5 w II fazie oraz przez immobilizowane komórki S5, aktywność analizowanych enzymów komórek immobilizowanych była znacznie wyższa niż wolnych komórek.

Przebieg biodegradacji naproksenu przez immobilizowane na gąbce Loofah komórki szczepu *Bacillus thuringiensis* B1(2015b) monitorowano w złożu biologicznym augmentowanym autochtoniczną mikroflorą pochodzącą z komory przepływowej osadnika Imhoff'a w Krupskim Młynie – Ziętek. Analiza wykazała, że immobilizowane komórki B1(2015b) zdegradowały 70% naproksenu w stężeniu 1 mg/L w nieaugmentowanym złożu biologicznym. Natomiast w obecności autochtonicznej mikroflory, unieruchomione komórki B1(2015b) w tym samym czasie rozłożyły 90% leku. Uzyskane wyniki ukazały synergistyczne oddziaływania pomiędzy autochtoniczną mikroflorą złoża biologicznego a wprowadzonymi komórkami B1(2015b), które skutkowały przyspieszeniem biodegradacji naproksenu. Dzięki analizie bakteryjnych regionów V3-V5 genu 16S rRNA z zastosowaniem elektroforezy w gradiencie czynnika denaturującego (DGGE), potwierdzono, że wprowadzony szczep *Bacillus thuringiensis* B1(2015b) był zdolny do przetrwania oraz namnażania się w złożu biologicznym po zakończonym procesie degradacji naproksenu. Ponadto przeprowadzono analizę zmian jakościowych populacji bakteryjnych oraz grzybowych autochtonicznej mikroflory złoża biologicznego po ekspozycji na naproksen, a także po wprowadzeniu

immobilizowanych komórek B1(2015b). Wykazano, że naproksen spowodował wyraźne obniżenie bioróżnorodności mikroflory bakteryjnej. Szczepy grzybowe charakteryzowały się mniejszą wrażliwością na lek. Natomiast w wyniku wprowadzenia immobilizowanych komórek B1(2015b), które były zdolne do szybkiej eliminacji leku, obserwowano wzrost bioróżnorodności mikroflory bakteryjnej oraz grzybowej.

V. Summary

More and more researches are reporting the presence of pharmaceuticals, especially non-steroidal anti-inflammatory drugs in the natural environment. One of them, naproxen is not fully degraded in the human body. Additionally, wastewater treatment plants are not adapted to its utilization. In recent years, bacterial strains which are characterized by increased naproxen degradation potential have been isolated and described. Therefore, the aim of the doctoral dissertation was to immobilize bacterial strains capable to degrade naproxen. The characteristics of the carrier used and bacterial biofilms formed on its surface were made. The effect of immobilization on the course of drug degradation in monocultural conditions, as well as in the presence of autochthonous microflora of the trickling filter was determined. In addition, changes in the activity of enzymes involved in naproxen degradation as a result of immobilization were investigated.

Studies on the effects of immobilization on the biodegradation of naproxen began with the optimization of the immobilization process. However, to correctly assess the physiological state of immobilized bacteria in the biofilm, the method based on the fluorescein diacetate hydrolysis was modified. The modification was to omit the detachment of the biofilm from the carrier and conducting the test on the intact biofilm together with the carrier. The developed procedure assumed shaking of samples in phosphate buffer with a pH in the range of 7.4-7.6 for 1 hour and the result was expressed as total metabolic activity (TEA). The sensitivity assay, during which changes in TEA as a result of starvation were measured, allowed to determine the minimal endogenous metabolism of the *Bacillus thuringiensis* B1(2015b) which was equal to 161-170 μg/g dry weight per hour. It was also observed that nutrient deficiency induced biofilm formation by B1(2015b) cells on the surface of polyurethane foam.

As a result of optimization of the immobilization of the strain *Planococcus* sp. S5 on Loofah sponge, it was observed that the highest TEA values (1250.26 ± 87.61 μg/g dry weight per hour) were achieved during 72-hour incubation in mineral salt medium (MSM; pH 7.2) additionally supplemented with glucose, NaCl and $MnSO_4$, shaken at 90 rpm at 30°C and with high cell concentration. Strain *Bacillus thuringiensis* B1(2015b) immobilized on the Loofah sponge showed the highest TEA values (790.14 ± 40.60 μg/g dry weight per hour) after 48-hour incubation in HTC medium (pH 8), supplemented with glucose and $MnSO_4$, shaken at 110 rpm at 20°C with low cell concentration.

Analysis of naproxen degradation by *Planococcus* sp. S5 strain showed an inhibitory effect at a concentration higher than 12 mg/L on free S5 cells. It was observed that free S5 cells were able to completely degrade the drug in a concentration of 6, 9 and 12 mg/L in 38, 44 and 62 days, respectively.

The degradation of the drug proceed in two phases. The first phase, lasting 29 days, was characterized by a slower naproxen degradation rate. During the second phase, drug degradation was twice as fast. Immobilized S5 cells on Loofah sponge were able to completely degrade the drug in all analyzed concentrations, and the degradation rate was constant, independent of the day of incubation and similar to degradation during phase II performed by free cells. Studies on the course of repeated cycles of naproxen degradation at the lowest analyzed concentration showed that as a result of immobilization, *Planococcus* sp. S5 cells maintained full degradation capacity for 55 days, during which degraded 3 doses of the drug. Additionally, during naproxen degradation, S5 cells secreted significant amounts of exopolysaccharides to increase the protective barrier against naproxen. Studies on the impact of the *Planococcus* sp. S5 immobilization on the activity of enzymes involved in naproxen degradation have shown that immobilization did not change the degradation pathway. However, significant changes were observed in the values of these activities. It was shown that the enzymatic activity in the first phase of drug degradation by free S5 cells was much lower than in the faster degradation phase. Despite the similar rate of drug degradation by free S5 cells in phase II and by immobilized S5 cells, the activity of the analyzed enzymes of immobilized cells was significantly higher than that of free cells.

The naproxen biodegradation by *Bacillus thuringiensis* B1(2015b) immobilized on the Loofah sponge was monitored in a trickling filter augmented with autochthonous microflora from the Imhoff tank flow chamber in Krupski Młyn – Ziętek. Analysis showed that immobilized B1(2015b) cells degraded 70% of naproxen at a concentration of 1 mg/L in the trickling filter without autochthonous microflora. However, in the presence of indigenous microflora, immobilized B1(2015b) cells at the same time degraded 90% of the drug. Obtained results showed synergistic interactions between the autochthonous microflora of the trickling filter and introduced B1(2015b) cells, which resulted in acceleration of naproxen biodegradation. By analyzing the bacterial V3-V5 regions of the 16S rRNA gene using denaturing gradient gel electrophoresis (DGGE), it was confirmed that the introduced *Bacillus thuringiensis* B1(2015b) was able to survive and multiply in the trickling filter after the process of naproxen degradation. In addition, an analysis of the qualitative changes of bacterial and fungal communities of autochthonous microflora after exposure to naproxen, as well as after the introduction of immobilized B1(2015b) was performed. Naproxen has been shown to cause a significant reduction in bacterial microflora biodiversity. Fungal strains were less sensitive to the drug. However, as a result of the introduction of immobilized B1(2015b) cells, which were able to quickly eliminate the drug, an increase in the biodiversity of bacterial and fungal microflora was observed.

VI. Oświadczenia doktoranta i współautorów

Katowice, dn. 29.05.2020

mgr Anna Dzionek
Instytut Biologii, Biotechnologii i Ochrony Środowiska
Wydział Nauk Przyrodniczych
Uniwersytet Śląski w Katowicach

Oświadczenie

W związku z wykorzystaniem poniższych prac, jako rozprawy doktorskiej, oświadczam, że mój udział procentowy, jako autora rozprawy szacuję na:

1. 80% - praca „Natural carriers in bioremediation: A review", opublikowana w Electronic Journal of Biotechnology (2016, 28-36). Wkład procentowy obejmuje współudział w napisaniu oraz zredagowaniu tekstu publikacji, przygotowanie tabel oraz wykonanie rysunków;

2. 85% - praca "Fluorescein Diacetate Hydrolysis Using the whole Biofilm as a Sensitive Tool to Evaluate the Physiological State of Immobilized Bacterial Cells", opublikowana w Catalysts (2018, 434). Wkład procentowy obejmuje zaprojektowanie i wykonanie doświadczeń, analizę wyników, przygotowanie tabel oraz rysunków, a także współudział w opracowaniu manuskryptu;

3. 70% - praca "Immobilization of *Planococcus* sp. S5 Strain on the Loofah Sponge and Its Application in Naproxen Removal", opublikowana w Catalysts (2018, 176). Wkład procentowy obejmuje zaprojektowanie i wykonanie doświadczeń, przygotowanie tabel oraz rysunków, analizę wyników oraz współudział w opracowaniu manuskryptu;

4. 75% - praca "Enchanced Degradation of Naproxen by Immobilization of *Bacillus thuringiensis* B1(2015b) on Loofah Sponge" opublikowana w Molecules (2020, 872). Wkład procentowy obejmuje zaprojektowanie i wykonanie doświadczeń, przygotowanie tabel oraz rysunków, analizę wyników, a także współudział w opracowaniu manuskryptu.

Anna Dzionek
.....

mgr Anna Dzionek

Katowice, dn. 25.05.2020

dr hab. Urszula Guzik, prof. UŚ
Instytut Biologii, Biotechnologii i Ochrony Środowiska
Wydział Nauk Przyrodniczych
Uniwersytet Śląski w Katowicach

Oświadczenie

W związku z wykorzystaniem przez mgr Annę Dzionek poniższych prac, jako rozprawy doktorskiej, oświadczam, iż mój udział procentowy szacuje się w nich na:

1. „Natural carriers in bioremediation: A review", opublikowanej w Electronic Journal of Biotechnology (2016, 28-36) – 10%, obejmujący współudział w opracowywaniu manuskryptu;

2. "Fluorescein Diacetate Hydrolysis Using the whole Biofilm as a Sensitive Tool to Evaluate the Physiological State of Immobilized Bacterial Cells", opublikowanej w Catalysts (2018, 434) – 5%, obejmujący współudział w opracowywaniu manuskryptu;

3. "Immobilization of *Planococcus* sp. S5 Strain on the Loofah Sponge and Its Application in Naproxen Removal", opublikowanej w Catalysts (2018, 176) – 10%, obejmujący współudział w analizie wyników i opracowywaniu manuskryptu;

4. "Enchanced Degradation of Naproxen by Immobilization of *Bacillus thuringiensis* B1(2015b) on Loofah Sponge" – opublikowanej w Molecules (2020, 872) – 10%, obejmujący współudział w projektowaniu doświadczeń i opracowywaniu manuskryptu.

Urszula Guzik

dr hab. Urszula Guzik, prof. UŚ

Katowice, dn. 25.05.2020

dr hab. Danuta Wojcieszyńska, prof. UŚ
Instytut Biologii, Biotechnologii i Ochrony Środowiska
Wydział Nauk Przyrodniczych
Uniwersytet Śląski w Katowicach

Oświadczenie

W związku z wykorzystaniem przez mgr Annę Dzionek poniższych prac, jako rozprawy doktorskiej, oświadczam, iż mój udział procentowy szacuje się w nich na:

1. „Natural carriers in bioremediation: A review", opublikowanej w Electronic Journal of Biotechnology (2016, 28-36) – 10%, obejmujący współudział w opracowywaniu manuskryptu;

2. "Fluorescein Diacetate Hydrolysis Using the whole Biofilm as a Sensitive Tool to Evaluate the Physiological State of Immobilized Bacterial Cells", opublikowanej w Catalysts (2018, 434) – 5%, obejmujący współudział w opracowywaniu manuskryptu;

3. "Immobilization of *Planococcus* sp. S5 Strain on the Loofah Sponge and Its Application in Naproxen Removal", opublikowanej w Catalysts (2018, 176) – 10%, obejmujący współudział w analizie wyników i opracowywaniu manuskryptu;

4. "Enchanced Degradation of Naproxen by Immobilization of *Bacillus thuringiensis* B1(2015b) on Loofah Sponge" – opublikowanej w Molecules (2020, 872) – 10%, obejmujący współudział w projektowaniu doświadczeń i opracowywaniu manuskryptu.

D. Wojcieszyńska

dr hab. Danuta Wojcieszyńska, prof. UŚ

Katowice, dn. 29.05.2020

dr hab. Małgorzata Adamczyk-Habrajska
Instytut Technologii i Mechatroniki
Wydział Nauk Ścisłych i Technicznych
Uniwersytet Śląski w Katowicach

Oświadczenie

W związku z wykorzystaniem przez mgr Annę Dzionek poniższych prac, jako rozprawy doktorskiej, oświadczam, iż mój udział procentowy szacuje się w nich na:

1. "Immobilization of *Planococcus* sp. S5 Strain on the Loofah Sponge and Its Application in Naproxen Removal", opublikowanej w Catalysts (2018, 176) – wkład procentowy 5% obejmujący wykonanie mikrografii SEM;

2. "Enchanced Degradation of Naproxen by Immobilization of *Bacillus thuringiensis* B1(2015b) on Loofah Sponge" – opublikowanej w Molecules (2020, 872) – wkład procentowy 5% obejmujący wykonanie mikrografii SEM;

.....

dr hab. Małgorzata Adamczyk-Habrajska

Katowice, dn. 25.05.2020

dr hab. Katarzyna Hupert-Kocurek, prof. UŚ
Instytut Biologii, Biotechnologii i Ochrony Środowiska
Wydział Nauk Przyrodniczych
Uniwersytet Śląski w Katowicach

Oświadczenie

W związku z wykorzystaniem przez mgr Annę Dzionek pracy pt. "Immobilization of *Planococcus* sp. S5 Strain on the Loofah Sponge and Its Application in Naproxen Removal", opublikowanej w Catalysts (2018, 176) jako rozprawy doktorskiej, oświadczam, iż mój udział procentowy, jako współautora, szacuję na 5% i obejmuje udział w korekcie manuskryptu.

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dr hab. Katarzyna Hupet-Kocurek

Katowice, dn. 29.05.2020

dr Jolanta Dzik
Instytut Technologii i Mechatroniki
Wydział Nauk Ścisłych i Technicznych
Uniwersytet Śląski w Katowicach

Oświadczenie

W związku z wykorzystaniem przez mgr Annę Dzionek pracy pt. "Fluorescein Diacetate Hydrolysis Using the whole Biofilm as a Sensitive Tool to Evaluate the Physiological State of Immobilized Bacterial Cells", opublikowanej w Catalysts (2018, 434) jako rozprawy doktorskiej, oświadczam, iż mój udział procentowy, jako współautora, szacuje się na 5% i obejmował wykonanie mikrografii SEM.

Jolanta Dzik

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dr Jolanta Dzik